PRINCIPLES *and* REACTIONS *of* PROTEIN EXTRACTION, PURIFICATION, *and* CHARACTERIZATION

Hafiz Ahmed

Ligand

Solid support

+

Ligand immobilized matrix

Crude proteins

Absorption

+

Wash column to remove unbound substances

Elution of bound substances

+

Pure substance

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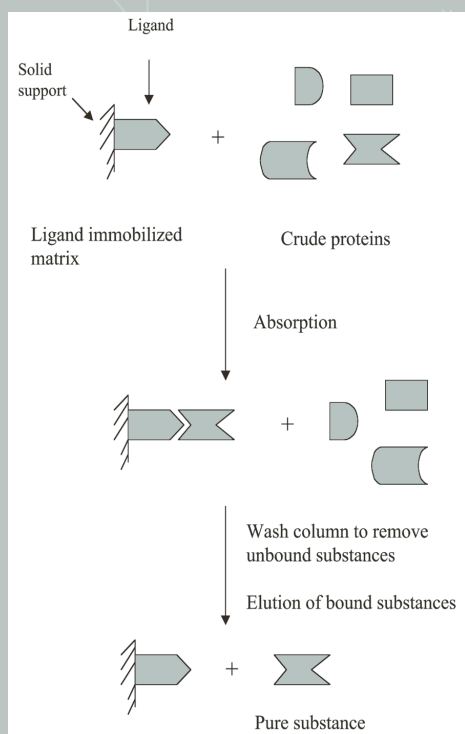# PRINCIPLES *and* REACTIONS *of* PROTEIN EXTRACTION, PURIFICATION, *and* CHARACTERIZATION

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Dedication

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Dedicated to my parents

Foreword

A protein chemistry book in the post-genomic era? Possibly more useful now than ever before, this excellent book is the product of many years of hands-on experience in the extraction, purification, and characterization of authentic and recombinant proteins. Dr. Hafiz Ahmed, an excellent biochemist, outstanding collaborator, and very productive scientist, conceived this project as a response to what he perceived as a need to address in a practical manner not only those questions and quick problem-solving arising during his own experimental endeavor, but also from the inquiries of the students, postdoctoral associates, and colleagues he has interacted with at the bench when dealing with purification of both native and recombinant proteins and glycoproteins. Accordingly, this book is not just a compendium of techniques for protein purification, and characterization, but rather a very well crafted learning tool that leads to a rational understanding of the basis of the procedures he has selected. And this is what makes this volume unique: it not only provides the most detailed and state of the art techniques, but also discusses in detail the underlying principles that have guided the development of such methodology, and provides a critical assessment of the advantages and disadvantages of alternative protocols from the most established to the most recent and innovative. Thus, it enables the reader not only to make a well-informed choice of methods, but also to apply those same principles to tailor them according to any specific goal.

The first chapter deals with a comprehensive discussion of methods for extraction of authentic and recombinant, soluble and membrane associated protein extraction, including techniques for cell lysis, and the use of protease inhibitors and detergents. These are critical steps in the isolation and purification of proteins, seldom found as the comprehensive and logically organized chapters provided in this volume. This is followed by three chapters consisting of critical assessments of the methods for estimating protein concentration, analytical electrophoretic techniques, and protein purification. These three chapters constitute the core of this manual, and wide array of methods, from the most classical to the most recent, are described in superb detail. The following methodology concerns the use of both conventional and monoclonal antibodies for protein isolation, analysis, and cell/tissue localization. The discussion of labeling techniques is particularly detailed, and constitutes an invaluable resource. Finally, the last chapter is devoted to the purification and characterization of glycoproteins, and area in which Dr. Ahmed is particularly skilled, and as expected, the information is presented in a well organized sequence leading from the most basic biochemical techniques to the biophysical approaches aimed at the structural elucidation of the oligosaccharide components. All chapters are fully supported by an extensive reference list that will enable the reader to further examine in depth the various aspects of the method of interest.

From the brief description above, it becomes clear that Dr. Ahmed has fully succeeded in bringing forth to the academic and industrial community a very well crafted book that will become an invaluable resource for students, technicians, and established scientists alike, who seek detailed information not only on the "hows" but also on the "whys" of protein purification and characterization.

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Preface

Several excellent protocol books on protein techniques have become available in recent years. These books adequately cover various practical aspects of protein techniques with sufficient protocols and methods. Here, I attempted to prepare a more comprehensive guide that is suitable to novices as well as experienced researchers. The book presents techniques (both classical and recent) for protein extraction, purification, and characterization, and discusses their underlying principles and working procedures including comments on their advantages and disadvantages. Thus, the reader can find useful advice on which technique to use or when to apply a particular method. To help the reader understand and compare different techniques, the book presents several tables, diagrams, and chemical reactions. The descriptions of numerous protein techniques in one book covering extraction, purification, and characterization are intended to make it convenient for researchers to understand the procedures involved in the course of an investigation. Readers will also get a broad picture of how many ways a certain study can be performed. The detailed table of contents should allow readers to retrieve a particular topic easily. I believe all these elements will help students and researchers to gain confidence and master the protocols.

Chapter 1 presents protein extraction methods, with background information on the preparation of buffers, and the use of protease inhibitors and detergents in protein extraction. Various cell lysis techniques with their principles are presented in one table, while another lists common extraction buffers and their advantages and disadvantages. Chapter 2 describes various methods (UV absorption, colorimetric, and fluorometric) for quantitation of protein, with advantages and disadvantages of most quantitation methods presented in a table. In Chapter 3, various electrophoretic methods for protein separation are described. A highlight of this chapter is the presentation of various polyacrylamide gel electrophoresis (PAGE) systems with their applications. Common problems encountered in isoelectric focusing gels and possible remedies are also listed. Characteristics, advantages, and disadvantages of most protein stains are also presented in a single display. Similarly, various protein-blotting membranes with their characteristics and applications are presented in a single table. Chapter 4 presents most procedures for protein purification. The two most common purification procedures, gel filtration and ion-exchange chromatography, are described with practical details. Additional techniques such as preparation of columns and loading samples for other chromatographic methods can be adapted from the first two procedures. Similarly, high performance liquid chromatography (HPLC) is described briefly, as this can be easily conducted once the researcher becomes familiar with the HPLC instrument. As in other chapters, comparative tables listing various HPLC features are included to assist in choosing the best method. Tables containing potential problems, possible causes, and remedies are shown to

help master the protocols. Antibodies and immunochemical techniques are described in two chapters (Chapters 5 and 6) as antibodies are widely used for characterization of proteins. Chapter 5 describes basic information on antibody response, antibody structure, antigen-antibody interactions, and antibody production under self-explanatory titles. Chapter 6 includes antibody labeling, antibody detection, and various immunochemical techniques. Diagrams and chemical reactions are shown to help the reader understand the principles behind the protocols. In Chapter 7, methods for purification and analysis (carbohydrate determination) of glycoproteins are presented, as glycosylation of proteins represents one of the most common post-translational phenomena. In the future, this chapter will be expanded to include other protein modifications.

Profile of the Author

The author received his Ph.D. degree (1986) in science from Jadavpur University, Calcutta, India for his work on the purification and characterization of carbohydrate-binding proteins (lectins) from plants, invertebrates, and bacteria. As a post-doctoral fellow at Max Planck Institute for Experimental Medicine (Gottingen, Germany), Roswell Park Cancer Institute (Buffalo, NY), and Center of Marine Biotechnology (University of Maryland Biotechnology Institute, Baltimore, MD) he has conducted studies on animal lectins, especially on structure and functions of β-galactoside binding lectins (galectins) and C-type lectins. He is currently an assistant professor at the Center of Marine Biotechnology.

Among his accomplishments are the development of a sensitive and reproducible solid phase assay for determining lectin-carbohydrate interactions, and co-authorship of the first report of the 3-D structure of an authentic galectin. He has published more than 50 papers in peer-reviewed journals. He is a member of Society for Glycobiology and American Society for Biochemistry and Molecular Biology. His recent research interest is on the developmental regulation and role of galectins.

Contents

1 Extraction of Protein

In order to isolate intracellular proteins, cells must be disrupted. Several disruption techniques, both mechanical and chemical, are available (Table 1.1). An efficient protocol for cell disruption must be developed to release the protein in a soluble form from its intracellular compartment. The disruption protocol should be as gentle as possible to the protein, as the extraction step is the starting point for all subsequent procedures. The success of cell disruption depends on a number of variables, such as the choice of buffers, the presence of protease inhibitors, and the osmolarity of the resuspension buffer. The condition and the constituent of the extraction buffer depend on the nature of the cell type, the target protein, and its intended application.

A great deal of strategy and consideration are required in choosing the right condition for extraction. Investigators need to have an answer prior to extraction for some common questions: How is the protein to be used? Is the protein required for peptide sequencing in order to perform cloning and sequencing the cDNA? Is the protein required for investigation of its properties and biological activities? For peptide sequencing, a few micrograms of denatured protein on polyacrylamide gel or on polyvinylidene difluoride (PVDF) membrane are adequate. But if the investigation of the proteins' properties and biological activities is intended, care should be taken to maintain the protein of interest in a stable and active form.

1.1 PREPARATION OF BUFFERS FOR PROTEIN EXTRACTION

Proteins are extremely heterogeneous biological macromolecules. Their properties can be severely affected by small changes in hydrogen ion concentration, and thus a stable pH of the protein environment is necessary.

1.1.1 THEORY OF BUFFERING

In order to ensure reproducible experimental results, it is important to maintain the protein solution at the constant pH. It has been observed that partially neutralized solutions of weak acid or weak bases are resistant to pH changes on the addition of small amounts of strong acid or strong base. This is known as "buffering."[1] Buffer solutions consist of a weak acid and its salt with a strong base, or of a weak base and its salt with a strong acid. The buffering capacity may be explained by the following equations:

$$HA \text{ (acid)} \leftrightarrow H^+ + A^- \text{ (incomplete)} \qquad (1.1)$$

$$NaA \rightarrow Na^+ + A^- \text{ (complete)} \qquad (1.2)$$
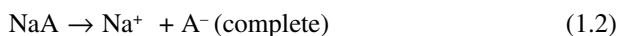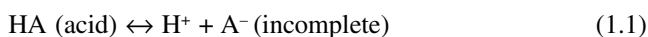
TABLE 1.1
Various Cell Lysis Techniques

Technique	Principle	Time of Lysis	Example
Enzyme digestion	Digestion of cell wall leading to osmotic disruption of cell membrane	15–30 min	Gram positive bacteria
Osmotic shock lysis	Osmotic disruption of cell membrane	<5 min	Red blood cells
Hand homogenization	Cells are forced through narrow gap leading to disruption of cell membrane	10–15 min	Liver tissue
Blade homogenizer	Large cells are broken by chopping action	5–10 min	Muscle tissue, animal tissue, plant tissue
Grinding with alumina or sand	Cell walls are ripped off by micro roughness	5–15 min	Bacteria
Grinding with glass beads	Cell walls are ripped off by rapid vibration of glass	10–20 min	Bacteria
French press	Cells are forced through small orifice at very high pressure. Shear forces disrupt cells.	10–30 min	Bacteria, plant cells
Sonication	Cell disruption by shear forces and cavitation caused by high-pressure sound waves	5–10 min	Bacteria

NaA is shown here as an example of salt of weak acid and strong base. A^- is called conjugate base of the acid HA. The addition of small amounts of strong acid (H^+) to the buffer shifts equilibrium (Equation 1.1) to the left using A^- supplied by Equation 2. Whereas the addition of small amounts of strong base (OH^-) combines with H^+ provided by equilibrium (Equation 1.1) moving to the right. In either case, change of H^+ concentration, hence pH, is unchanged.

The pH of a solution of a weak acid and or base is calculated as follows:

From Equation 1.1, the dissociation constant (K_a) is defined as

$$K_a = [H^+][A^-]/[HA]$$

$$pK_a = -\log K_a = -\log [H^+][A^-]/[HA]$$

$$= -\log [H^+] - \log [A^-]/[HA]$$

$$= pH - \log [A^-]/[HA]$$

$$pH = pK_a + \log[\text{basic form}]/[\text{acidic form}]$$

This is the Henderson-Hasselbalch equation.

The pH of a buffer is equal to the pK_a, when the concentrations of acidic and basic species are equal. The above equation is valid in the pH range of 3 to 11. Table 1.2 shows pK_a values for some common buffers. The buffering capacity is maximal at the pK_a values, and significantly lower at values one pH unit lower or higher than the pK_a value.[2] The reason is the following:

$$pH = pK_a + 1; \text{ where [basic form]} = 10 \times \text{[acidic form]}$$

$$pH = pK_a - 1; \text{ where [basic form]} = 0.1 \times \text{[acidic form]}$$

A buffer will have good buffering capacity when both forms are present in a reasonable amount. So it is desirable to work within about 0.5 unit of the pK_a.

TABLE 1.2
pK_a Values of Common Biological Buffers[a]

Trivial Name	Buffer Name	pK_a at 25°C
Phosphate (pK_{a1})		2.15
Citrate (pK_{a1})		3.06
Formate		3.75
Succinate (pK_{a1})		4.21
Citrate (pK_{a2})		4.76
Acetate		4.76
Pyridine		5.23
Citrate (pK_{a3})		5.40
Succinate (pK_{a2})		5.64
MES	2-(N-Morpholino)ethanesulfonic acid	6.15
Cacodylate	Dimethylarsinic acid	6.27
Carbonate (pK_{a1})		6.35
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)	6.76
Imidazole		6.95
MOPS	3-(N-Morpholino)propanesulfonic acid	7.20
Phosphate (pK_{a2})		7.20
TES	2-[Tris(hydroxymethyl)methylamino] ethanesulfonic acid	7.50
HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic acid	7.55
Tris	Tris(hydroxymethyl)aminomethane	8.06
Tricine	N-[Tris(hydroxymethyl)methyl]glycine	8.15
Borate		9.23
CHES	Cyclohexylaminoethanesulfonic acid	9.55
Glycine		9.78
Carbonate (pK_{a2})		10.33
CAPS	3-(Cyclohexylamino)propanesulfonic acid	10.44
Phosphate (pK_{a3})		12.43

[a] Adapted from Reference 2.

1.1.2 Selection of an Appropriate Buffer

Several factors are considered when choosing a buffer (Table 1.3). These are the pK_a and the effects of temperature, interactions with other components (such as enzymes and metal ions), compatibility with different purification techniques, ultra-violet (UV) absorption, permeability through biological membranes, and cost. When studying the properties of a protein, one should consider determining the pH optimum of the protein's activity in order to choose the best buffer. In order to determine the pH optimum, it is advisable to start with a series of related buffers that span a wide pH range. Once an optimal pH has been determined, different buffers within the same pH range can be examined for a specific buffer effect. Once an appropriate choice of buffer is made, it is best to work at the lowest reasonable concentration (around 50 mM) to avoid "non-specific" ionic strength effects. Buffers at 50 mM concentration are usually non-toxic to cells.[3] The buffer selection depends also on the separation methods employed. For example, in gel permeation chromatography, almost any buffer suitable for the protein of interest can be chosen. But for

TABLE 1.3
Limitations of Buffers Commonly Used in Extractions

Buffer	Advantage	Disadvantage
Phosphate	Compatible with gel permeation and cation-exchange chromatography Compatible with most cross-linking reagents Inexpensive	Weak buffering capacity in the pH 8–11 Precipitates in the presence of polyvalent cations Inhibits a wide variety of enzymes, including kinase, phosphatase, dehydrogenase Not suitable in anion-exchange chromatography
Tris	Suitable in gel permeation and anion-exchange chromatography Inexpensive	Poor buffer below pH 7 Passes through biological membrane Contains a reactive primary amine and thus forms Schiff base adduct with aldehyde and inhibits protein conjugation by amine-based cross-linkers Sensitive to temperature
Borate	Inexpensive	Forms complexes with the ribose moieties of nucleic acid, and other mono- and oligosaccharides
Citrate	Inexpensive	Binds to some proteins and forms metal complexes
Carbonate	Inexpensive	Limited solubility Since carbonate is in equilibrium with CO_2, studies should be carried out in a closed system
Good buffers (e.g., MES, MOPS, HEPES)	Relatively free of side effects Low UV absorbance Effect of temperature and ionic strength is minimum	Most Good buffers interfere with the Lowry protein assay All piperazine-based Good buffers (HEPES, PIPES, etc.) form radicals under various conditions and are thus not suitable for the systems where redox processes are being studied Expensive

anion-exchange chromatography, cationic buffers such as Tris (and for cation-exchange chromatography, anionic buffers such as phosphate) are preferred. However, these inorganic buffers do have some side effects. Phosphate buffers are shown to inhibit many enzymes including carboxypeptidase, urease, kinase, and dehydrogenase.[4] Tris and other primary amine buffers may form Schiff base adducts with aldehydes and ketones and inhibit protein conjugation by amine-based cross-linkers. Borate buffers may form covalent complexes with the ribose moieties of nucleic acids, and other mono- and oligosaccharides. However, the Good buffers (developed by Good et al.[5]) such as MES, PIPES, and MOPS have been shown to be relatively free of side effects. They have low UV absorbance and are minimally affected by temperature or ionic strength. Unlike inorganic buffers, the Good buffers are also useful with respect to low metal binding capabilities, retaining most metals essential to enzymatic activity.

1.1.3 PREPARATION OF BUFFERS

Once the selection of a suitable buffer is made, its pH may be adjusted directly at the temperature at which the buffer is to be used. However, before titrating a buffer solution, the pH meter must be calibrated and standardized at the working temperature (usually at 4°C). In practice, the buffer is usually prepared at room temperature and the pH adjusted so that it provides the correct pH after the solution is brought to the desired temperature. The pH of the working buffer should be tested after all the components (e.g., ethylenediamine tetraacetic acid [EDTA], dithiothreitol [DTT], Mg^{2+}) have been added, since the pH may change after such additions. Unless otherwise stated, the pH of a buffer is adjusted down with hydrochloric acid (HCl) and up with either NaOH or KOH. When both protonated and unprotonated forms of a buffer are readily available, one should mix solutions of the two forms at the same concentration until the desired pH is obtained. When the buffer requires the complete absence of metal ions, the pH of the buffer should be adjusted with tetramethyl ammonium hydroxide. Tris buffers should be avoided when a metal cofactor is required for protein activity or stability; for 2 mM Mn^{2+} in 100 mM Tris, 29% is found to be chelated by the buffer. Metal ion chelators such as EDTA are commonly used when it is necessary to limit metal effects.

   One can make a buffer of the desired pH simply by mixing components based on the available tables or calculations.[2] However, the pH of the final solution should be verified with a pH meter. Table 1.4 lists some common buffers, their pH range, and the components from which they can be made by mixing. Table 1.5 shows the preparation of a phosphate buffer of varying pH.

1.1.4 EFFECT OF TEMPERATURE ON pH

The carboxylic acid buffers (of low pK_a) are generally least sensitive to temperature, but the amine buffers (of high pK_a) have temperature-sensitive pK_a. For example, pK_a of Tris changes from 8.85 at 0°C to 8.06 at 25°C.[4] The effect of temperature

TABLE 1.4
Some Common Buffers with Wide Range of Buffering Capacity[a]

Buffer	pH Range	Reagents
Glycinate-HCl	2.6–3.6	Glycine, HCl
Citrate	3.0–6.2	Citric acid, sodium citrate
Acetate	3.6–5.6	Acetic acid, sodium acetate
Citrate-phosphate	2.6–7.0	Citric acid, sodium phosphate (dibasic)
Succinate	3.8–6.0	Succinic acid, NaOH
Cacodylate	5.0–7.4	Sodium cacodylate, NaOH
Phosphate	5.7–8.0	Monobasic sodium phosphate, dibasic sodium phosphate
Barbital	6.8–9.2	Sodium barbital, HCl
Tris	7.2–9.0	Tris (hydroxymethyl) aminomethane
Boric acid-borax	7.6–9.2	Boric acid, borax
Ammediol	7.8–10.0	2-Amino 2-methyl 1, 3 propanediol, HCl
Glycine-NaOH	8.6–10.6	Glycine, NaOH
Borax-NaOH	9.28–10.1	Borax, NaOH
Carbonate-bicarbonate	9.2–10.7	Sodium carbonate, sodium bicarbonate

[a] Adapted from Reference 2.

on pK_a values can be explained by the following thermodynamic equations as previously described (6):

$$d\ln K_a/dT = \Delta H^0/RT^2$$

or

$$dpK_a/dT = \Delta H^0/2.3RT^2$$

and

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT\ln K_a = 2.3\ RTpK_a$$

According to the above equations, ΔG^0 is large and positive for buffers of high pK_a. Considering the entropy of dissociation for these buffers, ΔH^0 also tends to be large and positive. Thus, the rate of change of pK_a with temperature is likely to be negative and larger for the higher pK_a values.

1.1.5 EFFECT OF CONCENTRATION ON pH

It is a common practice to prepare buffers as 10× to 100× stocks. This allows smaller storage volumes and further dilutes the bactericidal agents such as 0.02% sodium azide to an insignificant level before use. It is important to note that the dilution of the stock buffer solution may change the pH. For example, the pH of Tris decreases by 0.1 unit per tenfold dilution, while a tenfold dilution of 0.1 M phosphate buffer (pH 6.7) raises the pH to 6.9.[7] Table 1.6 shows the saturating solubilities of some buffers at 0°C.

TABLE 1.5
Preparation of Phosphate Buffer

Stock solutions:
x: 0.2 M of monobasic sodium phosphate (27.8 g of NaH_2PO_4 in 1 liter)
y: 0.2 M of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7 H_2O$ in 1 liter)

0.2 M buffer of varying pH can be obtained by mixing these two stock solutions as follows. But, it is advisable to verify the pH after mixing.

x	y	pH
93.5	6.5	5.7
92.0	8.0	5.8
90.0	10.0	5.9
87.7	12.3	6.0
85.0	15.0	6.1
81.5	18.5	6.2
77.5	22.5	6.3
73.5	26.5	6.4
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5
13.0	87.0	7.6
10.5	89.5	7.7
8.5	91.5	7.8
7.0	93.0	7.9
5.3	94.7	8.0

TABLE 1.6
Saturating Solubilities of Some Common Buffers

Buffer	Concentration (M)
MES	0.65
PIPES	2.3
MOPS	3.0
TES	2.6
HEPES	2.3
Tris	2.4
Phosphate	2.5

TABLE 1.7
Examples of Common Volatile Buffers[a]

Buffer	pH
87 ml glacial acetic acid + 25 ml of 88% formic acid in 1 liter	1.9
25 ml of 88% formic acid in 1 liter	2.1
5 ml pyridine + 100 ml glacial acetic acid in 1 liter	3.1
5 ml pyridine + 50 ml glacial acetic acid in 1 liter	3.5
25 ml pyridine + 25 ml glacial acetic acid in 1 liter	4.7
100 ml pyridine + 4 ml glacial acetic acid in 1 liter	6.5
Trimethylamine-formic acid	3.0–5.0
Trimethylamine-acetic acid	4.0–6.0
Triethylamine-formic (or acetic) acid	3.0–6.0
Triethanolamine-HCl	6.8–8.8
Ethanolamine-HCl	8.5–10.5
Ammonia-formic (or acetic) acid	7.0–10.0
24 g Ammonium bicarbonate in 1 liter	7.9
20 g Ammonium carbonate in 1 liter	8.9
Ammonium carbonate-ammonia	8.0–10.5

[a] From Reference 1.

1.1.6 PREVENTING BUFFER CONTAMINATION

In order to prevent bacterial or fungal growth, a buffer (a) can be filtered through a sterile ultrafiltration device; (b) can be mixed with 0.02% sodium azide; and (c) can be stored at 4°C. Microbial contamination is common to phosphate buffered saline (PBS), but this may be avoided at 1 M phosphate stock solution.[8]

1.1.7 VOLATILE BUFFERS

Volatile buffers are used in some applications when it is necessary to remove the buffer quickly and completely. Table 1.7 lists some volatile buffers. Volatile buffers are particularly useful in the digestion of proteins followed by separation of peptides or amino acids.[5] Most volatile buffers are compatible with ninhydrin.

1.2 USE OF PROTEASE INHIBITORS IN EXTRACTION

Proteolysis can be a major problem after extraction and at any stage of purification of the desired protein. It is a serious problem because, in addition to the complete inactivation of the desired protein, proteolysis may generate degraded proteins, partially retaining the biological activity. This results in erroneous conclusions about the nature of the protein (such as size and structure). Several classes of proteases are present in cells, and fortunately, various protease inhibitors are commercially available that can act on each class of protease. Some commonly used protease inhibitors and the recommended concentration are listed in Table 1.8. Phenylmethylsulfonyl fluoride (PMSF) is widely used to block serine proteases, probably due to its low cost. PMSF should be added at a regular interval to the protein extracts

TABLE 1.8
Common protease inhibitors

Inhibitors	Solubility	Recommended Stock	Concentration Used
Serine protease inhibitors			
Phenylmethylsulfonyl fluoride (PMSF)	2-propanol, ethanol	100 mM	0.1–1 mM
Benzamide	2-propanol, ethanol	100 mM	1 mM
Benzamidine-HCl	Water	100 mM	1 mM
ε-Amino n-caproic acid	Water	500 mM	5 mM
Aprotinin	Water	1 mg/ml	1–5 µg/ml
Cysteine (thiol) protease inhibitors			
Sodium p-hydroxymercuribenzoate (PHMB)	Water	100 mM	1 mM
Antipain	Water	1 mg/ml	1 µg/ml
Leupeptin	Water	1 mg/ml	1 µg/ml
Aspartate (acidic) protease inhibitors			
Pepstatin	Methanol	10 mg/ml	0.1 mg/ml
Diazoacetylnorleucine methyl ester (DAN)	Methanol	100 mM	5 mM
Metalloprotease inhibitor			
EGTA [ethylene glycol bis(β-aminoethyl ether) N, N, N′, N′-tetraacetic acid]	Water	500 mM	10 mM

and the following purification steps (if desired) because of its short half-life (about 100 min at pH 7).

It is advisable to use a mixture of inhibitors when working with a new protein extract. Recently, a mixture of inhibitors of appropriate concentration became available commercially (Roche Chemicals, Indianapolis, IN). Investigators should keep in mind that metal ions (such as Cu^{2+}), if used for acidic protease inhibitors, preclude the simultaneous use of chelators (such as EDTA and EGTA) that are required to inhibit metalloproteases. The use of aprotinin is incompatible with extraction buffers that contain a high amount of reducing agent. Aprotinin contains three disulfide bonds, and thus the reducing agent may inactivate the aprotinin. A mixture of benzamide and benzamidine may substitute for aprotinin, since the mixture is as active as aprotinin. Once the conditions for maintaining the desired protein in a stable form are established, selective protease inhibitors can be used.

1.3 USE OF DETERGENTS IN EXTRACTION

Detergents are generally added to the buffer for extraction and purification of membrane proteins, which are usually insoluble in an aqueous buffer. Detergents are a class of compounds characterized by their amphiphilic structure (both hydrophobic
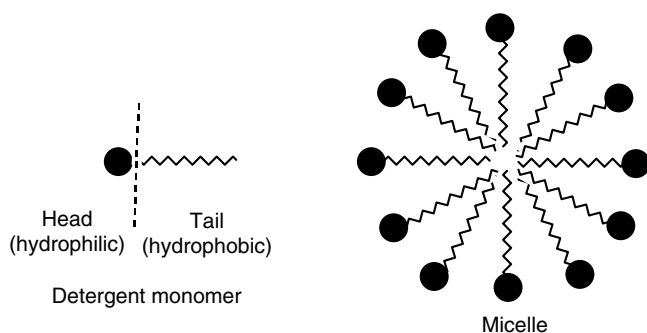
FIGURE 1.1 Schematic representation of a detergent monomer and a micelle.

and hydrophilic). The "tail" of the detergent molecule is hydrophobic, usually consisting of a linear or branched hydrocarbon, whereas the hydrophilic head may have diverse chemical structures (Figure 1.1).

1.3.1 Types and Properties of Detergents

Detergents are of three types: ionic, non-ionic, and zwitterionic (Figure 1.2 and Table 1.9). Ionic detergents contain head groups with either positive charges (cationic detergents) or negative charges (anionic detergents). Ionic detergents denature proteins; thus, these are not included in the extraction. Sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) are examples of anionic and cationic detergents, respectively.

Non-ionic detergents have uncharged hydrophilic head groups and have a polyoxyethylene polar region. These are generally used to isolate functional proteins, as they are far less denaturing than ionic detergents. Common examples of non-ionic detergents are Triton X-100 and Tween 20.

Zwitterionic detergents contain head groups with both positive and negative charges. These are bile salts or steroid-based detergents. These detergents are more effective than non-ionic detergents, as they prevent protein-protein interactions while causing less protein denaturation than ionic detergents. CHAPS is the most commonly used zwitterionic detergent.

An important property of detergents is their formation of "micelles." Micelles are clusters of detergent molecules in which the hydrophilic head portions face outward (see Figure 1.1). Solubilized membrane proteins form mixed micelles with detergents, which shield the hydrophobic (or transmembrane) domain of the protein from contact with the aqueous buffer. Critical micelle concentration (CMC) is one important parameter used to describe detergent behavior. The CMC is defined as the lowest detergent concentration at which micelles form (see Table 1.9). Several factors, such as pH, ionic strength, multivalent ions, and organic solvents, affect the CMC. Other parameters to describe the detergent behavior are the aggregation number (N), the critical micelle temperature (CMT), the cloud point (CP), and the hydrophile-lipophile balance (HLB) number.

Sodium dodecylsulfate (SDS)

$$H_3C-(CH_2)_{11}OSO_3^- \ Na^+$$

Sodium deoxycholate

Cetyltrimethylammonium
bromide (CTAB)

$$H_3C-(CH_2)_{15}-N-CH_3 \ Br^-$$

Triton X-100

$$(O-\underset{H_2}{C}-CH_2)_{9-10}-OH$$

Triton X-114

$$(O-\underset{H_2}{C}-CH_2)_{7-8}-OH$$

Octyl glucoside

Brij 35

$$O-(CH_2-\underset{H_2}{C}-O)_{23}-H$$

CHAPS

Zwittergent 3-14
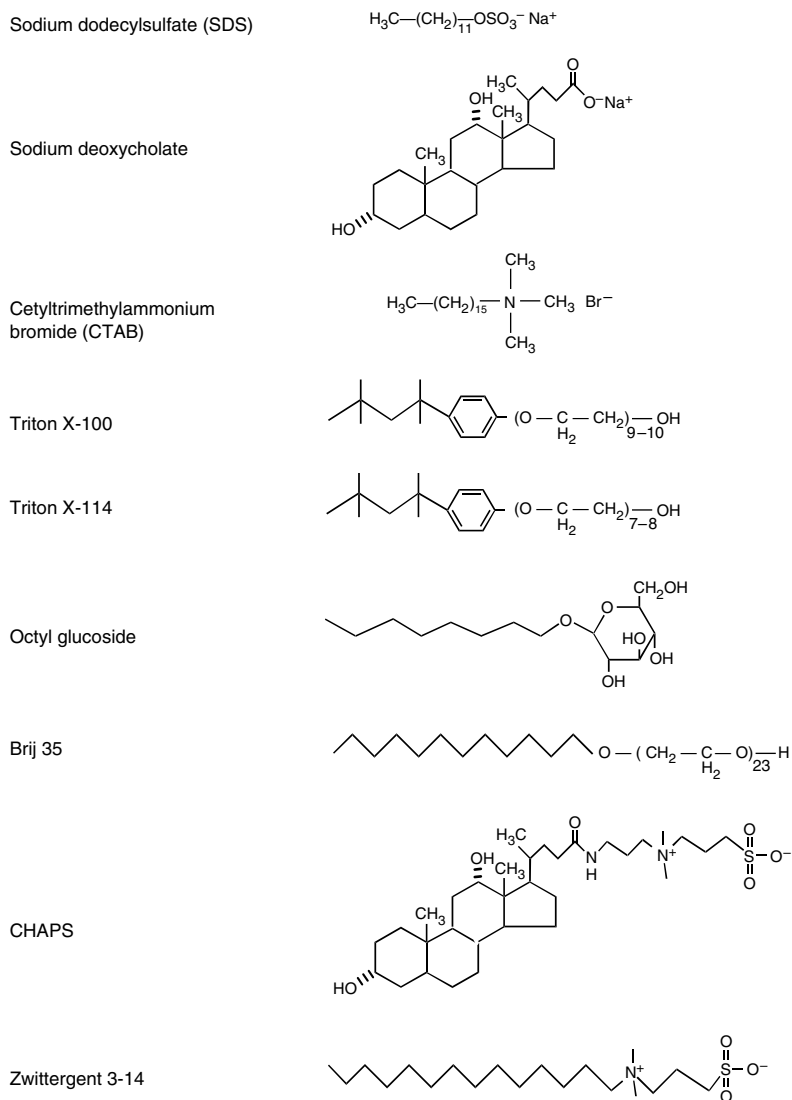
FIGURE 1.2 Structure of commonly used detergents.

The last two parameters are mostly applicable to non-ionic detergents. The aggregation number (N) is defined as the average number of detergent monomers in a micelle. At the CMT, detergent passes from a crystalline state to a micellar solution. CP is the temperature at which a cloudy solution clears upon cooling and defines when a detergent solution passes from a micellar system to a two-phase system. One phase is detergent depleted, and the other phase is detergent rich (rich in micelles). Triton X-114, because of its low cloud point (20°C), has been a popular detergent for solubilization of protein and lipid.[9] The HLB represents the overall

TABLE 1.9
Properties of Common Detergents[a]

Detergent	MW (Da)	CMC	N	HLB	Micelle MW (kDa)	Conc. for Solubilization	Specific Use
Ionic							
Sodium dodecyl sulfate	288.5	7–10 mM, 0.23%	60	40	18		Electrophoresis and electrofocusing Micellar chromatography
Sodium cholate	431	3–10 mM, 0.2%			1.8		Liposome preparation
Sodium deoxycholate	433	2–4 mM, 0.1%	20				Liposome preparation
Non-ionic							
Triton X-100	Approx. 628	0.3 mM, 0.02%	140	13.5	90	0.2–0.6 mg/mg protein	Selective solubilization of membranes Enzyme immunoassay
Triton X-114	Approx. 543	0.17 mM, 0.01%		12.4			Protein fractionation by phase separation Liposome preparation
Octylglucoside	292.4	15–25 mM, 0.5%			8	20–45 mM	Protein crystallization
Tween 20	1,230	50 μM, 0.006%	62	16.7	76		Enzyme immunoassay
Zwitterionic							
CHAPS	614.9	6.5 mM, 0.4%	10		6	6–10 mM	
Zwittergent 3-14	364	0.3 mM			30		In situ hybridization

[a] Taken from Reference 10.

hydrophilic properties of a detergent. Detergents with an HLB value higher than 7 are more soluble in an aqueous solvent than in an organic solvent. HLB values also indicate the nature of the detergent (denaturing or non-denaturing). Detergents with an HLB value between 12 and 16 are usually non-denaturing, while detergents with an HLB value above 20 are most likely to be denaturing. A detailed description of commonly used detergents is reported elsewhere.[10]

1.3.2 PRINCIPLE OF DETERGENT SOLUBILIZATION

The process of solubilization of membrane proteins depends on the amount of the detergent relative to membrane protein. Detergent extraction of a membrane protein occurs as follows: (a) binding of detergent to the membrane and initiation of lysis; (b) solubilization of the membrane in the form of detergent-lipid-protein complex; and (c) further solubilization of the detergent-lipid-protein complexes to give detergent-protein complexes and detergent-lipid complexes.[11,12] At a very low concentration of detergent, detergent monomers bind and partition into the membranes without a gross change in membrane structure. As the detergent concentration is increased to reach a critical concentration (approximately 1:10), the structure of the membrane is altered, leading to lysis (Figure 1.3). As the ratio of detergent concentration to membrane protein is progressively increased (approximately 1:1), small complexes of membrane lipid, membrane protein, and detergents are formed. At a high ratio of detergent to protein (10:1 to 20:1), individual detergent-protein complexes and detergent-lipid complexes are formed.

However, this is not always the case for all detergents. Highly bound lipids resist the solubilization process of the membrane protein. In practice, the best detergent and ideal conditions for solubilization are determined from trial and error. For each detergent tested, a series of detergent concentration (0.01 to 3%) is mixed with protein preparation. After incubation for a certain time, the mixture is then centrifuged at 105,000 g for 1 h.[13] The solubilized membrane protein is recovered in the supernatant and assayed for activity. The detergent that yields the highest specific and total activities for the desired protein should be chosen for further work.

1.3.3 CRITERIA FOR CHOOSING DETERGENTS

The choice of detergent for the solubilization of a membrane largely depends on the physical properties of the membrane proteins. It is important to consider how a detergent may affect native structures or activities of membrane proteins. Detergents that interfere with assays and are difficult to remove from the extract should not be considered.

1.3.3.1 General Consideration

The most important consideration in choosing an appropriate detergent is the non-denaturing property. Non-ionic detergents, such as Triton X-100 and octylglucoside, and zwitterionic detergents, such as CHAPS, are often used to solubilize membrane proteins.
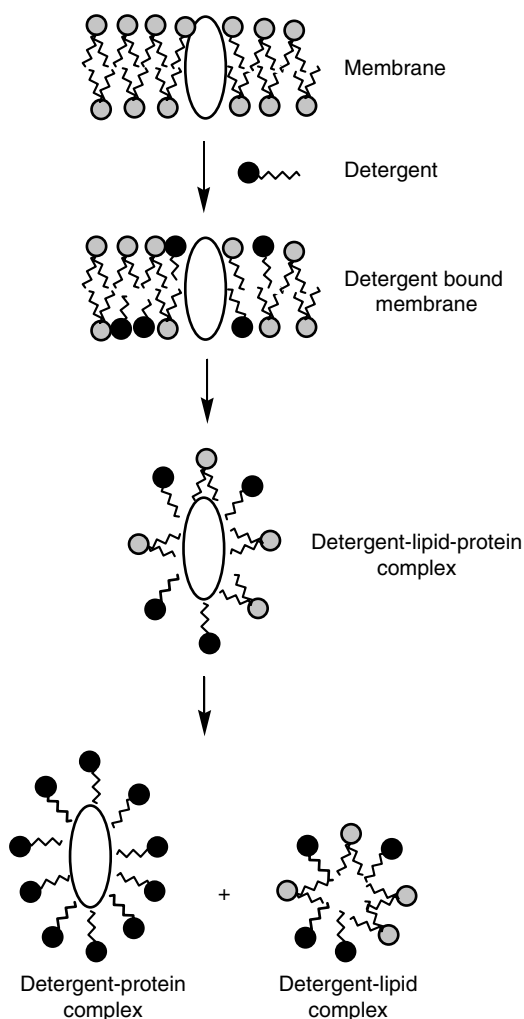
FIGURE 1.3 Schematic representation of membrane protein solubilization with detergents (adapted from Reference 12).

Spectral properties of the detergents are also considered, especially when the purification of the membrane protein is sought through a column chromatography, and UV monitoring of the fractions is desired. The detergents with aromatic groups such as Triton X-100 and Triton X-114 have substantial UV absorbance at 280 nm. Bile salts and their derivatives including CHAPS and CHAPSO do not have such interference.

Detergents with carboxylic acid such as N-lauryl sarcosinate, bile salts, and their carboxylic acid derivatives precipitate with divalent cations. Thus, one should consider avoiding this class of detergents in the sample when calcium or magnesium is

absolutely required in the purification or assay buffer. CHAPS and CHAPSO do not precipitate with divalent cations.

Ionic detergents interfere with charge-related procedures such as ion-exchange chromatography and isoelectric focusing. Thus, ionic detergents should be avoided if separation techniques based on charge difference are to be employed. If proteins are to be separated according to size by gel permeation chromatography, detergents with a smaller micelle size aggregation number (< 30) should be chosen. These detergents give better resolution, because size differences between protein containing micelles and non-protein containing micelles are greater.

1.3.3.2   Consideration of pH

Carboxylic acid-containing detergents, such as bile salts and N-lauryl sarcosinate, may not be suitable in some experiments related to separation techniques (isoelectric focusing, pH gradient elution from ion-exchange resin, chromatofocusing) where pH values vary. Detergents of this class are expected to protonate and become insoluble in aqueous media at weakly acid pH value. Zwitterionic detergents containing stronger acids such as sulfonic acid and sulfonate ester have pK values 0.2 and thus do not present such problems.

1.3.3.3   Consideration of Temperature

Detergents containing polyoxyethylene ethers such as Triton X-100 and Triton X-114 change micelle molecular weight with temperature. The micelle expands in an exponential fashion as the temperature increases. This process leads to a separation of detergent as a non-aqueous phase at a particular temperature, known as the cloud point. This temperature effect is advantageous for the extraction of membrane proteins but not in the electrophoretic techniques, such as isoelectric focusing, where heat is generated. Other non-ionic or zwitterionic detergents may be appropriate for such techniques.

1.3.3.4   Consideration of Electrical Properties

Unlike ionic detergents, both non-ionic and zwitterionic detergents do not move in electrical fields, don't bind to ion exchange resins, and do not contribute to the net charge of macromolecules to which they are bound. Thus, non-ionic or zwitterionic detergents should be used for charge-related separation techniques such as ion-exchange chromatography and preparative electrophoresis.

1.4   CHEMICAL LYSIS FOR PROTEIN EXTRACTION

Chemical lysis includes the treatment of cells with alkali, enzyme, or detergent. Chemical lysis methods minimize denaturation and expose the inner, cytoplasmic membrane by degrading the peptidoglycan cell wall of bacteria. The cell wall of Gram-positive bacteria is thick, containing several interconnecting layers of peptidoglycan (60 to 90% of the cell wall). In contrast, the cell wall of Gram-negative bacteria appears thin, containing two or three layers of peptidoglycan (10 to 20%

of the cell wall). In addition to this, Gram-negative bacteria contain an outer membrane composed of lipopolysaccharide, phospholipids, and lipoprotein. Lysozyme, a commercial lytic enzyme, is widely used to lyse Gram-positive cells in the presence of EDTA and detergent Brij 58. Lysozyme hydrolyzes N-acetylmuramide linkages, resulting in degradation of bacterial cell walls. The activity of lysozyme is optimal in the pH range of 6.7 to 8.6.

In contrast, gram-negative bacteria are less susceptible to lysozyme and detergents due to the presence of an asymmetric lipid bilayer. The outer membrane of the peptidoglycan acts as a permeability barrier to large molecules, and so the outer membrane needs to be permeabilized to expose the peptidoglycan layer for successful enzymatic lysis. The permeability barrier is, in part, due to the presence of polyanionic lipopolysaccharides (LPS) that provide a network interaction in the presence of divalent cations, such as Mg^{2+}. The chelators of divalent cations (e.g., EDTA), polycationic species, and small molecules (e.g., Tris) are suitable for permeabilizing the membrane in order to release LPS.

Working Procedure

Enzymatic Lysis of Gram-Positive Bacteria (e.g., Bacillus subtilis)

1. To the washed cells add 50 mM Tris-HCl (pH 7.6), 10% sucrose, 1 mM dithiothreitol to get 50% cell suspension.
2. To the cell suspension add lysozyme (300 μg/ml final conc.), EDTA (1 mM final conc.), and Brij 58 (0.1% final conc.).
3. Incubate on ice for 1 h.
4. Centrifuge for 30 min at 40,000 g and collect the supernatant by decanting. (**Note:** centrifugation is a technique that is applied to separate particles from a solution. Centrifugation procedures are of two types: preparative and analytical. In preparative centrifugation, specific particles are isolated. In analytical centrifugation, physical properties of a sedimenting particle are measured.
   a. Before centrifugation, tubes must be balanced across the central axis of the rotor. For example, in a six-place rotor, tubes 1 and 4, 2 and 5, and 3 and 6 should be of equal weight. Tubes are usually filled with the homogenate up to about 75% of their volume.
   b. During centrifugation, the particle sediments at a rate that is proportional to the centrifugal force. Sedimentation rate of the particle is also dependent on the viscosity of the sample solution and the physical properties of the particle. At a fixed centrifugal force and sample solution viscosity, the sedimentation rate of the particle is proportional to its molecular size and the difference between its density and the density of the solution. In the literature, the condition for centrifugation is quoted in terms of the relative centrifugal field [RCF], rather than as revolutions per minute [rpm].)

Enzymatic Lysis of Gram-Negative Bacteria (e.g., E. coli)

Reagents

1. Tris-sucrose buffer: 50 mM Tris-HCl (pH 7.5), 10% sucrose
2. Lysis solution: 0.3 M spermidine-HCl, 2 M NaCl, 10% sucrose, adjust to pH 7.5

Lysis Procedure

1. To the frozen *E. coli* cells, add prewarmed (at 37°C) Tris-sucrose-buffer (2.5 times of packed cell weight) and lysis solution (0.25 times of packed cell weight).
2. Once a thawed homogeneous mixture is achieved, add lysozyme (1 mg/g of packed cell). Incubate on ice for 1 h. Fresh solution of lysozyme should be used each time, as lysozyme activity is not stable to freezing.
3. Incubate the homogenate at 37°C water bath for about 4 min with gentle agitation.
4. Place the homogenate immediately on ice for a few minutes.
5. Centrifuge at 23,000 g for 1 h at 4°C. Decant and save the supernatant. (**Note:** the supernatant should yield 20 mg protein/ml if lysis is complete. If cells are not lysed completely, a more vigorous lysis is performed by adding EDTA [5 to 10 mM final conc.] to the Tris-sucrose buffer or by extending the lysis reaction up to 15 min.)

1.4.1 OSMOTIC SHOCK LYSIS

Cell lysis may be achieved by osmotic shock when suspended in a hypotonic solution (i.e., of a lower ionic strength than the cell cytoplasm). Cells that are not protected by cell walls are sensitive to osmotic shock. Red blood cells are an example of this type.

1.5 MECHANICAL LYSIS FOR PROTEIN EXTRACTION

Mechanical lysis means disruption of cells using sonication, a pressure cell, homogenizer, or bead beater. Mechanical lysis methods are economical and preferable for large-scale preparations because the addition of chemicals is not required. However, mechanical lysis produces heat, which needs to be controlled. Care should also be taken to avoid foaming, to prevent surface denaturation and oxidation. Mechanical lysis methods are two types: agitation and liquid shear methods.

Agitation with abrasives is commonly performed in bead mills (such as Dyno-Mill). Its chamber is filled with glass grinding beads, which rupture cells during rotation of beads. The suspension of broken cells exits the chamber, retaining the beads inside the chamber. The efficiency of cell lysis, however, depends on the rate

of agitation, cell concentration, concentration and diameter of glass beads, lysis time, and temperature.

Liquid shear lysis by French press or Manton-Gaulin press is the most common mechanical lysis method. Cells are lysed by high pressure (6,000 to 10,000 psi) followed by a sudden release of pressure. The release of pressure creates a liquid shear that causes lysis of cells. High-pressure operation raises temperature, and care should be taken to control it. For lysing small quantities of cells, sonication remains a convenient technique. It lyses cells by liquid shear and cavitations. To control heating during sonication, it is conducted in 30- to 40-sec pulses, with pauses for a few minutes on ice. Usually, mechanical lysis methods release nucleic acids that should be removed from the extract by phase partitioning or by treatment with RNAses or DNAses.

1.5.1 HOMOGENIZATION

Homogenization in a blender (Figure 1.4)[14] and in a Dounce are common and simple procedures for disrupting soft tissues such as liver, heart, brain, and muscle. These methods are rapid (5 to 10 min) and gentle to proteins. These homogenization procedures usually produce heat, and thus the blender and the associated container should be prechilled at 4°C. The homogenization should be performed in a cold room or on ice.

FIGURE 1.4 Blender.

Working Procedure

1. Cut tissue into small pieces with knife and transfer to an appropriate vessel according to the homogenization technique.
2. Add 5 volumes of buffer and homogenize:
   a. Using a blender, three pulses for 20 sec each at high speed, pausing for several seconds between pulses.
   b. Using a glass Teflon homogenizer at 500 to 1,500 rpm, 3 to 6 strokes, 5 to 10 seconds per stroke.
   c. Using a Dounce hand homogenizer, 10 to 20 passes.
3. Centrifuge at 23,000 g for 1 h at 4°C. Decant and save the supernatant.

1.5.2 SONICATION

Sonication (Figure 1.5) is most commonly used to disrupt various types of cells (prokaryotes and eukaryotes). Sonication creates vibrations that cause mechanical shearing of the cell wall. Sonication is performed at the highest allowable power setting, which is adjusted to a level slightly below that at which foaming occurs.

Working Procedure

1. Suspend tissue (small pieces) or cells in at least 2 volumes of buffer.
2. Sonicate at highest allowable setting for about 2 min in several cycles, pausing for 5 to 10 sec between cycles, to permit the sample to cool. Decrease the power setting if the solution starts to foam.
3. Centrifuge at 23,000 g for 1 h at 4°C. Decant and save the supernatant.

1.5.3 GRINDING WITH ALUMINA OR SAND

Grinding of cells such as *E. coli* with abrasive materials, such as sand or alumina, is a simple and inexpensive, yet effective, procedure for lysis of unicellular organisms (15). Cell lysis is performed by hand, using mortar and pestle.

Working Procedure

1. To a chilled mortar, add washed cells (up to 30 gram).
2. Add alumina or quartz sand (2 times cell weight).
3. Grind cells using the pestle.
4. Resuspend the cell paste in buffer (3 to 4 volumes of the original cell volume).
5. Centrifuge at 23,000 g for 1 h at 4°C to remove cell debris and sand or alumina. Decant and save the supernatant.
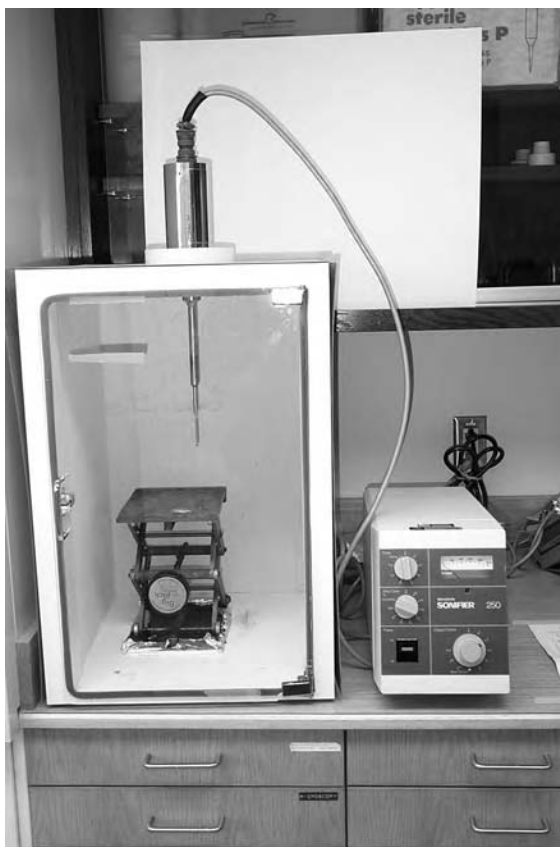
FIGURE 1.5 Sonicatore.

1.5.4 GRINDING WITH GLASS BEADS

Unicellular organisms, particularly yeasts, are extracted by vortexing with glass beads.[16] The abrasive action of the vortexed beads shears cell walls, releasing the cytoplasmic proteins. Small samples (up to 3 g) are usually carried out in a test tube. For larger samples, specialized apparatus such as Braun MSK Glass Bead Mill, Biospec Product Bead Beater, and Manton-Gaulin homogenizer are used. Glass beads are cleaned with concentrated HCl and extensively washed with water (monitored with pH paper).

Working Procedure

1. To washed cells (0.1 to 3 gram) in a polystyrene tube, add equal volume of lysis buffer.

FIGURE 1.6 French Press.

2. Add 1 to 3 gram of chilled glass beads per gram of cell weight. Vortex 3 to 5 times for 1 min, allowing 5 to 10 sec to cool between vortexing.
3. Centrifuge at 23,000 g for 1 h at 4°C. Decant and save the supernatant.

1.5.5 DISRUPTION USING FRENCH PRESS

In French press (Figure 1.6), cells are lysed at very high pressure, followed by a sudden release to atmospheric pressure. This rapid change in pressure causes cells to burst. The appropriate volume of extraction in a French press is about 10 to 30 ml. Extraction of a large volume of sample is time consuming, as the homogenate is passed through high pressure two to three times. On the other hand, extraction of a very small volume of sample becomes technically difficult. The cell of the French press should be thoroughly cleaned before and after use to prevent sample-to-sample contamination.

Working Procedure

1. Suspend the washed cells in lysis buffer in the ratio of packed cell volume to buffer volume 1:4.
2. Add cell suspension to the French press cell and apply pressure (usually 8,000 to 20,000 pounds per sq inch or 550 to 1,400 kg/cm^2). For *E. coli* the pressure used is in the range of 7,000 to 10,000 psi.
3. Adjust the outlet flow rate to about 2 to 3 ml per minute, while maintaining the pressure.

4. For more complete lysis, two to three passes (step 2) may be performed.
5. Centrifuge at 23,000 g for 1 h at 4°C. Decant and save the supernatant.

1.6 PREPARATION OF EXTRACTS FROM PROKARYOTES

As discussed in Sections 1.4 and 1.5, prokaryotes can be extracted by a variety of methods such as enzymatic and mechanical lysis. Enzymatic lysis usually yields a lysate free of chromosomal DNA. This is because lytic enzymes can create holes that are large enough to escape proteins only. But most extraction methods related to mechanical lysis cause a release of nucleic acids, which should be removed from the lysate because of viscosity problems and interference with subsequent chromatographic steps. The most common method for removal of RNA and DNA is the treatment of the lysate with protease-free RNAses and DNAses.

1.7 EXTRACTION OF RECOMBINANT PROTEIN FROM BACTERIA

Molecular cloning techniques allow high levels of expression of heterologous proteins in bacteria such as *E. coli.* They are grown in large scale to obtain the desired amount of protein. In some cases, bacteria secrete recombinant proteins into the media and thus eliminate the need to lysis the cells. But in most cases, lysis of the bacteria is required to extract the recombinant protein product. Lysozyme is generally used to cleave the glucosidic linkage of the polysaccharide present in the bacterial cell wall. Detergents, osmotic pressure, or other mechanical methods are then employed to disrupt the inner cytoplasmic membrane to release soluble recombinant proteins.

Unfortunately, over-expression of recombinant proteins often results in the formation of insoluble protein aggregates known as inclusion bodies.[17,18] Inclusion bodies are dense and granular structures distributed throughout the cytoplasm. As they form insoluble aggregates, they require further extraction for protein isolation. Inclusion bodies are usually formed from non-bacterial proteins. However, native bacterial proteins also aggregate when they are over-expressed at very high cellular levels.[19]

A number of strategies are available to minimize the formation of inclusion bodies by manipulating growth temperature, media composition, and host strain or by expression of fusion proteins.[20]

1.7.1 SOLUBILIZATION OF INCLUSION BODIES

The mechanism for inclusion body formation is unknown. However, the favorite theory for forming inclusion bodies is linked to the protein-folding pathway.[21,22] Other theories, such as sulfhydryl mispairing or expression of a protein content higher than the native solubility limit, do not seem to be convincing.[22,23]

A nascent polypeptide chain may undergo several intermediate conformational changes until it finds a correct native three-dimensional structure. When recombinant protein is expressed at very high concentration in the cytoplasm, intermediate folding conformations of protein may associate with each other faster than they can refold into their native conformations, leading to their precipitation into insoluble inclusion bodies.

Inclusion bodies offer some advantages over soluble recombinant protein. They contain high levels of protein expression and can be easily separated from the rest of the bacterial cytoplasmic proteins by centrifugation, facilitating purification of the recombinant protein. The major disadvantage of inclusion bodies is that the extraction of protein is generally performed in the presence of denaturing agents. This is problematic, since 100% of refolding is usually difficult to achieve. However, some inclusion bodies can be solubilized by altering pH and temperature,[24] but strong denaturing agents are required in most cases. Urea (8 M) and guanidine hydrochloride (6 M), water-soluble chaotropic agents, are commonly used to solubilize inclusion bodies. They are compatible with protein folding and most chromatographic procedures.[25,26] Refolding of the denatured protein is commonly achieved in the presence of thiol reagents and by dilution or dialysis to gradually reduce the concentration of urea and guanidine hydrochloride. Renaturation is sometimes facilitated by the addition of cofactors, ligands, or substrates that may stabilize the folding intermediates. On the other hand, SDS is also an effective solubilizing agent but difficult to remove from most proteins, and it can interfere with the subsequent refolding. SDS is also incompatible with ion-exchange chromatography. However, proteins used for antibody development generally do not require refolding unless antibodies to a tertiary structure are required.

Working Procedure

Reagents

1. Proteolytic inhibitor: prepare a stock solution of 100 mM PMSF in isopropanol and store at -20°C until use. Add PMSF to lysis buffer (final concentration of 1 mM) just before extraction.
2. Lysis buffer: prepare 50 mM Tris-HCl (pH 8) containing 1 mM EDTA, 50 mM NaCl, and 1 mM PMSF.
3. Lysozyme: freshly prepare 10 mg/ml of hen egg lysozyme.
4. DNAse I: prepare 1 mg/ml stock solution.
5. Sodium deoxycholate: prepare 20 mg/ml stock solution.
6. Solubilizing buffer: prepare 50 mM Tris-HCl (pH 8.0) containing 8 M urea and 1 mM DTT.
   (**Note:** urea solution should be prepared fresh. Previously made urea solution may form cyanate ions, which react with protein amino groups, resulting in carbamylated derivatives.)

Cell Lysis

1. Centrifuge bacterial cells at 1,000 g for 15 min at 4°C. Remove the supernatant and resuspend the cell pellet with lysis buffer (3 ml/g cell pellet).
2. Add lysozyme (final concentration of 300 μg/ml) and stir the suspension (30 min, 4°C). While stirring, add deoxycholate (final concentration of 1 mg/ml).
3. Place the suspension at room temperature and add DNAse I (final concentration of 10 μg/ml) and MgCl$_2$ (final concentration of 10 mM). Continue stirring for 15 min to remove the viscous nucleic acid.
4. Centrifuge at 10,000 g for 15 min at 4°C and collect the supernatant.
5. Resuspend the pellet in lysis buffer, as in step 1.
6. Analyze aliquots of both supernatant (step 4) and pellet suspension (step 5) on SDS-PAGE to determine if inclusion bodies are formed during the recombinant protein expression.

Solubilization of Inclusion Bodies

1. Prior to solubilization, inclusion bodies should be washed to remove contaminating proteins. For this purpose, resuspend the pellet in 9 volumes of lysis buffer containing 0.5% Triton X-100. After incubation at room temperature for 5 to 10 min, centrifuge at 12,000 g at 4°C. Save both supernatant and cell pellet. For effective washing, urea (1 to 4 M) may be added in the above washing buffer. All supernatant samples should be analyzed for the protein of interest on SDS-PAGE. The best washing buffer will be the one that strips most contaminating proteins, but little or none of the protein of interest.
2. Suspend the pellet in lysis buffer containing 8 M urea, 2 mM reduced glutathione, and 0.2 mM oxidized glutathione (9 ml/g pellet). Incubate the suspension at room temperature for 1 to 2 h. The mixture of reduced and oxidized glutathione accelerates refolding (renaturation) of the protein by formation of the correct pairing of disulfides.[24]
3. Centrifuge the suspension at 20,000 g for 30 min at 4°C to remove any remaining insoluble material. Save the supernatant. Renaturation of the protein of interest in the supernatant may be continued at room temperature for additional 1 to 4 h in the presence of the mixed glutathiones as described above. The pellet should be analyzed on SDS-PAGE for the presence of any remaining protein of interest. If the pellet still contains a substantial amount of the protein of interest, then increase the incubation time with the solubilization buffer or try a different solubilizing agent.

1.8 PREPARATION OF EXTRACTS FROM YEAST

Yeast cells can be lysed by both chemical and mechanical means.[27] The yeast cell wall can be digested with a variety of enzymes, such as zymolyase, lyticase, and β-glucuronidase. The yeast cell wall contains the carbohydrate glucan, mannoprotein, glycoprotein, and small amounts of chitin. Enzymatic reactions are usually carried out at room temperature to 37°C in the presence of sulfhydryl reagents in order to enhance the lysis. Lysis can be carried out directly, but in most instances spheroplasts are prepared as an intermediate step with these enzymes. Spheroplasts are then lysed in a variety of ways, such as detergent extraction, homogenization using glass beads, or French press. A protocol for yeast extract by isolating spheroplasts as an intermediate is described below.[27]

Working Procedure

Reagents

1. Cell suspension buffer: 1 M sorbitol-50 mM Tris-HCl (pH 7.5)
2. Sulfhydryl reagent: dithiothreitol
3. Lytic enzyme: zymolyase (5 mg/ml in 10% sucrose)

Procedure

1. Harvest yeast cells by centrifugation (3,000 g) at 4°C for 5 min.
2. Resuspend the cell pellet in 1 M sorbitol-50 mM Tris-HCl (pH 7.5) buffer containing 30 mM dithiothreitol (10^9 cells/ml). Incubate for 10 min at 30°C.
3. Collect cells by centrifugation as above and resuspend in an equal volume of the same buffer containing 2 mM dithiothreitol.
4. To the suspension, add zymolyase to a final concentration of 0.5 mg/ml. Incubate at 30°C for 30 min. Monitor spheroplasting by phase contrast microscopy. (The spheroplasts are small, round, bright, and refractile bodies, which burst within a few minutes if diluted in water. Over 95% of the cells yield spheroplasts.)
5. Collect spheroplasts by centrifugation at 1,000 g for 5 min at 4°C. Centrifugation at higher speed results in rupture of spheroplasts.
6. Wash spheroplasts twice with 4 volumes of the suspension buffer, as in step 3, to remove the zymolyase.
7. Resuspend the final spheroplast pellet in 50 mM Tris-HCl (pH 7.5)-2 mM dithiothreitol at 5×10^9 spheroplasts/ml. Spheroplasts are lysed in 1 mM EDTA (pH 8.0)/0.2% Triton X-100/0.2 M KCl with gentle mixing.
8. Centrifuge the lysate at 100,000 g for 1 h at 4°C and collect the supernatant.

1.9   PREPARATION OF EXTRACTS FROM EUKARYOTES

Like prokaryotes, several eukaryotes can be extracted by a variety of pressure cells, such as the French press, Eaton press, and Gaulin homogenizer. Extraction of eukaryotes by abrasive action of glass beads remains a very effective method for small to large scale. The percentage of cell breakage depends on the speed of agitation. For a small volume, agitation of glass beads with the use of a vortex mixer is a convenient method of extraction. Sonication with glass beads is also effective. In all cases, beads are separated by centrifugation or after decanting the supernatant.

Prior to extraction, solid tissues are cut into small pieces and homogenized with a homogenizer. The choice of extraction buffer often dictates by the nature of protein that needs to be analyzed. Proteins whose activities are sensitive to oxidation require extraction in the presence of a reducing agent such as dithiothreitol or mercapto-ethanol. For the isolation of some proteins, such as lectins, tissues are often extracted in the presence of their competing ligands for maximum yield. Chelating agents such as EDTA and EGTA are used in the buffer, because they protect enzymes from inactivation by heavy metals and from proteolysis by metalloproteases. Protease inhibitors are widely used in the extraction buffer to inhibit or suppress endogenous protease activities.

1.9.1   Subcellular Fractionation of Animal Tissues

Subcellular fractionation by centrifugation is a widely used method for separating cellular components. Subcellular fractionation is performed in three steps: (a) tissue or cell suspension into a homogenate, (b) separation of the components based on density or sedimentation coefficient, and (c) analysis of the isolated fractions.

Tissue or cell suspension is accomplished by any procedure previously described such as by grinding, by sonication, and by using osmotic properties. Suspension from soft tissues such as liver and kidney is usually achieved with the homogenizer (Figure 1.7). Mincer and a blender such as a Waring blender is used for tough tissues such as muscle. Tissue or cell disruption by osmotic methods is applied mostly on red blood cells and reticulocytes. Sonication is generally applied to disrupt bacterial cells and some animal cells. Suspension thus obtained contains whole cells, broken cells, nuclei, mitochondria, endoplasmic reticulum, etc. Fractionation of these constituents can be achieved by exploiting differences of their physical and chemical properties. Although alternative methods, such as electrophoresis, can be employed for this purpose, centrifugation is the most widely used, probably due to easy recovery of the sample at the end of the fractionation procedure. Fractionation of liver (rat) homogenate using differential centrifugation is described below.

Working Procedure

Reagent

Homogenization Buffer

0.2 M sucrose/5 mM imidazole-HCl, pH 7.4

FIGURE 1.7 Homogenizer.

Homogenization Methods

1. Remove the liver from a rat and wash with saline to remove blood and hair.
2. Mince the liver finely and add ice-cold homogenization buffer (0.2 M sucrose/5 mM imidazole-HCl, pH 7.4) to get 0.25% (w/v).
3. Homogenize using a Potter-Elvejhem homogenizer (six passes) and dilute the extract to 12.5% (w/v) with homogenization buffer.

Fractionation

1. Centrifuge the homogenate at 4°C at 600 g for 10 min. Collect supernatant and keep at 4°C. Pellet contains all nuclei, whole cells, partially intact cells, and plasma membrane sheets (Figure 1.8).
2. Resuspend the pellet in the same volume of homogenization buffer as previously used and centrifuge again at the same speed for additional 10 min. Collect the supernatant and combine with the first supernatant.
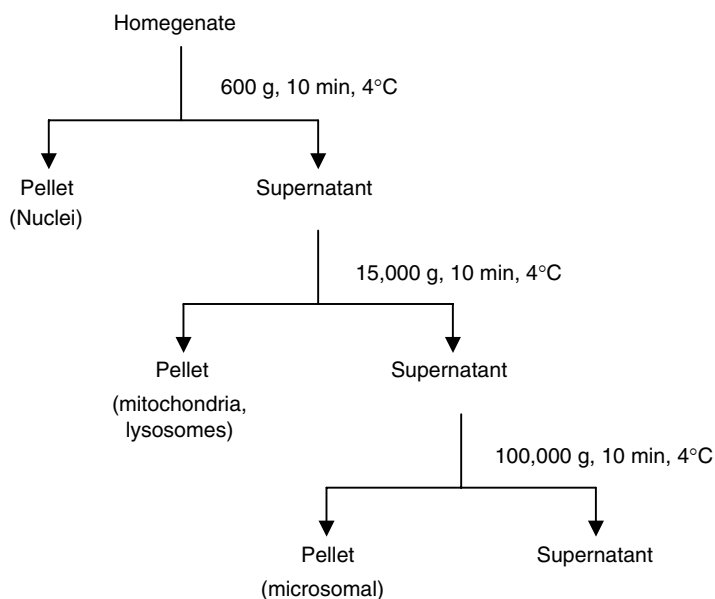
FIGURE 1.8 Subcellular fractionation.

3. Centrifuge the supernatant at 15,000 g for 10 min. Pellet contains mito-
   chondria and lysosomes. Collect supernatant for the next fractionation.
4. Resuspend the pellet in the same volume of homogenization buffer and
   centrifuge at 15,000 g for additional 10 min. Collect the supernatant and
   combine with the previous one (step 3).
5. Centrifuge the pooled supernatants at 100,000 g for 1 h at 4°C in an
   ultracentrifuge. The microsomal pellet contains vesicles derived from
   plasma membrane, endoplasmic reticulum, peroxisomes, endosomes,
   Golgi, stacks, and other membranous systems. The supernatant contains
   the remainder of the cellular components, i.e., the soluble components
   and smaller elements, such as free ribosomes.
6. Resuspend each of the pellets with a small volume of homogenization
   buffer and store at -20°C until use.

1.10 PREPARATION OF EXTRACTS FROM PLANTS

Plant cells possess a rigid cell wall and require a great deal of strategy for extraction.
Besides cytoplasm, plant cells contain four organelles: nucleus, plastid, mitochon-
drion, and vacuole. Vacuole, which occupies 90% of the cell volume, serves the
functions of both a vessel and a lysosome. Vacuoles contain alkaloids and various
hydrolases including proteases. Therefore, protease inhibitors should be used during
preparation of plant extract. The chloroplast is the second major organelle in the
green tissues. Procedures are available for the isolation of chloroplasts, mitochondria,
and nuclei from plant cells.[28]

1.11 PREPARATION OF MEMBRANE EXTRACTS

In order to isolate membrane proteins, the first step is to remove contaminating cytosolic proteins. Soluble cytoplasmic proteins are extracted by cell disruption in a neutral, isotonic, and detergent-free buffer and are removed after centrifugation. Membrane proteins remain associated with the insoluble components of the cell extract. In the second step of isolating membrane proteins, a highly purified membrane fraction is obtained. The starting material is further enriched, if the target protein is known to be associated with a specific subcellular membrane fraction such as plasma membrane, mitochondria, or nucleus.

Membrane proteins are classified into peripheral and integral membrane proteins. Peripheral membrane proteins are non-covalently bound to the membrane through the hydrophilic regions of integral membrane proteins or through the head group region of the lipid bilayer (Figure 1.9). Human erythrocyte glyceraldehyde-3-phosphate dehydrogenase,[29] cow heart mitochondria NADH dehydrogenase I,[30] and human erythrocyte peripheral proteins[31] are examples of peripheral membrane proteins. Peripheral membrane proteins are usually separated from the membrane by procedures used to dissociate soluble protein-protein interactions without total membrane disruption. Common dissociating reagents are high salt (1 M NaCl), chaotropic ions such as I$^-$, ClO4$^-$, SCN$^-$, and denaturing agents such as 8 M urea and 6 M guanidine hydrochloride.[29–31] In several cases, high pH favors the solubilization of peripheral membrane proteins. But, in most cases high pH also results in a loss of activity of many enzymes.[32] High salts decrease electrostatic interactions between proteins and charged lipids. Chaotropic ions disrupt hydrophobic bonds present in the membrane surface and promote the transfer of hydrophobic groups from non-polar environment to the aqueous phase. At low concentration, chaotropic ions cause selective solubilization, but at higher concentration they promote protein inactivation.

Integral or intrinsic membrane proteins interact with the hydrophobic moieties of the phospholipid bilayer (see Figure 1.9). Integral membrane proteins from various sources have one or more characteristic runs of apolar amino acids that span the lipid bilayer. Integral membrane proteins are further classified as type I, type II, etc., membrane proteins. In a type I membrane protein, the COOH terminus is embedded in the cytosol, while a type II membrane protein contains the NH$_2$- terminus in the cytosol. The domain of the membrane protein that is embedded in the membrane in contact with the hydrophilic lipid tail is composed of mostly hydrophobic amino acids. In order to solubilize integral membrane proteins, it is necessary to disrupt the lipid bilayer. Solubilization of the integral membrane protein may be accomplished by extracting the membrane with organic solvents[33] but is most commonly accomplished by treating them with detergents.

Working Procedure

Extraction with butanol

Reagent
N-butanol
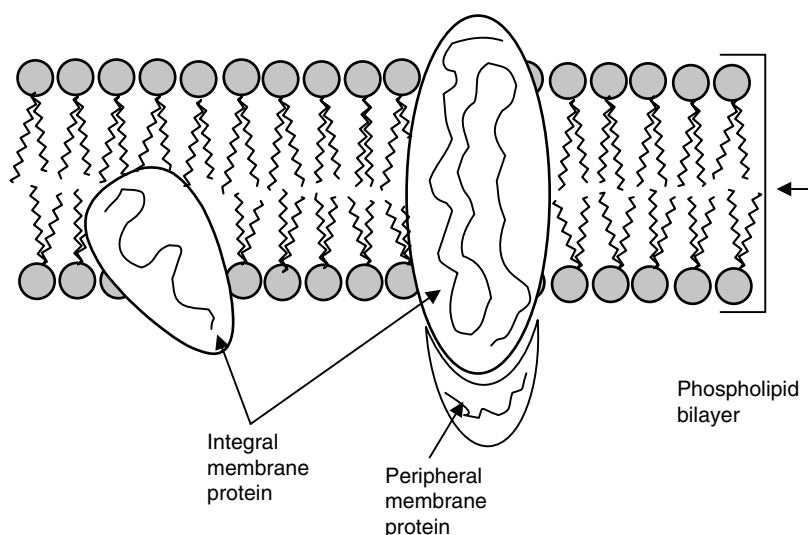
FIGURE 1.9 Schematic representation of integral and peripheral membrane proteins.

Procedure

1. To membrane particle suspension add equal volume of n-butanol. Mix and maintain the temperature at 4°C.
2. Centrifuge at 500 g for 10 min. This results in a two-phase separation. Upper phase consists of butanol and membrane lipids, and the lower aqueous phase contains solubilized proteins.
3. Collect the upper and the lower aqueous phase separately.
4. Dialyze the aqueous phase against a large volume of water or suitable buffer.

Extraction with Triton X-114

The low cloud point (20°C) of Triton X-114 has been exploited to solubilize membrane proteins. At cloud point, a microscopic phase separation most likely occurs due to secondary association of small micelles into large micelle aggregates. At higher temperature, it separates two phases, a detergent-depleted and detergent-enriched phase. Thus, integral membrane proteins that partition into the detergent phases can be separated from hydrophilic proteins.[9]

Reagents

1. Preparation of Triton X-114:
   a. Add 2 ml of Triton X-114 to 100 ml of cold phosphate buffered saline in a graduated cylinder.
   b. Mix until the suspension is homogeneous and let stand at 37°C overnight to separate into two layers.

     c. Aspirate off the detergent-depleted upper layer (80 to 90 ml) and discard.

     d. Adjust the detergent-rich lower layer to 100 ml with PBS and mix well. Let stand at 37°C overnight.

     e. Aspirate off the upper layer as before and save the detergent-rich lower layer. This is the stock solution of Triton X-114, 11.4%.

2. Triton X-114 lysis buffer: Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM EDTA)

3. Sucrose cushion buffer: 6% sucrose, 0.06% Triton X-114 in TBS.

Procedure

1. Prepare a cell lysate (5×10^6 cells) with 1 ml of Triton X-114 lysis buffer and agitate gently for 1 h at 4°C.

2. Centrifuge the cell lysate at maximum speed for 10 min in a microfuge at 4°C and collect the supernatant.

3. Load the supernatant directly onto 100 µl of sucrose cushion buffer. Incubate the tube at 37°C for 3 min until the contents of the tube became cloudy.

4. Centrifuge at 400 g at 37°C to separate the contents into two phases: aqueous on top and detergent at the bottom.

5. Transfer the aqueous phase to a fresh tube and incubate on ice. This forms a detergent-rich oily drop in the bottom of the tube (volume should be 50 to 100 µl).

6. Resuspend the detergent phase with 500 µl of cold TBS and continue incubation on ice.

7. Repeat steps 3 to 6, pool the second detergent-depleted aqueous phase with the first, and incubate on ice.

8. Resuspend the detergent-rich fraction in 2 ml of cold TBS and incubate on ice. This detergent-rich fraction contains integral membrane proteins.

9. Reextract the pool of detergent-depleted aqueous phase by adding 50 µl of 11.4% Triton X-114 stock solution as prepared above. Mix, incubate at 37°C for 3 min, and centrifuge at 400 g for 5 min at 37°C. Collect the aqueous phase, which contains proteins. Discard the oily pellet.

1.11.1 REMOVAL OF DETERGENTS FROM MEMBRANE PROTEINS

One should keep in mind that most membrane proteins precipitate upon complete removal of detergent. However, during the course of the purification of a membrane protein, it may be necessary to remove excess detergent or exchange detergent prior to separation by certain charge-based related techniques such as ion-exchange chromatography and isoelectric focusing. As the initial extraction is carried out with a high amount of detergent in order to give maximal dispersion of membrane proteins and lipids, removal of excess detergent at this stage usually facilitates the subsequent purification procedure. The detergent exchange is best achieved by absorbing of the detergent-membrane protein complex to a chromatographic support followed by extensive washing with a buffer containing the new detergent (Table 1.10).

TABLE 1.10
Various Reagents and Matrices Used for Removal of Detergents[a]

Reagent/Matrix	Principle	Remarks
Sephadex G-25	Gel permeation chromatography	Protein is collected in the void volume.
Dowex 1-X2	Ion-exchange chromatography	Protein-detergent solution is passed through the column in the presence of 6–8 M urea. Detergent is retained in the column. Solution of protein and urea is collected in the flow through. Urea is removed by dialysis.
Ligand immobilized Sepharose	Affinity chromatography	Protein must be active to bind to ligand.
Bio-Beads SM2 (Bio-Rad) Calbiosorb (Calbiochem) Extracti-Gel D (Pierce)	Hydrophobic interaction chromatography	Detergent is retained in the matrix.
Acetone	Precipitation	Detergent (e.g., SDS) remains in solution.
Buffer or water to dilute	Dialysis	Suitable for detergents with low micelle size and high CMC
High MW cut off membrane	Ultrafiltration	Solution is first diluted below detergent CMC
Sucrose	Density centrifugation	Separation is based on the size difference between the protein and the micelle.
Toluene/water PEG/water	Phase partition	Protein is precipitated from the micellar solution.

[a] From Reference 12.

Removal of excess detergent or exchange of detergent is also possible by dialysis. However, the effectiveness of dialysis depends on the CMC and micelle molecular weight (mMW). Most non-ionic and ionic detergents with linear alkyl hydrophobic groups have a high mMW value and do not pass through dialysis membranes. An example of this category is Triton X-100. Detergents with high CMC and a low mMW are removed by dialysis. Bile acids and their derivatives and octylglucoside are examples of such detergents.

Procedures for Removing Detergents from Protein Solutions

1. Remove detergents on a desalting column (such as Sephadex G-25).
2. Remove ionic detergent on an ion-exchange column. Run protein-detergent solution through an ion-exchange column in the presence of 8 M urea. Collect detergent-free protein solution in the flow through and dialyze to remove the urea.
3. Dilute the detergent-protein solution and dialyze to remove detergent when using detergent with low micelle size and high CMC.

4. Add Bio-Beads SM-2 (Bio-Rad) to the protein solution containing non-ionic detergent and stir gently. Bio-Beads have high surface area capable of absorbing organics with MW < 2,000 from aqueous solution.
5. Remove detergents through Extracti-Gel[R] D (Pierce). Extracti-Gel is an affinity matrix designed to trap detergent molecules.

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2 Estimation of Protein

Measurement of total protein is essential to monitor the progress of the purification of a desired protein. Total protein is typically measured in the supernatant following extraction and clarification by centrifugation. Various methods are employed for the estimation of protein, but none is free from shortcomings. A major disadvantage for most protein assays is the significant amount of variation among proteins (Table 2.1). Error is even greater if the protein contains non-proteinaceous groups such as carbohydrates. Dry weight determination gives a value for the whole molecule, but is not advantageous when one is working with sub-milligram-size samples.

The most accurate method for protein analysis is hydrolysis of the protein and determination of total amino acid residue weight following amino acid analysis. But this is a time-consuming process and not practical for day-to-day operation. The protein assay should be rapid and sensitive. Several sensitive and rapid methods are available, but the appropriate choice of method largely depends on the nature of the reagents present in the protein sample. Other criteria such as the amount of protein available to assay, the specificity, the reliability of the assay, and the ease of performing the assay are also considered in choosing the estimation procedure.

2.1   ULTRAVIOLET ABSORPTION METHODS

In ultraviolet (UV) absorption methods, proteins are measured directly without the addition of any reagents.[1] Proteins have two absorption maxima: 280 nm and 200 nm (Figure 2.1). In absorption spectroscopy, an electron absorbs photons. Photons with lower energy levels have longer wavelengths, and thus electrons that are excited at 280 nm have absorbed less energy than those at 200 nm. Electrons that are excited at 280 nm require less energy because they lie within the aromatic rings, which stabilize the excited state due to resonance.

Absorbance at 280 nm (A_{280})

The quantitation of protein by this method can only be applied to pure protein. Nonetheless, absorbance is widely used for monitoring purification progress and for generating a protein elution profile during column chromatography. The elution profile provides a guide for pooling fractions containing proteins. For A_{280}, the amino acids containing aromatic rings, such as tryptophan, tyrosine, phenylamine, and histidine, are involved.

TABLE 2.1
Advantages and Disadvantages of Some Common Methods for Protein Determination

Method	Range of Sensitivity	Minimum Vol./Amount	Destructive	Time of Assay	Protein-to-Protein Variation	Comments
A_{280}	0.2–2 mg/ml	100 µl/20 µg	No	Instant	Large	Interference by UV absorbing materials
A_{205}	10–50 µg/ml	100 µl/1 µg	No	Instant	Low	Interference by UV absorbing materials
Biuret	1–5 mg/ml	2 ml/2 mg	Yes	1 h	Low	Rapid color; interference by caustic reagents, NH_4^+
Lowry	0.01–1 mg/ml	0.5 ml/5 µg	Yes	40 min	Moderate	Many interfering substances; slow color development
BCA	10–1200 µg/ml (standard) 0.5–10 µg/ml (micro)	0.5 ml/250 ng	Yes	40 min – 2 h	Moderate	Warming required, interference by reducing agents
Bradford	25–200 µg/ml (standard) 5–40 µg/ml (micro)	100 µl/0.5 µg	Yes	10 min	Moderate	Interference by detergents and strong bases
Colloidal gold	20–200 ng 1–10 ng (Modified assay)	10 µl/1 ng	Yes	30 min	Moderate	Assay pH should be acidic (around pH 3)
Ninhydrin	0.1–1 mg/ml	10 µl/1 µg	Yes	>24 h	Low	Protein must be hydrolyzed
Fluorescamine	0.5–50 µg/ml (standard) 50 ng–25 µg/ml (micro)	100 µl/5 ng	Yes	<10 min	Moderate	Not compatible with buffers containing primary amines
o-phthalaldehyde	10–500 µg/ml (standard) 0.05–2 µg/ml (micro)	50 µl/2.5 ng	Yes	<10 min	Moderate	Not compatible with buffers containing primary amines
CBQCA	0.12–1.8 µg/ml	80 µl/10 ng	Yes	90 min	Moderate	Buffers containing primary amines and reducing agents are not compatible
Nano-Orange[R]	0.01–10 µg/ml	2 ml/20 ng	Yes	10 min at 95°C	Low	Not compatible with detergents
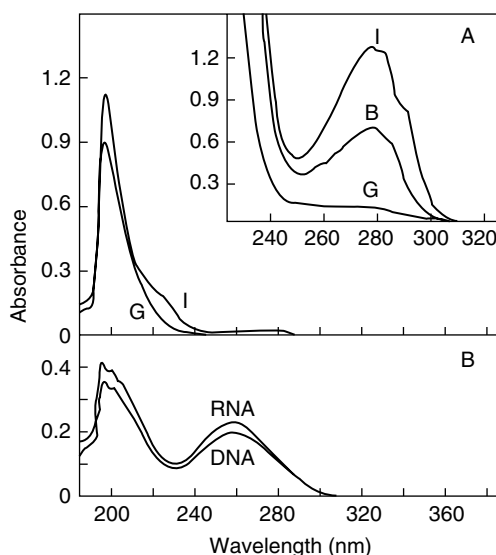
FIGURE 2.1 UV absorption spectra of proteins and nucleic acids. A: scan of 15 µg/ml solution of bovine immunoglobulin G (I) and gelatin (G). Inset shows scan of 1mg/ml solution of immunoglobulin G (I), bovine serum albumin (B), and gelatin (G). B: scan of 10 µg/ml of RNA and DNA. (Reprinted from Reference 1 with permission.)

Advantages

The method is simple, rapid, and non-destructive. The relationship between protein concentration and absorbance is linear.

Disadvantages

Despite its technical simplicity, this method is rather insensitive (0.2 to 2 mg/ml). Protein-to-protein variation is high. As the A_{280} is due to the aromatic rings, the sensitivity of the assay is dependent on the number of aromatic rings containing amino acids. For example, the assay is less sensitive for gelatin, which contains few aromatic amino acids. Low amounts of nucleic acids (1 µg) interfere with the A_{280} (see Table 2.2).

Absorbance at 205 nm (A_{205})

In the far UV region (190 nm), the peptide bond absorbs photons very strongly (about 50-fold more sensitive than at 280 nm). Thus, protein can be measured at this wavelength because of the large number of peptide bonds in a protein. But it is not possible to measure the peptide peak at this wavelength using routine spectro-photometers, because oxygen is absorbed in this region. Measurements at 205 nm give sufficiently accurate results, although the absorbance of the protein is about half at 205 nm than at 190 nm.[2]

TABLE 2.2
Concentration Limits of Chemicals on Commonly Used Protein Assays[a]

Chemical	A_{280}	A_{205}	Lowry	BCA	Bradford	Colloidal Gold	Fluorescent[b]
Buffers							
Acetate	0.1 M	0.01 M	—	0.2 M	0.6 M	—	0.02 M
Glycine	1 M	5 mM	2.5 mM	1 M	0.1 M	0.1 M	—
Citrate	5%	<0.01 M	2.5 mM	<1 mM	0.05 M	—	—
HEPES	—	<0.02 M	2.5 µM	0.1 M	0.1 M	0.0 M	0.01 M
MES	—	—	25 µM	0.05 M	0.7 M	—	—
MOPS	—	—	25 µM	0.05 M	0.2 M	—	—
Phosphate	1 M	0.05 M	0.25 M	0.25 M	1 M	0.1 M	0.02 M
Tris	0.5 M	0.04 M	0.25 M	0.1 M	2 M	—	—
Detergents							
Brij	1%	1%	—	1%	—	—	—
CHAPS	10%	<0.1%	—	1%	—	—	—
Deoxycholate	0.3%	0.1%	0.0625%	—	0.25%	—	—
Octylglucoside	10%	—	—	1%	—	—	—
SDS	0.1%	0.1%	1.25%	1%	0.1%	0.1%	0.01%
Triton X-100	0.02%	<0.01%	0.25%	1%	0.1%	1%	0.001%
Tween 20	0.3%	0.1%	0.1%	1%	—	1%	<0.001%

Chelators							
EDTA	0.03 M	—	125 µM	0.01 M	0.1 M	—	0.04 M
EGTA	OK	—	interfere	—	0.05 M	—	—
Reductants							
DTT	3 mM	0.1 mM	50 mM	<1 mM	1 M	1 µM	0.1 M
Mercaptoethanol	10 mM	<10 mM	1.8 µM	<1%	1 M	10 µM	0.1 M
Sugars							
Glucose	OK	—	30 mM	10 mM	OK	—	—
Sucrose	2 M	—	10 mM	1 M	1 M	—	0.01 M
Miscellaneous							
DMSO	20%	<10%	>6.2%	5%	—	20%	—
Glycerol	40%	5%	25%	10%	100%	10%	10%
KCl	0.1 M	0.05M	0.03M	<0.01 M	1 M	—	0.03 M
NaCl	>1 M	0.6 M	1.75 M	1 M	5 M	<1 M	0.02 M
Urea	>1 M	<0.1 M	>0.2 M	3 M	6 M	—	1 M
HCl	4 M	0.5 M	—	0.1 M	0.1 M	0.1 M	0.01 M
NaOH	4 M	0.025 M	—	0.1 M	0.1 M	0.1 M	0.01 M
DNA/RNA	1 µg	—	0.2 mg	0.1 mg	0.25 mg	10 ng	100 ng

a Taken from Reference 1.
b Taken from Reference 3.

Advantages

Sensitivity is higher at 205 nm compared to 280 nm. There is little variation between proteins at 205 nm, because peptide bonds are measured. Like A_{280}, A_{205} is linear with the protein concentration. The A_{205} is also rapid and non-destructive.

Disadvantages

The disadvantage of the A_{205} is that the compatibility of most chemicals is relatively low compared to A_{280} (see Table 2.2). For example, common buffers such as phosphate and Tris at 50 mM concentration interfere with the A_{205}. Among detergents, CHAPS is not compatible at > 0.1%. At A_{205}, glycerol, HCl, and NaOH are used at 8 to 16 times less concentration compared to A_{280}.

Working Procedure for A_{280} or A_{205}

Equipment

Spectrophotometer and quartz cuvettes

Reagents

Sample buffer for blank

Assay

1. Turn on the UV lamp of the spectrophotometer and warm up the machine (usually for 15 min). Adjust the wavelength at 280 nm or 205 nm.
2. Zero the spectrophotometer using the experimental buffer as a blank. The buffer should be compatible with the wavelength. Protein sample should be dissolved in the experimental buffer.
3. Measure the absorbance of the protein solution, using quartz cuvettes. The cuvettes should be filled with a sufficient volume to cover the aperture through which the light beam passes.

2.1.1 DETERMINATION OF PROTEIN CONCENTRATION USING A_{280}

For an unknown protein or protein mixture, the following formula can be used to obtain a rough estimate of protein concentration. Using this procedure, a protein of 20 µg/ml to 3 mg/ml can be measured.

$$\text{Concentration (mg/ml)} = A_{280}/\text{path length in cm}$$

Since nucleic acids absorb strongly at 280 nm, crude cell extracts containing RNA and DNA produce erroneously high protein estimates. In that case, the absorbance of the extracts at 260 nm (A_{260}) is measured as the procedure outlined above.

The protein concentration of the solution is corrected when nucleic acid is present using the following formula:

$$\text{Protein concentration (mg/ml)} = 1.55\ A_{280} - 0.76\ A_{260}$$

For a protein whose absorbance coefficient is known, protein concentration is determined by any of the following formulas, assuming the protein is measured with a cuvette of 1 cm path length. The absorption (extinction) coefficient is expressed as $E_{280}^{\ 1\ \text{mg/ml}}$, $E_{280}^{\ 1\%}$, or $E_{280}^{\ M}$.

$$\text{Concentration (mg/ml)} = \text{absorbance}/E_{280}^{\ 1\ \text{mg/ml}}$$

$$\text{Concentration (\%)} = \text{absorbance}/E_{280}^{\ 1\%}$$

$$\text{Concentration (M)} = \text{absorbance}/E_{280}^{\ M}$$

Protein content in mg/ml can easily be converted from the percentage protein and the molarity as follows:

$$\text{Concentration (mg/ml)} = \text{percentage protein}/10$$

$$\text{Concentration (mg/ml)} = \text{molarity/protein molecular weight}$$

2.1.2 Determination of Protein Concentration Using A_{205}

This method is almost 20 times more sensitive than the previous method, as protein absorbs much more strongly at 205 nm. In this wavelength, the protein-to-protein variability is less. Concentration of the protein may be roughly calculated as follows:

$$\text{Concentration (mg/ml)} = 31 A_{205}$$

2.1.3 Determination of Extinction Coefficient at 280 nm

At first, the protein is measured at absorbance 280 and 205 nm. For the latter, the protein should be diluted 30-fold, and the reading should be multiplied by the dilution factor. The extinction coefficient of the protein at A_{205} is obtained from the following formula:

$$E_{205}^{1\ \text{mg/ml}} = 27 + 120\ (A_{280}/A_{205})$$

The concentration of the protein is then determined from the extinction coefficient by the following formula previously described.

$$\text{Concentration (mg/ml)} = \text{absorbance at 205 nm}/E_{205}^{1\ \text{mg/ml}}$$

Finally, from this known concentration, the extinction coefficient at 280 nm can be determined as follows:

$$E_{280}{}^{1\ mg/ml} = \text{concentration (mg/ml)}/A_{280}$$

2.2 COLORIMETRIC METHODS

Colorimetric methods are relatively sensitive, and as little as 1 ng of protein can be detected by spectrophotometer by a 96-well plate reader. However, none of the available colorimetric methods can provide accurate estimation of the protein due to variable reactivity of the amino acids to the assay reagent. Most color reactions depend on the temperature and the incubation time. Thus, protein standards and unknowns should be assayed at the same time and in the same buffer. In many applications, reagents present in the extraction buffer may interfere with protein estimation (see Table 2.2). For this reason, a buffer blank is also incubated in the assay along with the protein sample. The value for the buffer is then corrected from the value of the protein sample. Several protein assay kits are available commercially, mostly based on Lowry and Bradford methods (Table 2.3). They cover a wide range of sample conditions (Table 2.4) and can be conveniently used.

Choice of Standard

Bovine serum albumin (BSA) has become the preferred standard in many laboratories, because it is inexpensive and readily available in a pure form. Moreover, it allows the results to be compared directly with previous studies that have used BSA as a standard. However, BSA may not be a good choice for use as a standard in the Bradford assay (see Section 2.2.4). Ovalbumin and bovine γ-globulin are used as

TABLE 2.3
Commercial Protein Assay Kits

Vendor	Name of the Kit	Principle (Based on)
Bio-Rad	Bio-Rad Protein Assay	Bradford Assay
	DC Protein Assay (detergent compatible)	Lowry Assay
	RC DC Protein Assay (reducing agent and detergent compatible)	Lowry Assay
Pierce	Coomassie Protein Assay	Bradford Assay
	Coomassie Plus Protein Assay	Bradford Assay
	Modified Lowry Protein Assay	Lowry Assay
	BCA Protein Assay	Lowry Assay
	Fluoraldehyde Protein/Peptide Assay	*o*-phthalaldehyde
Molecular Probes	CBQCA Assay	3-(4-carboxybenzoyl)-quinoline-2-carboxaldehyde
	NanoOrange[R]	Not known

alternative standards (see Section 2.2.4 for explanation). Optimally, it is always best to use a purified preparation of the same protein being assayed as a standard (see Table 2.4). A purified protein sample is not available when it is subjected to purification for the first time.

2.2.1 BIURET ASSAY

Among the colorimetric methods, this assay provides a fairly accurate measurement, with little variation in color from protein to protein. This is because the peptide chain, rather than the side groups, reacts with the reagent. This assay involves the use of cupric sulfate ($CuSO_4.5H_2O$), sodium potassium tartrate, and sodium hydroxide.[4]

TABLE 2.4
Some Common Questions and Recommendations

Question	Recommendation
Which protein assay should I use for a sample in a detergent-containing buffer?	Usually Lowry assay and assays based on Lowry method, such as BCA protein assay (Pierce) and DC Protein assay (Bio-Rad), are suitable for a sample in a detergent-containing buffer (see Table 2.2).
Which protein assay should I use for a sample mixed with a reducing agent?	Usually Bradford assay and assays based on Bradford method, such as Bio-Rad protein assay, Coomassie protein assay (Pierce), and Coomassie plus protein assay (Pierce), are suitable for a sample mixed with a reducing agent (see Table 2.2).
A buffer in the protein sample is not found in the reagent compatibility list. How do I know if it interferes with the assay?	One should run two standard protein curves: one with standard protein in the same buffer as the sample and one with standard protein in water. The two standard curves will have identical slopes if the buffer does not interfere. A low amount of buffer interference may be adjusted by incorporating the buffer alone to the standard curve for the actual protein assay.
Which standard protein should I use to prepare a standard curve to measure a crude protein sample (mixture of proteins)?	Regardless of the type of assay, the best protein to use as a standard is a purified preparation of the protein being assayed. However, a purified protein sample is not available when it is subjected to purification for the first time. In the absence of the purified protein, one should use a protein that yields a similar standard curve to that of the protein being assayed. In most applications, bovine serum albumin and bovine γ-globulin are used as standard proteins, of which the latter is preferred in Bradford assay (see Section 2.2.4).
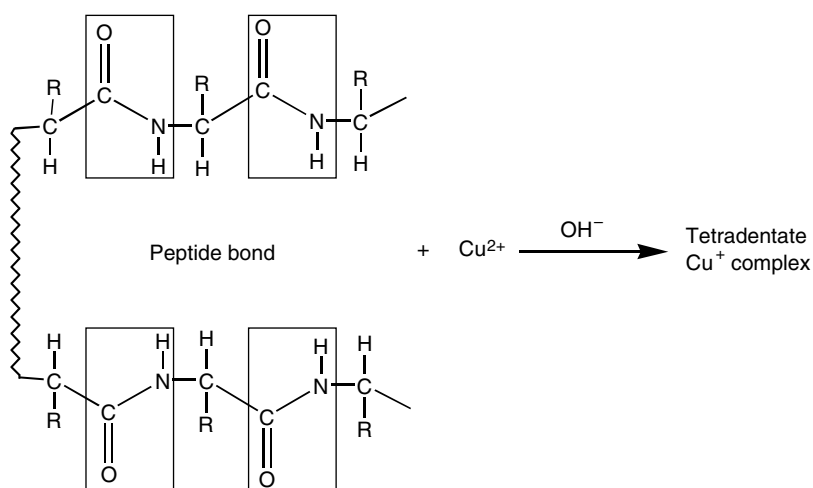
FIGURE 2.2 At alkaline condition, cupric sulfate reacts with the protein peptide bonds, resulting tetradentate-Cu$^+$ complex, which is measured at 550 nm.

Reaction

The mechanism of the Biuret reaction is not well understood. Smith et al.[5] theorized that cupric ion (Cu^{2+}) might react with the peptide bonds of proteins, producing cuprous ion (Cu$^+$). The Cu$^+$ ions then form a tetradentate complex with opposite pairs of peptide-bonded nitrogens (Figure 2.2). The complexes produce a blue color, which is measured at 550 nm.

Advantages

This method provides little protein-to protein variation and linear absorption/protein relationship.

Disadvantages

This assay limits its application because of low sensitivity (range 1 to 6 mg/ml). Low amounts of reducing agents (such as mercaptoethanol and dithiothreitol) and detergents interfere with the assay.

Working Procedure

Equipment

Spectrophotometer and plastic cuvettes.

Biuret Reagent

Dissolve 1.5 gram of $CuSO_4.5H_2O$ and 6.0 gram of sodium potassium tartrate $(NaKC_4H_4O_6.4H_2O)$ in about 500 ml of water. To this solution add 300 ml of 10% sodium hydroxide with constant stirring. Add distilled water to make 1 liter.

Assay

1. Set up several tubes containing standard protein (usually bovine serum albumin) in duplicate as follows:

   For BSA:

Amount (mg)	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
Final conc. (mg/ml)	0.5	1.0	1.5	2.0	2.5	3.0	4.0	5.0

   Take BSA (5 mg/ml):

Volume (ml)	0.2	0.4	0.6	0.8	1.0	1.2	1.6	2.0
Water to add (ml)	1.8	1.6	1.4	1.2	1.0	0.8	0.4	0

2. Set up unknown sample in at least two dilutions (2 ml).
3. Take 2 ml of sample buffer.
4. Take 2 ml of water as blank.
5. Add 8 ml of Biuret reagent to each tube and mix.
6. After incubation at room temperature for 30 min, read each reaction at 550 nm (see Table 2.5).
7. Correct data of the unknown sample by subtracting value for buffer as shown in Table 2.5.
   (**Note:** best results are achieved when the standards are prepared in the same buffer as the sample. In that case, step 3 is used as blank. Steps 4 and 7 are not necessary.)
8. Prepare standard curve for the known amount of standard protein and determine protein concentration of unknown sample from the plot as shown in Figure 2.3. For example, protein concentration of a sample showing absorbance of about 0.510 can be calculated as 2.5 mg/ml from the plot.

Modification of Biuret Assay

Itzhaki and Gill[6] described a micro-Biuret method, which was more sensitive (0.02 to 0.53 mg) than the original Biuret assay. The color was developed in less than 5 min.

Pelley et al.[7] adapted the Biuret method for samples containing thiols. The absorbance curve is linear up to 1 mg protein.

Watters[8] adapted the Biuret method for samples containing detergents. Protein gave a linear absorbance curve over the range of 0.4 to 8 mg/ml.

TABLE 2.5
Hypothetical Absorbance Values at 550 nm (A_{550}) for Various
Concentrations of Standard Protein

Sample	A_{550}	Corrected A_{550} for Sample
Standard solution (mg/ml)		
0.5	0.102	
1.0	0.213	
1.5	0.297	
2.0	0.385	
2.5	0.501	
3.0	0.590	
4.0	0.780	
5.0	0.942	
Unknown sample	0.528	0.510
Sample buffer	0.018	
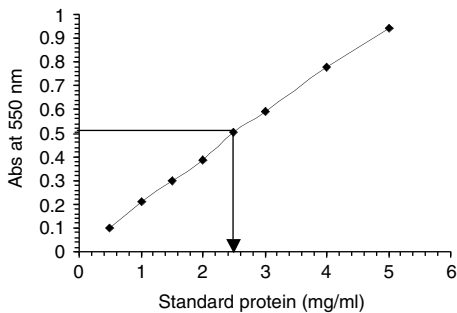
FIGURE 2.3 Standard curve for the Biuret assay. Curve is drawn from hypothetical absorbance values for the purpose of this demonstration. Protein concentration of the unknown sample (2.5 mg/ml) can be calculated from its absorbance (A_{550} 0.510) as shown by the arrow.

2.2.2 LOWRY ASSAY

The Lowry assay[9] is based on the Biuret reaction of proteins with cupric sulfate at alkaline conditions and the Folin-Ciocalteau phosphomolybdotungstate reduction. The use of Folin-Ciocalteau reagent greatly enhances the sensitivity of the assay (down to 10 µg/ml). The assay is pH dependent (optimum at pH 10 to 10.5), and thus it is important to maintain the pH during the assay. Color reaction is stable for several hours, and thus the reactions can be measured at any time after 10 min. But the Folin reagent is not very stable in alkaline condition and only reactive for the first few minutes after addition, and thus the mixing is critical to obtain reproducible results.

FIGURE 2.4 At alkaline condition, cupric sulfate reacts with the protein peptide bonds, producing Cu^{+}. Subsequent reduction of the Folin reagent by copper-treated protein yields a heteropoly-molybdenum blue product.

Reaction

In this assay, color develops in two steps. The peptide bonds of proteins first react with cupric sulfate under alkaline condition, producing Cu^{+}, and the subsequent reduction of Folin reagent (phosphomolybdotungstate) by the copper-treated protein to heteropolymolybdenum blue (5, Figure 2.4). Color develops in the second reaction (i.e., the reaction with phosphomolybdotungstate) and is primarily due to the amino acids tyrosine and tryptophan, and to a lesser extent cystine, cysteine, and histidine.[9,10] The blue color has the maximum absorbance at 750 nm.

Advantages

The Lowry assay is more sensitive than the Biuret assay. The range is 0.01 to 1 mg/ml.

Disadvantages

The disadvantage of this assay is the fact that many reagents interfere with the assay. Detergents and reducing agents (such as mercaptoethanol and dithiothreitol) interfere with this assay. These reducing agents reduce cupric to cuprous. Strong acids, high ammonium sulfate, and EDTA (which chelates the copper) are also not compatible with this assay (see Table 2.2). Diluting the sample is a good idea to reduce the interference, if the protein concentration is still in the linear range of detection sensitivity after dilution. Addition of SDS to the Lowry reagents reduces interference due to detergents, sucrose, and EDTA.[11]

Removal of Interfering Substances and Protein Estimation[12]

The protein sample can be precipitated with deoxycholate-trichloroacetic acid. This allows the removal of interfering substances as well as the determination of protein in dilute solution. The precipitation is as follows:

1. To 1 ml of protein sample, add 0.1 ml of 0.15% deoxycholate.
2. Vortex and allow the sample to stand at room temperature for 10 min.
3. Add 0.1 ml of 72% trichloroacetic acid. Vortex and centrifuge at 3,000 g for 30 min.

Remove the supernatant and dissolve the pellet directly in solution C (see below for working procedure) for protein estimation.

Working Procedure

Equipment
Spectrophotometer and plastic cuvettes

Reagents

1. Prepare 100 ml of solution A using 0.5 gram $CuSO_4.5H_2O$ and 1 gram sodium citrate $Na_3C_6H_5O_7.2H_2O$.
2. Prepare 1 liter of solution B with 20 gram sodium carbonate and 4 gram sodium hydroxide.
3. Mix 1 ml of solution A with 50 ml of solution B to obtain solution C.
4. Prepare solution D using 10 ml of Folin-Ciocalteu reagent and 10 ml of distilled water.

Assay

1. Set up several tubes containing standard protein (such as BSA) in duplicate as follows:

   For BSA:

Amount (µg)	5	20	50	100	300	400	500
Final conc. (µg/ml)	10	40	100	200	600	800	1,000

   Take BSA (1 mg/ml):

Volume (µl)	5	20	50	100	300	400	500
Water to add (µl)	495	480	450	400	200	100	0

2. Set up unknown sample in at least two dilutions (0.5 ml).

3. Take 0.5 ml of sample buffer.
4. Take 0.5 ml of water as blank.
5. Add 2.5 ml solution C to each tube. Mix well and incubate at room temperature for 5 to 10 min.
6. Add 0.25 ml solution D and mix.
7. After incubation at room temperature for 20 to 30 minutes, read each tube at 750 nm.
8. Correct data for unknown sample by subtracting value for buffer.
9. Prepare standard curve for the known amount of standard protein and determine protein concentration of the unknown from the plot.
   (**Note:** best results are achieved when the standards are prepared in the same buffer as the sample. In that case, step 3 is used as blank. Steps 4 and 8 are not necessary.)

Modification to Improve the Assay

A 20% increase in sensitivity of the assay is achieved when the Folin reagent is added in two portions, vortexing between additions.[13] The addition of the dithiothreitol 3 min after the addition of the Folin reagent enhances sensitivity by 50%.[14] Bio-Rad *DC* (detergent compatible) protein assay is based on the Lowry assay and can be used to assay protein in the presence of 1% detergent. Bio-Rad *RC DC* protein assay, also based on the Lowry assay, is compatible with reducing agent as well as detergent. But, the sensitivity of both assays is compromised (0.2 to 1.5 mg/ml) compared to the Lowry assay (0.01 to 1 mg/ml).

The Lowry assay can be performed in a few seconds by using microwave irradiation.[15] After irradiation, the absorbance of each sample and standard is read spectrophotometrically.

2.2.3 BCA PROTEIN ASSAY

Some of the interferences associated with the Lowry assay can be overcome by using bicinchonic acid (BCA), first described by Smith et al. (5). BCA is stable and highly specific for cuprous ion. A protein of 0.1 to 1.2 mg/ml can be measured using a standard assay. A microassay (0.5 to 10 μg/ml) is also available.[4]

Reaction

Like the Lowry assay, peptide bonds of protein first reduce cupric ion (Cu^{2+}) to produce tetradentate-cuprous ion (Cu^+) complex in alkaline medium. The cuprous ion complex then reacts with BCA (2 molecules per Cu^+ ion) to form an intense purple color that can be measured at 562 nm (Figure 2.5). The presence of four amino acids (cysteine, cystine, tryptophan, and tyrosine) in the protein is responsible for the color development.
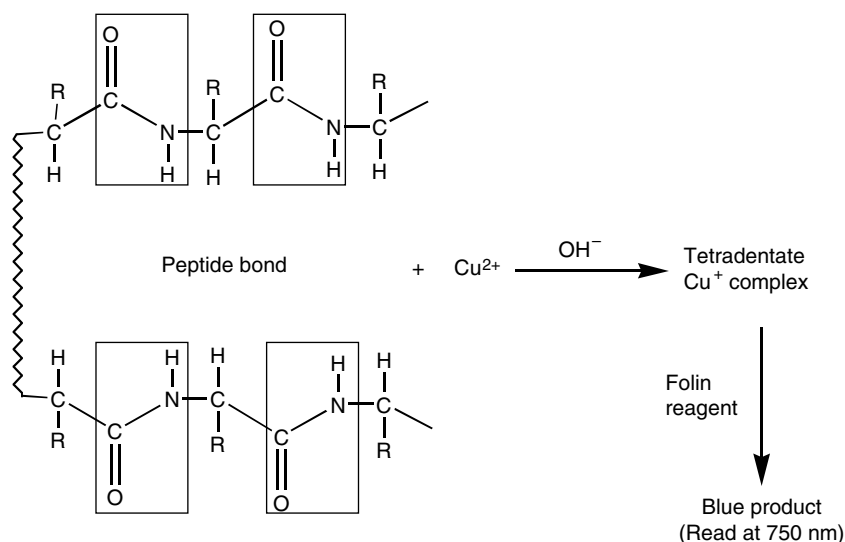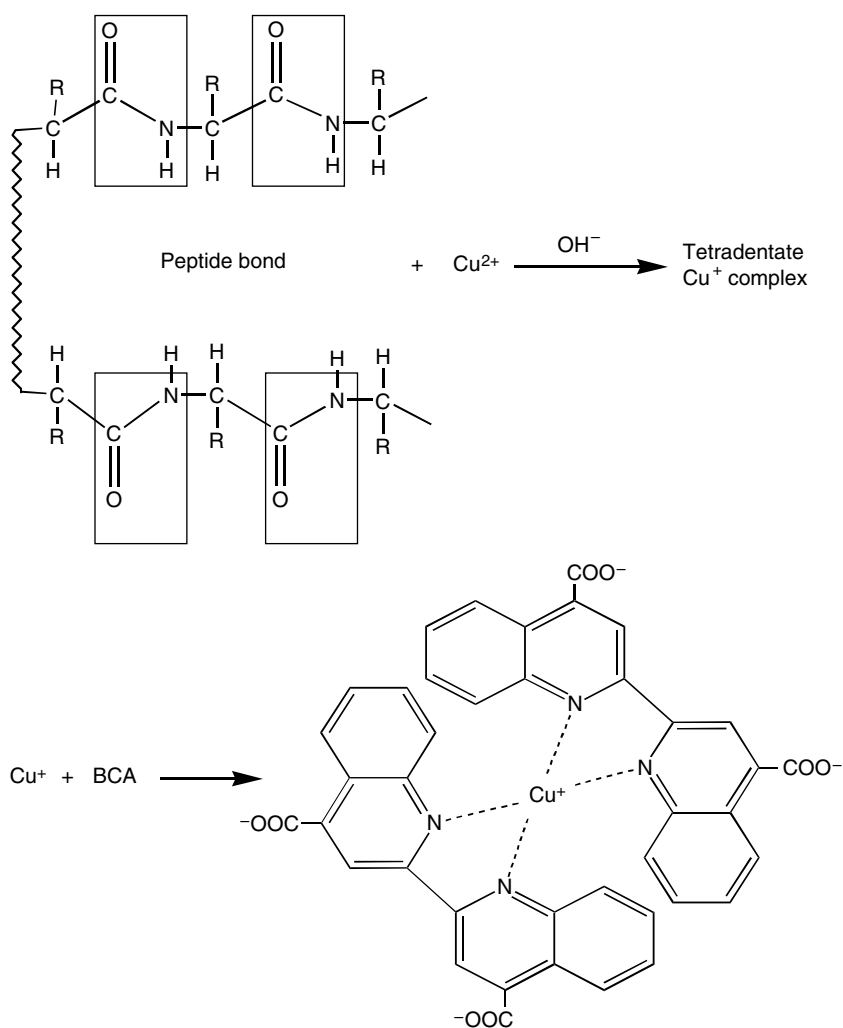
FIGURE 2.5 At alkaline condition, cupric sulfate reacts with the protein peptide bonds, producing Cu^+. Subsequent reaction of copper-treated protein with BCA results in an intense purple color, which is measured at 562 nm.

Advantages

Since BCA is stable in an alkaline condition, this assay can be carried out in one step, compared to two steps needed in the Lowry assay. Another advantage of the BCA assay is that it offers more tolerance toward the compounds that interfere with the Lowry assay. Particularly, detergents (such as SDS, Triton X-100, and Tween 20) of up to 1% concentration do not interfere with the assay (see Table 2.2).

Disadvantages

Like the Lowry assay, the BCA assay also has moderate protein-to-protein variation. Both assays respond poorly toward the protein gelatin. Compared to BSA, an equivalent amount of gelatin yields approximately 50% of the color when BCA assay is conducted at room temperature for 4 h (or at 37°C for 30 min). Unlike the Lowry assay, BCA assay is more sensitive to interference from reducing sugars (e.g., lucose) (see Table 2.2). The interference is probably due to the nature of the protocol, which allows the sugars more time to reduce Cu^{2+} to Cu^+. Like the Lowry assay, strong acids, EDTA, and reducing agents will interfere with this assay (see Table 2.2). The BCA assay was found to produce erroneously high values for protein when common membrane phospholipids were present in the protein sample.[16] This is because phospholipids and BCA have a similar absorbance peak.[16]

Modification to Improve the Assay

Protein-to-protein variability is reduced when the BCA assay is performed at high temperature (60°C for 30 min).[5] At this temperature, the response for gelatin is nearly 70% of the response for BSA. Based on this observation, Smith et al.[5] suggest that at least two sources contribute the total color yield. One source is the readily oxidizable amino acids (such as cysteine, tyrosine, and tryptophan), which contribute color independent of temperature. The second source of color, which is temperature-dependent, arises from the reaction of the peptide bonds with the cupric sulfate (Cu^{2+} ion). The contribution of the peptide bonds to total color development appears to be more significant at higher temperature than at room temperature and thus explains the reduction of protein-to-protein variation between proteins at 60°C.

A microassay is available to dilute protein samples (0.5 to 10 µg/ml) for test tubes[5] and microwell plates (Micro BCA™ Protein Assay, Pierce, Rockford, IL).

A rapid BCA assay may be performed by incubating the solution for 20 seconds in a microwave oven.[15]

Working Procedure for Standard Assay

Equipment

Spectrophotometer and plastic cuvettes

Reagents

1. Reagent A: dissolve 10 gram of BCA, 20 gram of sodium carbonate ($Na_2CO_3.H_2O$), 1.6 gram of sodium tartrate ($Na_2C_4H_4O_6.2H_2O$), 4 gram of sodium hydroxide, and 9.5 gram of sodium bicarbonate in 1 liter of water. Adjust pH to 11.25 with 10 M NaOH. Stable indefinitely at room temperature.

2. Reagent B: dissolve 0.8 gram of cupric sulfate ($CuSO_4.5H_2O$) in 20 ml of water. Stable indefinitely at room temperature.
3. Working reagent: prior to assay, mix 50 ml of reagent A with 1 ml of reagent B.

Assay

1. Set up several tubes containing standard protein (BSA, 100 to 1,000 µg/ml) in duplicate as follows:

For BSA:

Amount (µg)	10	20	30	40	60	80	100
Final conc. (µg/ml)	100	200	300	400	600	800	1,000

Take BSA (1 mg/ml):

Amount (µl)	10	20	30	40	60	80	100
Water to add (µl)	90	80	70	60	40	20	0

2. Set up unknown sample in at least two dilutions (100 µl).
3. Take 100 µl of sample buffer.
4. Take 100 µl of water as blank.
5. Add 2.0 ml of working reagent to each tube. Mix well and incubate at room temperature for 2 h or at 60°C for 30 min.
6. Cool to room temperature if incubated at 60°C and read each tube at 562 nm.
7. Correct data for unknown sample by subtracting value for buffer.
8. Prepare standard curve for the known amount of standard protein and determine protein concentration of the unknown from the plot.
   (**Note:** best results are achieved when the standards are prepared in the same buffer as the sample. In that case, step 3 is used as blank. Steps 4 and 7 are not necessary.)

Working Procedure for Microassay for Test Tube[5]

Equipment
Spectrophotometer and plastic cuvettes

Reagents

1. Reagent A: dissolve 8 gram of $Na_2CO_3.H_2O$, 1.6 gram of NaOH, and 1.6 gram of sodium tartrate in 100 ml of water. Adjust pH 11.25 with 10 M NaOH.
2. Reagent B: dissolve 4 gram of BCA in 100 ml of water.

3. Reagent C: dissolve 0.4 gram of $CuSO_4.5H_2O$ in 10 ml of water.
4. Working solution: mix 1 volumes of reagent C with 25 volumes of reagent B. To the mixture, add 26 volumes of Reagent A.

Assay

1. Set up several tubes containing standard protein (BSA, 0.5 to 10 µg/ml) in duplicate as follows:

For BSA:

Amount (µg)	0.5	1	2	4	6	8	10
Final Conc. (µg/ml)	0.5	1	2	4	6	8	10

Take BSA (10 µg/ml)

Amount (µl)	50	100	200	400	600	800	1,000
Water to add (µl)	950	900	800	600	400	200	0

2. Set up unknown sample in at least two dilutions (1 ml).
3. Take 1 ml of sample buffer.
4. Take 1 ml of water as blank.
5. Add 1 ml of working reagent to each tube. Mix well and incubate at room temperature for 2 h or at 60°C for 30 min.
6. Cool to room temperature if incubated at 60°C and read each tube at 562 nm.
7. Correct data for unknown sample by subtracting value for buffer.
8. Prepare standard curve for the known amount of standard protein and determine protein concentration of the unknown from the plot.
   (**Note:** best results are achieved when the standards are prepared in the same buffer as the sample. In that case, step 3 is used as blank. Steps 4 and 7 are not necessary.)

Working Procedure for Microassay for Microwell Plate

Equipment

Microtiter plate reader, 96-well microtiter plates

Reagents and Assay

Available from Micro BCA™ Protein Assay (Pierce, Rockford, IL)

2.2.4 BRADFORD ASSAY

The Bradford protein assay[17] has become the preferred method for many investigators, because it is simple and rapid compared to the Lowry method. Moreover, this assay is comparatively free from interference by common reagents except

detergents. The assay involves the use of Coomassie Brilliant Blue G-250, which reacts primarily to basic (especially arginine) and aromatic amino acids.[18] The Bradford protein assay is performed in two formats: standard assay (0.1 to 1 mg/ml) and a microassay (5 to 40 µg/ml) for use with a micro plate reader (19, 20).

Reaction

The assay is based on the immediate absorbance shift 470 nm to 595 nm that occurs when dye binds to protein in acidic solution. The dye is believed to bind to protein via electrostatic attraction of the dye's sulfonic acid groups (Figure 2.6 A). The mechanism of dye binding can be explained by the dye existing as three absorbing species, a red cationic species (A_{max} 470 nm), a green neutral species (A_{max} 650 nm), and a blue anionic species (A_{max} 595 nm). Color changes are due to successive loss of charge (Figure 2.6B). Stepwise addition of sodium hydroxide abolished absorption at 470 nm, but increased absorbance at 650 nm and finally replaced with a new peak at 595 nm (Figure 2.6 C).[21] Prior to protein binding, the dye molecules exist in doubly protonated (the red cationic dye form). Upon binding of the dye to protein, the blue anionic dye form is stabilized and is detected at 595 nm.[21]

Advantages

Color development is rapid. The assay can be performed in 10 minutes.

Disadvantages

Substances that have the ability to shift the above equilibrium (for example, strong bases) or agents that can form a complex with dye (for example, detergents) interfere with this assay (see Table 2.2).

Another disadvantage of this assay is the moderate protein-to-protein variation. Because of the specificity towards arginine residue, different proteins respond differently to this assay. Therefore, it is essential to specify the protein standard used when reporting measurement of the protein amount. However, several modifications can be adopted to reduce this protein-to-protein variability.[22–24] These modifications are largely on the pH and the dye content. Stoscheck[24] has reported increased uniformity in the response of the assay to different proteins after adjusting the pH by adding sodium hydroxide. This uniformity is probably due to an increase of free dye content in the blue form. However, the modification makes the assay more sensitive to interference from detergents in the sample. The second modification is to use the reagent with high dye content. The amount of soluble dye in Coomassie Blue G-250 appears to vary significantly from various sources.[25] Serva blue G is believed to have the highest dye content and should be used in the modified assay.[26]

Standard Protein

Although BSA is generally used to make a standard protein curve in most applications, it may not be a good choice to use as a standard in the Bradford assay. In this
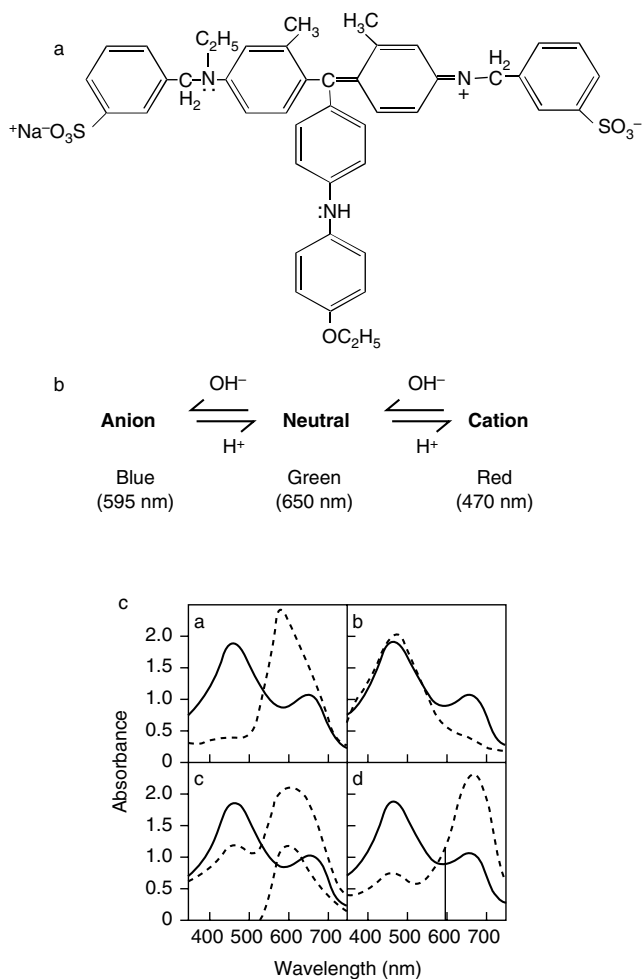
FIGURE 2.6 A: structure of Coomassie Brilliant Blue G-250 dye. B: the dye exists in three absorbing species: red cationic (A_{max} 470 nm), green neutral (A_{max} 650 nm), and blue anionic (A_{max} 595 nm) species. Color changes due to successive loss of protons. Upon binding with the protein, the dye changes to the unprotonated blue product. C: absorption spectra of (-) the Coomassie dye, (—) dye with (a) NaOH, (b) H_2SO_4 (pH 0.3), (c) 140 μg BSA (pH 0.78); (…..) same, after subtraction of blank value, (d) (….) dye with excess SDS (pH 0.77). Solid line represents 595 nm. (Figure 2.6 C reprinted from Reference 21 with permission.)

assay, BSA exhibits an unusually large dye response compared to other proteins (see Figure 2.7) and thus may underestimate the protein content of a sample. Ovalbumin and bovine γ-globulin are the better choice, as their dye binding capacities are approximately in the midpoint of those from different proteins (see Figure 2.7). However, it is always best to use a purified preparation of the same protein being assayed as a standard.
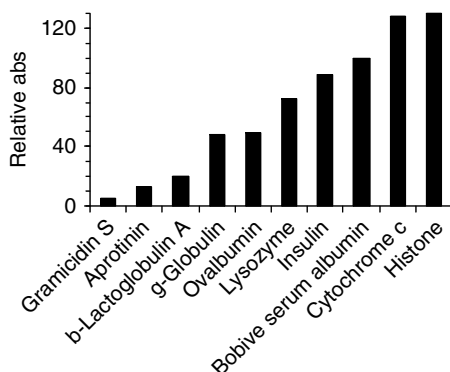
FIGURE 2.7 Relative absorbance of various proteins in the Bradford assay. The absorbance of each protein is expressed relative to that of the same concentration of bovine serum albumin. The plot is based on the values reported by Friendenauer and Berlet[23] and Stoscheck.[24]

Working Procedure for Standard Assay

Equipment

Spectrophotometer and plastic cuvettes

Reagent

Dissolve 100 mg of Coomassie Blue G-250 in 50 ml of 95% ethanol. Mix the solution with 100 ml of 85% phosphoric acid and make up to 1 liter with distilled water. Filter the final solution through Whatman No. 1 filter paper and then store in an amber bottle. The solution is stable for several weeks.

Assay

1. Set up several tubes containing standard protein (BSA, 0.1 to 1 mg/ml) in duplicate as follows:

For BSA:

Amount (μg)	10	20	30	40	50	60	80	100
Final conc. (μg/ml)	100	200	300	400	500	600	800	1000

Take BSA (1 mg/ml):

Volume (μl)	10	20	30	40	50	60	80	100
Water to add (μl)	90	80	70	60	50	40	20	0

2. Set up unknown sample in at least two dilutions (100 μl).
3. Take 100 μl of sample buffer.
4. Take 100 μl of water as blank.

5. Add 5 ml of Coomassie reagent to each tube. Mix well and incubate at room temperature.
6. Measure absorbance for each tube at 595 nm between 5 min and 1 h after mixing.
7. Correct data for unknown sample by subtracting value for buffer.
8. Prepare standard curve for the known amount of standard protein and determine protein concentration of the unknown from the plot.
   (**Note:** best results are achieved when the standards are prepared in the same buffer as the sample. In that case, step 3 is used as blank. Steps 4 and 7 are not necessary.)

Microssay Adapted from Bio-Rad Protein Assay[20]

Equipment
Microtiter plate reader, polystyrene 96-well microtiter plates

Reagents
Coomassie Brilliant Blue G-250 dye (Bio-Rad). Freshly dilute 2.5-fold with water for this assay.

Assay

1. Take standard protein (BSA, 5 to 40 µg/ml) in duplicate in microtiter plate as follows:

   For BSA:

Amount (µg)	0.5	1.0	1.5	2.0	2.5	3.0	4.0
Final conc. (µg/ml)	5	10	15	20	25	30	40

   Take BSA (100 µg/ml):

Volume (µl)	5	10	15	20	25	30	40
Water to add (µl)	95	90	85	80	75	70	60

2. Take unknown sample in at least two dilutions (100 µl).
3. Take 100 µl of sample buffer.
4. Take 100 µl of water as blank.
5. Add 100 µl of freshly diluted Coomassie dye (2.5-fold in water) to each well.
6. Incubate the plate at room temperature for about 10 to 15 min and read the plate at 595 nm on a plate reader.
7. Correct data for unknown sample by subtracting value for buffer.
8. Prepare standard curve for the known amount of standard protein and determine protein concentration of the unknown from the plot.
   (**Note:** best results are achieved when the standards are prepared in the same buffer as the sample. In that case, step 3 is used as blank. Steps 4 and 7 are not necessary.)

2.2.5 COLLOIDAL GOLD ASSAY

This assay[27] is most sensitive (range 2 to 20 µg/ml, as low as 20 ng, as the assay volume is 10 µl) among colorimetric protein determination methods. The lower detection limit can extend down to 1 ng in 10 µl (100 ng/ml) when the gold solution is stabilized with polyethylene glycol and adjusted to pH 3.8, and the assay is adapted to 96-well microtiter plates.[28] At very low concentrations, proteins tend to stick to glass and plastic surfaces, and thus any loss of protein can adversely affect the actual assay. In order to avoid protein absorption onto plastic wells, a small amount of detergents such as 0.001% Tween 20 can be added in the assay.

Reaction

In the colloidal gold protein assay, the binding of protein to the colloidal gold causes a shift in its absorbance. This absorbance is proportional to the amount of protein added to the assay. In order to ensure the binding of the protein to the negatively charged colloid, the pH of the solution must be acidic (around pH 3). In an acidic solution, proteins carry positive charges and facilitate binding to the negatively charged colloid.[29]

Advantages

The assay is sensitive. Most common reagents except thiols and SDS are compatible with the assay (see Table 2.2).[27]

Disadvantages

This assay has significant protein-to-protein variation.[27]

Working Procedure

Equipment

Spectrophotometer, plastic cuvettes

Reagents

1. Add 100 µl of chloroauric acid (40 µg/ml) to 80 ml boiling water and mix with stirring.
2. To the above gold mix, add 1 ml trisodium citrate dihydrate (40 mg/ml) with stirring.
3. Boil the solution for 30 min with refluxing and allow the mix to cool to room temperature.
4. Add 32 µl of 25% Tween 20 and mix.
5. Finally, add 400 µl of 1 M citric acid to the gold mix. The final solution should be a clear red.

Alternatively, ready-made colloidal gold solution can be purchased from vendors such as Aurodye from Hoefer (San Francisco, CA) and Quantigold™ from Diversified Biotech (Boston, MA).

Assay

1. In microfuge tubes take 10 µl of standard protein such as BSA (20 to 200 ng) and unknown sample in duplicate. Protein dilutions should be made in 0.01% Tween 20 to avoid absorption to the microfuge tube. Unknown protein sample should be diluted in at least two dilutions.
2. Add 0.8 ml gold reagent into each tube and vortex the mixture immediately.
3. After 30 min of incubation at room temperature, read the absorbance at 595 nm using the blank to zero the spectrophotometer.
4. Prepare standard curve by plotting absorbance against nanogram of BSA. Determine the protein concentration for unknown from the curve.

Modified Colloidal Gold Assay with Improved Sensitivity

Ciesiolka and Gabius[28] reported a modification of the colloidal gold assay by increasing the concentration of colloidal gold in the assay solution, changing the type and concentration of the stabilizer and the pH, and adapting the assay to microtiter plates. This modification resulted in about 10- to 20-fold enhancement in sensitivity for the quantitation of proteins (lower detection limit: 1ng in 10 µl).

Working Procedure

Equipment

Microplate reader

Reagents

1. Prepare colloidal gold granules (average particle diameter of 17 nm) by reduction of tetrachloroauric acid (Merck, Darmstadt, Germany) with sodium citrate. For this purpose, boil 40 ml of 0.05% $HAuCl_4$ solution in a flask equipped with a reflux condenser.
2. To the gold solution, add 5 ml of freshly 1% trisodium citrate solution (Merck) with vigorous stirring. Boil the resulting solution gently for another 8 min and cool to room temperature. The pH should be 5.5. Transfer the solution to polypropylene bottle and store in the refrigerator.
3. To the above solution, add freshly prepared and microfiltered Carbowax 20 M solution (polyethylene glycol, average MW 20,000, from Serva, Heidelberg, Germany) to a final concentration of 0.01% and adjust pH to 3.8 with 2.3 M citric acid solution. The solution is now ready to use for the modified assay.

Assay

1. Take 10 μl of BSA (1 to 10 ng) and unknown sample in duplicate into the wells of microtiter plate.
2. Add 190 μl gold reagent into each well and mix the solution gently.
3. After 30 min of incubation at room temperature, read the absorbance at 595 nm using the microplate reader.
4. Prepare standard curve by plotting absorbance against nanogram of BSA. Determine the protein concentration for unknown from the curve.

2.2.6 NINHYDRIN ASSAY

Starcher[30] described a ninhydrin-based microassay to quantitate protein based on the total amino acid content of protein hydrolysate. The assay is performed in the protein range 1 to 10 μg. The absorbance slope is very steep. The highest amount of protein (10 μg) hits the upper absorbance limit of the plate reader (closed to 4).[30]

Reaction

Ninhydrin (2,2-Dihydroxy-1,3-indanedione) reacts with α-amino acids to produce a purple-colored product (Figure 2.8). Ninhydrin removes two hydrogen atoms from the α-amino acid to yield α-imino acid (this reaction is called oxidative deamination) and gets reduced. The α-imino acid is rapidly hydrolyzed to form α-keto acid with the production of an ammonia molecule. This α-keto acid then undergoes a decarboxylation reaction at high temperature to form an aldehyde (one less carbon atom than the original amino acid) and carbon dioxide (Figure 2.8, upper panel). The reduced ninhydrin and ammonia thus produced react with another molecule of ninhydrin, forming a final purple complex (Figure 2.8, lower panel).

Advantages

This assay has minimum protein-to-protein variation. The assay gives a nearly accurate measurement of protein content.

Disadvantages

The assay is time consuming. Prior to the assay, protein is hydrolyzed for about 24 h, evaporated to dryness, and the residue is redissolved in water or assay buffer.

Working Procedure

Equipment

Microplate reader, Speed Vac
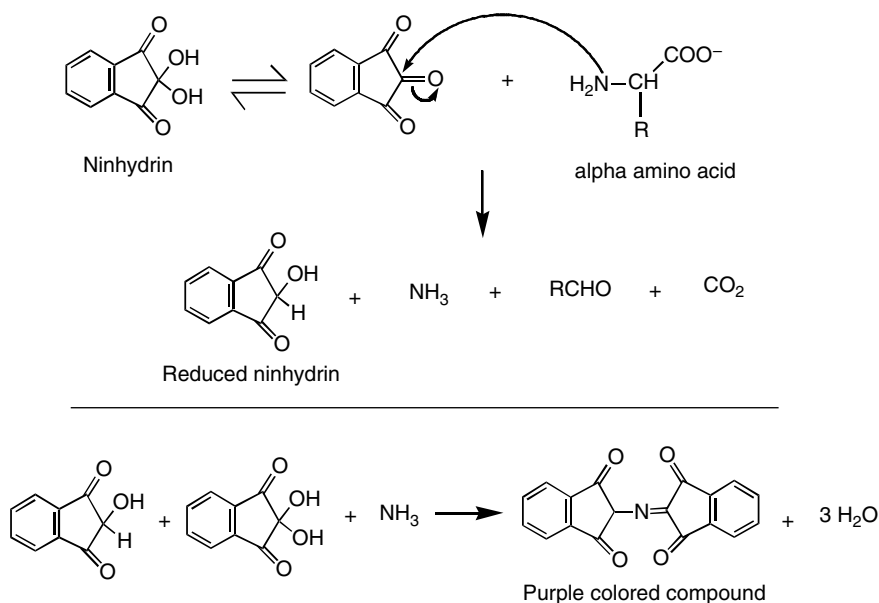
FIGURE 2.8 Ninhydrin (2,2-Dihydroxy-1,3-indanedione) reacts with α-amino acids to produce a purple-colored product.

Reagents

1. Protein hydrolyzing reagent: 6 N HCl.
2. 4 N sodium acetate buffer: dissolve 544 gram of sodium acetate trihydrate in 100 ml of glacial acetic acid. Add water up to 500 ml total volume.
3. Stannous chloride solution: take 50 mg of stannous chloride in 0.5 ml of ethylene glycol. This results in a cloudy solution or fine suspension.
4. Ninhydrin reagent: dissolve 200 mg of ninhydrin in a mixture of 2.5 ml of 4 N sodium acetate buffer and 7.5 ml of ethylene glycol and then add 250 μl of stannous chloride suspension with stirring. The resulting solution should be pale red in color and ready to use.

Assay

1. Take 5 to 10 mg of dry standard protein such as BSA in locking-cap microfuge tube and hydrolyze with 500 ml of 6 N HCl at 100°C for 24 h.
2. Hydrolyze tissue sample of about 10 mg as above.
3. Chill hydrolyzed BSA and unknown sample, evaporate to dryness in a Speed Vac, and redissolve in water to give a 10 mg/ml solution. Dilute this solution tenfold for the working protein standard.

4. Take 1 to 10 µg of BSA hydrolysate in wells of a flat-bottom microtiter plate and add 100 µl of ninhydrin reagent to each well. Also take an unknown sample hydrolysate in two to three dilutions and add 100 µl of ninhydrin reagent.
5. Float the plate on a boiling water bath for 10 min.
6. Remove the plate with forceps, blot with paper towels, and read absorbance at 575 nm on a microtiter plate reader.
7. Prepare standard curve by plotting absorbance against µg of BSA. Determine the protein content for unknown sample from the curve.

2.3   FLUORESCENT METHODS

The fluorescent methods for protein determination are usually more sensitive than colorimetric methods. Moreover, all colorimetric methods have a limited dynamic range because they are based on absorption measurements. In contrast, fluorescent methods have a broad dynamic range, besides their extreme sensitivity. They are usually suitable to a broad pH range and adaptable to the measurement of lipoproteins.

2.3.1   Fluorescamine Protein Assay

Bohlen et al.[31] reported a fluorescent assay based on the use of fluorescamine (4-phenylspiro[furan-2(3H), 1′-phthalan]-3. 3′-dione). The assay can detect as low as 500 ng of protein. The fluorescence is measured in a standard fluorometer with the excitation wavelength at 390 nm and the emission at 475 nm. Fluorescamine is used in large excess because of its high rate of hydrolysis.

Reaction

Fluorescamine (intrinsically non-fluorescent) reacts amino acids containing primary amines, such as lysine and N-terminal amino acid, to yield a highly fluorescent product (Figure 2.9). The reaction is very rapid. At room temperature, the reaction is complete in a fraction of a second, and the excess reagent is concomitantly destroyed to a non-fluorescent product.[32] The fluorescent product is stable for several hours and is proportional to the amine concentration.

Advantages

The assay is sensitive (detection of protein in nanogram range). The reaction is instantaneous, so the assay is performed in a few minutes.

Disadvantages

The assay has a moderate protein-to-protein variation. The reagent is hydrolyzed very rapidly, and thus rapid mixing is essential for reproducible results. As the
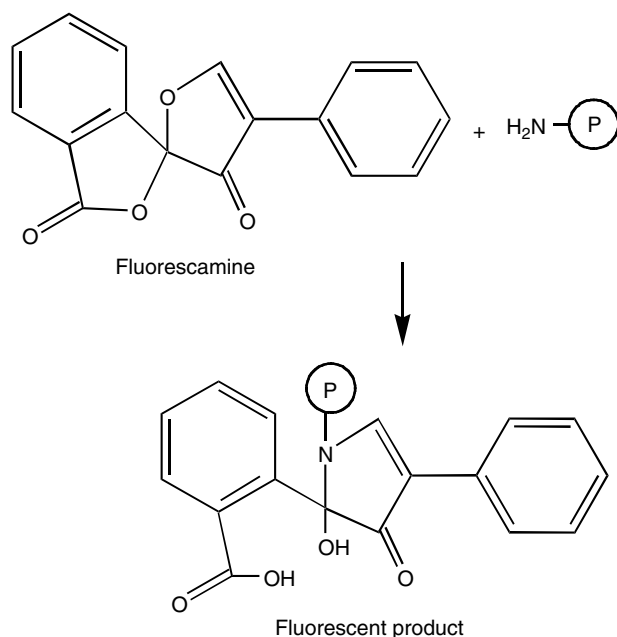
FIGURE 2.9 Non-fluorescent fluorescamine reacts with the primary amine of proteins, yielding a fluorescent product.

fluorescamine reacts with the primary amine-containing amino acids, primary amine buffers such as Tris and glycine are not compatible with the assay.

Working Procedure

Equipment

Spectrofluorometer

Reagents

1. Assay buffer: 0.05 M sodium phosphate buffer, pH 8.
2. Fluorescamine reagent: dissolve 30 mg of reagent in 100 ml dioxane or acetone.

Assay

1. Take standard protein (BSA, 0.5 to 50 μg) in duplicate in test tubes as follows:

For BSA:

Amount (μg)	0.5	2	5	10	20	30	40	50

Take BSA (1 mg/ml):

Volume (μl)	0.5	2	5	10	20	30	40	50
Assay Buffer (μl)	1499.5	1498	1495	1490	1480	1470	1460	1450

2. Take unknown sample (1 to 50 μl) and bring volume to 1.5 ml with the assay buffer.
3. Take same volume of sample buffer only as the unknown sample volume and bring volume to 1.5 ml with the assay buffer.
4. Take 1.5 ml of assay buffer as blank.
5. Rapidly add 0.5 ml of fluorescamine reagent to each tube and mix immediately since the reagent is hydrolyzed very rapidly.
6. After 5 min incubation at room temperature, measure the reaction tube in a spectrofluorometer with the excitation wavelength at 390 nm and the emission at 475 nm.
7. Correct data for unknown sample by subtracting value for sample buffer.
8. Prepare standard curve for the known amount of standard protein and determine protein concentration of the unknown from the plot.
   (**Note:** best results are achieved when the standards are prepared in the same buffer as the sample. In that case, step 3 is used as blank. Steps 4 and 7 are not necessary.)

2.3.2 *O*-PHTHALALDEHYDE PROTEIN ASSAY

The protein assay using *o*-phthalaldehyde is fast and sensitive. The reaction is complete in less than 1 min. In the standard assay, protein can be detected as low as 10 μg/ml. In the microassay the lower detection limit can be extended down to 50 ng/ml. Pierce's Fluoraldehyde Protein/Peptide assay is based on *o*-phthalaldehyde.

Reaction

This involves the use of *o*-phthalaldehyde, which can react with primary amines in proteins. Upon reaction with primary amines in the presence of mercaptoethanol, *o*-phthalaldehyde produces a blue fluorescent product (Figure 2.10) that has maximum excitation at 340 nm and maximum emission at 455 nm.[33] This method is good for protein free of tyrosine residue.

Advantages

The assay is sensitive (detection of protein in nanogram range). The reaction is instantaneous, so the assay is performed in a few minutes. Unlike fluorescamine, *o*-phthalaldehyde is stable. Reducing agents, metal chelators, and most detergents are compatible with the assay. But these should be included in the blank. Most common buffers and constituents are also compatible.
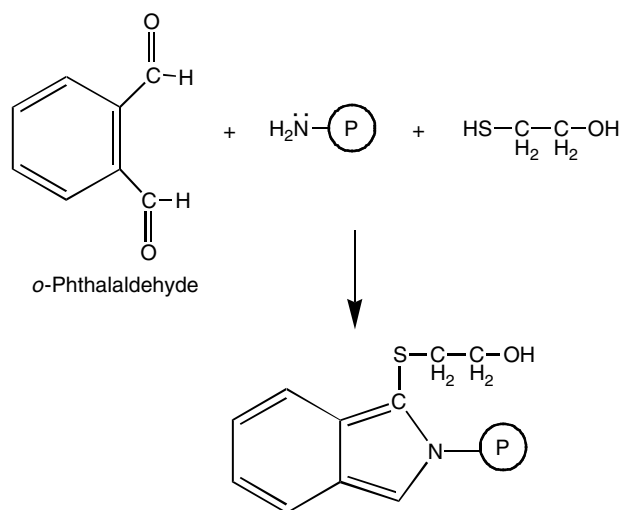
FIGURE 2.10 *o*-phthalaldehyde reacts with the primary amines in proteins in the presence of mercaptoethanol, producing a blue fluorescent product.

Disadvantages

The assay has a moderate protein-to-protein variation. As the fluorescamine reacts with the primary amine containing amino acids, primary amine buffers such as Tris and glycine are not compatible with the assay.

Working Procedure

Equipment

Spectrofluorometer

Reagents

1. Assay buffer: 0.1 M sodium phosphate buffer (pH 7.0).
2. *o*-phthalaldehyde reagent: dissolve 80 mg of *o*-phthalaldehyde in 2 ml 95% ethanol, 50 ml of 0.1 M sodium tetraborate, 5 ml of 20% (w/w) SDS, 0.2 ml of β-mercaptoethanol. Add water to get 100 ml. Store the solution at dark.

Assay

1. Take standard protein (BSA, 0.5 to 25 μg) in duplicate in test tubes as follows:

For BSA:

Amount (µg)	0.5	1	5	10	15	20	25

Take BSA (1 mg/ml):

Volume (µl)	0.5	1	5	10	15	20	25
Assay buffer (µl)	49.5	49	45	40	35	30	25

2. Take unknown sample (1 to 50 µl) in assay buffer.
3. Take 50 µl of assay buffer as blank.
4. Add 3 ml of *o*-phthalaldehyde reagent to each tube and mix immediately.
5. After 5 min incubation at room temperature, measure the reaction tube in a spectrofluorometer with the excitation wavelength at 340 nm and the emission at 455 nm.
6. Prepare standard curve for the known amount of standard protein and determine protein concentration of the unknown from the plot.

2.3.3 CBQCA Protein Assay

You et al.[34] developed a sensitive assay for quantitation of proteins by using 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA). The assay is marketed by Molecular Probes. The assay is linear and detects a broad range of protein from 10 ng to 150 µg. The assay is usually performed at high pH. The sensitivity decreases at low pH, possibly because amines are protonated at lower pH.

Reaction

CBQCA is intrinsically non-fluorescent but becomes highly fluorescent upon binding with amines in the presence of cyanide or thiols (Figure 2.11). The fluorescent product (CBQCA-protein adduct) has a broad absorption peak, excitable at 430 to 490 nm with maximum emission at around 560 nm.

Advantages

Assay is linear for a broad range of protein (10 ng to 150 µg). The assay works well in the presence of lipids known to interfere in most protein determination methods.

Disadvantages

Buffers containing primary amines (Tris, glycine), ammonium ions, or a high concentration of thiols (dithiothreitol, mercaptoethanol) are not compatible. The assay requires a long incubation (at least 90 min at room temperature).

Working Procedure

Equipment

Fluorescence microplate reader such as Cytofluor
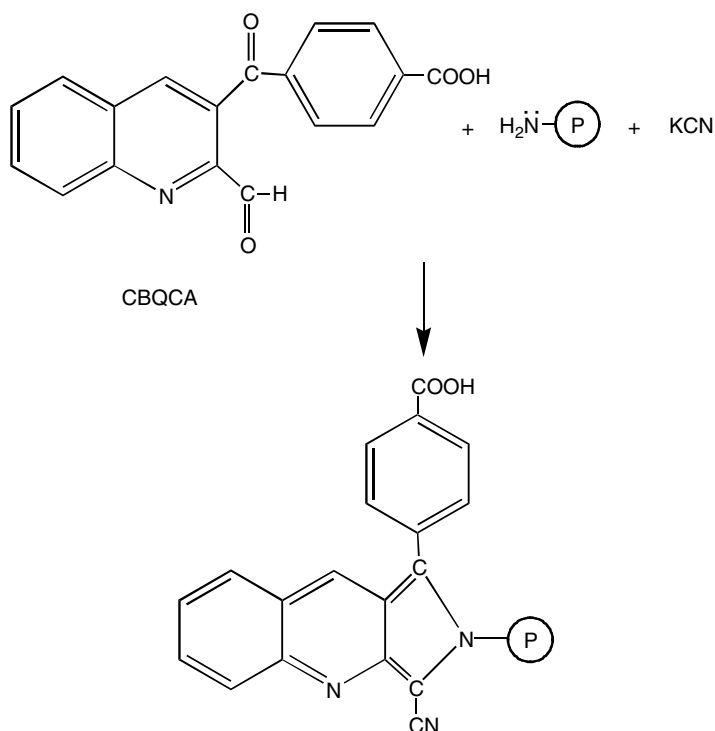
FIGURE 2.11 CBQCA reacts with the primary amine-containing amino acids in proteins to yield amine derivative of CBQCA.

Reagents

1. Assay buffer: 0.1 M sodium borate buffer, pH 9.3.
2. 10 mM KCN.
3. CBQCA reagent: prepare 40 mM CBQCA in dimethyl sulfoxide. Dilute eightfold in assay buffer to get 5 mM CBQCA.

Assay

1. Prepare standard protein solution such as BSA and unknown sample in assay buffer. Take standard protein (10 ng to the desired amount within the assay range) to the microtiter plate wells. Add buffer to obtain 80 µl.
2. Add 10 µl of 10 mM KCN (dissolved in water) to each well.
3. Add 10 µl of 5 mM CBQCA to each well.
4. Shake the plate and incubate at room temperature in the dark for 90 min. Alternatively, heat the plate in a microwave oven at full power (generally 1,140 w, 2,450 mHz) for 30 sec at a time for four times with short pause between heating. Place a beaker containing 100 ml of water in the

microwave oven at the same time, as recommended by Akins and Tuan.[35] Allow the plate to cool to room temperature and measure fluorescence intensity using Cytofluor fluorescence reader using 480±10 nm excitation filter and 530 ± 12.5 nm emission filter.

2.3.4 NanoOrange[R] Protein Assay

Molecular Probes' NanoOrange[R] protein assay can detect protein as low as 10 ng/ml. The assay also offers low protein-to-protein signal variability. For the assay, the protein sample is added to the diluted NanoOrange[R] reagent, and the mixture is then heated at 95°C for ten minutes. Fluorescence can be measured as soon as the temperature of the assay mixture drops to room temperature. The reagent product is stable up to 6 h, and thus the fluorescence can be measured any time before 6 h.

Advantages

The assay has low protein-to-protein signal variability. It is compatible with reducing agents.

Disadvantages

The assay is not compatible with detergents (see Table 2.2).

Working Procedure

Equipment

Fluorescence microplate reader such as Cytofluor

Reagents and Assay

The procedures for reagent preparation and assay are available from Molecular Probes.

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3 Electrophoretic Analyses of Protein

In electrophoresis, proteins are separated in an electric field by virtue of their charge and size. Electrophoresis of macromolecules is normally carried out by applying a thin layer of a sample to a solution stabilized by a porous matrix. The matrix can be composed of a number of different materials including paper, cellulose, acetate, or gels made of starch, agarose, or polyacrylamide. Agarose and polyacrylamide can act as a size-selective sieve in the separation. However, polyacrylamide is the most common matrix for separating proteins, probably due to its versatile applications.

3.1 DRIVING FORCE OF ELECTROPHORESIS

The movement of molecules in electrophoresis is dependent on the applied voltage (V), which equals the product of current (I) and resistance (R).

$$V = IR \text{ (Ohm's Law)}$$

The following power equations are also used in electrophoresis:

$$P = VI \text{ or } P = I^2R \text{ or } P = V^2/R$$

where P = power, which provides amount of heat produced in the circuit.

$$H = I^2RT, H = \text{heat produced over time (T).}$$

In electrophoresis, voltage and current are supplied by a DC (direct current) power supply, and the electrodes, buffer, and gel are considered to be resistors. Power supply is used to hold one electrical parameter (current, voltage, or power) constant. Most power supplies have more than one pair of outlets. When two gels are connected in parallel to one outlet of a power supply, gel currents are additive. When two gels are connected in series to one outlet of a power supply, gel voltages are additive. Gel currents are additive when two gels are connected in parallel to adjacent outlets of a power supply.

Choice of Driving Force: Constant Current or Constant Voltage?

The resistance of the circuit does not remain constant during electrophoresis. For example, in a discontinuous system (separating and stacking gels) of SDS-PAGE
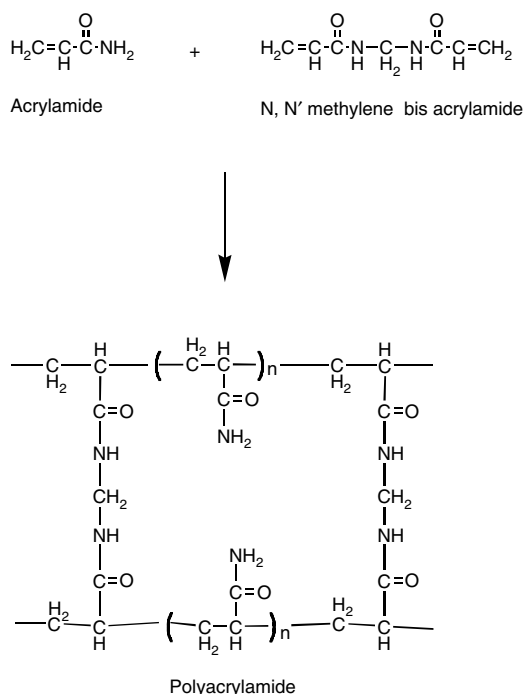
FIGURE 3.1 Polymerization of acrylamide.

running at constant current, resistance increases. Therefore, voltage will increase over time, leading to increased heat generation and may require active heat removal. When running SDS-PAGE at constant voltage, current drops as the resistance increases. This will not result in a high increase of heat, since the main determinant factor (square root of current) is decreased, although resistance is increased. In contrast, in a continuous system (only separating gel) of SDS-PAGE, resistance decreases, resulting in a heat gain when running at constant voltage.

3.2 POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide (Figure 3.1) gels are formed by copolymerization of acrylamide monomer, $CH_2 = CH\text{-}CONH_2$, and a cross-linking comonomer, N,N′-methylenebisacrylamide, $CH_2 = CH\text{-}CO\text{-}NH\text{-}CH_2\text{-}NH\text{-}CO\text{-}CH = CH_2$ (bisacrylamide).

Mechanism of Gel Formation

The mechanism of gel formation is vinyl addition polymerization and is catalyzed by a free radical-generating system composed of ammonium persulfate (the initiator) and an accelerator, N,N,N′,N′-tetramethylethylenediamine (TEMED). TEMED catalyzes the decomposition of the ammonium persulfate to yield a free radical (unpaired electron), which activates the acrylamide monomer. The activated

monomer then reacts with an unactivated monomer to begin the polymer chain elongation as shown below:

$$(NH_4)_2S_2O_8 + e^- \rightarrow (NH_4)_2SO_4 + SO_4^{-\cdot}$$
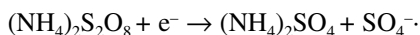
If S represents $SO_4^{-\cdot}$, its reaction with acrylamide monomer (A) can be written as follows:

$$S\cdot + A \rightarrow SA\cdot$$

$$SA\cdot + A \rightarrow SAA\cdot$$

$$SAA\cdot + A \rightarrow SAAA.$$
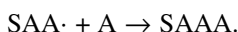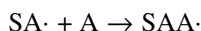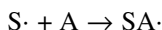
and so on.

During the polymer chain elongation, bisacrylamide is randomly cross-linked, resulting in closed loops and a complex "web" polymer (see Figure 3.1) with a characteristic porosity, which depends on the polymerization conditions and monomer concentrations.

In some applications (e.g., acid urea PAGE), riboflavin (or riboflavin-5′-phosphate) is used as an initiator of polymerization of acrylamide, as ammonium persulfate interferes with the stacking of the protein. In the presence of light and oxygen, riboflavin is converted to its leuco form, which is active in initiating polymerization.

Oxygen, a radical scavenger, interferes with polymerization, so that proper degassing to remove dissolved oxygen from acrylamide solutions is crucial for reproducible gel formation.

The effective pore size depends on the acrylamide concentration of a gel. The pore size decreases as the acrylamide concentration increases. Usually, gels are characterized by the two parameters, %T and %C, where %T refers to the total monomer (acrylamide + cross-linker), and %C is the ratio of cross-linker (i.e., bisacrylamide) to acrylamide monomer (w/w). The following formulas are used to calculate:

$$\%T = \frac{\text{Acrylamide}(g) + \text{Bisacrylamide}(g)}{\text{Volume}(ml)} \times 100$$

$$\%C = \frac{\text{Bisacrylamide}(g)}{\text{Acrylamide}(g) + \text{Bisacrylamide}(g)} \times 100$$

The effective pore size is established by the three-dimensional network of fibers and pores that are formed by cross-linking acrylamide with bifunctional bisacrylamide.

When the gel is poured into a tube or slab mold, the top of the solution forms a meniscus. If the meniscus is ignored, the gel will polymerize with a curved top,

TABLE 3.1
Various PAGE Systems and Their Applications

PAGE System	Application	Comment
SDS-PAGE (Laemmli)	Determination of subunit molecular weight Homogeneity test of a purified protein	Native protein activity is lost Not suitable for low molecular weight proteins/peptides (<10 kDa)
SDS-urea PAGE	Separation of membrane proteins Suitable for low molecular weight proteins	—
Non-denaturing PAGE	Homogeneity test of a purified protein	Native protein activity usually retained Not reliable for molecular weight estimation
Tricine PAGE	Separation of low molecular weight proteins/peptides (1 – 40 kDa range)	Protein band in the gel can be excised for amino acid sequencing without significant interference.
Non-urea SDS-PAGE (modified Laemmli)	Separation of low molecular weight proteins/peptides (as low as 5 kDa)	—
Acid-urea PAGE	Separation of basic proteins such as histones	Long run Proteins move toward cathode Electrode connection is opposite to the SDS-PAGE configuration
CTAB-PAGE	Determination of native molecular weight Native activity assay	Proteins move toward cathode Electrode connection is opposite to the SDS-PAGE configuration

which will cause the separated sample bands to have a similar curved pattern. To eliminate the meniscus, a thin layer of water, water-saturated n-butanol, or isopropanol is carefully floated on the surface of the gel mixture before it polymerizes. The layer of water or water-saturated butanol should be deaerated; otherwise, it will inhibit polymerization on the gel surface.

Various polyacrylamide gel electrophoresis (PAGE) systems are known, and the choice of PAGE depends on the nature of the protein sample and the applications after electrophoresis (see Table 3.1).

3.2.1 PAGE under Denaturing Conditions (SDS-PAGE)

Denaturing PAGE in the presence of sodium dodecyl sulfate (better known as SDS-PAGE) is a low-cost, reproducible, and rapid method for analyzing protein purity and for estimating protein molecular weight.[1] SDS-PAGE is also employed for the following: (a) monitoring protein purification; (b) verification of protein concentration; (c) detection of proteolysis; (d) detection of protein modification; and (e) identification of immunoprecipitated proteins. SDS-PAGE can also be performed in a preparative mode to obtain sufficient protein for further studies. After electrophoresis the protein of interest is recovered from polyacrylamide by electroelution
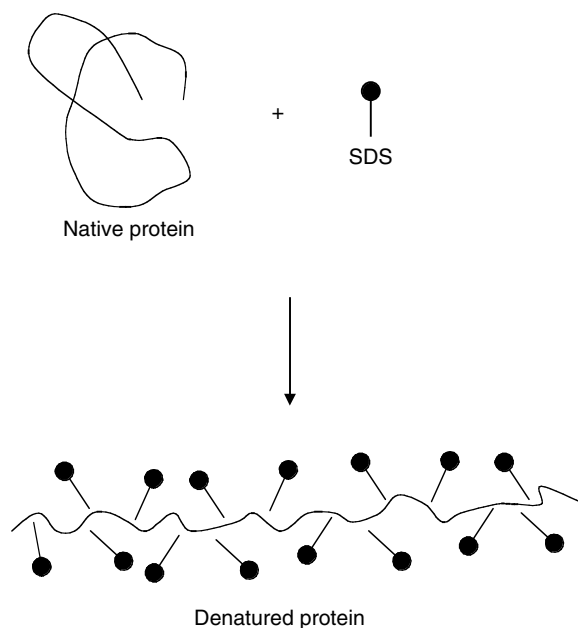
FIGURE 3.2 Denaturation of protein with sodium dodecyl sulfate, creating a highly negative charged molecule.

(see Section 3.2.1.7). The protein that is obtained by this process is generally used for raising antibodies or sequencing.

Mechanism

In SDS-PAGE, the sample applied to the electrophoresis has been treated with sodium dodecyl sulfate, an anionic detergent. This detergent denatures the proteins in the sample and binds strongly to the uncoiled molecule. Approximately one SDS molecule binds per two amino acids. The SDS molecules mask the surface charge of the native proteins and create a net negative charge resulting from the sulfate groups on the SDS molecule (Figure 3.2). Therefore, charge/size ratio is equal for all proteins, and separation can be achieved only on the basis of size. Low molecular weight proteins travel faster in the gel, and proteins of high molecular weight move slower in the gel. Because proteins are separated on the basis of size, their molecular weights can be estimated by running appropriate standard proteins of known molecular weights on the same gel.

The quality of the SDS is very important, because differential protein-binding properties of impurities such as C10, C14, and C16 alkyl sulfates can cause single proteins to form multiple bands in gels.

Electrophoresis can be performed in two buffer systems: continuous and discontinuous. A continuous system has only a single separating gel and uses the same buffer in the tanks and the gel. The widely used SDS-PAGE, a modification by
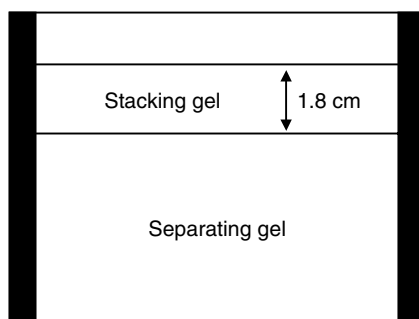
FIGURE 3.3 Gel plate, showing the location of separating and stacking gels.

Laemmli[1] from those described in Ornstein[2] and Davis,[3] is a discontinuous system consisting of two contiguous but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel (Figure 3.3). The two gels are cast with different porosities, pH, and ionic strength. In addition, different mobile ions are used in the gel and electrode buffers.

How Are Proteins Concentrated in the Stacking Gel?

The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in better resolution than is possible using the same sample volumes in gels without a stacking gel. Proteins, once concentrated in the stacking gel, are separated in the resolving gel. In SDS-PAGE, samples prepared in a low-conductivity buffer (0.06 M Tris-HCl, pH 6.8) are loaded between the higher conductivity electrode buffer (0.025 M Tris, 0.192 M glycine, pH 8.3) and the stacking gel buffer (0.125 M Tris-HCl, pH 6.8). When power is applied, a voltage drop develops across the sample solution, which drives the proteins into the stacking gel. During electrophoresis, glycinate ions from the electrode buffer follow the proteins into the stacking gel. Between the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear, a high-voltage gradient forms. This causes SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and the glycinate ions. Most proteins usually move in the stacking gel (3 to 4%) due to large pore size, but at the interface of the stacking and resolving gels, the proteins experience a sharp increase in retardation due to the restrictive pore size of the resolving gel. In the resolving gel, the glycinate ions overtake the proteins, which continue to be slowed by the sieving of the matrix. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular weight. The molecular weight ranges of proteins that are separated depend on the percentage of the acrylamide gel (Table 3.2).

SDS-PAGE yields the molecular weight of the subunit that is non-covalently linked. To obtain the molecular weight of the subunit that is linked by disulfide bond, the presence of a reducing agent, such as 2-mercaptoethanol or dithiothreitol

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TABLE 3.2
**Optimal Resolution Ranges of Protein Molecular Weights vs.
Percentage of Acrylamide Gel[a]**

Percentage (%) of Acrylamide Gel	Separating Resolution (kDa)
15	15–45
12.5	15–60
10	18–75
7.5	30–120
5	60–212

[a] Adapted from Reference 4.

(DTT), is necessary in the sample buffer. The reducing agent breaks the disulfide bonds in the protein as follows:

$$P_1\text{–S–S–}P_2 + RSH \rightarrow P_1\text{–SH} + P_2\text{–SH}$$
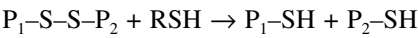
Of two gel formats (tube gel and slab gel), slab gels (formed between two sheets of supporting glass) are most widely used, since many samples can be run on the same gel, thereby providing uniformity during polymerization, staining, and destaining. Reagents for SDS-PAGE and electrophoresis cells of various designs are available from several vendors. For most analytical applications, the mini slab gel (8×10 cm) is generally used, due to the increased resolution and reduced amounts of time and materials needed for electrophoresis. The experimental procedures and reagents described below have been calculated for a mini gel system; however, working procedures for other systems are adapted easily.

3.2.1.1 Preparation of Gels

Polyacrylamide gels with various pore sizes are made by varying the concentration of acrylamide. The choice of acrylamide concentration is determined by the molecular weight range of proteins to be separated (see Table 3.2). Recipes for making gels of varying concentration are shown in Table 3.3. Gels of fixed acrylamide concentrations are typically used on a daily basis because they are simple to prepare. When proteins of broad molecular weight range or higher resolution are desired, gradient polyacrylamide gels can be made (see Section 3.2.3). In practice, acrylamide solution (from the recipe in Table 3.3) is poured into a cassette made by joining two gel plates (usually made of glass) to form a separating gel (Figure 3.4 A). Spacers are placed between plates to make the cassette. Once the separating gel is polymerized, stacking gel is then made on top of the separating gel (Figure 3.4 B). Gels can be made in various thicknesses according to the thickness of the spacers (0.75 mm and 1.5 mm are common). Single or multiple gels can be made at a time. Gel casters of various sizes are commercially available for this purpose.

TABLE 3.3
Recipes for Polyacrylamide Separating Gel

Stock Solutions	Final Acrylamide Concentration in the Separating Gel										
	5	6	7	8	9	10	12	13	15	17	20
30% acrylamide/0.8% bisacrylamide	2.50	3.00	3.50	4.00	4.50	5.00	6.00	6.50	7.50	8.5	10.00
4× separating gel buffer	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75
Water	8.75	8.25	7.75	7.25	6.75	6.25	5.25	4.75	3.75	2.75	1.25
10% ammonium persulfate	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
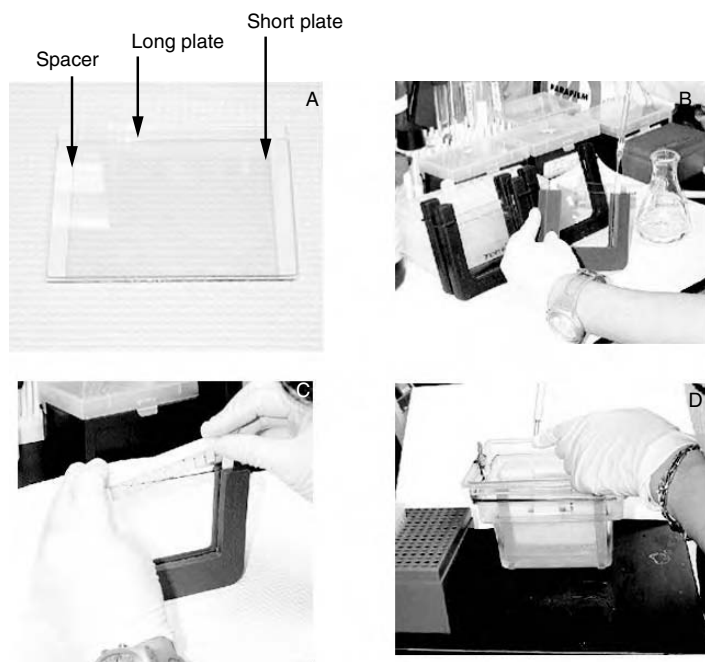
FIGURE 3.4 Preparation of gel and loading samples on the gel. A, Gel cassette; B, Pouring stacking gel on top of separating gel; C, Inserting comb into the stacking gel; and D, Loading samples into wells.

Working Procedure

Equipment

1. Glass plates
2. Spacers
3. Comb
4. Gel caster (Hoefer, Bio-Rad)

Reagents

1. Acrylamide, electrophoresis grade
2. Bisacrylamide (N,N′-methylene bisacrylamide)
3. Tris (2-hydroxymethyl-2-methyl 1,3 propanediol)
4. SDS (Sodium dodecyl sulfate or sodium lauryl sulfate)
5. TEMED (N,N,N′,N′-tetramethylethylenediamine)
6. Ammonium persulfate
7. 2-Mercaptoethanol
8. Glycerol
9. Bromophenol blue

10. Glycine
11. Hydrochloric acid (HCl)
12. Dithiothreitol

Stock Solutions

1. 2 M Tris-HCl (pH 8.8), 1 liter: weigh out 242 gram Tris base and add 900 ml distilled water. Adjust pH to 8.8 by adding concentrated HCl slowly and finally add distilled water to a total volume of 1 liter.
2. 1 M Tris-HCl (pH 6.8), 100 ml: to 12.1 gram of Tris base add 80 ml distilled water and adjust pH to 6.8 with concentrated HCl. Add distilled water to a total volume of 100 ml.
3. 10% SDS (w/v), 100 ml: weigh out 10 gram SDS and add distilled water to a total volume of 100 ml. Store solution at room temperature.
4. 50% glycerol (v/v), 100 ml: add 50 ml distilled water to 50 ml 100% glycerol.
5. 1% bromophenol blue (w/v), 10 ml: weigh out 100 mg bromophenol blue and add 10 ml distilled water. Stir until dissolved and filter to remove particulates.

Working Solutions

1. 30% acrylamide stock solution: dissolve 29.2 gram acrylamide and 0.8 gram bisacrylamide with distilled water to make 100 ml. The solution is stable at 4°C for months.
   (**Note:** unpolymerized acrylamide is a skin irritant and a neurotoxin and thus should be handled with gloves. Use of a mask is also recommended to avoid inhalation. Unused acrylamide solution should be polymerized and disposed of with solid waste.)
2. 4× separating gel buffer, 100 ml:
   a. 75 ml 2M Tris-HCl (pH 8.8); final conc. 1.5 M
   b. 4 ml 10% SDS; final conc. 0.4%
   c. 21 ml water
      (The solution is stable for months at 4°C.)
3. 4× stacking gel buffer, 100 ml:
   a. 50 ml 1 M Tris-HCl (pH 6.8); final conc. 0.5 M
   b. 4 ml 10% SDS; final conc. 0.4%
   c. 46 ml water
      (The solution is stable for months at 4°C.)
4. 10% ammonium persulfate, 5 ml: dissolve 0.5 gram ammonium persulfate in 5 ml distilled water. Aliquot 100 μl in 0.5 ml microfuge tubes and store at –20°C (stable for months).
5. Electrophoresis buffer, 1 liter:
   a. 3 gram Tris base; final conc. 25 mM
   b. 14.4 gram glycine; final conc. 192 mM

    c. 1 gram SDS; final conc. 0.1%

    d. Water to make 1 liter

       (pH should be approximately 8.3; 10× stock solution can also be made and stored at 4°C; stable for months.)

6. 5× sample buffer, 10 ml:

    a. 0.6 ml 1M Tris-HCl (pH 6.8); final conc. 60 mM

    b. 5 ml 50% glycerol; final conc. 25%

    c. 2 ml 10% SDS; final conc. 2%

    d. 0.5 ml 2-mercaptoethanol; final conc. 14.4 mM

    e. 1 ml 1% bromophenol blue; final conc. 0.1%

    f. 0.9 ml water

       (Aliquot in small volume in microfuge tubes and store at –20°C. Stable for months.)

Procedure

Making the Gel Sandwich

1. Clean the gel plates with detergent and dry.
2. Make a sandwich with two gel plates by placing spacers (0.75 or 1.5 mm) between them.
3. Assemble single or multiple sandwiches into the appropriate gel caster following the manufacturer's instructions (see Figure 3.4 A).
   (**Note:** preparation of multiple gels has advantages. Since all gels are identical, the effect of gel-to-gel variation in protein separation is minimum. Additionally, the time to make multiple gels compared to single gel is only slightly more.)

Pouring the Separating Gel

4. From the recipe in Table 3.3, combine the appropriate volume of gel components without ammonium persulfate and TEMED in a 250 or 500 ml Erlenmeyer flask with a side tube. The volume of the gel components depends on number and percentage of gels.
5. Cover the flask with a rubber cork and degas the solution under vacuum for 5 to 10 min by connecting the side tube to a laboratory vacuum line.
6. Add appropriate volume of ammonium persulfate and TEMED (see Table 3.3) and mix by swirling. Work without delay at this point, as polymerization begins upon addition of ammonium persulfate and TEMED.
7. Carefully pour the gel solution into gel sandwich using a pipette by touching with the surface of the glass plate (see Figure 3.4 B). Pouring the gel this way minimizes the formation of air bubbles. Do not fill the whole cassette. Leave empty about 1.5 cm from top of the sandwich for making stacking gel (on top of the separating gel).

8. Without any delay, gently layer 100 to 200 µl of isopropanol (for mini gel, 0.75 mm thickness) on top of the separating gel solution. This allows the gel surface to remain flat.

9. Allow gel to polymerize at room temperature for 30 to 60 min. During polymerization, the gel sandwich should not be disturbed. Leave the unused gel solution in the flask to allow polymerization and discard. This serves two purposes. Unlike acrylamide monomer, polyacrylamide is not toxic and thus can be considered as non-hazardous waste. Second, it serves as a check for polymerization. At this point, the gel sandwich can be checked for polymerization. Once the acrylamide solution has been poly-merized, a distinct interface should appear between the separating gel and the isopropanol. At this point, isopropanol can be flicked off, and after washing the gel surface with 1× separating gel buffer, stacking gel can be added. Separating gels can be stored for several weeks at 4°C. If separating gels are to be stored, replace isopropanol with 1× separating gel buffer and cover with parafilm or plastic wrap.

Pouring the Stacking Gel

10. Combine the following reagents in a 50 ml sidearm flask:
    a. 30% acrylamide stock solution: 0.65 ml
    b. 4× stacking gel buffer, pH 6.8: 1.25 ml
    c. Water: 3.05 ml
       (The solution is good for making two stacking gels for mini gels of 0.75 mm thickness).

11. Degas under vacuum for 10 to 15 min at room temperature.

12. Add 25 µl of 10% ammonium persulfate and 5 µl of TEMED, mix by swirling.

13. Rapidly add stacking gel solution on top of separating gel. Fill up the empty space completely.

14. Carefully insert comb into gel sandwich (Figure 3.4 C). Make sure not to trap air bubbles in between teeth. The distance between the separating gel and the bottom of the comb teeth should be about 0.5 cm.

15. Allow stacking gel to polymerize at room temperature for about 30 min.

3.2.1.2 Running Gels

Once a gel has been made, the comb is removed from the gel. After washing the wells with the electrophoresis buffer, the gel sandwich is placed in the electrophoresis tank. The tank is filled with the electrophoresis buffer following the manufacturer's instructions. Electrophoresis tanks from various commercial sources vary in size and shape. But, in all cases, the gel sandwich is submerged in the electrophoresis buffer and protein samples are loaded into wells through the buffer. Once samples are loaded, the electrophoresis lid is carefully closed; the chamber electrodes are attached to the power supply.

Working Procedure

Equipment

1. Mini gel apparatus (Hoefer, Bio-Rad, Life Technologies**)**
2. Power supply (capacity 200 V)
3. Heat block (100°C) or boiling water bath
4. Disposable gel loading tips for micropipette or Hamilton syringe
5. Eppendorf tubes
6. Plastic or glass container with lid
7. Rocking shaker
8. Gel dryer

Reagents

1. Electrophoresis buffer (see Working Solutions, Section 3.2.1.1)
2. Sample buffer (see Working Solutions, Section 3.2.1.1)
3. Protein markers (Bio-Rad, Sigma, Invitrogen)

Procedure

1. With marker place a line indicating the bottom of each well on the front plate. This marking provides a guide for loading the samples onto wells. Alternatively, bromophenol blue to a final concentration of 1 mg/ml in the stacking gel can be added to visualize the gel wells.[5]
2. Remove the comb from the stacking gel and rinse the wells with 1× running electrophoresis buffer.
3. Place the gel sandwich in the electrophoresis tank following manufacturer's protocol.
4. Fill up the electrophoresis chamber with 1× electrophoresis buffer according to the manufacturer's instructions.
5. Prepare the protein samples as follows:
   a. Mix protein sample or standard protein and 5× sample buffer (4:1 ratio) in Eppendorf tube. (For 10-well comb and 0.75 mm gel thickness, 20 µl of protein sample and 5 µl sample buffer can be mixed and after heating 5 to 20 µl can be loaded on each well. Amount of protein to be loaded depends on the gel thickness, size of the comb, and the staining method. For 0.75-mm-thick gel with 10-well comb, 5 to 25 µg of protein is recommended per well for complex protein when staining with Coomassie blue. If silver staining is used, 10- to 100-fold less protein can be applied.)
   b. Heat the mixture at 100°C for 2 to 5 min.
   c. Spin down protein solution for a second in a microfuge. The sample is now ready to load on gel.

6. Load protein sample (as prepared in the previous step) onto well using a disposable gel loading tip or a Hamilton syringe as shown in Figure 3.4 D. Carefully layer protein sample (without introducing any bubbles) onto bottom of the well.
7. Load also a mixture of protein standards into a separate well. Mixture of protein standards in various molecular weight ranges is commercially available, such as Sigma, Bio-Rad, and Novex.
8. Connect the power supply to the electrophoresis chamber. Ensure the electrodes connection is in correct polarity (red to red, black to black). In SDS-PAGE, all proteins move to the anode (+).
9. Turn on power supply to 100 volt when GIBCO BRL apparatus is used. Once the sample passes stacking gel, voltage can be increased to 150 volt. When running gel at constant current at 20 to 30 mA (e.g., Hoefer mini gel system), the temperature of the running buffer should be controlled (10 to 20°C) with a circulating cold-water bath (usually set at 10°C) to prevent "smiling" (curvature of the migratory band).
10. Disconnect the power supply when bromophenol blue tracking dye reaches the bottom of the separating gel.
11. Remove the gel sandwich and orient the gel so that the order of the sample wells is known.
12. Carefully slide one of the spacers halfway along the edge of the sandwich and, using the exposed spacer as a lever, pry open the glass plate to obtain gel.
13. Mark one corner by cutting gel (a small triangle) to avoid any loss of sample well orientation during staining.
14. Carefully remove the gel from the plate and proceed with the staining.

3.2.1.3 Detection of Proteins in Gel

After electrophoresis, proteins are detected on the gel by using various stains (Coomassie blue, silver, Amido Black, etc.). Staining with Coomassie blue is rapid and the most common protein stain for routine work (Table 3.4). Compared to Coomassie stain, silver staining is a time-consuming, but more sensitive, method for staining proteins in gels. Silver staining should be used to assess the purity of a protein preparation, such as antigen preparation for development of polyclonal antibodies. Reversible stains such as Ponceau and India ink are generally used to visualize protein bands in gels prior to Western transfer or on membranes prior to protein elution (see Section 3.5). Protein staining with Procion blue can be used for quantification of protein in gels.[6] Many stains are commercially available (Table 3.5).

3.2.1.3.1 *Coomassie Brilliant Blue Stain*
Coomassie Brilliant Blue dye (see Figure 2.6 A) is widely used to visualize proteins in polyacrylamide gels.[7] The staining is simple and can detect as little as 0.1 μg of protein in a single band. The dye binds primarily to positively charged amino acids, such as lysine and arginine. Thus, basic proteins tend to stain more strongly than acidic proteins.[8] The advantage of the Coomassie stain is that it is rapid and

TABLE 3.4
Characteristics of Various Protein Stains

Stain	Sensitivity	Time Required	Advantage	Disadvantage	Recommended Application
Coomassie	100 ng	5–10 min staining 1–3 h destaining	Rapid Low cost After Coomassie stain, gel can be silver stained	Accumulates large volume of methanol present in staining and destaining solution	Routine work
EZBlue™ (Sigma)	2 ng	5–10 min	No fixation step	Expensive	Routine work
Colloidal Coomassie	10 ng	5–10 min	No destaining step	Fixation required	Routine work
CBB in acid	20 ng	30–60 min	No fixation step No destaining step	Longer staining time	Quantitation of protein in gel
Silver (alkaline method)	0.1 ng	2 h	Most sensitive, when sensitized with glutaraldehyde prior to staining	Complex reagent preparation, unstable reagent	To assess purity of protein preparation
Silver (acid method)	0.6–1.2 ng	90 min	Few steps Simple reagent preparation	Less sensitive than alkaline silver stain method	To assess purity of protein preparation
Zinc	5 ng	25–40 min	No fixation step Elution of unstained protein	Multiple steps when toning reaction intended	Peptide sequencing, antibody development
Nile Red	100 ng	As little as 6 min	Rapid	UV light box and camera required for documentation	Routine work
Calconcarboxylic acid	10 ng (during electrophoresis) 25 ng (post electrophoresis)	30–70 min	Migration of stained proteins during electrophoresis	Simultaneous staining is less sensitive than post staining	Routine work
Eosin Y	10 ng	30 min	Antigenicity of the stained protein retained	Transilluminator required	Antibody development
Procion blue	100 ng	1.5 h staining 48 h destaining	Proteins in gel can be quantitated	Time consuming	Quantitation of proteins
Amido Black	>100 ng	2–4 h	—	Less sensitive	—
Fast Green FCF	200 ng	2–4 h	—	Less sensitive	—

TABLE 3.5
Commercial Stains of Protein on Gels

Based on	Stain	Vendor
Coomassie Blue	GelCode^R Blue Stain	Pierce
	(Colloidal properties of Coomassie G-250)	
	InstaStain™ Blue Gel Stain Paper	Pierce
	(Coomassie dye in a solid phase)	
	Coomassie	Bio-Rad
	Bio-Safe Coomassie	Bio-Rad
	EZ Blue™ Gel Stain	Sigma
Silver	GelCode^R Silver Stain	Pierce
	GelCode^R SilverSNAP™ Stain	Pierce
	Silver Stain (Meril)	Bio-Rad
	Silver Stain Plus (Gottlieb and Chavco)	Bio-Rad
Zinc Reverse	GelCode^R E-Zinc™ Reversible Stain	Pierce
	Zinc Stain	Bio-Rad
Fluorescent	SYBR Red (Based on Nile Red)	Molecular Probes
	SYPRO^R Tangerine	Molecular Probes
	SYPRO^R Orange	Molecular Probes
	SYPRO^R Red	Molecular Probes
Luminescent	SYPRO^R Ruby Stain	Molecular Probes

flexible. The gel can be stained in 5 to 10 min, followed by destaining that requires about 1 to 2 h. Coomassie blue turns the entire gel blue, and after destaining, the blue protein bands appear against a clear background. If staining appears to be incomplete after destaining, gel can be restained. If staining is not sensitive enough to detect all proteins, the gel can be rinsed and then subjected to the more sensitive silver staining procedure (see Section 3.2.1.3.2).

Working Procedure

Equipment

1. Plastic or glass container with lid
2. Rocking shaker

Reagents

1. Coomassie gel stain stock solution, 1 liter:

Coomassie Brilliant Blue R-250	1 g
Methanol	450 ml
Water	450 ml
Glacial acetic acid	100 ml

2. Coomassie gel destain stock solution, 1 liter:

Methanol	100 ml
Water	800 ml
Glacial acetic acid	100 ml

Procedure

1. After electrophoresis, transfer gel to a small container and add Coomassie stain enough to submerge.
2. Incubate the gel in staining solution for 5 to 10 min (for 0.75 mm). Thicker gels require longer incubation times (10 to 20 min for 1.5 mm gel). Gels can also be kept in staining solution overnight.
3. Discard the stain and incubate the gel in destaining solution (40 to 50 ml) with agitation. Strong bands become visible within a few minutes. To destain completely, agitate gel in several changes of fresh destaining solution. Complete destaining of the gel usually requires several hours. A piece of Styrofoam or a Kimwipe paper may be added in the container during destaining to facilitate the destaining. Styrofoam or Kimwipe paper absorbs Coomassie stain, which diffuses from the gel. Replace with a new foam when original becomes dark blue.

Other Variations of Coomassie Stain

Coomassie Brilliant Blue G (0.04%, w/v) in 3.5% (w/v) perchloric acid can be used to stain proteins on SDS and non-denaturing polyacrylamide gels as well as agarose gels.[9] Proteins are stained in 30 to 60 min. No destaining step is required. Fixation step is also not required as perchloric acid can fix proteins during staining. However, SDS-PAGE gels are pre-fixed to remove SDS prior staining.

EZBlue™ Gel staining reagent (Sigma-Aldrich) is a one-step ultrasensitive stain based on Coomassie Brilliant Blue G-250. EZBlue stain can detect as little as 2 ng protein. It fixes proteins during staining, and thus a separate fixing step is not required. Destaining is also not required with this stain, although a water rinse after staining enhances sensitivity.

A colloidal concentrate of Coomassie Brilliant Blue G is available from Sigma-Aldrich. After dilution, the suspension contains 0.1% Brilliant Blue G, 0.29 M phosphoric acid, and 16% saturated ammonium sulfate. The stain is about tenfold more sensitive than the regular Coomassie stain. A fixation step is required prior to staining.

An advantage of these variations of the Coomassie stain is the absence of methanol, which is a regulated chemical waste.

3.2.1.3.2   Silver Stain

Switzer et al.[10] introduced a silver stain, which is at least 100 times more sensitive than Coomassie stain. Several variations and modifications have been developed. However, silver staining is primarily achieved in two ways: an alkaline method based

on the use of ammoniacal silver or silver diamine, and the use of silver nitrate in weakly acidic solution. Both procedures are based on the reduction of cationic silver to metallic silver.[11] Amino groups, especially the epsilon-amino group of lysine and sulfur residues of cysteine and methionine, are believed to react with silver cations.[12,13]

Reaction

In the first method, ammoniacal silver or silver diamine is prepared by mixing silver nitrate with sodium hydroxide resulting in a precipitate of silver hydroxide, which is brought back into solution with the slow addition of ammonia as follows:

$$AgNO_3 + NaOH \rightarrow AgOH$$

$$AgOH + NH_3 \rightarrow Ag(NH_3)_2OH$$
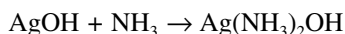
The gel is impregnated with silver diamine to allow the formation of a complex with the proteins. After removing the excess silver diamine from the gel, the complexed silver cations are reduced to metallic silver with formaldehyde in the presence of acid, usually citric acid.

The second method requires an initial gel soak in a weakly acidic silver nitrate solution and development with formaldehyde in the presence of alkali, usually sodium carbonate or sodium hydroxide. Sodium carbonate or other bases buffer the formic acid produced by the oxidation of formaldehyde, so that the silver ion reduction can continue until the protein bands appear in the gel.

Of the two methods, the alkaline silver nitrate method is more sensitive than the acidic silver nitrate method, but the former is more time consuming than the latter.

In some cases, prior to the silver nitrate step, gels are primed with a reducing agent like dithiothreitol or an oxidizing reagent like permanganate or dichromate. With Bio-Rad's Silver Stain, the formation of a positive image is enhanced by dichromate oxidation, which may convert protein hydroxyl and sulfhydryl groups to aldehydes and thiosulfates, thereby altering the redox potential of the protein. Complexes formed between the proteins and dichromate may further form nucleation centers for silver reduction.

Among the various modifications, a method combining the use of glutaraldehyde treatment and the use of silver diamine to soak the gel was found to be most sensitive.[14] The increased sensitivity is probably due to increased reduction rate of silver on the proteins.[15]

Before a protein gel can be stained, the proteins must be fixed, in order to minimize the diffusion of molecules in the gel. Fixation also elutes substances from the gel that may interfere with the establishment of the oxidation/reduction potential differences and with silver reduction. Ampholytes, detergents, reducing agents, initiators or catalysts, and buffer ion (glycine, chloride, etc.) must be removed. Water used in all silver stain reactions must be of 1 μmho conductance or less and free of organic contaminants.

Although silver staining normally produces a dark brown image, other colors may be produced when dense protein zones become saturated after prolonged development. Color production largely depends on the size and the distribution of the silver particles within the gel and the refractive index of the gel.[16]

Working Procedure

Silver Diamine Method

Reagents

1. Gel fixation solution: 20% (w/v) trichloroacetic acid solution.
2. Sensitization solution: 10% (w/v) glutaraldehyde solution.
3. Silver diamine staining solution: add 4 ml of 20% (w/v) silver nitrate dropwise with stirring to a mixture of 21 ml of 0.36% (w/v) NaOH and 1.4 ml of 35% (w/v) ammonia. Mixture will form a brown precipitate. Add a minimum amount of ammonia to dissolve the precipitate. Dilute the solution to 100 ml with water.
   (**Note:** the resulting silver diamine solution is unstable and should be used within 5 min.)
4. Developing solution: combine 2.5 ml of 1% citric acid and 0.26 ml of 36% (w/v) formaldehyde. Dilute the solution to 500 ml with water.
5. Stopping solution: 40% (v/v) ethanol/10% (v/v) acetic acid in water.
6. Destaining solution: 0.3% (w/v) potassium ferricyanide/0.6% (w/v) sodium thiosulfate/0.1% (w/v) sodium carbonate.

Procedure

All steps are performed at room temperature.

1. After electrophoresis, incubate the gel in 200 ml of fixing solution for at least 1 h. (**Note:** gels with a high percentage of acrylamide may require longer period of fixation. Alternatively, the gel can be soaked in fixing solution overnight.)
2. Wash the gel twice for 30 min each (2 × 30 min) with 200 ml of 40% ethanol/10% acetic acid in water.
3. Rehydrate the gel in excess water for 2 × 20 min.
4. Incubate the gel in sensitizing solution for 30 min.
5. Wash the gel in water for 3 × 20 min.
6. Incubate the gel in staining solution for 30 min.
   (**Note:** dispose silver diamine reagent after treating with 1 N HCl.)
7. Wash the gel in water for 3 × 5 min.
8. Develop the gel in developing solution until the appearance of proteins as dark brown zone (color development should be within 10 min).
9. Stop the staining with the stopping solution.

10. For destaining, wash the gel in water for 5 min and incubate the gel in destaining solution until the background clears.
11. Terminate destaining by incubating the gel in stopping solution.

Acidic Silver Nitrate Method

Reagents

1. Gel fixation solution:

   250 ml   ethanol
   60 ml   acetic acid
   0.25 ml   formaldehyde

   Add distilled water to 500 ml

2. Staining solution:

   100 mg   silver nitrate
   70 µl   formaldehyde

   Add distilled water to 100 ml

3. Developing solution:

   3 g   sodium carbonate (anhydrous)
   50 µl   formaldehyde
   1 mg   sodium thiosulfate ($Na_2S_2O_3.5H_2O$)

   Add distilled water to 100 ml

4. Stop solution: 0.01 M EDTA

(**Note:** all solutions should be made fresh except EDTA.)

Procedure

1. After electrophoresis, incubate the gel in fixative solution for 30 to 60 min.
2. Wash the gel three times for 20 min each with 50% ethanol.
3. Wash the gel in 0.01% (w/v) sodium thiosulfate ($Na_2S_2O_3.5H_2O$) solution for 1 min.
4. Wash the gel three times for 20 sec each with distilled water.
5. Incubate the gel in staining solution for 20 min.
6. Wash the gel three times for 20 sec each with distilled water.
7. Incubate the gel in developing solution until color develops.
8. Rinse the gel with distilled water for 20 sec and stop the reaction with the stopping solution.

3.2.1.3.3   Procion Blue Stain

Procion blue MX-2G-125 dye can be used to quantitate proteins on gels (6). The lower detection limit is about 1 µg per band.

Reaction

Procion dye contains a dichlorotriazine group which reacts with hydroxyl and amino groups of proteins.

Working Procedure

Reagents

1. Procion blue stain (0.2%, w/v): dissolve 0.4 gram dye in 100 ml methanol. Add 20 ml glacial acetic acid and 80 ml distilled water.
2. Destaining solution: to 800 ml distilled water, add 100 ml methanol and 100 ml glacial acetic acid.

Procedure

1. At the end of electrophoresis, stain gels in 0.2% Procion blue for 2 to 6 h at room temperature.
2. Clear the background with several changes of destain solution.
3. Measure the band intensity by scanning densitometry.
4. Determine the protein content from a standard curve of known amount of proteins.

3.2.1.3.4   Nile Red Stain

Staining of proteins with Nile Red (9-diethylamine 5 H-benzo [α] phenoxazine-5-one) (Figure 3.5) is rapid. It detects as low as 0.1 µg protein/band.

Reaction

Nile Red is a fluorescent hydrophobic dye. It binds protein-SDS complexes. Since it also interacts with SDS micelles, SDS-PAGE is usually performed at a lower SDS
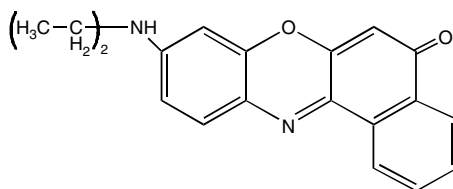

FIGURE 3.5 Diagram of Nile Red.

concentration (0.05% instead of typical 0.1%) in order to reduce background. This concentration is lower than the critical micelle concentration.

Working Procedure

Equipment

1. Transilluminator with midrange ultraviolet (UV) bulbs (approximately 300 nm).
2. Camera and photo films: Polaroid instant films such as 667 (black/white positive film), 665 (black/white positive/negative film), 669 (color positive film).
3. Optical filter: Wratten (Eastman Kodak) filters No. 9 (yellow) and 16 (orange) to eliminate the UV and visible light from the transilluminator. Filters are placed together, 16 facing camera lens and 9 facing gel.
4. Negative cleaning solution: 18% sodium sulfate. This is required when 665 films are used.

Reagent

Nile Red: 50× concentrated stock (0.4 mg/ml) in dimethyl sulfoxide. Stable at room temperature for at least 3 months. Nile Red is commercially available from vendors such as Sigma (St. Louis, MO) and Eastman Kodak (Rochester, NY).

Ready-to-use Nile Red solution is available from Molecular Probes (Eugene, OR) under the trade name SYBR Red Protein Gel Stain.

Procedure

1. At the end of electrophoresis, transfer gel to a plastic box.
2. Dilute Nile Red just before staining. Take 5 ml of concentrated Nile Red in 500 ml Erlenmeyer flask and quickly add 245 ml distilled water.
3. Soak the gel in diluted Nile Red and agitate vigorously using an orbital shaker (speed 300 rpm) for about 5 min.
   (**Note:** Nile Red is stable in DMSO, but it precipitates rapidly in aqueous solution. So as soon as the dye is diluted to 1× with water, this should be used immediately for staining protein bands with vigorous agitation. This ensures the staining of protein bands (reaction with SDS-protein) before it precipitates in the gel.)
4. Discard the starting solution and rinse the gel with distilled water (four times, 10 sec each) to remove the excess Nile Red precipitates.
5. Place the gel on a UV transilluminator and document the protein bands by photography.

3.2.1.3.5 Zinc Reverse Stain

Unlike traditional staining methods such as Coomassie and silver stains, reverse staining methods stain the whole gel except the area of the protein bands. The sensitivity of zinc stain is comparable to Coomassie stain. Zinc reverse staining is achieved in three steps: (a) incubate the gel in sodium carbonate, (b) incubation in imidazole, and (c) finally incubation with zinc sulfate. No fixative solution is used in this method. Reverse stain is particularly useful when elution of unstained protein is intended for further analyses. Usually, the gel is kept in water after staining. However, a toning reaction with a mixture of potassium ferricyanide, *o*-tolidine, and sulfuric acid is necessary if gels should be dried.

Reaction

At alkaline pH, Zn^{2+} forms a white insoluble precipitate with imidazole. The white precipitate turns into a deep blue with toning reaction.

Working Procedure

Reagents

1. Staining solution I: 1% (w/v) sodium carbonate
2. Staining solution II: 0.2 M imidazole containing 0.1% SDS (w/v)
3. Staining solution III: 0.2 M zinc sulfate
4. (Optional) Toning reaction I: 0.2% (w/v) potassium ferricyanide
5. (Optional) Toning reaction II: 0.2% (w/v) *o*-tolidine
6. (Optional) Toning reaction III: 0.36 N sulfuric acid

Procedure

1. At the end of electrophoresis, soak the gel in staining solution I for 5 min.
2. Soak the gel in staining solution II for 15 min.
3. Wash the gel briefly (about 10 sec) with water.
4. Soak the gel in staining solution III for about 40 sec. White background should develop in a few seconds, while protein bands remain transparent. (**Note:** incubation time should be limited to 40 sec, because the white precipitate in the gel surface can be redissolved with excess of Zn^{2+}.)
5. Wash the gel with abundant water to remove excess Zn^{2+} solution (twice 10 sec each and twice 5 min each).
6. Store the gel in water. Document the protein bands.
7. For toning reaction, mix equal volumes of toning reaction I and II and soak the gel for 4 min. In this step, the white background of the gel turns brown-yellowish, while the protein bands turn colorless to yellow.
8. Wash the gel briefly with water (10 sec). This will eliminate any trace amount of precipitate.
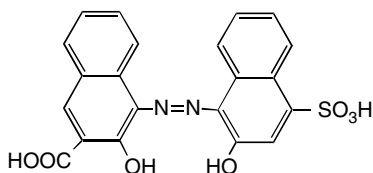
FIGURE 3.6 Diagram of calconcarboxylic acid.

9. Soak the gel in a mixture of equal volume of toning reaction II and III for 5 min. Within 2 min, the brown background of the gel turns blue, while the yellow protein bands become colorless again.
10. Wash the gel several times with water to remove excess reagents. Gels can now be dried.

3.2.1.3.6 Calconcarboxylic Acid Stain

Calconcarboxylic acid [1-(2-hydroxy-4-sulfo-1-naphthylazo)-2-hydroxy-3-naphthoic acid, CNN] (Figure 3.6) can be used for simultaneous as well as post-electrophoretic protein staining.[17] For simultaneous staining of proteins during electrophoresis, CNN is simply added in the upper reservoir. The sensitivity of this stain is about 10 ng and 25 ng by post-staining and simultaneous staining, respectively.

Reaction

Staining of proteins with CNN is pH dependent (intense staining at pH 1.6 to 4.4 and weak at alkaline pH). At acidic pH, various functional groups of CNN (carboxyl, sulfonic acid, hydroxyl) probably form electrostatic bonds with protonated amino groups in proteins. The lower staining intensity in simultaneous staining is probably due to alkaline pH of the electrophoresis buffer.

Working Procedure

Reagents

1. Destaining solution: 530 ml distilled water/400 ml methanol/70 ml glacial acetic acid.
2. Simultaneous staining solution: 1% (w/v) CNN. Dissolve 1 gram CNN in 100 ml reservoir buffer with stirring at 50 to 60°C.
3. Post-staining solution: 0.05% (w/v) CNN. Dissolve 0.05 gram CNN in 100 ml destaining solution.

Procedure for Simultaneous Staining

1. Load the sample electrophorese for about 10 min to allow protein migration into the stacking gel.

2. Turn off power supply and add 1% CNN in the upper reservoir to give a final concentration of 0.01 to 0.015%.
3. At the end of the electrophoresis, remove stained gel and destain for 30 min.

Procedure for Post-electrophoretic Staining

1. Soak the gel in post-staining solution with agitation for 30 min.
2. Remove staining solution and destain with destaining solution until the background clears.

3.2.1.3.7   Eosin Y Stain

The Eosin Y staining method detects proteins on gels as well as on membranes more rapidly than most Coomassie and silver staining methods. The stain can detect as little as 10 ng of protein. An advantage of this stain is that the antigenicity of the stained protein is retained.

Reaction

Protein staining may occur by means of hydrophobic interaction between aromatic rings of eosin Y and the protein and by hydrogen bonding between hydroxyl groups of eosin Y and the protein.

Working Procedure

Equipment

1. Black plastic board
2. Transilluminated fluorescent white light box

Reagents

1. Fixation solution: 10% glacial acetic acid/40% methanol
2. Staining solution: 1% (w/v) Eosin Y in 40% methanol and 0.5% glacial acetic acid
3. Destaining solution: 10% glacial acetic acid/40% methanol

Procedure

1. At the end of electrophoresis, fix the gel in fixation solution for 10 min.
2. Rinse the gel with distilled water twice.
3. Soak the gel in staining solution for 15 min with gentle agitation.
4. Remove the staining solution and soak the gel in destaining solution for about 15 sec.

5. Rinse with water.
6. View the stained protein bands by using transilluminated fluorescent light. Stained protein bands can also be viewed by placing the gel on a black plastic board with illuminated light on top of the gel. Stained gel can be kept in water for a month without losing sensitivity.

3.2.1.3.8   Amido Black Stain

Amido Black (also known as Naphthol Blue Black, Acid Black 1, or Buffalo Black NBR) can be used to stain proteins on gels. The detection sensitivity is lower than that of Coomassie Blue. Fixation is recommended for this stain.

Working Procedure

Reagents

1. Fixation solution: 10% glacial acetic acid/40% methanol
2. Staining solution: 0.1% (w/v) Amido Black in 7% (v/v) acetic acid
3. Destaining solution: 7% acetic acid

Procedure

1. At the end of electrophoresis, fix the gel in fixation solution for 10 min.
2. Rinse the gel with distilled water twice.
3. Soak the gel in staining solution for 2 h with gentle agitation.
4. Remove the staining solution and soak the gel in destaining solution.

3.2.1.3.9   Fast Green FCF

Fast Green FCF dye is used for protein staining in SDS-PAGE, native PAGE, and isoelectric focusing gels.[18] After electrophoresis, fixing is required for maximum sensitivity. Sensitivity is about two times less than Coomassie staining.

Working Procedure

Reagents

1. Staining solution: 0.1% (w/v) Fast Green FCF in 30% (v/v) ethanol and 10% (v/v) acetic acid
2. Destaining solution: 30% (v/v) ethanol and 10% (v/v) acetic acid

Procedure

1. At the end of electrophoresis, soak the gel in staining solution for about 2 h.
2. Destain the gel with several changes of destaining solution.

3.2.1.3.10   Other Stains

Vendors of several commercial protein stains (Molecular Probes, Bio-Rad, Pierce, Sigma) offer ready-to-use convenient packs (Table 3.5). The sensitivity of some of these is comparable to silver stain. For example, Molecular Probes' SYPRO[R] Tangerine, SYPRO[R] Orange, and SYPRO[R] Red are fluorescent-based stains (Ex/Em wave lengths are 490/640, 470/570, and 550/630, respectively) and can detect as little as 4 ng protein per band. SYPRO[R] Ruby stain (Molecular Probes) is an ultrasensitive luminescent stain for the detection of proteins on polyacrylamide gels (lower detection limit 75 fmol).

3.2.1.4   Determination of Molecular Weight

Subunit molecular weight of a protein is usually determined on SDS-PAGE, since the migration of protein is proportional to the mass. A standard curve is generated from proteins of known molecular weight (known as standard proteins), and the molecular weight of unknown protein is determined from the curve. The standard curve is obtained by plotting the relative mobility (R_f) value (in x-axis) and $\log_{10}$ of the molecular weight (in y-axis). R_f value is determined as follows:

$$R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

Following electrophoresis and staining, the migration of proteins and tracking dye (bromophenol blue) can be measured.

3.2.1.5   Quantitation of Proteins in Gels by Densitometric Scan

Following staining of proteins in gels, individual protein bands can be quantitated by densitometric scan over a limited range of protein concentration (1 to 10 µg/band). This technique clearly provides an advantage over the estimation of crude proteins (mixture of proteins) in solution where quantitation of individual proteins cannot be obtained. For densitometric quantitations, the most suitable protein stains are Procion blue stain,[6] zinc stain,[19] and colloidal Coomassie stain.[20] Staining of proteins with these procedures is discussed in Section 3.2.1.3. A standard curve is drawn from known amounts of proteins, and the amount of the unknown protein is then determined from the plot.

Alternatively, protein quantitation is achieved by eluting dye from the stained protein bands.[21]

3.2.1.6   Drying Gel

For long-term preservation, stained gels can be dried on thick paper backing under vacuum[22] or between sheets of cellophane at atmospheric pressure.[23] Gels dried between transparent sheets are useful for densitometry.

Working Procedure for Vacuum Drying

Materials

1. Acetate sheets or plastic kitchen wrap
2. Whatman 3 MM paper

Equipment

1. Gel dryer (Bio-Rad)
2. Vacuum pump

Procedure

1. Soak stained and destained gels in 5% glycerol overnight. This reduces the risk of cracking gel during drying.
2. Place the gel on Whatman 3 MM paper.
3. Cover the gel with an acetate sheet or plastic wrap, taking care not to trap air bubbles. A test tube can be used to roll out air bubbles.
4. Place the gel on the gel dryer. Acetate sheet or plastic wrap should face up.
5. Operate the gel dryer according to the manufacturer's instructions. Usually, gel is covered with sealing gasket and gel drying is performed at 60°C under vacuum.

Working Procedure for Air-Drying

Materials

Cellophane

Equipment

Gel drying frame (Figure 3.7)

Procedure

1. Soak stained and destained gels in 20% ethanol/10% glycerol for 30 min. This reduces the risk of cracking gel during drying.
2. Soak two cellophane sheets in water for about 2 min. This will soften the sheets.
3. Place one sheet of wet cellophane on the solid back plate, beveled edge down, taking care not to trap air bubbles between the plate and the cellophane.
4. Place gel on the center of the cellophane. Avoid trapping air bubbles. Layer a few milliliters of ethanol/glycerol solution on top of the gel.
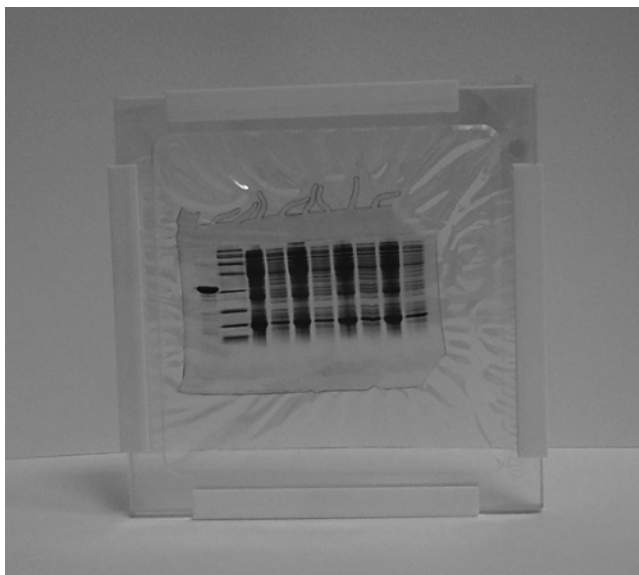
FIGURE 3.7 Drying gel using gel drying frame. The frame in figure is available from Diversified Biotech (Boston, MA).

5. Place a second sheet of wet cellophane on top of the gel. Match the edges of the cellophane with the edges of the backing plate. Avoid trapping air bubbles between the gel and the top sheet of cellophane.
6. Place the open frame over the stack, beveled edge up. Be sure the open frame matches the edges of the back plate and covers all edges of the cellophane.
7. Tie the back plate and open frame together by attaching plastic chips to all four sides of the frame.
8. Leave the frame assembly at room temperature overnight to allow the gel to air dry.

3.2.1.7   Extraction of Protein from Gel

Proteins from acrylamide gels can be extracted by electroelution[24] or protein diffusion.[25] For this purpose, stained gels (usually Coomassie or zinc reverse stained) containing protein bands are cut out with a razor blade, minced, and subjected to elution of proteins employing either method. Various electroelution devices are commercially available (from vendors such as Bio-Rad and Millipore) and should be operated following the manufacturer's instructions.

For extraction of proteins by diffusion, an appropriate buffer is added to the minced gel slice, incubated for 15 min to several hours, and centrifuged, and the supernatant is collected. Ball[21] described an efficient and simple procedure to isolate Coomassie stained protein from gel slices. In this procedure, the gel slice is incubated with 1 ml of 3% SDS in 50% isopropanol at 37°C for 24 h, and after centrifugation supernatant is collected.

3.2.2 SDS-Urea PAGE

SDS-urea PAGE is often used for proteins of low molecular weight and membrane proteins.[26,27] In SDS-PAGE, the migration of low molecular weight proteins may not be proportional to their molecular weight, as the protein charge properties become significant relative to the mass. SDS-urea PAGE is suitable for membrane proteins, as they may not be soluble at conditions used in SDS-PAGE.

Working Procedure

All procedures for SDS-urea PAGE are essentially similar to those described for SDS-PAGE except the composition of gels (both separating and stacking) and sample loading buffer. These should contain 8 M urea. The recipe for 10% separating gel is shown below as an example.

For 10 ml 10% Separating Gel

Acrylamide stock solution (see Section 3.2.1.1)	3.3 ml
4× separating gel buffer (see Section 3.2.1.1)	2.5 ml
Urea	4.8 g (equivalent to 3.6 ml)
Water	0.6 ml
10% ammonium persulfate	50 μl
TEMED	5 μl

For 4 ml 5% Stacking Gel

Acrylamide stock solution (see Section 3.2.1.1)	0.67 ml
4× stacking gel buffer (see Section 3.2.1.1)	1.0 ml
Urea	1.9 g
Water	0.93 ml
10% ammonium persulfate	30 μl
TEMED	5 μl

3.2.3 Gradient Gels

Although polyacrylamide gels of fixed concentrations are widely used for routine analyses, the use of gradient polyacrylamide gels (increasing acrylamide concentration and hence decreasing pore size) has at least two advantages over fixed-concentration acrylamide gels. First, a gradient gel allows the separation of proteins of a larger range of molecular weights compared to a fixed-percentage gel. The second advantage of the gradient gels over the fixed-percentage gels is that the proteins of very close molecular weights can be resolved as sharp bands. However, the gradient gel requires additional equipment (such as gradient maker, pump, and tubing) and special attention when pouring the gel mixture into the gel sandwich. Air bubbles lodged in the tubing or in the gradient maker can cause the gradient to form unevenly. Fortunately, precast gradient gels are commercially available from Pharmacia, Bio-Rad, Jule Inc., and other manufacturers. The two common ranges of gradient

gels are 3 to 30% and 5 to 20%, which resolve 13 to 950 kDa and 15 to 200 kDa, respectively.

Mechanism

In gradient gels, proteins of a wide molecular weight range enter the gel. Proteins of high molecular weight start to resolve immediately according to the pore size of the gel. Proteins of low molecular weight migrate freely in the beginning of the gel and start to resolve when they reach the appropriate percentage of gel with the smaller pore size. Proteins travel until they reach critical pore size (pore limit), which impedes further progress. At this point, the pattern of protein bands does not change significantly with time, although migration does not stop completely.[28]

Regarding the separation of two proteins of very close molecular weights, each protein travels through the gel until it reaches its pore size limit. At this point, the protein stacks up, as the gel pore is too small to allow further migration of protein. A similar protein but with slightly lower molecular weight is able to travel further before it reaches its pore size limit and stacks as a sharp band.

The following procedure shows the preparation of two 0.75-mm-thick gradient gels. The amounts of each component can be scaled up when multiple gels are to be prepared. However, it is important to assemble all gel sandwiches in a single gel caster.

Working Procedure

Equipment

1. Gradient maker (Bio-Rad, Hoefer, Pharmacia)
2. Peristalic pump (Millipore, Fisher, Pharmacia)
3. Tygon tubing

Reagents

1. 30% acrylamide stock solution (see Section 3.2.1.1)
2. 4× separating gel buffer (see Section 3.2.1.1)
3. 4× stacking gel buffer (see Section 3.2.1.1)
4. 10% ammonium persulfate
5. TEMED
6. Sucrose

Procedure

1. Prepare gel sandwich as shown in Figure 3.4 A.
2. Set up gradient maker as shown in Figure 3.8. Place a magnetic bar in the chamber attached to the outlet. This chamber receives heavy solution (higher percentage of acrylamide solution). Place a magnetic stirrer under this chamber separated by a sheet of Styrofoam.
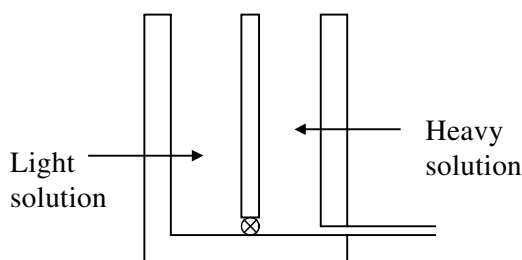
FIGURE 3.8 Diagram of gradient maker.

3. Prepare the heavy and light gel solutions without TEMED as follows. Example shows the amounts of solution for a 5 to 20% separating gel.

	5% (light solution)	20% (heavy solution)
30% acrylamide stock solution	1.67	6.67
4× stacking gel buffer	2.5	2.5
Water	5.8	0
Sucrose	0	1.5 g (equivalent to 0.8 ml)
10% ammonium persulfate	50 μl	50 μl
TEMED (To be added later)	5 μl	5 μl

4. Add 5 μl of TEMED to each separating gel solution, mix gently, and immediately transfer to the appropriate chamber as shown in Figure 3.8. (**Note:** work rapidly after the addition of TEMED as the polymerization is under way. To allow more time for pouring gradient gels [i.e., to delay polymerization] gel solution may be chilled. Alternatively, riboflavin [to a final concentration of 0.0005%] may be used to replace ammonium persulfate to allow more time for pouring. If riboflavin used, after pouring polymerization should be initiated by exposure of daylight or white or blue fluorescent lamp.)
5. Turn on magnetic stirrer, open connection between the chambers, and turn on the peristaltic pump to allow gradient gels to fill gel cassettes at a rate of about 3 to 5 ml/min.
6. When the level of the gel reaches about 3 cm from top of the gel cassettes, gently layer about 2 to 3 mm of water on top of gels.
7. Rinse out the gradient maker before the remaining gel solution polymerizes.
8. Allow gel to polymerize for about 30 min at room temperature.
9. Polymerized gels may be stored at 4°C for future use or set up for electrophoresis. For running gel, a stacking gel is made as described before (see Section 3.2.1.1).

3.2.4 NON-DENATURING PAGE

Non-denaturing PAGE, also called native PAGE, refers to the electrophoretic separation of the native protein. This can be performed following the standard Laemmli SDS-PAGE protocol described above, except the solutions contain no SDS or reducing agent. In non-denaturing PAGE, separation of proteins depends on many factors such as size, shape, and native charge. Native PAGE is mostly used to determine the homogeneity of the purified protein. Native PAGE is very useful to visualize enzyme or lectin activity after electrophoretic separation. Unlike SDS-PAGE, in which the denatured proteins are uniformly negatively charged and their mobilities are dependent on their molecular weights, determination of the native molecular weight using native PAGE is not reliable, as the mobility of the native proteins depends on both molecular weight and charge.

This difficulty is partly overcome by operating native PAGE at a high pH buffer (pH 8.8). At this pH, most proteins are negatively charged and thus move toward the anode. In order to determine molecular weight using non-denaturing gel electrophoresis, the protein should be run under a variety of acrylamide concentration (usually 4 to 12%). The results from these conditions are used to adjust the effect due to protein charge. In native PAGE, acrylamide concentration may vary from 5 to 15% and acrylamide:bisacrylamide ratio may vary from 20:1 to 50:1 to achieve different sieving effects. The ionic strength is an important factor in the native PAGE, especially when the protein's activity is to be investigated after electrophoresis. High ionic strength generates heat during electrophoresis, resulting in a loss of protein activity. However, if the ionic strength is too low, proteins may aggregate nonspecifically. Typically, ionic strength is kept in the range of 10 to 100 mM. All steps are usually performed at 0 to 4°C to minimize the loss of protein activity by denaturation and to reduce proteolysis.

Native PAGE is performed in two ways: (a) discontinuous: both stacking and separating gels like SDS-PAGE, and (b) continuous: no stacking gel. Continuous gel electrophoresis is simpler than discontinuous, as no stacking gel is involved. However, the lack of stacking gel often results in diffused or poorly resolved bands. In continuous native PAGE, ionic strength of the protein buffer is kept five- to tenfold lower than the gel buffer in order to obtain the sharpest bands. The volume of the protein sample is kept as small as possible. Thus, the protein concentration should be high (2 to 10 mg/ml). Buffers for continuous native PAGE may be the same as described below except that those pertaining to the stacking gel are omitted. Additional buffers are described elsewhere.[29] The procedure for discontinuous non-denaturing gel electrophoresis is described below.

Working Procedure

Equipment

1. Mini gel apparatus (Hoefer, Bio-Rad, Life Technologies)
2. Power supply (capacity 200 V)

3. Heat block (100°C) or boiling water bath
4. Disposable gel loading tips for micropipette or Hamilton syringe
5. Eppendorf tubes
6. Plastic or glass container with lid
7. Rocking shaker
8. Gel dryer

Reagents

1. Acrylamide, electrophoresis grade
2. Bisacrylamide (N,N′-methylenebisacrylamide)
3. Tris
4. Hydrochloric acid (HCl)
5. TEMED (N,N,N′,N′-tetramethylethylenediamine)
6. Ammonium persulfate
7. Glycine
8. Glycerol
9. Bromophenol blue

Stock Solutions

1. 2 M Tris-HCl (pH 8.8), 1 liter: weigh out 242 gram Tris base and add 500 ml distilled water. Adjust pH to 8.8 by adding concentrated HCl slowly and finally add distilled water to a total volume of 1 liter.
2. M Tris-HCl (pH 6.8), 100 ml: to 12.1 gram of Tris base add 50 ml distilled water and adjust pH to 6.8 with concentrated HCl. Add distilled water to a total volume of 100 ml.
3. 1% bromophenol blue (w/v), 10 ml: weigh out 100 mg bromophenol blue and add 10 ml distilled water. Stir until dissolved and filter to remove particulates.

Working Solutions

1. 30% acrylamide stock solution: Dissolve 29.2 gram acrylamide and 0.8 gram bisacrylamide with distilled water to make 100 ml. The solution is stable at 4°C for months.
   (**Note:** unpolymerized acrylamide is a skin irritant and a neurotoxin and thus should be handled with gloves. Unused acrylamide solution should be polymerized and disposed of with solid waste.)
2. 4× separating gel buffer, 100 ml:
   a. 75 ml 2M Tris-HCl (pH 8.8); final conc. 1.5 M
   b. 25 ml water
   (The solution is stable for months in the refrigerator.)

3. 4× stacking gel buffer, 100 ml:
   a. 50 ml 1 M Tris-HCl (pH 6.8); final conc. 0.5 M
   b. 50 ml water
   (The solution is stable for months in the refrigerator.)
4. 10% ammonium persulfate, 5 ml: dissolve 0.5 gram ammonium persulfate in 5 ml distilled water. Aliquot 100 µl in 0.5 ml microfuge tubes and store at –20°C (stable for months).
5. Electrophoresis buffer, 1 liter:
   a. 3 gram Tris base; final conc. 25 mM
   b. 14.4 gram glycine; final conc. 192 mM
   c. Water to make 1 liter
   (pH should be approximately 8.8; 10× stock solution can also be made and stored at 4°C; stable for months.)
6. 5× sample buffer, 10 ml:
   a. 3.1 ml 1M Tris-HCl (pH 6.8); final conc. 312.5 mM
   b. 5 ml glycerol; final conc. 50%
   c. 0.5 ml 1% bromophenol blue; final conc. 0.05%
   d. 1.4 ml water
   (Aliquot in small volume in microfuge tubes and store at –20°C. Stable for months.)

Preparation of Gel

The recipe for making gels of varying strengths is essentially identical to the amounts shown in Table 3.3. Preparation of gel cassettes, pouring separating and stacking gel into the gel cassettes, and running gels are similar to denaturing PAGE.

3.2.5 Tricine PAGE

Tricine PAGE is mainly used for the separation of low molecular weight peptides (range 40 to 1 kDa),[30] which cannot be resolved in Laemmli SDS-PAGE. In the Laemmli system, SDS and smaller proteins comigrate and thus obscure the resolution. In Tricine (N-Tris [hydroxymethyl] methylglycine) gel electrophoresis, Tricine separates SDS and peptides, thus improving resolution. The Tricine PAGE system has an additional advantage. Since glycine (which interferes with the amino acid sequence analyses) is replaced by Tricine in the electrophoresis buffer, protein bands in the gel can be excised for amino acid sequencing.

Working Procedure

Reagents

1. 30% acrylamide stock solution (see Section 3.2.1.1)
2. Separating/stacking gel buffer: 3 M Tris-HCl/0.3% SDS, pH 8.45
3. Glycerol

4. 10% ammonium persulfate
5. TEMED
6. Cathode buffer: 0.1 M Tris/0.1 M Tricine/0.1% SDS
7. Anode buffer: 0.2 M Tris

Procedure

1. Prepare gel sandwich as shown in Figure 3.4 A.
2. In a 100 ml side-arm flask, combine the following reagents for separating gel without ammonium persulfate and TEMED.

	Separating gel	Stacking gel
30% acrylamide stock solution	15.0 ml	1.62 ml
Separating/stacking gel buffer	10.0 ml	3.10 ml
Water	1.83 ml	7.78 ml
Glycerol	3.17 ml	
10% ammonium persulfate (to be added later)	50 μl	50 μl
TEMED (to be added later)	5 μl	5 μl

3. Degas under vacuum for 10 min. Add 50 μl ammonium persulfate and 5 μl TEMED to the gel solution, mix gently, and immediately pour gel solution into the gel cassette, leaving empty about 1.5 cm from top of the cassette as described in Section 3.2.1.1.
4. Once polymerized, combine the above recipe to prepare stacking gel, following protocols as described in Section 3.2.1.1.
5. For running gel, heat sample to 100°C for 3 to 5 min or 40°C for 30 to 60 min.

3.2.6 Non-Urea SDS-PAGE for Separation of Peptides

Okajima et al.[31] described a modification of Laemmli SDS-PAGE for separation of peptides as low as 5 kDa. In this modification, the concentration of buffers is increased to provide better separation between the stacked peptides and the SDS micelles.

Working Procedure

Reagents

1. 30% acrylamide stock solution (see Section 3.2.1.1)
2. Separating gel buffer: 3 M Tris-HCl, pH 8.8 (compared to Laemmli SDS-PAGE, this concentration is equivalent to 8×)

3. Stacking gel buffer: 0.5 M Tris-HCl, pH 6.8
4. 10% SDS
5. 10% ammonium persulfate
6. TEMED
7. SDS electrophoresis buffer: 0.05 M Tris/0.384 M glycine/0.2% SDS, pH 8.3 (compared to Laemmli SDS-PAGE, this recipe is 2×)

Procedure

1. Prepare gel sandwich as shown in Figure 3.4 A.
2. In a 50 ml side-arm flask, combine the following reagents for separating gel without ammonium persulfate and TEMED:

	Separating gel	Stacking gel
30% acrylamide stock solution	10.0 ml	0.65 ml
Separating gel buffer	3.75 ml	—
Stacking gel buffer		1.25 ml
10% SDS	0.15 ml	50 μl
Water	1.0 ml	3.0 ml
10% ammonium persulfate (to be added later)	50 μl	25 μl
TEMED (to be added later)	10 μl	5 μl

3. Degas under vacuum for 10 min. Add 50 μl ammonium persulfate and 10 μl TEMED to the gel solution, mix gently, and immediately pour gel solution into the gel cassette, leaving empty about 1.5 cm from top of the cassette as described in Section 3.2.1.1.
4. Once polymerized, prepare stacking gel, following protocols as described in Section 3.2.1.1.
5. Run gel in 2× SDS electrophoresis buffer.

3.2.7 ACID UREA PAGE

Panyim and Chalkley[32] introduced a continuous acetic acid urea PAGE for the separation of unmodified histone from its monoacetylated or monophosphorylated form. The procedure can separate similar basic proteins based on differences in effective charge as well as differences in size. Proteins of a slightly different charge such as unmodified and acidic acetylated derivative can be separated in acid urea PAGE. Urea is commonly used in this PAGE, mainly to disrupt any aggregation and to increase the density of the loading solution. In this system, riboflavin or riboflavin 5′-phosphate is used as initiator of photo-polymerization of acrylamide, as ammonium persulfate interferes with stacking of the proteins in the gel. Chloride ions also

interfere with the stacking system. Thus, protein samples and glycine used in the elctrophoresis buffer should be free of chloride salts.

Mechanism

In acid urea PAGE, the samples are electrophoresed in acetic acid buffer (pH around 3). At this pH, basic proteins with a high isoelectric point get positively charged and move toward the cathode under an electric field.[33]

Working Procedure

Reagents

1. Acrylamide stock solution: 60% (w/v) acrylamide in water.
2. N,N′-methylenebisacrylamide stock solution: 2.5% (w/v) in water.
3. Urea, ultrapure quality.
4. Glacial acetic acid: 17.5 M.
5. Concentrated ammonium hydroxide: approximately 15 M.
6. Riboflavin 5′-phosphate: 0.006% (w/v) in water. Stable for at least 6 months at dark (4°C).
7. TEMED.
8. Phenolpthalein indicator solution: 1% (w/v) in 95% ethanol.
9. Sample buffer: add 7.7 mg dithiothreitol in 0.9 ml 8 M urea solution and mix. Then add 50 μl 1% phenolphthalein and 50 μl NH₄OH to obtain pink sample buffer.
   (**Note:** urea should be free from ionic contaminants such as cyanate to avoid amino acid modification in the protein sample. For example, cysteine residues can be modified by cyanate. To obtain deionized urea, pass urea solution through a column of mixed resin [e.g., AG 501-X8, Bio-Rad]. Freeze dry the deionized solution and store at –20°C. Storage of urea at low temperature minimizes production of ionic contaminants.)
10. Methylene blue solution: 2% (w/v) in sample buffer.
11. Electrophoresis buffer: 1 M acetic acid/0.1 M glycine.

Procedure

1. Prepare gel sandwich as shown in Figure 3.4 A.
2. In a 100 ml side-arm flask, combine the following reagents for separating gel without riboflavin 5′-phosphate and TEMED. The recipe is good for making five mini gels.

		Final concentration
Acrylamide stock solution	17.5 ml	15%
Bisacrylamide stock solution	2.8 ml	0.1%
Glacial acetic acid	4.2 ml	1.0 M
Concentrated ammonium hydroxide	0.23 ml	0.05 M
Urea	33.6 g	8.0 M
Water to make 65 ml		
Riboflavin 5′-phosphate (to be added later)	4.67 ml	0.0004%
TEMED (to be added later)	0.35 ml	0.5%

3. Degas under vacuum for 10 min. Add 0.35 ml TEMED and 4.67 ml riboflavin 5′-phosphate to the gel solution, mix gently, and immediately pour gel solution into the gel cassette, leaving empty about 1.5 cm from top of the cassette as described in Section 3.2.1.1. Leave the gel cassette under white light or on the regular light box.
   (**Note:** since polymerization is initiated by light, high-intensity light boxes close to either side of the gel surface may be required for thick gels of greater than 1.5 mm.)
4. Once polymerized, pour the stacking gel on top of the separating gel as described in Section 3.2.1.1. For the preparation of stacking gel, combine the following gel components.

		Final concentration
Acrylamide stock solution	1.34 ml	4%
Bisacrylamide stock solution	1.28 ml	0.16%
Glacial acetic acid	1.14 ml	1.0 M
Concentrated ammonium hydroxide	0.07 ml	0.05 M
Urea	9.6 g	8.0 M
Water to make 18.6 ml		
Riboflavin 5′-phosphate (to be added later)	1.3 ml	0.0004%
TEMED (to be added later)	0.1 ml	0.5%

5. Prepare sample by adding 50 µl of sample buffer to the salt-free lyophilized protein (5 to 50 µg). Incubate for 5 min at room temperature for reduction.

    (**Note:** reduction by DTT [in sample buffer] for 5 min at room temperature is optimum for most samples. Longer incubation may modify cysteine residues in the protein by urea contaminant cyanate. To assure complete reduction of proteins by DTT, make sure the pH of the solution is above 8.0. If the pink phenolphthalein color disappears during sample incubation, add a few microliters of concentrated NH_4OH to obtain alkaline pH.)

6. Acidify the sample with 2.5 µl glacial acetic acid.

7. Add 2 µl methylene blue to each sample. Sample is now ready for loading onto the gel.

8. Once polymerized, set up the gel in the electrophoresis apparatus, fill the reservoir with the electrophoresis buffer, and load sample as described in Section 3.2.1.2.

9. Connect the electrodes to the power supply.

    (**Important note:** in the acid urea PAGE system, basic proteins are positively charged and thus move toward the cathode. So, upper electrode of the electrophoresis apparatus should be connected to the (+) lead of the power supply and lower electrode to the (-) lead of the power supply. This is opposite to the SDS-PAGE configuration.)

10. Run gel at 200 V in constant voltage mode.

Bonner et al.[34] described acid urea PAGE system in the presence of non-ionic detergent Triton X-100 for separation of core histones. This is based on the observation that core histones but not linker histones or any other protein bind Triton.[35]

3.2.8   CTAB PAGE

Although Laemmli PAGE is widely used for testing the purity of a protein and the determination of its subunit size, this procedure is not suitable to assess the biological activity of proteins treated with SDS. Cetyltrimethylammonium bromide (CTAB) PAGE allows the sample solubilization in CTAB and molecular size-dependent separation of proteins in an arginine/Tricine buffer, with the retention of native activity.[36,37] The following working procedure is based on Akins et al.,[37] who described CTAB PAGE in a discontinuous gel format.

Mechanism

In the CTAB PAGE system, proteins get positively charged and thus migrate toward the cathode under electric field. The arginine in the electrophoresis buffer also migrates toward the cathode, as arginine is positively charged at the electrophoresis buffer pH 8.2 (pI of arginine is 10.8). However, at the stacking gel (pH 9.96) arginine will have a lower net positive charge and will move slowly. In the interface zone between the upper tank buffer and the stacking gel/sample buffer, sodium ions

(Tricine-NaOH) move ahead of the slow-moving arginine. The CTAB-coated proteins migrate more quickly in this interface zone than in the sodium-containing zone and "stack" as the interface advances.

Working Procedure

Reagents

1. 40% acrylamide stock solution: 38.93 gram ultrapure acrylamide and 1.07 gram bisacrylamide in 100 ml of distilled water.
2. Separating gel buffer: 1.5 M Tricine-NaOH, pH 8.0. Dissolve 134.4 gram of Tricine in 400 ml distilled water. Add NaOH until the pH of the solution reaches 8.0 and finally bring the solution to a volume of 500 ml with distilled water.
3. Stacking gel buffer: 0.5 M Tricine-NaOH, pH 10.0. Dissolve 22.4 gram of Tricine in 200 ml distilled water. Add NaOH until the pH of the solution reaches 10.0 and finally bring the solution to a volume of 250 ml with distilled water.
4. Agarose stock solution: prepare a ready-to-use agarose stacking gel solution by combining 25 ml stacking gel buffer, 0.1 gram CTAB, 0.7 gram of electrophoresis grade agarose, and finally bringing the solution volume to 100 ml with distilled water.
5. Electrophoresis buffer: to prepare 1 liter of 5× buffer, dissolve 22.4 gram Tricine and 5 gram CTAB in 900 ml of distilled water. Add 1 M arginine solution (approximately 75 ml) until the pH of the solution reaches 8.2. Finally bring the solution to a volume of 1 liter with distilled water.
6. 10% ammonium persulfate.
7. TEMED.

Procedure

1. Prepare gel sandwich as shown in Figure 3.4 A.
2. In a 25 ml side-arm flask, combine the following reagents for separating gel without ammonium persulfate and TEMED. This makes one mini gel of 10%.

40% acrylamide stock solution	2.5 ml
Separating gel buffer	2.5 ml
Distilled water	4.89 ml
10% Ammonium persulfate (to be added later)	0.1 ml
TEMED (to be added later)	0.01 ml

3. Degas under vacuum for about 10 min. Add 100 µl ammonium persulfate and 10 µl TEMED to the gel solution, mix gently, and immediately pour gel solution to the gel cassette, leaving empty about 1.5 cm from top of the cassette as described in Section 3.2.1.1.

4. Once polymerized, pour the stacking gel on top of the separating gel as described in Section 3.2.1.1. For the preparation of stacking gel (4%), combine the following gel components.

40% acrylamide stock solution	1.0 ml
Stacking gel buffer	2.5 ml
Distilled water	6.39 ml
10% ammonium persulfate (to be added later)	0.1 ml
TEMED (to be added later)	0.01 ml

(**Note:** stacking gels are made from agarose when subsequent protein activity assay is performed, as these are shown to provide better results.[38] For this purpose, melt agarose stock solution in a microwave oven and pour to the gel cassette. Insert the comb immediately. Allow the agarose to cool before removing the comb.)

5. Once polymerized, set up the gel in the electrophoresis apparatus, fill the reservoir with 1× electrophoresis buffer, and load sample as described in Section 3.2.1.2.

6. Connect the electrodes to the power supply.
(**Important note:** in the CTAB PAGE system, proteins are positively charged with the CTAB and thus move toward the cathode. Therefore, the upper electrode of the electrophoresis apparatus should be connected to the [+] lead of the power supply and lower electrode to the [-] lead of the power supply. This is opposite to the SDS-PAGE configuration.)

7. Run gel at 100 V in constant voltage mode.

3.3   ISOELECTRIC FOCUSING

Isoelectric focusing (IEF) is an electrophoretic method in which amphoteric molecules are separated as they migrate through a pH gradient. Polyacrylamide gels are generally used for focusing proteins. However, for proteins larger than 200,000 dalton (Da), 1% agarose gels can be employed.

Mechanism

The net charge on a protein is the algebraic sum of all its positive and negative charges. At physiological pH, lysine, arginine, and histidine residues in a protein are positively charged, while aspartic acid and glutamic acid carry a negative charge. So the net charge of a protein at a specific pH depends on the relative number of positive and negative charges. The pH at which a protein carries no net charge (total

positive charge equal to total negative charge) is called its isoelectric point (pI). Below the pI the protein carries a positive charge, and a negative charge at pHs above pI. When protein is placed in a medium with varying pH and subjected to an electric field, it will initially move toward the electrode with the opposite charge. During migration through the pH gradient, the protein will either pick up or lose protons. As it does, its net charge and mobility will decrease, and at its pI the protein will stop moving. This type of motion is in contrast to conventional electrophoresis, in which proteins continue to move through the medium until the electric field is removed. In, proteins migrate to their steady-state positions from anywhere in the system, and thus the location of sample application is arbitrary.

The key to IEF is the establishment of stable pH gradients in electric fields. This is most commonly accomplished by means of commercially available, synthetic carrier ampholytes (amphoteric electrolytes). These compounds are mixtures of relatively small (600 to 900 Da), multicharged, amphoteric molecules with closely spaced pI values and high conductivity.[39] Under the influence of an electric field, carrier ampholytes partition themselves into smooth pH gradients, which increase monotonically from the anode to the cathode. The slope of the pH gradient is determined by the pH interval covered by the carrier ampholyte mixture and the distance between the electrodes. Isoelectric focusing is usually carried out in a denaturing gel system with urea. Charged denaturing agents such as SDS and sodium deoxycholate should not be used, as these interfere with the electrophoresis. Isoelectric focusing can also be carried out in a non-denaturing system, when functions of proteins (e.g., enzyme activity, lectin activity) are studied after focusing. Table 3.6 shows some common problems in isoelectric focusing and their remedies.

It is important to perform isoelectric focusing on a device where efficient gel cooling is achieved. This is required to maintain high-voltage gradient for better resolution of protein bands. Isoelectric focusing can be performed on either slab gels or tube gels. Several devices are commercially available for running slab gels (vertical and horizontal) and tube gels. A procedure for isoelectric focusing gel electrophoresis on a vertical slab gel format is described here.

Working Procedure

Equipment

1. Mini Protein II Gel System (Bio-Rad) or other slab gel electrophoresis apparatus
2. Power supply

Reagents

1. Acrylamide: 30% acrylamide stock solution (see Section 3.2.1.1)
2. Ampholyte solutions (pH 3.5 to 10; pH 4 to 6; pH 6 to 9; pH 9 to 11)
3. Urea, ultrapure
4. Ammonium persulfate

5. TEMED
6. Triton X-100
7. 2-mercaptoethanol
8. Phosphoric acid: 10 mM (made fresh)
9. Sodium hydroxide: 20 mM (made fresh)
10. Trichloroacetic acid (TCA): 10% and 1%

TABLE 3.6
Common Problems in Isoelectric Focusing Gels and Possible Remedy[a]

Problem	Cause	Remedy
High background staining	Incomplete removal of ampholytes from the gel	Increase time of fixing with 1% TCA
Wavy bands	High salt content in the sample	Dialyze the sample in low salt buffer
	Impurities in the ampholyte or electrolyte solutions	Use fresh ampholytes and electrolytes
	Dirty electrodes	Clean electrodes
Streaking bands	Protein aggregation or precipitation	Centrifuge samples before loading
	Presence of nucleic acids in the sample	Remove nucleic acids
	Modification of protein may occur such as oxidation of cysteine, deamination of asparagine and glutamine, carbamoylation of protein by isocyanate present in impure urea.	Remove isocyanate impurities by prerunning the gel. Handle sample properly to avoid other modification.
Overlapping bands	Complex protein mixtures	Change the pH range of the gel
Skewed bands	Faults in the pH gradient	Verify that the electrodes are clean for good contact
Fuzzy bands	Incomplete focusing	Increase voltage gradient incrementally towards the end of the run
	Large proteins and thus restricted mobility	Use more porous agarose gel for large proteins
Missing or faint bands	Proteins have not been denatured during fixation	Increase TCA concentration
Uneven pH gradient	Electrode contact is not parallel to the gel	Make sure electrodes are parallel to the gel
	Impurities within the gel	Use reagents of highest grade
	Ampholyte concentrations are too low	Increase ampholyte concentrations

[a] Based on Reference 40.

TABLE 3.7
Ratio of Ampholytes Required in the Preparation of Isoelectric Focusing Gels with Varying Ranges of pH[a]

Gels with pH Range	Ratio of Ampholytes	% in Final Gel Solution
pH 3.5–10	pH 3.5–10	2.4
pH 4–6	pH 3.5–10	0.4
	pH 4–6	2.0
pH 6–9	pH 3.5–10	0.4
	pH 6–8	1.0
	pH 7–9	1.0
pH 9–11	pH 3.5–10	0.4
	pH 9–11	2.0

[a] Based on Reference 41.

Procedure

Preparation of Gels

1. Prepare gel cassettes according to the manufacturer's instructions.
2. Combine the following gel components in 50 ml Erlenmeyer flask. The following recipe makes two mini gels (0.75 mm) with a gradient from pH 4 to 6. For making gels with other pH ranges, see Table 3.7.

Acrylamide stock solution (see Section 3.2.1.1)	2.0 ml
Water	5.4 ml
Ampholyte solution, pH 3.5-10	48 μl
Ampholyte solution, pH 4-6	240 μl
Urea, ultrapure	6 g

3. Swirl gently to dissolve urea.
4. Add 50 μl 10% ammonium persulfate and 20 μl TEMED to the above gel solution. Mix gently and immediately pour into assembled gel cassettes. Be careful to avoid trapped air bubbles. Fill the gel cassette completely with acrylamide solution.
5. Insert comb immediately, not trapping any air bubbles in the teeth of the comb.
6. Allow gels to polymerize at room temperature.

Set Up Gel

7. Remove comb carefully after gels have polymerized.
8. Attach gel to the electrophoresis tank according to the manufacturer's instructions.
9. Add catholyte (20 mM sodium hydroxide) to the upper buffer chamber and anolyte (10 mM phosphoric acid) to the lower buffer chamber.

Sample Preparation and Loading

10. Mix protein sample (5 to 10 µg per lane) with an equal volume of 2× loading buffer. To obtain 2× loading buffer, combine the following reagents.[42] This recipe makes 5 ml loading buffer. Aliquot the unused buffer into small volume and store frozen at –20°C until use.

		Final concentration
Urea, ultrapure	6 g	8 M
Ampholyte solution, pH 3.5 to 10	20 µl	
Ampholyte solution, pH 4 to 6		100 µl
20% Triton X-100	500 µl	2%
2-mercaptoethanol	5 µl	1%
1% bromophenol blue	200 µl	
Distilled water	1.7 ml	

(**Note:** protein sample should not contain high salt [not more than 50 mM]).
11. Centrifuge the sample, if necessary, for a few minutes in an Eppendorf centrifuge at highest speed to remove any aggregate.
(**Note:** aggregated protein may cause streaking in the gel).[43]
12. Apply the clear supernatant into the bottom of the well with a disposable gel loading tip or a Hamilton syringe.

Running Gel

13. Connect the electrophoresis chamber with the power supply (lower chamber with the anode, upper chamber with the cathode).
14. Run the gel at 150 V for 30 min and at 200 V for additional 2.5 h.

Cutting Gel Slices for pH Determination

15. After electrophoresis, cut a strip of gel into 0.5 cm slice, keeping track the distance from any electrode (e.g., anode). Place each slice into an Eppendorf tube and label by the distance (in cm) from the anode.
16. Incubate each slice in 1 ml 10 mM KCl for about 30 min.

17. Centrifuge and read pH of the clear supernatant. A standard curve is drawn by plotting pH of the gel slices and the distance from the anode. The pI of the unknown is determined from the plot.
(**Note:** alternatively, pI of the unknown can be determined from a plot of migration of proteins of known pIs [isoelectric focusing markers]. Marker proteins are loaded in the same gel where unknown protein is electrophoresed, and the migration from the electrode is determined after staining the gel.)

Fixing and Staining the Gel

18. Soak gels in 10% TCA for 10 min.
19. Replace with 1% TCA and incubate for 2 h to overnight to remove ampholytes. Removal of ampholytes in important to reduce background staining.
20. Stain gel with Coomassie blue for 10 min (see Section 3.2.1.3 for Coomassie stain composition).
21. Discard staining solution and replace with the destaining solution (see Section 3.2.1.3 for destaining solution).
22. Gel may be dried following protocols as previously described (see Section 3.2.1.6).

Native Isoelctric Focusing Gel

The working procedure for native isoelectric focusing is essentially identical to denaturing isoelectric focusing except some modifications in the recipe of the gel solution and the gel-loading buffer.

Recipe for Native Isoelectric Focusing Gel

Acrylamide stock solution (see Section 3.2.1.1)	2.0 ml
Water	9.7 ml
Ampholyte solution, pH 3.5 to 10	48 μl
Ampholyte solution, pH 4 to 6	240 μl
10% ammonium persulfate (to be added later)	50 μl
TEMED (to be added later)	20 μl

Recipe for Native Gel Sample Buffer (2×)

Glycerol	3.0 ml
Ampholyte solution, pH 3.5 to 10	33.3 μl
Ampholyte solution, pH 4 to 6	166.7 μl
Distilled water	1.8 ml

Isoelectric Focusing in Horizontal Slab Gels

Isoelectric focusing in a horizontal slab gel format has several advantages over the vertical format. Cooling of the gel during electrophoresis is very efficient, as the gel remains flat on the cooling plate. Only a few milliliters of electrode solutions (enough to soak electrode strips) are required for electrophoresis. The gel size can be adjusted as needed. Sample can be added at any position in the pH gradient. Larger sample volume (more than 10 to 15 μl) can be added on a slab gel. Precast isoelectric focusing gels (polyacrylamide gel on a plastic support film) with varying pH ranges are commercially available (Amersham Pharmacia). Electrophoresis should be performed according to the manufacturer's instructions.

3.4   TWO-DIMENSIONAL (2D) GEL ELECTROPHORESIS

In 2D gel electrophoresis, protein separates in two dimensions: the first dimension on the basis of pI and the second dimension based on subunit molecular weight.[44] Usually, isoelectric focusing is performed on a tube gel of very small diameter or on a thin gel strip, and after completion of the run the gel is placed horizontally onto the top of a polymerized slab gel for SDS-PAGE. In this system proteins are separated into many more components than is possible with conventional one-dimensional electrophoresis (Figure 3.9). Due to greater resolution, it is possible to quantitate differentially expressed proteins during a certain biological process. The powerful technique of protein separation and identification under the heading "Proteomics" is based on the principle of 2D electrophoresis. Procedures for 2D gel electrophoresis can be adopted from the isoelectric focusing and SDS-PAGE procedures described above. Additionally, 2D equipment and procedures for 2D electrophoresis are available from various commercial sources such as Hoefer, Bio-Rad, and Millipore.

3.5   WESTERN BLOTTING

Western blotting refers to the electrophoretic transfer of the resolved proteins or glycoproteins from a polyacrylamide gel to a membrane such as nitrocellulose and polyvinylidene difluoride (PVDF).[45] The immobilization of proteins on a membrane is more useful than working on the gel for a number of reasons: (a) proteins are more accessible, (b) membranes are easier to handle than gels, (c) smaller amounts of reagents are needed, and (d) processing time is shorter.[46] Following the transfer of proteins to a membrane, a wide variety of applications can be carried out on the immobilized proteins such as immunodetection (see Chapter 6), carbohydrate detection (in case of glycoprotein see Chapter 7), and amino acid analysis and protein sequencing. Other applications involved in immobilized proteins are (a) epitope mapping, (b) ligand binding, (c) cutting out protein band for antibody production, and (d) structural domain analysis (see Figure 3.10). In most applications, immobilized proteins or glycoproteins can be identified and visualized by using very specific and sensitive detection techniques (immunological or biochemical). For example, as
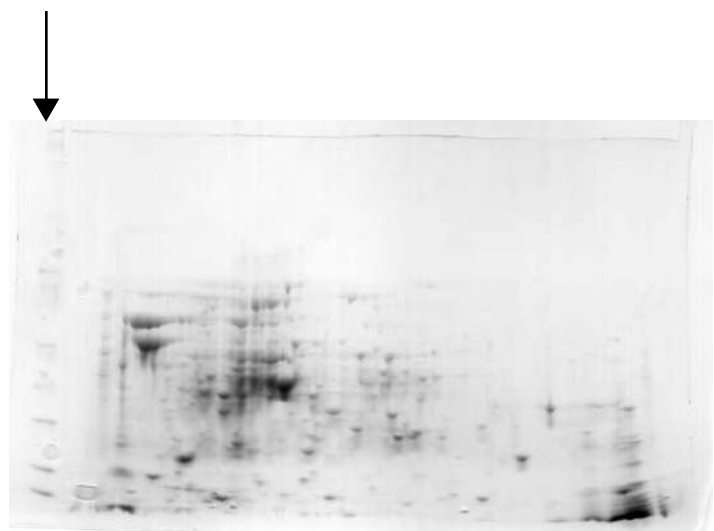
FIGURE 3.9 2D gel of an aqueous extract of *E. coli* stained with Coomassie Blue. Isoelectric focusing in the first dimension was performed in Protein cell (Bio-Rad, Hercules, CA) on IPG strips in the pH range 3 to 10. SDS-PAGE in the second dimension was performed on a 4 to 15% gradient gel (Bio-Rad). Protein markers are shown in the left (indicated by arrow).
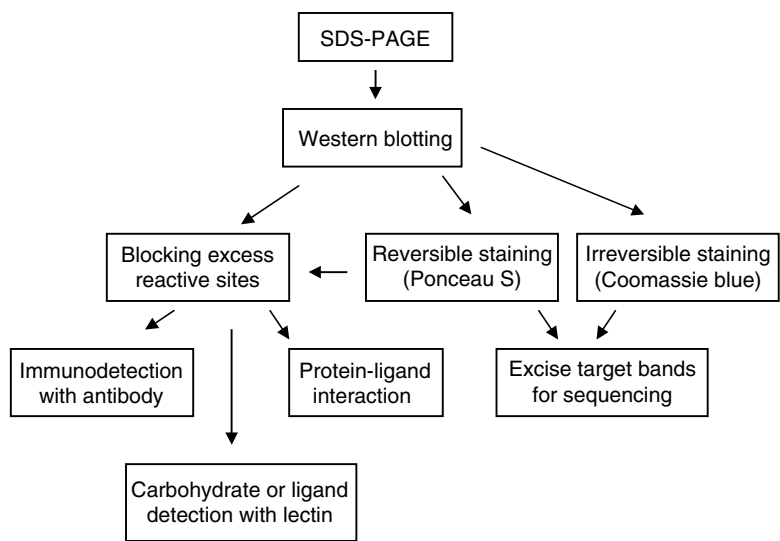

FIGURE 3.10 Flow diagram showing applications of immobilized proteins.

low as 1 to 10 pg of protein can be detected employing immunological techniques. Because of its wide applications and flexibility in protein detection methods, Western blotting has become a very popular and convenient method for analysis of denatured proteins.

Mechanism

Proteins are transferred from the SDS-PAGE gels, in which all proteins are negatively charged due to the SDS treatment. In an electric field, these negatively charge proteins migrate towards the positive and get immobilized on the membrane.

Protein transfer is usually accomplished by one of two electrophoretic methods: semi-dry blotting and wet blotting. In the former method, the gel and immobilizing membrane matrix are sandwiched between buffer-wetted filter papers, and a current is applied for 10 to 30 min. In wet blotting, the gel-membrane matrix sandwich is submerged in a transfer buffer and current is applied for 45 min to overnight. Due to its greater flexibility, wet blotting will be described here.

Blotting Membrane

Proteins in acrylamide gels can be transferred to nitrocellulose, PVDF, nylon, or carboxymethyl cellulose. However, for most applications, nitrocellulose and PVDF are preferred for the following reasons (see also Table 3.8). Nitrocellulose is relatively inexpensive, and its non-specific binding to the antibody can largely be blocked. PVDF is more expensive than nitrocellulose, but is ideal for N-terminal amino acid sequencing and amino acid analysis, since the membrane is resistant to acid and organic solvents. In contrast, blocking of the non-specific protein band in nylon is cumbersome because of the high-charge density of the matrices. Protein staining in nylon with common anionic dyes (Coomassie Blue, Amido Black, etc.) is not possible due to the positive charge of the nylon matrix. However, nylon is used when (a) higher protein binding is required, (b) a protein binds weakly to nitrocellulose (especially high molecular weight), and (c) greater resistance to mechanical stress is desired. The binding capacity of nylon is almost eightfold more than that of nitrocellulose (80 µg/cm^2).[46]

The efficiency of Western transfer depends on several factors such as composition of buffer, time, voltage, and size of the protein; percent acrylamide; and the thickness of the gel. An optimization is required for each protein, and the efficiency of transfer can be assessed by staining the blots with any blot stain (see Section 3.5.1). In general, proteins of low molecular weight transfer more easily than those of high molecular weight. Proteins transfer more effectively from low-percent acrylamide gels than from high-percent gels. Methanol improves protein binding to nitrocellulose membrane, but inhibits transfer. SDS is sometimes added to the transfer buffer to improve transfer of large proteins, but unfortunately it inhibits protein binding to the membrane. Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) is commonly used for most proteins. When the transferred protein is used for amino acid analyses, the Towbin buffer is replaced by the CAPS buffer

TABLE 3.8
Characteristics of Various Membranes Used in Western Blotting

Membrane	Commercial Source	Charge of the Membrane	Nature of Interaction between Membrane and Protein	Remarks	Recommended Applications of the Transferred Protein
Nitrocellulose	Bio-Rad	Negative	Hydrophobic and electrostatic forces	Low cost Mechanically fragile	Immuno detection
PVDF	Millipore	Negative	Hydrophobic forces	High cost	N-terminal sequencing, amino acid analysis, immunodetection, ligand binding
Nylon	Millipore	Positive	Electrostatic forces	Mechanically strong	Multiple reprobing
Carboxymethyl cellulose	Millipore	Negative	Ionic interactions	Very high capacity for histones Elution step is required before sequencing	Sequencing of basic proteins

(10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11.0) to avoid interference with the analyses due to the presence of glycine. When protein is transferred from acid urea gel or isoelectric focusing gel, acetic acid (0.7%) is used as a transfer buffer. The apparatus for Western transfer is available from several vendors such as Hoefer, Bio-Rad, and Pharmacia and can easily be operated following the manufacturer's instructions. A procedure for Western transfer using Hoefer apparatus is described below.

Working Procedure

Equipment

1. Western transfer apparatus
2. Power supply
3. Gel-membrane sandwich cassette
4. Sponges
5. Whatman 3MM paper
6. Blotting membrane (for example, PVDF)
7. Magnetic stirrer
8. Cooling water circulator
9. Plastic or glass tray to assemble gel-membrane sandwich

Reagents

1. Towbin transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol
2. Methanol

Procedure

1. Following SDS-PAGE, soak gel in Towbin buffer for 20 min at room temperature.
2. Fill up approximately two thirds of the transfer apparatus with the transfer buffer and cool down to 10°C with cold-water circulator.
3. Cut a piece of PVDF membrane slightly larger than the gel. Soak the membrane in 100% methanol for a few minutes (PVDF will not wet directly in Towbin buffer) and incubate the membrane in transfer buffer for 10 to 15 min.
4. Cut two pieces of Whatman 3MM filter paper slightly larger than the membrane and soak in transfer buffer. Leave gel, membrane, and papers in transfer buffer until a sandwich is made.
5. Unlock the empty cassette and place in a tray. Assemble gel-membrane sandwich as shown in Figure 3.11. In order to prepare sandwich, place a sponge (presoaked in transfer buffer) on top of one backing of the cassette.
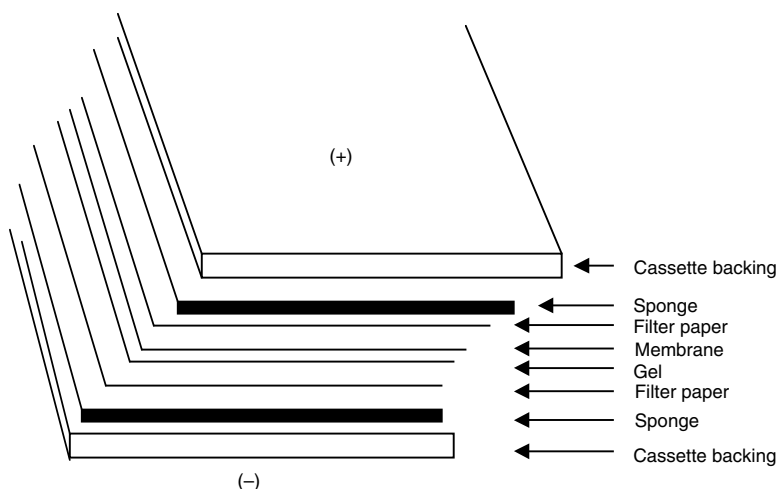
FIGURE 3.11 Preparation of sandwich for Western transfer.

Place one filter paper on top of the sponge. Place the gel on top of filter paper. Now layer carefully the membrane on top of the gel, not trapping any air bubbles. A test tube can be rolled over the membrane to remove bubbles. Be careful also not to dry out sponge, gel, or membrane during the assembly. It is helpful to put a few milliliters of transfer buffer in the tray. Place the second filter paper on top of the membrane and finally the second sponge on top of the filter paper. Now lock the cassette and place into the transfer apparatus, facing the membrane side towards the anode and the gel side towards the cathode. Add more transfer buffer if needed.

6. Close the lid of the electrophoresis apparatus and connect the electrodes to the power supply.
7. Run the transfer at 60 to 80 V or at 0.4 amps for 60 to 90 min. Most proteins up to 100 kDa are usually transferred by these conditions.
8. At the end of transfer, turn off power supply and remove the cassette from the transfer apparatus. Separate membrane from the gel and mark the side of the membrane to which the proteins were immobilized. The membrane is now ready for immunodetection or other applications as described. Treat Towbin buffer as MeOH hazardous waste. However, Towbin buffer can be reused three to four times.

3.5.1 Staining Proteins on Blot Transfer Membrane

Membrane-immobilized proteins are often visualized to monitor the efficiency of transfer prior to further processing. Several stains are used for blot membranes, but anionic dyes such as Amido Black or India ink are less satisfactory for nylon membranes.

Staining with Coomassie Brilliant Blue

Immobilized proteins can be visualized in a few minutes with Coomassie staining. As this staining is irreversible, blot membranes that are subject for immunodetection should not be stained with Coomassie. However, one important application of Coomassie blue is to stain a portion of the membrane and match the stained proteins with the immunodetected proteins.

Working Procedure

Reagents

1. Staining solution: 0.1% (w/v) Coomassie Brilliant Blue in 40% methanol/10% acetic acid
2. Destaining solution: 40% methanol/10% acetic acid

Staining Procedure

1. Stain the membrane with the staining solution for 1 to 5 min.
2. Destain with the destain solution until it clears the background.
3. Rinse with water and leave the membrane in water.

Staining with Amido Black

A protein band of lower microgram range can be detected with Amido Black satin.[47]

Working Procedure

Reagents

1. Amido Black 10B
2. Stain or destain solution: 25% isopropanol/10% acetic acid

Procedure

1. Stain the membrane (nitrocellulose or PVDF) with 0.1% Amido Black in staining solution for a minute.
2. Destain the membrane for 30 min with destaining solution and wash in water before drying.

Staining with India Ink

With this staining, protein bands appear as black on a gray background.[48] The stained membrane can be stored for at least one month without any loss of sensitivity.

Working Procedure

Reagents

1. Wash buffer: 0.3% Tween 20 in 10 mM sodium phosphate/150 mM NaCl, pH 7.2 (PBS-T)
2. India ink: 1% solution in wash buffer
3. KOH solution: 1%
4. PBS: 10 mM sodium phosphate/150 mM NaCl, pH 7.2

Procedure

1. Incubate the blot membrane in KOH solution for 5 min at 20°C.
2. Rinse the membrane twice with PBS (10 min each).
3. Wash the blot with wash buffer at 37°C (4 times, 5 min each). Rinse with water between wash.
4. Stain the blot with India ink solution with agitation for 15 min to overnight at room temperature.
5. Destain with multiple changes of PBS.

Staining with Ponceau S

Staining of immobilized proteins with Ponceau S is a reversible procedure, since the stain can be washed off completely with water. This stain is not very sensitive. Nonetheless, it is often used to monitor the transfer of protein prior to immunodetection or other applications. The stain is also used to identify bands for micro sequencing.

Working Procedure

1. At the end of transfer, soak the blot in Ponceau S solution (0.1 g Ponceau S/1 ml acetic acid/100 ml water) for 5 min with gentle agitation.
2. Destain with water for 2 min. Blot now can be photographed.
3. For immunodetection or other applications, destain the membrane completely in water for 10 min. Prior to destaining, mark the band and the molecular weight standard with a pencil or pinhole.

Other Stains for Blot Membranes

Several stains for blot membranes are commercially available. They are usually more sensitive than Ponceau or Amido Black. In most cases, the identities of the staining reagents are trade secrets. The staining procedures are available from vendors. MemCode™ Reversible Protein Stain (Pierce, Rockford, IL) is used to stain the protein band on nitrocellulose membranes. The stain on blots can be washed off quickly for immunodetection or other applications. This stain is not suitable for PVDF membrane. SYPRO^R Ruby protein blot stain (Molecular Probes, Eugene, OR) is a very sensitive reagent to detect proteins on both nitrocellulose and PVDF membranes.

3.5.2 RECOVERY OF PROTEINS FROM BLOT MEMBRANE

Recovery of proteins from membranes is often needed for many applications such as amino acid composition analysis, for protein sequencing, and as an immunogen. Several solvents can be used to elute protein from the membrane, and the choice of the solvent system depends on the intended application. For example, acetonitrile or n-propanol usually maintains the protein structure and thus can be used as an immunogen or antigen in radioimmunoassays. Detergent-based systems are used to elute proteins when proteolytic and analytical manipulations are desired. Detergent elution is more effective than elution with organic solvents.

Working Procedure

Elution with an Organic Solvent System

1. Cut out the band of interest from the membrane and place it in a microfuge tube.
2. Incubate with 500 μl elution buffer (40% acetonitrile in 0.1 M ammonium acetate buffer, pH 8.9) at 37°C with shaking for 3 h.
3. Centrifuge the tube for 10 min at highest speed in a microfuge. Collect the supernatant.
4. Incubate the same blot for a second time with 250 μl elution buffer. After centrifugation, pool the second supernatant with the first.
5. Lyophilize to remove the volatile solvent.

Elution with a Detergent-Based Solvent System

1. Cut out the band of interest from the membrane and place it in a microfuge tube.
2. Incubate with 500 μl elution buffer (50 mM Tris-HCl, pH 9.0 containing 2% SDS, 1% Triton X-100, and 0.1% dithiothreitol) at room temperature for 1to 3 h.

3. Centrifuge the tube for 10 min at highest speed in a microfuge. Collect the supernatant.
4. Incubate the same blot for a second time with 250 µl elution buffer. After centrifugation, pool the second supernatant with the first.

3.6 CAPILLARY ELECTROPHORESIS

In this procedure molecules such as proteins, glycoproteins, peptides, and DNA are separated in a capillary tube (usually made of silica, 10 to 100 µm diameter) under a potential difference produced at two ends. The most common type of capillary electrophoresis is capillary zone electrophoresis (CZE), which relies on simple instrumentation consisting of a capillary column, a detector, and a high-voltage power supply (Figure 3.12). The two ends of the capillary tube are immersed in reservoirs containing electrolytes, which serve as electrodes. These electrodes are connected to a high-voltage power supply. A sample is introduced at one end of the capillary (inlet), and upon applying an electric field, sample components are separated as they travel through the capillary toward the other end (outlet). At the far end of the capillary, the separated components are sensed by a detector, and output signal is recorded. Since the walls of the capillary have a standing charge, an electroosmotic flow of water is produced from anode to cathode (Figure 3.13). So migration of a positively charged molecule from anode to cathode depends on the applied voltage gradient and electroosmotic flow. Uncharged molecules are separated
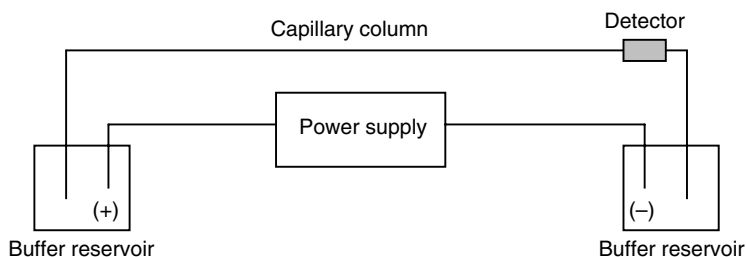
FIGURE 3.12 Schematic representation of capillary electrophoresis apparatus.
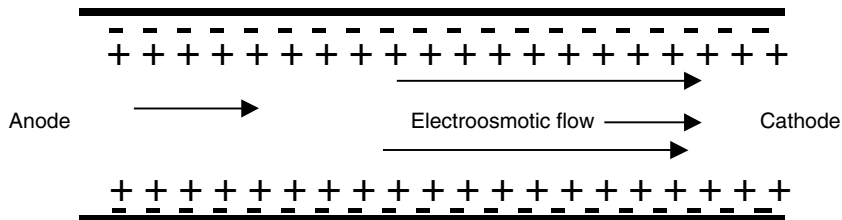
FIGURE 3.13 Separation of sample in the silica capillary.

$$\gg SiO^- \quad + \quad H^+ \quad \rightleftharpoons \quad \gg SiOH \quad \rightleftharpoons \quad \gg SiOH_2$$

Alkaline pH   Neutral   Acidic pH
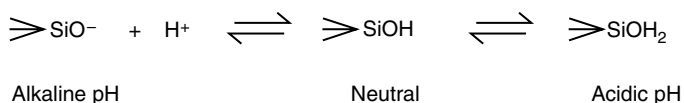
FIGURE 3.14 Silanol equilibrium.

on a silica capillary because of the electroendo-osmotic flow. For charged molecules, the apparent rate of migration is the algebraic sum of electrophoretic mobility and electroosmotic flow. Electrophoretic mobility is dependent on the mass/charge ratio.

The silica capillary columns are usually coated to reduce electroosmotic flow, resulting in improved separation. In the presence of electroosmotic flow, charged molecules migrate in an eliptical shape, but when migration is solely by the applied voltage gradient, the molecule front is plug shaped, resulting in a sharp peak.

The use of a buffer at extreme pH (high about pH 10 and low at about 2) results in a decrease in electrostatic adsorption. The silanol group is negatively charged at high pH and is protonated at acidic pH (Figure 3.14). At pH 10, most proteins (except very basic proteins) are negatively charged, and since the capillary wall is also negatively charged, the electrostatic interaction is minimized. Similarly at very low pH (about 2) both capillary wall and proteins are positively charged, resulting in a reduced electrostatic adsorption of proteins onto capillary wall. However, this practice is not popular because of possible denaturation and the loss of biological activities of proteins at extreme pH.

Detection of Protein

In contrast to standard liquid chromatography, where proteins are usually detected at 280 nm (the path length of the absorbance detector is usually 1 cm), detector signal in a capillary electrophoresis system is not satisfactory due to a very short detection path length (25 to 75 µm). Although the absorbance of protein at 200 nm is about 50- to 100-fold greater than that at 280 nm, detection in the low UV region is also not suitable for many applications in capillary electrophoresis. Alternatively, proteins can be detected by the intrinsic fluorescence of their tryptophan and tyrosine residues. However, the detection of intrinsic protein fluorescence requires very costly laser detectors (49). Thus, for capillary electrophoresis, pre- and postcolumn derivatization techniques have been developed to increase detection sensitivity of proteins.

Detection Using Precolumn Derivatization of Proteins

Precolumn derivatization is widely used for analysis of amino acids using a variety of reagents such as phenylisothiocyanate and *o*-phthalaldehyde, which react with the amino groups of the proteins. There are some inherent problems in derivatization of proteins prior to electrophoresis. In contrast to amino acids, which have one or two reaction sites, proteins can have multiple reactive sites producing multiple derivatization (heterogeneous) products with varying mobilities. This results in broadening of a protein peak. The production of heterogeneous derivatives can be

minimized, to some extent, by using either mild or drastic derivatization conditions. In the former condition, only the most reactive sites will be derivatized, while in the latter condition all possible reactive sites will be labeled.[49]

Capillary Coating

Capillary walls are coated in several ways in order to reduce the non-specific adsorption of protein onto the capillary wall. The capillary is generally deactivated by silanization, and the negatively charged silanol is then modified by a variety of groups such as methyl cellulose, polyacrylamide, polyethylene glycol, etc.

Capillary walls can be coated temporarily during electrophoresis by several buffer additives. High salts such as sulfates and phosphates of about 0.25 M compete with protein for adsorption, resulting in an improved separation. The only problem associated with high ionic strength buffer is the generation of Joule heat, which needs to be dissipated efficiently. Some zwitterionic salts such as betaine, sacrosine, and triglycine are shown to be advantageous up to 1 to 2 M without contributing significant change of conductivity. No single method is suitable for the separation of all types of proteins. Thus, the type of coating changes according to the nature of protein to be separated. For example, for the separation of hydrophobic proteins, non-ionic surfactants such as Tween 20 or Brij 35 are used to reduce the hydrophobicity of the coated capillary column. Similarly for the improved separation of the cationic proteins, the negative charge of the capillary wall is reversed by cationic surfactants such as CTAB.

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4 Purification of Protein

The purification of protein is an essential first step for the study of its molecular and biological properties in order to understand its biological function. There are several properties (such as molecular weight, charge, hydrophobicity, etc.) that can be exploited to purify or single out a protein from a mixture. Based on those properties, several chromatographic and non-chromatographic (electrophoretic, precipitation, membrane-filtration) procedures have become available (see Table 4.1).

4.1 GENERAL CONSIDERATION AND PURIFICATION STRATEGY

In protein purification, it is important to adopt procedures that do not cause denaturation of proteins, especially the protein of interest. The choice of purification methods is also influenced by factors such as how the purified protein is to be used in studies, the quantity of the purified protein needed, and the cost of the materials and reagents used in the purification. A purification step that may denature purified protein is not suitable for studies of its biological properties, but may be suitable for the determination of its primary structure, subunit size, etc. The purification protocols for obtaining a microgram level of purified protein (for partial peptide sequence in order to construct a gene probe) may be different from those that yield larger quantities of purified protein. The cost of ligands used for immobilization of matrix and for elution of a bound protein in affinity chromatography may be limiting factors for large-scale purification.

A protein may be purified by a single step (for example, affinity chromatography), or by a combination of several steps (for example, salt fractionation, ion exchange, gel filtration, etc.). In general, anion-exchange chromatography is employed for the purification of an acidic protein. Similarly, for the purification of a basic protein, cation-exchange chromatography is the better choice. Reverse-phase chromatography is suitable for a family of active proteins of similar charge.

Researchers should develop an assay for the determination of activity of the protein to be purified in order to monitor purification. Purification steps divide the total protein in the crude extract into several fractions, each of which is then assayed for activity and protein content. A fraction with high specific activity and purification-fold dictates the success of its purification step. Specific activity is defined as the total activity per milligram of protein per milliliter in a fraction. Purification-fold is the ratio of specific activity of a fraction to that of crude extract. Table 4.2 shows an example of a typical purification table of protein.

TABLE 4.1
Several Techniques for Protein Purification

Technique	Property Required	Remarks	When to Use (Recommended Application)
Membrane filtration	Molecular size	Fractionation as well as concentration. Loss of protein by non-specific adsorption.	At the beginning of a purification procedure. Particularly useful for concentrating large volumes of culture medium.
Centrifugation	Molecular size, shape, density	Commonly used for cellular fractionation	
Preparative isoelectric focusing	pI	Proteins precipitate in the rotofor chamber	
Preparative electrophoresis	Charge		
Size exclusion	Molecular size	Usually low resolution. Provide information about protein molecular weight.	At the end of a purification procedure
Ion-exchange	Charge	Protein binding capacity usually high	At the beginning of a purification procedure
Reversed phase	Hydrophobicity	Resolution varies according to gel size. Commonly used for peptide separation.	Used for separation of peptides, digested purified proteins, and other applications where loss of protein's biological activity is not a concern.
Hydrophobic	Hydrophobicity		After ammonium sulfate fractionation, but before ion-exchange chromatography
Hydroxyapatite	Charge		
Affinity	Binding ligand	Usually specific separation. Limited by availability of immobilized ligand. Expensive to scale up.	At the beginning of a purification procedure
Covalent	Thiol groups	Limited to thiol-containing proteins. Specific separation.	For thiol-containing proteins
Chromatofocusing	Charge, pI		Useful to separate isoforms of closely spaced pIs. Use after affinity chromatography.

TABLE 4.2
Purification of a Sialic Acid Binding Lectin from Human Placenta[a]

Step	Total Protein (mg)	Specific Activity	Total Activity	Recovery (%)	Purification (Fold)
Crude extract	1,340	0.6	804	100	1
Anion-exchange chromatography on DEAE-cellulose	928	0.8	742	92	1.3
Affinity chromatography on BSM-Sepharose	0.9	427	384	48	712
Gel filtration chromatography on Ultrogel AcA 44	0.12	800.0	96	12	1,333

Note: Specific activity is expressed as titer/mg protein/ml. Titer is the reciprocal of the highest dilution of the lectin showing visible agglutination.

[a] Adapted from Reference 1.

4.2 NON-CHROMATOGRAPHIC PURIFICATION OF PROTEINS

Prior to chromatographic techniques there are several fractionation procedures available that enable the concentration of a desired protein relatively easily.

4.2.1 FRACTIONATION

Fractionation of proteins by precipitation with ammonium sulfate is the most commonly used method to enrich a particular protein (Table 4.3). Proteins of very high molecular weight usually precipitate below 25% ammonium sulfate saturation. Crude extracts may be subjected to fractionation in three stages: 0 to 30%, 30 to 60%, and 60 to 80% ammonium sulfate saturation. Precipitated protein from each stage plus the 80% supernatant are then tested for activity. Precipitation of proteins by water miscible organic solvents such as alcohol or acetone is another technique, but is not commonly used, probably because of its tendency to denature protein at room temperature. Precipitation with polyethylene glycol (PEG) has been used with considerable success for several proteins. A disadvantage of using PEG is that it is not easily removed from protein solutions.

4.2.2 MEMBRANE ULTRAFILTRATION

Ultrafiltration of the crude extract through a membrane with a specific molecular weight cut-off (MWCO) is an alternative way of enriching a desired protein without significant loss of biological activity. Another advantage of membrane ultrafiltration is that proteins become concentrated in this process. In ultrafiltration, solutes are

TABLE 4.3
Amounts of Solid Ammonium Sulfate (in grams) Required per Liter of Solution from initial Percentage Saturation to Target Percentage Saturation at 0°C[a]

Initial % Saturation at 0°C	Target % Saturation at 0°C														
	20	30	40	45	50	55	60	65	70	75	80	85	90	95	100
0	106	164	226	258	291	326	361	398	436	476	516	559	603	650	697
10	53	109	169	200	233	266	301	337	374	412	452	493	536	581	627
20		55	113	143	175	207	241	276	312	349	387	427	469	512	557
30			56	86	117	148	181	214	249	285	323	362	402	445	488
40					58	89	120	153	187	222	258	296	335	376	418
45						59	90	123	156	190	226	263	302	342	383
50							60	92	125	159	194	230	268	308	348
55								61	93	127	161	197	235	273	313
60									62	95	129	164	201	239	279
65										63	97	132	168	205	244
70											65	99	134	171	209
75												66	101	137	174
80													67	103	139
85														68	105
90															70

[a] Adapted from Reference 2.

forced through the membrane, usually by nitrogen pressure. Air is not used, to avoid protein oxidation. Smaller solutes can pass through the pores of the membrane, leaving larger molecules in the retentate. The retention properties of ultrafiltration membranes are usually expressed as a molecular weight cut-off that rejects approximately 90% of a globular protein of stated molecular weight. If the ultrafiltration is performed first for the purpose of either concentrating or desalting the purified protein, then it is important to perform pilot experiments to verify membrane retentivity before use, because different manufacturers use different molecules to define the MWCO of their membranes.

However, molecules whose sizes are at least 50% larger than the stated cut-off size will usually be retained on the membrane. Although membrane filtration provides only two fractions (filtrate and retentate), it is nevertheless advantageous for enriching the protein of interest. For example, if the molecular size of the protein of interest is about 200 kDa, a membrane with 100 kDa cut-off can be used to remove most of the proteins of less than 100 kDa molecular size. This also allows the retentate to be concentrated to a smaller volume, which is essential for gel filtration chromatography.

Small volumes (0.5 to 30 ml) are conveniently filtered through the membrane by centrifugal force. The protein extract is loaded on top of the membrane and then centrifuged at the recommended speed. Proteins of molecular weight lower than the

TABLE 4.4
Common Ultrafiltration Devices

Supplier	Trade Name	Molecular Weight Cut-Off (kDa)	Maximum Sample Volume (ml)	Type of Membrane
Pressure device:				
Amicon	PM	10, 30	50, 200, 500	Polyethersulfone
	YM	1, 3, 10, 30, 100	50, 200, 500	Regenerated cellulose
	YC	500	50, 200, 500	Cellulose acetate
Centrifugal device:				
Amicon	Microcon, Centricon	3, 10, 50, 100	0.5, 2	Regenerated cellulose
Gelman Science	Nanosep Microsep Macrosep Jumbosep Delta spin	1, 3, 10, 30, 50, 100, 1000	0.5, 3.5, 15, 60	Modified polyethersulfone
Nalgene		4, 10, 30, 100	0.5, 4, 15	Polyethersulfone
Schleicher & Schuell	Centrex UF	3, 10, 30, 100	0.5, 2	Regenerated cellulose

membrane MWCO are collected at the bottom and higher molecular weight proteins are retained on the top of the membrane. Table 4.4 shows several types of membranes and their MWCO. Fractionation of proteins based on molecular weight is achieved by passing an extract through several membrane filtration devices with varying MWCO. Pall Gelman Sciences (Ann Arbor, MI) developed a unique device (DeltaSpin) where multiple filtration membranes with varying MWCO are stacked together in a decreasing molecular weight order from top to bottom. Protein extract is loaded on top of the first filtration device, and upon centrifugation proteins are compartmentalized according to their molecular weight. The most active fraction from either technique is then subjected to chromatographic separation. The disadvantage of using membrane filtration is that proteins are easily adsorbed nonspecifically onto the membrane. Adsorption of proteins may be reduced by using low-binding membrane (YM type, Amicon) and separating retentate protein from membrane as soon as filtration is done.

4.2.3 DIFFERENTIAL CENTRIFUGATION

The working principle of separation by differential centrifugation has been discussed in Chapter 1. This technique is widely used for subcellular fractionation of a tissue homogenate (see Chapter 1).

4.2.4 Preparative Electrophoresis

Preparative electrophoresis is a powerful technique, in which proteins are first separated by electrophoresis and then eluted from the polyacrylamide gel. Depending on the nature of studies of the purified proteins, native PAGE or SDS-PAGE is performed. For preparative electrophoresis, a special preparative comb is used that contains a wide single well and a narrow reference well for standard (reference) protein. After electrophoresis, a slice of the gel containing a protein band is cut out, and protein is extracted by simple diffusion into an appropriate buffer by electroelution. In electroelution, proteins are electrophoresed out of gel pieces into a Centricon chamber (Amicon, Millipore Corporation, Bedford, MA). A gel eluter is available from Bio-Rad for elution of proteins from gels and simultaneous collection of multiple protein bands. Bio-Rad's Prep Cell (Model 491) and Mini Prep Cell are continuous-elution electrophoresis systems that allow separation of proteins differing in molecular weight by as little as 2%. In a single chamber, molecules are electrophoresed through the cylindrical gel matrix, and as they exit the bottom of the gel, they are eluted in a stream of elution buffer to be collected as liquid fractions.

4.2.5 Preparative Isoelectric Focusing

The principle of isoelectric focusing is described in Chapter 3. Although isoelectric focusing is used widely as an analytical procedure, recent developments in electrophoretic apparatus make it possible to purify proteins on a larger scale. Bio-Rad's Rotofor® has been developed for free solution isoelectric focusing in the presence of non-ampholyte electrofocusing buffers, RotoLytes.[3] The advantages of using RotoLytes over ampholytes are that RotoLytes can easily be removed from proteins after electrophoresis, and RotoLytes neither interact with proteins nor interfere with protein estimation. RotoLytes consist of more than ten buffer pairs with closely spaced pK_as covering pH range 2.9 to 11.0, and generate shallow and linear pH gradients between their individual pK_as. The sample with RotoLytes is loaded into the Rotofor cell comprising 20 discrete compartments partitioned with polyester membrane screens. Upon application of voltage perpendicular to the membrane screens, proteins migrate through the screens to their isoelectric points (pIs). Focused proteins in the 20 compartments are collected into 20 tubes by a harvesting apparatus.

4.3   CHROMATOGRAPHIC PURIFICATION OF PROTEINS

All chromatographic systems consist of two phases: stationary phase and mobile phase.[4] The stationary phase may be a solid, gel, liquid, or a mixture of solid and liquid, whereas the mobile phase may be liquid or gaseous and flows through the stationary phase. All chromatographic purifications of proteins are based on an equilibrium achieved between the stationary phase and the mobile phase. Table 4.5 shows the medium of stationary phase and mobile phase for most chromatographies. Most chromatographic systems require some common equipment. These are buffer reservoir, tubing, peristaltic pump, column, UV detector, chart recorder, and fraction collector.

TABLE 4.5
Medium of Stationary and Mobile Phases of Most Chromatographies

Chromatography	Stationary Phase	Mobile Phase	Remarks
Gel filtration	Liquid inside gel beads	Liquid	
Ion-exchange	Solid	Liquid	Ion-exchange equilibrium
Chromatofocusing			
Reversed-phase	Liquid	Liquid	Partition equilibrium
Hydrophobic	Solid	Liquid	Adsorption equilibrium
Affinity covalent	Immobilized ligand	Liquid	

Buffer Reservoir

This stores washing or eluting buffer. The reservoir is connected to the column by tubing. The reservoir is usually a bottle or Erlenmeyer flask, but can also be a beaker.

Tubing

The tube used in chromatography is usually silicone, Tygon[R], or Teflon[R]. The buffer travels from the reservoir to the column through the tube by gravity or with the help of a peristaltic pump.

Peristaltic Pump

The pump controls the rate of buffer flow to the column. A pump is usually preferred over gravity, to maintain a uniform buffer flow rate.

Column

This is the most important part of the chromatography setup. Column hardware is usually a plastic cylinder filled with gel matrix. Gel matrices or packing materials vary based on the type of chromatography used. Different chromatographic separation methods depend on the nature and chemistries of the gel matrices. Column specification for each chromatography will be described later. In general, "dead space" after the column support should be minimum to reduce any sample remixing after the chromatographic separation ($< 0.1\%$ of the column volume is standard).

UV Detector

An ultraviolet wavelength detector (280 nm) is generally used to monitor the protein peaks.
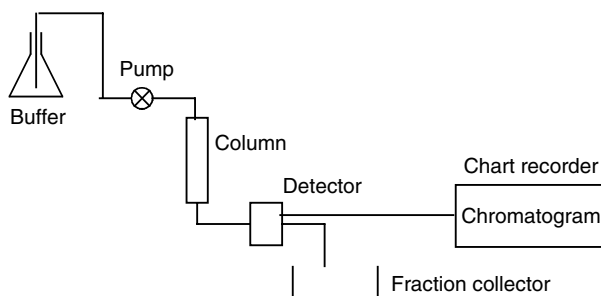
FIGURE 4.1 Diagram of basic gel filtration chromatography system.

Chart Recorder

The protein peaks measured by the UV detector are traced by the chart recorder. The UV detector and chart recorder save the researcher the time and effort of measuring each fraction's protein content by standard spectrophotometric methods.

Figure 4.1 shows a simple setup of chromatography. The sample is loaded on top of the column, and its top is connected to a reservoir of washing or eluting buffer. Samples are fractionated by the eluting buffer under gravity or by a peristaltic pump. The effluent passes through the UV monitor (usually at 280 nm) and is collected in tubes placed on a fraction collector. The length and diameter of the tube, which connects the bottom of the column, monitor, and fraction collector, should be minimized in order to prevent mixing of the fractions. Sometimes, good separations fail because of the mixing of fractions inside tubing with a large diameter.

Matrices for all chromatographic separations are available for conventional use or for use in high-performance liquid chromatography (HPLC). Conventional matrices are distinguished by slow flow rates and are less expensive, whereas high-performance columns are expensive and render high flow rates. While this chapter discusses the principles of various types of chromatographies, the focus will be on conventional columns of different chromatographies. Other types of columns widely used in HPLC will be discussed in the last section of this chapter.

4.3.1 GEL FILTRATION (SIZE EXCLUSION) CHROMATOGRAPHY

In gel filtration, proteins are fractionated based on their relative size. The term "filtration" is somewhat misleading, because unlike any filtration procedure, all proteins pass through the column, and there is no "retentate." Gel filtration chromatography is also recognized as gel permeation chromatography, size exclusion chromatography, and molecular sieving chromatography. Besides purification, gel permeation chromatography is also used to determine the molecular weight of the protein and to remove low molecular weight impurities (desalting).

Principle

In gel filtration, liquid inside the gel particles (stationary phase) is in equilibrium with a mobile phase. Solute molecules (proteins) are diffused by the Brownian
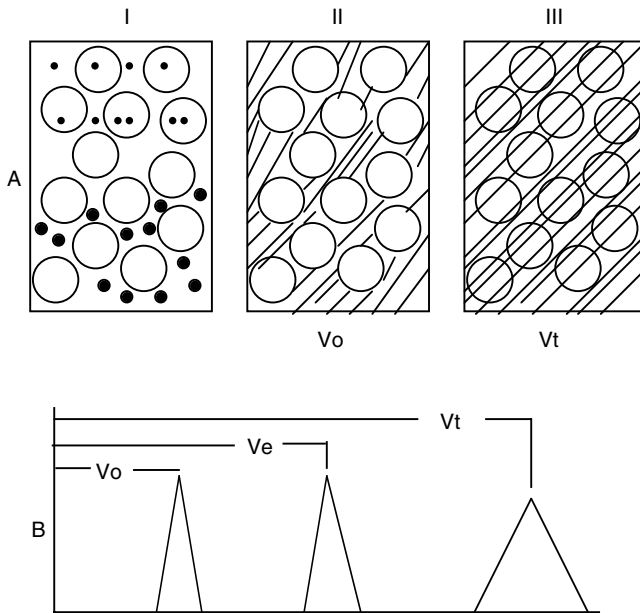
FIGURE 4.2 Gel filtration chromatography. A I: Migration of samples (shown in closed circle) on a gel filtration column. Small molecules enter the gel pore (gel beads shown in open circle), while large molecules cannot enter the pores of the gel and travel very fast through the interstitial space (Vo). A II: Interstitial space (Vo) is shown in shaded area. A III: Total column volume (Vt). B: Vo, Ve, and Vt are obtained by measuring the elution volumes as shown.

motion to both inside and outside of the gel particles. The molecules are separated because of their different abilities to enter the pores of the gel particles. Very large molecules cannot enter the pores of the gel particles and thus travel very fast through the interstitial spaces of the gel particles (Figure 4.2 A I). The interstitial space of the gel particle is known as the void volume, Vo, which is the elution volume of the very large or excluded molecules that do not enter the gel pores (Figure 4.2 A II). The void volume is usually one third of the total volume. Smaller molecules can enter the gel pores and spend some time in the stationary phase. They move slowly through the column, and their elution volumes are designated as Ve. The total volume (Vt) represents the sum of the external and internal volume within the beads (Figure 4.2 A III). Solute molecules are eluted in order of decreasing molecular weight. The elution volume (Ve) of standard protein can, therefore, be used to determine the molecular weight of the unknown protein. But Ve of a solute is not the independent parameter, since it varies with the total volume (Vt) of the packed gel bead, which can vary from experiment to experiment. The elution of a solute is best characterized by a distribution coefficient (Kd). Kd is the fraction of the stationary phase, which is available for diffusion of a particular solute. The volume of the stationary phase (Vs) is equal to the volume of liquid inside the gel particle and can be calculated as follows:

$$Vs = Vt - Vo - Vgel \text{ matrix}$$

$$So, \ Kd = (Ve - Vo) / Vs$$

In practice, Vs is rather difficult to measure, and thus Kd is not easily obtained. Kav is commonly used to determine the molecular weight of an unknown protein from a semi-logarithmic plot of the dependence of Kav on known molecular weights. Kav is calculated from the following equation:

$$Kav = (Ve - Vo) / (Vt - Vo)$$

Although Kav is not a true partition coefficient, for a given gel there is a constant ratio of Kav:Kd, which is independent of the nature of the solute or its concentration. In practice, Vo, Vt, and Ve are determined from gel filtration of solutes of high molecular weight, low molecular weight, and intermediate molecular weight, respectively, as shown in Figure 4.2 B. Blue dextran and small peptides are frequently used to determine Vo and Vt, respectively. For molecular weight determination of unknown proteins, gel filtration column is calibrated with several standard proteins, and the values of their Kav are obtained from their elution volumes (Ve). However, measurements of Vo, Vt, and Ve are not required if gel filtration chromatography is applied only for a purification purpose. The plot of Kav and molecular weight usually appears as a sigmoid whose central linear range (Kav values between 0.2 and 0.8) is suitable for protein separation. The span of linearity is dependent on the fractionation range of a gel filtration matrix. The higher the span, the greater the resolving power of the matrix.

4.3.1.1 Recommended Application of Gel Filtration Chromatography in a Protein Purification Procedure

As shown in Figure 4.2 B, fractions that elute first appear sharper than those that elute later. The largest size molecules spend less time in the column and thus spread less. Smaller molecules spend more time in the column and thus diffuse more. The sharpness of the eluted fractions also depends on the volume of the loading sample. The volume of the loading sample should be kept below 3% of the total volume. Better results are obtained when the loading volume is within 1% of the total volume. Usually, each component is eluted in a volume of about 8 to 10% of the column. Since the effective fractionation volume (Vt - Vo) is slightly higher than half of the total column volume, theoretically, not more than five to six proteins can be resolved from one another in the effluent from any gel filtration column. Thus, gel filtration is not suitable for fractionation of crude extracts.

It is rather wise to perform gel filtration at the end of a purification procedure when the numbers of undesirable proteins are minimum. Other purification procedures based on different properties, such as charge, hydrophobicity, or ligand affinity chromatography, are generally used prior to gel filtration. However, if the protein of interest is relatively large (e.g., over 150 kDa), gel permeation chromatography can be used as an initial step in the purification. With a proper gel matrix (e.g.,

Sephadex G-100), proteins over 150 kDa will be eluted in the void volume, leaving all smaller proteins in the column, achieving a partial purification of the desired protein.

4.3.1.2 Various Gel Filtration Matrices

Gel filtration matrices are generally cross-linked products of dextran, agarose, and polyacrylamide. Table 4.6 shows the various types of matrices and their chemistries. One of the most widely used matrices in gel filtration is Sephadex, which is prepared from the cross-linking of dextran by epichlorohydrin (Figure 4.3). Sephadex gels are usually stable in water, salt solutions, organic solvents, and buffers of a wide pH range, but their glycosidic linkages can be hydrolyzed in the presence of strong acid. Biogels and cross-linked polyacrylamide beads are available in different grades and have also been used widely to fractionate solutes of wide molecular weight range. Despite their chemical stability and fractionation capabilities, these are physically very fragile. During chromatography even slight pressure, including osmotic pressure, can cause distortion and irregular packing and thus create a poor flow. This problem is largely overcome by extra cross-linking of the dextran to acrylamide. For example, Sephacryl (Pharmacia) is a rigid gel prepared by cross-linking allyl dextran with N, N′-methylene bisacrylamide (Figure 4.4). The Ultrogels (LKB) are the cross-linked products of agarose gels with acrylamide. Sephacryls and Ultrogels confer greater rigidity and lower porosity than Sephadex and Biogels.

For fractionation of large molecules, agarose gels are suitable. Pharmacia's Sepharose is a bead-formed gel prepared from agarose, which is a polymer of Gal β1,4 [3,6]-anhydro-L-galactose (Figure 4.5). Natural agarose is a complex mixture of charged and neutral polysaccharides known as agar. Agarose is obtained by a purification process that removes most of the charged saccharides. To prepare Sepharose, a hot solution of agarose is cooled, allowing the individual polysaccharide chains to form double helices, followed by aggregation to form bundles and finally a stable gel. The gel is stable in most gel filtration applications in the presence of buffers at the pH range 4 to 9. Additionally, the presence of the unusual sugar 3,6-anhydro-L-galactose in Sepharose contributes to its resistance effect to biological degradation. The gel structure of Sepharose is stabilized by hydrogen bonding (not by covalent cross-linking, as for other matrices) and therefore not ideal for use with dissociating media such as guanidine hydrochloride, urea, etc. Pharmacia has introduced a more rigid cross-linked Sepharose, Sepharose CL, that is suitable in the presence of dissociating agents and chaotropic salts. Sepharose CL is prepared from a cross-linking of Sepharose with 2,3-dibromopropanol under alkaline conditions followed by desulphation (removal of charge) by alkaline hydrolysis under reducing conditions. Because of a very low content of charged groups present in Sepharose CL, it shows lower non-specific adsorption than the parent Sepharose. In addition to gel filtration chromatography, Sepharoses are also widely used in affinity chromatography where various ligands are immobilized on the matrix (see Section 4.3.6).

The types of matrices differ in their degree of cross-linking and hence in their fractionation range. The G-types of Sephadex differ in the degree of their cross-linking and thus their fractionation range. Usually, the higher the number, the higher

TABLE 4.6
Some Commonly Used Gel Filtration Matrices

Cross-Linking Chemistry	Matrix	Trade Code	Fractionation Range (kDa)	Manufacturer
Dextran	Sephadex	G10	<0.7	Pharmacia
		G25	1–5	
		G50	1.5–30	
		G100	4–150	
		G150	5–300	
		G200	5–600	
Dextran and polyacrylamide	Sephacryl HR	S200	5–250	Pharmacia
		S300	10–1,500	
		S400	20–8,000	
Agarose	Sepharose	2B	10–4,000	Pharmacia
		4B	60–20,000	
		6B	70–40,000	
Agarose	Biogel A	A5m	10–5,000	Bio-Rad
		A15m	40–15,000	
		A50m	100–50,000	
		A150m	1,000–150,000	
Polyacrylamide	Biogel P	P2	0.1–1.8	Bio-Rad
		P6	1–6	
		P30	2.5–40	
		P60	3–60	
		P100	5–100	
		P200	30–200	
		P300	60–400	
Agarose	Ultrogel A			IBF
Agarose and polyacrylamide	Ultrogel AcA			IBF
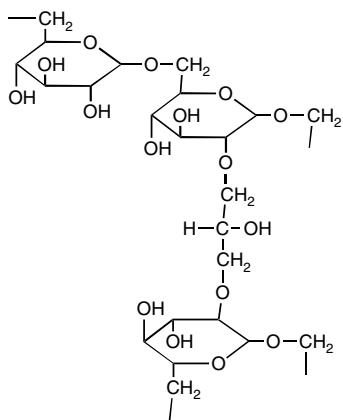
FIGURE 4.3 Partial structure of Sephadex.
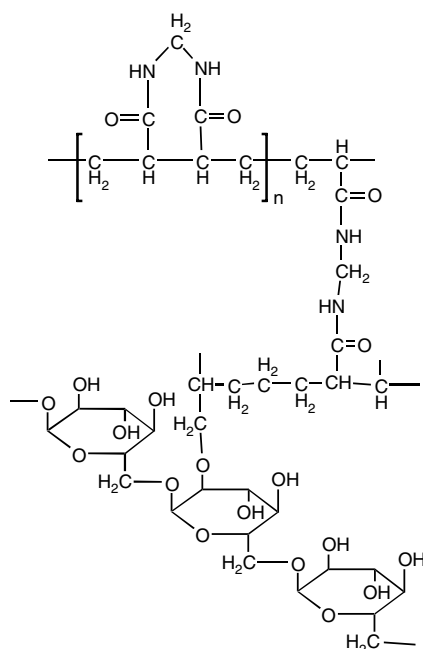
FIGURE 4.4 Partial structure of Sephacryl.
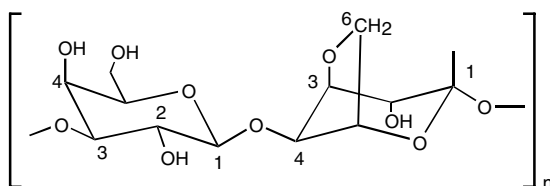
FIGURE 4.5 Partial structure of Sepharose.

is the fractionation range. Some matrices are available in different particle sizes. The highest resolution is obtained with the smallest particle size. For Sephadex, gel particles are designated as super fine, medium, and coarse. Operating pressure is highest with the smallest size or super fine particles. Large-size particles are generally used for preparative chromatography, where a high flow rate at a low operation pressure is essential.

4.3.1.3 Choosing the Buffers

Various buffers of moderate pH and ionic strength can be used in gel filtration chromatography. The choice of buffer depends on the nature and compatibility of the protein of interest. Phosphate and Tris buffers in the presence of sodium chloride of ionic strength 50 to 100 mM at physiological pH are typically used in gel permeation chromatography. There are some considerations about possible interactions between

the packing materials and the protein molecules including the target molecule. Buffers of the above ionic strength are suitable to avoid such interactions between the packing material and the protein molecules.

4.3.1.4   Choosing the Flow Rate

In gel permeation chromatography, maximum peak resolution is generally achieved using a long column with a low flow rate. The peak resolution increases as the flow rate decreases. However, when the flow rate is too low, diffusion plays a significant role, causing poor resolution. The typical flow rate for optimal resolution is in the range 2 to 10 cm/h, where cm/h (centimeter/hour) equals to $ml/h/cm^2$. The cm^2 represents cross-sectional area of the column. An increase in column diameter (i.e., cross-sectional area of the column) increases the column flow rate (ml/h).

4.3.1.5   Preparing the Column

Since a wide variety of prepacked gel filtration columns have become available from commercial sources, and they are conveniently used in high-performance liquid chromatography, columns are rarely packed in a laboratory. For separation of large volumes and quantities of samples, conventional gel filtration chromatography with a large column is still cost effective. Matrices are available in powder form as well as in hydrated form.

Swelling the Matrix

Powder form of the matrix is generally swollen according to manufacturer's instruction. For swelling the matrix, ten parts of the buffer are added to one part of the matrix, and the slurry is agitated on a rotary shaker. Magnetic stirrers should not be used, as beads become trapped between the magnetic bar and stirrer, resulting in a considerable breakage of the beads. Swelling can take an hour (when boiled) to overnight (at room temperature). When the matrix is not swelled by boiling, degassing may be necessary to reduce the formation of air bubbles in the column.

Degassing the Matrix

To degas, the gel slurry is placed in a flask with a side arm. The mouth of the flask is sealed with a rubber stopper and a vacuum (with a bench top vacuum line) is applied through the side arm of the flask for about 30 minutes with occasional agitation.

Pouring the Gel Slurry onto a Column

The degassed gel slurry is usually poured into a cylinder made of glass or transparent plastic. Various sizes cylinders are available commercially, and the ratio of the length of the cylinder to its diameter usually varies from 20 to 100. The cylinder is clamped in a vertical position and a funnel, gel reservoir, or column extension is attached
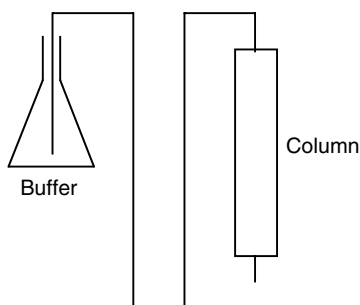
FIGURE 4.6 Tubing arrangement to prevent drying out the column, when not using a pump.

airtight on top of the cylinder. A gel slurry of 1.3 to 1.7 times of settled volume is made and poured into the cylinder in a single step as fast as possible, using a glass rod to guide the slurry into the cylinder. Rapid pouring in a single step prevents separation of large and small beads during the settling. During packing it is important to keep the column outlet tubing at a height not to exceed the recommended operating pressure. This is measured by the difference between the free surface of eluent in the reservoir and the outlet. After packing, two to three column volumes of eluent are passed through the column in order to stabilize the bed and equilibrate the column with the buffer.

At this point, the buffer can be passed through the column under gravity or with the help of a peristaltic pump. If the column is run under gravity, the recommended operating pressure (measured by the difference between the free surface of eluent in the reservoir and the outlet) is maintained throughout the chromatography. When using a pump, the buffer reservoir is not necessarily placed in a level above the column outlet. The flow of the buffer should be adjusted not to exceed the recommended operating pressure to the gel matrix. The column must never be run dry, as this produces channeling within the column and perturbs protein resolution. When running under gravity, drying of the column may be avoided simply by keeping the inlet tubing (the tube between the reservoir and the top of the column) at a level below the level of the column outlet (Figure 4.6). This arrangement stops the buffer flow before the column can run dry.

4.3.1.6 Checking the Column

Prior to chromatographic separation, the column should be checked for the presence of air bubbles or uneven column packing, as these result in poor resolution during chromatography. The column can be inspected visually for any crack or air bubbles with the help of a hand-held lamp. Alternatively, blue dextran (2 mg/ml) can be applied to the column. Blue dextran will migrate through the column as an evenly flowing blue band when column materials are packed well. A streaking blue dextran band indicates uneven or poor packing. An advantage of using blue dextran for this purpose is that the void volume (Vo) of the column can also be determined at the same time.

4.3.1.7 Loading and Eluting the Sample

When loading the sample, the concentration of sample proteins is kept very high (10 to 20 mg/ml), but the volume is kept as minimal as possible (usually 1 to 5% of the column bed volume). A load volume greater than 5% of the column bed volume results in a poor resolution. The protein sample should be free from debris, which may block the column flow. Clarification of the protein sample may be achieved by membrane filtration (0.2 μm pore size) or by centrifugation at 20,000 g for 3 to 5 min. The sample may be loaded in two ways: on a drained bed or under the eluent. The former one is the most common procedure of sample loading. In both cases, care must be taken not to disturb the gel-bed surface, as an uneven gel-bed surface may cause poor resolution. To load the sample onto a drained bed, the flow of the buffer to the column (inlet) is disconnected by opening the top cap of the column. After removing the residual buffer above the gel-bed surface (by letting it flow through the column or by withdrawing with a Pasteur pipette), the column outlet is closed.

The clarified protein sample is then gently applied onto the drained gel-bed surface, and the sample is allowed to enter the gel-bed. A small volume of the buffer is gently applied to the column and allowed to enter the gel. Care must be taken not to allow the gel-bed to run dry in any step during loading or chromatography. The top of the gel-bed is refilled with the buffer, and the column is now connected with the buffer reservoir to begin sample elution. When the sample is to be loaded under the eluent, the sample is prepared in the presence of 10% glycerol to increase viscosity and applied gently onto the top of the gel-bed with the column outlet closed. After the sample has been applied, the column outlet is opened. Elution of the sample is continued until one column volume of the buffer is collected.

4.3.1.8 Regenerating and Storing the Column

Gel permeation matrices usually do not bind proteins under standard operating procedures when clarified protein samples are applied. However, some carbohydrate-binding proteins (lectins) may adhere to glucose- and galactose-based matrices such as Sephadex and Sepharose. In such cases, Sephadex and Sepharose can be washed with 50 to 100 mM glucose and galactose solution, respectively, to remove the bound lectins. In general, washing gel matrices with dilute sodium hydroxide (0.1 to 0.5 M NaOH) or non-ionic detergent (1%) removes most bound materials.

The column should be stored in a buffer containing an antimicrobial agent such as 0.02% sodium azide. Azide is often used in the eluting buffer, when the protein of interest is not affected by its presence. In such cases, no additional treatment is required in order to store the column.

4.3.1.9 Problems and Remedies in Gel Filtration
          Chromatography

Table 4.7 shows some common problems in gel filtration chromatography and their remedies.

TABLE 4.7
Common Problems in Gel Permeation Chromatography, Possible Causes, and Their Remedies[a]

Problem	Cause	Remedy
Poor peak resolution	Short column	Use long column
	High flow rate	Use low flow rate
	Large sample volume	Keep sample volume below 5% of the column bed volume
	Large "dead space"	Reduce the length of the tube between end of the column and fraction collector
	Poor column packing	Check column packing with blue dextran
	Incorrect fractionation range and grade of matrix	Use a matrix with correct fractionation range and grade
Poor sample recovery	Sample may be precipitated due to very low or excessive salt concentration in the buffer	Check the solubility of the protein
	Protein adsorbed to the matrix	Include detergent or carbohydrate (in case of a lectin) in the buffer. Reduce ionic strength of the buffer
	Proteolysis	Include protease inhibitors in elution buffer
	Harsh elution conditions, which can remove essential cofactors or can dissociate protein subunits, resulting in a partial loss of activity	Try mild conditions
Elution profile not reproducible	Buffer composition may have changed since the previous experiment	Use fresh buffer
	Some components of the sample may have changed or precipitated during storage	Try other storage conditions
Low column flow rate	Column outlet may be partially closed	Check the column outlet and reopen
	Sample precipitate on top of the gel-bed	Scrape the surface of the gel-bed and remove the precipitate. To obtain even gel-bed surface, aspirate the top 1 – 2 cm of matrix and gently apply onto the column. Allow to settle the gel before continuing with the elution.
	Salt deposit inside tubing, obstructing flow rate	Change the tubing
	Gel matrix compressed due to excessive pressure	Repack column
	Air bubbles in the tubing resisting flow	Degas buffer and matrix before packing
	Leaky tubing	Check tubing and replace, if necessary

[a] Based on Reference 5.

4.3.2 Ion-Exchange Chromatography

Ion-exchange chromatography is widely used at the beginning of a purification scheme and is designed for the separation of ionic or ionizable compounds in the mobile phase by the counter ion of the opposite sign in the stationary phase (column packing). In ion-exchange chromatography, ionizable functional groups are covalently bound to the stationary phase (matrix).

Principle

Proteins are amphoteric; that is, they contain both positive and negative charges. The ionization of lysine and arginine residues render positive charges, while the ionization of aspartic and glutamic acid residues produce negative charges. The ionization of such groups is pH dependent, and therefore the net charge on a protein will be a function of the pH of the buffer. At a pH identical to the protein's pI, the net charge of a protein is zero. At a pH higher than pI, the protein is negatively charged, and at a pH lower than pI, the protein is positively charged. During chromatography, proteins of similar charge (either positive or negative) interact with opposite charges in the stationary phase, leaving other proteins of a charge identical to the charges of stationary phase. The bound proteins can then be eluted or displaced from the stationary phase by a new counter ion (usually NaCl) with a greater affinity for the fixed charges of stationary phase than the protein. Ion-exchange chromatography is named on the basis of the sign of the displaceable charges. Thus, in anion-exchange chromatography the fixed charges (stationary phase) are positive, and the displaceable charges (proteins) in the mobile phase are negative. Similarly, in cation-exchange chromatography, fixed charges are negative and displaceable charges are positive.

4.3.2.1 Various Ion Exchangers

Among the fixed stationary phase charges, diethylaminoethyl (DEAE) and carboxymethyl (CM) are widely used in conventional anion-exchange and cation-exchange chromatography, respectively. DEAE is a weak base containing a net positive charge, while CM is a weak acid containing a negative charge. In HPLC, relatively stronger ion exchangers have been used (e.g., quaternary amino ethyl [QAE] and quaternary ammonium [Q] for anion exchangers and sulpho propyl [SP] and methyl sulfate [S] for cation exchangers). Table 4.8 shows most of the functional groups used for ion exchangers. Strong ion exchangers are completely ionized over a wide pH range, whereas with weak ion exchangers, the degree of dissociation and thus exchange capacity varies much more markedly with pH. These charged (functional) groups are covalently bound to solid supports with high porosity and improved flow properties, such as Sephadex, Sepharose, Sephacel, polystyrene, etc. Colloidal Cellulose-based ion exchangers were the first used for the separation of biological materials, but have poor flow properties due to their irregular particle shape. Ion exchangers containing tightly cross-linked hydrophobic polymer matrices highly substituted with ionic groups have high capacities for small ions and are usually unsuitable for biological samples.

TABLE 4.8
Functional Groups Used for Ion Exchangers

Functional Group	Structure	Nature	Name of Ion Exchangers
Anion exchanger:			
Aminoethyl- (AE)	$-OCH_2CH_2NH_3^+$	Weak	—
Diethylaminoethyl- (DEAE)	$-OCH_2CH_2N^+H(CH_2CH_3)_2$	Weak	DEAE-Sepharose
Quaternaryaminoethyl- (QAE)	$-OCH_2CH_2N^+(C_2H_5)_2-$ $CH_2CH(OH)CH_3$	Strong	QAE-Sephadex
Quaternaryammonium- (Q)	$-CH_2N^+(CH_3)_3$	Strong	Q-Sepharose
Cation exchanger:			
Carboxymethyl- (CM)	$-OCH_2COO^-$	Weak	CM-Sepharose
Phospho-	$-H_2PO_4^-$	Moderate	—
Sulphopropyl- (SP)	$-CH_2CH_2CH_2SO_3^-$	Strong	SP-Sepharose
Methylsulphate- (S)	$-CH_2-SO_3^-$	Strong	SOURCE S

In Sephadex ion exchangers, functional groups are attached to glucose units of dextran. They are usually derived from Sephadex G-25 and G-50. Ion exchangers based on Sephadex G-25 are more tightly cross-linked than those based on Sephadex G-50 and therefore swell less and have greater rigidity. Ion exchangers based on Sephadex G-50 are more porous than those based on Sephadex G-25 and therefore have a better capacity for molecules with molecular weights larger than 30 kDa. Sephadex ion exchangers are stable in water, salt solutions, organic solvents, and alkaline and weakly acidic solutions. However, in strongly acidic solutions, hydrolysis of the glycosidic linkages may occur, and so pH values below 2 should be avoided, particularly at elevated temperatures.

Sepharose ion exchangers such as DEAE-Sepharose CL-6B and CM-Sepharose CL-6B are based on Sepharose CL-6B, with functional groups attached to the gel by ether linkages to the monosaccharide units. Because of its macroporous structure, the gel is good for molecules up to 1×10^6 Da in molecular weight.

Sephacel ion exchangers are produced from high-purity microcrystalline cellulose, which is a naturally occurring polymer of β 1,4-linked glucose residue. Cellulose polysaccharide chains are extensively hydrogen-bonded with interspersed amorphous regions that have less hydrogen bonding. Microcrystalline cellulose is produced by limited acid hydrolysis of cellulose, resulting in a loss of the amorphous regions. During the production of DEAE-Sephacel, the microcrystalline structure is broken down and the cellulose is regenerated to give a bead-formed gel-like structure. The gel is strengthened by cross-linking with epichlorohydrin, although the main structure-forming bonds are still hydrogen bonds. Functional groups are attached during the synthesis by ether linkages to glucose units of the polysaccharide chains.

Polystyrene beads are generally used to prepare strong ion exchangers. SOURCE Q and SOURCE S (Pharmacia) are based on monosized polystyrene/divinyl benzene beads that are substituted with quaternary ammonium groups and sulfonate groups, respectively.

4.3.2.2 Choosing the Ion Exchanger

The choice of the right ion exchanger and the pH of the working buffer largely depend on the isoelectric points of the sample components to be separated. Since proteins are amphoteric and become negatively charged at a pH above their pIs and positively charged at a pH below their pIs, one could in principle use either an anion exchanger or a cation exchanger by selecting the appropriate pH. In practice, the buffer pH at which the protein of interest is stable dictates the choice of ion exchanger. For example, if a protein of pI 5.0 is found stable in the range pH 5 to 8, then the only choice would be an anion exchanger. Similarly, if the pH of a working buffer is chosen at 7.0 for a protein of pI 8.0, the protein would become positively charged, and therefore a cation exchanger should be used for binding. In any case the pH of the starting buffer is usually kept at least 1 pH unit above the pI for an anion exchanger or at least 1 pH unit below the pI for a cation exchanger to facilitate adequate binding without requiring drastic conditions for elution.

The scale of the sample to be separated and the mode of separation (column vs. batch) are also important parameters when choosing an ion-exchange medium. For laboratory-scale separations, most ion exchangers are suitable. However, for large-scale applications, BioProcess media such as SOURCE or STREAMLINE are cost effective. If ion exchange is to be carried out using a batch process rather than in a column, the flow and bead size of the matrix are less important, and therefore economical and high-capacity Sephadex-based ion exchangers are the better choice.

4.3.2.3 Choosing the Buffer

The charge of the buffering ions should be similar to those of the functional groups of the ion exchanger, since the opposite charges will take part in the ion-exchange process and will cause local disturbances in pH. Several cationic buffers, such as Tris, alkylamines, ammonium, imidazole, ethyldiamine, pyridine, aminoethyl alcohol, etc., are recommended for an anion exchanger. Similarly, several anionic buffers, such as phosphate, acetate, citrate, glycine, barbiturate, etc., are used for a cation exchanger. However, in some cases where modification of amino groups of an acidic protein is to be carried out and the modified protein is to be separated by anion-exchange chromatography, the use of a single buffer such as phosphate is a good choice.

Non-ionic detergents can be used in ion-exchange chromatography because they carry no charge. However, the non-ionic detergent Triton X-100 has an absorption maximum at 280 nm wavelength (that of protein) and thus should be avoided.

4.3.2.4 Preparing the Ion-Exchange Column

Ion exchangers are usually available in both dry and gel forms. If purchased in dry form, the ion-exchange matrix must be hydrated with the column buffer or buffer of choice prior to packing the column. For swelling, the matrices are incubated with about 10 volumes of column buffer and boiled for several hours or left several days at room temperature.

Packing an ion-exchange column is essentially similar to a gel filtration column. Prior to packing the column, the matrix may be equilibrated with the working buffer. A 50 to 70% gel slurry is prepared with the equilibrating buffer and degassed. After packing, the column is washed further with several column volumes of equilibrating buffer to ensure the equilibrium with the buffer.

4.3.2.5   Loading Sample to an Ion-Exchange Column

Prior to loading a protein sample, make sure that the column matrix has been equilibrated. This can be tested by comparing the pH and conductivity of the equilibrating buffer to those of the column flow through. For loading, the protein sample is prepared in an equilibrating buffer, centrifuged (if necessary), and applied to the top of the gel-bed as previously described (see Section 4.3.1.7). When application of a large volume of protein sample is intended, a pump can be used to deliver the sample to the column. Unlike gel filtration chromatography, the volume of the sample to be loaded is not limited, as long as the amount of sample does not exceed the binding capacity of the ion-exchange column.

4.3.2.6   Eluting a Target Protein (Step and Linear Gradient)

After loading the sample, the column is washed with 5 to 10 column-bed volumes of the equilibrating buffer to remove unbound proteins. The washing is monitored through optical density (A_{280}) or protein assays to ensure complete removal of the unbound proteins. When the wash-through contains negligible or no protein, elution of bound target protein(s) may be initiated. Target proteins can be eluted in two ways: step elution and gradient elution (Figure 4.7). In step elution, ionic strength of the eluting buffer is increased stepwise (e.g., 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, and 1 M NaCl) (Figure 4.7 A). In gradient elution, ionic strength of the eluting buffer is gradually increased by the help of a gradient maker (Figure 4.7 B). Besides using a positive gradient of ionic strength, bound proteins can also be eluted from the column by varying the pH of the eluent. Elution of proteins by the gradient of pH (continuous or stepwise) is not frequently employed, since some proteins may not be stable or precipitate at some pHs. Moreover, in conventional ion-exchange chromatography, a continuous pH gradient is not easy to produce at constant ionic strength and cannot be achieved by mixing buffers of different pH in linear volume ratios, since simultaneous changes in ionic strength occur.

However, in some HPLC systems such as Akta purifier (Amersham Biosciences, Piscataway, NJ), pH gradient is easily obtained almost precisely. In conventional ion-exchange chromatography, elution of bound proteins is accomplished by applying salt solution (usually sodium chloride) in the equilibrating buffer. For a step elution, the salt solution of the next higher concentration in the step is introduced on to the column and maintained for at least 2 bed volumes to achieve an equilibrium or until all the proteins are eluted at this particular concentration. After that step, the solution of the next higher concentration is introduced and the process repeated. The salt solutions of several higher concentrations are introduced until all the proteins
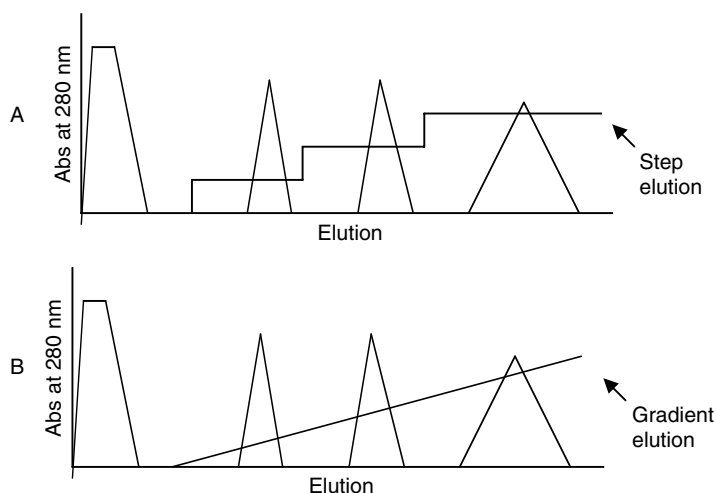
FIGURE 4.7 Step (A) and gradient (B) elution in ion-exchange chromatography.

are eluted. The concentrations of salt in each step can be determined by trial and error. Since most proteins are eluted between 0.1 and 0.4 M of sodium chloride, it is advisable for the first experiment to include steps at 25 to 50 mM increments until 0.4 M. Beyond 0.4 M salt concentration, two to three more steps until 1 to 2 M can be introduced to elute any residual bound protein.

If the salt is to be introduced as a gradient, two solutions are prepared, one of the low salt (usually equilibrating buffer) and the other of the high salt. With the aid of a gradient maker, the solution of the lowest concentration is introduced first onto the column followed by higher concentration. Resolution of proteins with either elution depends on the number of steps or the form of the gradient (i.e., steep vs shallow). Between the two procedures, the steep elution is the simpler and more rapid procedure, but often results in simultaneous elution of multiple protein peaks due to a large increase of ionic strength. Gradient elution is helpful when separation of proteins with close pIs is intended.

4.3.2.7 Regenerating the Ion-Exchange Column

Following elution of bound proteins, the column is regenerated with a high concentration of salt (e.g., 1 to 2 M NaCl). Salt of high concentration removes tightly bound contaminants from the matrix. After regeneration, the column is equilibrated with a working buffer for future separation.

4.3.2.8 Problems and Remedies in Ion-Exchange Chromatography

Table 4.9 shows some common problems in ion-exchange chromatography and their remedies.

TABLE 4.9
Common Problems in Ion-Exchange Chromatography, Possible Causes, and Their Remedies[a]

Problem	Cause	Remedy
No protein adsorption to the matrix	Column may not be equilibrated	Continue washing column with the equilibration buffer
	Ionic strength of the equilibration buffer may be high	Decrease ionic strength of the equilibration buffer
	pH of the equilibration buffer may not be suitable	Change pH of the buffer (for anion-exchange chromatography, increase the pH; for cation-exchange chromatography, decrease the pH)
Poor yield of the target protein	Protein remains adsorbed to the column	Increase salt concentration of the eluent
	Protein may be precipitated. Precipitation may occur due to the lack of salt in the buffer or pH of the buffer is very close to the pI of the target protein.	Try alternative ionic strength and pH for the equilibration buffer
Loss of activity after purification	Cofactor or part of a protein complex may have lost	Remix and test protein activity
	Protein may not be stable in the elution buffer	Try alternative elution conditions
Poor protein resolution	Gradient is too steep	Elute column using a shallower gradient
	Speed of elution is too fast	Try a slower elution rate
	Proteins may have remixed after leaving column	Minimize the volume between bottom end of the column and the fraction collector
Elution profile not reproducible	Incomplete column equilibration	Be sure to equilibrate the column. Compare the ionic strength and pH of the equilibrating buffer and column flow through.
	Ionic strength and pH of the eluting buffer may have changed from the previous experiment	Use fresh buffer

[a] Based on Reference 5.

4.3.3 CHROMATOFOCUSING

Chromatofocusing (isoelectric focusing by ion-exchange chromatography) is a procedure in which the biomolecules separate according to their isoelectric points in an ion-exchange column.[6,7] In this procedure, the proteins are allowed to bind to the ion-exchange bed in an equilibrating buffer of low ionic strength and eluted with a polybuffer (from Pharmacia) at a pH lower than that of the starting buffer. The polybuffer, containing cationic and amphoteric buffering species, makes a pH gradient, which is used to elute bound proteins from the ion-exchange resin in order of their isoelectric points. Two ion exchangers, PBE 94 and PBE 118, are used in

chromatofocusing. PBE 94 is used most commonly with the amphoteric buffers Polybuffer 96 (pH range 9.0 to 6.0) and Polybuffer 74 (pH range 7.0 to 4.0). For basic proteins, PBE 118 with buffer Pharmalyte (pH range 8.0 to 10.5) can be used. Proteins with pI differences as small as 0.05 pH unit can be resolved in chromato-focusing.

Chromatofocusing is useful to separate isoforms of closely spaced pIs after affinity chromatography.

Principle

In chromatofocusing, a mixture of proteins is loaded on an ion-exchange column that is equilibrated at an alkaline pH or a pH slightly above the assumed pI of the target protein. Like conventional ion-exchange chromatography, proteins with a net charge opposite to that of the column remain bound in the equilibrating buffer, but proteins of like charge are washed out. The ion-exchange resin containing bound proteins is then titrated with a polybuffer of a more acidic pH. In practice, a pH gradient is generated in the column by a continuous flow of an acidic polybuffer. In this process, when the pH of the gradient approaches to the pI of the target protein, it starts to desorb from the column. Since the flow rate of the eluting buffer is faster than the formation of the pH gradient, a protein with a particular pI desorbs through the length of the column and elutes as a single peak.

Working Procedure

A detailed description of chromatofocusing is reported elsewhere.[7,8]

Column
PBE 94 ion exchanger (Pharmacia)

Reagents

1. Equilibrating buffer: 0.025 M ethanolamine, pH 9.4 (adjusted with glacial acetic acid)
2. Eluting buffer: 10% (v/v) Polybuffer 96, pH 6.0

Procedure

1. Equilibrate the column with equilibrating buffer.
2. Apply sample (0.5 bed volume) to the column and elute proteins.
3. Assay each fraction for protein of interest.

4.3.4 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

In hydrophobic interaction chromatography (HIC), proteins are separated on the basis of varying strengths of their hydrophobic interactions with a solid phase containing uncharged hydrophobic groups.

Principle

In this chromatography, proteins and peptides interact with the hydrophobic surfaces of the matrix by adsorption in an aqueous buffer. In the equilibration buffer, salting out ions are maintained at a high concentration to decrease the availability of water molecules, which in turn enhances hydrophobic interactions. Ammonium sulfate (0.8 to 1 M) is often used in the equilibrating buffer to increase the hydrophobic interaction of the proteins. Anions that favor the hydrophobic interactions are PO_4^{3-} > SO_4^{2-} > CH_3COO^- > Cl^- > Br^- > NO_3^- > ClO_4^- > I^- > SCN^- and for cations are NH_4^+ > Rb^+ > K^+ > Na^+ > Cs^+ > Li^+ > Mg^{2+} > Ca^{2+} > Ba^{2+}.[9]

Hydrophobicity is the repulsion between a non-polar compound and a polar medium, e.g., water.[10] Membrane glycoproteins are bound to the lipid bilayer of biological membranes via hydrophobic interactions. Hydrophobic interactions can be utilized to separate biomolecules that possess hydrophobic moieties. In proteins, the hydrophobic amino acids are tryptophan, tyrosine, leucine, valine, methionine, and alanine, in decreasing order of hydrophobicity. In hydrophobic chromatography proteins are usually adsorbed on phenyl- or octyl-immobilized support in the presence of high salt. Phenyl-immobilized resin is usually a good choice for most applications such as strongly hydrophobic and uncharacterized proteins. Octyl-immobilized resin is generally used for weakly hydrophobic proteins, but may not be suitable for strongly hydrophobic proteins. After washing off unbound proteins, bound proteins are eluted by reducing the concentration of salt in a negative gradient. However, elution of proteins from hydrophobic columns can also be achieved with a positive gradient of chaotropic ions or detergents or by raising the pH.

HIC can be conveniently performed after ammonium sulfate fractionation, but before ion-exchange chromatography. As the target protein is eluted in a low salt buffer, the eluate can then readily be subjected to ion-exchange chromatography without the need of a dialysis step.

Separations by both reversed-phase (see Section 4.3.5) and hydrophobic chromatographies are based on the surface hydrophobicity of the proteins, but in reversed-phase the bound proteins are eluted with an organic solvent, while in hydrophobic chromatography the proteins are desorbed with an aqueous salt solution.

Working Procedure

Column
Phenyl Sepharose CL 4B

Reagents

1. Loading buffer: 20 mM sodium phosphate (pH 7.0) containing 50% ammonium sulfate
2. Elution buffer: 20 mM sodium phosphate (pH 7.0) containing 50 to 0% ammonium sulfate

Procedure

1. Wash the column (5 ml bed) with 10 column volumes of loading buffer.
2. Dissolve protein sample (200 to 400 mg) in loading buffer and apply to column.
3. Wash column with loading buffer until absorbance at 280 nm returns to background (usually 3 to 5 column volumes).
4. Elute bound protein with 2 column volumes each of elution buffer with decreasing concentrations of ammonium sulfate.
5. Regenerate column with successive additions of water, 1 M NaCl, and water (5 column volumes each).

4.3.5 REVERSED-PHASE CHROMATOGRAPHY

In reversed-phase chromatography (RPC), the matrix is silica that has been substituted with n-alkyl chains, usually C_4, C_8, and C_{18}. The mobile phase is usually a mixture of water and a less polar organic solvent. The name "reversed-phase" is introduced to distinguish it from "normal phase" chromatography, in which the matrix is silica and the mobile phase is a non-polar solvent such as hexane. In a reversed-phase system, water present in the mobile phase is more polar that the stationary phase, C_8 or C_{18}-derivatized silica. RPC is rarely used for the purification of biologically active protein molecules. However, this chromatography is widely used to separate peptides obtained from chemically or enzymatically digested purified protein and other applications where loss of the protein's biological activity is not a concern. Separation of peptides is commonly achieved on reversed-phase columns using a high-performance liquid chromatography system rather than a conventional chromatography system. The HPLC system will be described at the end of this chapter.

Principle

RPC is based on hydrophobic interactions between proteins or peptides with the packing hydrophobic surfaces. This chromatography provides continuous partitioning of small molecules, such as peptides, between the mobile phase and the hydrophobic stationary phase. As soon as the peptides enter the column, they adsorb to the hydrophobic surface and remain adsorbed until the concentration of organic modifier reaches the critical concentration necessary to cause desorption.[11]

In RPC, the elution of the peptides by an organic modifier has usually been accomplished in the presence of an ion-pairing agent or a buffer. While the organic modifier desorbs polypeptide from the stationary phase, the ion-pair agent or buffer sets the eluent pH and enhances the hydrophobic nature of peptides by neutralizing their charges. Trifluoroacetic acid (TFA) is widely used as an ion-pairing agent because it is volatile and thus easily removed from the eluted fractions, and it has negligible UV absorption at 215 nm. TFA is normally used at concentrations of about 0.1%. As the elution shifts from aqueous to non-aqueous solvent, the change in dielectric constant affects pi-pi electron interactions, which in turn alters absorption of peptides at lower wavelengths. To prevent the shift in absorption spectra from causing baseline drift, the concentration of TFA in solvent B is kept 15% less than in solvent A (i.e., 0.085% TFA in solvent B).

Comparison between RPC and HIC

Both chromatographies are based on the interactions between the hydrophobic moieties of a sample and an insoluble immobilized hydrophobic group. In HIC, the immobilized matrix is hydrophilic (e.g., Sepharose) substituted with short chain phenyl or octyl non-polar groups. In RPC, the matrix is silica substituted with longer n-alkyl chains such as C_8 and C_{18}. In HIC, the mobile phase is an aqueous salt solution, whereas in RPC, the mobile phase is usually a mixture of water and a less-polar organic modifier. Separation on HIC occurs in non-denaturing conditions, but separation on RPC is achieved in a mixture of aqueous and organic solvents, which often denature proteins.

4.3.6 AFFINITY CHROMATOGRAPHY

The most powerful procedure for the purification of proteins is affinity chromatography. Under ideal conditions the target protein can be purified in a single step. Moreover, the purified protein is obtained in a biologically active form, as the purification is based on its biospecific interaction with an immobilized ligand. The procedure involves the adsorption of crude protein extract onto a ligand conjugated solid support (commonly called a matrix). Proteins in the extract having a binding site complementary to the ligand remain bound to the matrix, while the unbound components are removed by washing the matrix with a buffer. The bound protein is eluted with an elution buffer.

Principle

In affinity chromatography, proteins are specifically bound to an immobilized ligand. When crude extract is passed through the column, the target molecule for which the ligand possesses affinity remains bound to the column (Figure 4.8). Upon washing the column, the unbound proteins are removed from the column. Finally, the bound protein is displaced by incorporating the free ligand that competes for protein binding sites. Alternatively, the displacement of the bound protein can be achieved by
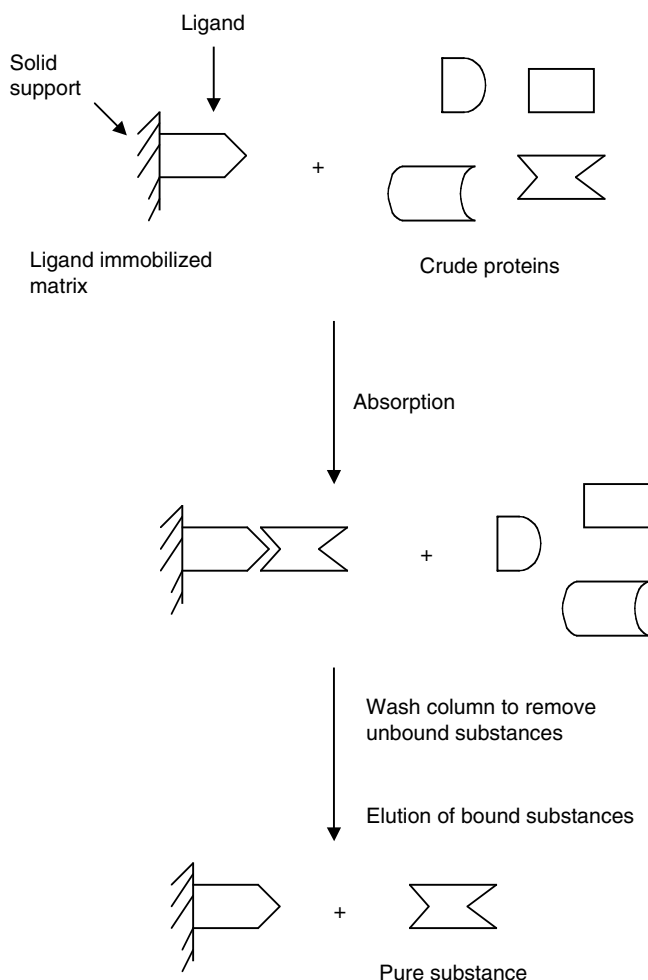
FIGURE 4.8 Principle of affinity chromatography.

changing the pH or increasing the ionic strength of the buffer or, in the case of metal binding proteins, by adding a metal chelator, such as EDTA or EGTA.

4.3.6.1 Preparation of Affinity Matrices

A variety of affinity matrices are commercially available. They are cost effective for many applications. However, for specialized applications, affinity matrices are readily synthesized with ligands of choice. The following points should be considered in order to make successful affinity matrices: (a) the ligand to be immobilized to solid support should be specific to the target molecule; (b) the immobilized ligand should be accessible to the target molecule; (c) a "spacer arm" may be helpful to keep the ligand away from the solid support in order to gain accessibility to the target molecule; (d) linkage between the ligand and solid support should be stable;

TABLE 4.10
Common Reagents for Matrix Activation and Immobilizing Ligands

Reagent for Matrix Activation	Length of Spacer Introduced (Atom)	Interacting Group with the Activated Matrix	Ligand
Cyanogen bromide	1	$-NH_2$	Protein, glycoprotein
N-hydroxysuccinimide ester	8	$-NH_2$	Protein, glycoprotein
Divinylsulfone	5	$-OH, -NH_2, -SH$	Carbohydrate, polysaccharide, protein
Bis oxirane	11	$-OH, -NH_2, -SH$	Carbohydrate, polysaccharide
Epichlorohydrin	3	$-OH, -NH_2, -SH$	Carbohydrate, polysaccharide
Tosyl/tresyl chloride	0	$-NH_2$	Protein

and (e) there should be little or no non-specific interactions of unrelated proteins with the immobilized ligand.

The affinity matrix is usually prepared by covalent conjugation of a ligand to solid support. Agarose or Sepharose, Sephadex, and starch are good candidates for solid supports because they can be easily derivatized with various ligands. However, agarose gels are mostly used as the immobilizing solid supports, as they are highly porous, allowing free access to macromolecules.

To prepare an affinity column, the matrix must be activated to allow covalent attachment of the ligand. In some cases, a spacer arm is introduced during activation, and then the ligand is allowed to bind covalently. The covalent linkage of the solid support with ligands can be achieved by several methods. Table 4.10 shows some common reagents for activation of matrices and the immobilizing ligands. Additional reagents for the activation of matrices and the working procedures are described elsewhere.[12] However, various activated matrices can be purchased commercially and conveniently used to conjugate ligands of choice.

4.3.6.1.1 Ligand Immobilization through Cyanogen Bromide Activation

The most common reagent for linking the ligand to solid support is cyanogen bromide (CNBr). It reacts with hydroxyl groups of Sepharose at alkaline pH.

Reaction

The reactions of CNBr are complex. Figure 4.9 shows the plausible mechanism of CNBr activation of agarose or other hydroxyl-containing matrices. At high pH (about 11), CNBr reacts with hydroxyl groups of matrices to produce a major reactive component, cyanate ester,[13] and other minor components, imidocarbonates (cyclic and acyclic), carbamates, and carbonates.[4,13] However, cyclic imidocarbonates
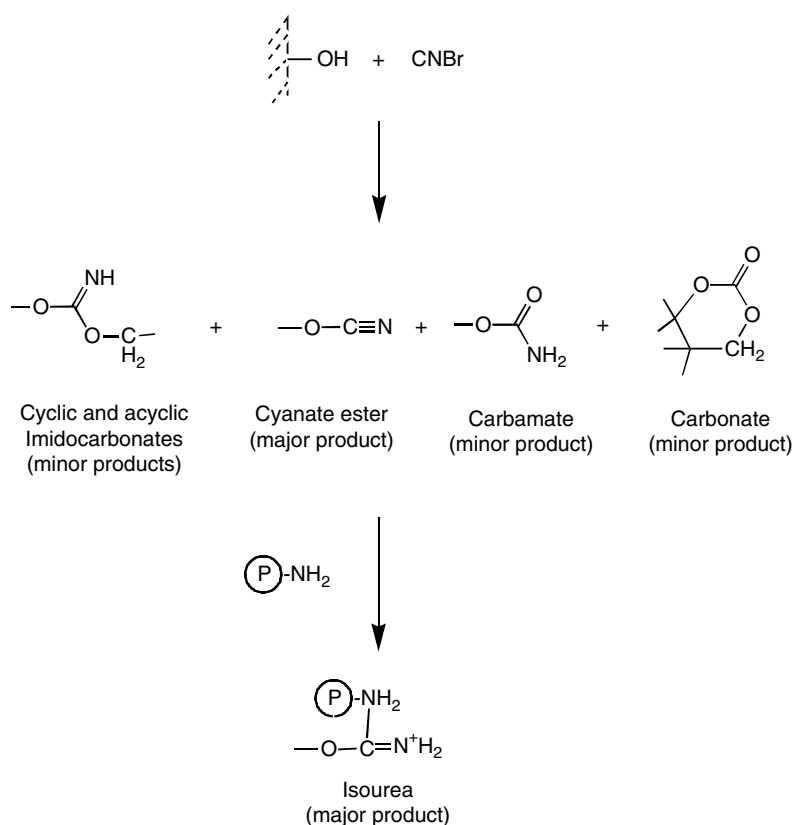
FIGURE 4.9 CNBr activation of matrix.

predominate upon activation of cross-linked dextrans and cellulose.[12] In mild alkaline conditions (pH 9 to 10), cyanate esters and cyclic imidocarbonates then readily react with primary amines of the ligands, resulting in an isourea derivative and substituted imidocarbonate, respectively (see Figure 4.9). The amino groups act as nucleophiles in this reaction, so it is important to carry out the reaction at pH around 8 to 10, where the amino groups remain unprotonated. The coupling reaction should not be performed in a buffer containing primary amine, such as Tris, glycine, or ethanol-amine, as these reagents compete with the immobilizing ligand. The buffer of choice is usually sodium carbonate/bicarbonate, and coupling is optimum at pH 8.3. After ligand conjugation and removal of excess ligand, the residual active groups in the matrix can be blocked by adding an excess of small primary amines, such as Tris, glycine, ethanolamine, etc.

Advantages

CNBr activation is simple and mild for conjugating sensitive biomolecules such as enzymes, lectins, and antibodies. CNBr activation can be employed not only with agarose, dextran, or Sephadex, but also with synthetic polymers containing hydroxyl

groups. Once activated, small ligands as well as high molecular weight ligands containing primary amines can be coupled.

Disadvantages

One main drawback of CNBr-activated affinity matrices is that the immobilized ligand leaches continuously. This leakage is primarily due to the instability of the isourea bond between the activated matrix and the ligand. Another drawback of CNBr-activated affinity matrices is that they may act as weak anion exchangers, promoting non-specific binding. This is because the isourea derivative is positively charged at neutral pH.

Working Procedure

Reagents

1. Cyanogen bromide (Aldrich, Milwaukee, WI)
2. Sepharose 4B or 6B (Pharmacia)

Activation
(**Note:** CNBr is highly toxic. Carry out CNBr activation in a well-ventilated hood.)

1. Wash 100 ml packed Sepharose 4B with 1 liter deionized water in a sintered glass funnel. Suction dry to a wet cake.
2. Suspend gel in 100 ml 2 M sodium carbonate in a beaker.
3. Dissolve 10 gram CNBr in 5 ml acetonitrile and add CNBr solution to the gel suspension with constant stirring, using an overhead paddle stirrer. (**Note:** do not use magnetic stirrer to avoid breakage of gel beads.)
4. Allow the activation reaction to continue for exactly 2 min at room temperature.
5. Quickly wash the activated gel with 1 liter ice-cold water followed by cold coupling buffer (0.1 M sodium bicarbonate containing 0.5 M NaCl, pH 8.5).
6. Proceed to ligand immobilization immediately.

Ligand Immobilization

7. Suspend the activated gel in coupling buffer (0.1 M sodium bicarbonate containing 0.5 M NaCl, pH 8.5) containing protein (5 to 10 mg per ml gel). Continue coupling reaction for 2 h at room temperature (or overnight at 4°C) with constant stirring, using paddle stirrer.
8. Wash the coupled gel with coupling buffer to remove unreacted ligand.
9. Block the remaining active groups on the gel by suspending in 100 ml of 1 M ethanolamine or 0.2 M glycine, pH 8.0 for 2 h at room temperature.

10. Wash the gel extensively with coupling buffer followed by 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl followed by coupling buffer.
11. Finally wash the gel with water followed by buffer of choice for affinity chromatography.

Ligand Immobilization of AH-Sepharose and CH-Sepharose

In some affinity matrices, such as AH-Sepharose and CH-Sepharose, ligands can be linked through spacer arms. A spacer arm is used to increase the accessibility of large proteins that need to be bound to small ligands. AH-Sepharose is prepared by covalent binding of 1,6-diaminohexane to Sepharose by CNBr (Figure 4.10). AH-Sepharose can be reacted with several other reagents to form alternative functional groups, such as diazonium, bromoacetamidoalkyl, and thiol derivatives (Figure 4.11). CH-Sepharose is the product of a reaction of Sepharose and 6-aminohexanoic acid activated by CNBr (Figure 4.12).

CH-Sepharose must be activated before coupling with the ligands. It can be activated with carbodiimide and can be coupled with ligands containing either carboxylic acid or amino groups. Carbodiimide initiates a reaction between a free amino and a free carboxylic group to form a peptide link by acid catalyzed removal of water. CH-Sepharose can also be activated by the esterification of the carboxyl group of CH-Sepharose using N-hydroxysuccinimide. Unprotonated amino groups at an alkaline pH attack the electron-deficient carbonyl group, leaving N-hydroxysuccinimide (Figure 4.13).

4.3.6.1.2   Ligand Immobilization through Divinylsulfone Activation

Divinylsulfone is widely used to activate hydroxyl-containing matrices such as Sepharose and agarose.[13] Reactive vinyl groups of divinylsulfone can couple to hydroxyls, sulfhydryls, and amines. Activation and coupling are very efficient at a high pH, but immobilized ligands prepared by this method are also unstable at high pH and are hydrolyzed over time. So the duration of activation and the coupling of the activated Sepharose with ligands should be compromised. The immobilized products are equilibrated with a suitable buffer of pH 6 to 7.5. For monosaccharides and oligosaccharides, hydroxyl at C-1 (for oligosaccharides, C-1 OH of the reducing sugar) usually participates in this coupling.
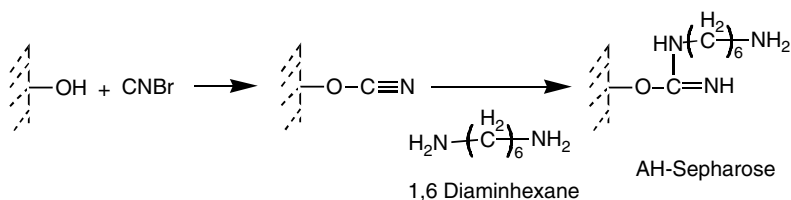
FIGURE 4.10 Preparation of AH-Sepharose.
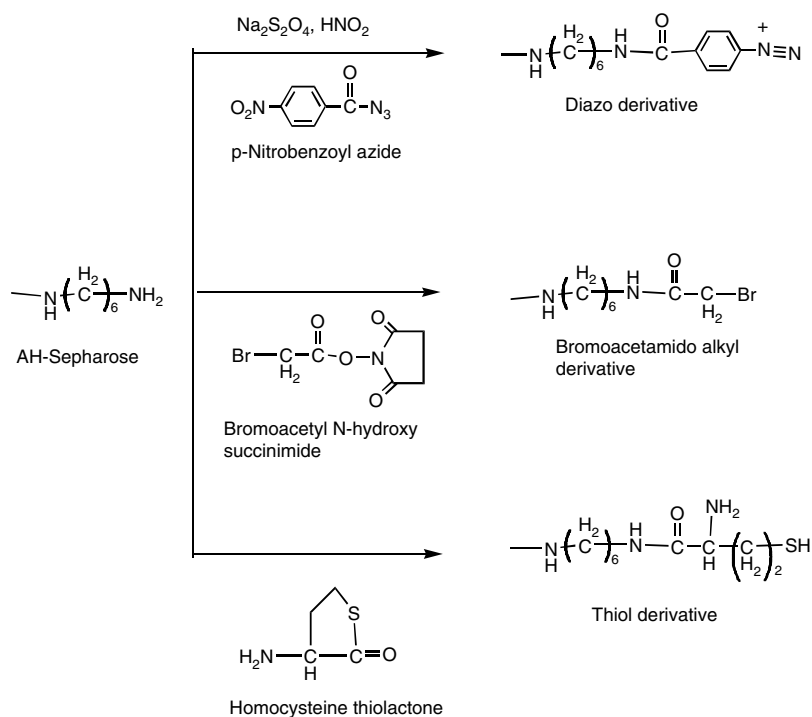
FIGURE 4.11 Reactions of AH-Sepharose.
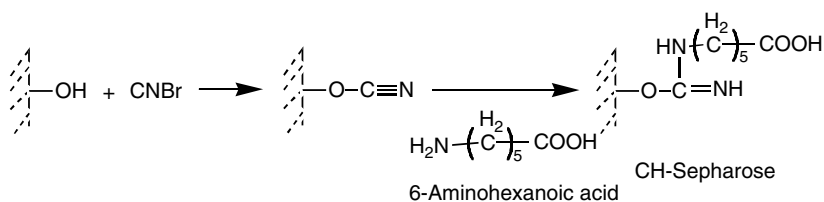
FIGURE 4.12 Preparation of CH-Sepharose.

Reaction

Divinylsulfone, a bifunctional coupling reagent, reacts with hydroxyl-containing matrices such as Sepharose or agarose through its reactive vinyl group at one end (Figure 4.14). Activated agarose can then react with hydroxyls, sulfhydryls, and amino groups of a ligand through the second reactive vinyl group.

Advantage

The procedure is simple and efficient.
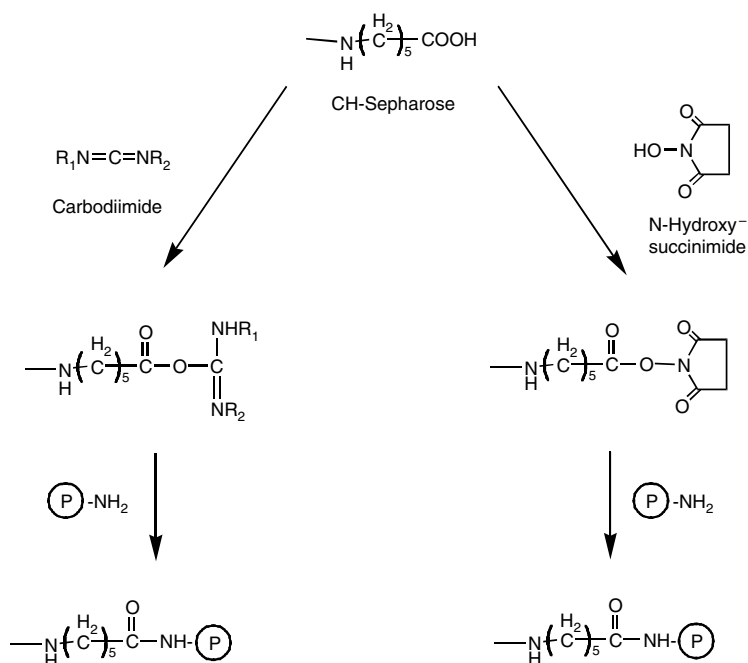
FIGURE 4.13 Reactions of CH-Sepharose.
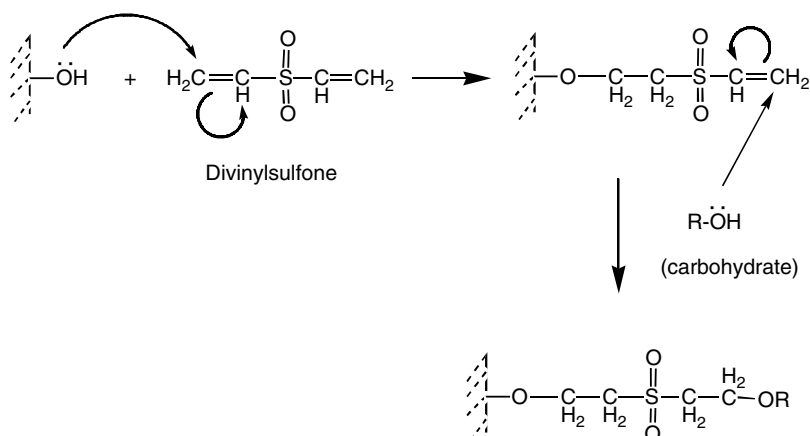
FIGURE 4.14 Divinylsulfone activation of matrix.

Disadvantage

Immobilized ligand is alkali labile. Reaction of a ligand with a divinylsulfone-activated matrix will introduce a positive charge into the linkage.

Working Procedure

Reagents

1. Divinylsulfone (Aldrich)
2. Sepharose 4B or 6B (Pharmacia)

Activation

(**Note:** divinylsulfone is highly toxic. Activation should be performed in a well-ventilated hood.)

1. Wash 100 ml packed Sepharose 4B with 1 liter deionized water in a sintered glass funnel. Suction dry to a wet cake.
2. Suspend the gel in 100 ml 0.5 M sodium carbonate in a beaker and add 10 ml divinylsulfone dropwise over a 15 min period with constant stirring, using an overhead paddle stirrer.
   (**Note:** do not use magnetic stirrer to avoid breakage of gel beads.)
3. Allow the activation reaction to continue for additional 1 h at room temperature.
4. Wash the activated gel extensively with water until the filtrate is no longer acidic.

Ligand Immobilization

5. Suspend the activated gel in an equal volume of coupling buffer (0.5 M sodium carbonate, pH 10) containing 20 gram carbohydrate (e.g., monosaccharide, oligosaccharide) or thiol compound. Continue coupling reaction at room temperature for 24 h with constant stirring, using a paddle stirrer.
6. Wash the coupled gel with coupling buffer to remove unreacted ligand.
7. Block the remaining active groups on the gel by suspending in 100 ml of coupling buffer containing 5 ml 2-mercaptoethanol for 2 h at room temperature.
8. Wash the gel extensively with 1 M NaCl and water.

4.3.6.1.3 Ligand Immobilization through "Epoxy" Activation

Epoxy (bisoxirane)-activated Sepharose containing a long hydrophilic spacer is ideal for conjugating ligands through hydroxyl, amino, or thiol groups. Two reagents, epichlorohydrin and 1,4-butanediol diglycidyl ether (a bisoxirane), are commonly

used to introduce epoxy group. Upon activation, epichlorohydrin introduces a 3-atom spacer arm, while bisoxirane adds an 11-atom spacer arm.

Reaction

Hydroxyl groups of Sepharose react with this bisoxirane at alkaline pH to form a stable ether bond with the matrix (Figure 4.15). The other end of the bisoxirane is capable of reacting primary amines, sulfhydryls, as well as hydroxyls of ligands. Primary amines of ligands react with the activated matrix to form a very stable secondary amine linkage. But amine linkage can be protonated at pHs below 8.
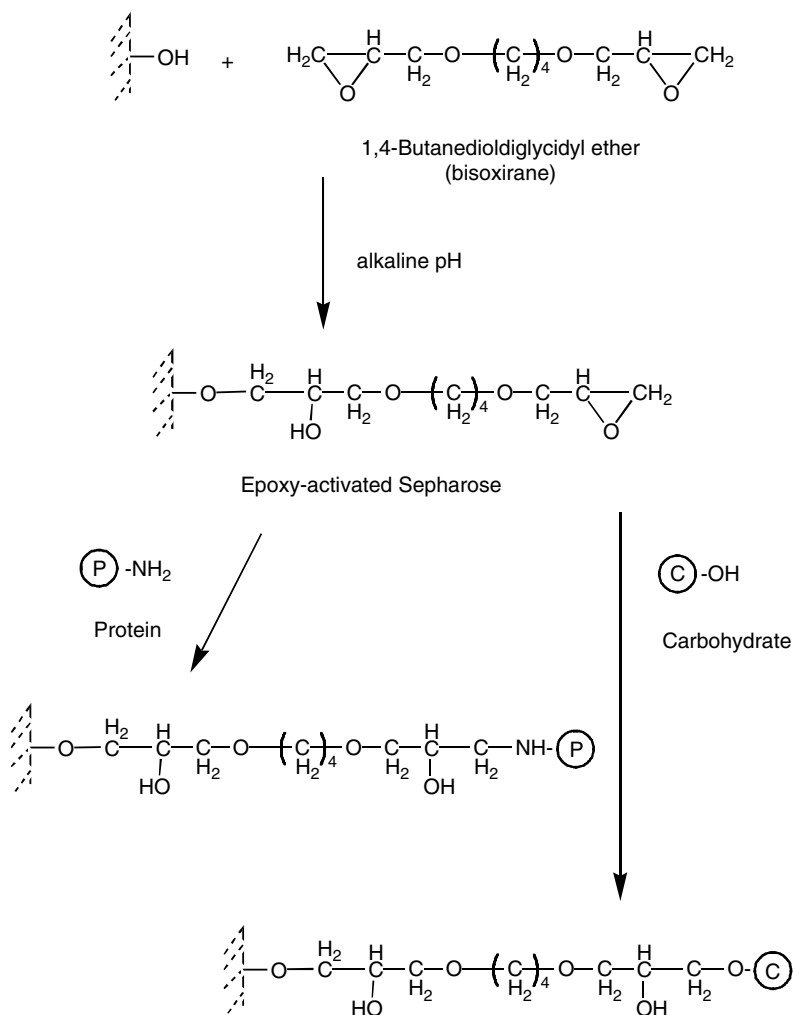
FIGURE 4.15 Epoxy activation of matrix.

Epoxy groups also react rapidly with the sulfhydryls, but slowly with the hydroxyls. At a high pH, a hydroxyl-containing ligand reacts with an epoxy-activated matrix, resulting in a stable, uncharged ether linkage. For monosaccharide hydroxyl at C-6 and for oligosaccharide C-6 OH of the reducing sugar usually participates in this process. But, unlike oxygen linkage (through hydroxyls), thioether linkage (through sulfhydryls) is unstable at alkaline pH.

Advantages

Immobilized ligands through amine and oxygen (hydroxyl group) linkages are stable. A hydroxyl-containing ligand results in an uncharged ether linkage.

Disadvantages

Immobilized ligands through thioether linkage (sulfhydryl group) linkages are unstable. Reaction of a primary amine ligand with epoxy-activated matrices will introduce a positive charge into the linkage.

Working Procedure (Based on Reference 11)

Reagents

1. 1,4-Butanediol diglycidyl ether (Aldrich)
2. Sepharose 4B or 6B

Activation

(**Note:** 1,4-Butanediol diglycidyl ether is highly toxic. Activation should be performed in a well-ventilated hood.)

1. Wash 100 ml packed Sepharose 4B with 1 liter deionized water in a sintered glass funnel and transfer the gel in 75 ml of 0.6 N NaOH containing 150 mg sodium borohydride.
2. Slowly add 75 ml 1,4-Butanediol diglycidyl ether with constant stirring, using an overhead paddle stirrer.
   (**Note:** do not use magnetic stirrer to avoid breakage of gel beads.)
3. Allow the activation reaction to continue for 10 h to overnight at room temperature.
4. Wash the activated gel extensively with water until no evidence of an oily film on the surface of the gel.
   (**Note:** washing the gel with acetone helps remove excess bisoxirane quickly. The gel is washed with water prior to coupling to ligand.)

Ligand Immobilization

5. Suspend the activated gel in 100 ml of coupling buffer (0.1 M Na_2CO_3 containing 0.5 M NaCl, pH 10) containing carbohydrate or protein (5 to 10 mg/ml gel). Continue coupling reaction at room temperature for 24 to 48 h with constant stirring, using an overhead paddle stirrer.
6. Wash the coupled gel with 1 M NaCl and water to remove unreacted ligand.
7. Block the remaining active groups on the gel by suspending in 100 ml of 0.1 M ethanolamine, pH 9.0 for 6 h at room temperature with stirring.
8. Wash the gel extensively with 1 M NaCl followed by water.

4.3.6.1.4   Ligand Immobilization through Tosyl/Tresyl Activation

Toluene sulfonyl chloride (tosyl chloride) and the more reactive 3,3,3-trifluoro-ethanesulfonyl chloride (tresyl chloride) are monofunctional reagents that react with a hydroxyl-containing matrix such as agarose. These reagents do not add any spacer to the matrix. Activation of the matrix is performed in dry acetone to prevent hydrolysis of tosyl chloride/tresyl chloride. Activated gel is then used to couple to the ligand in either aqueous or organic solvents. Ligands are immobilized to tosylated gels at high pH (9.0 to 10.5), whereas coupling of the ligand to tresylated gels can be performed at neutral pH.

Reaction

Both tosyl chloride and tresyl chloride react with hydroxyl group of the matrix (Figure 4.16). The primary amine in the ligand then readily reacts with the activated matrix, resulting a very stable secondary amine linkage.

Advantage

The immobilized ligand is more stable than the isourea produced by cyanogen bromide.

Disadvantage

At neutral pH, the secondary amine linkage is positively charged.

Working Procedure (Based on Reference 12)

Reagents

1. Tosyl chloride/tresyl chloride (Aldrich)
2. Pyridine
3. Sepharose 4B or 6B
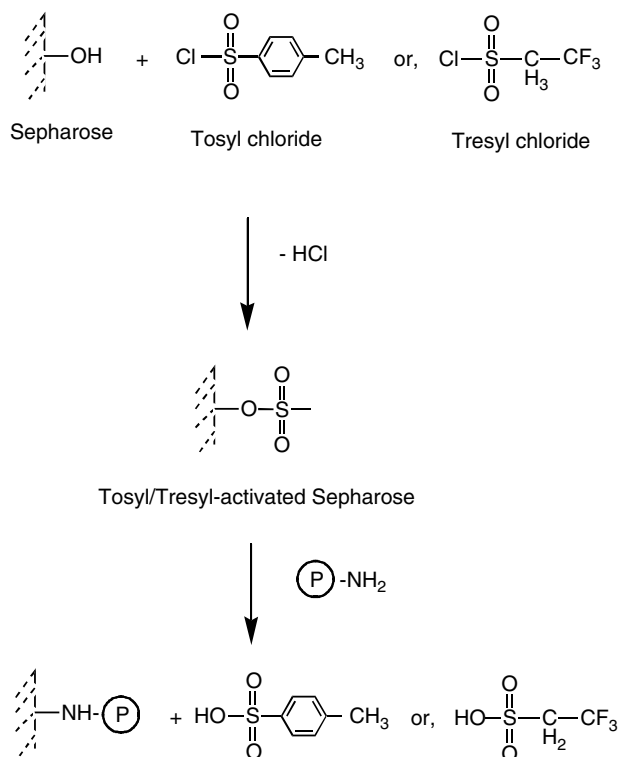
FIGURE 4.16 Tosyl/Tresyl chloride activation of matrix.

Activation

Activation should be carried out in a well-ventilated hood.

1. Using a glass-sintered funnel attached with a vacuum source, sequentially exchange 100 ml water-swollen packed Sepharose 4B into dry acetone with 40 ml each of acetone/water mixture at the ratio 30:70 (v/v), followed by 60:40, 80:20, and finally with dry pure acetone (three times).
2. Resuspend the acetone washed gel in 100 ml dry acetone containing 6 gram tosyl chloride.
   (**Note:** for tresyl chloride activation, add dropwise 1 ml tresyl chloride to the gel suspension in 100 ml dry acetone over a period of 1 min.)
3. Add 10 ml dry pyridine to neutralize the liberated HCl and continue stirring at room temperature for 1 h (10 min for tresyl chloride activation) using an overhead paddle stirrer.
   (**Note:** do not use magnetic stirrer to avoid breakage of gel beads.)
4. Wash the activated gel with 1 liter of acetone (twice) followed by 30:70, 50:50, 70:30 of 1 mM HCl:acetone. Wash the gel finally with 1 mM HCl.

Ligand Immobilization

   5. Suspend tosyl-activated gel (100 ml) in an equal volume of 0.25 M sodium carbonate, pH 9.5 (tresyl activated gel in 0.2 M sodium phosphate, pH 7.5) containing 5 to 10 mg/ml protein. Allow coupling reaction to continue at 4°C for 24 h with constant stirring using an overhead paddle stirrer.
   6. Wash the coupled gel with coupling buffer to remove unreacted ligand.
   7. Block excess active groups of the gel by suspending the gel in 100 ml 1 M ethanolamine, pH 9.0 for 1 h at room temperature.
   8. Wash the gel extensively with 1 M NaCl and water.

4.3.6.1.5 Ligand Immobilization through N-Hydroxysuccinimide Ester Activation

N-hydroxysuccinimide ester (NHS) activation of hydroxyl-containing matrices can be performed using N, N′-disuccinimidyl carbonate in a non-aqueous environment.[14] NHS activation is a fairly simple method and can be easily performed in the laboratory. However, two types of NHS activated cross-linked agarose matrices (Affi-Gel 10 and Affi-Gel 15) are available from Bio-Rad (Hercules, CA). Affi-Gel 10 contains a non-charged spacer and is recommended for conjugating nearly neutral and basic proteins of pI 6.5 to 11. Affi-Gel 15 contains a spacer arm with positively charged nitrogen and is suitable for coupling acidic proteins.

   The coupling of a ligand with an NHS-activated matrix can be accomplished in either an aqueous or a non-aqueous solution. The choice largely depends on the ligand solubility. For proteins or biomolecules, an aqueous system is preferable, as they tend to denature in a non-aqueous system. Coupling of primary amine-containing ligands to an NHS-activated matrix requires an uncharged primary amine group, and thus coupling is carried out at pH 7.5 to 8.0. Prior to coupling, the activated matrix is brought from organic solvent to an aqueous system. At this point, the ligand should be added to the activated matrix without delay, as the activated matrix is readily hydrolyzed in an aqueous system.

Reaction

N, N′-disuccinimidyl carbonate reacts with hydroxyl-containing matrices. The activated matrix can then form amide bonds with primary amines (Figure 4.17).

Advantage

Amide linkage is uncharged.

Disadvantage

At alkaline pH, amide linkage is unstable.
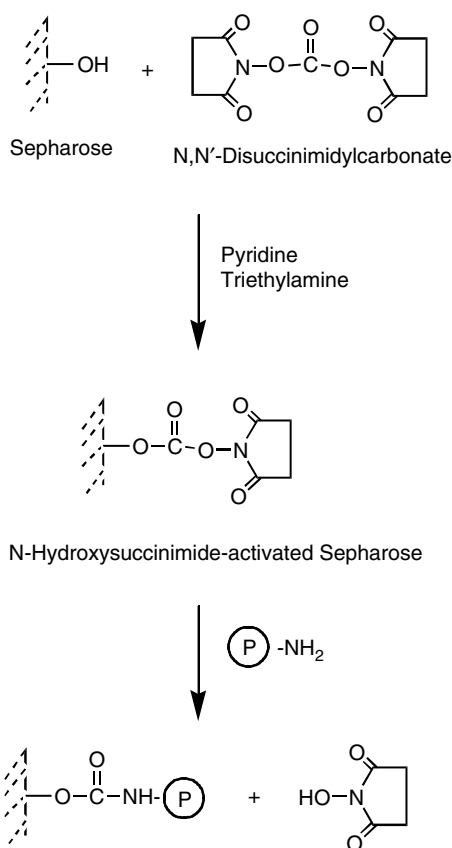
FIGURE 4.17 N-hydroxysuccinimide ester activation of matrix.

Working Procedure (Based on Reference 12)

Reagents

1. N, N′-disuccinimidyl carbonate (Aldrich)
2. Pyridine
3. Triethylamine (Aldrich)
4. Sepharose 4B or 6B

Activation

1. Using a glass-sintered funnel attached with a vacuum source, sequentially exchange 100 ml water-swollen packed Sepharose 4B into dry acetone with 40 ml each of acetone/water mixture at the ratio 30:70 (v/v), followed by 50:50, 70:30, and finally with dry pure acetone (three times).

2. Resuspend the acetone washed gel in 100 ml dry acetone containing 8 g of N, N′-disuccinimidyl carbonate.
3. Add dropwise 100 ml dry pyridine containing 7.5 ml of anhydrous triethylamine over a period of 30 to 60 min with constant stirring using an overhead paddle stirrer. Continue stirring for 1 h.
   (**Note:** do not use magnetic stirrer to avoid breakage of gel beads.)
4. Wash the activated gel with dry acetone three times (200 ml each) and finally with dry isopropanol three times (200 ml each). Activated gel (in isopropanol) is stable at 4°C and remains active for several months.

Ligand Immobilization in Aqueous Solution

5. Dissolve the ligand (5 to 20 mg/ml gel) to be coupled in 10 ml coupling buffer (0.1 M NaHCO$_3$, pH 8.0).
   (**Note:** alternate coupling buffers: 0.1 M MOPS, pH 7.0; 0.1 to 0.2 M phosphate, pH 7.5; and 0.1 M sodium borate, pH 8.5. Do not use buffers containing primary amine such as Tris and glycine, as they interfere with the coupling reaction.)
6. To isopropanol slurry of activated gel (10 ml packed gel), add 20 ml of ice-cold degassed deionized water. Quickly filter wash the gel with ice-cold degassed deionized water (three times, 20 ml each).
   (**Note:** washing the gel with cold deionized water is preferable to direct exchange with cold coupling buffer. Washing with cold water avoids precipitation of buffer salts and slows down hydrolysis of activated gel matrix.)
7. Transfer the washed gel in ligand solution and allow the coupling reaction at 4°C for 4 to 16 h with constant stirring, using an overhead paddle stirrer.
8. Wash the coupled gel with coupling buffer to remove unreacted ligand. Store the gel slurry at 4°C in the presence of 0.02% sodium azide until use.

4.3.6.2 Group Specific Affinity Matrices

Table 4.11 lists some biomolecules and their preferred ligands. The binding property can be utilized to prepare the ligand-immobilized affinity matrix to purify the interacting protein.

Immobilized sugars (neutral and amino), polysaccharides, and glycoproteins by CNBr or divinylsulfone cross-linking to insoluble support are generally used to purify lectins from plants, bacteria, and animals.[15–17]

Conversely, lectins can be immobilized onto solid support, and the matrix can be utilized to purify glycoproteins, glycolipids, and polysaccharides that have lectins' ligands. For example, Con A-Sepharose is used to purify glycoproteins that contain α-D-glucopyranosyl or α-D-mannopyranosyl, or both residues.[18]

Protein A from *Staphylococcus aureus* interacts with the fragment crystallizable (Fc) portion of immunoglobulin G (IgG) molecules from most mammalian species,[19] and thus the Protein A immobilized matrix (e.g., Protein A-Sepharose from Pharmacia) can be used to purify IgG molecules. Usually adsorption is performed

TABLE 4.11
**Some Common Ligands Used in Affinity
Chromatography**

Ligand	Target Molecule
Lectins	Glycoproteins, polysaccharides
Carbohydrates	Lectins
Protein A	Antibodies
Protein G	Antibodies
NAD, NADP	Dehydrogenase
Histones	DNA
Growth hormone	Growth hormone receptor
Gelatin	Fibronectin
Benzamidine	Serine proteases
Cibacron blue	Kinases, dehydrogenases, phosphatases
Heparin	Lipoproteins, DNA, RNA
Calmodulin	Kinases

at alkaline pH around 7 to 8 and elution of bound IgG is accomplished by lowering pH at about 3.

Lysine-Sepharose has been used to purify plasminogen.[20] Lysine is immobilized onto Sepharose by CNBr coupling, where the alpha-amino group is bound to solid support, leaving ε-amino and α-carboxyl groups free to interact with plasminogen.[21]

4.3.6.2.1 Lectin Affinity Chromatography

Lectins are proteins or glycoproteins that bind carbohydrates.[22] Lectin-carbohydrate interactions are very selective and reversible. Table 4.12 shows some common lectins and their carbohydrate specificities. *In vivo*, glycoproteins, glycolipids, or glycosaminoglycans can serve as endogenous ligands of lectins. Therefore, these interactions of lectin-carbohydrates or lectin-carbohydrate-containing glycoproteins can be exploited to purify glycoproteins, polysaccharides, or glycosaminoglycans on lectin-immobilized supports.[23,24] The lectin affinity chromatography consists of four steps: equilibration of the lectin-immobilized column with a buffer optimal to the lectin's binding properties; application of a mixture of glycoconjugates to the column; washing off the column with the equilibrating buffer to remove unbound glycoconjugates; and finally elution of adsorbed (bound) glycoconjugate with the saccharide inhibitor of lectin binding (usually hapten sugars such as monosaccharides and oligosaccharides). Some lectins require divalent cations for their binding (see Table 4.12). Cations at a final concentration 1 to 5 mM should be added in the binding buffer if these lectin columns are used. The advantage of using these types of lectin-immobilized columns is that after washing the columns, bound glycoconjugate can be removed from the column with metal chelators such as EDTA, avoiding using the expensive hapten sugars.

Lectin-immobilized columns can be made by employing several activating reagents as previously described (Section 4.3.6.1). But the immobilization reaction

TABLE 4.12

Some Commonly Used Immobilized Lectins and Their Carbohydrate Specificities

Lectin	Carbohydrate Specificity[a]	Metal Requirement
Con A (Concanavalin A)	α-D-Man> α-D-Glc	Mn^{2+}, Ca^{2+}
RCA (*Ricinus communis* agglutinin I)	β-D-Gal	—
WGA (wheat germ agglutinin)	β-D-GlcNAc, NeuAc	Mn^{2+}, Ca^{2+}
PNA (peanut agglutinin)	Galβ1,3GalNAc	—
Jacalin (jackfruit agglutinin)	α-D-Gal	—
SBA (soybean agglutinin)	D-GalNAc	—
UEA (*Ulex europaeus* agglutinin)	α-L-Fuc	Mn^{2+}
DBA (*Dolichos biflorus* agglutinin)	α-D-GalNAc	Ca^{2+}
SNA (*Sambucus nigra* agglutinin)	Neu5Acα2,6Gal	—
MAA (*Maakia amurensis* agglutinin)	Neu5Acα2,3Gal	—
GNA (*Galanthus nivalis* agglutinin)	Terminal Man	Mn^{2+}
BS-I (*Bandeira simplicifolia* I)	α-D-Gal	Ca^{2+}, Mg^{2+}

[a] Man, Mannose; Glc, Glucose; Gal, Galactose; Fuc, Fucose; GlcNAc, N-Acetylglucosamine; GalNAc, N-Acetylgalactosamine; NeuAc, N-Acetylneuraminic acid (sialic acid).

of lectins with the activated matrix should be performed in the presence of the lectins' carbohydrate ligands (50 to 100 mM) in order to protect the lectins' carbohydrate binding sites. Several lectin-immobilized columns are commercially available from vendors such as Sigma, Pierce, and EY Lab. Purification of glycoprotein using Con A-Sepharose is described here for an example.

Working Procedure

Column

Con A-Sepharose

Reagents

1. Equilibrating buffer: 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$
2. Eluting buffer: equilibrating buffer containing 0.1 M methyl α-D-mannoside

Purification

1. Equilibrate the column with 5 to 10 column volumes of equilibrating buffer.
2. Dissolve the sample in equilibrating buffer, centrifuge to remove particulate, and apply the clear supernatant to column.

3. Wash the column with 10 column volumes of equilibrating buffer or until A_{280} reaches baseline.
4. Elute the bound glycoprotein with the eluting buffer.

4.3.6.2.2 Carbohydrate Affinity Chromatography

The binding affinities of lectins towards carbohydrates can be exploited to purify lectins on carbohydrate-immobilized affinity columns. This situation is just opposite to that of the lectin affinity chromatography, where lectin is immobilized to solid support and the column is used to purify carbohydrates (in practice, glycoproteins and polysaccharides). Carbohydrate affinity columns can be prepared through divinylsulfone activation of Sepharose, followed by mixing the activated Sepharose with the carbohydrates such as monosaccharides (e.g., glucose, galactose) and disaccharides (e.g., lactose, melibiose). Several lectins from plants, invertebrates, and vertebrates have been purified employing carbohydrate-immobilized columns.[15,17] Purification of jacalin, a α D-galactoside-binding lectin from jackfruit (*Artocarpus integrifolia*), by affinity chromatography on a melibiose-agarose column[25] is shown here as an example.

Working Procedure

Column

Melibiose (Galα1,6Glc)-agarose (Sigma)

Reagents

1. Equilibrating buffer: phosphate buffered saline (PBS, 20 mM PO_4, 130 mM NaCl)
2. Elution buffer: 0.2 M melibiose in equilibrating buffer

Purification

1. Equilibrate the melibiose-agarose column with 5 to 10 column volumes of equilibrating buffer.
2. Dissolve sample in equilibrating buffer, centrifuge to remove particulate, and load the clear supernatant to the column.
3. Wash the column with 10 bed volumes of equilibrating buffer or until A_{280} reaches baseline.
4. Elute the bound protein in elution buffer. Dialyze the protein containing fractions with PBS.

4.3.6.2.3 ProteinA/G Affinity Chromatography

Bacterial cell wall proteins, Protein A (for *Staphylococcus aureus*) and Protein G (for *G. streptococci*), specifically bind the Fc portion of immunoglobulin G (for the structure of immunoglobulin G, see Figure 5.5). Thus, antibodies can be purified on

a Protein A or Protein G immobilized column. The binding of antibodies to Protein A or Protein G can be reversed by changing pH, salt concentration, or temperature.

Working Procedure

Reagents

1. Protein A-Sepharose: the column can be made using CNBr activated Sepharose following the protocols described in Section 4.3.6.1. It is also available commercially from several vendors such as Pharmacia, Sigma, and Pierce.
2. Equilibrating buffer: 0.1 M Tris-HCl (pH 7.5) containing 0.1 M NaCl.
3. Eluting buffer: 0.1 M glycine-HCl (pH 2.5).
4. Neutralizing buffer: 1 M Tris-HCl (pH 8.0).

Procedure

1. Equilibrate Protein A-Sepharose column (5 ml bed) with at least 5 column volumes of equilibrating buffer.
2. Dilute 5 ml of antibodies with an equal volume of equilibrating buffer and centrifuge, if necessary, to remove any precipitated proteins.
3. Apply the clarified sample to column.
4. Wash the column with approximately 10 column volumes of equilibrating buffer or until the absorbance at 280 nm reaches the baseline.
5. Elute the bound protein with about 5 column volumes of eluting buffer. Collect 1 ml fraction and add immediately 0.1 ml of neutralizing buffer.
6. Regenerate column by cleaning with 5 column volumes of eluting buffer followed by 5 column volumes of equilibrating buffer.

4.3.6.2.4 Immunoaffinity Chromatography

The specific recognition of antigens and antibodies can be exploited to purify antigens on an antibody-immobilized column. For this purpose, IgGs can be coupled to Sepharose by CNBr activation (see Section 4.3.6.1.1). Like other affinity chromatography, immunoaffinity chromatography is a powerful method, allowing purification of a protein in a single step. The method is particularly useful when purification of a native protein is sought using antibodies made against recombinant protein.

The success of immunoaffinity purification relies on at least four factors. The most important one is that antibodies to be immobilized to solid support must be made against a pure antigen. As polyclonal antibodies are heterogeneous, antibodies raised against an antigen with a slight impurity can be problematic and undermine the success of immunoaffinity purification. For this reason, monoclonal antibodies are preferred over polyclonal antibodies, as the former represent a homogeneous antibody population with specificity for a single epitope (see Chapter 5 for a description of antibodies).
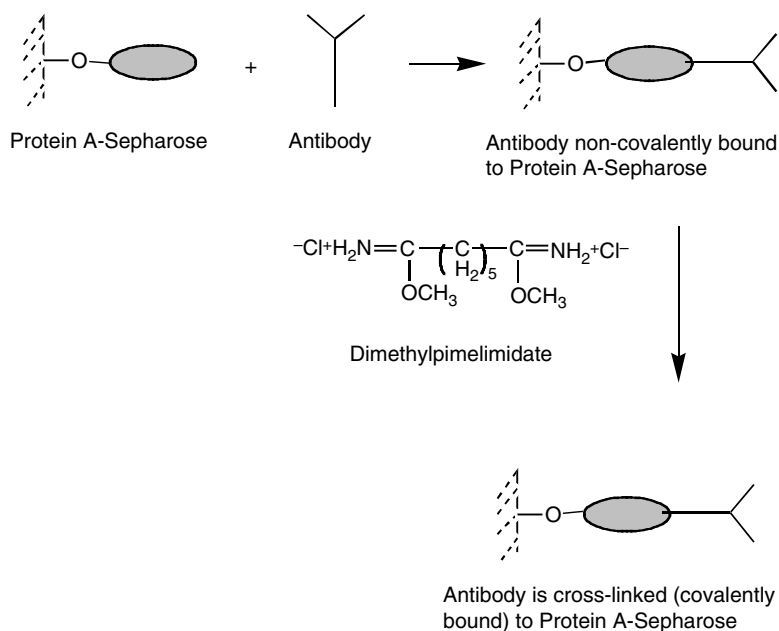
FIGURE 4.18 Cross-linking of antibody to Protein A-Sepharose with dimethylpimelimidate.

The second factor for the success of immunoaffinity purification is the affinity of the antibodies (regardless of the type of antibodies, mono- or polyclonal) for the antigens.

Another critical factor for successful immunoaffinity purification is the accessibility of the antigen binding sites in the antibodies. Direct coupling of antibodies (even antibodies with high affinity) to the matrix often restricts accessibility of the antigen binding sites, resulting in an inefficient immunoaffinity column. This problem can largely be overcome by using antibodies-Protein A/G-Sepharose, which is made by mixing Protein A- or G-Sepharose beads with antibodies (Figure 4.18). Protein A or G specifically binds to the Fc domain of immunoglobulin G, resulting in antigen-binding sites more accessible for maximal interactions with the antigens. To avoid leaching of antibodies from Protein A/G-Sepharose beads, the interaction can be stabilized with a cross-linking reagent[26] (see Figure 4.18).

The fourth factor for successful immunoaffinity purification is to eliminate non-specific interactions between immobilized antibodies and proteins in the sample. The non-specific interactions can largely be avoided by passing the crude sample through a similar immunoaffinity column made with normal IgG (from pre-bleed serum from an animal prior to immunization). Affinity columns can also be blocked with standard blocking agents used in Western blot such as non-fat dry milk (5%), bovine serum albumin (1 to 3%), horse serum (1 to 3%), Tween 20 (0.1%), and gelatin (0.25%) for several hours at room temperature prior to loading sample to the column.[12]

Like other affinity chromatography, immunoaffinity chromatography consists of four steps: equilibration of the column, loading the sample to the column, washing the column to remove unwanted proteins, and elution of the bound protein from the column. Specific elution of antigens (with excess small antigen peptides) from an antibodies-immobilized column is not common due to cost and lack of the availability of purified antigens. However, small inorganic substances such as acids and chaotropic agents can be used as eluting agents. Chaotropic agents can dissociate antigen-antibody complexes by disrupting hydrogen bonding, ionic interactions, and hydrophobic interactions. In most applications, elution with acids (usually 0.1 M glycine-HCl, pH 2.5 and sodium citrate, pH 2.5) is effective to dissociate antigen-antibody interactions. At this pH, binding constants of antigen-antibody interactions are very low.

Working Procedure

Reagents

1. Protein A-Sepharose (Pharmacia)
2. Dimethylpimelimidate (DMP) (Pierce)
3. Ethanolamine

Antibody Coupling to Protein A-Sepharose

1. Using a glass sintered funnel, wash Protein A-Sepharose with PBS, pH 7.5.
2. To washed Protein A-Sepharose, add polyclonal or monoclonal antibodies (2 to 5 mg/ml packed beads). Incubate the mixture at room temperature for 1 h with gentle rocking.
3. Wash the beads twice with 0.2 M sodium borate, pH 9.0 (10 ml/ml beads).
4. Resuspend beads in 10 volumes of borate buffer and add solid DMP to make a final concentration of 20 mM. Allow cross-linking reaction for 30 to 45 min at room temperature with gentle rocking.
5. Wash the beads with 0.2 M ethanolamine, pH 8.0. Resuspend beads in 0.2 M ethanolamine and incubate for 2 h at room temperature with gentle rocking.
6. Wash the beads with PBS.
7. Wash the beads with 100 mM glycine-HCl, pH 3.0 to remove any antibodies that remain non-covalently bound, but not cross-linked.
8. Finally, wash the beads with PBS. The beads are now ready for purification of antigens.

Purification of Antigens Using Antibodies-Protein A-Sepharose or Antibodies-Sepharose

1. Pack the beads into a column and equilibrate with PBS.
2. Load the sample in equilibrating buffer. Allow antigens to bind for 3 to 4 h at room temperature (overnight at 4°C).
3. Wash the column with 10 bed volumes of equilibrating buffer or until A_{280} reaches baseline.
4. Elute bound protein with 0.1 M glycine-HCl, pH 2.5. Collect 1 ml fractions in tubes containing 0.1 ml 1 M Tris-HCl, pH 8.0 to neutralize bound protein.
5. To regenerate the column, wash with 10 volumes of 0.2 M glycine-HCl, pH 2.5 followed by 10 to 20 volumes of PBS.

4.3.6.2.5 Dye Ligand or Pseudo Affinity Chromatography

Several dyes such as Cibacron Blue F3-GA, Procion Blue HB, Procion Red HE-3B, Procion Yellow H-A, and Procion Green H-E4BD are used as affinity adsorbents although they do not have characteristics of a true ligand. For this reason, dye ligand chromatography is also known as pseudo affinity chromatography.[4] Dye ligand or pseudo affinity chromatography has been useful in purifying nucleotide-dependent proteins such as kinases, hydrolases, and polymerases.[27] Dyes usually contain one or two reactive groups, monochloro-triazine or dichloro-triazine, which resemble the nucleotide structure of NAD (Figure 4.19). The reactive triazine group of the dye facilitates immobilization of the dye to Sepharose by nucleophilic displacement of the chlorine atom. However, immobilized dyes are commercially available.

Working Procedure

Column

Blue-Sepharose CL 6B (Cibacron Blue F3-GA linked to Sepharose CL-6B) from Pharmacia
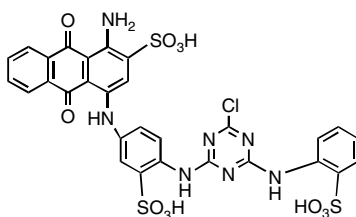
FIGURE 4.19 Structure of Cibacron blue dye.

Reagents

1. Equilibration buffer: 20 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl
2. Elution buffer: 20 mM Tris-HCl (pH 8.0) containing 1 M NaCl.

Procedure

1. Equilibrate Blue-Sepharose CL-6B with 5 to 10 column volumes of equilibrating buffer.
2. Dissolve protein sample (10 to 40 mg per ml of matrix) in equilibrating buffer and load to column.
3. Wash 5 to 6 column volumes or until A_{280} reaches baseline.
4. Elute with eluting buffer.
5. Regenerate column with 3 column volumes of 1 M NaOH followed by washing with 10 column volumes of equilibrating buffer.

4.3.6.2.6   Nucleic Acid Affinity Chromatography

Nucleic acid affinity chromatography is generally used to purify regulatory proteins that are involved in the control of gene expression and gene repair. This chromatography is also used to isolate enzymes, which require NAD or NADP cofactors. For successful affinity columns, the size of nucleotides should be within 14 and 51 bases.[28] In most cases, nucleotides longer than the recognition sequence are used to make an affinity column. Longer nucleotides can serve as spacers.

In nucleic acid chromatography, non-specific binding is often problematic. However, this problem can be avoided by passing the sample through a non-specific affinity precolumn (an affinity column containing a random oligonucleotide sequence) or by using an additional affinity column employing alternative flanking sequences.

Oligonucleotide-Sepharose can be prepared by CNBr activation as previously described (Section 4.3.6.1.1).

Working Procedure

Reagents

1. Equilibrating buffer: 20 mM Tris-HCl, pH 8.0 containing 150 mM KCl and 1 mM EDTA
2. Elution buffer: equilibrating buffer containing 0.2 to 1 M KCl

Purification

1. Equilibrate the column with 5 to 10 column volumes of equilibrating buffer.

2. To protein sample in equilibrating buffer, add non-specific DNA such as sonicated DNA from salmon sperm and calf thymus (100 µg/mg protein). Apply the mixture to affinity column (15 ml/h).
3. Wash the column with 10 bed volumes of equilibrating buffer.
4. Elute bound proteins with increasing concentrations of KCl (0.2 to 1 M) in equilibrating buffer (1 column volume each).

4.3.6.3 Metal Chelation Affinity Chromatography

This chromatography developed by Porath et al.[29] involves the immobilization of metal ions, such as zinc, copper, nickel, cobalt, and calcium, onto an insoluble support such as Sepharose via chelating groups, such as iminodiacetic acid and tris(carboxymethyl) ethylenediamine. The chelating group is usually attached to Sepharose via a long hydrophilic spacer arm that provides easy access of interacting proteins to immobilized metals. The metal of choice must have a higher affinity for the matrix than for the interacting proteins. The metal chelation chromatography is based on the fact that some amino acids like histidine, tryptophan, and cysteine can coordinate transition metal ions by their electron donor groups on the amino acid side chain.[30] The HiTrap Chelating column (Pharmacia) is available for this chromatography. The column is charged with an appropriate divalent cation. After loading the sample and washing the column, the elution is normally achieved using a lower pH or increasing concentration of imidazole. A list of proteins that have been isolated by different metal chelate columns is shown elsewhere.[31]

Working Procedure

Column

Iminodiacetate substituted Sepharose

Reagents

1. Divalent cation solution: $ZnCl_2$ or $CuSO_4.5 H_2O$ (1 mg/ml)
2. Equilibration buffer: 20 mM sodium phosphate containing 0.5 M NaCl (pH 7.5)
3. Elution buffer: 0.1 M sodium acetate (pH 4-6) containing 0.5 M NaCl
4. Chelating buffer: 50 mM EDTA in 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl

Procedure

1. Charge the column matrix with either cation solution.
2. Equilibrate the column with the equilibration buffer.
3. Apply protein sample (dissolved in equilibration buffer) to the column.
4. Wash the column with the equilibration buffer.

5. Elute the bound protein with elution buffer. Usually elution is more effective at lower pH.
6. Use chelating buffer to elute more tightly bound proteins.
7. Wash the column with 50 mM EDTA to regenerate column.

4.3.7 Covalent Chromatography for Thiol-Containing Substances

Activated Thiol-Sepharose and Thiopropyl-Sepharose are generally used to separate thiol-containing substances. Activated Thiol-Sepharose is formed from a reaction of CNBr-activated Sepharose with glutathione and 2,2′-dipyridyl disulfide (Figure 4.20). Thiopropyl-Sepharose is a mixed disulfide prepared from 2,2′-dipyridyl disulfide and activated Sepharose.[32] Unlike other matrices used in affinity chromatography, the covalent chromatography involves the covalent attachment of the thiol groups to the activated thiolated matrices. After removing the unbound substances, the covalently bound thiol-containing substances can reversibly be eluted by passing small substances containing thiol groups, such as L-cysteines, mercaptoethanol, dithithreitol, etc. (see Figure 4.20). The matrices can be regenerated by passing of 2,2′-dipyridyl disulfide at alkaline pH around 8.

Working Procedure (21)

1. Equilibrate activated Thiol-Sepharose 4B or Thiopropyl-Sepharose 6B (Pharmacia) with 0.1 M tris-HCl, pH 7.5 containing 0.5 M NaCl and 1 mM EDTA.
2. Load sample (thiol containing) to the column.
3. Wash the column to remove unbound protein.
4. Elute the bound protein with 0.025 M cysteine in equilibrating buffer.
5. Regenerate column by passing 1 or 2 bed volumes of saturated solution (1.5 mM) of 2,2′-dipyridyl disulfide at pH 8.0 through the gel.

4.3.8 Hydroxyapatite Chromatography

Tiselius and his coworkers[33] introduced hydroxyapatite chromatography for protein purification. Later Bernardi[34] extensively studied this chromatography for a variety of proteins using a small solvent system. However, due to low capacity and difficulty in predicting chromatographic behavior, this chromatography has a limited use.

Principle

Hydroxyapatite is the calcium phosphate crystal that provides a mosaic of positive (due to Ca^{2+}) and negative (due to PO_4^{3-}) sites. When the column is equilibrated with a phosphate buffer, the surface of the column becomes negative because of the neutralization of the positive calcium ion by phosphate, and the column, in turn, is
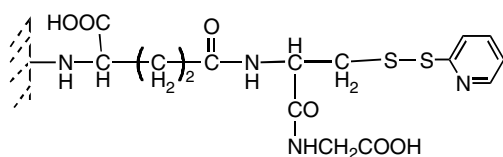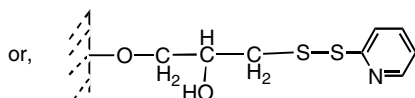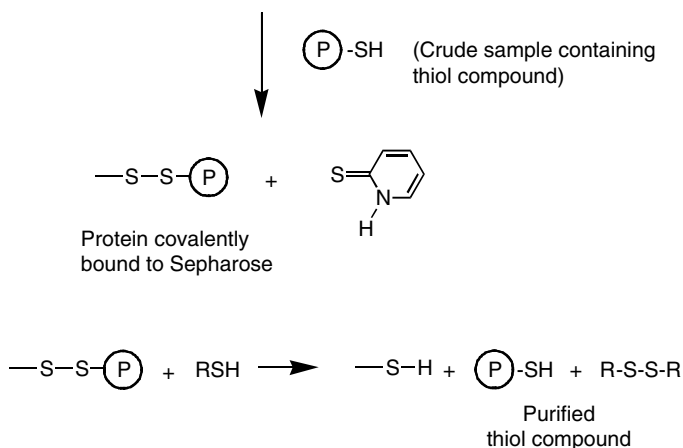
FIGURE 4.20 Purification of thiol-containing substances by covalent chromatography.

capable of binding basic proteins.[33] After washing off the unbound protein by the equilibrating buffer, the bound protein can be eluted with excess negative charge, such as F^-, Cl^-, $ClO4^-$, SCN^-, or PO_4^{3-}, or by specific displacement with Ca^{2+} or Mg^{2+} that complexes with column phosphates. Conversely, acidic proteins bind by complexation to calcium sites on the column, and the elution of the bound proteins can then be achieved by ions (e.g., F^-, PO_4^{3-}) that form stronger complexes with calcium loci than do carboxyl groups of proteins. Thus Cl^- is not an effective eluent for acidic proteins because it does not complex with calcium.

Loading and Eluting

A detailed description of this chromatography and guidelines for the use of hydroxyapatite columns are reported elsewhere.[35] Prior to loading the sample, the column should be equilibrated with any of the following three ways depending

on the objective. If the objective is to retain mostly basic proteins, the column is equilibrated with dilute phosphate buffer (1 mM, pH 6.8). For binding of acidic proteins, the column is equilibrated with unbuffered NaCl solution (1 mM). For binding of some acidic proteins which do not bind to an NaCl column (column that is equilibrated with NaCl), the column should be equilibrated with unbuffered $MgCl_2$ or $CaCl_2$ solution (1 mM). After loading, the column is washed with the same buffer as that on the column. Elution is performed with 5 mM $MgCl_2$ (or a gradient of 1 to 5 mM) to displace basic proteins, a wash with 1 M NaCl (or a gradient of 0.01 to 1 M) to displace proteins with pI around neutrality, and finally a wash with 0.3 M phosphate (or a gradient of 0.1 to 0.3 M) to displace acidic proteins.

Working Procedure for Purification of a Basic Protein

Column

Hydroxyapatite (Bio-Rad)

Reagents

1. Equilibrating buffer: 20 mM potassium phosphate, pH 7.0
2. Eluting buffer: 0.3 M phosphate, pH 7.0

Purification

1. Equilibrate the column with 5 bed volumes of equilibrating buffer.
2. Load protein sample previously dialyzed in equilibrating buffer.
3. Wash the column with 10 bed volumes of equilibrating buffer or until A_{280} reaches baseline.
4. Elute the column with a linear gradient made from equilibrating and eluting buffer.

4.3.9 High-Performance Liquid Chromatography

Unlike other chromatographies described above, HPLC (also known as high-pressure liquid chromatography) does not represent any new chromatography chemistry; rather, it refers to an automated, programmable, and computer-controlled system, which can be used to perform all types of chromatographies discussed above. Gel matrices used in HPLC are very homogeneous, and small particles provide better resolution of sample peaks with reproducibility and improved physical and chemical stability than the conventional gel matrices. Since columns are packed with high-resolution small gel beads (range between 8 and 40 micron), resistance to column buffer flow becomes very high. Thus, HPLC equipment has been designed to provide such high pressure to deliver solvent to the column by a more sophisticated pump. Table 4.13 shows several prepacked columns of different applications. Several HPLC systems and chromatographic columns are commercially available (for example,

TABLE 4.13
Some Commonly Used Prepacked HPLC Columns

Column	Chemistry	Vendor
Gel filtration:		
Superose	Cross-linked agarose	Pharmacia
Protein Pak	Bonded silica	Waters
Synchropak	Bonded silica	Synchrom
TSK-SW	Bonded silica	Toyo-Soda
Zorbax	Bonded silica	DuPont
Ion-exchange:		
Mono Q	Quaternary aminoethyl-	Pharmacia
Mono S	Sulphopropyl-	Pharmacia
Biologic S	Sulphopropyl-	Bio-Rad
Chromatofocusing:		
Mono P	Phospho-	Pharmacia
Reversed-phase:		
C_4	Butyl substituted silica	Vydac
C_{18}	Octadecyl substituted silica	Vydac
Delta Pak C_{18}	Octadecyl substituted silica	Waters

AKTA purifier from Amersham Biosciences, BioLogic Duo-Flow and BioLogic HR systems from Bio-Rad). Although the column chemistry is based on the type of purification procedure, operation of HPLC varies from vendor to vendor. HPLC should be operated according to the manufacturer's instructions. In general, HPLC systems are equipped with two pumps, mixer, injection unit, column detector, fraction collector, and a computer to control pump, injection, and data acquisition (Figure 4.21). Additional features such as conductivity and pH meters are available in some systems to monitor chromatogram.

General Practice

Column guards are generally used between the injector and the column. Column guards filter the sample and protect the column from particle deposits. Buffers should be degassed and filtered using a 0.2 μm filter to prevent the formation of air bubbles. If the buffers are stored at 4°C, they should be brought to working temperature prior to use. The pump should be primed to ensure that no air is trapped in the pump head. The sample should be clarified by centrifugation and membrane filtration to reduce backpressure increase due to particle deposit. When injecting the sample, a pointed needle should be avoided as this may damage the injection valve. For multiple injections, the column should be re-equilibrated with the starting buffer prior to another injection. Sample should be stable in both equilibrating and eluting buffer. After run, equipment should be washed thoroughly to avoid any salt deposits.
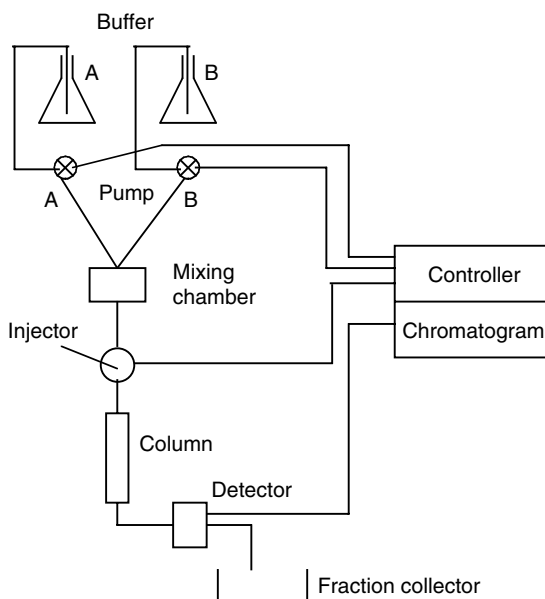
FIGURE 4.21 Basic setup of HPLC system.

Gel Filtration HPLC

In gel filtration HPLC, proteins are separated based on the principle similar to conventional gel filtration chromatography (i.e., molecular size). Like conventional chromatography columns, HPLC gel filtration columns may interact non-specifically with the protein sample. These interactions are minimized by the increasing ionic strength of the buffer. Some lectins such as D-galactose-binding lectins often are retarded in agarose-based columns, resulting in an underestimation of their molecular sizes. To avoid such lectin-matrix interactions, free D-galactose (10-20 mM) can be added in the column buffers (both equilibrating and eluting buffers).

Ion-Exchange HPLC

In ion-exchange HPLC, strong ion exchangers such as Mono Q and Mono S are generally used. Mono Q can be used in the pH range 5.5 to 9.5 (typical pH 7 to 8), whereas Mono S is used in the pH range 3.8 to 7.6. Ion-exchange HPLC has a few advantages over conventional ion-exchange chromatography. Since ion-exchange columns can be operated at relatively high speed, multiple runs can be performed in a short period of time. Another advantage of ion-exchange HPLC method is the formation of salt or pH gradient, which may not be precise in the conventional chromatography.

Working Procedure

Column

Mono Q (Pharmacia)

Reagents

1. Buffer A: 10 mM Tris-HCl, pH 7.0. Filter (0.22 μm membrane) and degas.
2. Buffer B: 10 mM Tris-HCl, pH 7.0 containing 1 M NaCl. Filter and degas.

Purification

1. Disconnect column from the system and prime pumps A and B with buffer A and B, respectively.
2. Set up the pressure limit on both pumps below the maximum pressure limit for the column.
3. Equilibrate the column with 10 column volumes of buffer A.
4. Prepare the sample by dissolving or dialyzing in buffer A. Filter the sample (0.20 μm membrane filter).
5. Apply the sample to the column.
   (**Note:** in ion-exchange chromatography, any volume of sample can be applied as long as the amount of sample does not exceed the binding capacity of the ion-exchange column. Typically, small volume up to 10 ml is applied through the injection loop. Higher volumes can be loaded through the pump using a separate buffer line.)
6. Wash the column with 5 column volumes of buffer A to remove unbound protein.
7. Elute bound protein with a 0 to 100% gradient of buffer B over a period of 25 min as follows: 0 to 50% B for 20 min, 50 to 100% B for 5 min, 100% B for 5 min, 100 to 0% B for 5 min.
   (**Note:** improve protein resolution by changing the percentage of B for different periods of time [i.e., by creating gradients of varying degree of shallowness].)
8. Monitor elution at 280 nm and by determining activity of protein fractions.
   (**Note:** try cation exchanger such as Mono S if protein does not bind to the anion exchanger.)

Reversed-Phase-HPLC

Reversed-Phase (RP)-HPLC has been widely used for the separation and analysis of peptides. Like standard RP chromatography, several bonded phases such as C_4, C_8, and C_{18} are available for the separation of peptides by RP-HPLC. Typically, columns with particle sizes of 2 to 10 μm (commercially available) are used in RP-HPLC. In RP-HPLC, a short fat column is more useful than a thin long column.

When separation of sub-microgram sample is desired, columns of 1 and 2.1 mm internal diameter (micro bore and narrow bore, respectively) are suitable. For samples of microgram to low milligram range, analytical column (4.6 mm internal diameter) is recommended.

Working Procedure

Column

C_{18} (Vydac, Hesperia, CA)

Reagents

1. Buffer A: 0.1% trifluoroacetic acid
2. Buffer B: 0.085% TFA in acetonitrile: water (70:30)

Purification Procedure

1. Disconnect column from the system and prime pumps A and B with buffer A and B, respectively.
2. Set up the pressure limit on both pumps below the maximum pressure limit for the column.
3. Equilibrate the column with 10 column volumes of buffer A.
4. Dissolve sample in buffer A and apply sample to the column.
5. Wash the column with 5 column volumes of buffer A to remove unbound peptides.
6. Elute bound peptides with a 0 to 100% gradient of buffer B over a period of 40 min.
7. Monitor elution at 214 nm.

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5 Antibodies: Structures, Interactions, and Production

Antibodies are produced primarily by plasma cells and the precursor B lymphocytes in response to the presence of foreign molecules (antigens) in the body.[1,2] The antibody response by the lymphocytes does not occur in all living organisms and is known only to vertebrate recognition systems. The circulating antibodies bind to the specific antigen against which they are produced, and the resulting antigen-antibody complexes are removed from circulation by macrophages through phagocytosis. Antibodies are widely used in all areas of biological research, including clinical diagnostics, because of their specific interactions with antigens. Since antigens can specifically detect molecules in complex mixtures and in tissues, varieties of immunochemical techniques have been manipulated for investigation.

5.1 IMMUNE SYSTEM AND ANTIBODY RESPONSE

The vertebrate immune system protects the body from infectious organisms. The surveillance of the immune system is served by circulating proteins and cells that recognize foreign materials and destroy the invaders. Vertebrates defend themselves against pathogens via two major mechanisms: innate immunity and adaptive response. Innate immunity is the first line of defense system and is mediated by cells that respond non-specifically to foreign molecules. Several systems such as phagocytosis by macrophages, secretion of lysozyme by bacterial cells, and cell lysis by natural killer cells contribute to innate immunity. However, the phagocytes of the innate immune system cannot recognize many pathogens and thus fail to eliminate infectious organisms.

To overcome this problem, the lymphocytes of the adaptive immune system have evolved to provide protection from pathogens in a sophisticated way. Adaptive or acquired immunity is achieved in two ways: humoral and cell mediated. The humoral immunity results in the generation of circulating antibodies that bind to foreign antigens and is mediated by B lymphocytes in conjunction with helper T lymphocytes. B and T lymphocytes are the two main players of the adaptive immune response. B lymphocytes mature in the bone marrow, while T lymphocytes mature in the thymus. Cell-mediated immunity is achieved by the binding of cytotoxic T lymphocytes to foreign or infected cells and subsequent lysis of these cells. In addition to killing or destroying pathogens, lymphocytes of the adaptive immune system provide another level of protection from a re-infection with the same pathogen.

When an antigen gets entry into a naive animal, the first step in the generation of immune response is the phagocytosis of the antigen. Foreign antigens are engulfed non-specifically by macrophages or other cells including Langerhans cells in the skin, dendritic cells in the spleen, and lymph nodes in the blood. The engulfed antigen is then partially degraded by proteases in the acidic chamber of endosome in the cytosol (Figure 5.1). Since these cells process antigens in the same manner, they are collectively referred to as antigen-presenting cells (APCs). The APCs
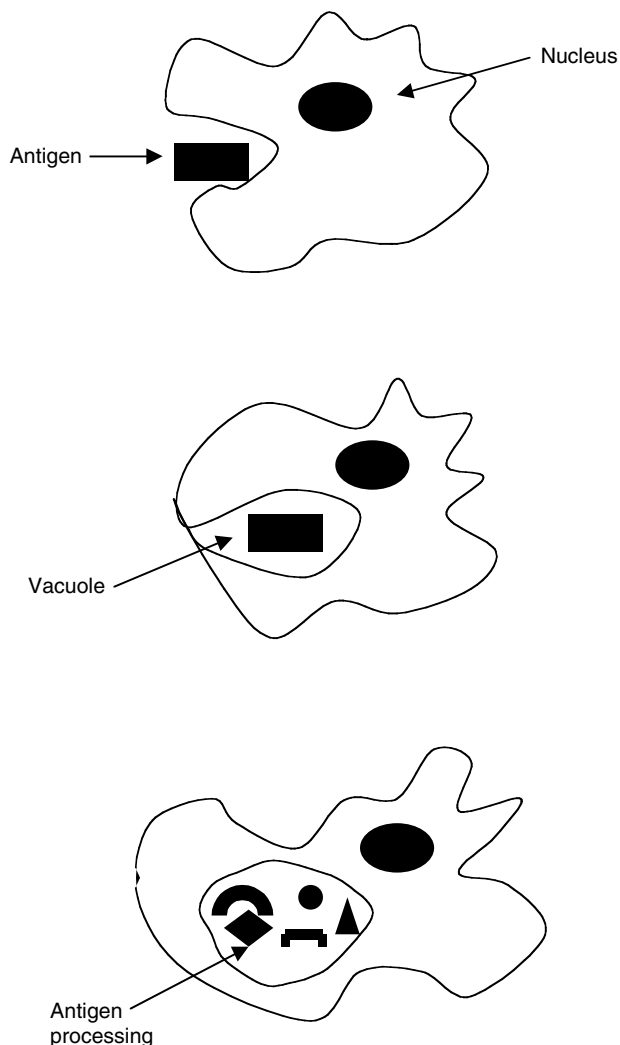
FIGURE 5.1 Antigen processing. Foreign antigens or pathogens are engulfed by the phago-cytes (macrophages or other cells). The phagosomes are then fused with other organelles (lysosomes) where engulfed antigens are degraded by hydrolytic enzymes.

present antigen on their surface, thus exposed to antibody attack. For most antigens, this non-specific uptake is essential to initiate an effective response. The *in vitro* immunization techniques are based on the efficiency of phagocytosis. The better the immunogen, the greater the degree of phagocytosis.

CD4⁺ (Helper T, T$_H$) Cell Activation

The degraded antigens appear on the cell surface of the APC by a cell surface glycoprotein known as the major histocompatibility complex (MHC) class II protein. The mechanism of this process is not well understood. Class II molecules are heterodimers containing polypeptides of α and β subunit. MHC class II molecules are "self" molecules that have a cleft region that binds "non-self" pathogen (degraded) particles and present them on the surface of the cell (Figure 5.2).
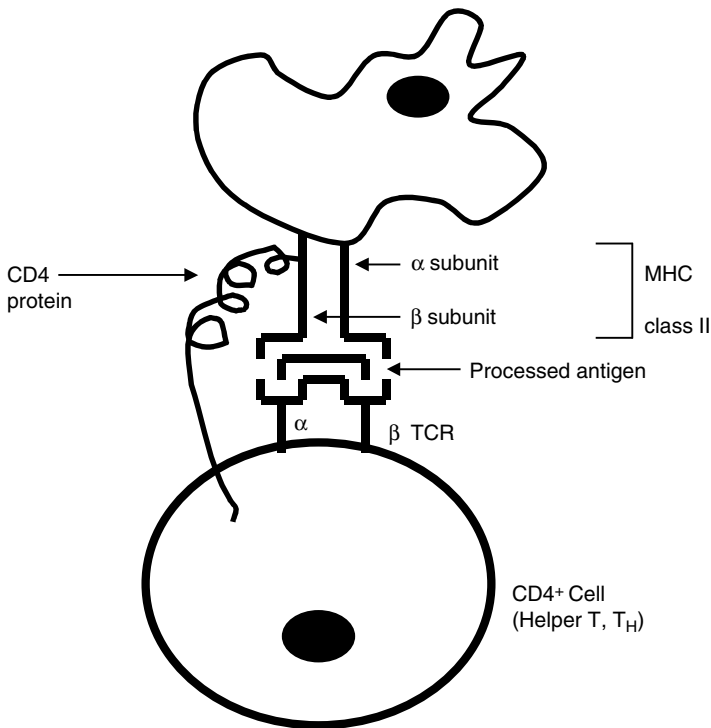
FIGURE 5.2 CD4⁺ (helper T, T$_H$) cell activation. In antigen presenting cells (APCs), the degraded antigens are appeared on the cell surface of the APC by a cell-surface glycoprotein, major histocompatibility complex (MHC) class II protein, and presented to CD4⁺ cells (usually considered helper T, T$_H$ cells). This process and the release of a helper monokine, interleukin-1 (IL-1), activate CD4⁺ cells.

Helper T Cell Proliferation and Differentiation

In the next step of the antibody response, helper T cells bind to the antigen fragment-class II protein complexes on the APC (Figure 5.2). This binding is mediated through T cell receptors on the surface of the helper T cells. The T cell receptor is a heterodimer containing polypeptides known as α and β chains. Binding of a helper T cell to an APC starts a cascade of events leading to T cell proliferation and differentiation. The first stage of the cascade, activation, requires two signals. First, helper T cells must bind to the correct antigen fragment-class II protein complex. This binding ensures that only helper T cells that are able to recognize the correct antigen are stimulated. The second signal is mediated by the binding of a soluble polypeptide growth factor known as interleukin 1 (IL-1). The production of IL-1 by the APC is non-specific, but within the helper T cell population, only T cells that are bound to an APC will respond.

The next step in the proliferation and differentiation of helper T cells involves a 15 kDa polypeptide growth factor, interleukin 2 (IL-2). It is secreted by T cells in response to the combined stimulation of antigen binding and IL-1. This combined stimulus also induces the helper T cells to synthesize IL-2 receptors. Helper T cells divide exponentially in the presence of IL-2; in the absence of IL-2, the cells stop dividing. The expression of simultaneous IL-2 receptors is also important. In the absence of IL-2 receptors, proliferation of the helper T cells ceases, even if IL-2 is still present.

CD8⁺ (Cytotoxic T, T$_C$) Cell Activation

CD8⁺ T cells (known as cytotoxic T cells, T$_C$) are programmed to kill infected cells in which bacterial pathogens or viruses replicate inside cells, where they cannot be detected by antibodies. Activated cytotoxic cells respond in cell-mediated immunity reactions by recognizing the antigen on the target cells (virally infected cells, foreign tissues, or tumor cells) associated with class I MHC molecules (Figure 5.3). MHC I is generally on the surface of every cell in the body.

B Cell Activation

Helper T cells initiate B cell response. During proliferation of helper T cells, antigen-specific B cells also start dividing by the similar events. Like APC, antigens are processed by B cells, and degraded fragments appear on the cell surface bound to MHC class II protein. But in contrast to non-specific phagocytosis by APC, the uptake of antigen by B cells is specific. First, the antigen is captured by a modified antibody that presents on the surface of virgin B cells. The antigen-antibody complex is then internalized and the antigen is processed (Figure 5.4). The degraded and processed antigen bound to a class II protein complex is now target for binding by helper T cells through T cell receptors. The complex of antigen fragment and class II protein on B cells is identical to the analogous complex on APCs. The contact between B cells and T cells provides a key stimulus that leads to B cell division and the production of an antibody.
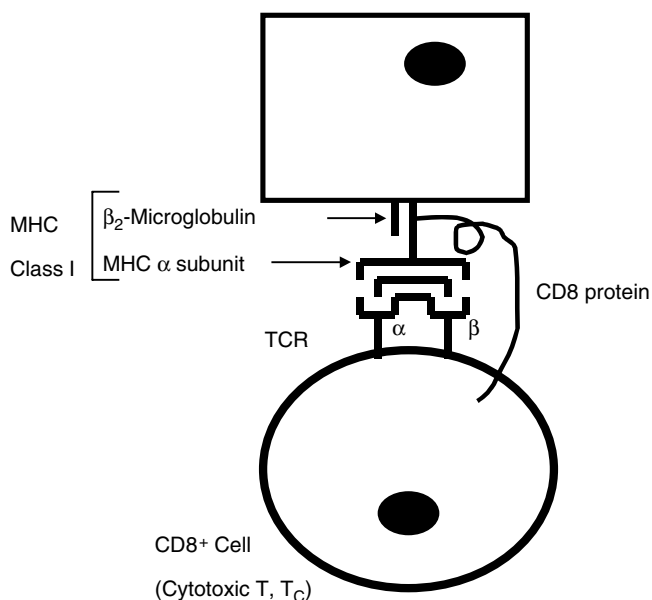
FIGURE 5.3 CD 8+ (cytotoxic T, T_c) cell activation. Activated CD8+ cells (known as cytotoxic T cells, T_c) respond in cell-mediated immunity reactions by recognizing the antigen on the target cells associated with MHC class I proteins.

The following three steps are important in this process. First, the antigen must bind to the surface antibody on the B cell. Only cells that bind the antigen are stimulated, thus ensuring the selection of the correct antibody secreting clones (i.e., secreting antibody will have the same antigen-binding site as the surface antibody). Second, the processed antigen fragments must bind to the class II proteins, providing the basis of recognition by T cells. Third, the antigen fragment-class II protein complex must bind to the cell receptor, ensuring that B-cell stimulation by helper T cells is specific. The properties of physical interactions explain why some compounds make good antigens and others do not. In order to induce an antibody response, a compound must have an epitope complementary to virgin B cell surface antibody, and upon degradation the compound must generate fragments that can be bound simultaneously to both the class II protein and the T cell receptor.

B Cell Proliferation and Differentiation

The binding of helper T cells to B cells induces synthesis of the B cell growth factor called interleukin-4 (IL-4). After binding IL-4, B cells start to synthesize the receptor for a B cell differentiation factor known as interleukin-5 (IL-5). By a poorly understood mechanism, the binding of IL-5 in conjunction with other signals induces differentiation of the dividing B cells into plasma cells and memory cells. Plasma cells are highly specialized and devoted to secrete large amounts (typically 40% or more of the total protein synthesis) of antibody, providing humoral immunity. They
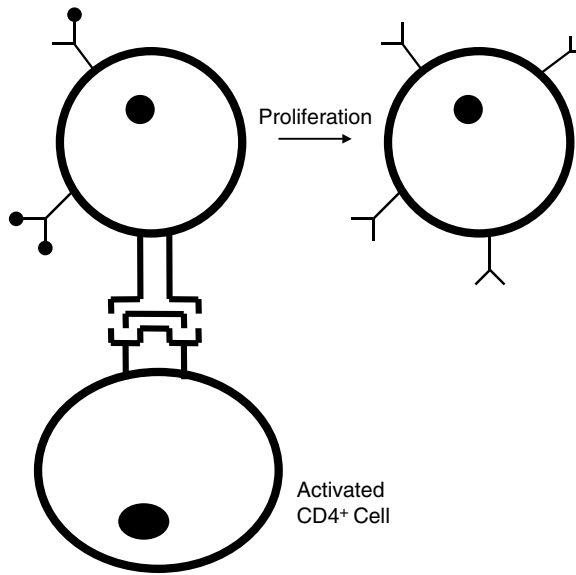
FIGURE 5.4 B cell activation and proliferation. Unlike APC, antigen presentation by B cells is specific. The antigen is captured by a modified antibody that presents on the surface of virgin B cells. The antigen-antibody complex is then internalized, and the antigen is processed. The degraded and processed antigen is then presented to activated CD4+ (helper T) cells by MHC class II proteins, analogous to APC. The contact between cells and helper T cells provides a key stimulus that leads to B cell proliferation and production of the antibodies.

are terminally differentiated and live primarily in the lymphoid organs for only three to four days. Some B cells are converted to B memory cells. They are long lived and remain in the circulation. They do not secrete antibody but retain cell-surface antibody as their specific antigen receptor and are primed to respond to subsequent exposure to the antigen.

5.2   STRUCTURE OF ANTIBODIES

The first structural studies on antibodies by Tiselius and Kabat suggested that the antibodies are least negatively charged γ-fraction of globulin (called immunoglobulin G, IgG) as they migrate very slowly towards the anode when immune serum is electrophoresed. However, two scientists, Gerald Edelman and Rodney Porter, were awarded the 1972 Nobel Prize in Medicine for their structural studies of antibodies. Edelman obtained two peaks on size exclusion chromatography of an IgG molecule that was treated with a reducing agent (dithiothreitol or mercaptoethanol) followed by an alkylating agent (such as iodoacetamide). He designated the large peak (50 kDa) as the heavy (H) chain and the smaller peak (23 kDa) as the light (L) chain. Since the molecular weight of the native IgG molecule is 150 kDa, he concluded that IgG consists of two heavy and two light chains (Figure 5.5).
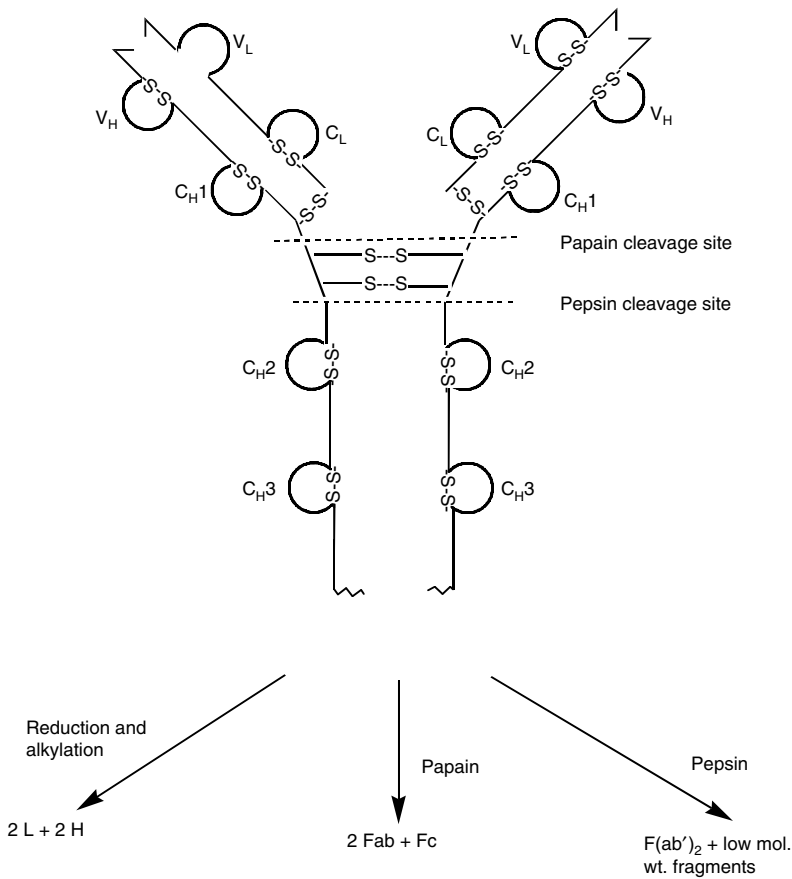
FIGURE 5.5 Schematic representation of immunoglobulin G and its chemical and enzymatic (papain and pepsin) cleavage.

On the other hand, Porter treated IgG with a proteolytic enzyme papain and produced three fragments of similar molecular weight (50 kDa). When subjected to ion-exchange chromatography, of three fragments, the first two were found to bind antigen, while the third one was unable to bind antigen, but was readily crystallized during cold storage. Porter called the first two fragments the Fab fragments (for fragments of antigen-binding) and the third one the Fc fragment (for fragment crystallizable).

Similarly, IgG when treated with pepsin yielded one large fragment of molecular weight 100 kDa and many small fragments. Since the large fragment retained its antigen-binding property and was two times heavier than the Fab fragment (characterized by Porter), the fragment was called F(ab′)$_2$. The two Fab fragments and Fc fragment give the antibody a Y shape, in which the stem of the Y is known as the hinge region. The hinge region allows segmental flexibility of the molecule. While Fab is responsible for antigen binding, the Fc region is involved in activation of the complement system and binding to cell surface receptors.

Further studies on the fine structure revealed that the amino acid sequence of the carboxyl-terminal end of either light chain or heavy chain of different antibodies (even from unrelated species) are almost identical. This region is called the constant (C) region. In contrast, the amino-terminal end of different antibodies shows great variability in amino acid sequence and thus is called the variable (V) region. In the light chain, the C-region (C_L) is about the same size as the V-region (V_L), but three to four times larger than the V-region in the heavy chain. The V-regions of both light and heavy chains actually provide antigen-binding sites. The most variable parts of the V-region in the light and heavy chains form three hypervariable regions or complementarity-determining regions (CDRs), which determine the specificity of the antigen-binding site. The comparatively invariant regions between the CDRs (about 85% of the V-region) are called frame work residues.

The antigenic determinants of antibodies show three levels of variability, called isotypic, allotypic, and idiotypic, and classify antibody molecules into isotypes, allotypes, and idiotypes, respectively. Five isotypes of antibodies are known based on their heavy-chain C domains. These immunoglobulins are called IgG, IgA, IgM, IgD, and IgE, each with distinctive heavy chain designated $\gamma, \alpha, \mu, \delta$, and ϵ, respectively. While there are five different types of heavy chains, there are only two types of light chains, κ and λ. Thus, isotypic determinants also can be used to classify antibody light chains into κ and λ chains.

Isotypic differences can be used to partition classes into subclasses. IgG has four subclasses, called $\gamma 1, \gamma 2, \gamma 3$, and $\gamma 4$ or IgG1, IgG2, IgG3, and IgG4; IgA has two subclasses designated $\alpha 1$ and $\alpha 2$ or IgA1 and IgA2. Allotypic determinants are carried out by only some individuals with a given species and are inherited in a Mendelian fashion. Idiotypic determinants are individual-specific and represent the antigen-combining site of an antibody. IgG constitutes about 80% of the antibody in serum. IgA, although constituting 13% of the antibody in human serum, is the major antibody in extravascular secretions. The IgA present in secretions (tears, saliva, nasal secretions, etc.), called secretory IgA, is a dimeric that is attached by a glycopeptide J chain and a glycoprotein called a secretory component (Figure 5.6). IgM is a pentameric polypeptide chain that makes up about 8% of the antibody in the serum. The five monomeric IgM molecules are arranged radially, the Fab fragments pointing outward and Fc fragments pointing to the center of the circle (see Figure 5.6). It is the first antibody to appear during an immune response and the first formed by a developing fetus. IgD remains membrane-bound and somehow regulates the activation of cells. IgE is found in trace amounts in the blood and triggers allergies.

5.3 ANTIGEN-ANTIBODY INTERACTIONS

In vivo antibody functions by combining with antigen. The interaction of an antigen with its corresponding antibody is very specific. The shape of the antigenic determinant must complement to the cleft or groove of the binding site in an antibody (Figure 5.7), and this fitting may be undestood best with the analogy of a "key" inside a "lock." The precise fit of an antibody (lock) with an antigenic determinant (key) indicates a high degree of specificity. An antibody directed against one epitope
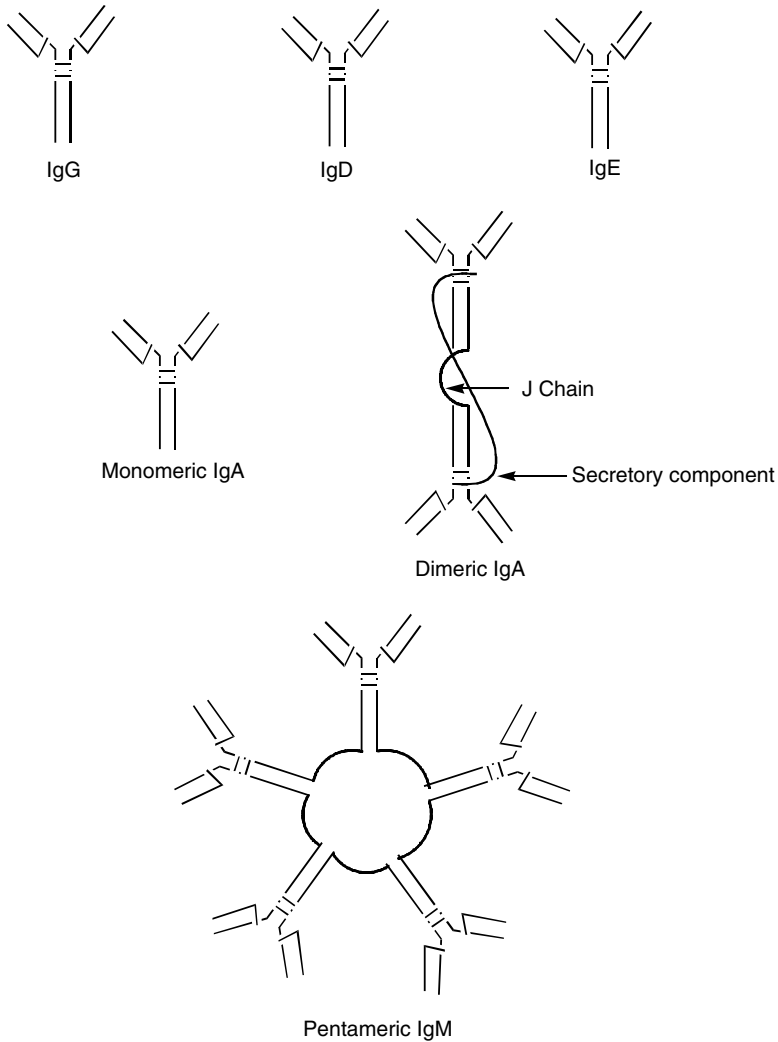
FIGURE 5.6 Several classes of immunoglobulins.

of the antigen is not complementary to epitopes of another antigen and thus fails to react with another antigen. If two different antigens share common epitopes, then an antiserum can recognize and bind these shared antigenic determinants; this phenomenon is called cross-reactivity. Antigen-antibody interactions are widely used in clinical and research applications.

Several non-covalent forces work to hold antigenic determinants within the binding groove of antibodies. These forces are coulombic (ionic or electrostatic) forces, Van der Waals forces, hydrogen bonding, and hydrophobic interactions. The strength of these forces between a single antigenic epitope and the single binding site of an antibody is known as antibody affinity. In contrast, the binding strength
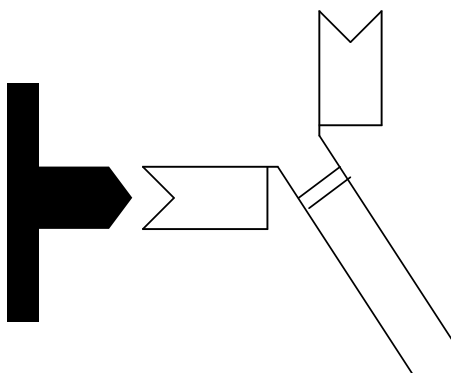
FIGURE 5.7 The antigen-antibody binding. The antibody reacts with antigen, analogous to a key matching a lock.

between a multivalent antibody and a multivalent antigen refers to avidity. Since the non-covalent binding between an antigenic epitope and antibody is reversible, the affinity can be calculated thermodynamically from the following reaction (called the intrinsic binding reaction):
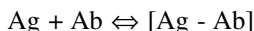
$$Ag + Ab \Leftrightarrow [Ag \text{ - } Ab]$$

where [Ag – Ab] is the concentration of complexed antigen, Ag is the concentration of free antigen, and Ab is the concentration of free binding sites of antibody at equilibrium.

Affinity constant K can be obtained as follows:

$$K = [Ag – Ab] / [Ag] [Ab]$$

The higher the value of K, the more affinity an antibody has for an antigen.

5.3.1 Two Types of Antigen-Antibody Interactions: Primary and Secondary

Interaction of antigen with antibody may be categorized into two stages. The first interaction of an antigenic determinant with its antibody is called a primary antigen-antibody reaction. The primary antigen-antibody reaction is generally rapid and invisible, but more sensitive than the latter stage. When the invisible primary antigen-antibody reaction is followed by an aggregation of antigen-antibody complexes into macroscopically visible clumps, the aggregation is called the secondary antigen-antibody reaction. Unlike the primary antigen-antibody interaction, the secondary antigen-antibody interaction is dependent on the electrolytes for maximum formation and requires hours to days to reach a maximum.
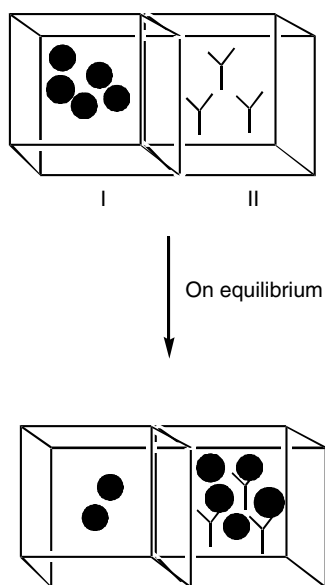
FIGURE 5.8 Equilibrium dialysis. Initially haptens are added in chamber I and antibody in chamber II. Two chambers are separated by a semipermeable membrane. With time only haptens are migrated through semipermeable membrane to chamber II and are bound by antibody molecules. Antibody molecules cannot migrate through the membrane. In equilibrium, the amount of free haptens in both chambers is the same; chamber II contains more haptens compared to chamber I because some remain bound to antibody molecules.

5.3.1.1 Equilibrium Dialysis: A Method to Measure Affinity of Primary Antigen-Antibody Interaction

Equilibrium dialysis is the classical and very simple method to measure the affinity constant of the antigen-antibody interaction. Because of the nature of this technique, the antigen should be small enough to be dialyzed. Equilibrium dialysis is accomplished in an apparatus having two chambers that are separated by a semipermeable membrane (Figure 5.8). Small molecules or haptens of known concentration are taken in one chamber of the dialysis apparatus and purified antibody into the other. During dialysis haptens are diffused across the semipermeable membrane, and an equilibrium is achieved. Theoretically, at equilibrium the free hapten concentration [H] is the same in both chambers, but the total [H] is higher in the antibody chamber because some hapten is bound to the antibody. Similar results are obtained if hapten and antibody are placed on the same side of the membrane and a buffer solution on the other side. At equilibrium, since the free [H] are the same on both chambers, the bound hapten $[H_{Ab}]$ can be calculated as follows:

$$[H_{Ab}] = [H_{total}] - 2\,[H_{free}]$$

From the equilibrium dialysis, it is possible to determine the antibody valence (number of combining sites) as well as the association constant by the following equation:

$$r/c = nK - rK$$

where r equals the number of moles of hapten bound per mole of antibody; c equals the number of moles of free hapten; and n equals the number of binding sites. A straight line curve is obtained when r/c is plotted as a function of r according to Scatchard[3] (Figure 5.9). Since the curve results in a straight line, n (where r/c = 0) can be obtained by extending the curve to abscissa. The intercept of the curve with the ordinate is equal to nK, while the slope of the equation equals to $-K_a$, where K_a is the association constant. Dissociation constant $(K_d) = 1/K_a$.

Scatchard analyses of equilibrium dialysis experiments on IgG and IgM produce straight line curves that produce antibody valence 2 and 10, respectively. This indicates that IgM is pentameric. Scatchard analyses of conventional antibodies usually do not produce a straight line, because antibodies produced by conventional immunization are not homogeneous. For heterogeneous antibodies, it is advisable to determine average K (called K_o), which is defined by $[H_{free}]$ required to occupy half of the antibody-combining sites. K_o equals the reciprocal of the $[H_{free}]$ when r is substituted by n/2 in the above equation (Figure 5.9).

5.3.1.2 Methods of Detecting Primary Antigen-Antibody Interactions: Enzyme-Linked Immunosorbent Assay, Immunofluorescence, Radioimmunoassay, and Fluorescence Quenching

The methods of detecting primary antigen-antibody interactions are usually sensitive and quantitative. Most methods require purified antigen or antibody and the ability to separate free antigen or antibody from antigen-antibody complex before detecting the interaction.

ELISA

Several methods are known to detect primary antigen-antibody reactions; of them, enzyme-linked immunosorbent assay (ELISA) is widely used. In this assay, bound antigen is detected by an antibody that is covalently coupled with a receptor enzyme such as peroxidase or alkaline phosphatase (Figure 5.10). Quantification of antigen-antibody binding is achieved by measuring the color intensity of the colored product generated by the enzyme and the added substrate. The intensity of the color is equivalent to the amount of the labeled antibody bound to the antigen. Direct detection of an antigen with the labeled antibody is not popular because of low sensitivity. In the indirect method, the antibody with specificity for the desired antigen is unlabeled, and a second enzyme labeled antibody with a specificity for the first antibody (an antibody to an antibody) is then added to the mixture
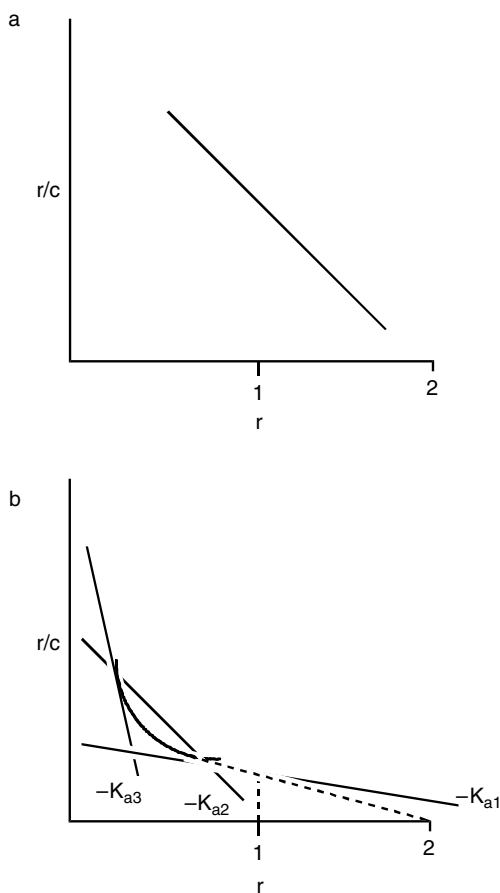
FIGURE 5.9 Scatchard Equation. (A) The plot of r/c versus r makes a straight line of slope $-K_a$; (B) the mixture of antibodies produces a curved line with an x-axis intercept of 2. The average association constant (K_o) for the mixture of antibodies is determined from the slope of this line at a concentration of antigen where 50% of the binding sites are occupied (at $x = 1$). If an individual antibody is purified from the above mixture and is tested, several straight lines of distinct slope ($-K_{a1}$, $-K_{a2}$, $-K_{a3}$) are produced.

(Figure 5.11). A detailed working procedure for ELISA will be described later (see Section 6.3.1). Several labeling techniques of antibodies and enzyme detection systems will also be described (see Section 6.1).

Immunofluorescence

Immunofluorescence is another method to detect a primary antigen-antibody reaction (Figure 5.12). It is widely used to detect or localize the antigen on the surface of intact cells or the internal antigen within tissue and cell sections. The most commonly used fluorochromes in immunofluorescence are fluorescein and rhodamine. These
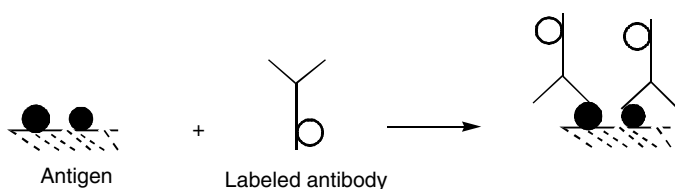
FIGURE 5.10 Direct binding. The antigen is absorbed onto a solid support such as plastic wells or membrane, and the absorbed antigen is directly detected by labeled antibody.
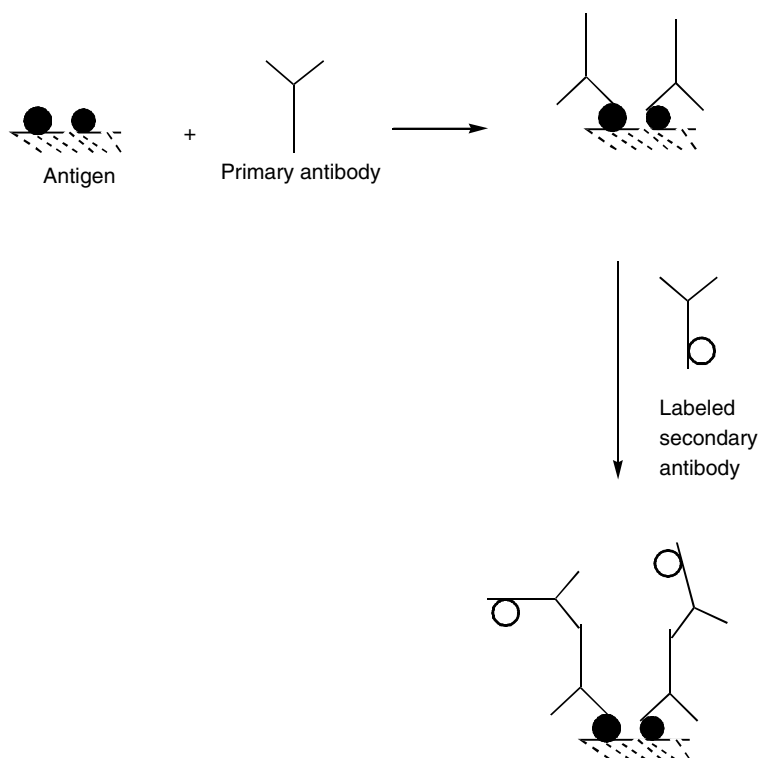
FIGURE 5.11 Indirect binding. The antigen is bound with its antibody followed by labeled secondary antibody that binds to primary antibody.

compounds emit fluorescence (visible light of characteristic wavelength) when they are irradiated with light of a different color (or shorter wavelength). Fluorescein's absorption maximum is at 490 to 495 nm, and emission with characteristic green color at 517 nm. Rhodamine emits its characteristic red color at 580 nm when excited at 490 to 495 nm (absorption maxima). The labeling of antibody with fluorochromes and their detection will be described later.
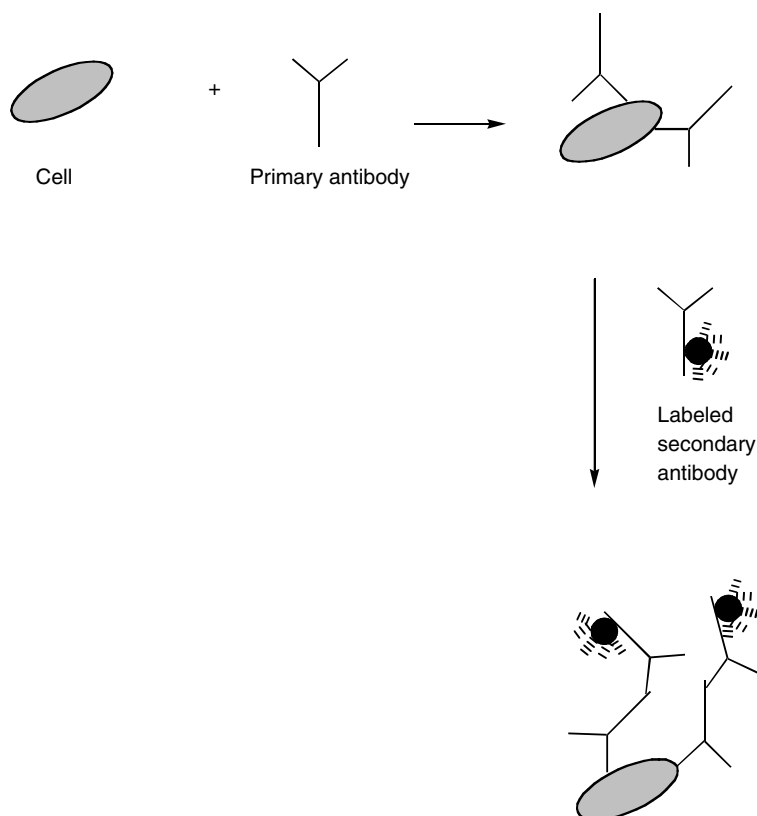
FIGURE 5.12 Immunofluorescence. Cell surface antigen is bound with primary antibody followed by fluorescent labeled secondary antibody.

RIA

Another very sensitive method, radioimmunoassay (RIA), was popularized in the 60s, but is becoming less attractive, probably because it is potentially hazardous and tedious. The assay was originally developed to detect trace amounts of peptides and hormones in plasma or biological fluids. In this assay, antibodies are immobilized and the radiolabeled (usually ^{125}I) and cold antigen are allowed to compete for binding to the immobilized antibodies (Figure 5.13).

Fluorescence Quenching

Another method to detect primary antigen-antibody reactions is fluorescence quenching. In this method, an antigenic determinant fits into an antibody-combining site to block fluorescence. The amino acids phenylalanine, tryptophan, and tyrosine, present in antibody, absorb ultraviolet at 280 nm. This absorption excites electrons from the ground state, resulting in emission of visible light at 350 nm (called
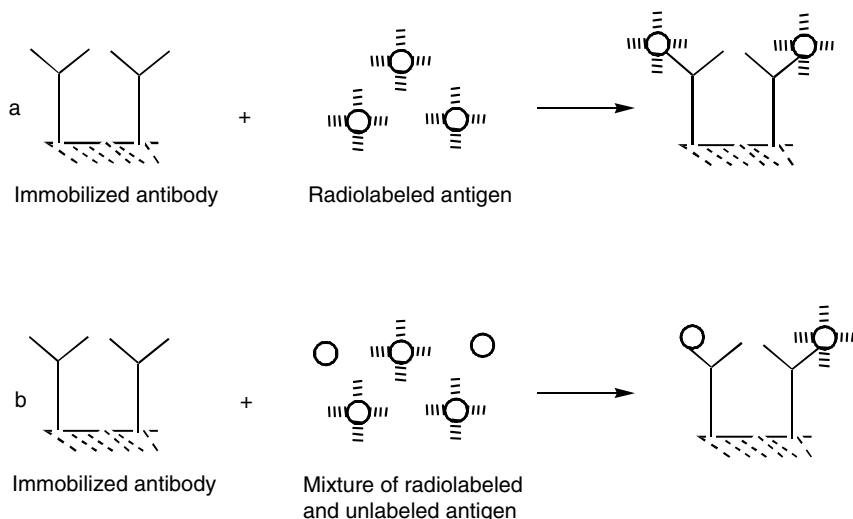
FIGURE 5.13 Radioimmunoassay direct binding (A); competitive binding (B).
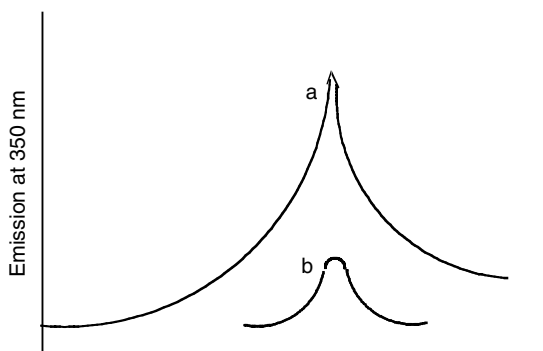
FIGURE 5.14 Quenching of antibody fluorescence with hapten. The emission spectra of antibody upon excitation at 280 nm (A). The emission is reduced upon addition of hapten to the antibody (B).

fluorescence). The amount of fluorescence can be measured as a function of the amount of hapten added to the antibody (Figure 5.14).

5.3.2 TWO TYPES OF SECONDARY ANTIGEN-ANTIBODY INTERACTIONS: PRECIPITATION AND AGGLUTINATION

Precipitation and agglutination are the two visible secondary antigen-antibody inter-actions. Precipitation occurs when a soluble antigen is combined with a specific antibody, leading to the formation of an insoluble aggregate. Agglutination is the combination of a particulate (insoluble) antigen (such as cells) with specific antibody,
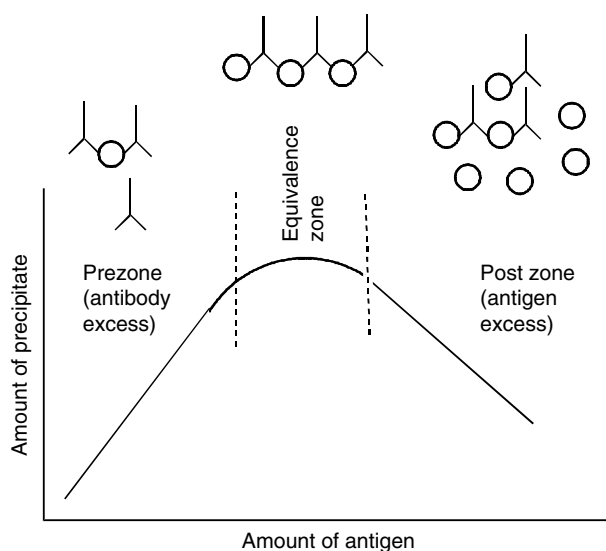
FIGURE 5.15 The precipitation curve shows the optimal precipitation of antigen-antibody complex in the equivalence zone. Reactions are performed by adding an increasing amount of antigen to a fixed amount of antibody.

leading to a clumping of particles. Aggregate formation is best explained by the formation of lattice, which is a network of alternating antigen and antibody molecules. The lattice theory is based on the fact that antigen and antibody molecules are multivalent. Maximum precipitation is achieved in the presence of salts. Salts (or electrolytes) promote hydrophobic protein-protein interactions, resulting in a decrease of protein-water interactions. The fact that the precipitation reactions are reversible is explained by zone phenomenon (Figure 5.15). It divides the precipitation reaction into three zones: (a) antibody excess (prezone), (b) equivalence zone, and (c) antigen excess zone (post zone). In the equilibrium zone, all antigen and antibody molecules are in the lattice, leading to maximum immune precipitation. In the prezone, the lattice has a high ratio of antibody molecule to antigen. The precipitate formed is less than maximum, since not all antibody molecules are able to interact with antigen. In the post zone phenomenon when antigen is present in excess, the ratio of antibody to antigen in the lattice falls below a threshold. Antigen-antibody complexes become small, and the lattice begins to solubilize and thus the precipitation is less than maximum.

5.3.2.1 Common Methods for Detecting Precipitation Reactions in Gels: Immunodiffusion and Immunoelectrophoresis

Immunodiffusion

Precipitation reactions can be tested in several ways either in fluid or gel — but in all reactions, optimal lattice formation occurs in the zone of equivalence. The double
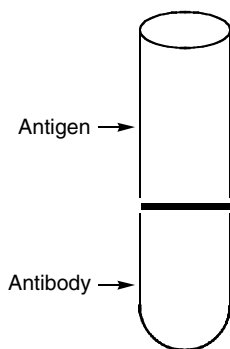
FIGURE 5.16 Double diffusion in a tube. Antigen solution is carefully layered over an antibody solution. Antigen and antibody diffuse and form a visible band at the interface.
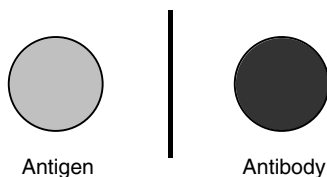
FIGURE 5.17 Double diffusion of antigen and antibody. Precipitation occurs at the equivalence point. In this case the precipitation is in the form of a line in the gel.

diffusion method in a capillary tube is one of the classical tests of fluid-phase precipitation. In this method an antigen solution is carefully layered over an antibody solution, and these are allowed to diffuse toward each other without mixing the solution. Antigen and antibody react at the interface, leading to the formation of a visible band or a cloudy opaque ring (Figure 5.16). The procedure is qualitative; nevertheless, it is a very simple procedure to detect antigen-antibody reaction very quickly.

Ouchterlony double diffusion and immunoelectrophoresis are widely used methods of precipitin reactions in gel. In the Ouchterlony method, antigen and antibody are placed on two separate wells in 0.5 to 1% agarose gel. They diffuse toward one another, and precipitin lines form where antigen and antibody meet in equivalence zone proportions, leading to an opaque white precipitate in a transparent background (Figure 5.17). In the Ouchterlony method, diffusion rates of antigens depend on their concentration and on the intrinsic properties of the antigens such as size and shape. The qualitative comparisons of antigens, such as whether two antigens are the same, somewhat related, or distinct, are obtained also from the Ouchterlony method (Figure 5.18). Since both antigen and antibody diffuse to meet and precipitate, this method is also known as double diffusion. In radial immunodiffusion technique, a monospecific antiserum to the liquified gel (after pouring into a plate) is allowed to solidify by cooling to room temperature. The wells are cut into the agar, and antigens are added. Since the antigen diffuses in all directions from the well, a ring is formed
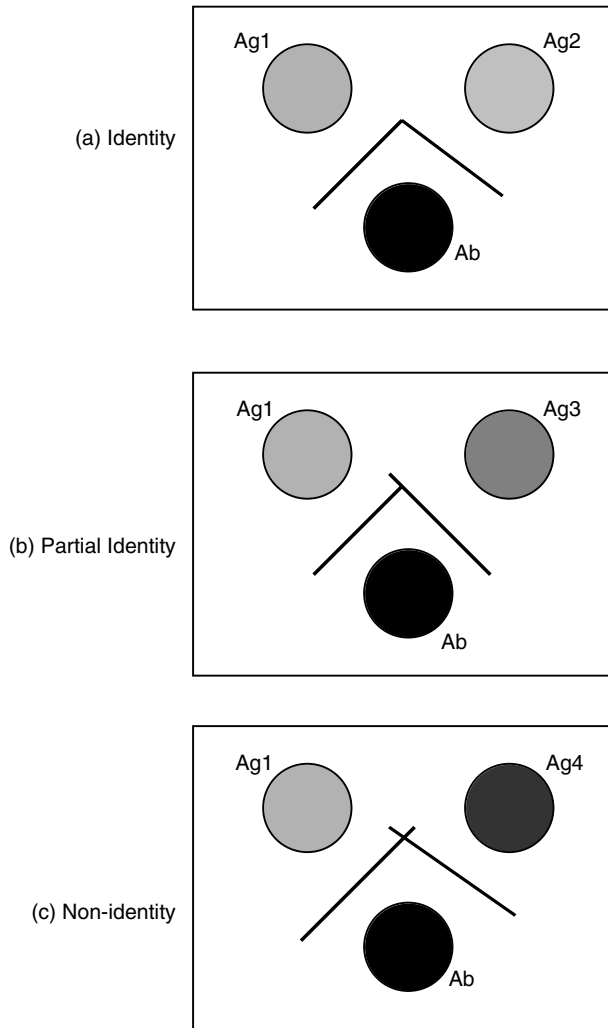
FIGURE 5.18 Double diffusion that shows identity (A), partial identity (B), and non-identity (C). In (A) both antigens (Ag1 and Ag2) are identical since two precipitin lines meet and stop. In (B) two precipitin lines meet, but one crosses beyond the meeting point, indicating that antigen 1 and antigen 3 are partially identical. In (C) both precipitin lines cross beyond the meeting point, indicating that antigens 1 and 4 are not identical.

close to antigen-antibody precipitation (Figure 5.19). Since only antigen diffuses, this is also called single diffusion. The diameter of the ring can be used to determine the concentration of an unknown sample from a standard curve of the same antigen of known concentration. Diameters of rings are measured after one of two incubation times: kinetic diffusion or endpoint diffusion. In kinetic diffusion, the diameter of the rings is measured at 18 h and plotted in x-axis. Y-axis represents the logarithm of the concentration of the standard antigen (Figure 5.20 A). The concentration of
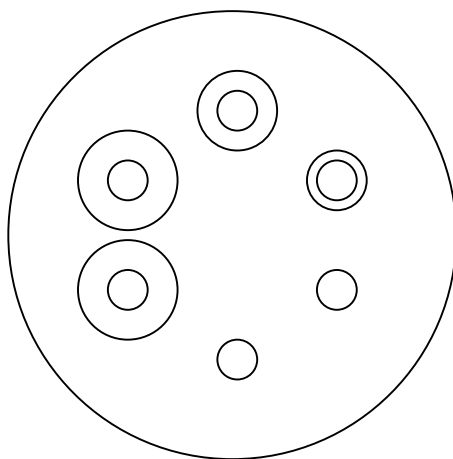
FIGURE 5.19 Radial immunodiffusion. The known volume of antigen with varying concentration is loaded on wells cut into a gel that contains an antibody.

the antigen in an unknown sample is obtained from a straight line curve. In the endpoint method, the diameter of the precipitation ring is measured at 72 h or at a predetermined time where maximal precipitation is achieved. In this method the square of the diameter of the ring is plotted against the concentration of the antigen on a linear graph paper; from the straight line curve, the concentration of the antigen in an unknown sample is determined (Figure 5.20 B).

Working Procedure for Immunodiffusion in Solutions

1. Add 100 µl of twofold serially diluted antigen solution (1 mg/ml to 1 µg/ml in phosphate buffered saline) in microfuge tubes.
2. Add 100 µl of a fixed concentration of antibody solution (100 µg/ml) to each tube.
3. Incubate all tubes at 37°C or at room temperature overnight.
4. Centrifuge to isolate precipitate.

Working Procedure for Ouchterlony Method

1. Prepare 1.2% agarose in saline (or in PBS) and after boiling pour the gel on a glass petri dish.
2. After solidification, cut out gel to make wells as in Figure 5.18.
3. Add antigen (10 to 20 µl of 1 to 5 mg/ml) in one well and antibody (1 to 5 mg/ml) in another well.
4. Incubate the plate at room temperature for 24 to 72 h or till a precipitin line appears.
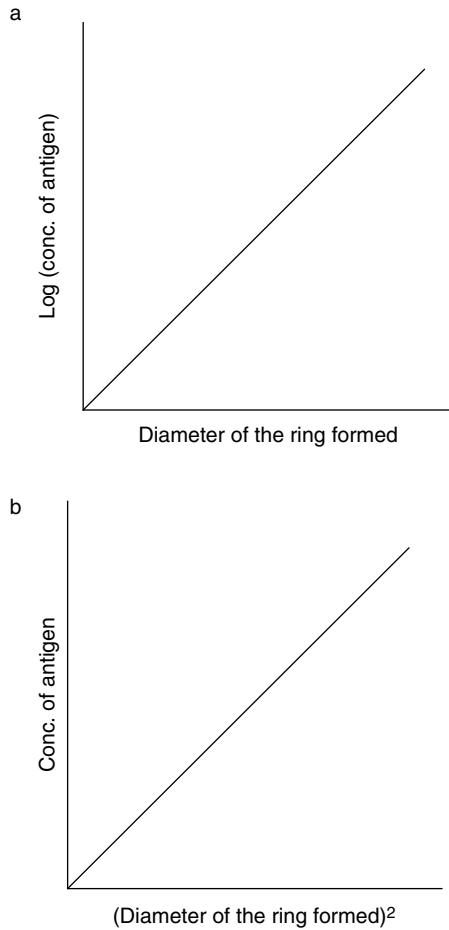
FIGURE 5.20 Determination of antigen concentration by two methods: kinetic diffusion (A) and end point (B)

Immunoelectrophoresis

In immunoelectrophoresis, antigens are first electrophoresed (to separate by charge) and allowed to diffuse (Figure 5.21). After electrophoresis, the antibody is placed in another trough and allowed to diffuse, to form precipitin arcs at the zone of equivalence between the antigen and the specific antibody. This is advantageous over Ouchterlony diffusion, because complex antigen-antibody systems are difficult to interpret in the Ouchterlony method.

Rocket technique is another electrophoretic technique to quantitate antigen. This has been developed mostly to avoid the lengthy and time-consuming radial immuno-diffusion. It is essentially similar to radial immunodiffusion, which uses electro-phoresis to migrate antigen. Like radial immunodiffusion, the antibody is incorpo-rated into the gel except that the agar must be in a buffer capable of carrying an
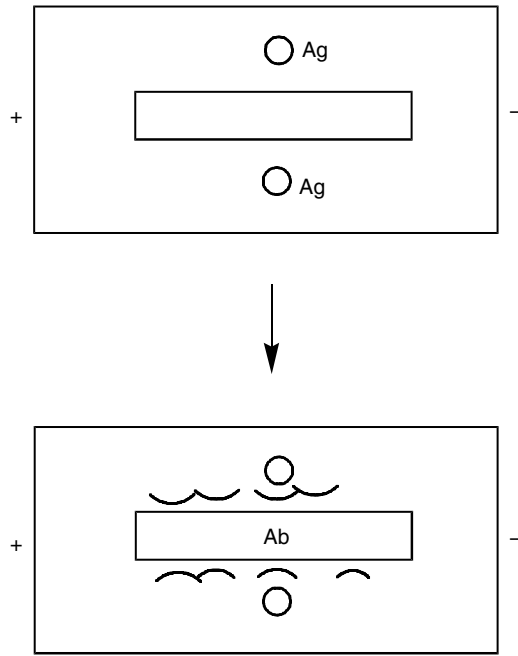
FIGURE 5.21 Immunoelectrophoresis. Antigens are electrophoresed in an alkaline buffer solution (usually at pH 8.5), and after electrophoresis antibody is added in the trough cut parallel to the migration of electrophoresed antigens. Antigens and antibody diffuse and precipitate in the form of an arc in the equivalence zone.
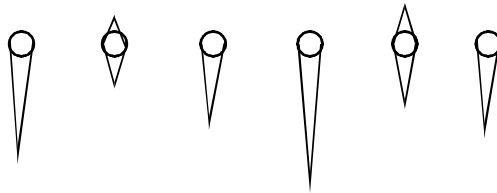
FIGURE 5.22 Rocket immunoelectrophoresis. The wells are cut into an agar gel containing a specific antibody. Proteins (antigens) are loaded into the wells and electrophoresed at pH 8.6 conditions, under which antibodies do not usually migrate, but most proteins move towards the anode; resulting in precipitation in equivalence zone in the form of a rocket or peak. The size of the rocket is proportional to the original concentration of the antigen.

electric current, usually at pH 8.6. At this pH, the migration of antibodies is minimum. But the test proteins (antigens) migrate and move towards the equivalence zone by electrophoresis, thereby resulting in the precipitin lines characteristic as peaks or "rockets" in the gel (Figure 5.22). The quantitation of an unknown sample may be obtained from the calibration curve constructed by plotting peak height versus concentration of antigen.

Working Procedure for Immunoelectrophoresis

1. Prepare 1.2% agarose solution in 0.075 M barbital buffer II, pH 8.6 (BioRad) and after boiling pour the gel on a film to solidify.
2. Cut out the gel to make wells as in Figure 5.20.
3. Add antigen solution (1 to 5 mg/ml in barbital buffer II) in the circular well and electrophores at constant current (0.7 Amp). Electrophoresis is usually performed in an isoelectric focusing unit.
4. After electrophoresis, add antibody in the rectangular well and incubate to allow diffusion of antigen and antibody to form precipitin line.

5.3.2.2 Two General Types of Agglutination: Direct and Indirect

In contrast to precipitation (which is quantitative), agglutination methods are qualitative or semiquantitative. Agglutinations are used in many applications because they have a high degree of sensitivity. Agglutination occurs when antibodies react with an antigen that is attached on the surface of a large particle (e.g., cells or bacteria), causing the cells or bacteria to clump. Agglutination reactions are two types: direct and indirect. When antigenic determinant is a normal constituent of the particle surface (such as bacterial cell surface or human blood cells), then the agglutination is called direct agglutination. Hemagglutination is one type of agglutination where the particle is a red blood cell (RBC). In direct agglutination, an antigen molecule that is originally soluble is attached to a particle and rendered insoluble. The attachment of a soluble antigen to a particle surface makes a change from precipitation reaction to agglutination reaction. When the antibody is attached to a particle and the agglutination occurs with a specific antigen, the procedure is called indirect agglutination. Agglutination procedures are performed in several ways such as in slide, tube, and microtiter wells. Figure 5.23 shows agglutination. Hemagglutination is applied for blood typing. In this case the target antigens are on the surface of red blood cells, and antibodies against antigen cause the clumping reaction.

Working Procedure for Agglutination

1. Add 25 µl of twofold serially diluted antibody solution to each well of a 96-well (U-shaped) plate.
2. Add 25 µl of 1% cell suspension to each well and incubate the plate for 1 h at room temperature to observe agglutination (clumping of cells).

5.4 PRODUCTION OF ANTIBODIES

Polyclonal antibodies are heterogeneous populations of antibodies that are produced by various cell lines (or clones) of B lymphocytes in response to various epitopes of the same antigen. Production of antibodies with high titer and specificities is
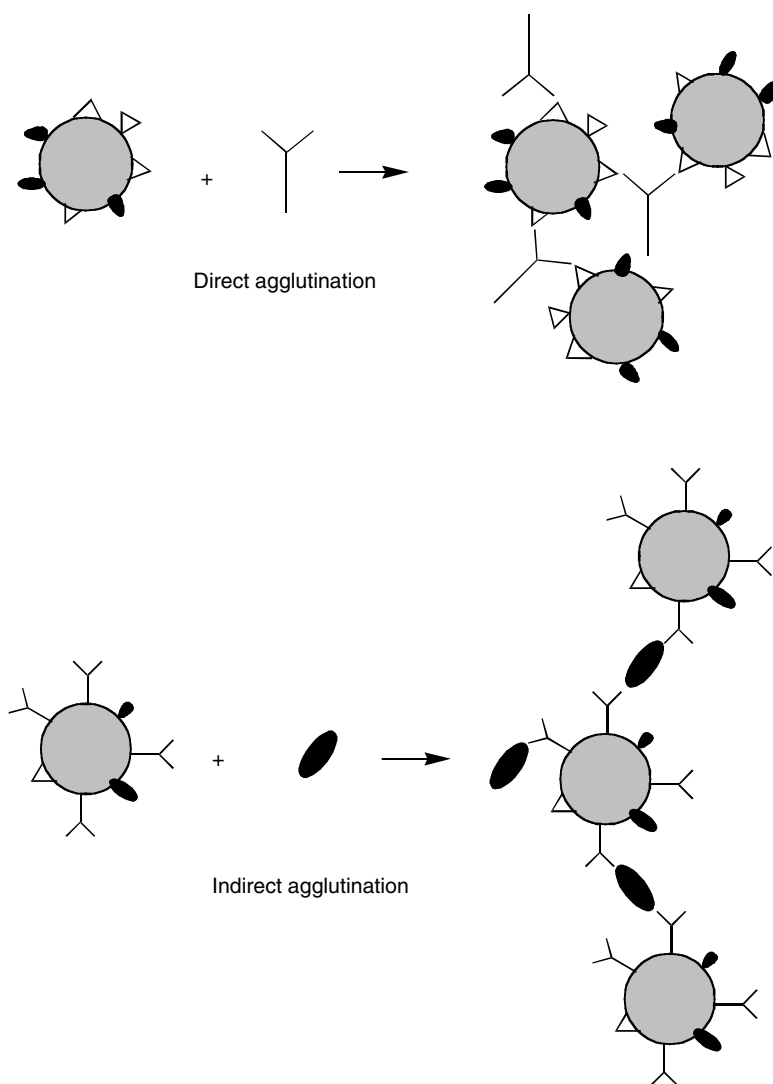
FIGURE 5.23 Agglutination: direct and indirect. Direct agglutination occurs when an antibody attaches an immobilized antigen. In indirect agglutination, an antigen binds to an immobilized antibody.

partly dependent on the type of antigens, stimulants such as type of adjuvants, the route of entry, and number of injections.[4,5] All procedures regarding immunization of animals such as injection of immunogen and bleeding of animals require skill, patience, and practical training. The care and maintenance of laboratory animals must meet the guidelines of the Institutional Animal Care and Use Committees (IACUC).

5.4.1 IMMUNOGENS MUST HAVE A FEW CHARACTERISTICS, SUCH AS FOREIGNNESS, HIGH MOLECULAR WEIGHT, CHEMICAL COMPLEXITY, AND SOLUBILITY

In order to produce an antibody, an antigen must be immunogenic. Immunogen differs from antigen in a way that the former can trigger an immune response and interact with the sensitized cells and antibody produced, while the latter can bind with the antibodies, but is not capable of producing an immune response. So, all immunogens are antigen, but not all antigens are immunogens. A good immunogen contains three intrinsic characteristics: 1. It must have an epitope that is recognizable by the B cell surface antibody molecule. 2. After processing, the degraded immunogen must offer at least one site that can be recognized simultaneously by an MHC class II protein and by a helper T cell receptor. 3. Immunogen must be degradable.

In order to be immunogenic, a substance must have four characteristics: foreignness, high molecular weight, chemical complexity, and solubility.

Foreignness

For immunogenic response the antigen should look foreign to the host animal. For example, mammalian serum proteins are stronger immunogens to rabbits, but poor in closely related species. Generally, compounds that are part of an animal are not immunogenic to that animal.

High Molecular Weight

Large Molecules

Large molecules, because of their higher degree of conformation and structural rigidity, usually produce a stronger immunogenic response. Polysaccharides, because of their complex carbohydrate structures, are usually very good immunogens. Various homopolymers of amino acids, although they are large molecules, are not sufficiently chemically complex, and so they are not good immunogens. Lipids usually are not immunogenic, but can be immunogenic if they are conjugated to carrier protein.

Small Molecules (Haptens) Coupled to a Carrier Protein

Haptens are small molecule antigens that can bind to an antibody, but cannot elicit an adaptive immune response. Haptens must be chemically linked to protein carriers to initiate an immune response. So, a hapten is antigenic, but not immunogenic by itself. Since an immunogen must have an epitope or antigenic site and a class II T cell receptor binding site, there is a minimum size necessary for a molecule to be an immunogen. Natural immunogens usually have a molecular weight greater than 5 kDa.[5] Small peptides, glycopeptides, and carbohydrates are some examples of haptens.[6] The most commonly used carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA).[6] Other carriers include *Maia squinada*

hemocyanin (MSH), thyroglobulin, and egg albumin. KLH, usually obtained from arthropods and mollusca, contains many available lysine groups for effective conjugation. BSA, although it is much smaller than KLH, also contains about 50 lysine residues and is used to couple with small molecules.

For the specific detection of haptens by immunoassays of anti-hapten-carrier protein antibodies, a non-relevant protein should be used for conjugating and coating haptens onto microtiter wells. For example, BSA may be used as a non-relevant protein in enzyme immunoassays of anti-hapten-KLH antibodies. Since antibody response will be directed against the carrier protein as well as the hapten, hapten-KLH conjugates cannot be used in microtiter plates because of its interference with the measurement of anti-hapten antibodies. So, when KLH is used as a carrier, hapten-BSA can be used in microtiter plates because they do not interfere with the measurement of anti-hapten antibodies (Figure 5.24). Haptens can be conjugated to carrier proteins in several ways, by using homobifunctional and heterobifunctional cross-linkers. These procedures and chemistries are described in Chapter 6 (see Section 6.1.4). Several immunogen conjugation kits are also available from Pierce. It is necessary to obtain conjugates that contain high hapten content to increase the antigenic response.

Chemical Complexity

Immunogenic molecules must have some degree of chemical complexity, because large substances such as nylon or polyacrylamide with repeating units of a single amino acid are not immunogenic.

Solubility

Immunogens must be biodegradable. Cells must interact with antigens and be degradable in order to enhance the immunogenecity of the antigenic substance.

5.4.2 ADJUVANTS ARE NON-SPECIFIC STIMULATORS OF IMMUNE RESPONSE AND WIDELY USED WITH IMMUNOGEN

Adjuvants, the non-specific stimulators of the immune response, are widely used for routine production of antibodies in animals. There are several types such as bacterial derivatives, aluminum salts, vehicles, and surface active agents. Antigens are mixed with adjuvants and administered to the recipient animal. Adjuvants potentiate antibody production by increasing the efficiency of antigen presentation for a long period of time, by protecting the antigen from rapid removal and breakdown.[7,8] In addition to adjuvants' ability to release antigen slowly from the depot at the injection site, adjuvants may also target antigens to antigen-presenting cells, such as Langerhans' cells, follicular dendritic cells, and B cells.
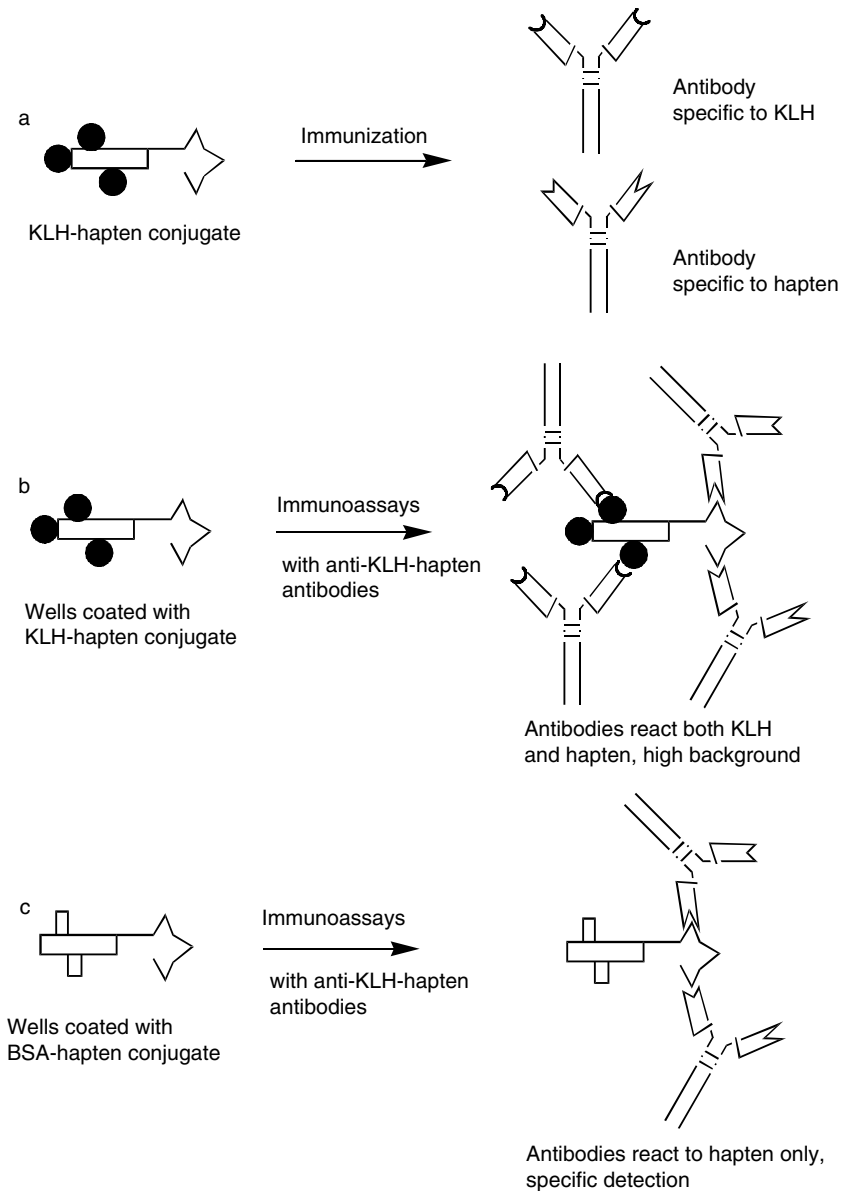
FIGURE 5.24 Immunization of KLH-hapten conjugate (A) and immunoassays with anti-KLH-hapten antibodies (B, C). When an animal is immunized with KLH-hapten conjugate, antibodies are made against both hapten and KLH. (A) For the specific detection of haptens by immunoassays, antibodies are tested with a different conjugate that is made with haptens and an unrelated protein (usually BSA). (C) Immunoassays on the same conjugate result in high background because antibodies bind to both hapten and KLH (B).
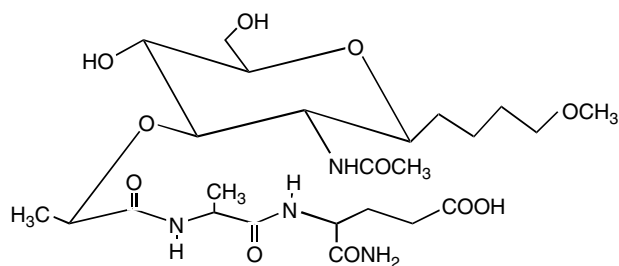
FIGURE 5.25 Muramyl dipeptide. It is the smallest active compound in Freund's complete adjuvant and can be used to replace *M. tuberculosis* in Freund's complete adjuvant.

Bacterial Derivatives: Freund's Complete Adjuvant and Bordetella pertusis

Among the bacterial derivatives class of adjuvants, Freund's complete adjuvant is used the most. This consists of a mixture of heat-killed *Mycobacterium tuberculosis*, mineral oil (Bayol F), and an emulsifier Arlacel A. Antigens in solution are emulsified with these components to produce an antigen/water microdroplet phase within the oil phase.[5] When injected intramuscularly, emulsion forms a depot of immunogen that slowly becomes available to recipient cells. Mycobacteria in emulsion stimulate cells of the immune system at local lymph nodes. Freund's adjuvant is most commonly used and is one of the best adjuvant for stimulating strong and prolonged responses. Sometimes it may result in side effects (granuloma). To avoid the side effect, only the primary injection is given in complete Freund's adjuvant, while all boosts are given in incomplete Freund's adjuvant, which lacks *M. tuberculosis.*

Muramyl dipeptide, N-acetylmuramyl-L-alanyl-D-isoglutamine (Figure 5.25), is shown to be the smallest active compound in Freund's complete adjuvant and can be used to replace *M. tuberculosis* in Freund's complete adjuvant.[9]

Bordetella pertusis and lipopolysaccharide from *Esherichia coli* are the other bacterial derivatives that are used in injections with antigen to potentiate antibody response.

Working Procedure for Preparation of Antigen with Freund's Complete Adjuvant

1. Shake well before using Freund's adjuvant, as mycobacteria may settle upon storage. Mix protein antigens, preferably in saline, with an equal volume of the adjuvant oil and an emulsion using one of the following methods: vortex, two syringes, and tissue homogenization.
2. For a small volume, vortex the mixture of antigen and adjuvants vigorously until a thick emulsion develops. For an intermediate volume, take equal volume of the antigen and the adjuvant into two different syringes to fill approximately one half of the syringe capacity. Remove all air and connect the syringe through the luer fitting, as shown in Figure 5.26 A. Alternately,
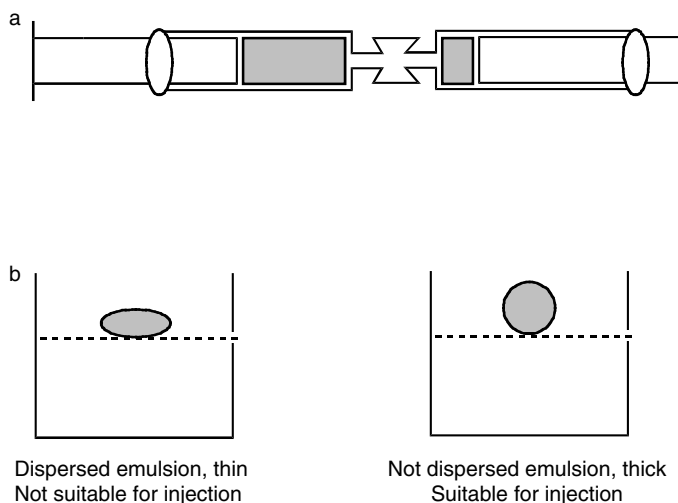
FIGURE 5.26 Preparation of adjuvant. Equal volumes of antigen and adjuvant are taken into two syringes separately. Syringes are connected and by pushing the plungers alternatively, the adjuvant and antigen are mixed until a thick emulsion is formed (A). The emulsion is ready for injection if it does not disperse when placed on the surface of water or a saline solution (B).

push the plungers, mixing the adjuvant and immunogen solution until the mix forms an emulsion (at that point syringe plungers are difficult to push). For a large volume, take the adjuvant and homogenize for a short period. Then add aqueous immunogen and homogenize again until a thick emulsion develops. The emulsion in all methods should be very thick and not disperse when a drop of it is placed on the surface of a saline solution (Figure 5.26 B). Thick emulsion is now ready for injection.

Vehicle

Freund's incomplete adjuvant and liposomes are the vehicle type adjuvants. Freund's incomplete adjuvant is commonly used for booster immunizations to a recipient animal. Like the Freund's complete adjuvant, water phase immunogen can be emulsified with this adjuvant.[5]

Aluminum Salts

Alum (aluminum potassium sulfate, $AlK(SO_4)_2.12H_2O$), the most popular aluminum salt, is sometimes used at alkaline pH to replace Freund's adjuvant to avoid harmful side effects. At alkaline pH, alum produces aluminum hydroxide, which can absorb antigen. When injected, the precipitate provides the depot effect and prolongs antibody response.

Working Procedure

1. To 10 ml of 10% potassium alum, add 22.8 ml of 0.25 N NaOH dropwise with stirring.
2. After incubation at room temperature for 10 min, centrifuge the precipitate at 1,000 g for 10 min.
3. After removing the supernatant, add 50 ml of water to the aluminum hydroxide pellet and resuspend. Allow antigen to bind with aluminum hydroxide (1 mg of aluminum hydroxide will bind approximately 50 to 200 µg of protein antigen).
4. After incubation at room temperature for 20 min, centrifuge at 10,000 g for 10 min. Collect the supernatant that contains antigen bound aluminum hydroxide and use for injection.

Surface Active Agents

Surface active agents such as saponins or dimethyl dioctadecyl ammonium bromide are used to produce a number of veterinary vaccines.

5.4.3 CHOICE OF ANIMAL DEPENDS ON SEVERAL FACTORS, SUCH AS AMOUNT AND TYPE OF ANTISERUM (POLYCLONAL OR MONOCLONAL) NEEDED

Several animals such as rabbits, mice, rats, hamsters, guinea pigs, chickens, horses, and sheep are used for the production of antisera. The selection of animal depends on the amount of serum needed, source and availability of antigen, and type of antiserum (polyclonal or monoclonal). Rats and mice are used for the production of monoclonal antibodies. They are not suitable for the production of polyclonal antibodies, because 40 to 50 ml of antiserum is usually desired for most immunological procedures, and a single bleed from mice and rats will yield only 0.1 to 0.2 ml and 1 to 2 ml of antiserum, respectively. Hamsters and guinea pigs are also not popular for polyclonal antibodies, but sometimes they are used for a limited antigen. For most purposes, rabbits are a good choice for the routine production of polyclonal antibodies, because 30 to 50 ml of serum is conveniently obtained from a single bleed, and the animals are relatively easy to maintain. Horses, donkeys, sheep, goats, and pigs are used commercially when large volumes of antibodies are needed. Since the immune systems have the property of self-tolerance, which protects the animal from auto-immune damage, rabbit serum albumin, for example, is not immunogenic to rabbits but is to goats. In practice, immunizations are performed in animals that are not evolutionarily related to the source of antigen. For highly conserved mammalian antigens that may be weakly immunogenic to mammals, chickens are a good choice.

5.4.4 PREPARATION OF ANTIGENS

Before starting antigen preparation for the immunization, one should consider how the antibodies will be used or if a monoclonal antibody will finally be developed.

If the final aim is to make a monoclonal antibody, then further purification of the antigen may not be warranted. If the aim is to make polyclonal antibodies that should recognize specifically to antigen, then the antigen must be purified to homogeneity.

The purified antigen can be obtained from standard purification techniques, such as ion-exchange and size exclusion chromatography. However, these time-consuming and labor-intensive procedures can be avoided if the desired protein is needed for the purpose of immunization only. One simple method to obtain a pure antigen is to separate the crude sample on SDS-polyacrylamide gels and electroelute the desired proteins. In that case, a tool must be available to identify the desired protein on a gel by employing its activity assay, or the protein of interest on the gel must be recognized based on its known molecular weight.

5.4.4.1 Preparation of Antigens from Polyacrylamide Gels

Following electrophoresis, the protein band of interest is located on the gel and the gel slice excised for immunization. If the desired protein is abundant and well separated from the contaminating protein band, it is a good idea to stain a strip of the gel cut from the side. The stained band is then aligned with the unstained gel to locate the protein of interest and excise the gel slice. In this way, the fixation of the desired protein can be avoided. If the desired protein is not abundant or not well separated from the contaminating protein, the protein band can be visualized by immersing the gel in sodium acetate[10] or copper chloride[11] (see also Chapter 3 for additional stains). The sensitivity of the sodium acetate stain is about 1 to 2 µg/band. The copper chloride stain can detect as low as 0.1 µg/band on 0.5 mm gel.

Working Procedure for Sodium Acetate Stain

1. After electrophoresis, wash the gel briefly with water (several changes).
2. Incubate the gel with 4 M sodium acetate (10 volumes) for 40 to 80 min with gentle shaking.
3. View bands against black background and excise the protein band of interest with a razor blade or scalpel.
4. Re-equilibrate the excised gel with the elution buffer if the sample is to be made gel free by electroelution.

Working Procedure for Copper Chloride Stain

1. After electrophoresis, wash the gel briefly with water (several changes).
2. Incubate the gel with 0.3 M $CuCl_2$ for 5 min with gentle shaking.
3. View bands against black background and excise the band of interest with a razor blade or scalpel.
4. If the sample is to be made gel free by electroelution, destain the excised gel with 0.25 M EDTA, 0.25 M Tris (pH 9.0) (three changes, 10 min each) and re-equilibrate with elution buffer.

5.4.4.1.1   Processing of the Excised Gel for Immunization

For injections, the gel slice can be processed in several ways. 1. The gel slice is minced by passing it repeatedly through a syringe, and the animal is immunized with the gel slurry. 2. The gel slice can be dried and ground into powder. 3. The protein sample is electroeluted from the gel. 4. The protein sample is electrotransferred to a suitable membrane, and then the membrane is processed for injection. However, the first two approaches are used in large animals, such as rabbits, and are not suitable for mice or other small animals.

Working Procedure for Electroelution of Protein Sample from Gel[12]

1. Add 10 ml of 0.2 M Tris/acetate (pH 7.4), 1% SDS, 10 mM dithiothreitol per gram of wet polyacrylamide gel and transfer the gel and buffer to a dialysis tube (6 to 8 kDa MWCO). The MWCO size of the dialysis membrane should be at least 4 to 5 kDa less than the MW of the protein to be electroeluted in order to retain the protein inside the dialysis bag.
2. Submerge the dialysis bag in a horizontal electrophoresis chamber containing electrophoresis running buffer (50 mM Tris/acetate, pH 7.4, 0.1% SDS, 0.5 mM sodium thioglycolate) and run at 100 volts.
3. After 2 h, remove the gel by opening one end of the dialysis tubing and reclose. Dialyze the eluted protein with 0.2 M sodium bicarbonate, 0.02% SDS. Gel can be stained with Coomassie Blue to ensure the complete transfer of the protein.
4. Collect the protein sample from the dialysis bag and lyophilize.
5. Resuspend in 0.1 volume of the original gel volume in water for immunization.

Working Procedure for Electrophoretic Transfer of Protein to Membrane

1. After electrophoresis, transfer proteins to nitrocellulose membrane by Western blot method as previously described (see Chapter 3).
2. Stain the nitrocellulose paper with India ink or Ponceau S as previously described (see Chapter 3).
3. Identify the band of interest and excise by a razor blade.
4. Cut the excised membrane into very fine pieces and sonicate with 200 µl of PBS for 10 bursts at 10 sec. Cool between sonication steps.

5.4.5   Example of Immunization Schedule

The following procedure is an example of an immunization schedule and is not necessarily the best procedure. Researchers should intervene and tailor the response to a particular antigen by modifying the dose of the antigen, the use of adjuvants, the route and number of injections, and the time between injections.

1. For each rabbit, mix 0.5 to 1 ml of native antigen solution (50 to 100 μg in saline) with equal volume of Freund's complete adjuvant until an emulsion is formed and inject subcutaneously under rabbit's neck.
2. On day 14, repeat injection with a mixture of equal amount of native antigen and its reduced and alkylated form with Freund's complete adjuvant, but at leg muscle.
3. On day 21, bleed the rabbit (about 5 to 10 ml) and test for immune response. (Antibody response can be examined by several ways, such ELISA, dot blot, and Western blot. See Section 6.3.1 for these procedures.)
4. On day 28, repeat injection with reduced and alkylated antigen at leg muscle, but with incomplete adjuvant.
5. On day 35, bleed the rabbit (10 to 20 ml) and test for antibody titer.
6. On day 42, bleed the rabbit (30 to 40 ml).
7. On day 49, bleed the rabbit (30 to 40 ml) and repeat injection, if necessary, with incomplete adjuvant.

5.4.6 POTENTIAL PROBLEMS AND REMEDIES

Some antigens may not be immunogenic. If no response is detected after three or four injections, several alternative approaches should be considered. Table 5.1 lists some causes of no antibody response and their possible remedies.

5.4.6.1 Modification of Antigens for Strong Immune Response

Many compounds are not able to elicit strong antibody response. These compounds can be made strongly immunogenic with small changes in their structures. The common methods for making weak antigens strong include the denaturation of the antigen by treating with heat and/or SDS. Other methods include the addition of small modifying groups, such as dinitrophenol or arsenate to the molecules, coupling the antigens with small synthetic peptides that are good sites for T cell receptor and

TABLE 5.1
Causes of No Antibody Response and Their Possible Remedies

Cause	Remedy
No class II protein binding site, therefore no antigen fragment presentation	Conjugate with class II binding site. Try other animals.
No T cell receptor binding site	Conjugate with T cell receptor binding site. Try other animals.
Small size	Conjugate with carrier protein (e.g., KLH).
Weak response	Denature the antigen. Add small modifying groups, such as dinitrophenol and arsenate. Couple antigen to agarose beads or red blood cells.
No epitope	No remedy.

class II protein binding, and conjugating the antigens to large particles, such as agarose beads and sheep red blood cells.

Protein Denaturation

Many protein antigens are made more immunogenic by denaturation. Denaturation usually exposes new epitopes by changing the structures. Antibodies that are generated from denatured protein are particularly good for immunoblots.

Working Procedure

1. To the protein solution (0.5 to 2 mg/ml in PBS), add SDS to 0.5% final concentration.
2. Heat to 80°C for 10 min. Allow the protein solution to cool down to room temperature. If large aggregates occur, break by pipetting.
3. Dilute fivefold with PBS. Sample is now ready for injection. (Alternatively, treat protein antigen with 8 M urea for 30 min at room temperature. Dilute eightfold with PBS before injection.)

Dinitrophenyl Conjugation (13)

Dinitrophenyl is commonly used to modify antigens to make them more immunogenic.

Working Procedure

1. Dialyze antigen (approximately 2 mg/ml) with 0.5 M sodium carbonate (pH 9.5).
2. Freshly prepare a solution of 4 mg/ml of sodium dinitrobenzene sulfonic acid in 0.5 M sodium carbonate (pH 9.5) and add to the antigen solution (1:1).
3. Stir the mixture overnight at 4°C in the dark and dialyze extensively with PBS. The modified antigen is now ready for injection.

Arsynyl Conjugation (14)

In this method the protein antigen is conjugated with arsanilic acid. At first, the diazotized arsanilic acid is prepared (see the Reaction section), and the reagent is coupled to the protein. The diazotized arsanyl group reacts primarily with tyrosine side chains. It also reacts less frequently with histidine, sulfhydryl group, and free amino groups. Arsanilation usually creates a new site for T cell recognition and thus breaks T cell tolerance.

Working Procedure

1. Dialyze the protein antigen (approx. 5 mg/ml) with 0.1 M sodium borate (pH 9.0).
2. Dissolve 0.21 gram of p-arsanilic acid (1 mmole) in 30 ml of 80 mM HCl containing 0.25 gram of sodium bromide (8 mM).
3. Place the arsanilic acid solution on an ice/salt bath. Add slowly freshly prepared and prechilled 10 ml of 0.7% sodium nitrate to the arsanilic acid solution with mixing.
4. After incubation on ice for 30 min, add 60 ml of cold water.
5. Slowly add the cold diazotized arsanilic acid solution to the diazotized protein solution (0.5 ml/ml of protein). Adjust pH to 9.0 if necessary.
6. After incubation at 4°C for 4 h, dialyze with PBS. During incubation check the pH periodically and readjust to pH 9.0, if necessary. The sample is now ready for injection.

Coupling Antigens with Small Synthetic Peptides Containing Binding Sites for T Cell Receptor and Class II Protein

Antigens can be made immunogenic by coupling a known class II-T cell receptor site to them. A list of synthetic peptides can be found elsewhere (4). Coupling can be performed following the procedures described elsewhere (see Chapter 6).

5.5 DEVELOPMENT OF MONOCLONAL ANTIBODIES

Conventional antibodies are heterogeneous and have specificities for many different antigens. Table 5.2 describes the advantages and disadvantages of conventional and monoclonal antibodies. Because of this heterogeneity, the results obtained by using these mixed populations of antibodies are difficult to interpret. Therefore, the need of homogeneous antibodies with a defined specificity was sought in immunochemical research. This goal was achieved with the development of the technology for hybridoma production that yields homogeneous antibodies known as monoclonal antibodies. Besides their homogeneity and specificity to bind single epitopes, hybri-

TABLE 5.2
Advantages and Disadvantages of Conventional and Monoclonal Antibodies

Antibodies	Advantages	Disadvantages
Conventional	Inexpensive	Heterogeneous; sometimes results are difficult to interpret
	Requires 45–60 days	
	Good for almost all immunochemical techniques	Antigen must be purified
		High background
Monoclonal	Specific to single epitope	Expensive
	Unlimited supply of antibodies	Labor intensive
	Antigen need not to be purified	Time consuming, requires 3-12 months

doma technology has two unique advantages over conventional antibodies. First, hybridoma cell lines can provide an unlimited supply of antibodies. The second advantage is that antigens need not to be purified, because hybridomas are single-cell cloned prior to use. Despite many advantages, production of monoclonal antibodies is not necessarily the best choice for certain immunochemical procedures, because the preparation of monoclonal antibodies is often costly and time consuming (3 to 12 months). Researchers should be certain that they need these antibodies before they start constructing hybridoma cell lines.

5.5.1 THEORY OF MONOCLONAL ANTIBODY PRODUCTION

In 1957 Burnet[15] postulated that each B lymphocyte is predetermined to make only one particular antibody molecule after the immunogenic response known as clonal selection theory. If an individual B lymphocyte (commonly known as a clone) could be propagated continuously *in vitro*, the culture supernatant would contain homogeneous antibody molecules (monoclonal antibody) that recognize a single antigenic determinant. Unfortunately, B lymphocytes have a limited life span and so cannot be grown long term in culture. In 1975, Kohler and Milsten[16] immortalized antibody-secreting lymphocytes by fusing them with cells from a continuously growing cell line and then cloned individual hybrid cells to produce lines of cells (hybridomas), each of which produce one particular antibody molecule. The immortal myeloma cells derived from a mutant cell line of a tumor of B lymphocytes are chosen to fuse with the cell suspension of spleen from the immunized animal in the presence of polyethylene glycol (PEG). The PEG probably promotes the membrane fusion.

5.5.2 SELECTION PROCEDURES TO PRODUCE A CONTINUOUS HYBRID CELL LINE

A selection procedure is required to produce a continuous hybrid cell line from two cells. The hybridomas are selected on a growth medium containing hypoxanthine, aminopterin, and thymidine (HAT). Aminopterin, which is a folic acid antagonist, blocks the normal biosynthetic pathways for guanosine production. When the normal biosynthetic pathway for guanosine is blocked by aminopterin, there is an alternative "salvage" pathway in which the nucleotide metabolites hypoxanthine or guanine are converted to guanosine monophosphate by the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). Cells lacking HGPRT (HGPRT$^-$ cells) die in a HAT medium because both the main and salvage pathways are blocked. However, an HGPRT$^-$ cell can grow in a HAT medium if it is provided the missing enzyme by fusion with an HGPRT$^+$ cell. The B lymphocytes provide the HGPRT enzyme to HGPRT$^-$ myeloma cells. Heterokaryons are produced from binucleate fusion in the presence of polyethylene glycol. The two nuclei fuse at the next division, generating hybrid cells. Hybrid cells grow in a HAT medium, but both unfused spleen and myeloma cells die (Figure 5.27). To select HGPRT$^-$ cells, a toxic base analogue such as 6-thioguanine or 8-azaguanine is used in the medium. HGPRT$^+$ cells will incorporate a toxic base into their DNA and will die, but HGPRT$^-$ cells will survive.
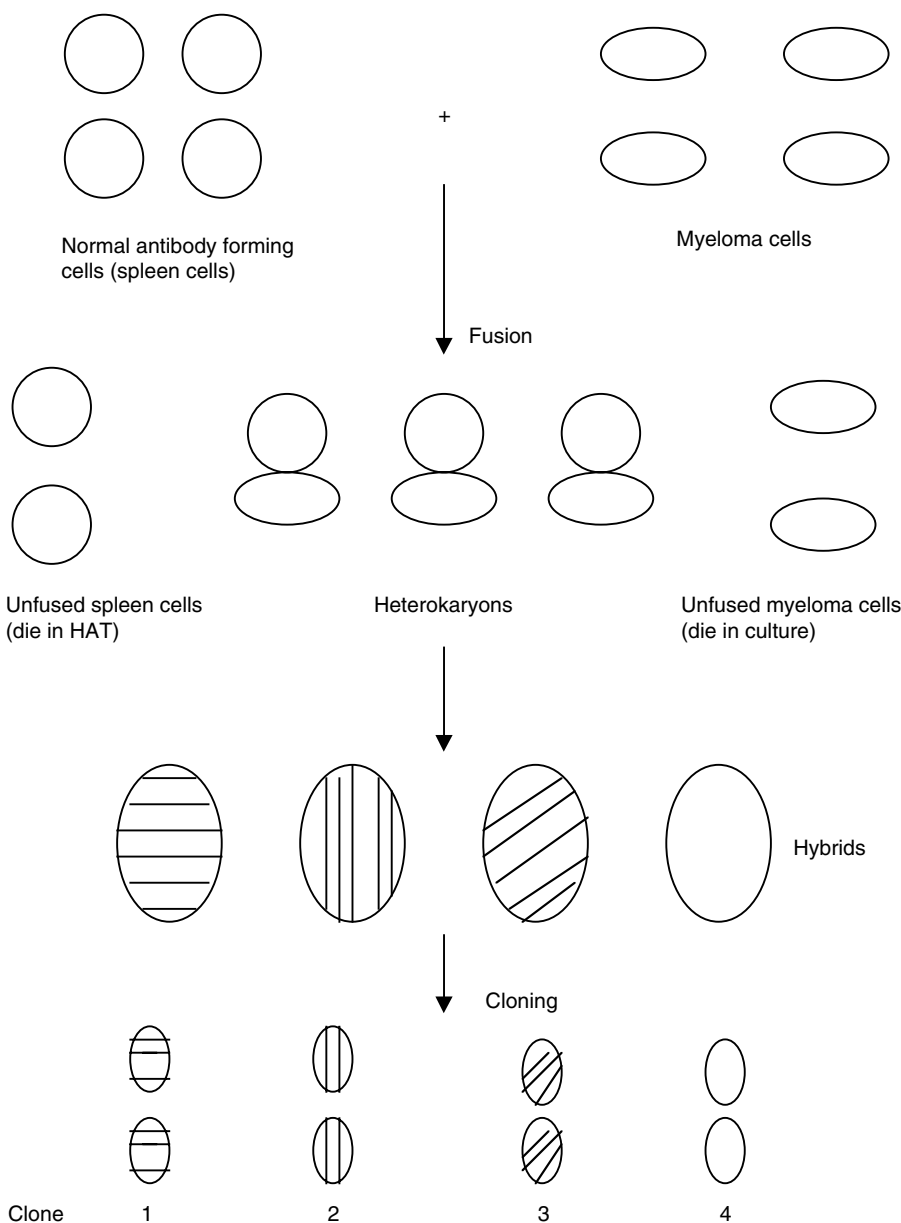
FIGURE 5.27 Production of hybridomas. Spleen cells from immune mice are fused with myeloma in the presence of polyethylene glycol to produce heterokaryons that grow in a HAT medium, producing antibodies.

5.5.3 Development of Monoclonal Antibodies Consists of Several Stages, such as Immunization, Testing Sera Prior to Fusion, Fusion for the Production of Hybridoma, Single-Cell Cloning, and Production of Monoclonal Antibodies

Production of monoclonal antibody is time consuming and requires about 3 to 12 months. First, animals are subjected to antigen injection. The sera from the immunized animals are then screened for good antibody response. Once an appropriate antibody response has been established, antibody-secreting cells are then fused with the myeloma cells. After the fusion, cells are diluted in a multi-well tissue culture plate with selective medium and tested for antibody response. Cells from positive wells are grown and then single-cell cloned. Finally, a monoclonal antibody is obtained from the supernatant of culture or from ascites culture in mouse peritoneum.

Immunization

Mice are frequently used for immunization for this purpose because they are cheap, they are easy to handle, and they can be responded to a lower antigen level. Immunization schedule, dose of antigen, and route of injection depend on several criteria such as availability of antigen. If the antigen is not limiting, then intraperitoneal injection (ip) is a choice of introducing antigens, because a larger volume of the antigen can be introduced into the large volume of the peritoneal cavity. Particulate antigens can also be used to immunize mice, since ip injections do not deliver the antigen directly into the blood system. In any case, the immunization protocol should be approved by the local Animal Care Committee. For the injection 5 to 50 µg of antigen is taken in 250 µl of suitable buffer or saline and mixed with an equal volume of Freund's complete adjuvant as described in Section 5.4.2. The use of adjuvants are avoided when live cells are to be introduced into the intraperitoneal cavity in order to make anti-cell surface antibodies. The ip route is also good for antigen bound beads such as agarose or polyacrylamide. Antigens are coupled to beads in order to increase the immune response. Subcutaneous injection requires only one fifth the maximum used for ip injection and is used for soluble or insoluble antigens into a local environment. This route is often used for tumorigenic cells and nitrocellulose bound antigen. Protein antigens immobilized on nitrocellulose are usually good immunogens due to their slow release from the membrane, thus mimicking the role of the adjuvant. For immunization, antigen-immobilized nitrocellulose is washed with phosphate buffered saline and implanted under the skin of the mouse's neck. For this purpose, the mouse is anesthetized by injecting a suitable drug (50 µl of sodium pentobarbitone), and after a few minutes, a 1.5 cm incision in the neck skin is made with sterile scissors, and the antigen-immobilized membrane (about 0.5 cm^2) is inserted.

Intravenous injections (ivs) to mice tails are generally used to deliver the final boost just before a hybridoma fusion, to ensure that the antigen is seen by the immune

system. In this route, antigens are processed quickly and immune response is short lived. Freund's adjuvant is avoided in this route because injection will deliver the antigen directly into a vital organ.

A Typical Immunization Schedule

1. Mix antigen solution (1.5 ml) with an equal volume of Freund's complete adjuvant to prepare emulsion and inject 0.5 ml ip to each of five BALB/c female mice.
2. After two weeks, repeat ip injections using incomplete Freund's adjuvant.
3. On day 24, collect tail bleeds for testing immune response.
4. On day 35, repeat ip injection with incomplete Freund's adjuvant.
5. On day 45, test tail bleeds again.
6. On day 56, inject 100 μl antigen both iv and ip to the mouse that shows best response on test screening. Inject ip with incomplete Freund's adjuvant to the rest of mice.
7. On day 59, fuse splenocytes from best responder.
8. On day 66, start screening of hybridoma cells.

Testing Sera Prior to Fusion

Test bleeds are collected from the tail of each mouse and checked for the desired antibodies. Several assays such as dot blot assay and ELISA can be performed to test the antibody titer (see Section 6.3.1 for these procedures). When satisfactory immune response is obtained using an appropriate screening protocol, fusion can be performed; otherwise, a boost injection is given.

Fusions for the Production of Hybridomas

Construction of hybridomas begins when the animal shows a good immune response. For the fusion, antibody-screening cells are isolated from a lymphoid tissue (usually from the spleen) and mixed with myeloma cells in the presence of polyethylene glycol. The fused cells are diluted with a suitable culture medium and plated in multi-well tissue culture dishes. After 7 to 10 days, culture supernatants are removed and tested for the presence of antibodies. Cells showing positive response are grown, single-cell cloned usually by limiting dilution, and frozen.

Working Procedure for the Fusion

1. Have ready at least seven flasks of SP2 myeloma cells in DMEM with 16% FBS (D16). (Feed and split every day three days prior to fusion.)
2. Three days prior to fusion, boost the animal iv and ip.
3. On the day of fusion, have all materials and surgical tools sterile and place in hood.

4. Anesthetize mouse with 1 to 2 ml of halothane and monitor. Spray ethanol with squirt bottle on the mouse. Cut skin and diaphragm to expose heart. Puncture left ventricle and bleed. Centrifuge 10 min at 400 g and collect serum for ELISA to test antibody response.

5. With sterile instruments (scissors, forceps, etc.) cut skin away and remove spleen without breaking fascia. Spray the spleen with 70% ethanol and place in a petri dish with serum-free medium (D16). Trim fat and transfer to the other petri dish. Flush spleen with medium. With a medium-full syringe fitted with 18-gauge needle prick the spleen (with needle) and deliver medium to the spleen to break up lump. Repeat this five to six times and draw the spleen cell suspension with the syringe and gently transfer to 50 ml sterile tube.

6. Centrifuge SP2 and spleen cell suspension for 10 min at 400 g. Aspirate supernatant with vacuum, gently break the pellet with 40 ml serum-free medium, mix well, and spin again. Remove supernatant and resuspend cells with 20 ml serum-free medium.

7. Using an aliquot of each, count cells. For counting spleen cells, take 30 µl of cell suspension in 270 µl of acetic acid in 5 ml tube. (Acetic acid will lyse red cells leaving lymphocytes intact.) After 1 min, load 10 µl of the mix in each side of counting chamber and count 25 squares. Take the mean of count for both sides of grids.
   (For counting SP2 cells, take 15 µl of SP2 cell suspension and add 15 µl of DPBS and 10 µl of Trypan blue. Count live and dead cells (dead cells will appear bluish).

8. Mix spleen and SP2 cells in a ratio of 4:1 and centrifuge 10 min at 400 g. Aspirate the supernatant thoroughly.

9. Have 50% PEG (that has been autoclaved before) in water bath at 40°C and a tube with D16 at 37°C.

10. For fusion take 1 ml of 50% PEG and, inside hood, add to the mixed cells dropwise during 1 min with continuous mixing. Continue mixing for another 45 sec. With a fresh sterile pipet take 1 ml of serum-free medium and add dropwise during 45 sec with continuous mixing.

11. With a fresh sterile pipet, add 20 ml of serum-free medium during 5 min.

12. Centrifuge the tube (at approx 8 min after the fusion starts) at 400 g for 7 min. Aspirate the supernatant, break the pellet gently and add 30 ml of D16 in several portions mixing in between additions.

13. Prepare three 96-well plates and plate 10 ml of fusion per plate. Plate unfused SP2 cells in four control wells. Place plates in incubator.

14. At day 1 (next day), add two drops of HAT medium (prewarmed at 37°C) to each well including the control wells and return plates to incubator.

15. At day 2, aspirate half volume of each well using sterile pipet and add two drops of HAT medium (prewarmed at 37°C) to each well and return plates to incubator.

16. Repeat feeding with HAT as day 2 at days 3, 5, 8, and 11.

17. At day 14, perform ELISA to test antibody response and feed the plates with HAT. Coating of the 96-well plate with the antigen can be performed on the previous day.
18. At day 15, transfer the wells that show positive response to 24-well plate. For this first take 1 ml of D16 to each well of 24-well plate. Designate this plate as "Plate a." Feed the fusion plates (96-well) with D16.
19. At day 17, aspirate half of the supernatant from each well of the fusion plates and add two drops of D16. Do not feed 24-well plate.
20. At day 21, perform ELISA on the three fusion plates and transfer the best 24 wells to a new 24-well plate labeled with the new wells and labeled "Plate a" with the new date. Feed fusion plates with 2 drops of D5. Split the first 24-well "Plate a" from Day 15 to the new "Plate b." Feed the "Plate a" with 0.5 ml of D5.
21. Continue feeding fusion and 24-well plates based on cell densities. Perform ELISA on fusion plates and transfer any new positives to new 24-well plates.

Single-Cell Cloning

Hybridoma cells are screened for the production of a specific antibody, and an individual clone is isolated. Several assays (see Section 6.3.1) are available to test the antibody titer. Since the method requires the selection for an individual clone that produces the antibody against a specific antigenic epitope, unpurified molecules can be used to immunize mice.

Isolation of an individual clone is performed in several ways, such as by the soft agar method, by limiting dilution, or by direct isolation of single cells. In the soft agar method, a low concentration of agar (0.5%) is usually incorporated into the medium of cultures containing diluted hybridoma cells. The culture is overlaid with agar of higher concentration (1%) and is observed for single-cell distribution. The soft agar method may not result in a monoclonal antibody, as there is no way to determine if a particular colony originated from one or more cells. In the limiting dilution method, a fixed volume of diluted cell suspension containing about one cell (based on a theoretical calculation) is dispensed into a number of wells, and these are observed for single-cell distribution. The direct isolation of single cells is tedious, but avoids the uncertainties for clonal isolation. Alternative methods for selecting and cloning hybridomas are also suggested elsewhere.[17]

Production of Monoclonal Antibodies

After the selection of the desired clone, antibodies can be obtained from either ascites or supernatant of culture. Ascites is intraperitoneal fluid from mice with peritoneal tumors that has been induced by injecting single-cloned hybridoma cells. In mouse peritoneum, the hybridoma cells grow to high densities and secrete the antibody of interest. Alternatively, hybridoma cells of the desired clone are grown in culture flasks, and the antibody of interest is obtained in the supernatant after centrifugation

of cells. The *in vitro* cultivation of hybridoma cells produces low antibody concentration (5 to 70 μg/ml), and growing in large scale may be disappointing. Ascites culture is the most commonly used system for generation of monoclonal antibodies because of its much better yield (usually 1 to 10 mg/ml). It is important to match the genetics of the animal with the myeloma cell line and immune lymphocyte donor. In the event of no match, hybridomas are rejected and no ascitic fluid is generated. Most mouse hybridomas are produced using BALB/c mouse and BALB/c-derived myeloma. However, the ascites culture has some disadvantages. *In vivo* production is limited when scaling up is needed, because this requires a large number of animals. Ascites preparation is also contaminated with the host antibodies.

5.6 PURIFICATION OF ANTIBODIES

Like other proteins, separation of the Ig fraction from a mixture of antibody serum can be achieved following salt fractionation and chromatography procedures of protein purification as previously described (Chapter 4). Certain organic compounds such as Ethodine, Rivanol, and caprylic acid are used to enrich IgG, which is precipitated selectively upon the addition of these organic compounds to the serum. Fractionation of serum proteins with ammonium sulfate is a simple and inexpensive procedure to enrich IgG fraction. An anion exchanger (such as DEAE-cellulose) is often used to purify IgG. The reason of using this positively charged matrix (DEAE-cellulose) is that most proteins of the serum (except IgG) bind strongly to the matrix at pH around 7, and thus IgG can be recovered in the early fraction of a gentle elution with a salt gradient. The advantage of DEAE over an affinity column such as Protein A is that extremes of pH are not necessary for purification, as salt gradient may be used.

However, affinity chromatography methods for purification of Ig are becoming increasingly popular because of its simplicity and high yield. Affinity columns are either Protein A immobilized to solid support such as Sepharose or specific antigen immobilized Sepharose. IgG subclasses except IgG3 are usually purified by affinity chromatography on a protein A-immobilized matrix (see Chapter 4 for the principle of affinity chromatography). Protein A, a cell-wall component of *Staphylococcus aureus*, contains four binding sites that are capable of interacting with the Fc region from IgG.[18] On either case, serum is usually passed through the column at pH around 8, and after washing the column, the bound Ig is recovered upon dissociation of the complex at acidic pH or with denaturing agent. Jacalin, an α D-galactose and T-antigen binding lectin from jackfruit (*Artocarpus integrifolia*),[19] can bind with IgA; thus, a jacalin-immobilized column can be used to purify IgA specifically.[20]

5.6.1 ASSESSMENT OF PURITY

The purity of the IgG can be assessed by measuring absorbance at 278 nm. The A_{278} of the pure IgG is about 2.5 to 3 times higher than A_{251}, in contrast to other serum proteins, where the ratio is about 1 to 1.5. Analysis on SDS-PAGE under reducing

TABLE 5.3
Sensitivity to Proteolytic Digestion of Igs from Various Sources

	Time (h) Required for Digestion of IgG[a]		
IgG	Papain	Pepsin	Trypsin
Human			
IgG1	4	24	48–72
IgG2	48	6	48–72
IgG3	4	1	48–72
IgG4	24	2	48–72
Horse	48	—	—
Bovine	48	—	—
Sheep	48	—	—
Rabbit	24	20	—
Mouse			
IgG1	27	12	—
IgG2a	4	4–8	—
IgG2b	4	4–8	—
IgG3	—	15 min	—

[a] From Reference 22.

and non-reducing conditions[21] is also a convenient way to determine if it is contaminated with other serum proteins.

5.6.2 PREPARATION OF FAB

In some cases, Fab fragments are preferred over whole IgG. Fab fragments usually offer lower background. In immunohistochemistry, Fab fragments penetrate the tissues easily and eliminate non-specific adsorption by Fc receptors. In an enzyme immunoassay, both Fab and IgG from the same serum can be used in different layers without interference, since the conjugated anti IgG antibody recognizes the Fc fragment only. The major disadvantage of Fab fragments is a loss of avidity.

Proteolytic enzymes, such as pepsin and papain, are used conveniently to cleave IgG into Fab and Fc fragments. Igs from various sources differ in their sensitivity to proteolytic digestion (see Table 5.3).

5.6.3 PREPARATION OF F(AB′)₂

F(ab′)$_2$ can be prepared using immobilized pepsin (available from Pierce, Rockford, IL). Purified IgG (10 mg in 1 ml 20 mM sodium acetate buffer, pH 4.0) is mixed with 0.5 ml gel slurry containing immobilized pepsin (packed bed volume 0.2 ml) pre-equilibrated with 20 mM sodium acetate buffer, pH 4.0. After incubation at 37°C for 4 h with shaking, the digested F(ab′)$_2$ is separated from Fc fragments and undigested IgG using a Protein A-Sepharose column. Fc fragments and undigested IgG remain bound in the column, and F(ab′)$_2$ is collected in the flow through.

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6 Antibody Labeling, Antibody Detection, and Immunochemical Techniques

Antibodies are labeled for the detection of antigens. Antigens can be detected in two ways: direct method and indirect method (see Figure 5.10 and Figure 5.11). In the direct method, the antigen is detected with the labeled primary antibodies. Therefore, it is necessary to purify antibodies prior to labeling. In the indirect method of detection, labeling is performed in the secondary antibodies that bind to the unlabeled primary antibodies. Labeled secondary antibodies are commercially available. The choice of the direct or the indirect method depends on several factors. Both methods have some advantages and disadvantages (Table 6.1), but the indirect method is used for most applications. This chapter describes several procedures of how antibodies can be labeled and the labeled antibodies can be detected. Some immunochemical techniques are also described.

6.1 METHODS OF LABELING ANTIBODIES

Labeling of the primary antibody is necessary when a direct assay is performed. For an indirect assay, labeling is performed in the secondary antibody that binds to the unlabeled primary antibody. Most labeled secondary antibodies and the procedures of their detection are commercially available. Nonetheless, a great deal of chemistries are involved in the labeling procedures of the antibodies and their detection systems. There are several ways by which antibodies can be labeled. The choice of label depends on the immunochemical techniques. For example, iodine labeling of the primary antibody is performed for quantitative results. Fluorescent labels are essential for immunocytochemistry or cell sorting analyses. For immunoblot, immuno-histology, and immunoassays, enzyme-labeled antibodies are used the most. Table 6.2 describes advantages and disadvantages of most labels and their major applications.

6.1.1 RADIOIODINATION

In radioiodination, iodine-125 is usually incorporated chemically or enzymatically into certain amino acids such as tyrosine, lysine, and histidine in proteins or antibodies. Because of potential health hazards, researchers should comply with the

TABLE 6.1
Direct versus Indirect Antibody Detection Methods: Advantages and Disadvantages

Method of Detection	Advantages	Disadvantages
Direct	Fewer steps	Requires purified antibodies
	Less prone to background	Need to label every antibody to be studied
	Quantitative	Activity may be lost during labeling
		Less sensitive
Indirect	More sensitive than the direct method	More steps
	No need to purify primary antibody	Background problems higher than the direct methods
	Labeled secondary antibodies are available commercially	
	The labeled secondary antibodies can be used to detect large range of antigens	
	Since primary antibody is not labeled, the loss of activity is avoided	

rules and regulations of radioactivity use. Reaction must be performed behind a protective shield in a ventilated hood to avoid exposure of γ-irradiation and volatile iodine. The following are some methods by which antigens can be radiolabeled. Some iodination methods may denature proteins. Table 6.3 lists advantages and disadvantages of most iodination methods.

6.1.1.1   Using Chloramine T

Radioiodination by chloramine T (N-chloro-p-toluene sulfonamide) is rapid and efficient. In this procedure, oxidant chloramine T is added to a solution of antibody and iodide-125 (normally supplied as sodium iodide).[1,2] After stopping the reaction with metabisulfite, the iodinated protein is usually separated from the unincorporated label by gel permeation chromatography on Sephadex G-10 or G-25.

Reaction

Iodide-125 is oxidized by chloramine T to iodine-125 (I_2), which reacts tyrosyl and histidyl side chains of proteins (Figure 6.1). Iodination reaction is generally stopped by using free tyrosine and sodium metabisulfite, which reduces the volatile iodine to iodide.

The disadvantage of chloramine T is that some proteins may denature in this process. Pierce (Rockford, IL) offers a milder version of chloramine T in the form of iodobeads. These beads are made by immobilizing N-chloro benzene sulfonamide to polystyrene beads. The iodobeads are conveniently used to label the protein simply by mixing with the protein solution. The iodination is easily terminated by separating iodobeads from the reaction mixture.

TABLE 6.2
Advantages and Disadvantages of Most Antibody Labels and Their Major Applications

Label	Method of Detection	Advantages	Disadvantages	Major Applications
Iodine-125	Gamma counter X-ray film	Highly sensitive Quantitative Easy labeling	Potential hazardous Short half life	Immunoassays (quantitative and qualitative) Immunoblots
³H	Scintillation counter X-ray film	Easy labeling Long half-life	Low sensitivity	Immunoassays Immunoblots
Biosynthetic	Scintillation counter X-ray film	No damage to antibody	Requires hybridoma Low sensitivity	Immunoassays Immunoblots
Enzyme	Color reaction Visual detection Absorbance	High sensitivity Direct visualization possible Long shelf life Applicable to electron microscopy	Multiple steps Long assays Background problematic Endogenous enzyme activity Some substrates hazardous Poor resolution in cytochemistry	Immunoassays Immunoblots Immunohistochemistry
Biotin	Avidin/streptavidin conjugated various labels	High sensitivity Multiple detection methods Long shelf life	Multiple steps Endogenous biotin Background	Immunoassays Immunoblots Immunohistochemistry
Fluorescent	Fluorimeter Fluorescence Microscope	Good resolution in immunocytochemistry Long shelf life	Low sensitivity Autofluorescence Quenching Multiple steps	Immunocytochemistry Flow cytometry
Colloidal gold	Light microscope SEM			Immunohistochemistry

TABLE 6.3
Advantages and Disadvantages of Most Iodination Methods

Method	Target Amino Acid Residue	Advantages	Disadvantages
Chloramine T	Tyrosine Some histidines	Easy labeling	Free iodine generation Oversubstitution Oxidation damage
Iodogen	Tyrosine Some histidines	Easy labeling	Free iodine generation Oversubstitution Oxidation damage
Bolton-Hunter reagent	Lysine Free amino groups	Easy labeling	Low specific activity
Lactoperoxidase	Some tyrosines Some histidines	Gentle Detects surface tyrosine	Multiple steps
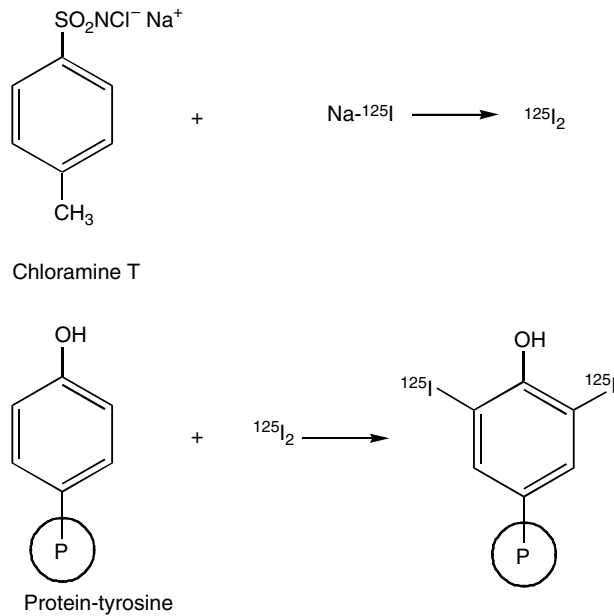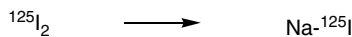
FIGURE 6.1 Iodination by chloramine T. Iodide-125 is oxidized to iodine-125 (I_2), which reacts with tyrosyl and histidyl side chains of proteins.

1. To 10 μg of protein in 25 μl of 0.5 M sodium phosphate (pH 7.5), add 500 μCi of Na^{125}I.
2. Add 25 μl of 2 mg/ml chloramine T (chloramine T can be diluted up to 0.02 mg/ml for an oxidation-sensitive protein).
3. After a minute at room temperature, add 50 μl of stop solution (a mixture of 12.4 mg/ml sodium meta bisulfite and 10 mg/ml tyrosine).
4. Separate labeled antibody or protein from the iodotyrosine on a desalting column and store at 4°C. Use within six weeks.

6.1.1.2   Using Iodogen

In this procedure, oxidant iodogen (1,3,4,6-tetrachloro-3 α, 6α-diphenylglycoluril) is coated into a reaction vessel or bead, and the mixture of protein and Na^{125}I is added. Coating of iodogen is performed in a volatile organic solvent (chloroform or methylene chloride) and allowed to evaporate. The iodogen is insoluble in water, and therefore the reaction can be efficiently terminated by removing the mixture from the tube or by removing iodogen coated beads from the reaction tube.[3]

Reaction

In this procedure, iodogen oxidizes Na^{125}I to free ^{125}I$_2$, which then reacts with tyrosine and histidine residues of the protein (Figure 6.2).

Some proteins are sensitive to oxidation, which results in a decrease of their activities. Antibodies are not generally affected by the oxidation, and therefore this method is helpful for antibody labeling. However, for oxidation-sensitive proteins, a modification of this approach is applied. In this modification, the iodide is oxidized to iodine in the absence of proteins. The oxidant is then removed, and the protein is added to the iodine.

Working Procedure

1. To prepare iodogen-coated tube, dissolve iodogen in chloroform (0.5 μg/ml) and add 20 μl per tube. Allow the chloroform to evaporate in a fume hood. (Iodogen-coated beads can be purchased from Pierce.)
2. In a ventilated fume hood and behind an appropriate shield, add 10 to 50 μg of antibody (in 50 μl PBS) and 500 μCi of Na^{125}I to the iodogen-coated tube. Incubate 1 to 2 min. (For oxidation-sensitive proteins, allow oxidation in the absence of proteins for 1 to 2 min and transfer the contents of the tube to the protein solution. Leave the reaction for 1 min.)
3. Add 50 μl of stop solution (10 mg/ml of tyrosine) and mix.
4. Separate the iodinated antibody from the iodotyrosine on a desalting column.
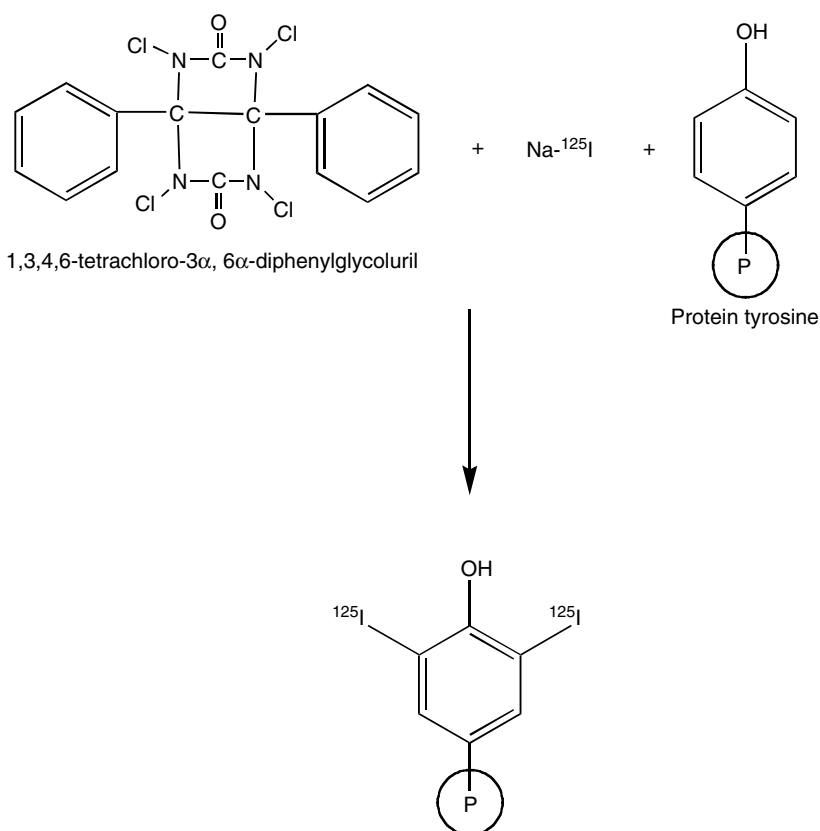
FIGURE 6.2 Iodination by iodogen. Iodogen oxidizes iodide-125 to iodine-125, which then reacts with tyrosine and histidine residues of the protein.

6.1.1.3   Using Lactoperoxidase

In this procedure, the enzyme lactoperoxidase catalyzes the oxidation of iodide-125 to iodine-125 in the presence of the oxidant hydrogen peroxide (H_2O_2).[4,5] For this reaction, hydrogen peroxide can be obtained from a chemical stock or can be produced enzymatically, usually by glucose oxidase. The reaction is terminated with free tyrosine residues.

Reaction

In the presence of H_2O_2, the lactoperoxidase catalyzes the oxidation of iodide-125 to iodine-125, which then reacts with tyrosine and histidine residues of the protein (Figure 6.3).

   Note: sodium azide should be avoided in the phosphate buffered saline (PBS) buffer, because it inhibits the lactoperoxidase activity.
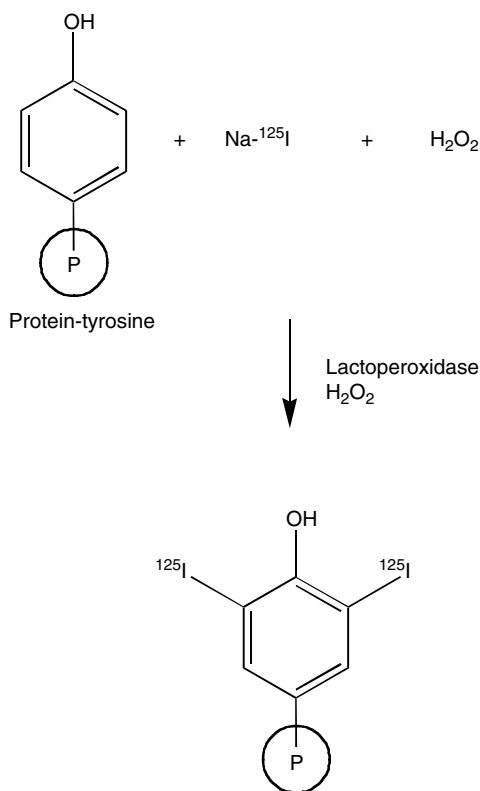
FIGURE 6.3 Iodination using lactoperoxidase. Lactoperoxidase catalyzes the oxidation of iodide-125 to iodine-125 in the presence of the oxidant hydrogen peroxide (H_2O_2).

Working Procedure

1. In a ventilated hood and behind a proper shield, to 10 μg of protein or antibody solution (in 10 μl of PBS), add 10 μl of lactoperoxidase (1 U/ml) and 500 μCi of Na^{125}I.
2. Dilute stock hydrogen peroxide (30%) to 20,000-fold with PBS. Add 1 μl of the diluted peroxide to the reaction mixture at every minute for a period of 4 minutes. (If hydrogen peroxide is produced enzymatically, add 5 μl of 4 U/ml glucose oxidase.)
3. Stop the reaction with 25 μl of stop buffer (10 mg/ml tyrosine).
4. Separate the labeled antibody on a desalting column.

6.1.1.4 Using Bolton-Hunter Reagent

Bolton and Hunter[6] developed a method for iodination of proteins that do not contain exposed tyrosine residues. In this method, N-hydroxysuccinimide (NHS) ester of 3-(4-hydroxyphenyl)propionic acid (Bolton-Hunter reagent) is mixed with chloramine T and Na^{125}I.

Reaction

Iodide-125 is oxidized by chloramine T to iodine-125 (I_2), which reacts with the Bolton-Hunter reagent (Figure 6.4). The ^{125}I-labeled Bolton-Hunter reagent then reacts primarily with free amino acids of lysyl side chains and the amino-terminal residue of the protein at pH 8.5. The lone pair of nitrogen in amino group attacks the electron-deficient carbon atom of the carbonyl group in NHS-ester, resulting the removal of NHS group.

The conjugation of protein with the radio-labeled Bolton-Hunter reagent can also be carried out in phosphate buffered saline.

Note: buffers containing primary amines such as Tris and glycine are not suitable since the conjugation occurs via amino groups of proteins.

Working Procedure

1. Behind a proper shield in a ventilated hood, add 10 µg of antibody (in 10 µl of 0.1 M sodium borate, pH 8.5) to 500 µCi of Bolton-Hunter reagent at 0°C. Leave the reaction on ice for 15 min.
2. Add 100 µl of 0.5 M ethanolamine (pH 8.5) to stop the reaction for 5 min at room temperature.
3. Separate the labeled antibody on a desalting column.

6.1.2 RADIOLABELING WITH ^{3}H

Antibodies can be labeled with ^{3}H by reacting carbohydrate residues with NaB^3H_4.

6.1.3 BIOSYNTHETIC LABELING OF MONOCLONAL ANTIBODIES

Biosynthetic labeling offers a convenient way of obtaining radiolabel antibodies when conventional labeling technique causes loss of activity. But this is possible only for monoclonal antibodies whose hybridoma cells are available. Monoclonal antibodies are labeled by growing hybridomas in a medium containing ^{3}H-leucine, ^{14}C-leucine, or ^{35}S-methionine.[7]

Working Procedure

1. Collect healthy and rapidly growing cells (approximately 2×10^6) by centrifugation (400 g for 10 min).
2. Wash the cells once by resuspending the cell pellet in methionine-free media (prewarmed to 37°C). Centrifuge at 400 g for 10 min and discard the suspension.
3. Resuspend the cells in methionine-free media/2% fetal bovine serum (10^6 cells/ml). Add [^{35}S] methionine (100 µCi) and incubate overnight at 37°C in a CO_2 incubator.
4. Centrifuge the cell suspension at 8,000 g for 10 min and collect the supernatant. Add 1/20 volume of 1 M Tris (pH 8.0) and sodium azide (final concentration 0.02%). The labeling usually yields 10^5 to 10^6 cpm of activities.
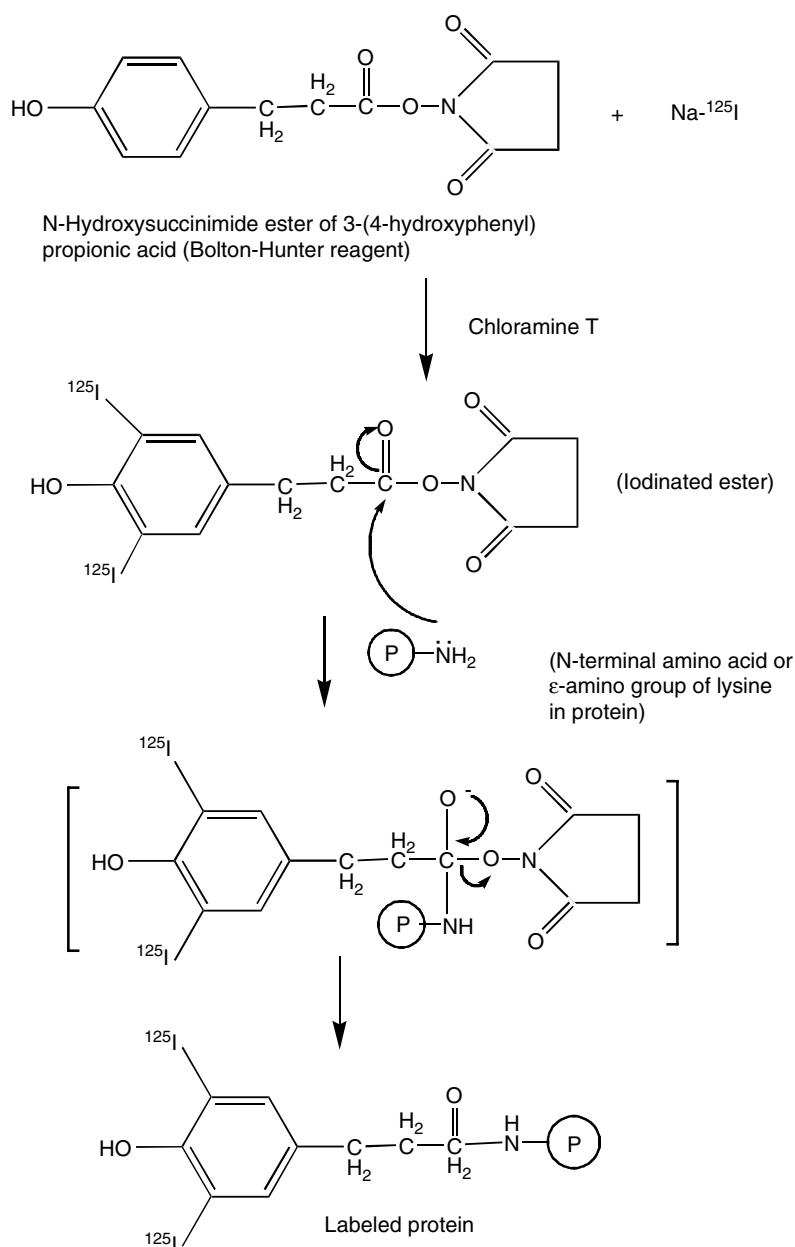
FIGURE 6.4 Iodination using the Bolton-Hunter reagent. The Bolton-Hunter reagent is mixed with chloramine T and iodide-125 to produce iodinated Bolton-Hunter reagent, which reacts primarily with lysine residues and the amino-terminal residues.

6.1.4 ENZYME LABELING

Antibodies can be readily conjugated to the enzyme by covalent coupling.[8,9] The most commonly used enzymes to label antibodies are horseradish peroxidase (HRP), alkaline phosphatase, β-galactosidase, and glucose oxidase. The criteria for good enzymes are high substrate activity, high stability, low cost, and ease of conjugation. It should have a wide range of substrates that yield both soluble products (enzyme immunoassays) and insoluble products (immunoblotting, immunocytochemical techniques). Table 6.4 describes advantages and disadvantages of commonly used enzyme conjugates.

Principle of Enzyme Conjugation by Bifunctional Cross-Linkers

Enzymes are usually conjugated with antibodies by the cross-linkers. Cross-linking is essentially a chemical modification process by a bifunctional agent, where the reactive groups are located at the two ends of the reagent with a connecting backbone. The relative reactivity of the amino acid side chains toward the reactive ends of the cross-linker dictates its specificity. Since the nucleophilic reactions mediated by amino acid side chains are most common, the cross-linkers are chosen in such a way that should favor the nucleophilic reactions.

The two main characteristics, electrophilicity and the leaving group reactivity, are important for choosing the cross-linker. Since the nucleophiles amino acid side chains attack the electron-deficient or slightly positive-charged carbon atom (electrophile), and thus have the greater electrophilicity, the reactivity will be greater. Usually alkyl and acyl carbon atoms serve as an electrophile, since the electrons of the carbon atom are drawn to the more electronegative atom (usually oxygen), rendering the carbon atom positively charged.

The rate of nucleophilic reaction also depends on the stability of the leaving group as a free entity after leaving. The more stable the leaving group, the easier to leave and thus the higher the reactivity. The following is the approximate order of leaving capability for groups attached to saturated carbon atoms: $RN_2^+ > ROTs$ (tosyl) $> ROSO_2OR > RI > BBr > ROH_2^+ > RCl > RO(H^+)R > RONO_2 > RNR'_3^+ > ROCOR'$, and for groups attached to the carbonyl carbons: $RCOCl$ (acyl halide) $> RCOOCOR'$ (anhydride) $> RCOOAr$ (aryl ester) $> RCOOH$ (carboxylic acid) $> RCOOR$ (alkyl ester) $> RCONH_2$ (amide).

The reactive groups in a cross-linker can be identical, called homobifunctional, or different, called heterobifunctional. Reactive groups that can be targeted using a cross-linker include primary amines, sulfhydryls, carbonyls, carbohydrates, and carboxylic acids. The length of the cross-linker is important, since the degree of cross-linking depends on the length of the cross-linker. Shorter spacer arms (4 to 8 Å) are often used in intramolecular cross-linking studies. Intermolecular cross-linking is favored, with a cross-linker containing a longer spacer arm. Many factors, such as cross-linker-to-protein molar ratio, hydrophobicity, and hydrophilicity of the reagent, should be considered to choose and design the bifunctional reagent. For preparing immunogen conjugates, a high degree of conjugation is normally desired to increase the immunogenicity of the antigen. In contrast, for the conjugation of an antibody,

TABLE 6.4
Advantages and Disadvantages of Commonly Used Enzyme Conjugates

Enzyme Conjugate	Advantages	Disadvantages	Detection	Recommended Application
Peroxidase	Stable, cheap Good substrates for immunohistochemistry Ultrasensitive substrates for immunoblot	Endogenous activity Moderate sensitivity moderate for soluble substrates	Several soluble substrates (e.g., ABTS, TMB) Several insoluble substrates (e.g., DAB, chloronaphthol)	Immunoassays Immunoblot Immunohistochemistry
Alkaline phosphatase	Excellent soluble substrates Good insoluble substrates for immunoblot Ultrasensitive substrates available	Endogenous activity Large conjugate	Soluble substrate (PNP) Insoluble substrate (BCIP/NBT)	Immunoassays Immunoblot
β-Galactosidase	Very low endogenous activity Good soluble and insoluble substra	Sensitive to inactivation Large conjugate	Soluble substrate (ONPG) Insoluble substrate (BCIG)	Immunoassays Immunoblot Immunocytochemistry

TABLE 6.5
Examples of Homobifunctional Cross-Linkers and Their Characteristics[a]

Cross-Linker	Solubility	Target Amino Acid	Recommended Reaction pH
Glutaraldehyde	Water	Lysine, N-terminal amino acid	Around pH 7
Dimethyladipimidate. 2 HCl (DMA)	Water	Lysine, N-terminal amino acid	pH 8–9
Dimethylsuberimidate. 2 HCl (DMS)	Water	Lysine, N-terminal amino acid	pH 8–9
Disuccinimidyl suberate (DSS)	DMF Water (sulfo derivative)	Lysine, N-terminal amino acid	pH 7–9
1,4-Di-[3′-(2′-pyridyldithio)-propionamido)]butane (DPDPB)	DMSO	Cysteine	Around pH 7
Bis-[β-(4-azidosalicylamido)ethyl] disulfide (BASED)	DMSO	Tyrosine	Around pH 7

[a] From Reference 10.

an enzyme, or a lectin, a low-to-moderate degree of conjugation may be optimal to ensure the maximum biological activity. A protein in a hydrophobic environment may require a hydrophobic reagent for membrane permeability. A description about the choice and design of cross-linking reagents and a comprehensive list of both homobifunctional and heterobifunctional cross-linkers are cited elsewhere.[11] Pierce (Rockford, IL) commercializes a good number of homobifunctional and heterobifunctional cross-linkers. Table 6.5 and Table 6.6 list some commonly used homoand heterobifunctional cross-linkers.

In general, one of the two proteins (often the carrier) is treated with an excess amount of the coupling agent. High concentrations of the coupling agent usually prevent auto cross-linking of the protein. Once the first reaction is over, the cross-linker is then removed by dialysis or gel filtration and an equal molar amount of the second protein is then added to allow binding to the cross-linked protein. Finally, if needed, the conjugate can be purified from the unconjugated components on the basis of molecular weight (size exclusion chromatography), charge (ion-exchange chromatography), or activity (for example, affinity chromatography).

6.1.4.1 Glutaraldehyde Coupling

Among the homobifunctional cross-linkers, glutaraldehyde was the sole option for most immunologists in the 1970s for the conjugation of an enzyme to an antibody.[12] The conjugate can be obtained from the reaction of either the one-step or two-step method. In the one-step method, enzyme and antibody are mixed in 1:1 or 2:1 ratio, and glutaraldehyde is then added. After incubation for 2 to 3 h at room temperature, the reaction is stopped by the addition of excess lysine, and the conjugate is separated from the unlabeled protein by gel permeation chromatography.

TABLE 6.6
Common Heterobifunctional Cross-Linkers and Their Characteristics[a]

Cross-Linker	Reactive Groups	Solubility	Target Amino Acid	Recommended Reaction pH
Succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC)	NHS ester and maleimide	DMSO; sulfo derivative in water	Primary amine and sulfhydryl	Reaction of maleimide group with sulfhydryl around pH 7.0 Reaction of NHS ester group with primary amine around pH 8.0.
m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS)	NHS ester and maleimide	DMSO; sulfo derivative in water	Primary amine and sulfhydryl	Reaction with sulfhydryl around pH 7.0 Reaction with primary amine around pH 8.0.
N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB)	NHS ester and haloacetyl	DMSO; sulfo derivative in water	Primary amine and sulfhydryl	Both NHS ester and iodoacetyl react around pH 8.0
N-succinimidyl-3-(2-pyridyldithio)-propionate	NHS ester and pyridyldithio	DMSO	Primary amine and sulfhydryl	Pyridyldithio reacts around pH 7.0 NHS ester reacts around pH 8.0

[a] From Reference 10.

In the two-step procedure, the enzyme is first activated with the cross-linker (glutaraldehyde), and after removing the excess cross-linker from the activated enzyme, the antibody is then added. The two-step method allows the most efficient coupling (mostly enzyme-antibody), whereas in the one-step method, enzyme-enzyme and antibody-antibody coupling also occur, along with enzyme-antibody. Alkaline phosphatase has also been successfully conjugated to antibody using glutaraldehyde coupling.[8,13] Eukaryotic alkaline phosphatase is a better choice than a bacterial one, since the former can easily be inactivated with EDTA to stop the reaction. The bacterial enzyme is difficult to stop and thus causes high background due to overdevelopment.

Reaction

At alkaline pH, glutaraldehyde reacts with the free amino acid of the lysine residues and the N-terminal amino acid (Figure 6.5).

Note: since the conjugation occurs through the primary amines, Tris and glycine buffers are not compatible for this procedure.

Working Procedure for the Conjugation of Peroxidase to IgG by Glutaraldehyde in One Step[8]

1. Dialyze the enzyme and antibody solution with 100 mM phosphate buffer, pH 6.8.
2. Mix the enzyme and antibody at 2:1 ratio by weight and add dropwise 1/20 volume of 1% glutaraldehyde.
3. After incubation at room temperature (RT) for 2 to 3 h, add an excess of L-lysine (1/20 volume, 1 M) to block the remaining active sites.
4. Dialyze the conjugate with PBS to remove reagents.

Working Procedure for the Conjugation of Peroxidase to IgG by Glutaraldehyde in Two Steps[14]

1. Dissolve 10 mg of good-quality HRP (OD at 403 nm: OD at 280 nm should be 3.0) in 0.2 ml of 1.25% glutaraldehyde in PBS (pH 6.8) and leave the reaction overnight at room temperature.
2. Remove excess-free glutaraldehyde through a desalting column (Sephadex G-25) (10 ml bed volume) pre-equilibrated with PBS.
3. Collect and pool the fractions that look brown. These fractions contain activated HRP.
4. Add the activated HRP (approx. 1 ml) to 1 ml antibody (5 mg/ml in 0.1 M sodium carbonate, pH 9.5) and leave the reaction at 4°C for 24 h with stirring.
5. Incubate the mixture with 0.1 ml of 0.2 M L-lysine or ethanolamine (pH 7.0) for 2 h at 4°C to inactivate the excess functional groups.

(a) One-step method:
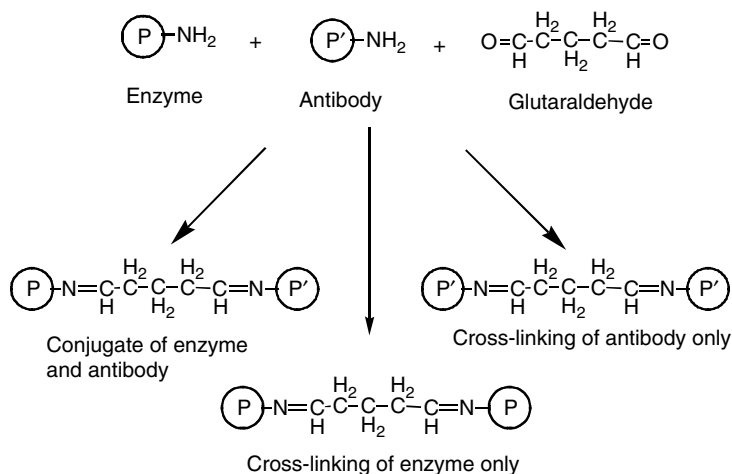
(b) Two-step method:
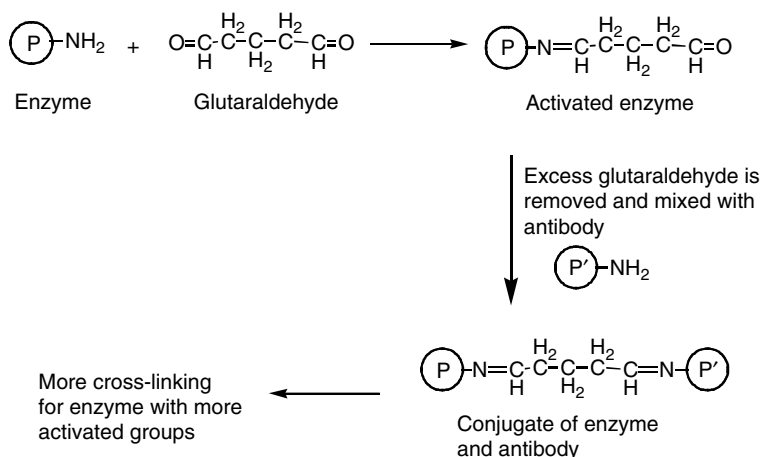
FIGURE 6.5 The coupling of an enzyme to an antibody by glutaraldehyde. In the one-step method (A), glutaraldehyde is added to a mixture of enzyme and antibody. In the two-step method (B), the enzyme is first reacted with the cross-linking agent, and after removing the excess reagent, the activated enzyme is then allowed to react with the antibody.

6. Dialyze the conjugate with PBS.
7. If necessary, separate conjugate from free antibody and free HRP on a gel filtration column.

6.1.4.2 Coupling by Other Homobifunctional Cross-Linkers

Among the other homobifunctional amine-reactive cross-linkers, imidoesters and N-hydroxysuccinimide esters are commonly used for conjugation (see Table 6.5).
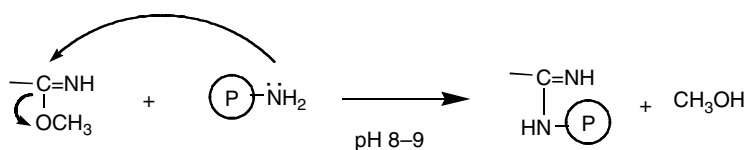
FIGURE 6.6 Reaction of imidoester cross-linker with amines

Imidoester Homobifunctional Cross-Linkers

Dimethyladipimidate. 2 HCl (DMA), dimethylpimelimidate. 2 HCl (DMP), and dimethylsuberimidate. 2 HCl (DMS) are the examples of this kind. One of the popular uses of the imidoester cross-linkers is to immobilize proteins onto solid support.[15] They are also useful for oligomer formation.[16]

Reaction

At alkaline pH around 8 to 9, imidoester cross-linkers react with primary amines on proteins, resulting in an amidine bond (Figure 6.6).

Note: buffers containing primary amines (e.g., Tris and glycine) are not compatible, as these buffers will compete with the cross-linking reaction.

Working Procedure

1. To protein or IgG sample (5 mg/ml in 0.2 M triethanolamine, pH 8.0), add tenfold molar excess of the cross-linker. Incubate the mixture at room temperature for 30 to 60 min.
2. Stop the reaction with 20 to 50 mM Tris or glycine. Dialyze the conjugate with PBS.

N-Hydroxysuccinimide-Esters Homobifunctional Cross-Linkers

Cross-linking of proteins with NHS-ester is relatively efficient at physiological pH. Examples of this type of cross-linker are disuccinimidyl suberate (DSS), dithiobis(succinimidyl propionate) (DSP), ethylene glycobis(succinimidylsuccinate) (EGS), disuccinimidyl tartarate (DST), and their sulfonate derivatives. Although the primary amine is the prime target for NHS-ester, in the event of inaccessibility of alpha-amine groups, the reaction with side chains of amino acids (especially ε-amines) becomes important. In contrast to imidoesters, which have short half-lives, NHS-esters have longer half-lives (4 to 5 h at pH 7.0) and are more stable at pH around 5. The NHS-esters are classified into two types: water-soluble and water-insoluble forms. The sulfonate ($-SO_3$) derivative of NHS-ester is water soluble and is advantageous to proteins that do not have tolerance to organic solvents. The water-soluble NHS-ester cross-linkers are recommended for surface conjugation because they will not permeate the membrane.
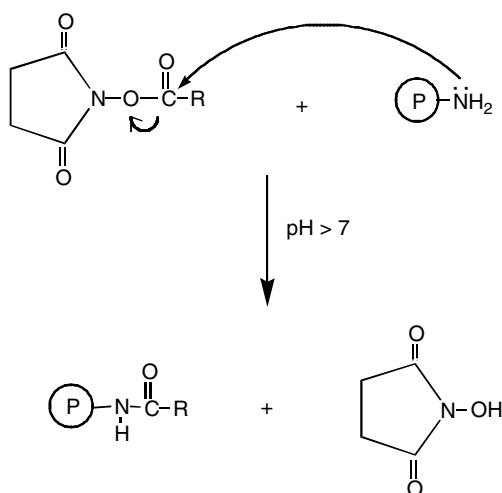
FIGURE 6.7 Reaction of N-hydroxysuccinimide-ester cross-linker with amines.

Reaction

A covalent amide bond is formed when the NHS-ester reacts with primary amines, releasing N-hydroxysuccinimide (Figure 6.7).

Note: NHS-ester cross-linking reactions must be performed in buffers that do not contain primary amines, and so phosphate, borate, HEPES, and bicarbonate/carbonate are the choice of buffers. Tris and glycine contain primary amines and are therefore unacceptable, but they can be used at the end of the reaction to quench.

Working Procedure

1. To 5 mg/ml protein or IgG solution in a cross-linking buffer, such as 20 mM phosphate buffer containing 0.15 M NaCl (pH 7.5), 20 mM HEPES (pH 7.0), or 50 mM borate (pH 8.5), add tenfold molar excess of the cross-linker. Incubate the reaction mixture at room temperature for 30 min.
2. Stop the reaction with 20 to 50 mM Tris or glycine. Dialyze the conjugate with PBS.

Sulfhydryl-Reactive Homobifunctional Cross-Linkers

1,4-Di-[3′-(2′-pyridyldithio)-propionamido)]butane (DPDPB) is a sulfhydryl-reactive homobifunctional cross-linker that contains two dithiopyridyl groups. These cross-linkers introduce sulfhydryl groups into protein, and the resultant conjugate is cleavable. The reaction facilitates between pH 4 and 5, but can be performed at physiological pH. The rate is slower at physiological pH.
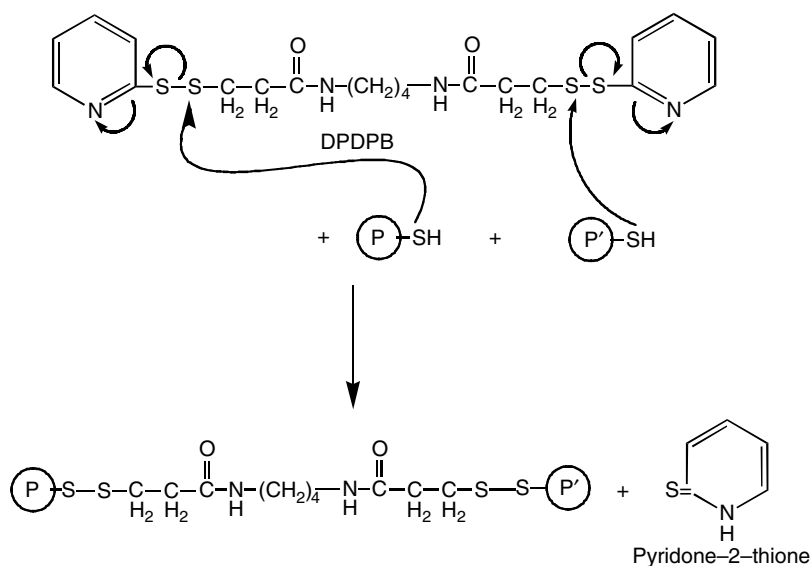
FIGURE 6.8 Reaction of DPDPB cross-linker with protein sulfhydryl groups

Reaction

DPDPB reacts with protein sulfhydryl groups, releasing pyridine-2-thione (Figure 6.8). The released pyridine-2-thione is measured at absorbance 343 nm (molar extinction coefficient is 8.08×10^3 M^{-1} cm^{-1}).

Other sulfhydryl-reactive cross-linkers are maleimides, alkyl and aryl halides, and α-haloacyls. Maleimides and α-haloacetyls react with sulfhydryl groups at physiological pH. Bismaleimidohexane (BMH) and 1,5-difluoro-2,4-dinitrobenzebne (DFDNB) are such examples.

Reaction

Maleimides, alkyl and aryl halides, and α-haloacyls react with sulfhydryls to form thiolether bonds (Figure 6.9). The iodoacetyl group reacts with a sulfhydryl, resulting in a nucleophilic substitution of iodine with a thiol producing stable thioether linkage (Figure 6.10)
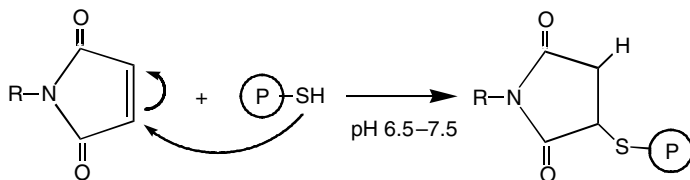
FIGURE 6.9 Reaction of maleimide cross-linker with protein sulfhydryl groups

$$\underset{\text{H}_2}{-\overset{\overset{\displaystyle O}{\|}}{C}-C}-I \quad + \quad HS-\boxed{P} \quad \longrightarrow \quad \underset{\text{H}_2}{-\overset{\overset{\displaystyle O}{\|}}{C}-C}-S-\boxed{P} \quad + \quad HI$$

pH >7.5
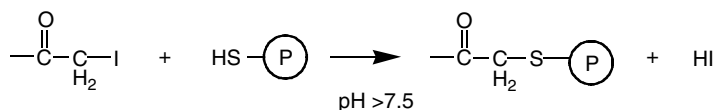
FIGURE 6.10 Reaction of α-haloacetyl cross-linker with protein sulfhydryl groups

Note: thiols should not be included in buffers when sulfhydryl-reactive cross-linkers are used to conjugate proteins, because they compete with the proteins. Thiols can be used at the end of the reaction to quench the excess sulfhydryl-reactive cross-linkers. EDTA can be included in the conjugating buffer to prevent the oxidation of sulfhydryls.

Working Procedure for DPDPB

Reduction of IgG

1. To IgG (5 mg in 1 ml 0.1 M sodium phosphate buffer containing 5 mM EDTA, pH 6) solution, add 2-mercaptoethylamine HCl (6 mg) and incubate at 30°C for 30 min.
2. Remove the excess reducing agent by dialysis with PBS (10 mM sodium phosphate/100 mM NaCl, pH 7.5) or on a desalting column equilibrated with PBS.

Reaction of DPDPB with the Reduced IgG and Enzyme (e.g., β-galactosidase)

1. To the dialyzed or desalted IgG, add 100 µl of DPDPB (10 mg/ml in dimethylsulfoxide [DMSO]) and incubate at 30°C for 30 min.
2. Remove the excess cross-linker by dialysis with PBS or on a desalting column equilibrated with PBS.
3. To the dialyzed or desalted activated IgG, add 2 mg of β-galactosidase and incubate the reaction at 30°C for 1 h.
4. Apply the reaction mixture on a Sephadex G-200 column equilibrated with PBS. Collect the fractions containing conjugate.

Nonselective Homobifunctional Cross-Linker

Bis-[β-(4-azidosalicylamido)ethyl] disulfide (BASED) (Pierce Chemical Co.) can be used for conjugating functional groups, such as hydroxyls for which specific cross-linkers are not available. It has a long spacer arm and two aromatic rings, which make it very hydrophobic with a limited solubility in aqueous systems. This cross-linker permeates membranes before conjugation initiates because of its large diffusion capacity, and it is ideal for receptor location. Two photoreactive phenyl
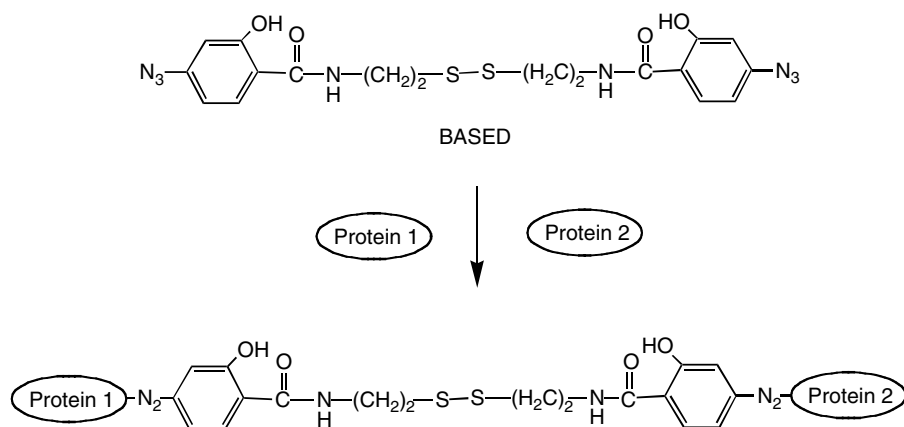
FIGURE 6.11 Reaction of BASED with proteins

azides are iodinatable so that, after the photolysis reaction, each protein will have a radioactive label. The radiolabel remains on each protein after reduction by dithiothreitol or mercaptoethanol.

Reaction

BASED (Figure 6.11) reacts non-specifically with proteins upon photo activation.

Working Procedure

1. To the IgG or protein solution (16 to 20 nmoles), add radiolabled (iodine) cross-linker, BASED. Radio iodination of the BASED (1mg/ml in DMSO) was performed with Na-^{125}I as previously described (see Section 6.1.1).
2. Photoactivate the mixture with a longwave UV light (366 nm) for 15 min at a distance of 3.5 cm.

6.1.4.3 Coupling by Heterobifunctional Cross-Linkers

A wide variety of heterobifunctional cross-linkers are available commercially (mostly from Pierce, Rockford, IL) for conjugation of antibodies with enzymes (see Table 6.6). These reagents contain two components (amine-reactive group and thiol-reactive group), and these two are separated by a spacer arm. The spacer arm prevents steric hindrances and also confers stability of the reactive groups. In heterobifunctional cross-linkers, N-hydroxysuccinimide usually plays as an amine-reactive group. At alkaline pH, it reacts with primary amine (lysine ε groups) of proteins, resulting in the formation of an amide bond. Among the thiol-reactive groups, maleimides or pyridyl disulfides are common. Under neutral conditions, maleimides react with sulfhydryl (cysteine residues) of proteins to form a stable thioether bond, while pyridyl disulfides form a disulfide bond. Heterobifunctional cross-linkers have

distinct advantages over the homobifunctional cross-linkers. Homobifunctional cross-linkers sometimes result in unwanted polymerization or self-conjugation. In case of heterobifunctional cross-linkers, the most labile group is allowed to react first, at such condition where the other group is non-reactive and thus ensures effective cross-linking and avoids unwanted polymerization.

Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate

Succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) is a heterobifunctional cross-linker containing an NHS-ester group and a maleimide group connected with a spacer arm. This can be dissolved in dimethylsulfoxide or dimethylformamide and diluted with the conjugating buffer to a final concentration of solvent of 10%. But its sulfo derivative (sulfo-SMCC) is water soluble. Cross-linking is performed in three steps: (a) reduction of IgG or protein to create free sulfhydryls, (b) maleimide activation of enzyme, and (c) and conjugation by mixing reduced IgG and activated enzyme.

Reaction

The NHS-ester group of the cross-linker reacts with primary amines of the protein at pH 7 to 9; while the maleimide group reacts with sulfhydryls at pH 6.5 to 7.5 (Figure 6.12). The enzyme is usually activated with the cross-linker around pH 7.0. At this condition, the maleimide reaction with sulfhydryls is favored.

Working Procedure

Reduction of IgG

1. To 8 mg of IgG solution (in 900 µl PBS/5 mM EDTA), add 100 µl of 0.5 M 2-mercaptoethylamine (in PBS/5 mM EDTA). Incubate at 37°C for 90 min.
2. Desalt the reduced IgG on a desalting column. Wash the column with PBS and collect 500 µl/fraction.
3. Identify the protein peak by protein assay and pool the fractions containing protein.

Activation of Enzyme

1. To 8 mg of enzyme (e.g., alkaline phosphatase) in 1 ml PBS (pH 7.0), add 1 mg of sulfo-SMCC cross-linker in 50 µl PBS (or 1 mg of SMCC in 50 µl DMSO). Incubate at room temperature for 1 h (or at 37°C for 30 min).
2. Desalt the activated enzyme solution on a desalting column. Wash the column with PBS and collect 500 µl/fraction.
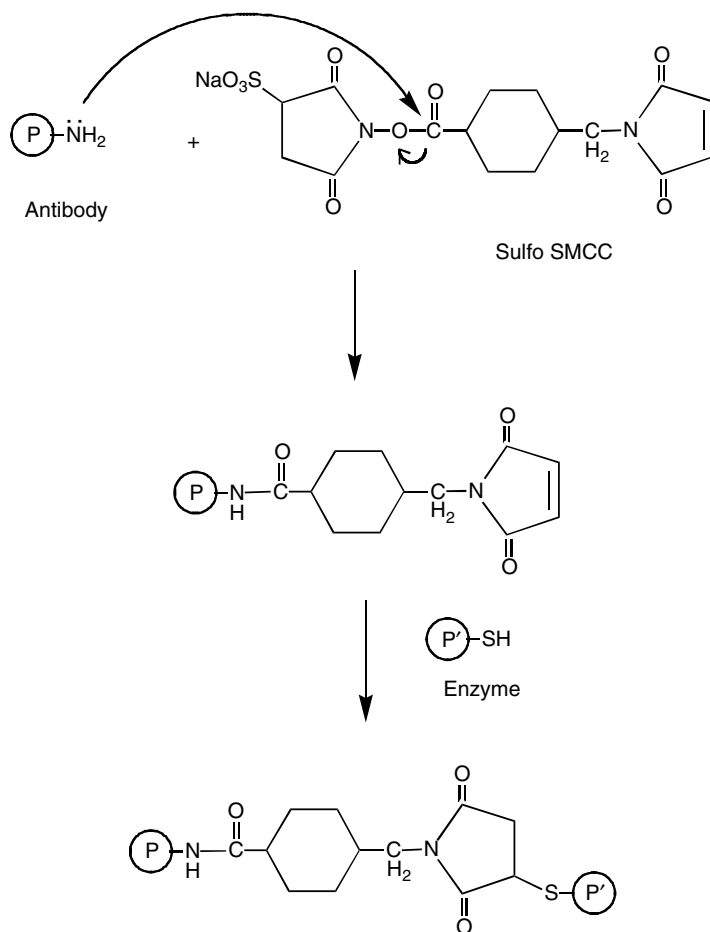
FIGURE 6.12 Cross-linking of an antibody and an enzyme with sulfo SMCC, a heterobi-functional cross-linker. The primary amine of the antibody molecule reacts with N-hydroxy-succinimide ester of sulfo SMCC to form an amide bond between SMCC and the antibody protein, with the release of N-hydroxysuccinimide. The sulfhydryl group on the enzyme then reacts with the activated antibody to form the conjugate.

3. Identify the protein peak by protein assay and pool the fractions containing protein.

Conjugation of the Activated Enzyme with the Reduced IgG

1. Mix the activated enzyme solution with the reduced IgG and incubate at 4°C for 2 h.
2. Desalt the conjugate on a desalting column using a buffer of choice. For example, alkaline phosphatase conjugate may be desalted using TBS

(25 mM Tris/0.15 M NaCl, pH 7.6) containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$.

N-Succinimidyl(4-iodoacetyl)aminobenzoate and its Sulfo Derivative

These cross-linkers consist of an NHS-ester group and an iodoacetyl group. N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB) is water insoluble and membrane permeable. SIAB is first dissolved in organic solvents (e.g., DMSO and dimethylformamide, or DMF) and then aliquoted into the aqueous reaction buffer. Sulfo-SIAB is water soluble and thus membrane impermeable. Conjugation is performed in two stages to avoid excessive polymerization, which is favored in one stage. Hydrolysis of the ester is a major concern, and the rate of hydrolysis increases at higher pH. For this reason, the NHS-ester reaction is performed first to minimize hydrolysis.

Reaction

The NHS-ester of the cross-linker reacts with the primary amines of the IgG to make an iodoacetyl-activated protein (Figure 6.13). The iodoacetyl group of the activated IgG is then replaced by the enzyme thiol group by nucleophilic substitution, resulting in a stable thioether linkage.

Working Procedure

1. To 1 ml IgG solution (1 mg/ml) in borate buffer (50 mM sodium borate, pH 8.3, 5 mM EDTA), add 10 µl of cross-linker (1.4 mg SIAB in 1 ml DMSO or 1.7 mg sulfo-SIAB in 1 ml water). Incubate at room temperature for 30 min.
2. Desalt the reaction mixture on a desalting column equilibrated with borate buffer. Collect 0.5 ml protein fractions.
3. Analyze each fraction with protein assay reagent and pool protein containing fractions.
4. Add 4 mg enzyme to the pool and allow the reaction at room temperature for 1 h at dark.
5. Block the excess active group with 5 mM cysteine at room temperature for 15 min at dark. Dialyze the conjugate with PBS.

6.1.4.4 Periodate Coupling

This procedure is very efficient for conjugating glycoprotein to antibodies. In this procedure (reductive amidation), the carbohydrate moiety of the enzyme provides the attachment to the antibody or protein.[17] It was originally developed for the conjugation of horseradish peroxidase to antibodies,[17] but can be applied to other glycoproteins. About five to six peroxidase molecules were conjugated with each IgG molecule.
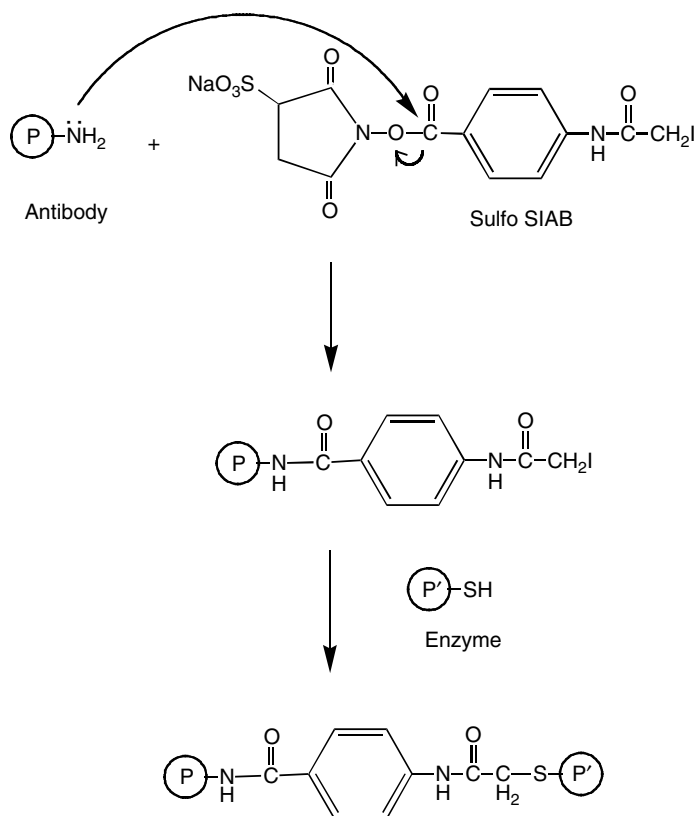
FIGURE 6.13 Cross-linking of an antibody and an enzyme with sulfo SIAB, a heterobifunctional cross-linker. The primary amine of the antibody molecule reacts with N-hydroxysuccinimide ester of sulfo SIAB to form an amide bond. The sulfhydryl group on the enzyme then reacts with the activated antibody to form a stable thioester bond.

Reaction

In this procedure, all the amino groups of the peroxidase are first blocked with 1-fluoro-2,4-dinitrobenzene (Sanger's reagent), and the carbohydrates of the enzyme are then oxidized with meta periodate to produce aldehyde and carboxyl groups (Figure 6.14). The aldehyde groups then form Schiff's bases with the free amino groups of added antibodies. Finally, these unstable amino-carbonyl adducts are stabilized by reduction with sodium borohydride.

Working Procedure for Coupling Peroxidase to Antibodies

1. To 5 mg of HRP in 1 ml of 0.3 M sodium bicarbonate (pH 8.1), add 0.1 ml of 1% 1-fluoro-2,4-dinitrobenzene (in ethanol). Incubate the mixture at room temperature.
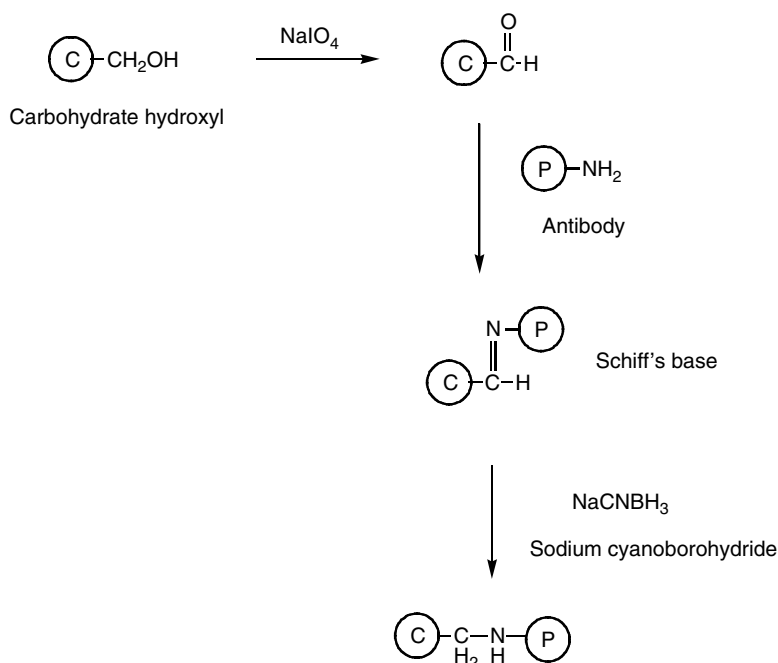
FIGURE 6.14 Periodate coupling of an antibody with an enzyme. Sodium periodate oxidizes the carbohydrate hydroxyl to form an aldehyde, which then reacts with the antibody primary amine to form a Schiff's base. Schiff's base is then reduced with sodium borohydride to form a stable covalent linkage.

2. To the mix, add 1 ml of 40 to 80 mM $NaIO_4$ (in distilled water) and incubate for 30 min at room temperature. The brown solution turns to yellow-green.
3. Add 1 ml of 160 mM ethylene glycol (in distilled water) to the mixture and incubate for 1 h at room temperature.
4. Dialyze activated peroxidase at 4°C with 10 mM sodium carbonate buffer, pH 9.5.
5. Add 5 mg of IgG (in 10 mM sodium carbonate buffer, pH 9.5) to the activated peroxidase solution and incubate for 3 h at room temperature.
6. Add 5 mg sodium borohydride (in water) and incubate at 4°C for 3 to 18 h.
7. Dialyze the conjugate with PBS.

6.1.5 BIOTIN LABELING

Several derivatives of biotin such as biotin-N-hydroxysuccinimide, biotin hydrazide, biotin maleimide, and iminobiotin-N-hydroxysuccinimide can be used for biotinylation of antibodies or proteins (Table 6.7). Most of these reagents are available from Pierce (Rockford, IL). Biotinylated antibodies can be detected with preformed avidin-biotin-enzyme complexes (ABC) in enzyme-linked immunosorbent assay (ELISA), dot blotting, and immunohistochemistry. A variety of biotinylation

TABLE 6.7
Common Biotinylating Agents and Their Characteristics[a]

Biotinylating Reagent	Solubility	Target Amino Acid	Recommended Reaction pH
NHS-biotin	DMSO, sulfo derivative in water	Primary amine	Around pH 8.0
NHS-LC-biotin	Water	Primary amine	Around pH 8.0
Biotin-BMCC	DMSO	Sulfhydryl	pH 6–7
Biotin-HPDP	DMSO	Sulfhydryl	pH 6–7
Iodoacetyl LC-biotin	DMSO	Sulfhydryl	pH 6–7
Biotin hydrazide	DMSO	Carboxylic acid	Around pH 5.5

[a] From Reference 10.

reagents are available for targeting most functional groups in proteins, such as primary amines, sulfhydryls, carboxyls, and carbohydrates.

6.1.5.1 Amine-Reactive Biotinylation Reagents

Among the amine-reactive biotinylation reagents, N-hydroxysuccinimide esters of biotin and iminobiotin-N-hydroxysuccinimide are commonly used.[18,19] The reagents are water insoluble, but their sulfo derivatives are water soluble. The water-insoluble reagents are usually dissolved in dimethylsulfoxide or dimethylformamide and then aliquoted into the aqueous reaction mixture. Most proteins are compatible up to 20% final concentration of DMSO/DMF.

Reaction

Both NHS- and sulfo-NHS-esters react with primary amines of the protein, resulting an amide bond (Figure 6.15 and Figure 6.16). The amine reacts with the ester by nucleophilic attack, and N-hydroxysuccinimide (or N-hydroxysulfosuccinimide) is released as a byproduct. Because the NHS-ester is targeted by the nucleophile primary amine, the reaction is facilitated at pH neutral or above where the amines remain deprotonated. However, hydrolysis of NHS-ester is problematic at higher pH. The half-life of hydrolysis for NHS-ester (or sulfo-NHS-ester) is 2 to 4 h at pH 7, but the half-life decreases to a few minutes at pH 9.

Note: buffers containing amines (such as Tris and glycine) should not be used because they compete with the reaction.

Working Procedure for Biotin-Labeling Using Succinimide Ester

1. To 1 ml of antibody solution (1 to 3 mg/ml in 0.1 M sodium borate buffer, pH 8.8), add 2.5 to 25 µl of N-hydroxysuccinimide biotin (10 mg/ml in dimethylsulfoxide). Leave the reaction at room temperature for 4 h with stirring.
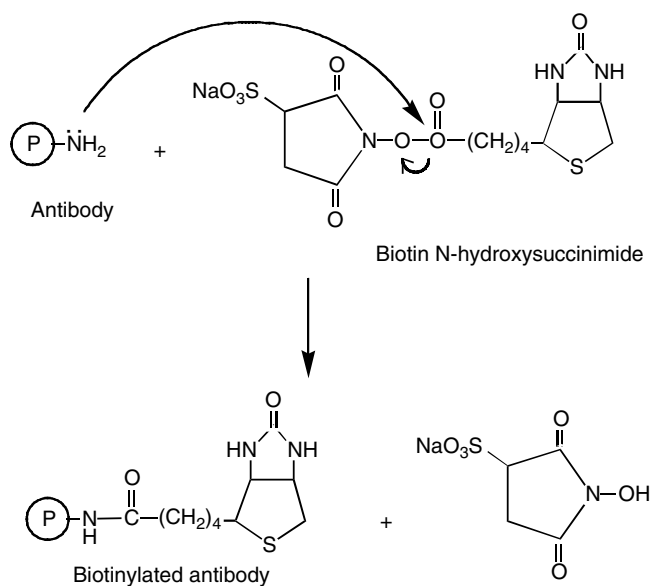
FIGURE 6.15 Biotin labeling of an antibody with biotin N-hydroxysuccinimide. In this case, the primary amine on the antibody molecule reacts with hydroxysuccinimide ester of the biotin, producing a stable covalent amide bond, with the release of N-hydroxysuccinimide.
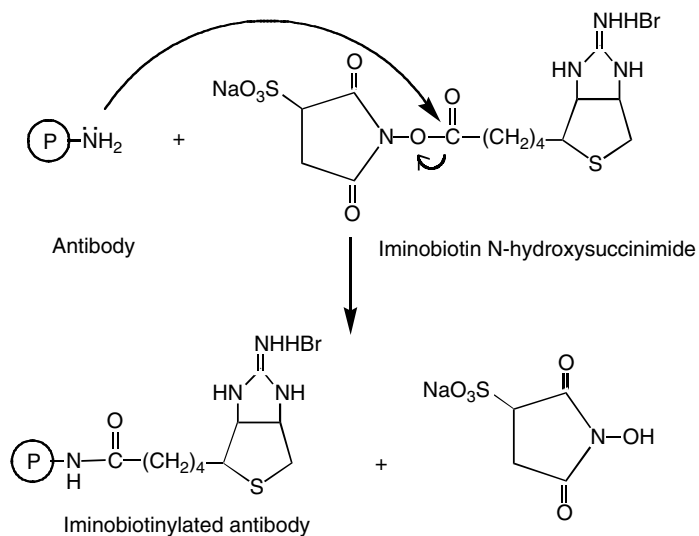
FIGURE 6.16 Biotin labeling of antibody with iminobiotin N-hydroxysuccinimide. The primary amine on the antibody molecule reacts with hydroxysuccinimide ester of iminobiotin producing a stable covalent amide bond with the release of N-hydroxysuccinimide.

2. Add 1 µl of 1 M ammonium chloride per 12.5 µg of biotin ester and incubate at room temperature for 10 min.
3. Dialyze the labeled antibody with PBS to remove unreacted biotin.

6.1.5.2 Sulfhydryl-Reactive Biotinylation Reagents

Several biotin derivatives, such as biotin maleimide, biotin dithiopyridyl, and biotin α-haloacyl, are available for reaction with sulfhydryl groups. Specific examples of these derivatives are 1-biotinamido-4-[4′-(maleimidomethyl)-cyclohexane-carbox-amido)butane (biotin-BMCC), N[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio)propi-onamide (biotin-HPDP), and N-iodoacetyl-N-biotinyl hexylene diamine (iodoacetyl-LC-biotin) (Pierce, Rockford, IL). Sulfhydryl group biotinylation may provide an advantage in an application where N-hydroxysuccinimide-mediated con-jugation become problematic. For example, amines are often found at the active site of the proteins, and thus modification of amines with N-hydroxysuccinimide results in the inactivation of the protein.

Note: conjugation of proteins with sulfhydryl specific biotinylation reagents should not be performed in buffers containing thiols, such as β-mercaptoethanol, dithiothreitol, and mercaptoethylamine.

Biotin-BMCC (Pierce, Rockford, IL)

Biotin-BMCC is a water-insoluble maleimide containing a biotinylation reagent that reacts with the free sulfhydryl groups about 1,000 times faster than the amines at pH 7. Above pH 7, cross-reactivity toward primary amines can occur. At higher pH, hydrolysis of maleimide can also occur, resulting in an inactive maleamic acid derivative.

Reaction

The maleimide group of Biotin-BMCC reacts with free sulfhydryl groups of protein, resulting in a thiol conjugate (Figure 6.17).[20]

Working Procedure

1. Reduce 2.5 mg IgG (in 100 mM sodium phosphate/5 mM EDTA, pH 6) with 6 mg mercaptoethylamine at 37°C for 90 min.
2. Remove the excess mercaptoethylamine on a desalting column.
3. To the reduced IgG solution, add 0.5 mg biotin-BMCC in 100 µl DMSO. Incubate the reaction at room temperature for 2 h.
4. Remove excess biotin-BMCC by dialyzing or desalting.
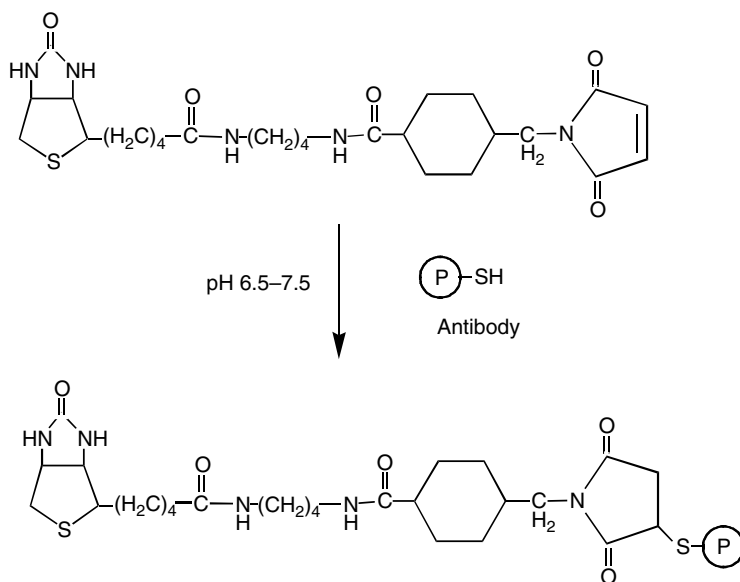
FIGURE 6.17 Biotin labeling of an antibody with biotin BMCC (1-biotinamido-4-(4′-[male-imido-ethylcyclohexane]-carboxamido) butane (Pierce). The sulfhydryl group on the antibody molecule reacts with maleimide of biotin to form a stable covalent bond.

Biotin-HPDP (Pierce, Rockford, IL)

Biotin-HPDP is a water-insoluble, sulfhydryl-reactive, and cleavable biotinylation reagent. The reagent offers several advantages. The reaction of Biotin-HPDP with protein thiols can be easily followed by measuring the reaction's leaving group, pyridine-2-thione at 343 nm (molar extinction coefficient is 8.08×10^3 M^{-1} cm^{-1}). After biotinylation, the conjugate can be cleaved from the biotin.

Reaction

Biotin-HPDP reacts with free sulfhydryls of protein, releasing pyridine-2-thione (Figure 6.18).

Working Procedure

1. Reduce 2.5 mg IgG (in 100 mM sodium phosphate/5 mM EDTA, pH 6) with 6 mg mercaptoethylamine at 37°C for 90 min.
2. Remove the excess mercaptoethylamine on a desalting column.
3. To the reduced IgG solution, add 0.22 mg Biotin-HPDP (in 0.1 ml dimethyl-formamide). Incubate the mixture at room temperature for 90 min. The progress of the reaction can be monitored by measuring absorbance of the released pyridine-2-thione at 343 nm. Purify the conjugate on a desalting column.
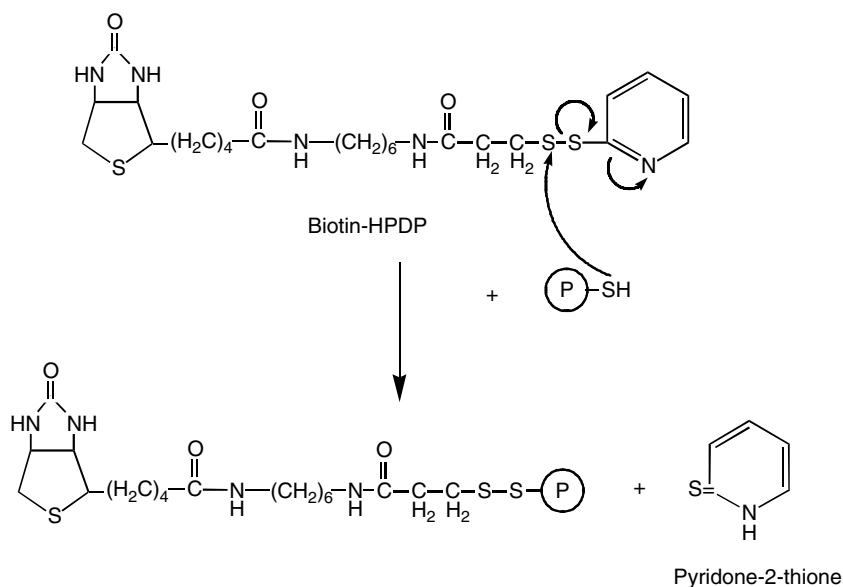
FIGURE 6.18 Reaction of biotin-HPDP with protein sulfhydryls.

Iodoacetyl-LC-biotin (Pierce, Rockford, IL)

Iodoacetyl-LC-biotin is a water-insoluble and sulfhydryl-reactive biotinylation reagent that reacts mainly with thiol groups at pH 7.5 to 8.5. The protein or IgG is first reduced to obtain free sulfhydryl groups. After the reduction, the excess reducing agent must be removed prior to conjugation to avoid any competition with the conjugating agent.

Reaction

In this reaction, a stable thioether bond is formed by nucleophilic substitution of iodine with a thiol group (Figure 6.19).

Working Procedure

1. Reduce 4 mg IgG (in 200 μl of 100 mM sodium phosphate/5 mM EDTA, pH 6) with 1.4 mg 2-mercaptoethylamine at 37°C for 90 min.
2. Remove the excess mercaptoethylamine on a desalting column.
3. To the reduced IgG solution, add 15 μl of 4 mM iodoacetyl-LC-biotin (in dimethylformamide). Incubate the mixture at room temperature for 90 min in the dark. Purify the conjugate on a desalting column.
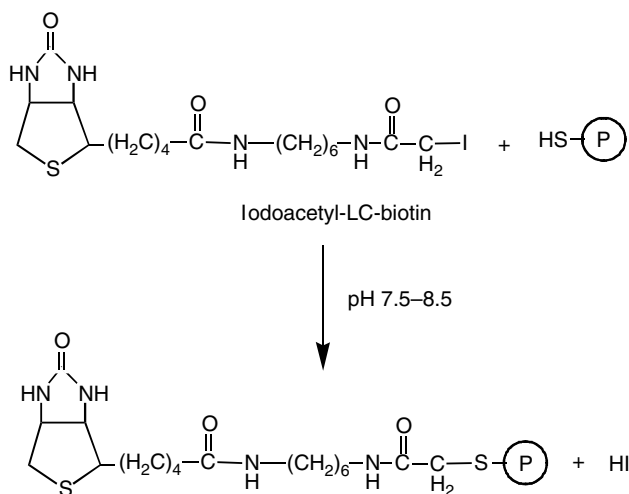
FIGURE 6.19 Reaction of iodoacetyl-LC-biotin with protein sulfhydryls.

6.1.5.3 Carboxyl- and Carbohydrate-Reactive Biotinylation Reagents

Biotin hydrazide and biotin-LC-hydrazide (Pierce, Rockford, IL) are used to conjugate IgG molecules by targeting their carboxyl groups.

Reaction

Biotin hydrazide reacts with carboxylic acid groups in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (Figure 6.20) (21). Biotin hydrazide reacts also with the aldehyde group, which can easily be generated from an immunoglobulin with sodium periodate (Figure 6.21).

Working Procedure

1. Prepare 5 mg/ml protein solution in 0.1 M MES buffer [(2-N-morpholino)ethanesulfonic acid], pH 5.5.
2. Add 25 μl of 50 mM biotin hydrazide (in DMSO) and 12.5 μl of 100 mg/ml EDC (in MES buffer) to 1 ml of protein solution and mix.
3. Incubate the reaction mixture overnight at room temperature with mixing.
4. Dialyze the biotinylated protein with PBS.
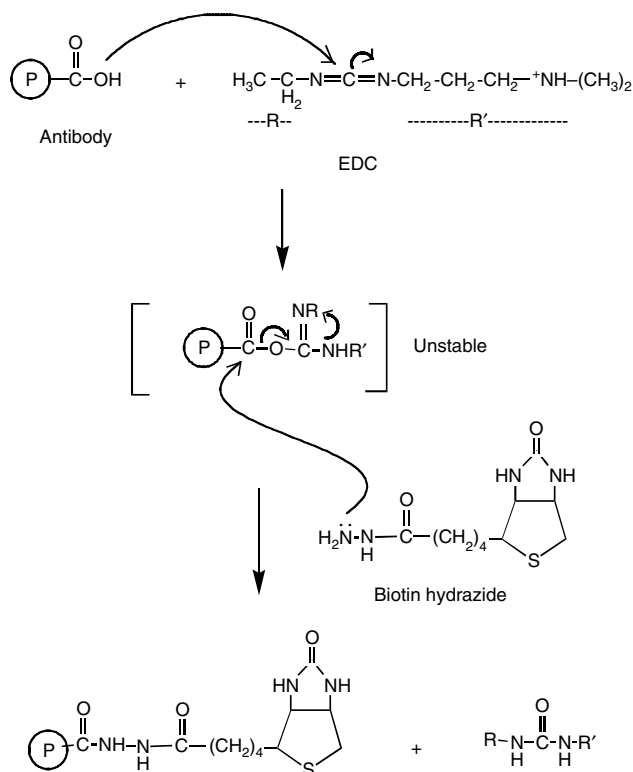
FIGURE 6.20 Biotin labeling of an antibody with biotin hydrazide. The carboxylic acid of the antibody molecule reacts with EDC to produce an unstable adduct, which then readily reacts with primary amine of biotin hydrazide to form the conjugate.

Biotinylation of IgG after Periodate Oxidation

1. Prepare 2 mg/ml IgG solution in 0.1 M sodium acetate buffer, pH 5.5 and keep on ice.
2. Prepare 20 mM sodium meta-periodate solution in 0.1 M sodium acetate buffer, pH 5.5 and keep on ice at dark.
3. To 1 ml of cold IgG solution, add 1 ml of cold sodium meta-periodate solution. Mix well and incubate on ice for 30 min in the dark.
4. Stop the oxidation reaction with glycerol (final concentration 5 mM) for 5 min on ice.
5. Dialyze the oxidized sample with 0.1 M sodium acetate buffer, pH 5.5.
6. To the dialyzed sample, add biotin hydrazide to a final concentration of 5 mM. Incubate the reaction mixture at room temperature for 2 h.
7. Dialyze the biotinylated IgG with PBS.
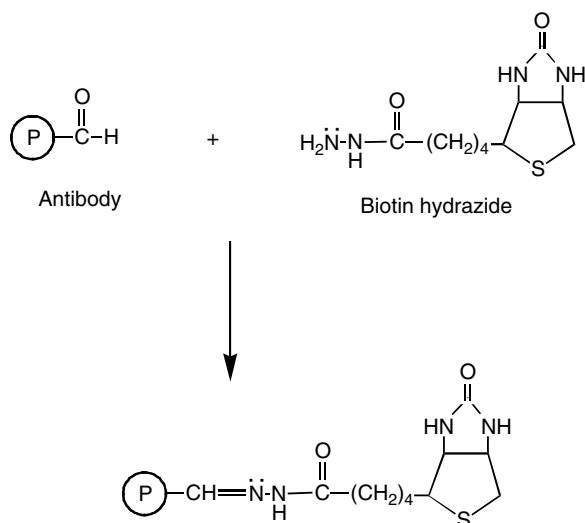
FIGURE 6.21 Biotin labeling of an antibody with biotin hydrazide. The aldehyde on the antibody molecule reacts with biotin hydrazide, with the release of a water molecule. The reactive aldehyde group on the antibody can easily be generated with sodium periodate.

6.1.6 FLUORESCENT LABELING

Several fluorescent compounds such as fluorescein isothiocyanate (Figure 6.22), fluorescein N-hydroxysuccinimide, and rhodamine N-hydroxysuccinimide (Figure 6.23) are available commercially for the attachment to the antibodies. The conjugation is usually performed at pH 8.5 at room temperature for 4 h.[22,23] The number of fluorescein molecules bound to the protein is estimated by measuring the absorbance of the labeled protein at 495 and 280 nm.

Reaction

The amine-reactive fluorescent compounds react with the free amino groups of proteins, resulting in fluorescent conjugates (Figure 6. 22 and Figure 6.23).

Working Procedure

1. To 1 ml of antibody solution (2 mg/ml in 0.1 M sodium carbonate, pH 8.5), add 50 µl of fluorochrome (1 mg/ml in DMSO) slowly in 5 µl aliquots with gentle stirring. Leave the reaction in the dark at 4°C.
2. After 8 h, add ammonium chloride to get 50 mM and after incubation for 2 h at 4°C, separate the labeled antibody on a desalting column.
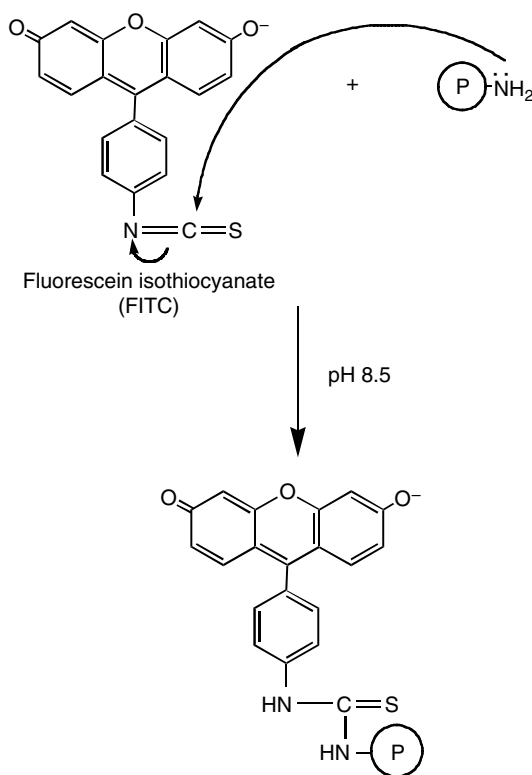
FIGURE 6.22 Fluorescent labeling of antibody with FITC

6.1.7 COLLOIDAL GOLD LABELING

Gold-labeled antibodies are used in immunohistochemistry,[24] and their binding is visualized by scanning electron microscopy (SEM) as well as light microscopy. The size of the gold particles usually dictates the choice of microscope. For example, large particles (15 to 50 nm diameter) are used in SEM because of the lower resolution of the scanners, whereas small particles (1 to 5 nm diameter) are preferred in light microscopy because of greater penetration of the antibodies into cells through membranes.

Gold sols are prepared from a super-saturated solution of metallic gold, which is formed by the reduction of gold chloride ($HAuCl_4$). As the reduction proceeds, the gold atoms cluster and form the seeds or nuclei of the gold particles. The size of the gold particles and the number of nuclei depend on the speed of reduction. Thus, it is essential to use a controller of the reduction process. In this process, sodium citrate is used as the reducing agent and tannic acid as the controller. The desired size range of gold particles is obtained by altering the amount of tannic acid in the reducing mixture.
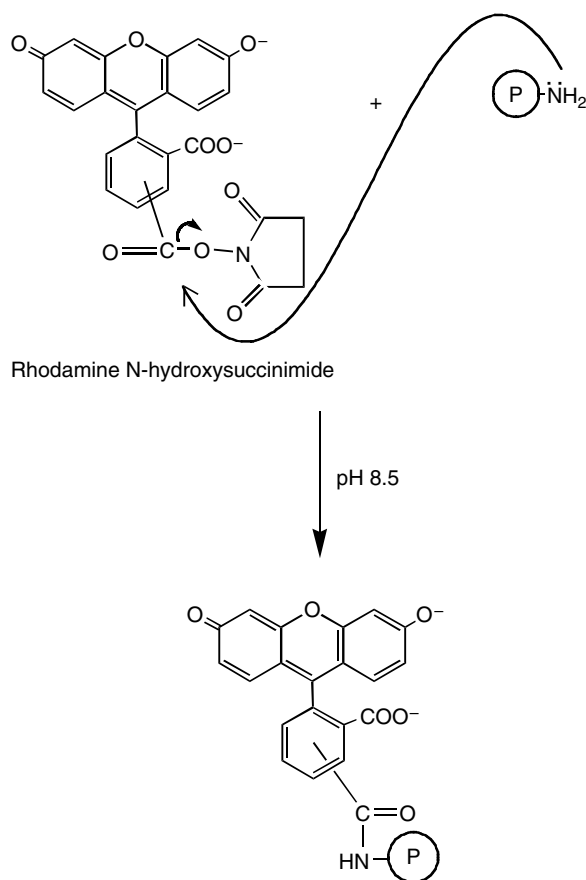
FIGURE 6.23 Fluorescent labeling of an antibody with rhodamine N-hydroxysuccinimide.

Working Procedure for the Preparation of Gold Sols[25]

The procedure described below yields golds of particle size 4 to 5 nm diameter.

1. Prepare 80 ml of 0.0125% gold chloride solution in distilled water.
2. Prepare 20 ml of reducing mixture containing 0.2% trisodium citrate, 0.1% tannic acid, and 2.5 mM potassium carbonate.
3. Warm the two solutions above to 60°C on the hot plate and add the reducing mixture to the gold chloride solution while stirring. Change of color to red is indicative of the sol formation.
4. Finally, heat the mixture to boiling and then cool.

Non-covalently linked gold-protein conjugates are readily formed (1) by using a minimum quantity of the protein, barely enough to stabilize the gold sol, and (2) at conjugation pH, which is 0.5 units higher than the pI of the protein. The amount of protein is determined from a titration experiment in which twofold serially diluted protein is added to a fixed volume of pH-adjusted gold solution. Sodium chloride (usually 10%) is then added to each mix to produce flocculation of gold solution. The correct ratio is determined in the tube, where red gold sol starts to flocculate and the red color changes to blue.

1. To prepare gold-labeled protein or antibody, add the exact amount of protein as estimated above to the gold sol.
2. After 5 min, add 1 ml of 1% aqueous polyethylene glycol (PEG) (Carbowax 20, Union Carbide) as the stabilizer.
3. Centrifuge the mixture at 125,000 g for 45 min (speed varies according to the size of the particles) to separate in two phases.
4. Remove top loose phase of the pellet and resuspend in PBS containing 0.2 mg/ml PEG. The conjugate can be stored at 4°C for up to 1 year.

6.2   DETECTION OF ANTIBODY BINDING

Once the labeled antibody is attached to the antigen directly or via a primary antibody, the bound antibody is then detected by one of several methods such as autoradiography, colorimetry, fluorometry, or microscopy.

6.2.1   Detection of Iodine-Labeled Antibodies

Iodine-labeled reagents are detected by a gamma counter and autoradiography for immunoassay and immunoblotting, respectively. For autoradiographic detection, the blot containing the iodine-labeled sample is placed in direct contact with an X-ray film at –70°C with an intensifying screen.

6.2.2   Detection of Enzyme-Labeled Antibodies

A wide range of colorimetric substrates are available to detect the reporter enzymes in a solution assay, such as in microtiter wells or in a solid phase assay such as in a membrane.

6.2.2.1   Detection of Peroxidase Conjugate with Two Types of Substrates (Hydrogen Donors): Soluble and Precipitating

Hydrogen peroxide is widely used as a substrate for peroxidase (hydrogen-peroxide oxidoreductase, EC 1.11.1.7); many substances are available for hydrogen donors (DHs), but the choice of hydrogen donor should be dependent on the type of assay

TABLE 6.8
Detection of Peroxidase Conjugates

Substrate	Nature of Substrate	Color of the End Product	Application
ABTS	Chromogenic, soluble	Bluish-green	ELISA
TMB	Chromogenic, soluble	Yellow	ELISA
OPD	Chromogenic, soluble	Yellowish-orange	ELISA
4-CN	Chromogenic, precipitating	Purple	Blotting
DAB	Chromogenic, precipitating	Brown	Immunohistochemistry
DAB metal enhanced	Chromogenic, precipitating	Black	Immunohistochemistry
4-CN/DAB	Chromogenic, precipitating	Black	Blotting
TMB-blotting	Chromogenic, precipitating	Blue	Blotting
Luminol	Chemiluminiscent	Chemiluminiscence	Blotting

(Table 6.8). For example, in ELISA application of HRP conjugate-soluble hydrogen donors, TMB or ABTS is used, but for Western blot and histochemistry hydrogen donors, DAB or chloronaphthol is used to achieve an insoluble colored product.

Principle

Peroxidase transfers hydrogen from hydrogen donors to hydrogen peroxide as follows:

$$H_2O_2 + 2\ DH \rightarrow 2\ H_2O + 2\ D$$
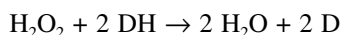
The plausible catalytic properties of peroxidase is shown in Figure 6.24.[27] Peroxidase loses two electrons from the oxidation by peroxide-forming compound I, which, in turn, returns to its initial state by gaining two successive electrons from the reduction by hydrogen donor. The reaction results in the formation of an oxidized product that is detectable colorimetrically. The amount of substrate (H_2O_2) is critical for peroxidase activity, since an excess of substrate inactivates the enzyme. Hydrogen donors that are suitable for assays should have several characteristics. The oxidized state of the donor must be stable and can be quantitated spectrophotometrically in the case of ELISA. Hydrogen donors should have a negligible oxidation rate in the absence of an enzyme. The donors should have a much slower reaction rate with the enzyme than the reaction rate between the enzyme and substrate. The oxidation reaction of the hydrogen donors should be almost pH independent.

Note: cyanide, sulfide, fluoride, and azide inhibit enzyme activity and thus must be removed from the reaction mixture prior to enzyme detection.
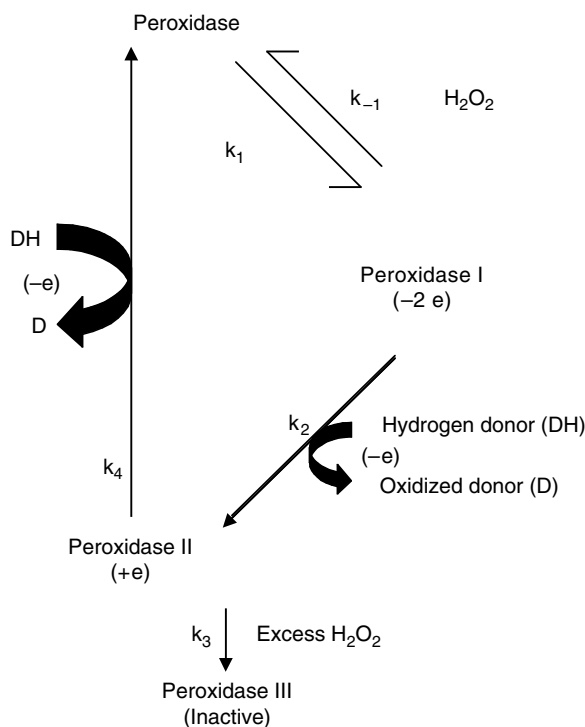
FIGURE 6.24 Catalytic actions of horseradish peroxidase. The peroxidase is oxidized by hydrogen peroxide to peroxidase I, with the release of two electrons. Peroxidase I is then returned to the initial state following two successive reductions by hydrogen donor (DH).

6.2.2.1.1   Soluble Substrates (Donors) for Peroxidase

Several soluble substrates (donors) are available for HRP, but the selection of a suitable substrate is usually made based on its sensitivity, solubility, and the wavelength at which the final product is to be read.

3,3′,5,5′ tetramethylbenzidine

3,3′,5,5′ tetramethylbenzidine (TMB) is often used with horseradish peroxidase in ELISA applications. TMB can be used to measure the reaction at two conditions: unstopped, which produces a blue product (absorption at 650 nm), and stopped, which produces a yellow product with sulfuric acid (absorption at 450 nm). The plate reader should have both filters to measure unstopped and stopped reactions. However, optimum sensitivity of this reagent (two- to four-fold increase) occurs at acidic condition (pH 5), and the absorbance of the substrate appears to be unaffected by the diluent.
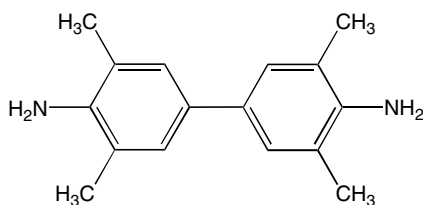
FIGURE 6.25 Structure of TMB.

Reaction

In this assay, HRP catalyzes H_2O_2 oxidation of TMB (Figure 6.25) to yield the colored product.[28] TMB has a maximum absorbance at 285 nm, but the loss of one electron will yield a blue product with major absorbances at 370 nm and 652 nm. A loss of two electrons under acidic conditions will yield a yellow product, with maximum absorbance at 450 nm. A green reaction product may result from partial conversion to the yellow product from the blue intermediate.

Working Procedure

1. For the detection of bound peroxidase-labeled antibody, prepare TMB substrate solution by adding 0.1 ml of 1 mg/ml (in dimethylsulfoxide) to 9.9 ml of 0.1 M sodium acetate. Add 3.3 µl of 30% hydrogen peroxide to the substrate solution and incubate each microtiter well with 50 µl of substrate solution for 10 to 20 min at room temperature until it appears pale blue.
2. Add 50 µl/well 1 M sulfuric acid which changes positive reaction to yellow.
3. Read at 450 nm.

o-phenylenediamine

Like TMB, the peroxidase activity with o-phenylenediamine (OPD) (Figure 6.26) hydrogen donor can also be detected at two wavelengths (450 nm for unstopped and 490 nm for stopped reaction). Like TMB, the sensitivity of OPD increases by two- to fourfold at acidic condition.

Reaction

It forms a soluble product that can be measured at 450 nm but yields an orange product that is easily detectable at 490 nm after the reaction is stopped with sulfuric acid.[29]

o-Phenylenediamine (OPD)
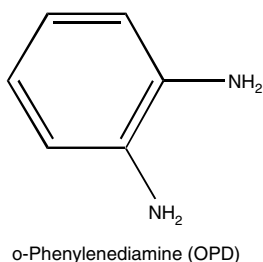
FIGURE 6.26 Structure of OPD.

Working Procedure

1. Prepare 0.5 to 1 mg/ml OPD solution in 50 mM phosphate, pH 5. Add 1 μl 30% hydrogen peroxide per ml of OPD solution.
2. Add 100 μl of the above solution to each ELISA well and incubate for 10 to 30 min.
3. Stop the reaction with 100 μl 2.5 M sulfuric acid and read at 490 nm.

2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) Diammonium Salt

2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (absorbance maximum at 340 nm) is a colorless water-soluble HRP hydrogen-donor that has a lower sensitivity than OPD or TMB. Its color development is slower, and so the reaction can be followed with a kinetic reader.

Reaction

ABTS yields a green end product upon reaction with peroxidase and hydrogen peroxide[30] (Figure 6.27). The green product has two major absorbance peaks: at 410 nm and 650 nm. ABTS is less sensitive than OPD or TMB, and its color development is slower.

2, 2′−Azinobis (3−ethylbenzothiazoline−6−
sulfonic acid) diammonium salt (ABTS)
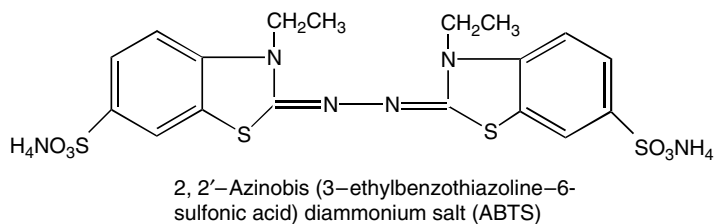
FIGURE 6.27 Structure of ABTS.

1. Prepare 1 mg/ml ABTS in 50 mM phosphate-citrate buffer, pH 5. Add 1 μl of 30% hydrogen peroxide per ml of ABTS solution.
2. Add 100 μl of substrate solution to each ELISA well and incubate at room temperature for 30 to 60 min.
3. Stop the reaction by adding 100 μl of 1% SDS solution to each well.
4. Read the wells at 405 to 410 nm.

6.2.2.1.2 Precipitating Substrates for Peroxidase

Several precipitating substrates are available for use in immunochemical and immunoblotting studies.

3,3′ Diamino Benzidine Tetrahydrochloride

3,3′ Diamino Benzidine Tetrahydrochloride (DAB) (Figure 6.28) is one of the most sensitive substrates for HRP and is compatible with a wide range of common histological stains including electron microscopy. Pierce's metal-enhanced DAB substrate is about 50 times more sensitive than only DAB and is excellent for use in immunohistochemistry because of its high signal-to-noise ratio. The DAB substrate is not suitable for blotting, since the brown colored product by DAB does not photograph well. However, the combination of 4-chloro-1-naphthol (4-CN) and DAB (kit available from Pierce) has a synergistic effect, providing higher sensitivity and producing a black-colored product suitable for photographs.[31]

Reaction

Electrons are transferred by HRP from the DAB to the peroxide, yielding an insoluble brown-colored end product.[32] The color can be intensified with the addition of metals, such as nickel, copper, silver, and cobalt, which form complexes. The brown insoluble marker can also be readily chelated with osmium tetroxide. This property makes DAB ideal for electron microscopy.

3, 3′–Diaminobenzidine terahydrochloride
(DAB)
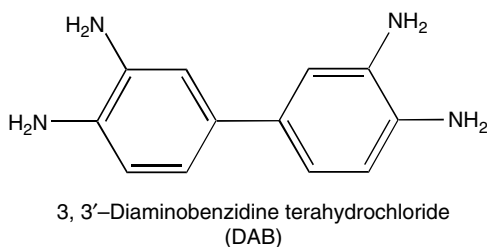
FIGURE 6.28 Structure of DAB.

Working Procedure

1. Dissolve 6 mg of DAB in 10 ml of 50 mM Tris (pH 7.6) and filter to remove any precipitate.
2. Add 10 μl of 30% H_2O_2 to the DAB solution and use the mix to detect enzyme activity on a membrane or tissue specimen.
3. Continue color development until it yields a dark brown color.
4. Stop reaction by rinsing with PBS.
5. When DAB is used in the presence of metal ion, 6 mg of DAB is dissolved in 9 ml of Tris (50 mM), pH 7.6 and add 1 ml of 0.3% $NiCl_2$ or $CoCl_2$. The solution is then mixed with 10 μl of 30% H_2O_2.

4-chloro-1-naphtho

4-chloro-1-naphtho (Figure 6.29) is also used for chromogenic detection of HRP in Western blotting and immunohistochemistry.[33] It is less sensitive than DAB. The color reaction should be photographed immediately, because color fades on storage.

Reaction

Electrons are lost from 4-CN due to HRP reaction in the presence of H_2O_2, resulting in a blue/black precipitate.

Working Procedure

1. Dissolve 60 mg of 4-chloro-1-naphthol in 20 ml of methanol.
2. Add 60 μl of 30% H_2O_2 in 100 ml of 0.1 M Tris buffer (pH 7.4) and mix with 4-CN.
3. Place the blot in the substrate solution and continue development until a blue-black reaction product is formed.
4. Stop reaction by rinsing with PBS.

4-Chloro-1-naphthol
(4-CN)
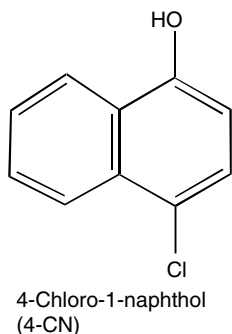
FIGURE 6.29 Structure of 4-CN.
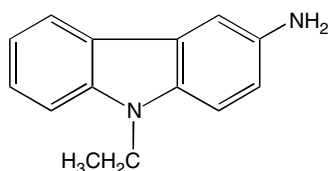
3-Amino-9-ethyl carbazole

FIGURE 6.30 Structure of AEC.

3-amino-9-ethyl carbazole

3-amino-9-ethyl carbazole (AEC) (Figure 6.30) (Pierce) is an excellent secondary marker[34] when DAB or 4-CN are used as the primary markers in multiple staining procedures. The reddish-brown color of AEC contrasts well with the brown-colored product from DAB and the blue product from 4-CN. AEC is also less sensitive than DAB.

Reaction

AEC yields a reddish-brown product upon reaction with peroxidase and hydrogen peroxide.

Working Procedure

1. For the detection of HRP activity, add 0.67 ml of 0.4% AEC to 10 ml of 0.1 M sodium acetate (pH 5.2) and filter, if necessary. Add 10 µl of 30% H_2O_2 to AEC solution.
2. Place the blot in the substrate and detect HRP enzyme activity in 1 to 5 min.

6.2.2.2   Detection of Phosphatase Conjugate with Two Types of Substrates: Soluble and Precipitating

Alkaline phosphatase transfers the phosphoryl residue via a phosphoryl-enzyme intermediate, as shown in Figure 6.31. Table 6.9 shows several soluble and insoluble colorimetric substrates for alkaline phosphatase (orthophosphoric mono ester phosphohydrolase, alkaline optimum, EC 3.1.3.1), and like peroxidase, the choice of susbtrate is dependent on the type of assay.

6.2.2.2.1   Soluble Substrates for Phosphatase

Alkaline phosphatase catalyses the hydrolysis of phosphate esters in alkaline conditions (pH 8 to 10). The assay should not be performed in PBS, since inorganic phosphates compete with the substrate.

APase + ROP

k_1 k_{-1}

APase.ROP

k_2 k_{-2}

APase.P + ROH
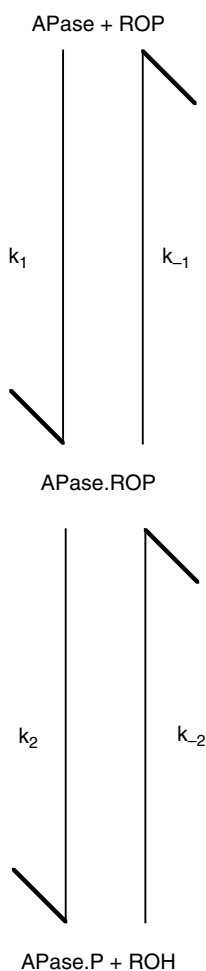
FIGURE 6.31 Catalytic actions of alkaline phosphatase. The phosphate ester (ROP) is hydrolyzed by alkaline phosphatase (APase).

Disodium Salt of p-nitrophenyl Phosphate

Disodium salt of p-nitrophenyl phosphate (PNP) is widely used as a soluble substrate.

Reaction

The enzyme converts the p-nitrophenyl phosphate to yellowish p-nitrophenol in alkaline condition[35] (Figure 6.32 a). p-nitrophenol behaves as acid due to the presence of a strong deactivating group ($-NO_2$), which pulls electrons and thus facilitates the release of H^+ by dissociating the -OH group (see Figure 6.32 b).

TABLE 6.9
Detection of Phosphatase Conjugate

Substrate	Nature of Substrate	Color of the End Product	Application
p-nitrophenyl phosphate	Chromogenic, soluble	Yellow	ELISA
Phenolphthalein monophosphate	Chromogenic, soluble	Red	ELISA
NADP/INT	Chromogenic, soluble	Purple	ELISA
4-methylumbelliferyl phosphate	Fluorescent, soluble	Fluorescence	ELISA
BCIP/NBT	Chromogenic, precipitating	Blue	Blotting, histochemistry
Fast red/naphthol AS-MX-phosphate	Chromogenic, preceipitating	Red	Histochemistry
Lumigen	Chemiluminiscent, precipitating	Chemiluminescence	Blotting

Working Procedure

1. Prior to developing the enzyme, wash the plate with 10 mM diethanolamine (pH 9.5) and detect the enzyme activity with 50 µl of 0.1% p-nitrophenyl phosphate (in diethanolamine) in each well.
2. Continue development until the positive wells appear bright yellow.
3. Add 50 µl of stop solution (0.1 M EDTA) and read the plate at 405 nm.

Phenolphthalein monophosphate

Phenolphthalein monophosphate (PMP) is another soluble susbstrate for alkaline phosphatase and is used in ELISA. PMP is usually dissolved in 1.1 M diethanolamine-HCl, pH 8.6 containing 2 mM $MgCl_2$.

Reaction

Alkaline phosphatase catalyzes PMP to produce a red product that is measured at 550 nm.

Working Procedure

1. Prepare 3 mM PMP solution in the above diethanolamine buffer.
2. Add 100 µl PMP solution into each microtiter well to detect phosphatase activity.
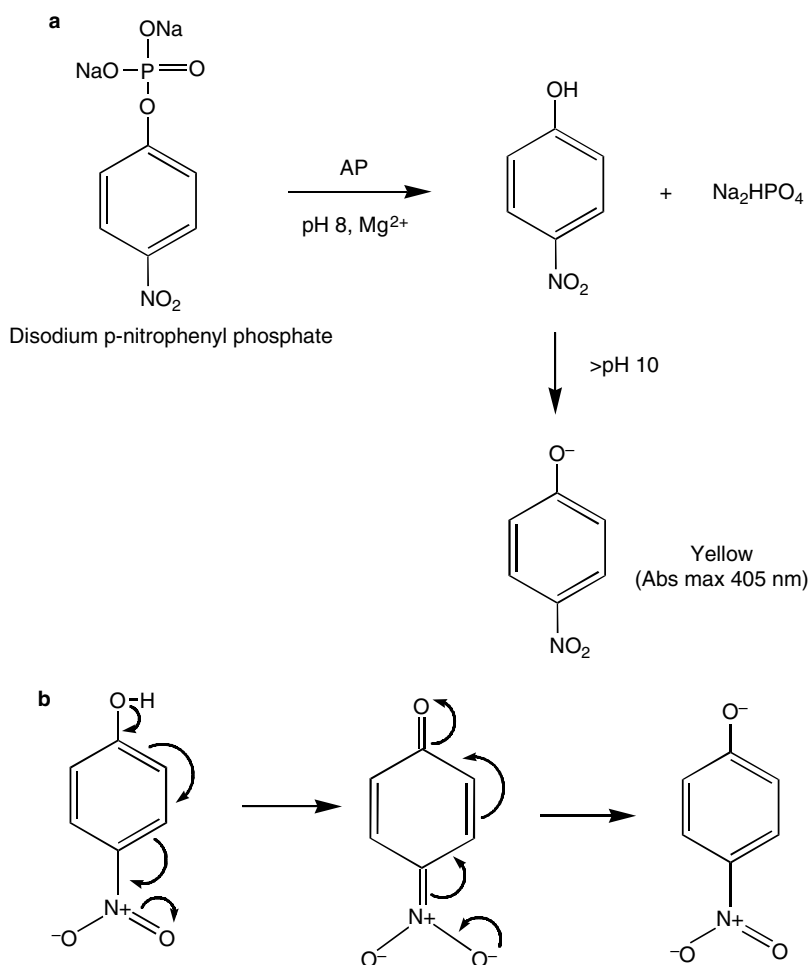
FIGURE 6.32 Reaction of alkaline phosphatase with disodium p-mitrophenyl phosphate, resulting a yellowish p-nitrophenol at alkaline condition (a); tautomerism of p-nitrophenol (b).

Nicotinamide adenine dinucleotide phosphate

Nicotinamide adenine dinucleotide phosphate (NADP) is used in an ELISA system for the detection of alkaline phosphatase. Together with two other enzymes, alcohol dehydrogenase and diaphorase, this substrate provides a very sensitive assay for alkaline phosphatase (detectable in the pg range). A kit including a working procedure is available from Koma Biotech Inc., Japan (koma@komabiotech.biz).

Reaction

In this procedure, antibody-conjugated alkaline phosphatase dephosphorylates NADP to produce nicotinamide adenine dinucleotide (NAD) which, in turn, is used
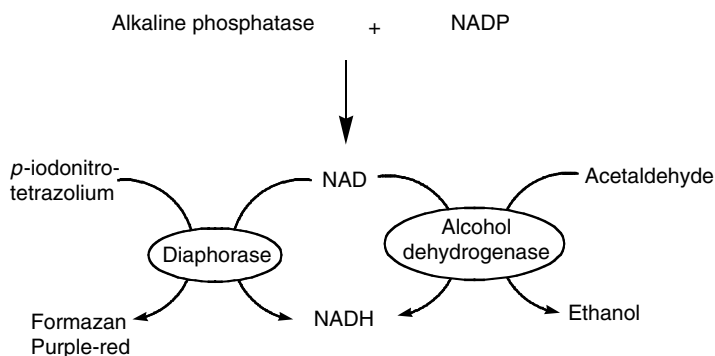
FIGURE 6.33 Reaction of alkaline phosphatase with NADP, resulting in NAD, which, in conjugation with alcohol dehydrogenase and and diaphorase, turns p-iodonitrotetrazolium to a purple-red formazan.

by alcohol dehydrogenase and diaphorase (Figure 6.33). The latter enzymes produce a pair of cycling redox reactions, which result in the reduction of a colorless p-iodonitrotetrazolium to a purple-red formazan (measured at 492 nm).[36]

Blue Phos[R]

Blue Phos[R] (Kirkegaard & Perry Laboratories, Gaithersburg, MD), a soluble form of BCIP (5-bromo-4-chloro-3-indolylphosphate) (see Figure 6.34 for the structure of BCIP), is a more sensitive substrate than p-nitrophenylphosphate.

Reaction

In the presence of phosphatase, Blue Phos[R] produces a blue reaction product with an absorption maximum at 620 nm.

Working Procedure

1. Wash microtiter plate wells with 10 mM diethanolamine (pH 9.5).
2. Detect the enzyme activity with BluePhos substrate according to the manufacturer's instructions.

6.2.2.2.2   Precipitating Substrates for Phosphatase

5-Bromo-4-Chloro-3′-Indolyphosphate p-Toludine Salt and Nitro-Blue Tetrazolium Chloride

The combination of 5-Bromo-4-Chloro-3′-Indolyphosphate p-Toludine salt (BCIP) and Nitro-Blue Tetrazolium Chloride (NBT) yields an insoluble black-purple stain
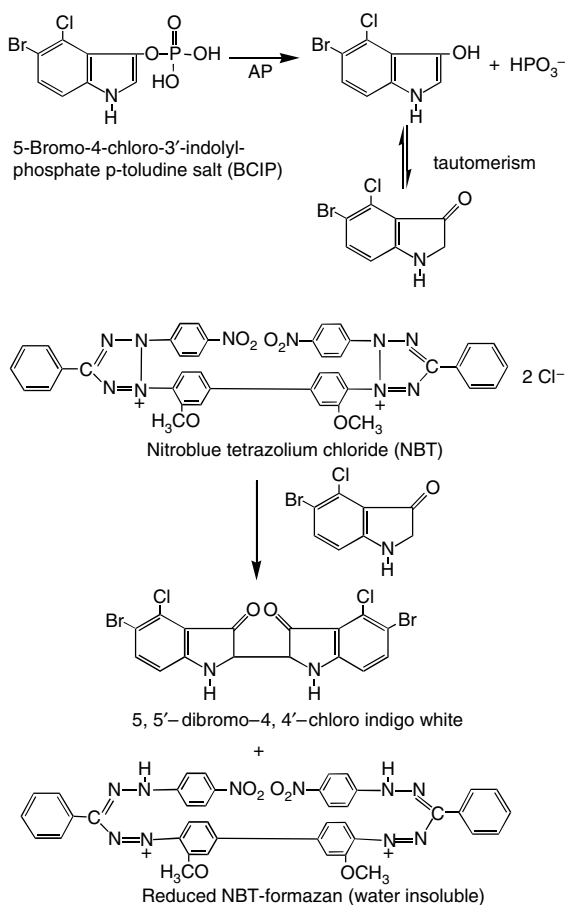
FIGURE 6.34 Catalytic reaction of alkaline phosphatase with BCIP. The hydrolyzed product is oxidized by NBT, which itself is reduced to an insoluble purple diformazan product.

when reacted with alkaline phosphatase in tissues or on blots.[37,38] BCIP alone can work as a substrate, which can be hydrolyzed by the enzyme and slowly oxidized at alkaline pH, resulting in the formation of blue insoluble 5,5′-dibromo-4,4′-dichloro indigo. However, the oxidation of the hydrolyzed product can be facilitated by the addition of oxidation catalysts such as nitroblue tetrazolium, copper sulfate, and ferri- and ferrocyanide.

Reaction

When BCIP is used in combination with NBT, the hydrolyzed product of BCIP is oxidized by the NBT to produce the dimerized indigo dye, and NBT itself gets reduced to purple diformazan, which precipitates along with the indigo product (Figure 6.34).

Working Procedure

1. Prepare 5% NBT in 70% dimethylformamide (50 mg in 1 ml) and store at 4°C.
2. Prepare 5% BCIP (disodium salt) in 100% DMF (50 mg in 1 ml) and store at 4°C.
3. Prior to detecting bound alkaline phosphatase add 66 µl of NBT and 33 µl of BCIP solution to 10 ml of enzyme buffer (0.1 M diethanolamine/0.1 M NaCl/0.005 M $MgCl_2$, pH 9.5) and develop the blot until it appears black-purple.
4. Stop the reaction with PBS containing 20 mM EDTA.

Fast Red TR/AS-MX

Pierce offers Fast Red TR/AS-MX substrate, which results in a bright red precipitate when reacted with alkaline phosphatase. The substrate is good for double staining in immunohistochemical studies, when used with peroxidase DAB substrate.

Reaction

The enzyme hydrolyzes the naphthol phosphate ester substrate (naphthol phosphate AS-MX) to a naphtholic compound and phosphate (Figure 6.35). The anionic naphthol couples to the colorless chromogen, which is a diazonium salt (Fast Red TR) and results in a precipitating, colored azo dye.[39]

Working Procedure

1. Prepare Fast Red TR solution (10 mg in 10 ml substrate buffer).
2. Add 1.5 ml of naphthol AS-MX phosphate (5 mg/ml) to the above Fast Red TR solution and mix.
3. Use the mixture within an hour.

6.2.2.3 Detection of Preformed Soluble Enzyme Immune Complexes such as Peroxidase Anti-Peroxidase and Alkaline Phosphatase Anti-Alkaline Phosphatase

These methods are first used in histochemical localization of a tissue antigen. The detection of an antigen through the soluble enzyme immune complex (PAP, APAAP) offers a sensitive procedure compared to the two-step indirect method. In the two-step indirect method, a labeled secondary antibody is added to the primary antibody (see Figure 5.11). In Peroxidase anti-peroxidase (PAP) and alkaline phosphatase anti-alkaline phosphatase (APAAP) methods, a labeled secondary antibody is replaced by an unlabeled secondary antibody, which is then allowed to bind to either a soluble immune complex of peroxidase and anti-peroxidase or a soluble immune complex of alkaline phosphatase and anti-alkaline phosphatase antibody
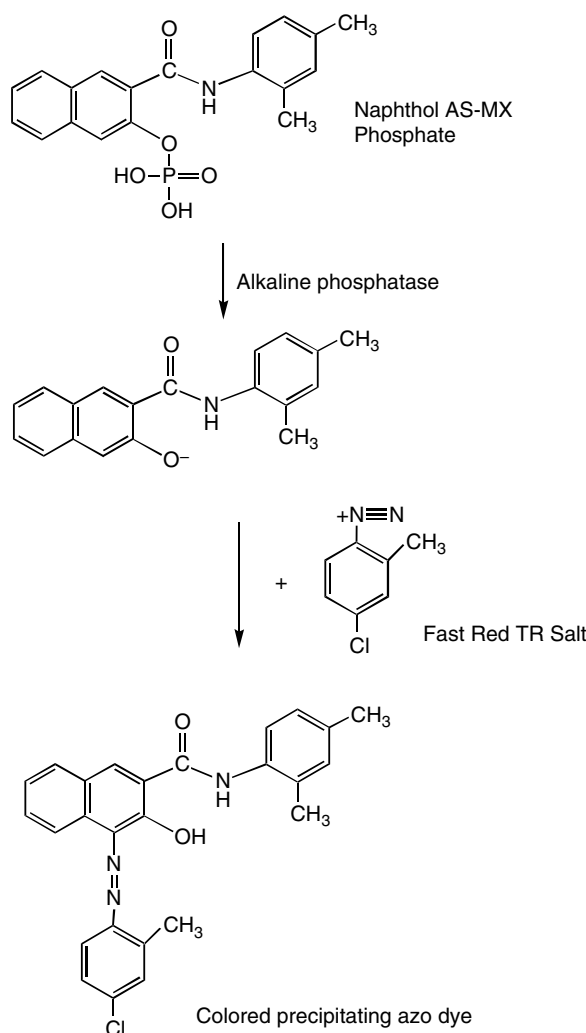
FIGURE 6.35 Hydrolysis of naphthol phosphate, resulting in a naphthol, which then reacts with Fast Red TR to produce an insoluble azo dye.

(Figure 6.36). The colorimetric detection is then achieved with the addition of a soluble substrate as described above. The PAP complex contains three peroxidase molecules to two anti-peroxidase antibody molecules, whereas the APAAP complex has two molecules of phosphatase and one anti-phosphatase antibody.[40] Since the soluble immune complex uses an increased amount of label on the third layer, the primary antibody to be used in the first layer can be diluted several-fold to reduce non-specific staining. The major advantage of using a soluble enzyme immune complex is that no labeling of the secondary antibody is needed. However, there are some disadvantages. Both methods require more time than the two-step indirect method. Penetration into tissue is inhibited because of the large immune complex.
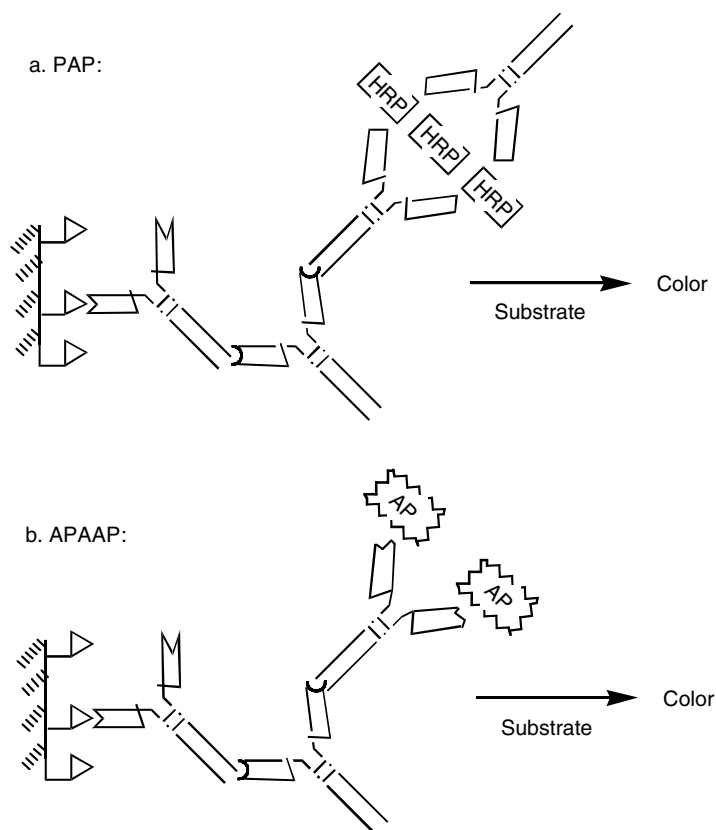
FIGURE 6.36 Diagram showing the detection of an antigen through the soluble peroxidase-anti-peroxidase (PAP) complex (a) or alkaline phosphatase-anti-alkaline phosphatase complex (b).

Working Procedure for the Formation of PAP Complex[40]

1. To anti-peroxidase antibody (40 ml antiserum), add peroxidase (4 mg/ml in 150 mM NaCl), which contains 1.5 times the amount of "equivalence" for 40 ml of serum.
2. After incubation for 1 h at room temp, collect the precipitate by centrifugation.
3. Wash the precipitate with cold NaCl (150 mM) and resuspend the precipitate in a solution of peroxidase (4 mg/ml), using a volume 4 times that used in step 1.
4. Adjust the pH to 2.3 with dropwise addition of HCl (2 drops of 1 N HCl, followed by a few μl of 0.1 N HCl). Precipitate will dissolve at this pH.
5. Neutralize with NaOH following the same approach as step 4.
6. Add 1/10 volumes of a solution of sodium acetate (80 mM) and ammonium acetate (150 mM) and chill on ice.

7. Centrifuge (10 min at 4,000 g) and collect supernatant.
8. To the supernatant add slowly an equal volume of saturated ammonium sulfate with stirring.
9. Collect precipitate by centrifugation and wash once with a half-saturated ammonium sulfate solution.
10. Dissolve the precipitate in water and dialyze with a solution containing NaCl (135 mM), sodium acetate (7.5 mM), and ammonium acetate (15 mM).

6.2.2.4 Detection of β-Galactosidase Conjugate

Table 6.10 shows soluble and precipitating substrates for β-galactosidase.

TABLE 6.10
Detection of β-Galactosidase Conjugate

Substrate	Nature of Substrate	Color of the End Product	Application
o-nitrophenyl-β-D-galactopyranoside	Chromogenic, soluble	Yellow	ELISA
Chlorophenyl red-β-galactoside	Chromogenic, soluble	Red	ELISA
Resorufin-β-D-galactopyranoside	Chromogenic, fluorescent, soluble	Magenta, fluorescence	ELISA, fluorometric assay
4-methylumbelliferyl-β-D-galactopyranside	Fluorescent Soluble		Fluorimetric assay
Pararosaniline/naphthol-AS-BI-β-D-galactopyranoside	Chromogenic, precipitating	Red	Histochemistry
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside	Chromogenic, precipitating	Blue	Immunoblotting, histochemistry

6.2.2.4.1 *Soluble Substrate for β-Galactosidase*

O-nitrophenyl β-D-galactoside

O-nitrophenyl β-D-galactoside (ONPG) is commonly used for β-galactosidase assay.

Reaction

The enzyme reacts with the substrate, yielding a soluble yellow product that can be detected at 405 nm (Figure 6.37).
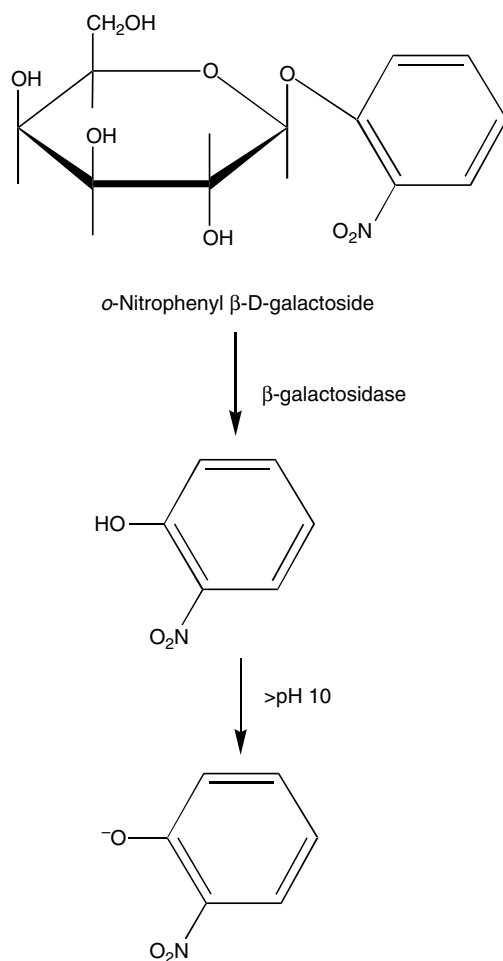
FIGURE 6.37 Reaction of β-galactosidase with o-nitrophenyl β-D-galactoside, resulting in a soluble yellow product.

Working Procedure

1. To the bound labeled antibody add o-nitrophenyl β D-galactoside (3 to 4 mM) and incubate at room temperature for 1 h.
2. Add 2 M sodium hydroxide and read the plate at 405 nm.

4-methylumbelliferyl β-galactoside

4-methylumbelliferyl β-galactoside is commonly used for fluorimetric immunoassay for β-galactosidase.[41]
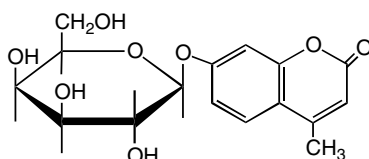
FIGURE 6.38 Reaction of β-galactosidase with 4-methylumbelliferyl β-D-galactoside, resulting in a soluble fluorescent product.

Reaction

The enzyme reacts with this substrate, yielding a soluble fluorescent product (Figure 6.38). The product is excited at 340 nm, and the emission is read at 440 nm.

Working Procedure

1. Prepare 0.1 mM 4-methylumbelliferyl β-galactoside in 10 mM potassium phosphate/150 mM NaCl/2 mM MgCl$_2$ (pH 7.0) containing 0.1% BSA and add to the bound labeled antibody.
2. Incubate at room temperature for 1 h and measure the reaction product using fluorometer.

6.2.2.4.2   Precipitating Substrate for β-Galactosidase

On a Western blot membrane, the binding of β-galactosidase-labeled antibody is conveniently detected with the substrate 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (known as X-Gal).[42] In combination with isopropyl β D-galactopyrano-side (IPTG), X-Gal is extensively used in recombinant DNA technology to detect vectors, plasmids, or DNA fragments that contain a β-galactosidase gene. IPTG enhances the expression of genes in expression vectors.

Reaction

The reaction yields an intense blue product that is stable and insoluble in alcohol and water (Figure 6.39).

Working Procedure

1. To prepare X-Gal solution, dissolve 4.9 mg of X-Gal in 0.1 ml of dime-thylformamide and mix the solution with 10 ml of PBS containing mag-nesium chloride (1 mM) and potassium ferrocyanide (3 mM) and filter to remove any particle.
2. Incubate specimen for 10 to 40 min at room temperature until color develops.

5-Bromo-4-chloro-3-indolyl-
β-D-galactopyranoside (X-Gal)
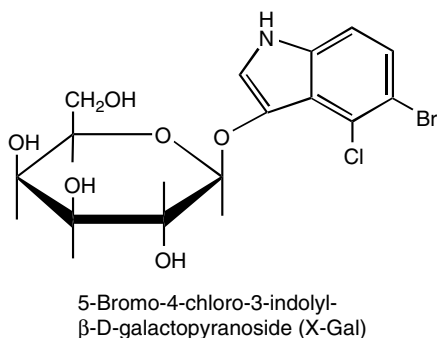
FIGURE 6.39 Reaction of β-galactosidase with BCIG, producing an insoluble 5-bromo-4-chloro-3-indole.

6.2.2.5 Detection of Glucose Oxidase

6.2.2.5.1 Soluble Substrate for Glucose Oxidase

Glucose oxidase reacts with glucose, producing no color. So enzyme activity is usually detected by the addition of an indicator such as ABTS or OPD that is oxidized by the hydrogen peroxide in the presence of peroxidase. Glucose oxidase is very specific for β D-glucose and almost no specificity toward α D-glucose. Free glucose solution is generally used for this enzyme, since β-anomer of glucose predominates in solution.

Reaction

A green product is formed when ABTS is used with glucose (see Figure 6.27 for oxidized ABTS).

Working Procedure

1. Dissolve 7.5 mg/ml of glucose in 50 mM citrate phosphate buffer, pH 5.3. Incubate for 1 h at room temperature. This will allow mutarotation of glucose. In equilibrium β-anomer (65%) of glucose predominates.
2. Add ABTS (0.1 mg/ml) or OPD (0.5 mg/ml) and horseradish peroxidase (0.1 μg/ml).

6.2.2.5.2 Precipitating Substrate for Glucose Oxidase

A precipitating substrate for glucose oxidase is a mixture of glucose and tetrazolium (see Figure 6.34 for structure).

Reaction

A purple-black precipitate is formed from this reaction.[43]

Working Procedure

1. Prepare 41.7 mM D-glucose in 0.1 M sodium phosphate, pH 6.9 and leave at room temperature for at least 1 h to allow mutarotation of glucose.
2. To the above solution, add tetrazolium salt to a final conc. of 0.5 mg/ml.

6.2.2.6 Chemiluminiscent Probes for the Detection of Peroxidase Conjugate

A very sensitive visualization technique based on the chemiluminiscent reactions has recently been developed (detection limit as little as 1 pg of antigen).

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione)

Luminol provides sensitive detection based on the HRP-catalyzed oxidation in the presence of hydrogen peroxide.[44,45] This substrate is used for immunoblotting (detected on light-sensitive photographic film) or chemiluminiscence immunoassay (measured using a luminometer).

Reaction

Upon oxidation, luminol produces an unstable α-amino phthalic acid (Figure 6.40). This reaches the excited state and decays to the ground state, emitting light at a maximum wavelength of 425 nm.

Note: sodium azide interferes with the chemiluminiscent reaction and thus should be avoided in the assay buffer.

Working Procedure

1. Dissolve luminol in 0.1 M NaOH and dilute to 10 nM with an assay buffer such as phosphate.
2. Add 30% H_2O_2 solution to the luminol solution to get a final concentration of 0.03%.
3. To the bound conjugate add 100 µl of the substrate solution.
4. Read the plate within 2 to 5 min.
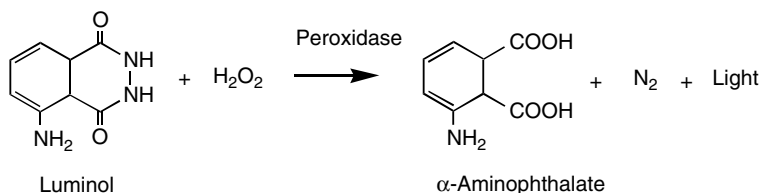
FIGURE 6.40 HRP catalyzed oxidation of luminol results in an unstable α-amino phthalic acid and light.

Enhanced Chemiluminiscent

Various phenols can be added to luminol to enhance luminescence intensity by approximately 1,000-fold.[46] Amersham's ECL Western Blotting System is based on this enhanced chemiluminiscence. Similar chemiluminiscent detection kits are also available from other vendors as follows: Super Signal Western Blotting kit (Pierce, Rockford, IL), LumiGlow (KPL, Gaithersburg, MD), and BM Chemiluminiscence (Roche, Indianapolis, IN).

6.2.2.7   Chemiluminiscent Probes for the Detection of Phosphatase Conjugate

Phenyl Phosphate Dioxetane

Dioxetane substances offer a very sensitive detection of phosphatase with a low background. In contrast to fluorescence, which requires an external light source for excitation energy, in chemiluminiscence the energy required for excited state formation is generated internally upon dioxetane decomposition (Figure 6.41).[47] The susbstrate is used both for immunoblotting and chemiluminiscence immunoassays. Immunoblots are developed on a light-sensitive photographic film, and immunoassays are monitored using a luminometer. Lumi-Phos Plus (Lumigen, Inc., Southfield, MI), a chemiluminiscent system for alkaline phosphatase, utilizes Lumigen[R] PPD in the presence of an enhancer. The enhancer system consists of fluorescent micelles formed by cetyltrimethylammonium bromide (CTAB) and a fluorescent-derivatized co-surfactant, 5-N-tetradecanoyl-aminofluorescein. The enhancer system provides an almost 370-fold increase in the chemiluminiscence efficiency. Light emission has a long half-life, so immunoblot can be exposed for a long time, if required.

Reaction

Alkaline phosphatase catalyzes dephosphorylation of the Phenyl Phosphate Dioxetane (PPD) substrate (Lumigen[R] PPD), resulting in stable hydroxy dioxetane, which around pH 10 produces a metastable phenolate anion intermediate (Figure 6.41). The phenolate anion then decomposes and emits light at a maximum wavelength of 466 nm.

Working Procedure

1. To the bound alkaline phosphatase-labeled antibody, add 100 ml of Lumi-Phos Plus to each microtiter well.
2. Incubate the reaction at 37°C for 30 min and read chemiluminiscence using a luminometer.
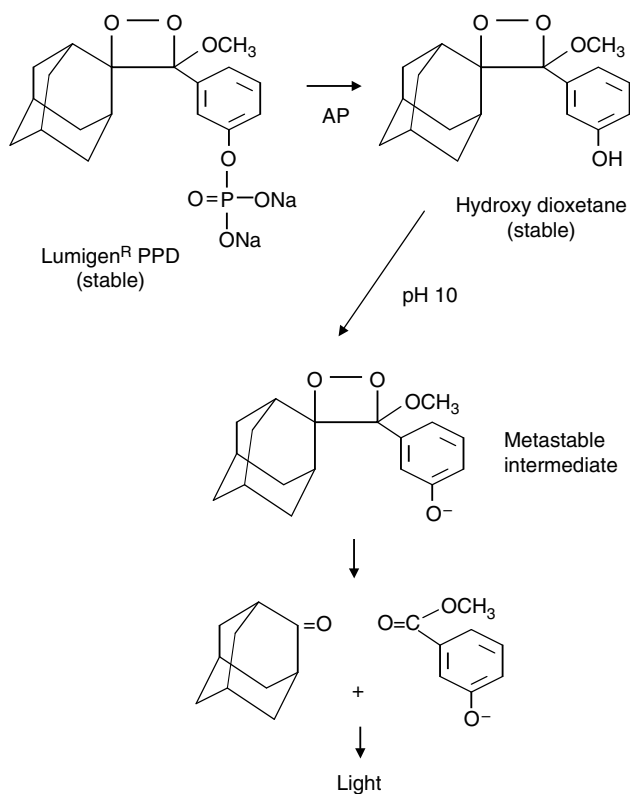
FIGURE 6.41 Reaction of alkaline phosphatase with Lumigen^R PPD.
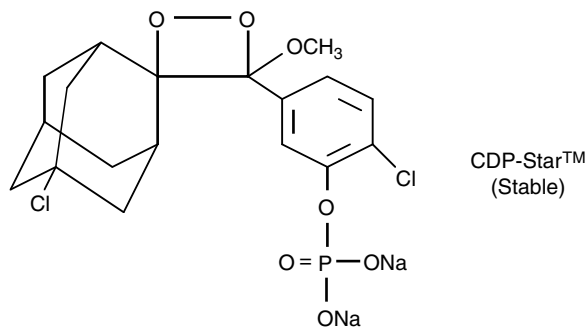
FIGURE 6.42 Structure of CDP-Star^{TM}.

CDP-Star^{TM}, CSPD^R, and AMPPD^R

Other examples of 1, 2-dioxetane substrates for phosphatase are CDP-Star^{TM} (Figure 6.42) (marketed by Tropix, Bedford, MA, and Bio-Rad under trade names Western-Star^{TM} and Immun-Star, respectively), CSPD^R (Figure 6.43) (marketed by
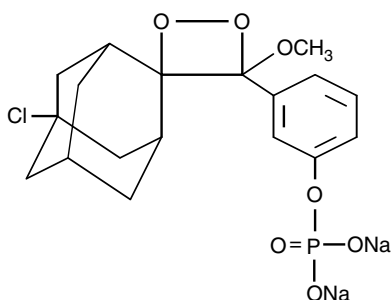
FIGURE 6.43 Structure of CSPD™.

Tropix, under trade name Western-Light™), and AMPPD[R 48] (Tropix). The detection sensitivity can be increased with the use of a biotinylated secondary antibody, followed by streptavidin-alkaline phosphatase conjugate.[49] The working procedures are available from the vendor.

LuciGLO™ (KPL, Gaithersburg, MD) is another chemiluminiscent substrate for detection of phosphatase activity in a microwell as well as in a membrane. LuciGLO™ is hydrolyzed by alkaline phosphatase, and the hydrolyzed product with lucigenin produces the emission of light that can be read in a luminometer.

6.2.2.8 Fluorogenic Substrate for the Detection of Enzyme Conjugate

Detection of Peroxidase Conjugate

Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR) can be used to detect peroxidase activity in ELISA. The assay uses Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine).

Reaction

In the presence of peroxidase, Amplex Red reagent reacts with hydrogen peroxide to yield a red fluorescent oxidation product, resorufin (Figure 6.44). The product has excitation and emission maxima at 563 nm and 587 nm, respectively. The product has also strong absorption at 565 nm, and thus the assay can also be performed spectrophotometrically.

10-Acetyl-3,7-dihydroxyphenoxazine
(Amplex Red)
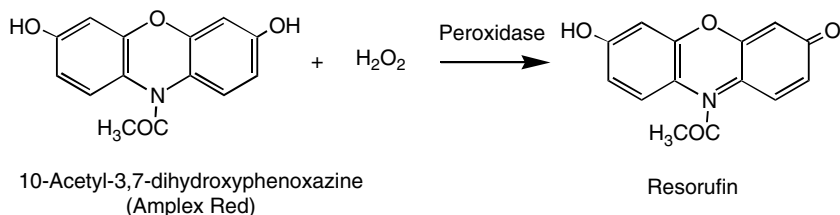
Resorufin

FIGURE 6.44 HRP catalyzed oxidation of the Amplex Red reagent results in a red fluorescent product, resorufin.

Working Procedure

A detailed protocol is available from the vendor. Briefly,

1. Prepare a 10 mM stock solution of Amplex Red reagent with dimethyl-sulfoxide.
2. Prepare a 20 mM H_2O_2 solution in ELISA reaction buffer, usually PBS.
3. Prepare a working solution containing 50 μM Amplex Red reagent and 200 μM H_2O_2.
4. Add 100 μl of the working solution to each of the microtiter plate wells and incubate for 30 min at room temperature.
5. Detect peroxidase activity either fluorometrically or spectrophotometrically.

Detection of Phosphatase Conjugate

4-methylumbelliferyl phosphate is used for fluorometric assays of alkaline phosphatase.[50]

Reaction

At pH 10.3, the enzyme cleaves the substrate to 4-methylumbelliferone (Figure 6.45). The resultant fluorescent product emits at 448 nm when excited at 364 nm.

Working Procedure

1. Prepare 0.1 mM 4-methylumbelliferyl phosphate in 50 mM 2-amino-2-methyl-1,3 propanediol, pH 10.3.
2. To the bound alkaline phosphatase conjugate, add 100 μl of the above substrate solution and incubate at room temperature for 30 to 60 min.

Detection of β-Galactosidase Conjugate

Molecular Probes' FluoReporter lacZ/Galactosidase Quantitation Kit uses 3-carboxyumbelliferyl β-D-galactopyranoside (CUG).
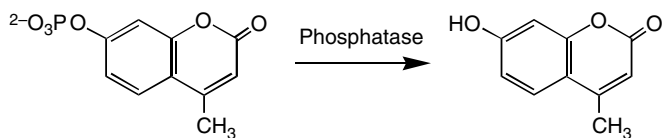
FIGURE 6.45 Reaction of alkaline phosphatase with 4-methylumbelliferylphosphate, producing 4-methylumbelliferone.

Reaction

The enzyme cleaves the CUG susbstrate to 3-carboxyumbelliferone, which has excitation and emission maxima of 386 nm and 446 nm, respectively.

Working Procedure

1. Prepare a 1.1 mM working solution of the CUG substrate reagent in reaction buffer (0.1 M sodium phosphate, pH 7.3/1 mM $MgCl_2$/45 mM β-mercaptoethanol).
2. Add 100 μl of the CUG working solution to each well and incubate for 30 min at room temperature.
3. Add 50 μl of stop solution (0.2 M Na_2CO_3) to each well.
4. Measure fluorescence of the solution using a fluorescence microplate reader (such as Cytofluor).

Detection of Glucose Oxidase Conjugate

Molecular Probes' Amplex[R] Red Glucose/Glucose Oxidase Assay Kit can be used to detect glucose oxidase activity in the presence of peroxidase.

Reaction

In this assay, glucose oxidase first reacts with D-glucose to form D-gluconolactone and H_2O_2 (Figure 6.46). H_2O_2, in the presence of peroxidase, then reacts with the Amplex Red reagent (10-acetyl-3,7-dihydroxy phenoxazine) to yield the oxidation product, resorufin. The product can be measured either spectrophotometrically (at 563 nm) or fluorometrically (excitation and emission wave lengths are 563 nm and 587 nm, respectively).

Working Procedure

A detailed protocol is available from the vendor. Briefly,

1. Prepare a working solution containing 10 μM Amplex Red reagent, 0.2 U/ml horseradish peroxidase, and 100 mM glucose in reaction buffer.
2. Add 50 μl of the working solution to each microtiter plate well that has glucose oxidase conjugate.
3. Incubate the reaction for 30 min at room temperature.
4. Read the plate using either regular plate reader or fluorescence plate reader.

6.2.3   DETECTION OF BIOTIN-LABELED ANTIBODIES

Biotin-labeled antibodies are detected using avidin or streptavidin that binds tightly to biotin ($Kd = 10^{-15}$ M). The binding is stable at extremes of pH, in buffer salts, or
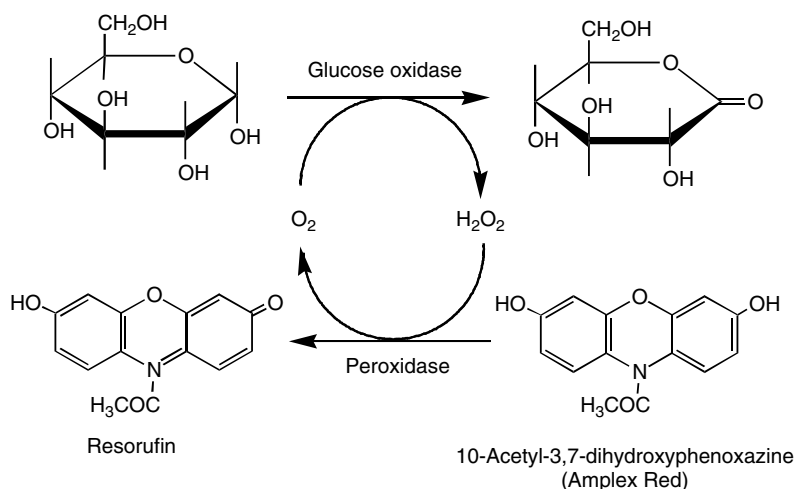
FIGURE 6.46 Glucose oxidase reacts with D-glucose to yield D-gluconolactone and hydrogen peroxide, which then reacts with the Amplex Red reagent in the presence of peroxidase to form fluorescent resorufin.

even in the presence of chaotropic agents, such as guanidine hydrochloride (up to 3 M). The avidin-biotin system, because of their strong interaction, is used in most immunochemical techniques. However, when used in immunohistochemical staining of a tissue specimen, endogenous avidin and biotin activities must be removed. Both avidin and streptavidin can be labeled with iodine or enzyme and subsequently detected by a gamma counter or with a corresponding substrate. In the direct assay, a biotinylated primary antibody is detected, while in the indirect assay the detection occurs in a labeled (biotinylated) secondary antibody that binds to the primary antibody. The indirect method is commonly employed in most immunochemical techniques. A biotinylated antibody can be detected in several ways: labeled avidin-biotin (LAB) method, bridged avidin-biotin (BRAB) method, and avidin-biotin complex (ABC) method.

6.2.3.1 Labeled Avidin-Biotin Method

The LAB and BRAB methods were developed by Guesdon and colleagues.[51] In the LAB method (Figure 6.47), the biotinylated secondary antibody (that bound to tissue antigen through an antigen-specific antibody) is detected with an enzyme-labeled avidin followed by a corresponding substrate.

6.2.3.2 Bridged Avidin-Biotin Method

The BRAB method is more sensitive than the LAB method. In the BRAB method (Figure 6.48), the avidin is not conjugated to an enzyme, but is used as a bridge between the biotinylated secondary antibody and the biotinylated enzyme. The four biotin-binding sites on the avidin allow more biotinylated enzyme to be complexed, resulting in an enhancement of the substrate reaction.
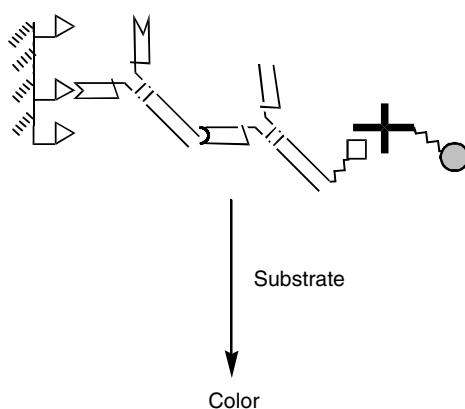
FIGURE 6.47 The labeled avidin-biotin (LAB) method. In this method, the antigen-bound primary antibody is reacted with the biotinylated secondary antibody, and after washing, the bound biotinylated antibody is then detected with the enzyme-labeled avidin, followed by a suitable substrate.
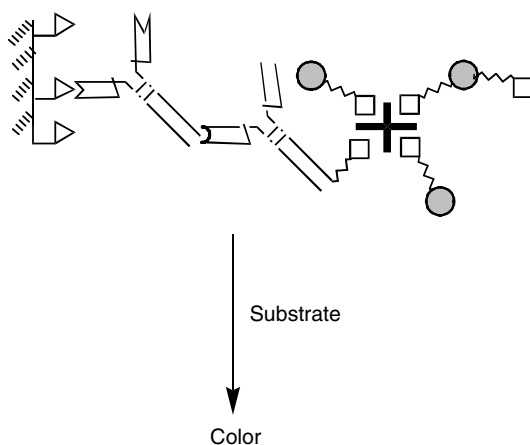
FIGURE 6.48 The bridged avidin-biotin (BRAB) method. Unlike the LAB method, the avidin is not labeled with an enzyme. The unlabeled avidin is used as a bridge between the biotinylated secondary antibody and the biotinylated enzyme.

6.2.3.3   Avidin-Biotin Complex Method

The ABC method is one of the most sensitive methods of antigen detection that utilize the avidin-biotin system.[52,53] The ABC method (Figure 6.49) is similar to the BRAB system, except that the biotinylated enzyme is preincubated with the avidin to form large three-dimensional complexes, which then are reacted with the biotinylated antibody. An avidin-biotin-labeled enzyme complex is usually premade by incubating the avidin with the biotinylated enzyme in a specified ratio for about 30 min at room temperature. Enzymes used are HRP, alkaline phosphatase, glucose
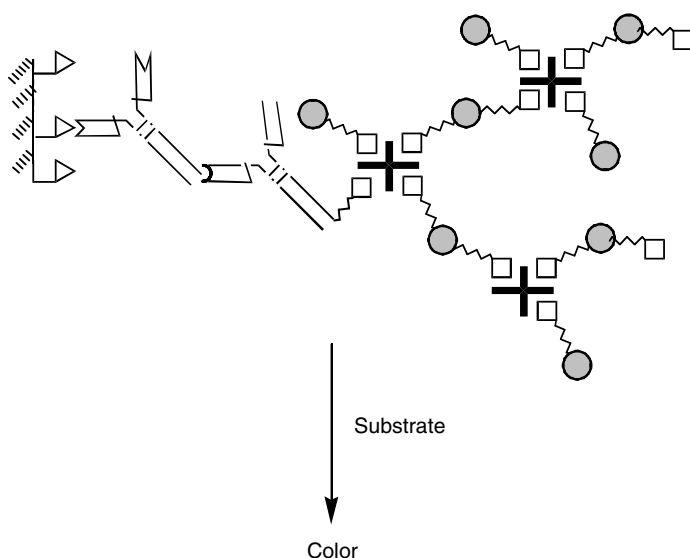
FIGURE 6.49 The avidin-biotin complex (ABC) method. This method is similar to BRAB, except that the biotinylated enzyme is preincubated with the avidin to form large three-dimensional complexes, which then are reacted with the biotinylated antibody.

oxidase, etc. Reagents for ABC or a kit are available from several vendors, such as Vector Laboratories (Breton, Peterborough, UK) and Pierce (Rockford, IL). There are some disadvantages of using the avidin-biotin system. Avidin is a basic glycoprotein of pI 10.5 and therefore has a tendency to bind the negatively charged plasma membrane because of its positive charge. However, at assay pH of around 7, successive treatments of a tissue section with avidin (0.001 to 0.1%) and biotin (0.001 to 0.01%) mostly eliminates non-specific binding. Second, because of its carbohydrate moiety, avidin may interact with endogenous lectins, resulting in a increase of background. These problems are largely solved by using streptavidin, an equivalent protein isolated from *Streptomyces avidinii* because of its neutrality at pH 7, and because it lacks carbohydrate.

Working Procedure for Preparing Avidin-Biotin Complexes

1. Saturate a column of iminobiotin-agarose (commercially available) with avidin. One of four binding sites is occupied by the iminobiotin.
2. Add biotinylated peroxidase on the column. The remaining three binding sites of avidin will interact with biotinylated peroxidase.
3. Add avidin to saturate additional free biotin.
4. Elute complexes from the column with 50 mM ammonium acetate (pH 4).

Avidin and biotinylated peroxidase are also commercially available. Vector Laboratories (Burlingame, CA) supplies these in two convenient bottles. Simply add 2 drops from each bottle to 5 ml of PBS. Incubate 30 min at room temperature.

6.2.4 Detection of Fluorochrome-Labeled Reagents

The procedure to detect antigen with fluorochrome-labeled reagents (such as FITC or rhodamine-labeled antibody) is commonly called the immunofluorescence method.[54–56] This is widely used to detect an antigen in fixed or living cells. The detection of bound fluorochrome is visualized through a fluorescence microscope in which the exciting radiation is transmitted through the objective lens onto the surface of the specimen. Filters are used to select radiation of the correct wavelength for excitation. Upon excitation, electrons of the fluorochrome are shifted to the higher energy level. When these electrons return to their ground state, they emit a light of characteristic wavelength and can be seen in the microscope. Since each fluorochrome has a characteristic excitation and emission spectra, it is possible to detect more than one antigen at the same time by choosing a fluorochrome whose emission spectra do not overlap. Table 6.11 lists some fluorochromes that are generally used for labeling antibodies.

Working Procedure

1. Incubate cells (live or fixed) or fixed-tissue specimen with blocking solution (3% BSA) for 30 min. After standard fixation certain tissue antigens become inaccessible to antibodies and therefore, before blocking, they require permeabilization with Triton X-100 (usually 0.1 to 0.2%) or partial digestion of the tissue proteins with proteinase.
2. After blocking incubate the cells or tissue sections with primary antibody (appropriate dilution) for 1 h at room temperature.
3. After washing two to three times with PBS-1% BSA, incubate the cells or tissue sections with fluorochrome-labeled secondary antibody (1,000 to 10,000 dilution in PBS-1% BSA).
4. Wash the cells or tissue sections three times with PBS-1% BSA and observe the reaction by fluorescence microscopy.

6.2.5 Detection of Gold-Labeled Antibodies

Microscopic (both electron and light) visualization of a specific antigen on a cell surface or tissue is demonstrated by using gold-labeled antibodies and Protein A[57,58] (Figure 6.50). In the gold conjugates, gold particles are not covalently bound to the proteins, rather they are held tightly by the proteins at the pH value close to their pIs. Gold is biologically inert and thus becomes the probe of choice in many applications. The detection offers usually higher resolution than enzyme-based methods and avoids the problems associated with endogenous activity. Conjugated colloidal gold particles are available in several sizes ranging from 1 to 40 nm in diameter. For example, AuroProbe BL Plus and AuroProbe One antibodies (Amersham

TABLE 6.11
Properties of Fluorochromes and Their Common Uses

Fluorochrome	Derivative Used for Labeling	Characteristic Excitation (nm)	Characteristic Emission (nm)	Color of Fluorescence	Common Use
Fluorescein	Fluorescein isothiocyanate (FITC)	495	520	Green	Immunohistochemistry/flow cytometry
Rhodamine	Tetramethyl rhodamine isothiocyanate (TRITC)	545	580	Red	Double labeling in immunohistochemistry
7-Amino-4-methyl coumarin 3-acetic acid (AMCA)	AMCA	355	440–460	Blue	Double or triple labeling
R-Phycoerythrin	N-hydroxysuccinimide ester	480, 545, 565	578	Orange-red	Double labeling in flow cytometry
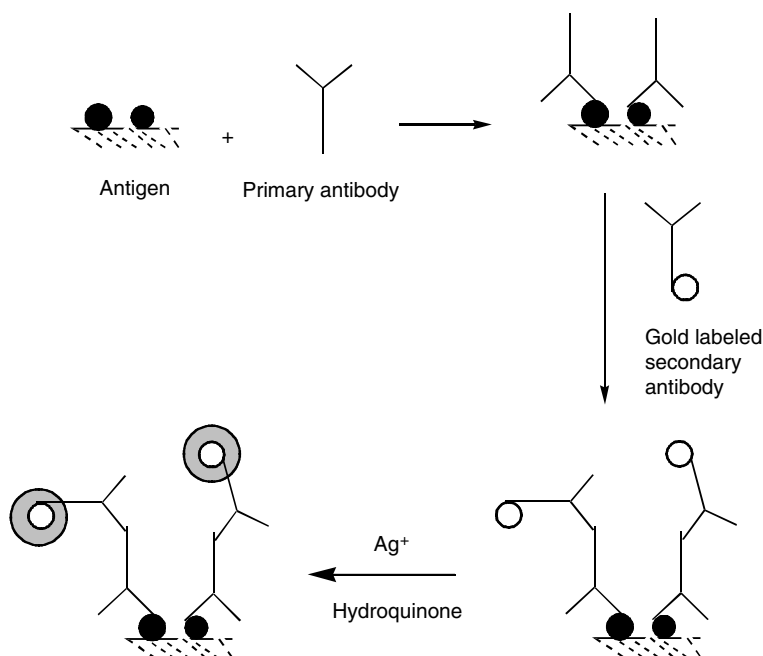
FIGURE 6.50 Schematic representation of antigen detection with gold-labeled antibodies. Visualization of bound gold-labeled antibodies can be enhanced by silver lactate, which precipitates on the gold surface, generating an intense signal.

International) are labeled with 10 nm and 1 nm gold particles, respectively. Gold particles of a small size allow increased labeling efficiency with the antibodies compared to larger gold particles.[59] The resulting gold conjugate can provide greater sensitivity probably due to the large number of gold particles per antigen molecule.[59]

Visualization of the gold label by light microscopy is further enhanced by silver. The enhanced sensitivity is achieved by using silver lactate and hydroquinone in a gold solution at acidic pH 3.5.[60] Gold particles catalyze the reduction of silver ion to metallic silver by the hydroquinone (see Figure 6.50). The silver atoms thus formed are deposited in layers on the surface of the gold particle, providing an intense signal. However, the system develops high background due to instability to light and self-nucleation. Self-nucleation is a spontaneous phenomenon whereby the reduction of silver ions occurs in solution to form silver particles, resulting in a silver deposit. However, a silver enhancement reagent, Intense™ BL (Amersham International), can be used to overcome these problems. It is not sensitive to light and exhibits delayed self-nucleation. A tenfold increase in sensitivity is achieved with this enhancement reagent.

Depending on the strength of reaction, gold particles appear pink to dark red under the light microscope using bright field illumination. However, with silver enhancement they become coated with metallic silver and appear as a black-brown label on bright field optics.

1. To the acetone-fixed frozen tissue section, add blocking solution (PBS-3% BSA) for 10 to 15 min at room temperature.
2. Incubate the slide with primary antibody (1,000- to 10,000-fold dilution) for 1 h at room temperature.
3. Wash off excess primary antibody with PBS (pH 7.2) for 5 min (four to five times).
4. Incubate the section with gold conjugated secondary antibody or protein A/G (1,000- to 10,000-fold dilution) for 30 to 60 min at room temperature.
5. Rinse the slide in distilled water and incubate with silver enhancer (0.85% hydroquinone, 0.11% silver lactate in 0.1 M sodium citrate, pH 4.0) for 10 to 20 min at room temperature. Rinse slide in water and examine tissue section under the microscope. Continue silver enhancement until it appears black under microscope.
6. Rinse briefly in 1% acetic acid and fix in a standard photographic fixer for a few minutes.
7. Counterstain with hematoxylin-eosin (if necessary).
8. Mount the section for visualization on a light microscopy.

6.3 IMMUNOCHEMICAL TECHNIQUES: A WIDE RANGE OF IMMUNOASSAYS FOR DETECTING AND QUANTITATING (SEMI) ANTIGEN-ANTIBODY REACTIONS

The immunochemical techniques comprise several immunoassays such as ELISA, radioimmunoassay, dot blot, immunoblot, etc. Immunoassays can be classified into three basic types: (a) antibody capture assay, where an antigen is immobilized and an antibody is allowed to bind, (b) antigen capture assay, where an antibody is immobilized and an antigen is allowed to bind, and (c) two-antibody sandwich assay, where one antibody is immobilized and an antigen is allowed to bind, and the bound antigen is detected with a second antibody (Figure 6.51). Thus, immunoassays can be performed in many ways depending on what is to be detected (i.e., antigen or antibody). The availability of antibody type (whether monoclonal or polyclonal) and the nature of the antigen (pure or impure) often dictate the choice of assay. Most sensitive assays make use of a primary antibody, which is unlabeled, and a labeled secondary antibody, which binds a primary antibody. Finally, the labeled secondary antibody is detected depending on the nature of labeling. Therefore, the various labeling techniques and detection systems make immunoassays very flexible to the user. The labels include radio-iodination, fluorescent probes such as fluorescein and rhodamine, enzyme conjugates such as horseradish peroxidase and alkaline phosphatase, or colloidal gold (Figure 6.52).

The detection systems are usually based on radioactivity, fluorescence emission, chemiluminiscence, or colorimetric reaction. The detection of iodine-labeled reagent is performed in a gamma counter or on X-ray film, fluorochromes by fluorimeter or
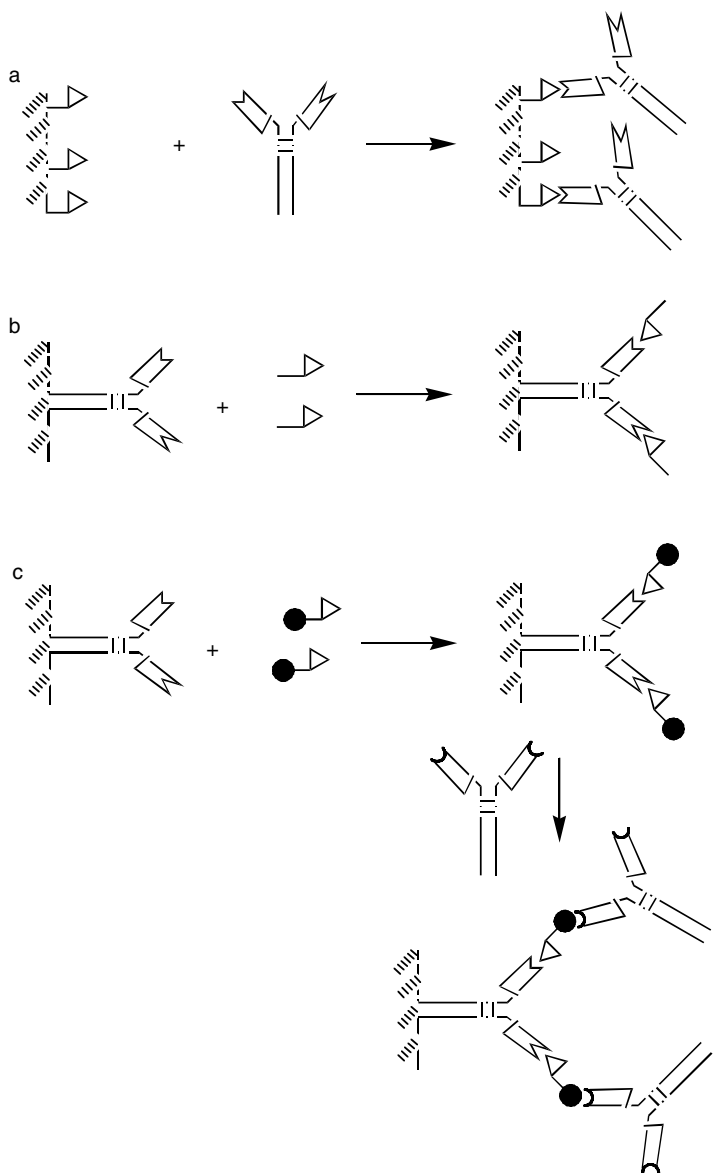
FIGURE 6.51 Three classes of immunoassays: antibody capture assay (a), antigen capture assay (b), and two-antibody sandwich assay (c).

fluorescence microscope, chemiluminiscence by luminometer or X-ray film, whereas the detection of enzymes is generally visualized using a color reaction on an enzyme-linked immunosorbent assay or on a membrane. Recently, the sensitivity of the detection is substantially increased using the avidin-biotin system, chemiluminiscent probes, and colloidal gold.
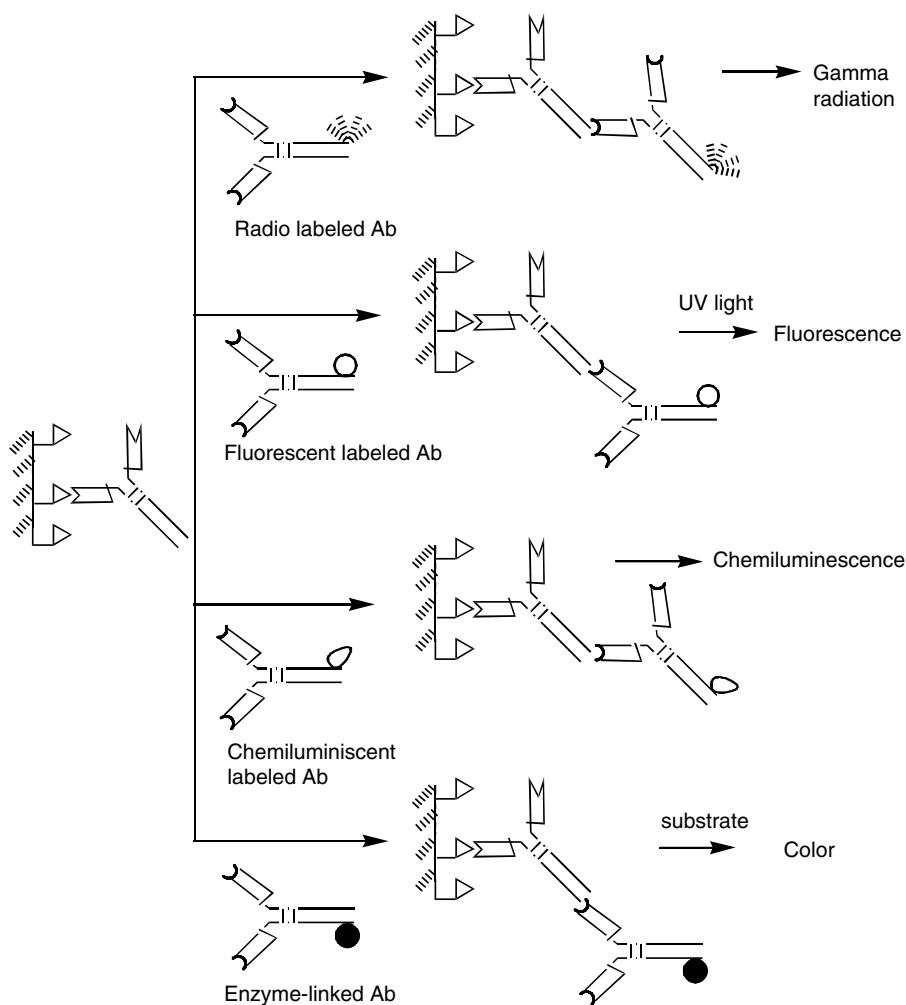
FIGURE 6.52 Various detection systems for immunoassays.

6.3.1 Antibody Capture Assay: ELISA, Immunoblotting, and Western Blot Are the Examples of this Type

These assays are probably the most popular and are widely used to detect and quantitate an antibody as well as an antigen. In this assay, an immobilized antigen is detected directly by a labeled antibody (i.e., in a single step) or indirectly by an unlabeled antibody followed by a labeled secondary antibody that will specifically recognize the first antibody (Figure 6.53). The indirect method is usually more sensitive than the direct one. The indirect antibody capture assay is widely used under different methods such as ELISA, immunoblot, Western blot, etc.
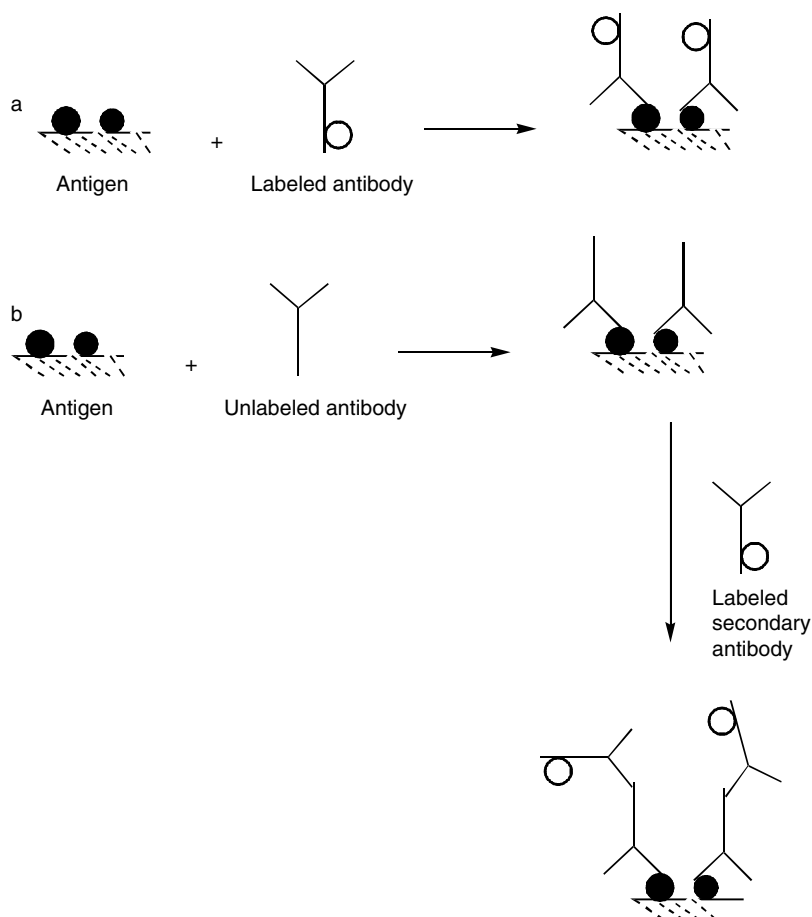
FIGURE 6.53 Antibody capture assays: direct (a) and indirect (b). In the direct method, a labeled antibody is allowed to bind with the immobilized antigen. In the indirect method, an unlabeled antibody is allowed to bind to the immobilized antigen, and after washing the complex, the labeled secondary antibody is then added.

ELISA

ELISA, introduced by Engvall and Perlman,[61] is distinguished from other immunoassays such as radioimmunoassay by the use of an enzyme label antibody to detect an antigen-antibody reaction. Although ELISA is popular and one of the most commonly used techniques, it requires a great deal of consideration in most steps in order to achieve optimal results (Figure 6.54).

Plates

ELISAs are usually performed in 96-well microtiter plates (Figure 6.55), which are arranged in 8 rows (horizontally) by 12 columns (vertically). The plates are generally
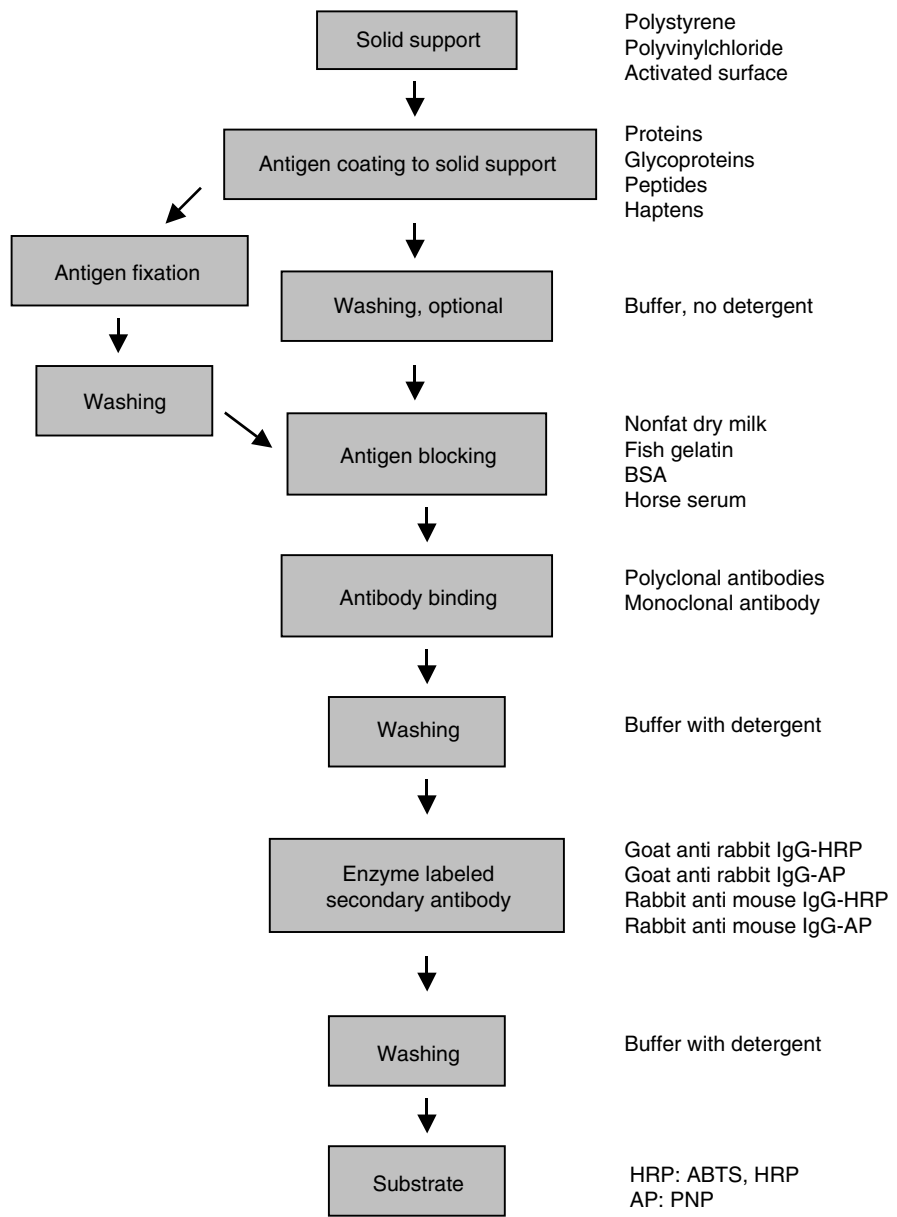
FIGURE 6.54 Typical steps of ELISA and components used in each step.

available in either polystyrene (rigid) or polyvinylchloride (PVC) (flexible). Flexible PVC plates are good for radioimmunoassays, because they can be cut up easily for counting of the radioactive content. For most ELISAs, rigid polystyrene plates are used, probably because they are easy to handle. Plates are available from many vendors, and their protein-binding capacity may vary. Therefore, it is advisable to
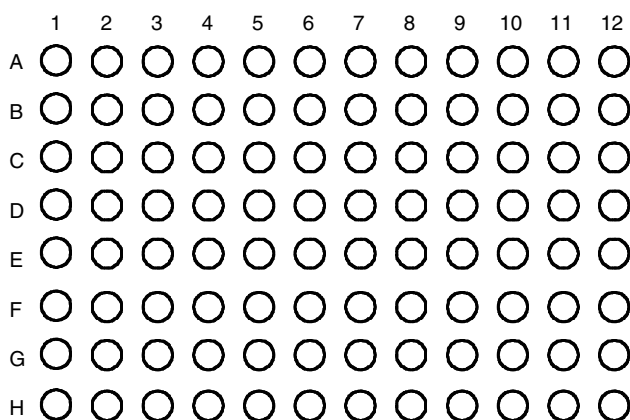
FIGURE 6.55 Diagram of 96-well microtiter plate.

stick with one vendor throughout the whole project. It is also a good idea to buy plates in bulk and avoid using different batches. If new batches are needed, it is a good idea to test new batches in parallel with old ones to find out any batch-to-batch variation.

Antigen Coating

The first step of ELISA is to attach antigen by passive adsorption, commonly called coating. Adsorption or coating is achieved due to hydrophobic interactions between the non-polar protein structure and the plastic surface, although charge may also play a role.[62] Different antigens such as proteins, peptides, and carbohydrates adsorb differently to the wells of the plate. While proteins and glycoproteins readily bind to plastics in the presence of a regular coating buffer (0.1 M sodium carbonate, pH 9.6) and small molecules (such as haptens or small antigens), drugs do not bind to the same surface under same condition. They require chemically activated ELISA plates such as maleic anhydride and hydrazide groups, which result in stable covalent bond formation between the surface and haptens. Covalent binding plates are commercially available (Pierce).

Reaction

Several peptide antigens can be covalently attached to a hydrazide-derivatized plastic surface in the presence of glutaraldehyde (Figure 6.56).

Alternatively, haptens are conjugated with a carrier protein such as BSA and KLH, and the hapten conjugate is allowed to bind plastic wells in the same way proteins and glycoproteins bind. However, it is essential that the protein of the protein-hapten conjugate that is used in the plate should be different from the protein-hapten conjugate that is used for the production of immunogen. Immobilization of the same protein conjugate will lead to increased non-specific binding and seriously compromised sensitivity, since antibodies will also bind to the carrier protein (see Figure 5.24).
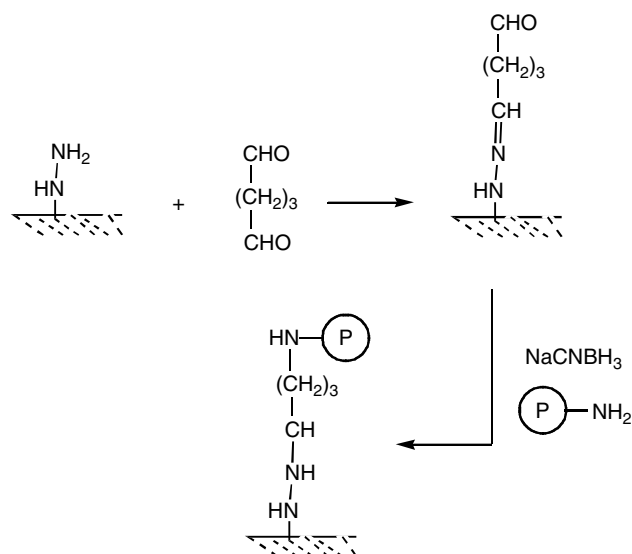
FIGURE 6.56 Covalent binding of a small peptide antigen to a modified plastic surface. The peptide antigen is linked with a hydrazide-modified plastic surface in the presence of glutraldehyde.

As hydrophobic interactions play a role in antigen coating, any condition (such as an increase of ionic strength of the buffer) that increases the hydrophobicity of the protein can be employed for this purpose. A concentration range of 1 to 5 µg/ml of protein, in a volume of 100 µl, is a fair guide to saturate a plastic well of 96-well microtiter plate. However, a preliminary experiment is needed to optimize the amount of antigen. A check board experiment (see Competitive Assay) is usually performed for this purpose by varying antigen and antibody concentrations. The determination of optimal antigen concentration is particularly critical to competitive ELISA (see Competitive Assay). Reducing the concentration of the antigen should improve sensitivity, while increasing the concentration will increase the limit of detection. But the excess antigen coating is not useful, either. High-density coating of antigen may not be accessible to antibody binding due to steric hindrance. The widely used coating buffers are 0.1 M carbonate, pH 9.6 and 20 mM Tris-HCl, pH 8.5. It is unknown, however, how pH affects immobilization of antigen. Although most reports in the literature make use of a buffer of high pH, a range of proteins are also shown to be immobilized using buffers under pH around 7.[63] The rate of the hydrophobic interactions increases at high temperature. Typically, the coating involves incubation at 37°C for 1 to 3 h or overnight at 4°C.

Blocking Buffer

Once the antigen is coated to the wells, they need to be blocked prior to the addition of the antibodies to the wells in order to prevent non-specific binding of the antibodies to the wells. Several blocking reagents, such as non-fat dry milk (usually

5%), fish gelatin (3%), BSA (1%), and horse serum (10%) in PBS, pH 7 to 8, are used. But researchers are urged to optimize the blocking condition to obtain the best results. The plate can be washed before the blocking step. In that case, detergent should not be used in the washing buffer, as detergent may leach some of the immobilized antigen, producing inconsistent results. Fixing the immobilized antigen before the washing and blocking steps usually provides consistent and reproducible results. For fixing, excess antigen solution is removed, and the wells are usually incubated with 2% formaldehyde in PBS for 30 min at 37°C. The wells are washed and blocked as described.

Binding Buffer

After blocking, antibodies specific to the antigen are added to the wells and incubated for 1 h at 37°C. A blocking buffer is normally used to dilute the antibodies. However, best results (high signal-to-background ratio) are usually obtained when the mixture of 0.05% Tween 20 and the blocking buffer is used as a binding buffer of antigen-antibody reaction.

Washing Buffer

Following binding of the antibodies to the antigen, the excess antibodies should be removed from the wells prior to the addition of the secondary antibody-labeled conjugate. For this purpose, the wells are washed with a plate washer or immersed in a tank of buffer. The washing buffer usually contains 0.1 to 0.5 M NaCl and 0.05% Tween 20 to block ionic and hydrophobic interactions, respectively, of the proteins with the plate. Washing is done by plate washer or by immersion in a tank of buffer or by using a squirt bottle.

Competitive Assay

For the development of a competitive assay (Figure 6.57), it is critical to determine the lowest concentration of antigen to be coated in wells in order to ensure that the antibody is the limiting factor. For this purpose, check board titrations are performed. In this experiment, one reagent is serially diluted horizontally and the other reagent is diluted vertically. For example, all horizontal rows (A to H) of the plate receive twofold serially diluted antigen starting at 100 μg/ml to 0.2 μg/ml in wells 2 to 11, respectively. A twofold serial dilution of antibody (starting concentration 1:200) is added from A to H. Results obtained from the colorimetric detection are graphically presented, as shown in Figure 6.58. Check board titration usually results in a characteristic binding pattern. The beginning of each binding curve for each antibody dilution is linear till it reaches a plateau (see Figure 6.58). The amount of antigen needed to coat the subsequent plate should be within the range that results in linear binding with the antibody (usually upper end of the range, 0.1 μg from Figure 6.58). The concentration of antibody is usually chosen such that it yields absorbance of around 1.5. Once the concentration of primary antibody is established, the same
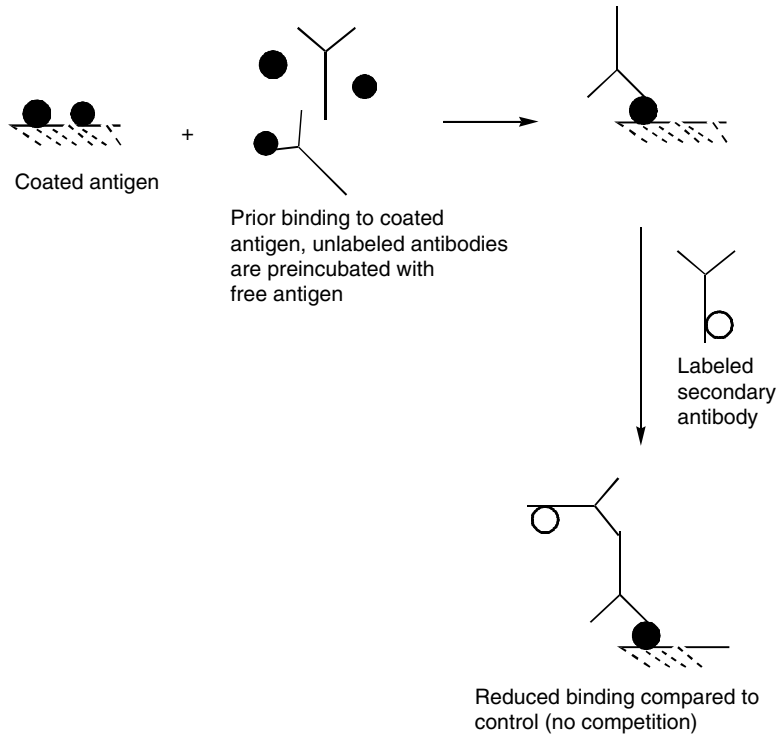
FIGURE 6.57 Competitive indirect ELISA assay. In this assay, primary antibodies are preincubated with antigen, and the mixture is then added to antigen-coated microtiter wells.
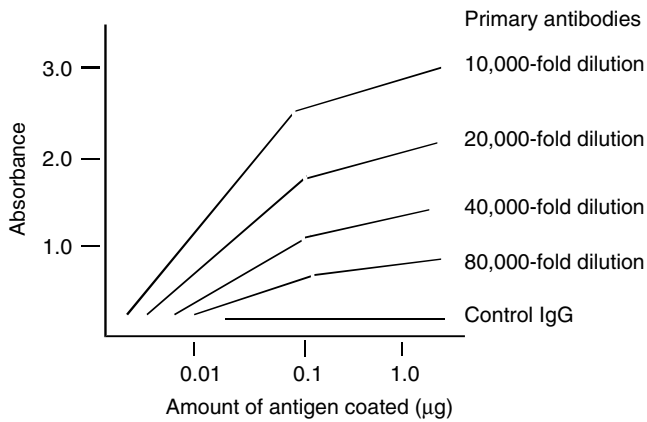
FIGURE 6.58 Binding of variable concentration of antibodies to a variable amount of antigen.
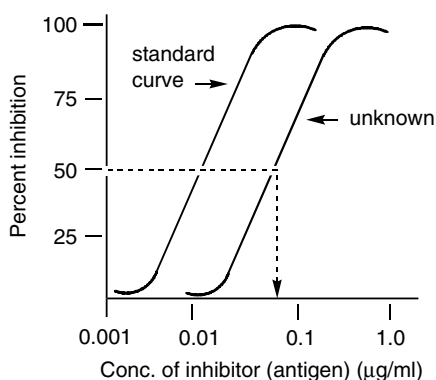
FIGURE 6.59 Inhibition curve. Inhibition of an antibody binding to a plastic-coated antigen by various known concentrations of the antigen shown in a standard curve. The amount of antigen in the unknown sample can be determined from a similar curve from its I_{50} (50% inhibition) value as shown by the arrow.

antibody concentration is usually incubated with an antigen of varying concentration, and the mixture is then added to the antigen-immobilized plastic wells. Typically a fixed volume of antibody (whose concentration is determined from check board titration) is mixed with a twofold serial dilution of known antigen.

After incubation at 37°C for an hour, each mixture is then transferred to the plastic wells and incubated for another hour at 37°C. After washing the plates, the bound antibody is then detected with the enzyme-labeled secondary antibody, followed by the substrate as described in the Working Procedure. Control wells contain only primary antibody without inhibitor antigen. The percentage of binding inhibition for each antigen inhibitor concentration is calculated based on control well, which is considered as 100% binding. Figure 6.59 shows the inhibition curve. To determine the antigen concentration in a mixture, a similar curve is obtained. Comparison of the curve with the standard curve that is obtained from a known amount of antigen allows the determination of the amount of antigen in an unknown sample (see Figure 6.59).

Incubation

The rotation of plates during incubation steps is recommended to avoid the effects of viscosity, time differences, and temperature. For most applications, incubations are done at 37°C. If incubation has to be done at room temperature, it is a good idea to note the temperature as room temperature varies during the year.

Substrate

The temperature of the substrate solution is important because the rate of color reaction varies with temperature. Substrate should be added always at the same temperature to minimize the plate-to-plate variation.

Stopping Reaction

Stopping the enzymatic reaction is usually performed at a time when the relationship between the enzyme-substrate product is in the linear phase. This is obtained from the check board experiment. The OD reading may be linear up to 3. But, for most applications, the reaction is stopped at OD 1 to 1.5. Stopping of the enzymatic reaction is usually performed by adding a molar concentration of strong acids or strong bases, which quickly denatures the enzyme. Other stopping reagents are enzyme specific. For example, sodium azide is a potent inhibitor of horseradish peroxidase, whereas EDTA inhibits alkaline phosphatase by chelation of metal ion cofactors. During the addition of stopping solution, the volume must be kept accurate throughout the plate for two reasons: (a) the addition of stopping solution may increase the sensitivity of an ELISA, and (b) the substrate readings are affected if the total volume in plate wells varies. This is because the plate reader reads plate wells perpendicularly, and the path length changes when the reaction volume in the wells changes.

In some cases, the addition of stopping agents may alter the absorption spectrum of the reaction product, and therefore, the absorption peak after stopping must be known. Table 6.12 lists the absorption maxima for some common substrates before and after the addition of stopping solution.

Internal Standard

Because ELISA consists of multiple steps and some steps are sensitive to temperature, there is a possibility of obtaining variable results in plate-to-plate and day-to-day operations. To normalize these variations, the use of an internal standard in every plate is recommended. A positive control should be included to ensure all reactants are working. Similarly, an appropriate negative control should also be included to check for non-specific binding. Preimmune serum or an irrelevant primary antibody can serve as a negative control.

The Use of ELISA

ELISA can be used for several purposes such as to screen test bleeds during antibody development, to detect or quantitate antigen in a solution, and to determine the relative activity of haptens. In the latter two cases, a fixed amount of primary antibody is mixed with various concentrations of antigen. The higher the antigen concentration, the lower the detection of secondary antibody binding.

Working Procedure

1. Add antigen (100 µl of 5 to 10 µg/ml in 0.1 M carbonate buffer, pH 9.6) into plastic wells of microtiter plate and incubate plate at 37°C for 3 h.

TABLE 6.12
Absorption Maxima for Common Enzymes before and after Stopping Solutions

Enzyme Label	Substrate	Stopping Solution	Color		Absorption Maxima (nm)	
			Before Stopping	After Stopping	Before Stopping	After Stopping
Peroxidase	TMB	1% SDS	Blue	Yellow	650	450
	OPD	1.25 M sulfuric acid	Green/orange	Orange	450	492
	ABTS	2 M citric acid	Green	Green	414	414
Alkaline phosphatase	PNP	2 M sodium citrate	Yellow/green	Yellow/green	405	405
β-galactosidase	ONPG	2 M sodium citrate	Yellow	Yellow	420	420

2. Flick over the plate over a sink or a waste container and wash three times with PBS/0.05% Tween 20 with a 500 ml squirt bottle. Add 200 μl/well blocking buffer (3% BSA/PBS/Tween 20) and incubate at 37°C for 1 h.
3. Flick off wells and incubate with primary antibody diluted 1,000- to 5,000-fold in blocking buffer (100 μl/well) at 37°C for 1 h. One set of control wells contains same dilution of preimmune serum. Another set of control wells contains antibody or preimmune serum in wells that are not immobilized with antigen.
4. Wash wells three to five times with PBS/T$_{20}$ and incubate with secondary antibody coupled with enzyme (such as horseradish peroxidase, alkaline phosphatase) diluted 1,000- to 2,000-fold with the blocking buffer at 37°C for 1 h. The nature and source of the secondary antibody used depends on the source of the primary antibody. For example, for primary antibodies raised in rabbits, secondary antibodies should be anti-rabbit IgG raised in a higher animal such as goat, sheep, etc.
5. Wash wells three to five times and incubate with the corresponding substrate (100 μl/well) at 37°C for about 30 min. When HRP is used as reporter enzyme, azide should be avoided in PBS buffer, since azide inhibits enzyme activity.

Problems and Solutions in ELISA

Researchers may experience problems during development of ELISA. Table 6.13 shows common problems and possible remedies.

Immunoblotting

Two immunoblotting techniques, dot blot and Western blot, are widely used to detect the presence of antigen in a mixture. In dot blot assay, antigens in small volume (1 to 5 μl) are dotted, usually on the nitrocellulose sheet. Alternatively, antigens are dotted in a dot blot apparatus by inserting a nitrocellulose sheet. The blotted antigens are then detected with the antibodies. Dot blot assay can be performed relatively quickly compared to Western blot. Nonetheless, Western blot has at least two advantages. First, it provides the relative molecular weight (subunit) of the antigen, because detection precedes gel electrophoresis (in the presence of SDS) of the test sample. Second, since proteins separate on electrophoresis, more samples can be loaded and thus a very small representation of antigen in a mixture can be detected. Since SDS-gel electrophoresis involves denaturation of an antigen, antibodies must recognize the denatured antigenic epitope in order to achieve successful detection. Most polyclonal antibodies bind to denatured antigen, but many monoclonal antibodies do not. The procedures for gel electrophoresis and Western blot are previously described (see Chapter 3).

TABLE 6.13
Common Problems in ELISA and Their Possible Remedies

Common Problems	Cause	Remedy
High background color	Non-specific attachment of antibodies	Optimize blocking condition
Color develops very quickly	Antibody-enzyme conjugate too concentrated	Dilute the conjugate
Color develops too slowly	Antibody conjugate very diluted	Increase concentration
	Incubation temperature low	Incubate at recommended temperature
	Incorrect substrate pH	Check the pH
	Inhibitory component present in substrate solution (e.g., sodium azide inhibits peroxidase activity)	Avoid wrong preservative in working buffer
No/very little color for HRP conjugate after incubation with substrate	H_2O_2 is not added	Add H_2O_2
	H_2O_2 stock is inactivated	Use fresh stock
	Wrong dilution of H_2O_2	Check the dilution
Color inconsistent	Variable coating of antigen	Check the homogeneity
	Poor washing	Wash carefully
	Poor pipeting	Practice pipeting
	Poor mixing of reagents	Ensure good mixing
	Serial dilution poorly done	Practice pipetting
Unexpected results	Plate format incorrect	Check the format
	Color does not match plate reader results	Check for appropriate wavelength

Working Procedure for Dot Blot Assay

1. Blot antigen (10 to 50 μg/ml) into a nitrocellulose sheet at 100 μl/cm² manually or using dot blot apparatus (available from Bio Rad, Pharmacia, etc.).
2. Wash the membrane three times with PBS and incubate in a solution of 3% BSA in PBS at 37°C for 1 h to block non-specific binding of antibody.
3. Transfer the membrane to another container box containing primary antibody (conventional or monoclonal). For screening different test bleeds or hybridoma clones, the assay is performed in dot blot apparatus.
4. Wash the paper three to five times with PBS/0.05% Tween 20 and incubate with secondary antibody (enzyme-labeled or radio-labeled) in blocking buffer (1 to 2 ml/cm²) at 37°C for 1 h.
5. After washing three to five times with PBS/T$_{20}$, detect the bound secondary antibody with the corresponding substrate in case of enzyme-labeled antibody or exposed to X-ray film in case of radio-labeled antibody.
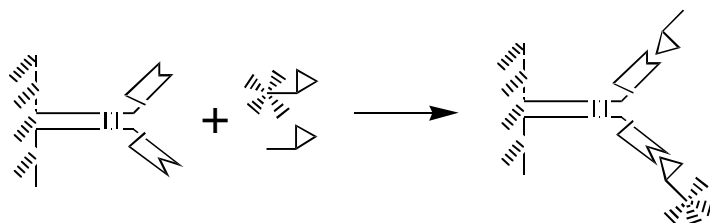
FIGURE 6.60   Radioimmunoassay.

6.3.2 ANTIGEN CAPTURE ASSAY

In this assay, antigens are detected and quantitated, but these should be prelabeled (see Figure 6.51). This assay is convenient to detect an antigen in an extract of cells that are metabolically labeled. The assay can be conveniently performed in both nitrocellulose membrane and microtiter plates. The most common example of this type is radioimmunoassay (RIA).

RIA

RIA is a very sensitive assay for detecting small amounts of antigens. In the 60s, this technique became very popular in many areas of clinical research, such as the measurement of the concentration of hormone in serum. However, because of the requirement to use hazardous radioactive substances, RIAs are frequently being replaced by other non-radioactive immunoassays employing sensitive fluorescent or chemiluminiscent substrates.

In RIA, radioactive antigen (^{125}I-labeled) is mixed with antibodies against that antigen (Figure 6.60). When this interaction is allowed in the presence of "cold" (unlabeled) antigen, the latter competes for the binding site of the antibodies. In order to prepare a standard curve, a varying amount of unlabeled antigen is added to a mixture of labeled antigen and antibodies. At an increasing amount of unlabeled antigen, an increasing amount of radioactive antigen is displaced from the antibody molecules. The antibody-bound antigen is separated from the free antigen and the radioactivity is measured. The unknown samples are assayed in parallel, and the antigen concentrations are determined from the standard curve.

6.3.3 TWO-ANTIBODY SANDWICH ASSAY

These assays are performed to determine the concentration of antigen in unknown samples (see Figure 6.51 c). Using a purified antigen, a standard curve can be obtained and the amount of antigen in an extract can be determined. One limitation is that not all antibodies can be used for this purpose. The two antibodies are to be selected so that they should not bind to the same epitope. Two antibodies can be either two monoclonal antibodies or one monoclonal antibody and one polyclonal antibody. The assay is usually performed in a microtiter plate.
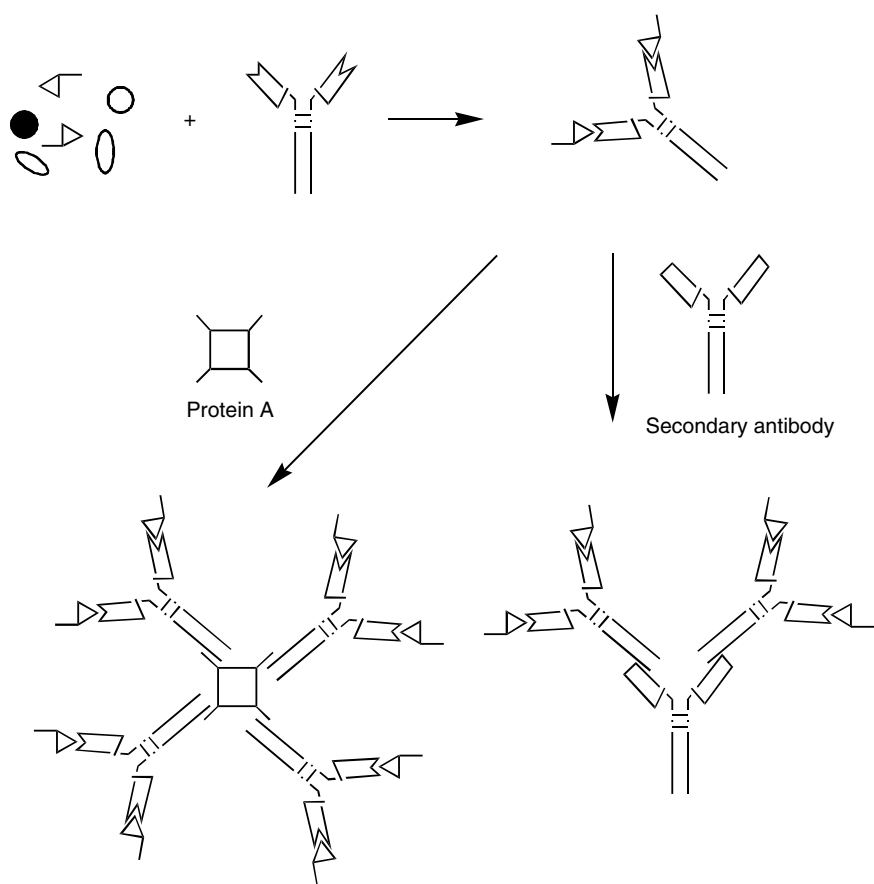
FIGURE 6.61 Immunoprecipitation.

6.4 IMMUNOPRECIPITATION

Immunoprecipitation is a process by which an antigen is isolated from a mixture by precipitating with the antibody. Immunoprecipitation is one of the most commonly used immunochemical techniques. It usually consists of several steps: (a) extraction of the cells for antigen solubilization, (b) formation of the antigen-antibody complexes, and (c) isolation of the antigen-antibody complexes. The isolation of immune complexes can be performed by precipitating with the anti-IgG antibodies followed by centrifugation, or by purification of the antigen-antibody complexes with an immobilized Protein A (Figure 6.61). Following purification, the complexes are usually subjected to SDS-polyacrylamide gel electrophoresis.

In most cases, the antigen is metabolically labeled prior to the extraction. For the isolation of antigen-antibody complexes, immobilized Protein A is more commonly used than a secondary antibody, which reacts with a primary antibody. Earliest immunoprecipitation techniques used anti-IgG antibodies to produce a large

aggregate (commonly known as a lattice) of antigen-antibody-anti-IgG antibodies. The insoluble lattice that precipitates out from the solution can then be removed from the solution by centrifugation. However, this procedure has some disadvantages. The molar ratio of antibody and anti-IgG antibodies must be determined for the formation of aggregates large enough to be precipitated and the precipitate quantitatively removed by centrifugation. The size of the lattice also depends on the amount and type of antigen (monomeric vs. multimeric) and the type of antibody (monoclonal vs. polyclonal) used. The use of staphylococcal Protein A as a solid phase instead of anti-IgG antibody to the immune complexes was suggested as a solution to this problem.[64] Since Protein A is found on the cell wall of *Staphylococcus aureus*, fixed cell wall proteins serve the same purpose. However, this matrix often results in high background because of the presence of other proteins. The background is greatly reduced when purified protein A-immobilized beads (such as protein A-Sepharose) are used.

6.4.1 LABELING OF ANTIGENS

Of several available techniques for labeling protein antigens, metabolic labeling of tissue culture cells is the most commonly employed. Other methods of labeling antigens are the iodination of surface proteins, treatment of surface proteins with radioactive sodium borohydride, or iodination of the protein after immunoprecipitation. However, antigens from the cells need not be labeled prior to immunoprecipitation, when immunoprecipitated antigens are detected by staining on SDS-PAGE or by immunoblotting, or tested for enzymatic activities.

Metabolic Labeling of Cells

Proteins from cells in tissue culture can be conveniently labeled by growing cells in the presence of radioactive amino acids. Since growing cells require amino acid for protein synthesis, radioactivity is incorporated as new proteins are synthesized. [^{35}S]-methionine is commonly used for metabolic labeling. During labeling with [^{35}S]-methionine it is important that cold methionine (non-radioactive) present in the culture medium should be substituted by radioactive methionine. There are several advantages of the use of ^{35}S label over other labels such as ^{3}H or ^{14}C. The signal of [^{35}S]-methionine can easily be enhanced using fluorography. The [^{35}S]-methionine can easily be incorporated into protein, because the intracellular pool of methionine is smaller than many other amino acids. The incorporation of [^{35}S]-methionine is linear over a wide range of added labels.

Working Procedure for Metabolic Labeling

For Monolayer Culture

1. When cells are approximately 75% confluent, remove the medium, wash the monolayer once with the prewarmed (37°C) methionine-free medium, and discard.

2. Add the appropriate volume of prewarmed methionine-free medium per dish (2 ml/100 mm dish; 0.5 ml/60 mm dish; 0.25 ml/35 mm dish; 0.2 ml/well of 24-well plate; 0.02 ml/well of 96-well plate).

3. Add [^{35}S]-methionine (5 to 500 µCi depending on the abundance of molecules to be detected) to the culture medium and incubate at 37°C for 2 to 4 h.

4. Remove the medium and wash the monolayer once with PBS. Dispose supernatant and wash solution. However, spent medium and wash solution should be saved for immunoprecipitation if the antigen protein is expected in the spent medium.

5. Cells are now ready for lysis.

For Suspension Culture

1. Pellet cells by centrifugation (400 g for 5 min) and resuspend the pellet in prewarmed (37°C) methionine-free medium. Wash once with the same medium, resuspend the cells in the same medium (10^7 cells/ml), and transfer to a small tissue culture plate.

2. Add [^{35}S]-methionine to the culture medium as before and incubate.

3. Collect cells and centrifuge at 400 g for 5 min. Remove the medium and wash once with PBS (by resuspending and centrifuging). Discard or collect spent medium and wash as needed.

4. Extract cells for antigen isolation.

Iodination of Cell Surface Proteins Using Lactoperoxidase

This method is commonly employed to label surface proteins that are not abundant and are not easily detected using metabolic labeling (see Section 6.1.1.3 for Reaction). Of several iodination procedures, this is most suitable for labeling surface proteins because most plasma proteins remain protected.

Note: sodium azide inhibits lactoperoxidase activity and thus should be avoided in PBS.

Working Procedure

1. For monolayer culture, remove the medium from rapidly growing cells, wash the monolayer twice with PBS, and finally add 0.5 ml of PBS for 100 mm dish. For suspension culture, centrifuge cells (approximately 10^7) at 400 g, remove the medium, wash cells twice with PBS by resuspension followed by centrifugation. Finally resuspend the cells in 0.5 ml of PBS.

2. Add 100 µl of lactoperoxidase (1 unit/ml) followed by the addition of 500 µCi of Na^{125}I. Mix.

3. Add 1 µl of H_2O_2 prediluted to 20,000 to initial reaction. Mix. Continue addition of 1 µl of diluted H_2O_2 at 1 min interval for another 4 min.

4. Terminate the reaction with an equal volume of dithithreitol (1 mM) and tyrosine (1 mg/ml) in PBS.
5. Wash the cells twice with PBS. For suspension cells, wash cells by centrifuging and resuspending in PBS. Cells are now ready for lysis.

6.4.2 Lysis of Cells

Cells can be lysed in several ways (see Chapter 1 for details) such as with detergents, by mechanical disruption, or by freezing and thawing. However, it is important to choose a lysis buffer that offers an efficient release of antigen and is suitable for an antigen-antibody reaction. Quantitative release can easily be determined on immunoblots by testing the amount of antigen from lysates as well as in the cell debris. NP-40 detergent is commonly used in lysis buffer.

6.4.3 Preclearing of the Lysate

Prior to immunoprecipitation, preclearing of the lysate is often employed to reduce background resulted from immunoprecipitation. This is necessary when the antigen represents a minor component of the cellular proteins. Preclearing is usually performed by treating the lysate with the control antibodies. For polyclonal antibodies, the best control should be pre-bled from the same animal used for immunization. For monoclonal antibodies, the control should be from the same source as the specific antibody. For example, if a monoclonal antibody is obtained from a spent medium, control should also be obtained from the spent medium that contains a non-specific antibody. Similarly, for a monoclonal antibody that is obtained from ascites, control should be from other ascites. Preclearing requires all steps that mimic the immunoprecipitation. But unlike actual immunoprecipitation, the supernatant must be saved.

Working Procedure

1. To 1 ml of lysate add 50 µl of normal rabbit serum and incubate 1 h at 4°C.
2. Mix the lysate with Protein A Sepharose (200 µl) or fixed *S. aureus* Cowan I (500 µl) prewashed with PBS or lysis buffer. Resuspend and incubate at 4°C for 30 min.
3. Centrifuge the mix on an Eppendorf centrifuge and carefully remove the supernatant. Save the supernatant for actual immunoprecipitation.

6.4.4 Formation of Immune Complexes

Once the lysate is pretreated with the control antibody, it is then incubated with the specific antibody for actual immunoprecipitation.

Working Procedure

1. To the precleared lysate add specific antibody (serum 0.5 to 5 µl; hybridoma tissue culture suspension 10 to 100 µl; and ascites fluid 0.1 to 1 µl). Incubate 1 h at 4°C.
2. To antigen-antibody mix, add fixed *S. aureus* Cowan I (50 µl, 10%) or Protein A Sepharose (50 µl, 10%) prewashed with lysis buffer. Incubate at 4°C for 30 min.
3. Centrifuge in a microfuge for 1 min. Remove supernatant by aspiration.
4. Wash pellet three times with lysis buffer by resuspending and centrifuging.
5. If immune complexes are to be applied on SDS-polyacrylamide gel electrophoresis, Laemmli sample buffer can be used to dissociate antigen from Protein A-Sepharose. For this purpose add 50 µl of Laemmli sample buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris, pH 6.8, and 0.001% bromophenol blue. Heat at 85°C for 10 min. Centrifuge and remove supernatant for loading onto gel.

Immunoprecipitation in combination with SDS-PAGE provides information on the purified antigen such as relative molecular weight, quantity, its rate of synthesis or degradation, presence of some post-translational modifications, and its interaction with proteins or ligands.

6.5 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (also known as immunocytochemistry) is a combination of anatomical, immunological, and biochemical techniques for the identification of specific tissue components by manipulating antigen-antibody reaction tagged by a visible label. This can be applied to a variety of tissue and cell samples, cell smears, frozen tissue sections, and sections prepared from paraffin- and plastic-embedded tissues. Figure 6.62 shows the general scheme of histochemical detection. In order to detect a specific tissue antigen, a primary antibody (polyclonal or monoclonal) is added and after removing the unbound antibody, a labeled secondary antibody (conjugated with fluorescent compound or enzyme) is then used and visualized directly (in case of a fluorescently labeled antibody) or with substrate (in case of an enzyme-labeled antibody) under light microscope. Among the enzymes that are labeled with secondary antibodies, the most popular enzymes are horseradish peroxidase and alkaline phosphatase (AP).[65]

6.5.1 SPECIMEN PREPARATION

In histochemical analysis, it is very important to maintain the morphology of the tissues and cells for proper accessibility of the antigenic site. Fixation of tissues and cells usually preserves the morphology by preventing autolysis caused by lysosomal enzymes. It also helps to prevent any bacterial or mold growth. A fixative step is

Antigen

↓

Antigen specific antibodies

↓

Labeled antibodies that
bind primary antibodies

For light microscopy:

a) enzymes - HRP, AP
b) fluorochromes
        - fluorescein
        - rhodamine
c) gold particles with silver
    enhancement

**For immunofluorescence
microscopy:**

Fluorochromes

For electron microscopy:

a) gold particles
b) ferritin
c) HRP
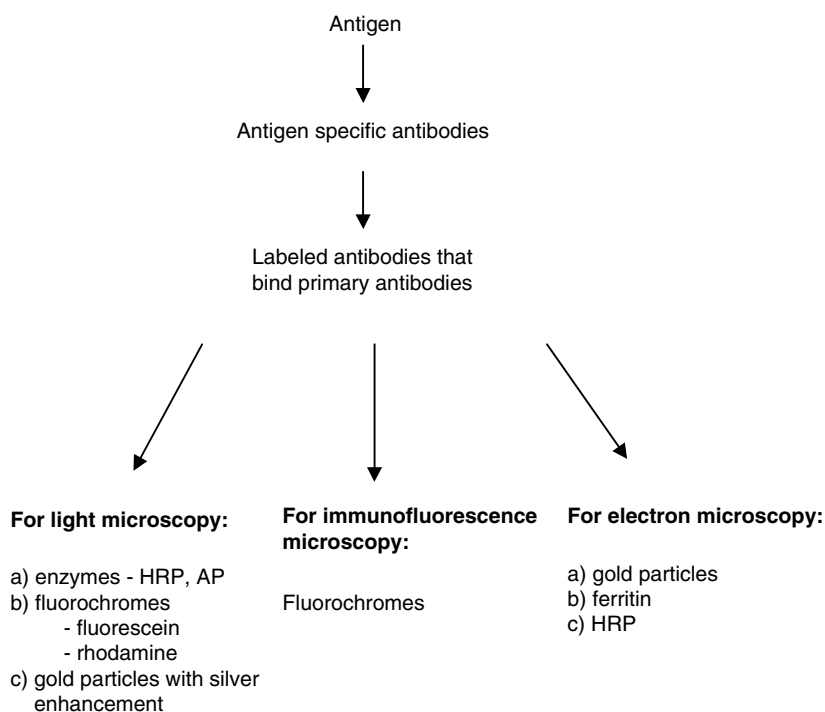
FIGURE 6.62 General scheme of histochemical detection.

required prior to the immunostaining procedure. Fixation is carried out either of the
tissue sample itself (followed by sectioning if necessary), or of the tissue section if
cut from an unfixed specimen (Figure 6.63).

6.5.2 SPECIMEN FIXATION

Several fixative reagents can be used for this purpose, but the choice of fixative
agent should be dependent on the nature of the target antigen.[39] Additionally, each
fixative agent needs serious optimization of concentration, pH, and temperature
based on the target antigen. For example, treatment of tissues with an improper
fixative agent may cause difficulty in penetrating the immunoassay components.
While a particular fixative may provide an optimal access of one epitope, it may
lose other epitopes on the same antigen. Tissue fixatives are generally two types:
aldehyde-based fixatives (for example, formaldehyde, glutaraldehyde, etc.) and pre-
cipitating fixatives (for example, methanol, methanol, acetone, etc.). The former type
causes chemical cross-linking of proteins, while the latter immobilizes proteins by
precipitation. Table 6.14 shows a list of target antigens and the recommended fixative
reagents. The recipes of each fixative and working procedures are described
elsewhere.[66]
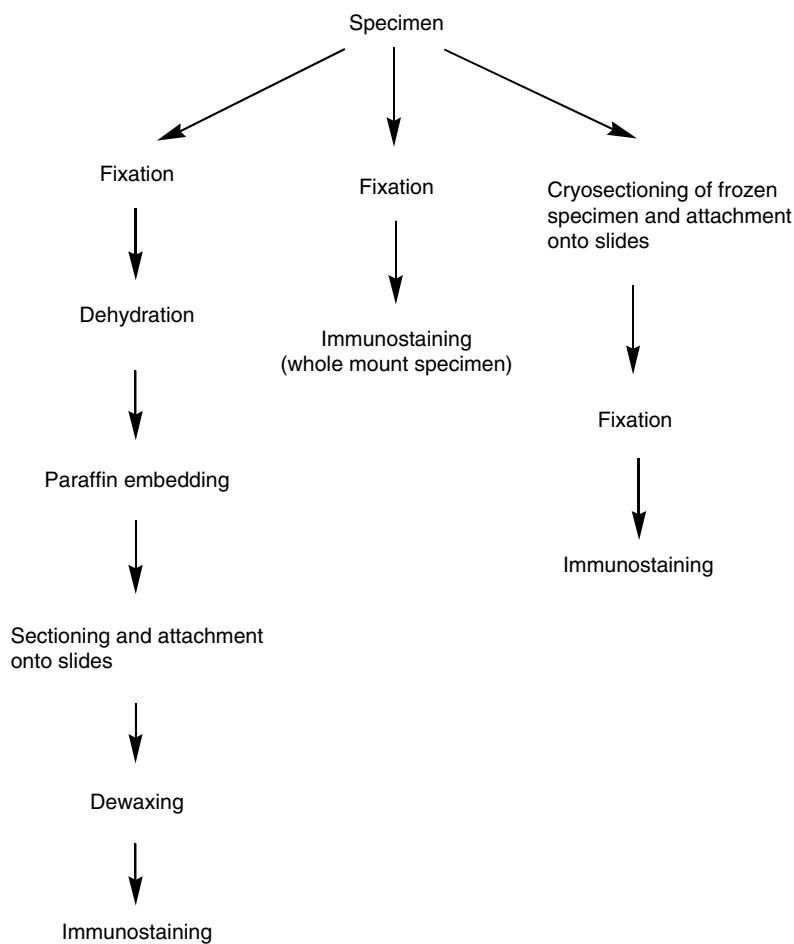
FIGURE 6.63 Fixation.

TABLE 6.14
Target Antigens and the Recommended Fixative Agents

Antigen	Fixatives
Proteins, peptides, enzymes	Formaldehyde, paraformaldehyde, paraformaldehyde-picric acid, glutaraldehyde
Glycoproteins	Periodate-lysine-paraformaldehyde
Membrane proteins	Acetic acid-zinc chloride
Large protein molecules	Ethanol, methanol, acetone

6.5.2.1   Aldehyde-Based Fixatives

Formaldehyde

Formaldehyde-based fixatives are usually mild to tissues. They allow good penetration, but fixation can lead to partial loss of antigenicity. Thus the detection of an antigen with a monoclonal antibody may not be a good choice since it recognizes a single epitope. Detection with a polyclonal antibody is less susceptible, since polyclonal antibodies are directed against a battery of epitopes. Examples of formaldehyde-based fixatives are Phosphate Buffered Neutral Formalin (4% [w/v] formaldehyde in phosphate buffered saline — i.e., 10% formalin), pH 7.4 and Bouin's Solution (a saturated solution of picric acid containing 4% paraformaldehyde and 1% acetic acid).

Formaldehyde (HCHO) is a gas, but is commercially available in two forms: a 40% (w/v) aqueous solution, which is called formalin, and a solid polymer known as paraformaldehyde $[(CH_2O)n]$. In an aqueous solution, formaldehyde exists predominantly in its hydrated form, methylene glycol $[CH_2(OH)_2]$.

Reaction

Formaldehydes fix tissues by reacting primarily with basic amino acids to produce cross-linking methylene bridges (-CH2-). Under reaction conditions, formaldehyde (HCHO) gets protonated to form reactive carbonium ion ($^+CH_2OH$). This electrophile then reacts with the electron-rich region of the proteins[67] (Figure 6.64).

Glutaraldehyde

Glutaraldehyde ($CHOCH_2CH_2CH_2CHO$) is a stronger cross-linker than formaldehyde, but its penetration to tissue is slower than formaldehyde. Glutaraldehyde-based fixatives can modify tissue structure, resulting in a considerable loss of antigenicity.

Reaction

They react primarily with amino groups and sulfhydryl groups of proteins (see Figure 6.5). Following fixation, the excess reactive aldehyde should be blocked with ethanol amine or lysine to prevent covalent bonding with amino group of antibodies. The

$$R-H + CH_2O \rightleftharpoons R-CH_2OH$$

$$R-CH_2OH + R-H \rightleftharpoons R-CH_2-R + H_2O$$

$$R-NH_2 + CH_2O \rightleftharpoons R-NH-CH_2OH$$
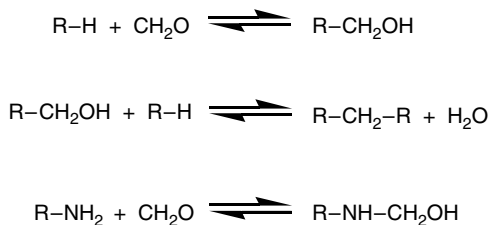
FIGURE 6.64 Fixation with formaldehyde.

examples of glutaraldehyde-based fixatives are as follows: (a) 0.1% glutaraldehyde in 0.1 M phosphate buffered saline (pH 7.4) containing 4% paraformaldehyde and (b) 5% glutaraldehyde in 0.1 M cacodylate and 1% sodium metabisulfite (pH around 7.5).

Dimethyl Suberimidate

Dimethyl Suberimidate (Pierce, Rockford, IL), a bifunctional imidoester cross-linking reagent, provides an alternative to glutaraldehyde-based fixatives. Dimethyl Suberimidate reacts with α and ε-amino groups of proteins. The advantage of using this cross-linker is that it maintains the net electronic charge on the protein after cross-linking. Unlike glutaraldehyde, this fixative does not require blocking because of the lack of aldehyde group. It preserves immunoreactivity of the antigen and is useful for both light and electron microscopes.

Mercuric Chloride

Mercuric chloride-based fixatives can be used as substitutes for formaldehyde-based fixatives because of their good penetration and preservation of cytological detail. Sometimes, these can be mixed with other fixatives. Zenker's Solution and B5 are examples of mercuric chloride-based fixatives. Zenker's working solution is a mixture of 1 part of acetic acid and 20 parts of Zenker's stock solution (5% mercuric chloride/2.5% potassium dichromate/1% sodium sulfate). B5 working solution is a mixture of 1 part of 40% formalin and 10 parts of B5 stock solution (6% mercuric chloride/1.25% anhydrous sodium acetate).

6.5.2.2 Precipitating Fixatives

Alcohol and Acetone

Methanol, ethanol, and acetone precipitate large proteins and are good for cytological preservation. The advantage of using precipitating fixatives is that they do not usually mask antigenicity. But they result in poor penetration. They can cause tissue shrinkage and thus are not suitable for electron microscopy work. Methanol and ethanol both compete with water for hydrogen bonding. Alcohols reduce the dielectric constant of the protein solution and thus aggregate or precipitate proteins at or near their isoelectric points.[68]

Picric Acid

Picric acid (2,4,6-trinitrophenol) is used as a constituent of a number of fixatives. The dipole induction of the nitro groups probably induce the formation of weak bonds between picric acid and surface groups of proteins. Picric acid may produce intermolecular salt links by inducing polarity in the amino acid side chains between two proteins.[69]

Osmium Tetroxide

Osmium tetroxide is mostly used as a fixative for electron microscopy.

6.5.3 SPECIMEN PROCESSING

For immunostaining, fixed tissues can be processed in a number of ways such as whole mounts, vibratome sections, sliding microtome frozen sections, cryostat sections, and paraffin sections. Table 6.15 lists several sectioning procedures and their advantages/disadvantages.

TABLE 6.15
Specimen Sectioning Procedures and Their Advantages and Disadvantages

Specimen Sectioning Procedure	Type of Specimen	Advantages	Disadvantages
Whole mount preparations	Fixed	No sectioning required No use of organic solvents, high temperature, or freeze/thawing Provides 3-D data	Poor penetration
Vibratome sections	Fixed	No use of organic solvents, high temperature, or freeze/thawing Free-floating or embedded tissue processing High-resolution cytological details obtained	May result in poor penetration Vibratome required May produce vibration lines Reconstruction of 3-D structure may be difficult
Sliding-Microtome frozen sections		Sections obtained easily compared to vibratome	Freeze/thawing may result in morphology Sucrose infusion makes sections sticky and transfer difficult
Paraffin sections		Easy to perform	Cutting procedure compresses sections that must be spread out Required to extract, dehydrate, and heat
Cryostat sections	Unfixed	Obtains high-quality sections Antigen preservation higher than with paraffin sections Faster procedure	Requires freeze/thawing, cryoprotection Difficult to operate May occur tearing May reduce morphological detail and resolution

In whole mount sectioning, a small block of tissue is processed without any sectioning, and thus 3-dimensional data can be obtained. The disadvantage of this process is poor penetration.

In vibratome sectioning, tissues are processed by a sharp razor blade in a vibratome apparatus. The sliding microtome frozen section technique cuts tissue with a sliding, freezing microtome.

In cryostat sectioning, tissues are frozen first and sectioned prior to fixing and immunostaining. During snap-freezing, cells and tissues are immobilized quickly and hardened, making sectioning easy. Cryosectioning usually overcomes the problems associated with cross-linking fixatives. Sometimes ice crystals that are formed during freezing may damage tissue morphology. For this reason, several cryoprotectants (e.g., sucrose, glycerol, dimethyl sulfoxide) are used to prevent the formation of ice crystals. The frozen tissues are sectioned with a cryostat and transferred to cold slides.

In paraffin sectioning, tissues are embedded in paraffin after dehydration and subsequently cut into ribbons for mounting onto slides.

6.5.4 Attachment of Specimen onto Slides

Once the specimen has been sectioned, it needs to be attached to a slide firmly. The method of attachment is critical, since the specimen may detach during lengthy staining procedures. Although tissue sections can be adhered to clean glass slides through capillary attachment, in most procedures, glues such as egg white glycerin and glycerin-gelatin mixtures are used to ensure complete attachment. Frequently, slides are coated with poly-L-lysine or amino alkyl silane, when trypsin digestion on the tissues is required to unmask the antigen. Poly-L-lysine- and aminoalkysilane-coated slides attach tissue sections, probably by binding through negative charges of the proteins.

Working Procedure for Paraffin Embedding

The following procedure is based on a protocol published elsewhere.[70] Alternative procedures are also used for this purpose.[71,72]

1. Dehydrate fixed tissue by immersion in increasing concentration of ethanol (50%, 70%, and 95%) for 1 to 2 h each at 0 to 4°C.
2. Immerse in absolute alcohol, three changes, 1 h each, last change at RT.
3. Immerse in xylene, two changes, 30 min each at RT.
4. Embed in paraffin wax or a synthetic wax such as diethylene glycol distearate.

Working Procedure for Dewaxing Fixed Paraffin-Embedded Sections

Once the paraffin-embedded specimens are sectioned and attached onto slides, they need to be dewaxed before immunostaining. Place slides in a rack and immerse in as follows:

1. Xylene, 20 min.
2. Xylene, 5 min.
3. 100% ethanol, 3 min each.
4. 95% ethanol in distilled water, 3 min.
5. 70% ethanol in distilled water, 3 min.
6. Distilled water, three changes, 1 min each.
7. Rinse in PBS prior to immunostaining.

6.5.5 ENZYME DIGESTION FOR UNMASKING OF ANTIGENS

Overfixation with aldehyde-based fixatives may cause excessive cross-linking and may mask some antigenic epitopes, making them inaccessible to antibodies. Optimization of the fixing procedures can avoid this problem. However, if fixation still masks the antigen, proteolytic enzymes such as trypsin, pepsin, chymotripsin, and pronase are applied to the tissue section for a certain period of time. The proteolytic enzymes unmask epitopes, probably by breaking some of the cross-linkages.[66,73,74] However, over-digested tissue with proteolytic enzymes may reveal new cross-reactive sites, and thus several preliminary experiments of proteolytic unmasking should be performed in order to obtain optimal conditions (e.g., reaction time, temperature, concentration).

6.5.6 INHIBITION OF ENDOGENOUS PEROXIDASE

Since the immunoproxidase method is commonly used as a final step of immunostaining cells and tissues, the presence of endogenous peroxidase activity in cells and tissues makes the staining methods complicated. With the addition of substrate, the endogenous peroxidase also reacts, resulting in a non-specific staining that is indistinguishable from the immunostaining of the antigen of interest. Since heme-bearing proteins such as hemoglobin, myoglobin, and cytochrome c are widely distributed, the inhibition of endogenous peroxidase activity is essential for the correct localization of an antigen when the immunoperoxidase method is a choice of immunostaining procedure.

The fixed tissue should be reacted with a peroxidase substrate to determine the presence of endogenous peroxidase activity (by the formation of any colored precipitate). The endogenous peroxidase activity can be inhibited by several methods. Treatment of the fixed tissue with 3% hydrogen peroxide or hydrogen peroxide in methanol is the most common method for this purpose. Other reagents such as methanol, nitroferricyanide, periodic acid, phenylhydrazine, sodium azide, and cyclopropane are also known to inhibit endogenous peroxidase activity. The peroxidase inhibitor should be applied after incubation with the primary antibody, prior to the peroxidase conjugate.

6.5.7 BLOCKING OF ENDOGENOUS AVIDIN-BIOTIN ACTIVITIES

Endogenous avidin-biotin activity is most common in cryostat sections of liver, adipose tissue, kidney, and mammary gland. To block this endogenous avidin-biotin activity, the cryosection is treated with avidin followed by biotin.

Working Procedure

1. Incubate tissue section with 0.1% avidin for 20 min at room temp.
2. Wash with PBS.
3. Incubate with 0.01% biotin for 20 min at RT.
4. Wash with PBS.

6.5.8 BLOCKING OF NON-SPECIFIC INTERACTIONS

Blocking of the unwanted reactive sites is essential in histochemical reaction for better signal-to-noise ratio. Normal serum or BSA is normally used for this purpose. Blocking serum reacts to protein-binding sites by either non-specific adsorption or by specific, but not desired, interactions. The use of non-ionic detergent such as Tween 20 in washing buffer or carrier protein helps to reduce background staining by reducing hydrophobic interaction between tissue antigens and antibodies.

For an indirect binding technique, preimmune serum from the host species, providing the second antibody is usually added to the tissue sample at the beginning of the procedure. For a direct binding technique, the blocking serum is usually from the species providing the primary antibody. Blocking serum is generally applied to the tissue sample 10 to 30 min prior to the addition of the primary antibody and, after draining off, is reapplied with the primary antibody and all subsequent steps until the final step (development with substrate).

6.5.9 BINDING WITH LABELED ANTI-BODY

Once the tissue section is fixed, the antigen is unmasked, if necessary. The tissue is blocked to avoid non-specific interaction, and then treated with the primary antibody. Endogenous peroxidase or avidin-biotin activity is then blocked depending on the immunostaining method. After washing the tissue section, a secondary antibody-peroxidase conjugate is added.

Working Procedure for Indirect Immunofluorescence

1. Incubate fixed tissue section with the unlabeled primary antibody for 1 h.
2. Wash three times for 5 min per wash with PBS.
3. Incubate with fluorochrome-labeled secondary antibody.
4. Wash three times as step 2.
5. View on fluorescence microscope.

6.6 FLOW CYTOMETRY

In flow cytometry, physical and chemical characteristics of biological particles such as cells, viruses, fungi, and parasites are measured as they pass through a detector device in a fluid system. When viable cells of specific surface properties are sorted

by antibodies conjugated to a fluorophore, the process is called fluorescence-activated cell sorting (FACS).

A flow cytometer consists of several components such as an optical device, electronic and nuclear radiation sensors, and a computer for data analyses. The availability of monoclonal antibodies against cell surface antigens makes flow cytometry a very popular technique not only to isolate and characterize subpopulations of cells within heterogeneous populations, but also to quantify distinct subpopulations of cells. Typically, fluorochrome attached to antibody molecules to the cells (in immunofluorescence flow cytometry) is excited at a particular wavelength of light as the cells pass through (in a single-cell stream) a light source, usually laser beams. As the fluorochrome decays, light of another wavelength is emitted and is detected by an optical device. Light scattering thus collected over several angular ranges is converted into electronic signals by photomultiplier tubes. Magnitudes of electronic signals are proportional to the intensity of the emitted and scattered light. Electronic signals are amplified and evaluated.

A variety of instruments are available for analytical cytology. They differ mostly in their light source, such as arc lamps (high-pressure mercury and xenon lamps) or lasers. The type of model of laser also varies by sizes and shapes. Among lasers, argon (wavelength 488), krypton (576), and helium-neon (633) ion lasers are common. The instruments are classified as two types: flow cytometer and cell sorter. A flow cytometer measures the physical and chemical characteristics of cells, but a cell sorter, in addition to the flow cytometer's function, can also selectively separate different cell populations.

Among the fluorochromes, fluorescein isothiocyanate (FITC) (absorption 495 nm, emission 530 nm) is mostly used in flow cytometry, since many of the antibodies that FITC conjugates are commercially available. Phycoerythrin (PE) (absorption 490 nm, emission 575 nm) is used with FITC in most cases where two-color flow cytometry is needed. This is because PE is excited at the same wavelength as FITC, but emitted at a different wavelength. However, other fluorochromes such as Texas Red (absorption 568 nm, emission 590 nm) can also be used with FITC for two-color flow cytometry, but it would require a second laser since its excitation wavelength differs from that of FITC. Researchers are urged to consult the manufacturer's literature about the specification of a light source. Beckton Dickinson (Mountain View, CA) and Coulter Corporation (Hialeah, FL) are the popular commercial sources that house varieties of flow cytometers for a number of applications.

Working Procedure

Staining of Cells

1. Centrifuge a healthy cell suspension at 400 g for 5 min and after discarding the supernatant, resuspend in FCM buffer (3% heat-inactivated fetal calf serum and 0.1% sodium azide in balanced salt solution) at 2×10^7 cells/ml. Keep the cell suspension on ice.

2. Aliquot 50 µl cell suspension to microfuge tubes and add 50 µl FITC conjugated antibody (for direct assay) or unlabeled primary antibody (for indirect assay) to each well. Appropriate dilutions of antibody should be predetermined based on its intensity of staining at several dilutions. Incubate the mixture on ice for 30 min. Set up controls with preimmune serum.
3. Centrifuge tubes at 400 g for 5 min and discard supernatant.
4. Wash the cells with ice-cold FCM buffer three times (200 µl each), followed by centrifugation as in step 3.
5. Add 50 µl FITC-labeled secondary antibody and incubate on ice for 30 min.
6. Wash the cells as in step 4.
7. If cells are to be stored before running, resuspend the cells in 100 µl FCM buffer and fix with 2% paraformaldehyde.

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7 Purification of Glycoproteins and Analyses of their Oligosaccharides

Glycoproteins constitute the class of glycoconjugates in which glycans (carbohydrate moieties) are covalently associated with the proteins, usually through the N atom of asparagines (N-glycans) or the O atom of serine/threonine (O-glycans). Glycosylation of proteins represents one of the most common and important post-translational phenomenons. Most proteins in an organism are glycoproteins. Glycans in glycoproteins are known to play several biological roles. Some examples are the protection of the protein from proteolysis attack; cell-cell interactions through lectins; association with enzymes, bacteria, and viruses; and maintaining protein conformation in a biologically active form.

It has been established that glycosylation of proteins is developmentally regulated. In cancer cells, the structure of glycans is known to alter during cancer progression. In host-parasite systems, carbohydrate structures play important roles for parasitic infections. Thus, understanding glycan structure is necessary in order to determine biological roles of glycoproteins.

7.1 DIAGRAMS AND STEREOCHEMISTRY OF MONOSACCHARIDES AND OLIGOSACCHARIDES

Glycan moieties of a glycoprotein are composed of various monosaccharides linked in a linear or branched fashion. The monosaccharide is a glycan's smallest block, which cannot be hydrolyzed into smaller units. In most glycoproteins, monosaccharides of six carbon atoms (hexoses) are the most common. Glucose, galactose, and mannose are such examples. Examples of five-carbon-atom monosaccharides (pentoses) are arabinose, ribose, and xylose. However, glyceraldehyde is the simplest monosaccharide, containing one chiral (asymmetric) carbon atom (C-2) (Figure 7.1). Chiral molecules show optical isomerism and can exist in two non-superimposable mirror images known as enantiomers (see Figure 7.1). Enantiomers have identical physical properties except for the direction of optical rotation. Monosaccharides that have a clockwise rotation of the plane of polarized light are called dextro rotatory, denoted as "d" or (+). Similarly, monosaccharides that have counterclockwise rotation of the plane of polarized light are called laevo rotatory, denoted as "l" or (−). Besides these (+) and (−) forms, glyceraldehydes can also be presented in two
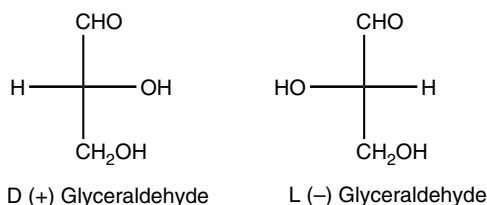
FIGURE 7.1 Enantiomers of glyceraldehyde.

additional forms: "D" and "L." By definition, a D-monosaccharide is a carbohydrate in which the hydroxyl group farthest from (or adjacent to, in the case of glyceraldehydes) the carbonyl group points to the right in a Fischer projection. A Fischer projection is a drawing of a monosaccharide in which chiral carbon atoms are represented as a pair of perpendicular lines being C-1 (carbon number 1) at the top. Vertical lines represent bonds projecting behind the plane of a paper. Horizontal lines represent bonds projecting out from the plane of a paper. For glyceraldehydes, D is the dextro rotatory, while L is laevo rotatory. The + or – sign is indicated between parentheses, immediately after D or L; e.g., D (+) glyceraldehydes. The direction of optical rotation of a monosaccharide is experimentally determined. Naturally occurring monosaccharides in animal cells possess the D configuration except for fucose and iduronic acid, which are in L configuration.

Pentose and hexose usually form cyclic structures. The hemiacetal produced by the reaction of the aldehyde group at C-1 with the hydroxyl group at C-5 results in a six-membered ring, called a pyranose (Figure 7.2). Five-membered rings, formed by the reaction of the C-1 aldehyde with the C-4 hydroxyl, are called furanose. Hexoses usually form pyranose rings, while pentoses form furanose rings. The formation of the ring produces an additional chiral center at C-1, forming two additional isomers, called anomers (designated α and β). In glycans, monosaccharides are linked to each other via anomeric centers, called α and β linkages. The ring form of a hexose can have 32 structures, since it contains five chiral carbon atoms ($2^5 = 32$). Monosaccharides with identical formulas but different spatial distribution of –OH and H are called diastereoisomer (e.g., galactose and mannose). When two monosaccharides differ only in the configuration of a single chiral carbon, they are called epimers (e.g., glucose and mannose differing at C-2). Besides the Fischer projection, monosaccharides are also shown in the Howarth representation (see Figure 7.2). In this form, a six-membered ring is drawn with the oxygen at the upper right corner, with the groups attached to the carbons above or below the ring. All groups that appear to the right in the Fischer projection are drawn below the plane of the ring in the Howarth representation. In α-anomer of the D sugar, the –OH at C-1 is drawn below the ring. In β-anomer of the L sugar, -OH at C-1 is placed above the ring

However, neither the Fischer projection nor the Howarth representation of monosaccharide depicts the real conformation. The pyranose form of a monosaccharide in solution preferentially adopts a "chair" conformation (see Figure 7.2). Each group (H or OH) in this conformation can adopt either "axial" or "equatorial" position.
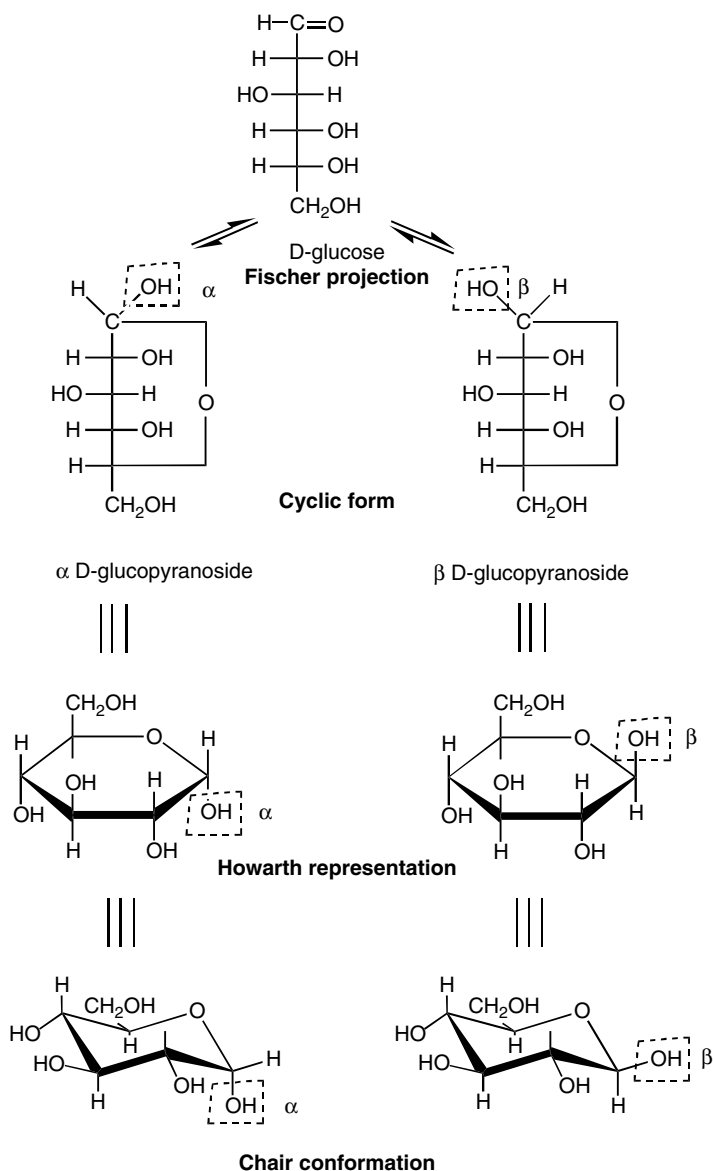
FIGURE 7.2 Open and ring forms of D-glucose.

An oligosaccharide is commonly described by the abbreviated form of its monosaccharide components and the type of linkage that connects each monosaccharide. For example, A-tetrasaccharide can be written as follows:

α D-GalNAc 1,3[α L-Fuc*p*1,2]β D-Gal*p*1,4 D-Glc*p*
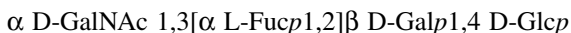
In practice, the D and L configuration and the nature of ring structures are assumed, and thus the formula is written as follows:

$$\text{GalNAc}\alpha1,3(\text{Fuc}\alpha1,2)\text{Gal}\beta1,4\text{Glc}$$

or:

$$\text{GalNAc}\alpha1,3\text{Gal}\beta1,4\text{Glc}$$
$$\Big|_{1}^{2}$$
$$\text{Fuc } \alpha$$
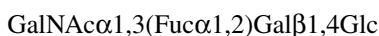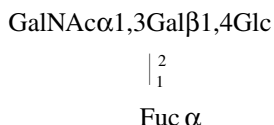
Types of Glycan-Peptide Linkages in Glycoproteins

Glycan moieties are usually conjugated to peptide chains through two types of covalent linkages (N-glycosyl and O-glycosyl). The most common N-glycosyl linkage in glycoprotein is N-acetylglucosaminyl-asparagine (GlcNAcβ1-N Asn). N-glycans can be of several types such as high mannose, complex, and hybrid types, but they all have a common pentasaccharide inner core (Figure 7.3).

For an O-glycosyl bond, a wide variety of linkages are known in glycoproteins. The most common examples of this type are (a) mucin type (GalNAcα1,3 Ser/Thr) found in numerous glycoproteins, (b) collagen type found in collagens (Galβ1,5 Lys), (c) proteoglycan type (Xylβ1,3 Ser) involved in the acidic mucopolysaccharide-peptide bond of proteoglycans, and (d) extension type (Arafβ1,4 Pro) identified in plant glycoproteins.
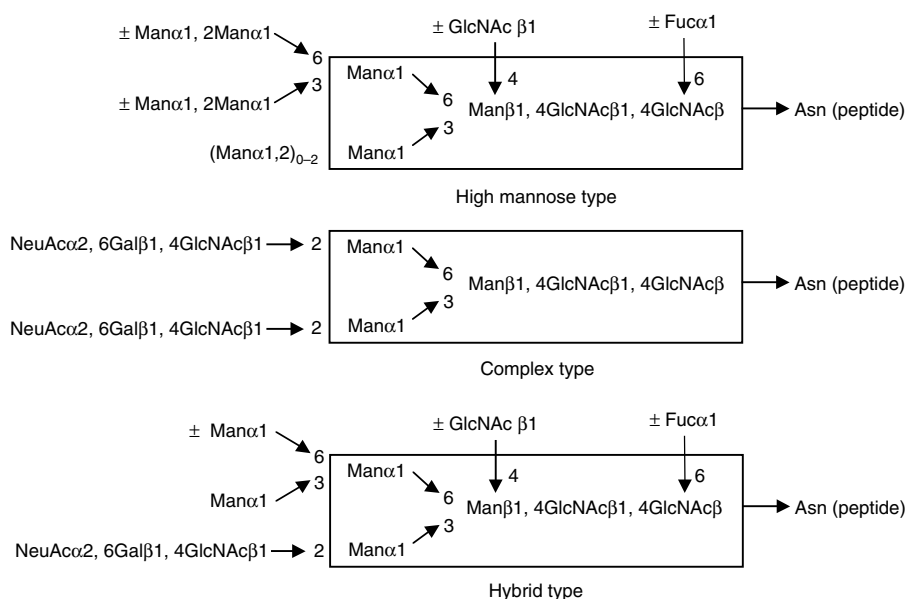
FIGURE 7.3 Structures of various types of N-glycans.

7.2   PURIFICATION OF GLYCOPROTEINS

Glycoproteins may be purified by utilizing the properties of proteins such as charge, hydrophobicity, etc. However, purification of glycoproteins is most frequently performed by employing affinity of the carbohydrate moieties toward lectins (i.e., lectin affinity chromatography).[1,2] Several lectin-immobilized columns are commercially available. Some most commonly used columns are Con A-Sepharose, WGA-Sepharose, and RCA-Sepharose.

Purification of Glycoproteins on a Con A-Sepharose

Con A (Canavalin A from jack bean *Canavalia ensiformis*) binds mannose and glucose residues, and thus glycoproteins containing terminal mannose or glucose can be purified using a Con A-Sepharose column. Since mannose is common in many glycoproteins, multiple glycoproteins can bind to the column and so additional purification procedures are often required to separate the glycoprotein of interest.

Working Procedure

1. Pour Con A-Sepharose gel slurry (available from various vendors such as Sigma, Pharmacia, Pierce, and EY Laboratories) onto a column.
2. Wash the column with 5 bed volumes of equilibrating buffer (0.01 M Tris-HCl, pH 7.5 containing 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$).
3. Load the glycoprotein solution slowly on the column to allow binding (2 mg of glycoprotein should bind per ml of packed Con A-Sepharose).
4. Wash the column with 10 bed volumes of equilibrating buffer until A_{280} of the flow through reaches baseline.
5. Elute the bound protein 3 to 5 bed volumes of 0.2 M α-methyl D-mannoside in equilibrating buffer.
6. Regenerate the column by washing with 10 volumes of equilibrating buffer.

Purification of Glycoproteins on a WGA-Sepharose

Wheat Germ Agglutinin (WGA)-Sepharose is employed to purify glycoproteins that contain terminal N-acetylglucosamine (GlcNAc) or sialic acid residues.

Working Procedure

1. Pour WGA-Sepharose (available from Sigma, Pharmacia, and EY Laboratories) on to a column.
2. Wash the column with 5 bed volumes of equilibrating buffer (phosphate buffered saline, PBS).

3. Load the glycoprotein solution slowly on the column to allow binding (2 mg of glycoprotein should bind per ml of packed WGA-Sepharose).
4. Wash the column with 10 bed volumes of equilibrating buffer until A_{280} of the flow through reaches baseline.
5. Elute the bound protein 3 to 5 bed volumes of 0.1 M D-GlcNAc in equilibrating buffer.
6. Regenerate the column by washing with 10 volumes of equilibrating buffer.

7.3   RELEASE OF OLIGOSACCHARIDES FROM GLYCOPROTEINS

Oligosaccharides can be released from glycoproteins either chemically or enzymatically.

7.3.1   RELEASE OF N-LINKED OLIGOSACCHARIDES BY CHEMICAL TREATMENT

Chemical cleavage of N-glycans is achieved by hydrazine[3] or by drastic alkali conditions.[4]

Hydrazinolysis

Hydrazine cleaves the N-glycosidic linkages of the glycoproteins, releasing the N-deacetylated glycans as their hydrazone (Figure 7.4). The released glycans are then re-N-acetylated and reduced in order to stabilize the molecules.

Working Procedure

1. Add 1 to 100 mg of glycoprotein and 0.2 to 1 ml of anhydrous hydrazine (Pierce, Rockford, IL) to a screw-cap tube with a Teflon disc seal.
2. Heat the tube at 100°C for 12 to 14 h.
3. Remove excess of hydrazine by evaporation in a desiccator connected with a vacuum pump through cold trap, drying trap, H_2SO_4 trap, and NaOH trap. Remove the last traces of hydrazine by co-evaporation with a few drops of toluene.
4. Purify the digested N-glycans by gel filtration on a Biogel P2 column. Pool the carbohydrate fractions (monitored by carbohydrate estimation, see Section 7.5) and lyophilize.
5. Dissolve the glycans in ice-cold saturated $NaHCO_3$ solution (1 ml/mg of glycan) and re-N-acetylate with the addition of 5 aliquots of 10 ml of acetic anhydride at 5 min intervals.
6. Desalt the reaction mixture on a Dowex 50×8 (25 to 50 mesh; H$^+$) column. Collect glycan-containing fractions and freeze dry.
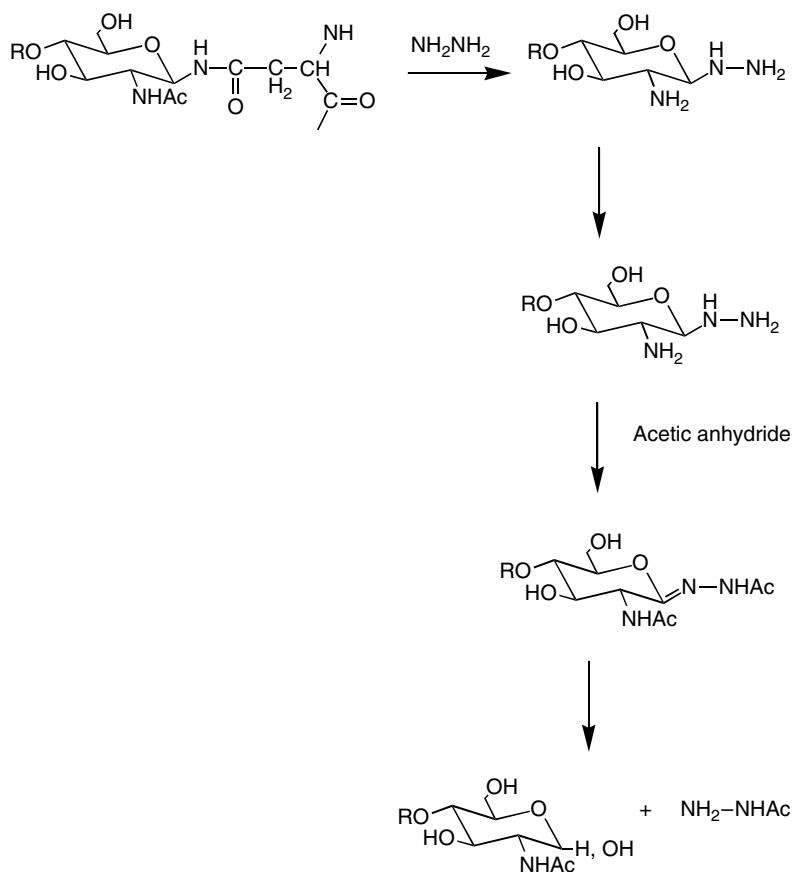
FIGURE 7.4 Hydrazinolysis of N-glycans.

7. Dissolve the glycan in 0.05 M NaOH (1 ml/mg), and reduce with $NaBH_4$ (5 mg/mg of sugar) for 16 h at 20°C.
8. Stop the reaction by adding Dowex 50×8.
9. Filter glycan and resin mix through glass-sintered funnel and collect the filtrate.
10. Remove boric acid by repeated co-distillation with methanol under vacuum.
11. Purify the glycan on a Biogel P2 column.

Alkaline Cleavage

N-glycosidic linkages are stable in the mild alkaline conditions of β-elimination. However, under drastic alkaline conditions (1 M NaOH at 100°C for 6 to 12 h), N-glycosidic linkages can be cleaved quantitatively.[4] In this procedure, GlcNAc residues are deacetylated, and thus the released glycans are re-N-acetylated.

Working Procedure

1. Dissolve 50 mg glycoprotein containing N-glycan in 1 ml 1 M NaOH/1 M $NaBH_4$ in a screw-cap tube with a Teflon disc seal.
   (**Note:** reaction is performed in the presence of a reducing agent $NaBH_4$ in order to prevent peeling reaction of released N-linked glycans).
2. Heat the tube at 100°C for 6 h.
3. Keep the tube on ice and neutralize the reaction mixture with 50% acetic acid to a pH around 6.0.
4. Purify the digested N-glycans by gel filtration on a Biogel P2 column. Pool the carbohydrate fractions (monitored by carbohydrate estimation, see Section 7.5) and lyophilize.
5. Dissolve the glycans in ice-cold saturated $NaHCO_3$ solution (1 ml/mg of glycan) and re-N-acetylate with the addition of 5 aliquots of 10 ml of acetic anhydride at 5 min intervals.
6. Desalt the reaction mixture on a Dowex 50x8 (25 to 50 mesh; H^+) column. Collect glycan containing fractions and freeze dry.

7.3.2 ENZYMATIC RELEASE OF N-LINKED OLIGOSACCHARIDES

Conditions for chemical cleavage can damage the 3D structure of the protein or cleave the labile groups from the carbohydrate chain. In contrast, conditions for enzymatic cleavage of N-glycans usually preserve intact the 3D structure of both protein and glycans.[5,6] However, complete removal of oligosaccharides from a protein often requires the denaturation of proteins, because the susceptible bonds of many oligosaccharides are not exposed in native proteins.

Two classes of endoglycosidases can be used to release N-glycans from glycoproteins. One class includes peptide-N-(N-acetyl β-glucosaminyl) asparagines amidase (PNGase F: EC 3.5.1.52), which removes the complete glycans from a wide variety of glycoproteins by hydrolyzing glycosylation linkages (Figure 7.5). The second class of endoglycosidases such as Endo F and Endo H cleave sugar-sugar glycosidic bonds (see Figure 7.5).

Working Procedure for PNGase F Digestion

1. To 75 to 100 µg of glycoprotein in a microfuge tube, add 25 µl of 0.1 M 2-mercaptoethanol/0.5% SDS solution.
2. Denature the glycopeptide at 100°C for 3 to 5 min.
3. Cool and centrifuge the tube to bring down the solution to the bottom.
4. For PNGase F digestion, add the following reagents in order:
   25 µl 0.5 M Tris-HCl, pH 8.0
   10 µl 0.1 M 1, 10 phenanthroline
   10 µl 10% non-ionic detergent such as Triton X-100 and Tween 20
   5 µl 200 to 250 U/ml PNGase F (Roche, Indianapolis, IN)
5. Incubate the tube at 37°C overnight.
6. Inactivate Endo H at 100°C for 5 min.

		Types of oligosaccharides			
	Endoglycosidas	High mannose	Hybrid	Bi-antennary	Tri/tetra-antennary
	PNGase F	+	+	+	+
	Endo H	+	+	–	–
	Endo F2	+	–	+	–
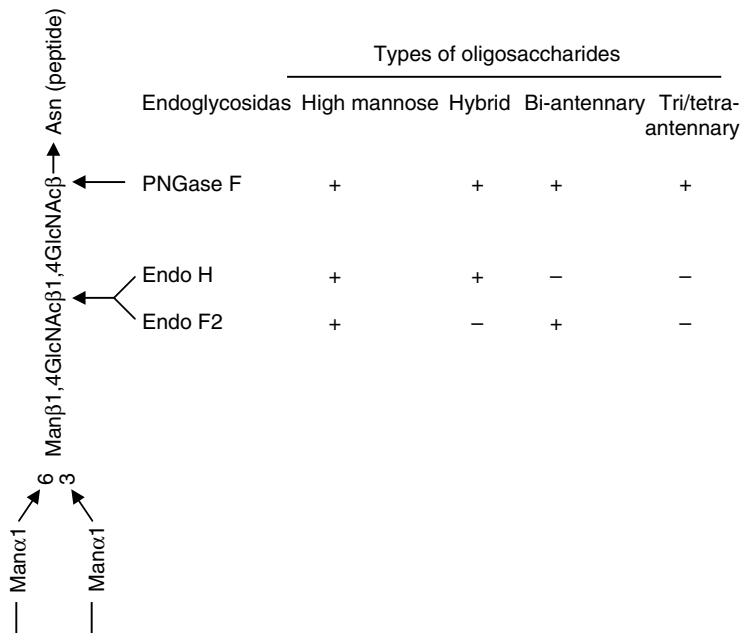
FIGURE 7.5 Schematic representation showing cleavage sites of various endoglycosidases.

Working Procedure for Endo H Digestion

1. To 15 to 20 µg of glycoprotein solution in a microfuge tube, add 25 µl of 0.1 M 2-mercaptoethanol/0.1% SDS solution.
2. Denature the glycopeptide at 100°C for 3 to 5 min.
3. Cool and centrifuge the tube to bring down the solution to the bottom.
4. For Endo H digestion, add the following components to the tube:
    15 µl 0.5 M sodium acetate, pH 5.5
    75 µl H$_2$O
    5 µl 10% PMSF (dissolved in isopropanol)
    5 µl 0.5 U/ml Endo H (Roche, Indianapolis, IN)
5. Incubate the tube at 37°C overnight.
6. Inactivate Endo H at 100°C for 5 min.

Working Procedure for Endo F2 Digestion

1. To 30 to 50 µg of glycoprotein solution in a microfuge tube, add 10 µl of 0.1 M 2-mercaptoethanol/0.5% SDS solution.
2. Denature the glycopeptide at 100°C for 3 to 5 min.
3. Cool and centrifuge the tube to bring down the solution to the bottom.

4. For Endo F2 digestion, add the following reagents in order:
    10 μl 0.5 M sodium acetate, pH 5.0
    3 μl 1, 10 phenanthroline in methanol
    5 μl 10% non-ionic detergent
    1 μl 200 U/ml Endo F2 (Roche, Indianapolis, IN)
5. Incubate the reaction mixture overnight at 37°C.
6. Inactivate Endo H at 100°C for 5 min.

7.3.3 RELEASE OF O-LINKED OLIGOSACCHARIDES

O-glycosidic linkages between glycans and the β-hydroxyamino acids Ser or Thr are cleaved in the presence of dilute alkali solution (0.05 to 0.1 M NaOH in mild conditions (40 to 45°C for five to six days) by β-elimination reaction[7] (Figure 7.6). The β-elimination reaction is performed in the presence of a reducing agent (0.8 to 2 M NaBH$_4$) in order to prevent the destruction of the released glycans by a "peeling reaction" (Figure 7.7).

Working Procedure

1. To 100 μg to 1 mg of glycoprotein in a screw-capped tube, add 100 μl 50 mM NaOH containing NaBH$_4$.
2. Incubate the reaction mixture at 45°C for 16 h.
3. Keep tube on ice and neutralize the reaction mixture with 50% acetic acid to pH around 6.0.
4. Purify the released glycan by gel filtration on a Sephadex G-50 or Biogel P4 column.
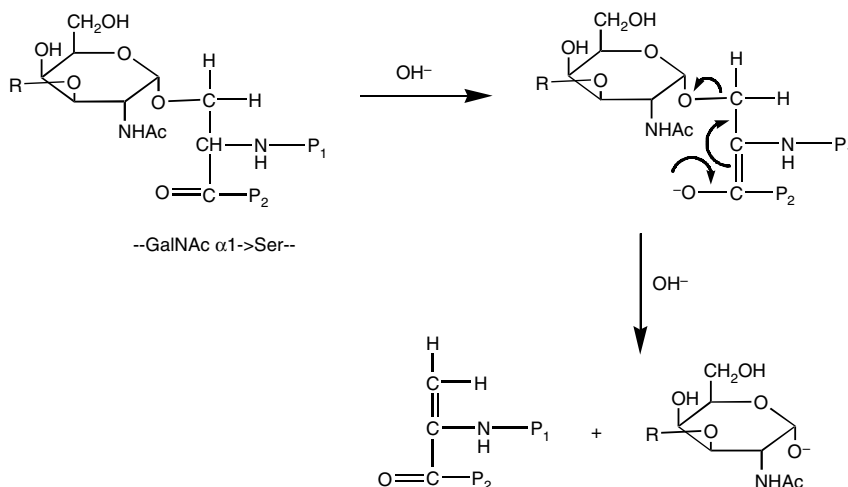
FIGURE 7.6 Release of O-glycan from glycoproteins and glycopeptides by β-elimination reaction. R represents oligosaccharide extension. P1 and P2 represent peptide extension.
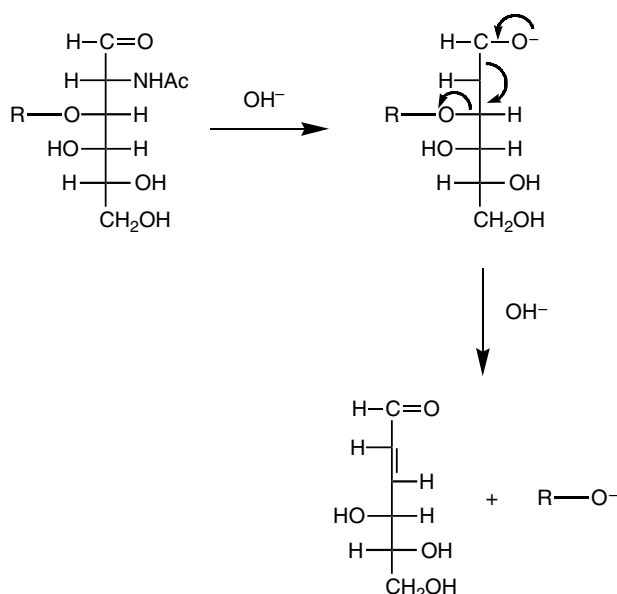
FIGURE 7.7 Peeling reaction of the oligosaccharide released by β-elimination reaction. R represents oligosaccharide extension.

7.4 PREPARATION OF GLYCOPEPTIDES BY PROTEOLYTIC CLEAVAGE

Glycopeptides from glycoproteins can be generated by proteolysis of glycoproteins. This approach is useful when proteins have multiple glycosylation sites. Two types of proteolysis can be performed. One type is an extensive proteolysis with pronase or proteinase K, resulting in oligosaccharides containing one or a few amino acid residues. The other type is a limited proteolysis with a specific endoproteinase (such as trypsin, V8 protease, and α-chymotrypsin), resulting a larger peptide attached to the oligosaccharide.

Working Procedure for Extreme Proteolytic Digestion of Glycoproteins

1. Add purified glycoprotein (0.1 to 1 mg) in 1 ml of 0.1 M Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ in a screw-cap tube.
2. Add 100 μl of pronase solution (10 mg/ml in 0.1 M Tris-HCl, pH 7.5 containing 10 mM $CaCl_2$) to the tube. Prior to use pronase solution, incubate 2 h at 50°C to inactivate contaminating glycosidases.
   (**Note:** for proteinase K digestion, take 0.1 to 1 mg of purified glycoprotein in 1 ml 0.1 M Tris-HCl buffer, pH 7.5 in a screw-cap tube. Add 100 μl of proteinase K solution (2 mg/ml in 0.1 M Tris-HCl, pH 7.5 containing 2 mM $CaCl_2$.)

3. Incubate the tube at 50°C for 2 to 4 h (for either digestion).
4. Add 100 µl of either protease to the tube and continue digestion at 50°C for another 12 to 16 h.
5. Separate digested glycopeptides by gel filtration on a Sephacryl S-200 column. Collect carbohydrate containing fractions.

Working Procedure for Limited Proteolytic Digestion of Glycoproteins with Endopeptidase

1. To the purified glycoprotein (1 mg), add 1 ml of 0.5 M Tris-HCl buffer (pH 8.6) containing 2% SDS. Add 17 mg dithiothreitol and boil the sample for 3 min.
2. Incubate the reaction mixture at 37°C for 1 h.
3. Add 46 mg iodoacetamide to the reaction mixture and incubate at room temperature in the dark for 1 h. Check the pH with pH paper immediately after iodoacetamide addition. Adjust to pH 8 to 9, if necessary, by adding a few drops of 1 NNaOH.
   (**Note:** reduction and alkylation prior to endopeptidase digestion is recommended to prevent cross-linking of peptides by disulfide bonds.)
4. Stop the reaction with 50 µl of 2-mercaptoethanol and apply the reaction mixture to a Sephadex G-50 column. Collect carbohydrate containing fractions and lyophilize.
5. Redissolve the dried sample in 0.5 ml water and precipitate the reduced and alkylated glycoprotein with 8 volumes of ice-cold acetone overnight at –20°C.
6. Centrifuge (3,000 g for 15 min) and discard the acetone. Rinse the pellet with 1 ml of 85% ice-cold acetone and centrifuge as before. Discard acetone with aspiration and finally dry the pellet with gentle stream of nitrogen.
7. Redissolve the sample in 0.5 to 1 ml digestion buffer (for trypsin or α-chymotrypsin 0.1 M ammonium bicarbonate, pH 8.0 adjusted with NH_4OH; for V8 protease 50 mM ammonium bicarbonate containing 2 mM EDTA, pH 7.8 adjusted with NH_4OH) and add 25 to 50 µl protease stock solutions. For trypsin stock, dissolve 10 mg TPCK-treated trypsin (Worthington) in 100 µl 1 mM HCl. For V8 protease stock, dissolve 10 mg staphylococcal V8 protease in 1 ml digestion buffer (50 mM ammonium bicarbonate containing 2 mM EDTA, pH 7.8).
8. Incubate the sample for 8 to 12 h at room temperature.
9. Add a second aliquot of protease (25 to 50 µl) and continue digestion for another 12 h.
10. Stop the protease reaction by boiling for 5 min.
11. Centrifuge the sample (3,000 g for 10 to 15 min) and collect the supernatant.

Glycopeptides can be separated by employing general purification procedures such as gel filtration, ion-exchange, and affinity chromatography. However, affinity chromatography on immobilized lectins may be advantageous, since carbohydrate moieties in the glycopeptides are more accessible for interactions with lectins.

7.5   ESTIMATION OF CARBOHYDRATES

Several colorimetric assays of varying sensitivities are available for neutral and amino sugars and sialic acids.

7.5.1   ESTIMATION OF NEUTRAL SUGARS

7.5.1.1   Phenol Sulfuric Acid Assay

A number of assays are known based on the action of concentrated sulfuric acid that causes hydrolysis of glycosidic linkage. The hydrolyzed neutral sugars (pentoses and hexoses) are then partially dehydrated, with the elimination of three molecules of water, to form furfural or a derivative of furfural[8] (Figure 7.8).

Furfural and its derivative cause condensation with a number of several phenolic compounds such as phenol,[8] α-naphthol, orcinol,[9] and anthrone[10] to form colored compounds (Table 7.1). Since 2-OH of the sugar participates with the formation of a five-membered heterocyclic ring, the amino sugars containing the -NH$_2$ group at 2-C (e.g., glucosamine, N-acetylglucosamine, etc.) are not sensitive to this test. Other reducing agents, cysteine, heavy metal ions, and sodium azide usually interfere with the assay.
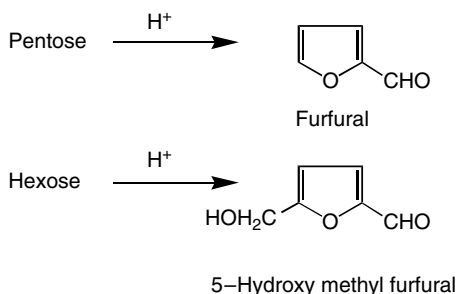
FIGURE 7.8 Reactions of neutral sugars with phenol and sulfuric acid

TABLE 7.1
Various Colorimetric Assays for Sugars Based on Phenolic Compounds

Name of Test	Phenol	Concentrated Mineral Acid	Positive Reaction	Color of Product
Molisch's	α-naphthol	H_2SO_4	All carbohydrates	Purple
Seliwanoff's	Resorcinol	HCl	Ketoses and sucroses	Red
Bial's	Orcinol	HCl	Pentoses and uronic acids	Green
Tollen's	α-naphthol-resorcinol	HCl	Uronic acids	Blue

Working Procedure

1. Set up several glass tubes containing 5 to 100 μg neutral hexose such as glucose in 200 μl of water. Take unknown carbohydrate sample in 200 μl water.
2. Add 200 μl phenol (5% w/v in water) to each tube.
3. Add 1 ml concentrated sulfuric acid rapidly and directly on the sample. (**Note:** do not allow sulfuric acid to touch the side of the tube. Leave the solution undisturbed for 10 min.)
4. Shake vigorously and continue incubation for another 30 min.
5. Read each tube at 490 nm. Determine the concentration of carbohydrate in the unknown sample from a standard plot.

7.5.1.2 Dinitrosalicylic Acid Assay

The reducing sugars are conveniently quantitated by the reaction of alkaline 3,5-dinitrosalicylic acid.[11] The reduced product is measured at 570 nm.

Working Procedure

1. Set up several glass tubes containing 5 to 500 μg glucose in 100 μl water. Set up tube for unknown sample in 100 μl water.
2. Prepare the assay reagent by dissolving 0.25 gram 3,5 dinitrosalicyclic acid and 75 gram sodium potassium tartrate in 50 ml 2 M NaOH. Dilute to 250 ml with water.
3. Add 1 ml assay reagent to each tube. Mix well.
4. Incubate at 100°C for 10 min.
5. Cool to room temperature and determine absorbance at 570 nm.

7.5.1.3 Nelson-Somogyi Assay

This assay for reducing sugar originally developed by Somogyi[12] is based on the reduction of cupric ions, best known as Fehling solution. Fehling solution is an alkaline mixture of cupric sulfate and sodium potassium tartarate. After heating the sugars with Fehling solution on the boiling water bath, a mixture of ammonium molybdate and sodium arsenate is added. Ammonium molybdate and sodium arsenate in the presence of sulfuric acid form arsenomolybdate complex. The reducing sugar reduces cupric salts to cuprous salts, which, in turn, reduce the arsenomolybdate complex to molybdenum blue. The production of molybdenmum blue can be quantitated at 520 nm.

Working Procedure

Reagents

1. Dissolve 15 gram sodium potassium tartrate and 30 gram of anhydrous sodium carbonate in 300 ml water. Add 20 gram sodium bicarbonate to this solution and dissolve. Dissolve 180 gram of anhydrous sodium sulfate in 500 ml boiling water and cool. Mix the two solutions and add water to get final volume of 1 liter.
2. Dissolve 5 gram $CuSO_4.5H_2O$ and 45 gram anhydrous sodium sulfate in about 200 ml water and add water to get final volume of 250 ml.
3. Mix 4 volumes reagent 1 with 1 volume reagent 2 just before use.
4. Dissolve 25 gram ammonium molybdate in 450 ml water. To this solution, carefully add 21 ml concentrated sulfuric acid with stirring. Dissolve 3 gram sodium arsenate ($Na_2HAsO_4.7H_2O$) in 25 ml water and add to the above molybdate solution. After incubation 24 to 48 h at 37°C, store the solution in a brown glass bottle. Prior to use, dilute this reagent with 2 volumes of 0.75 M H_2SO_4.

Assay

1. Set up tubes containing 10 to 100 µg glucose in 1 ml of reagent 3. Set up also unknown sample in 1 ml of reagent 3.
2. Heat each tube at 100°C for 15 min.
3. After cooling to room temperature add 1 ml of reagent 4. Mix well.
4. Add 3 ml water and mix well.
5. Measure absorbance for each tube at 520 nm.

7.5.1.4 Tetrazolium Blue Assay

This sensitive assay for reducing sugars is performed by heating the mixture of sugars and alkaline solution of tetrazolium blue at 100°C for 30 sec.[13] The production of reduced formazan salt is extracted with 2 volumes of tolune and then

Tetrazolium salt
(colorless, water soluble)

Reduced tetrazolium
(colored, water insoluble)
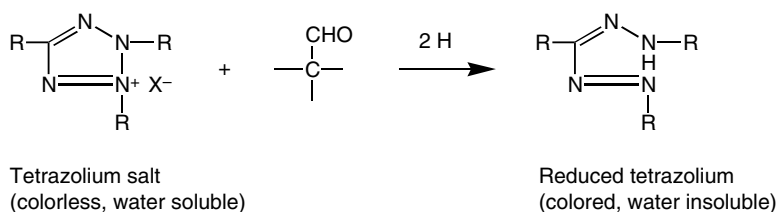
FIGURE 7.9 Reaction of tetrazolium salt with reducing sugars

quantitated at 570 nm (Figure 7.9). Extended heating of the reaction mixture containing oligosaccharides increases absorbance because of alkaline hydrolysis of the oligosaccharides.

Working Procedure

Reagent

To 1 volume of tetrazolium blue (1% w/v in water), add 3 volumes of 0.3 M NaOH. Stir the mixture to dissolve completely. Dilute the solution with 5 volumes of distilled water. Store at 5°C in the dark.

Assay

1. Set up tubes containing 1 to 10 μg glucose in 100 μl of water. Set up a tube for unknown sample.
2. Add 900 μl of assay reagent to each tube and mix well.
3. Heat the tubes at 100°C for 30 sec.
4. After cooling to room temperature add 1 ml toluene to each tube to extract color.
5. Measure absorbance at 570 nm.

7.5.1.5   Ferric-Orcinol Assay

This assay is for pentose only, although hexose may interfere.[14] Heating of a solution of orcinol in hydrochloric acid, in the presence of ferric ions pentose, produces a green color.

Working Procedure

Reagents

1. 10% (w/v) trichloroacetic acid solution in water.
2. Assay reagent: freshly prepare solution of ferric ammonium sulfate (1.15% w/v) and orcinol (0.2% w/v) in 9.6 M HCl.

Assay

1. Set up tubes containing 0.2 to 40 µg xylose in 200 µl of reagent 1. Set up tube for unknown sample.
2. Heat each tube at 100°C for 15 min.
3. After cooling to room temperature, add 1.2 ml of reagent 2. Mix well.
4. Heat the solution at 100°C for 20 min.
5. Cool the solution to room temperature and read the absorbance at 660 nm.

7.5.1.6 Phenol-Boric Acid-Sulfuric Acid Assay

This assay is for ketose, although slight interference occurs with non-ketose sugars.[15] In this procedure, the sample is mixed with the assay reagent, and concentrated sulfuric acid is added directly to the surface. After incubation at 37°C for 1 h, the pinkish product is measured at 568 nm. The reagent is made with phenol, followed by the addition of acetone and boric acid.

Working Procedure

Reagent

Dissolve 5 gram phenol (recrystallized from methanol and ethanol) in 100 ml water. Add 2 ml acetone dropwise with constant stirring. Continue stirring for 10 min at room temperature. Add 2 gram of boric acid in the mixture and mix well.

Assay

1. Set up tubes containing 0.1 to 10 µg fructose in 100 µl water. Set up tube for unknown sample.
2. Add 0.5 ml assay reagent to each tube and mix well.
3. Rapidly add 1.4 ml concentrated H_2SO_4 directly on the sample without touching the side of the tube. Mix the solution thoroughly.
4. Incubate the solution for 5 min at room temperature followed by incubation at 37°C for 1 h.
5. Measure absorbance at 568 nm.

7.5.2 Estimation of Amino Sugars

7.5.2.1 Morgan-Elson Assay

This assay is generally used to estimate 2-deoxy sugars.[16] The sample is taken in tetraborate and heated at 100°C. After cooling a mixture of 4-(N,N-dimethylamino) benzaldehyde, acetic acid and concentrated hydrochloric acid is added to the sample. After incubation for 20 min at 37°C, the absorbance is read at 585 nm. In this assay, D-GlcNAc responds three times more than D-GalNAc. Production of color depends on two independent processes: (1) the formation of an intermediate compound (glucoxazoline) produced by heating the amine sugar with alkali, and

(2) the reaction of this intermediate with 4-(N, N-dimethylamino) benzaldehyde during the development of color in an acid medium.[16]

Working Procedure

Reagent

1. Dipotassium tetraborate tetrahydrate (6.1% w/v in water)
2. Dissolve 10 gram of 4-(N,N-dimethylamino)benzaldehyde in HCl-acetic acid solution (11 ml conc. HCl/87.5 ml glacial acetic acid/1.5 ml water). Prior to use, dilute 10 ml of the solution to 100 ml with glacial acetic acid.

Assay

1. Set up tubes containing 0.06 to 6 µg D-GlcNAc in 250 µl water. Set up tube for unknown sample.
2. Add 50 µl of reagent 1 to each tube and mix well.
3. Heat each tube at 100°C for 3 min.
4. After cooling to room temperature, add 1.5 ml reagent 2 to each tube. Mix well.
5. Incubate each tube at 37°C for 20 min.
6. Cool down to room temperature and measure absorbance at 585 nm.

7.5.2.2   Benzothiazoline Assay

Glucosamine and galactosamine respond equally with this procedure under mild acid treatment. The procedure involves the deamination of hexosamines to produce 2, 5-anhydrohexose, which then interacts with 3-methyl-2-benzothiazoline hydrazone hydrochloride, resulting in a blue product.[17]

Working Procedure (Based on Reference 17)

Reagent

1. 0.5 M HCl
2. 2.5% sodium nitrite
3. 12.5% ammonium sulfamate
4. Freshly prepare 0.25% 3-methyl 2-benzothiazolone hydrazone hydrochloride (Eastman Chemicals)
5. Freshly prepare 0.5% ferric chloride

Assay

1. Set up tubes containing 0.1 to 10 µg D-GlcNAc in 200 µl of 0.5 M HCl. Set up tube for unknown sample.
2. Vortex and heat tubes at 110°C for 2 h.
3. Cool to room temperature in a water bath.
4. Under a fume hood, add 400 µl of reagent 2. Vortex and allow to stand at room temperature for 15 min.
5. Add 200 µl of reagent 3 to each tube and vortex. Allow to stand at room temperature for 15 min.
6. After liberation of excess sodium nitrite, add 200 µl of reagent 4. Vortex and incubate at 37°C for 30 min.
7. Add 200 µl of reagent 5 and incubate at 37°C for additional 5 min.
8. Cool the reaction mixture to room temperature and determine absorbance at 560 nm.

7.5.3 ESTIMATION OF URONIC ACIDS

7.5.3.1 Carbazole Assay

The uronic acid is generally quantitated by carbazole.[18]

Working Procedure

Reagents

1. Dissolve 0.9 gram sodium tetraborate decahydrate in 10 ml water. Add 90 ml of ice-cold 98% concentrated H_2SO_4 carefully to form a layer and, without disturbing, incubate overnight to allow slow mixing.
2. Carbazole (recrystallized from ethanol): 0.1% w/v in ethanol.

Assay

1. Set up tubes containing 0.2 to 20 µg D-galacturonic acid in 250 µl water. Set up tube for unknown sample.
2. Cool the tubes in ice bath and add carefully 1.5 ml of ice-cold reagent 1 to each tube. Mix well.
3. Incubate each tube at 100°C for 10 min.
4. Cool the mixture in ice bath and add 50 µl of reagent 2 to each tube. Mix well.
5. Incubate the tubes at 100°C for 15 min.
6. After cooling down to room temperature, measure absorbance at 525 nm.

7.5.3.2   *m*-Hydroxydiphenyl Assay

In this assay, the sample is heated in sulfuric acid and sodium tetraborate, and after cooling the mixture is allowed to interact with the *m*-hydroxydiphenyl reagent to produce a pinkish product (maximum absorbance at 520 nm).[19]

Working Procedure

Reagents

1. Prepare 0.0125 M solution of sodium tetraborate in concentrated sulfuric acid.
2. Prepare 0.15% (w/v) solution of *m*-hydroxydiphenyl in 0.5% sodium hydroxide (stable at 4°C for a few months).

Assay

1. Set up tubes containing 0.5 to 20 µg of uronic acids in 200 µl water. Set up tube for unknown sample.
2. Add 1.2 ml of tetraborate solution (reagent 1) to each tube.
3. Cool the tubes on ice and shake vigorously.
4. Incubate each tube at 100°C for 5 min.
5. Cool the mixture on ice bath and add 20 µl of reagent 2 to each tube. Mix well.
6. Determine the absorbance at 520 nm.

7.5.4   Estimation of Sialic Acids

7.5.4.1   Warren Assay

This assay is for free sialic acids that can easily be obtained from very mild acidic condition. The sample is mixed with sodium meta-periodate in the presence of concentrated ortho-phosphoric acid.[20] After 20 min at room temperature, a mixture of sodium arsenite, sodium sulfate, and potassium iodide in diluted sulfuric acid is then added and shaken vigorously to expel the yellow-colored iodine. To the reaction mix, thiobarbituric acid in sodium sulfate is then added and heated at 100°C for 15 min. Finally cyclohexanone is used to extract the colored product (upper layer) and read at 549 nm. The reaction involves the oxidation of sialic acid or N-glycolylneuraminic acids with periodic acid resulting in the formation of formyl pyruvic acid. This reacts with 2-thiobarbituric acid to form a pink-colored product. The purpose of adding the mixture of sodium arsenite, sodium sulfate, and potassium iodide is to remove excess periodate that reacts with sodium arsenite to produce arsenate. Arsenate oxidises iodide into molecular iodine, which is expelled by sodium sulfate. The Warren assay is very similar to the Aminoff method, with a minor difference in sensitivity.[21]

Working Procedure

Reagents

1. Dissolve 4.278 gram sodium meta periodate in 20 ml water. Add 58 ml concentrated ortho phosphoric acid. Add water to get a final volume of 100 ml.
2. Dissolve 10 gram sodium arsenate, 7.1 gram sodium sulfate, and 10 mg potassium iodide in 100 ml of 0.1 M sulfuric acid.
3. Dissolve 0.6 gram of 2-thiobarbituric acid and 7.1 gram of sodium sulfate in 100 ml water.

Assay

1. Set up tubes containing 0.08 to 8 µg N-acetyneuraminic acid in 80 µl water. Set up tube for unknown sample.
2. Add 40 µl of reagent 1 to each tube and mix well.
3. After 20 min incubation at room temperature, add 400 µl reagent 2. Vortex the tube to expel the yellow-colored iodine and incubate for 5 min at room temperature.
4. Add 1.2 ml reagent 3 to each tube and vortex.
5. Incubate at 100°C for 15 min.
6. Cool to room temperature and extract the chromophore with 1 ml cyclohexane (redistilled) by vortexing.
7. Centrifuge the solution to separate two layers and measure the absorbance of the upper cyclohexane layer at 549 nm.

7.5.4.2   Periodate-Resorcinol Method

The periodate-resorcinol method involves a periodate oxidation step prior to heating with resorcinol reagent.[22] Total, free, or bound sialic acid can be determined using this assay. The periodate-resorcinol method is more sensitive than the resorcinol procedure.

Working Procedure

Reagents

1. Prepare a stock solution of 0.4 M periodic acid in water. Stock solution is stable for several weeks at –19°C in the dark. Dilute tenfold prior to use.
2. Prepare a stock solution of 6% resorcinol in water. Stock solution is stable for several weeks at –19°C in the dark. Prepare assay reagent by combining 10 ml resorcinol stock, 60 ml of 28% HCl, 30 ml water, and 25 µmoles CuSO$_4$.
3. 95% tertiary butyl alcohol.

Assay

1. Set up tubes containing 0.02 to 0.2 μmole of N-acetyneuraminic acid in 0.5 ml water. Set up tube for unknown sample.
2. Add 0.1 ml of 0.04 M periodic acid solution to each tube and mix well.
3. After 20 min incubation in an ice bath, add 1.25 ml of resorcinol assay reagent to each tube, mix well, and place in an ice bath for 5 min.
4. Heat the tubes at 100°C for 15 min.
5. Cool to room temperature and add 1.25 ml of tertiary butyl alcohol. Vigorous mixing will give a single phase solution.
6. Place the tubes in a 37°C water bath for 3 min to stabilize the color.
7. Cool to room temperature and measure the absorbance at 630 nm.

7.6   CHROMATOGRAPHIC SEPARATION AND DETECTION OF SUGARS

Several chromatographic procedures for the separation of sugars are available both for analytical and preparative purposes. In some cases, unknown monosaccharides are identified by comparing the standard sugars.

7.6.1   THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is used for the preliminary examination of the carbohydrate mixtures, mostly for monomer to decamer. The detailed working procedures including the selection of solvents, and the methods of detection are described elsewhere.[23–25] Among the several solid supports, cellulose and silica gel are found useful. In cellulose TLC, sugars are separated by liquid-liquid partition, where the distribution of sugar between the mobile phase and the cellulose-bound water complex depends on the solubility of the sugar in the eluent and its ability to enter through the structures of the cellulose-bound water complex.

In silica gel, the separation of the carbohydrates occurs in a similar fashion. Sometimes when the gels are impregnated with salt solution such as phosphate or citrate, the separation of the carbohydrates is influenced by the gradient that formed with salt and the eluent.

TLC plates can be prepared in the laboratory by layering a slurry of the chromatographic material on glass plates. TLC plates coated with cellulose, silica gel, or impregnated silica gel are also commercially available. Either plate should be dried in an oven at 100°C and stored in a desiccator until use.

Various elution solvent systems are used in TLC. However, optimum elution solvent is achieved on a trial basis. An optimum solvent can separate as many as ten carbohydrates. In general, a solvent system is composed of two to four components including water, which is usually 10 to 20% by volume. Some common solvent systems for use in TLC to separate monosaccharides and some disaccharides are butanol:pyridine:0.1 M HCl (50:30:20); ethylacetate:pyridine:water (100:35:25); and butanol:pyridine:water (100:30:30).

After chromatographic separation, carbohydrates are detected with spray reagents.

Sulfuric acid is probably the classical reagent for this purpose. After chromatographic separation, sulfuric acid is sprayed and heated at 100°C until the carbohydrate forms char. However, cocktail spray reagents (Table 7.2) such as diphenylamine-aniline-phosphoric acid and naphthoresorcinol-ethanol-sulfuric acid are useful, probably because of the appearance of different colors for different carbohydrates.[26]

General TLC Procedure

1. Dissolve 0.05 to 2 mg dry sample or standard mixture in 1 ml water/iso-propanol mixture (0.5 ml each).
2. With a Hamilton syringe or capillary tube, spot sample or standard mixture (1 to 5 µl) on the TLC plate approximately 1 cm away from the edge (Figure 7.10 A). For application of large volume, spot the sample in small aliquot several times, drying the previous spot before applying the second. Sample spot can be dried by blowing warm air from a hand-held hair dryer.
3. After sample application, place dry plate (sample side down) in a chromatographic tank prefilled up to 0.5 to 0.7 cm with elution solution to saturate its atmosphere (Figure 7.10 B). To saturate tank atmosphere with the elution solution, a filter paper can be placed along on the side of the tank.
4. At the end of run, remove the plate from the tank. Dry the plate thoroughly in a stream of warm air.
5. Using a spray bottle, spray reagent on the plate and heat to detect carbohydrates (see Table 7.2).

7.6.2 PAPER CHROMATOGRAPHY

Paper chromatography (PC) is a simple and inexpensive method of carbohydrate separation. The principle of PC is similar to TLC on cellulose except the time of elution, which is longer than TLC.[27] In general, solvent systems for carbohydrate elution and their detection methods used in TLC can also be employed in PC. But unlike TLC, descending chromatography is frequently used in PC, preferably on Whatman No. 1 filter paper.

7.6.3 REVERSED PHASE CHROMATOGRAPHY

By this procedure, neutral oligosaccharides are fractionated on C18-bonded silica column. The sample is applied in water and the retarded oligosaccharides are eluted by a gradient of acetonitrile.[28]

TABLE 7.2
Spray Reagents for Detecting Carbohydrates in TLC

Spray Reagent	Preparation of Reagent	Development Condition	Color Developed		Detection Sensitivity
Diphenylamine/aniline/ phosphoric acid	Combine diphenylamine (100 ml 4% w/v in acetone), aniline (100 ml 4% v/v in acetone), and orthophosphoric acid (20 ml 85%)	10 min at 100°C	Aldose Ketose	Blue grey Red	1 μg
Naphthoresorcinol/ethanol/ sulfuric acid	Dissolve 0.2 gram of naphthoresorcinol in 100 ml of 95% ethanol. To prepare spray reagent, add 4 ml concentrated sulfuric acid to 96 ml of the above naphthoresorcinol solution.	5 min at 100–150°C	Aldose Ketose Uronic acid	Blue or violet Pink or red Blue	0.1 μg (L-sorbose) to 4 μg (D-glucose)
Ceric sulfate/sulfuric acid	Combine 5 ml 0.1 M ceric sulfate and 100 ml 15% (v/v) sulfuric acid	15 min at 120°C	Hexose Deoxyhexose Pentose	Blue grey Brown Pinkish grey	1 μg (hexose and pentose) to 10 μg (hexosamine)
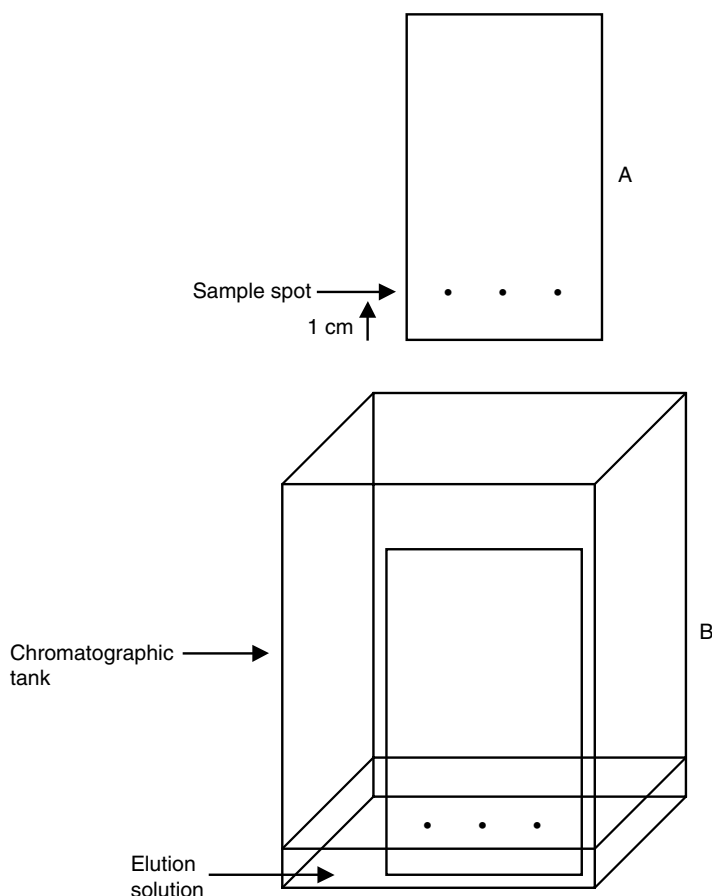
FIGURE 7.10 Schematic representation of TLC plate (A) and elution of sample in a chromatographic chamber (B)

7.6.4 GAS LIQUID CHROMATOGRAPHY

In gas liquid chromatography (GLC) volatile derivatives of carbohydrates are separated on liquid phases and then detected by means of flame ionization. Several liquid phases (most polar to non-polar) are available for this purpose. For GLC, glycans or oligosaccharides are cleaved into its smallest components, monosaccharides, and these non-volatile monosaccharides are then derivatized to volatile forms. Separation depends upon the differential extractive distillation of the derivatives in the mixture. Common cleaving methods are acid hydrolysis, methanolysis, and solvolysis by hydrogen fluoride.

The cleavage of glycosidic bonds by acid hydrolysis does not occur in an identical manner.[29] The rate of release is dependent on the nature of monosaccharides as well as their position and anomeric linkages. For example, hexosaminyl linkages are not generally cleaved under conditions sufficient for the hydrolysis of

neutral hexosyl linkages. Moreover, hydrolyzed monosaccharides differ in their susceptibility to destruction by acid. Therefore, glycan bond cleavage by acid hydrolysis often requires a compromise between allowing complete hydrolysis and preserving the released monosaccharides. Additionally, the drastic acidic condition required to release hexosamine results in a loss of N-acetyl groups from the amino sugars, and thus subsequent re-N-acetylation is required prior to derivatization. In practice, more than one set of hydrolysis is preferred to optimize the condition.

Glycosidic bond cleavage by methanolysis (methanolic-HCl) overcomes the problems of aqueous HCl hydrolysis. There is less destruction of carbohydrates in the methanolic HCl compared to aqueous acid hydrolysis, resulting in high yields of the carbohydrates. However, GlcNAc attached to asparagine is not efficiently cleaved.

Solvolysis using anhydrous hydrogen fluoride cleaves most glycosidic linkages without affecting N-acyl moieties of the acylamido sugars and the N-glycosidic linkage between asparagines and GlcNAc.[30] However, this procedure is not frequently used because it requires a special apparatus to handle highly toxic and corrosive hydrogen fluoride.

Derivatization

Preparation of volatile derivatives of carbohydrates is required prior to separation. Volatile derivatives are usually obtained by two common derivatization methods: formation of alditol acetates and trimethylsilylation. The former method requires several steps, but results in the formation of a single derivative per monosaccharide. In contrast, the trimethylsilylation method requires fewer steps, but results in the formation of multiple derivatives of each monosaccharide (a mixture of pyranose form, furanose form, α-anomer, and β-anomer), since monosaccharides remain in ring form during this procedure. These multiple derivatives produce a characteristic pattern for each monosaccharide.

Working Procedure

Preparation of Alditol Acetate Derivatives of Neutral Sugars and Their Analyses by GLC

Hydrolysis

1. Hydrolyze dried salt-free glycoconjugate or oligosaccharide containing 5 to 500 μg of carbohydrate with 0.5 ml of 2 M trifluoroacetic acid (TFA) in a Teflon-lined screw cap for 2 h at 121°C.
2. Remove the acid by evaporating under a stream of nitrogen while incubating the samples in a water bath at 40 to 50°C. Remove remaining traces of acid with 0.25 ml of 2-propanol and by drying under a stream of nitrogen. Repeat the propanol addition and drying once more.

Reduction

3. Add 2 to 20 µg of myo-inositol to the hydrolyzed sample and then reduce the mixture in 0.25 ml of 1 M ammonium hydroxide containing 2.5 mg of sodium borohydride for 2 to 18 h at room temperature. Add myo-inositol to the standard mixture also and carry through the entire procedure in parallel with the sample.

   (**Note:** myo-inositol serves as an internal standard.)

Removal of Borate

4. To destroy excess borohydride, acidify the sample by adding glacial acetic acid dropwise until bubbling (release of hydrogen gas) ceases. Evaporate to dryness under nitrogen.
5. Remove borate ions by adding 0.5 ml of methanol/acetic acid (9:1) mixture and drying under a stream of nitrogen.
6. Repeat step 5 four more times, except the last one use methanol instead of methanol/acetic acid.

Acetylation

7. Acetylate the alditol acetates by the addition of 0.1 ml acetic anhydride and 0.1 ml pyridine in a screw-capped tube for 20 min at 121°C.
8. Dry the sample under a stream of nitrogen at room temperature or up to 40°C. Remove the last traces of reagent by adding 50 to 100 µl of toluene to the sample and evaporate to dryness.

Sample Preparation

9. Dissolve the alditol acetate derivative in 0.5 ml chloroform. Add 0.5 ml water to the sample and vortex.
10. Centrifuge at 1,000 g for 2 min at room temperature to evaporate the organic and aqueous phase.
11. Transfer the organic (bottom) layer to a clean tube without transferring any of the aqueous phase. Dry sample (organic phase) under a stream of nitrogen.
12. Dissolve the dried sample in 50 to 100 ml of acetone. The sample is now ready for GLC analysis.

Analysis of Alditol Acetate Derivatives by GLC

13. Inject 1 ml of sample on a gas chromatograph equipped with a Supelco SP-2330 (Bellefonte, PA) silica capillary column and detect alditol acetate derivatives by a flame ionization detector.
14. Identify monosaccharides by coupling their retention times with those of standard sugars.

Preparation of Alditol Acetate Derivatives of Amino Sugars and Their Analyses by GLC

Derivatization of amino sugars is essentially similar to the procedure described for neutral sugars except that the hydrolysis of glycosidic bonds of amino sugars requires stronger acid conditions. Additionally, re-N-acetylation of the amino groups (resulted on hydrolysis) is required prior to reduction step. A mixture of standard amino sugars is prepared in parallel. Like the previous procedure, myo-inositol is added to the sample and standard mixture as an internal standard.

Hydrolysis (Based on Reference 29)

1. Hydrolyze dried salt-free glycoconjugate or oligosaccharide with 0.5 ml of 4 N HCl in a Teflon-lined screw cap for 18 h at 100°C.
2. Remove the acid by evaporating under a stream of air or nitrogen.
3. Add 0.5 ml water and evaporate under air or nitrogen.
4. Repeat step 3 once more.

Re-N-acetylation

5. To the dried sample, add 100 µl deionized water, 25 µl freshly prepared 5% (v/v in water) acetic anhydride, and 25 µl saturated sodium bicarbonate. Incubate the solution at room temperature for 3 min.
6. Add acetic anhydride and sodium bicarbonate (25 µl each) to the solution and incubate at room temperature for 3 min.
7. Repeat step 6, but incubate for 20 min instead of 3 min.
8. Stop reaction by heating at 100°C for 4 min and after cooling to room temperature, dry the solution under a stream of nitrogen or air.

Reduction, Removal of Borate, and Acetylation

9. Identical to those for neutral sugars.

Analysis of Alditol Acetate Derivatives by GLC

10. Inject sample onto a gas chromatograph using a Supelco DB 1 fused silica capillary column and identify amino sugars by comparing their retention time with those of standard amino sugars.

7.6.5 ANION-EXCHANGE CHROMATOGRAPHY

Oligosaccharides containing negatively charged sialic acid, sulfate, and phosphate groups can be separated by an anion-exchange column such as Mono Q (Pharmacia)

on HPLC. However, the separation is not suitable for free monosaccharides obtained by acid hydrolysis because of poor resolution of individual monosaccharides.

7.6.6 HIGH pH ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION

High pH anion-exchange chromatography (HPAEC) can be used to analyze covalently linked carbohydrates as well as monosaccharides when coupled with pulsed amperometric detection (PAD).[31] A major advantage of HPAEC coupled with PAD is that neutral sugars and amino sugars for acid hydrolysates of glycoproteins can be separated isocratically in a single run, and derivatization of monosaccharides or oligosaccharides is not necessary prior to chromatographic analyses. HPAEC has been an effective method for analyzing N-linked hybrid, oligomannosidic, and sialylated lactosamine-type oligosaccharides.

Principles

Carbohydrates, being polyhydric compounds, are weak acids with pK_a values of 12 to 14. In the presence of strong alkali, monosaccharides and oligosaccharides become negatively charged, forming oxyanion. As they become negatively charged, they can bind on strong anion-exchange columns. For monosaccharides, there is a good correlation of the retention time with their pK_a values (the lower the pK_a value, the higher the retention time).

A detailed protocol for this Working Procedure is described elsewhere.[32]

Working Procedure

Equipment

Chromatograph (Dionex) consisting of a gradient pump, a PAD-II and an eluent degas module (EDM), a sample injector Spectra Physics SP 8880 autosampler (Fremont, CA) with a 200 µl sample loop, controller Dionex AI 450 software for data collection; and an anion-exchange column. Columns are Carbo Pac PA1 (Dionex) for monosaccharides and Carbo Pac PA 100 for oligosaccharides analyses.

Eluent

Four eluent bottles (E1 to E4) are needed. E1 contains 2 to 4 liter deionized water. E2, E3, and E4 contain 200 mM NaOH, 100 mM NaOH, and 1 M sodium acetate in 100 mM NaOH, respectively. Sodium hydroxide solutions should be prepared from 50% NaOH stock solution, not from a NaOH pellet. In a pellet, NaOH adsorbs CO_2 from the air, producing a thin film of sodium carbonate, which will interfere with the ionization of carbohydrates. In 50% NaOH solutions, any CO_2 adsorbed by the NaOH will precipitate and can easily be avoided.

Neutral Hexose and Hexosamine Analyses

1. In a 1.5 ml microfuge tube, hydrolyze glycoprotein in 400 µl 2 M trifluoroacetic acid at 100°C for 4 h.
2. At the end of incubation, centrifuge the tubes. Remove the cap and evaporate to dryness using a Speed Vac.
3. Add 200 µl of water to each tube and transfer the sample to an autosampler vial.
4. For separation of hexose and hexosamine set up gradient as follows:

$$
\begin{aligned}
&t = 0 \text{ min,} \quad && E1 = 92\%, E2 = 8\% \\
&t = 25 \text{ min,} \quad && E1 = 92\%, E2 = 8\% \\
&t = 27 \text{ min,} \quad && E1 = 0\%, E2 = 100\% \\
&t = 37 \text{ min,} \quad && E1 = 0\%, E2 = 100\% \\
&t = 39 \text{ min,} \quad && E1 = 92\%, E2 = 8\%
\end{aligned}
$$

5. Compare the elution profile with that of the standard sugars.

7.7   ELECTROPHORETIC SEPARATION

Charged oligosaccharides can be separated by paper electrophoresis under high voltage. Polyacrylamide gel electrophoresis (PAGE) has recently been adopted for the separation and identification of monosaccharides that are prelabeled with charged fluorescent substances such as ANTS or AMAC: a process known as Fluorophore Assisted Carbohydrate Electrophoresis (FACE™) (Millipore, Glyko). The terminal reducing sugar of N-linked oligosaccharide reacts with the fluorescent tag ANTS (8-aminonaphthalene-1,3,6-trisulfonic acid) or AMAC (2-aminoacridonel) to form Schiff's base (Figure 7.11). The Schiff's base is then reduced by sodium cyanoborohydride to yield the stable fluorescently labeled derivative. Since ANTS has three sulphonic acid groups that remain ionized at the pH of PAGE buffers, it allows separation of neutral sugars in an electric field as well as detection as low as 0.2 pmol (emission maximum 515 nm). On the other hand, AMAC confers no charge onto fluorophore-labeled saccharides, and thus only naturally charged saccharides will move under the electric field. So AMAC can be used to distinguish between neutral and charged saccharides.

7.8   GLYCAN DIFFERENTIATION BY LECTINS

Since lectins have binding affinities to certain carbohydrate structures, they can be employed for investigation of carbohydrate structures. Use of various lectins allows limited identification of carbohydrates and their linkages. For this purpose, glycoproteins or glycopeptides are blotted onto membrane, and the blot is incubated with the labeled lectins. Lectins can be labeled and detected in various ways such as radio labeling (^{125}I); enzyme labeling (horseradish peroxidase, alkaline phosphatase) followed by chromatographic detection or chemiluminiscent visualization; biotin
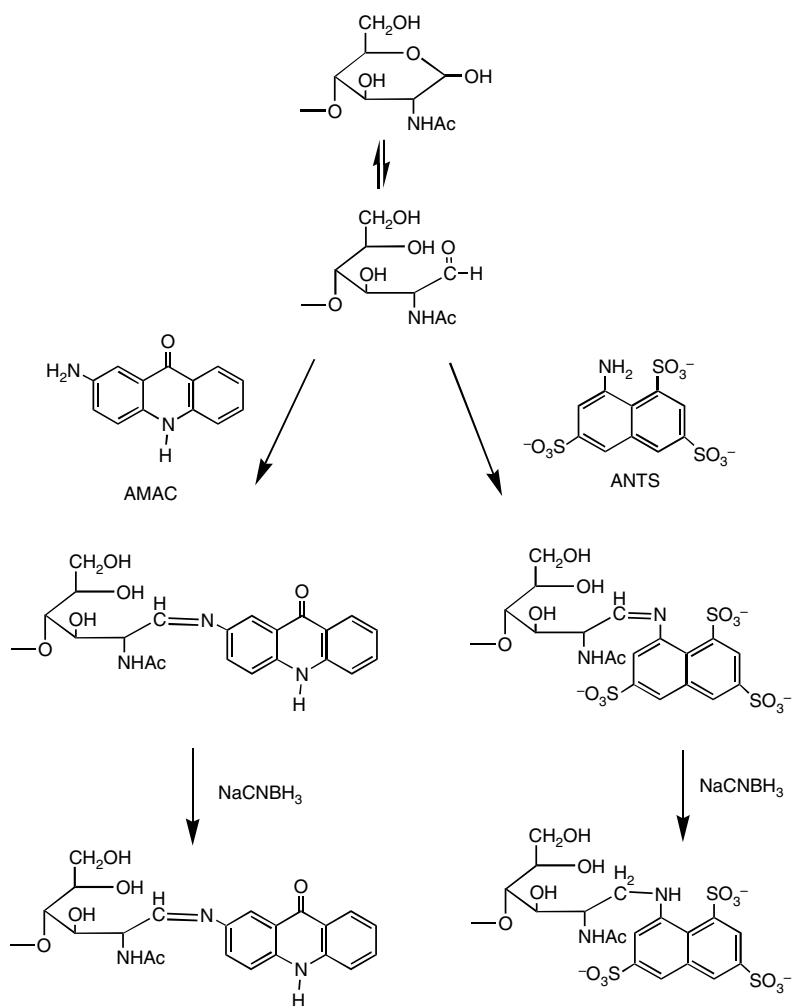
FIGURE 7.11 Labeling of sugars with fluorescent substances (ANTS and AMAC).

labeling followed by a second incubation with enzyme conjugated avidin; or digoxigenin labeling followed by a second incubation with anti-digoxigenin antibodies. Several commercial kits and working procedures are available for glycan analyses such as Glycan Differentiation kit (Roche), Lectin Staining kit (EY Laboratories), and Lectin Link kit (Genzyme). Glycan Differentiation kit is briefly described below. Table 7.3 shows the lectins used in the kit and their carbohydrate specificities. From the positive reaction of a known lectin with glycoconjugate, a pattern of carbohydrate sequence in glycoconjugate can be predicted.

TABLE 7.3
Lectins Used in Glycan Differentiation Kit

Lectin	Specificity	Reaction with Glycoprotein (Provided in the Kit)
GNA (*Galanthus nivalis* agglutinin)	α-mannose (binds terminal mannose α1,2, α1,3, or α1,6,linked to mannose) High mannose N-glycan	Carboxy-peptidase Y
SNA (*Sambucus nigra* agglutinin)	Sialic acid α2,6 linked to galactose O-glycan structure	Transferrin, fetuin
MAA (*Maackia amurensis* agglutinin)	Sialic acid α2,3 linked to galactose O-glycan structure	Fetuin
PNA (Peanut agglutinin)	Galactose β1,3 linked to N-acetylgalactosamine O-glycan structure	Asialofetuin
DSA (*Datura stramonium* agglutinin)	Galactose β1,4 linked to N-acetylglucosamine in complex and hybrid N-glycans; N-acetylglucosamine in O-glycans	Fetuin, asialofetuin, and weakly with transferring

Working Procedure

1. Separate glycoproteins on a SDS-PAGE and transfer onto a membrane. Include standard glycoproteins (provided in the kit) for positive controls.
2. Incubate the blot for 30 min at room temperature in 20 ml blocking solution.
3. Wash the blot twice for 10 min in 50 ml TBS (0.05 M Tris-HCl, pH 7.5 containing 0.15 M NaCl) and once with buffer 1 (TBS containing 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 1 mM $CaCl_2$).
4. Cut the blot into multiple strips and incubate each strip for 1 h at room temperature with individual lectin solution in buffer 1.
5. Wash each blot three times with 50 ml TBS.
6. Incubate each blot with anti-digoxigenin-alkaline phosphatase conjugate (10 μl in 10 ml TBS) for 1 h at room temperature.
7. After washing the blots three times with 50 ml TBS, develop with staining solution (200 μl 4-nitro blue tetrazolium chloride/5-bromo-4 chloro-3-indolyl-phosphate in 10 ml of 0.1 M Tris-HCl, pH 9.5 containing 0.05 M $MgCl_2$ and 0.1 M NaCl).

7.9   ANALYSES OF CARBOHYDRATE LINKAGE

The structural analyses of sugar chains can be determined by enzymatic and chemical methods.

7.9.1 ENZYMATIC METHODS

Several exoglycosidases with strict linkage specificity can be applied to elucidate the primary structure of glycans by sequential degradation (33, 34).

Neuraminidase (EC: 3.2.1.18; N-acetylneuraminyl hydrolase)

Neuraminidase from *Vibrio cholerae*, *Clostridium perfringens*, and *Arthrolacter ureafasciens* are commonly used for cleaving N-acetylneuraminic acids (sialic acids) that are linked α2,3 and α2,6 to galactose in the glycans. O-substitution (e.g., 4-O-acetyl derivative) in the N-acetylneuraminic acid is generally less sensitive to neuraminidase. N-acetyl neuraminyl N-acetylgalactosamine linkages are generally resistant to neuraminidase cleavage.

Working Procedure

1. Incubate the glycan or glycoconjugate with 0.1 to 1 U of neuraminidase (0.1 U of enzyme/μmol of N-acetylneuraminic acid to be released) in 50 mM sodium acetate buffer at the optimum pH (usually pH 5.0) for 10 min to 48 h at 37°C.
   (**Note:** in case of the neuraminidase from *Vibrio cholerae*, the buffer includes additionally 10 mM $CaCl_2$ and 0.1 M NaCl.)
2. Stop the hydrolysis in a boiling water bath for 3 min.
3. Determine the amount of released sialic acid by colorimetric methods.

β D-Galactosidase (EC: 3.2.1.23; β D-galactoside galactohydrolase)

β-D-Galactosidases are obtained from various sources such as plants and bacteria. Usually they are characterized by two groups. One group of β-D-galactosidases has high affinity toward the lactose (e.g., the enzymes from *E. coli, K. lactis*), but resistant to glycoconjugates. Another group of β-D-galactosidases is active on the β-glycosidic linkages present in glycoconjugates. Some β-D-galactosidases present a very strict linkage specificity. Generally, β-D-galactosidases are resistant when the monosaccharide to which the galactose is conjugated is substituted (e.g., lacto-N-fucopentaose II: Galβ1,3(Fucα1,4)GlcNAcβ1,3Galβ1,4Glc).

Working Procedure

1. Incubate the glycan or glycoconjugate with β-D-galactosidases (0.4 U of enzyme/μmol of galactose to be released) in 50 to 100 μl of 20 mM sodium phosphate/10 mM acetic acid buffer (pH 3.5 to 4.0) at 37°C for 10 min to 48 h.
2. Stop the hydrolysis in a boiling water bath for 3 min.
3. Determine the free galactose by standard method such as GLC, HPLC, or galactose dehydrogenase assay.

N-acetyl β D-hexosaminidase (EC: 3.2.1.52; 2-acetamido-2-deoxy-β D-hexoside acetamido deoxyhexohydrolase)

Generally, both N-acetyl β-D-glucosaminyl and N-acetyl β-D-galactosaminyl linkages are cleaved by N-acetyl β-D-hexosaminidases. However, the rate of hydrolysis depends on the source, the type of linkage, and the molecular conformation.

Working Procedure

1. Incubate 1 μmol of substrate in 100 to 200 ml of Mclevaine buffer (0.2 M sodium phosphate/0.1 M citric acid), pH 5 with 1.5 to 5 U of enzyme for 6 to 48 h at 37°C.
2. Stop the hydrolysis in a boiling water bath for 3 min.

α D-mannosidase (EC: 3.2.1.24; α D-mannoside mannohydrolase)

α-D-mannosidases from jack bean and Aspergillus niger are most commonly used. The α-D-mannosidase from jack bean hydrolyzes Manα1,2Man or Manα1,6Man linkages more easily than Manα1,3Man linkage (15-fold slower). The enzyme from A. niger is very active on Manα1,6Man, Manα1,4Man, or Manα1,4GlcNAc; weakly active on Manα1,2Man; and inactive on Manα1,3Man.

Working Procedure

1. Incubate the glycan or glycoconjugate (in 50 mM sodium acetate, pH 4.2 containing 0.1 to 5 mM $ZnCl_2$) with α-D-mannosidase (1.5 U of enzyme/μmol of mannose to be released) at 37°C for 30 min to 48 h. (**Note:** Zn^{2+} stabilizes all α-D-mannosidases except that from A. niger.)
2. Stop the hydrolysis in a boiling water bath for 3 min.

β D-mannosidase (EC: 3.2.1.25; β D-mannoside mannohydrolase)

β-D-mannosidases from A. niger is specific for β1,4Man linkages (34).

Working Procedure

1. Incubate 1 μmol of substrate in 100 to 200 μl 20 mM sodium acetate buffer (pH 3.5) with 5 mU of β-D-mannosidase (from A. niger) at 37°C for 1 to 24 h.
2. Stop the hydrolysis in a boiling water bath for 3 min.

α L-fucosidase (EC: 3.2.1.51; α L-fucoside fucohydrolase)

α-L-fucosidases are available from various sources, but the rate of hydrolysis depends on the origin of the enzyme. The enzyme for *Turbo cornutus* acts in the following order: Fucα1,4GlcNAc > Fucα1,2Gal > Fucα1,3GlcNAc, while the enzyme of *Charinia lampas* hydrolyzes as follows: Fucα1,2Gal > Fucα1,4GlcNAc > Fucα1,3GlcNAc. The α-L-fucosidases from *Clostridium perifringes*, *A. niger*, or *Streptococcus sanguis* are very specific for α1,2Fuc linkages.

Working Procedure

1. Incubate the substrate in 50 to 100 μl of 0.1 M phosphate citrate buffer, with α-L-fucosidase (1.0 U of enzyme/μmol of fucose to be released) at 37°C for 3 to 48 h.
2. Stop the hydrolysis in a boiling water bath for 3 min.

7.9.2 CHEMICAL METHODS

7.9.2.1 Methylation Analysis

Among the available chemical methods, methylation analysis is widely used to determine the position of linkage between component monosaccharide residues in oligosaccharides. In this method, oligosaccharides are completely methylated and hydrolyzed to a mixture of monosaccharides; the methylated monosaccharides are reduced to alditols; the alditols are acetylated to alditol acetate derivatives; and finally the methylated alditol acetates are identified by GLC or GLC-mass spectrometry. The types of glycosidic linkages are determined from the nature of the methylated alditol acetates. Information on the anomeric configuration of the glycosidic linkages as well as information on the sequence of the monosaccharide residues in the oligosaccharides is not obtained from this method.

In methylation analysis, it is important to methylate completely all of the hydroxyl groups of oligosaccharide or polysaccharide prior to hydrolysis. A successful methylation reaction, developed by Hakomori,[35] can be achieved using a strong base, methylsulfinyl methyl sodium, to ionize free hydroxyl groups of the oligosaccharides and using methyl iodide for methylating these groups. Generally, neutral oligosaccharides or polysaccharides are completely methylated by the method described below. However, polysaccharides containing uronic acid or hexosamine residues are difficult to methylate and may yield side products, which require special analytical techniques for identification.[36] Methylation can be performed at ambient temperature and at a faster rate when sodium hydride is replaced by potassium hydride.

Reaction

Sodium hydride and methyl sulfoxide are allowed to react at 50°C to form methylsulfinyl carbanion, which is then reacted with oligosaccharide (Figure 7.12). The
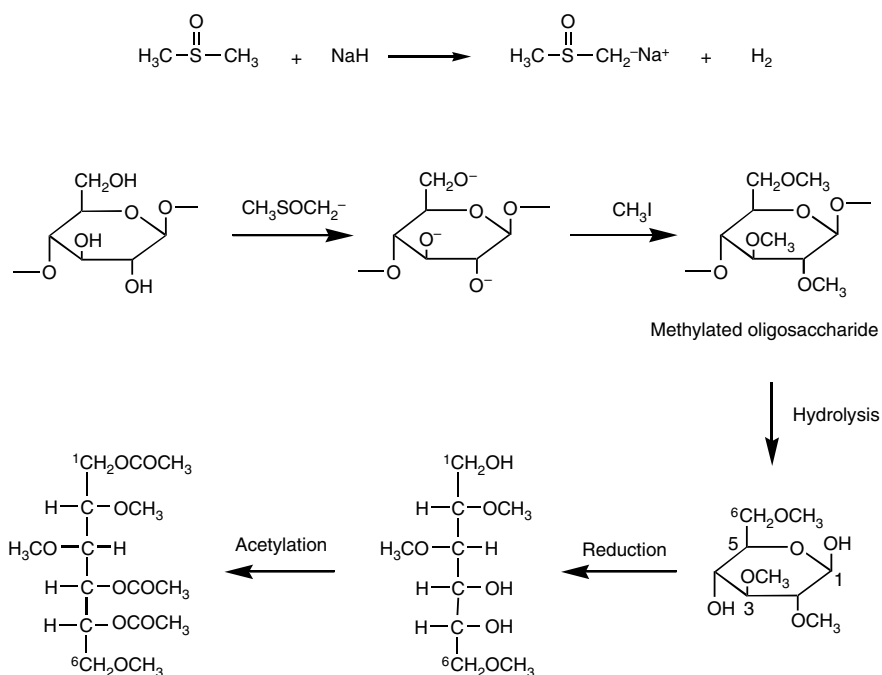
FIGURE 7.12 Reaction of dimethylsulfoxide and sodium hydride produces methylsulfinyl methyl sodium, which is then reacted with oligosaccharide to form an oligosaccharide anion. The oligosaccharide anion is methylated, hydrolyzed, reduced, and acetylated.

resulting oligosaccharide anion is then methylated by dropwise addition of methyl iodide under vigorous stirring.

Working Procedure

Preparation of Methylsulfinyl Methyl Sodium

1. To a three-necked 250 ml flask, add 1.5 gram 57% suspension of sodium hydride in mineral oil and 10 ml of anhydrous ether.
2. Flush the flask with nitrogen three times under vacuum and add 30 ml of dried DMSO from a syringe through the serum cap.
   (**Note:** DMSO should be dried thoroughly by stirring 500 ml of reagent-grade DMSO with excess calcium hydroxide for several hours at 65°C, followed by distillation under nitrogen at reduced pressure.)
3. Attach a glass syringe to the flask through the rubber cap and sonicate the reaction mixture in a water bath (at 50°C) for 2 to 3 h. The solution turns transparent green when the synthesis of methylsulfinyl methyl sodium is complete.

4. Transfer the product in several 5 ml serum bottles that are capped with serum caps and flushed with nitrogen. Store bottles in the dark in the frozen state at 4°C.

Methylation

1. Place 2 to 5 mg oligosaccharide or polysaccharide sample into a dry 25 ml round-bottom flask.
2. Attach the flask with a rubber serum cap, evacuate for 1 h, and finally flush the flask with nitrogen three times.
3. Add 1 ml dry DMSO using a dry glass syringe and sonicate the reaction mixture for 20 min at room temperature.
4. Thaw methylsulfinyl methyl sodium and remove 0.4 ml of the sample through the rubber cap using a dry glass syringe and directly add to the flask containing reaction mixture (step 7). Sonicate the reaction mixture for 20 min at 25°C.
5. Add 0.3 ml dry methyl iodide to the reaction mixture and continue sonication for 15 min at 25°C. Leave the reaction mixture for 1 to 6 h at room temperature.
6. Remove the serum cap from the flask, add a small amount of water to neutralize excess methylsulfinyl methyl sodium, and transfer the reaction product into a 12 ml clinical centrifuge tube.
7. Extract the methylated product with 4 ml chloroform. Remove and discard the top aqueous layer.
8. Add 3 ml water three times to the chloroform layer, extract and centrifuge. Remove and discard the top water layer after each washing.
9. To remove last traces of water, add anhydrous magnesium sulfate. To remove magnesium sulfate, filter the solution through glass filter in a Pasteur pipette.
10. Concentrate the methylated product to approximately 1 ml by evaporating the chloroform under a stream of nitrogen.
11. Clean up the methylated product on a column of Sephadex LH-20 (2 gram) pre-equilibrated with a mixture of chloroform and acetone (2:1, v/v). Methylated polysaccharide elutes as a light pink band. Remove the solvent by evaporation under a stream of nitrogen.

Hydrolysis of Methylated Polysaccharide

1. Suspend methylated sample in 1 ml 90% formic acid in a Teflon-coated screw-capped tube and incubate at 105°C for 1.5 h.
2. Evaporate the formic acid by heating to 40°C, add 2 ml 0.15 M H_2SO_4, and incubate at 105°C for 12 to 18 h.
3. Neutralize the acid with barium carbonate and 0.1 M NaOH to precipitate.
4. Centrifuge and collect supernatant.

Reduction

1. Reduce the hydrolyzed methylated monosaccharides with 1 mg of sodium borohydride for 12 h at room temperature.
2. Neutralize the reaction mixture by acidification with Dowex 50 (H⁺) ion-exchange resin and evaporate in a rotary evaporator. Remove borate with methanol under repeated evaporation (in a rotary evaporator).

Acetylation

1. Add 0.25 ml each of acetic anhydride and pyridine to the sample in a screw-capped (Teflon-coated) tube and incubate at 105°C for 2 h.
2. Evaporate the solvent in a stream of nitrogen at 40°C. Remove acetic anhydride and pyridine with ether by repeated evaporation.
3. Dissolve the sample in a small amount of chloroform for analysis by GLC and MS.

7.9.2.2 Periodate Oxidation

Periodate oxidation of oligosaccharides and polysaccharides has been a valuable analytical technique for determining the structure and sequence of oligosaccharides. In this procedure, carbohydrate residues containing glycol groups on adjacent carbon atoms are oxidized to dialdehydes by periodic acid, resulting in the cleavage of carbon-carbon bonds (Figure 7.13). The resulting aldehydes are converted to alcohols that can be split by mild acid hydrolysis without cleaving the glycosidic bonds of unoxidized sugars. So by applying several cycles of degradation, the linkages and sequence of sugar chains can be determined.
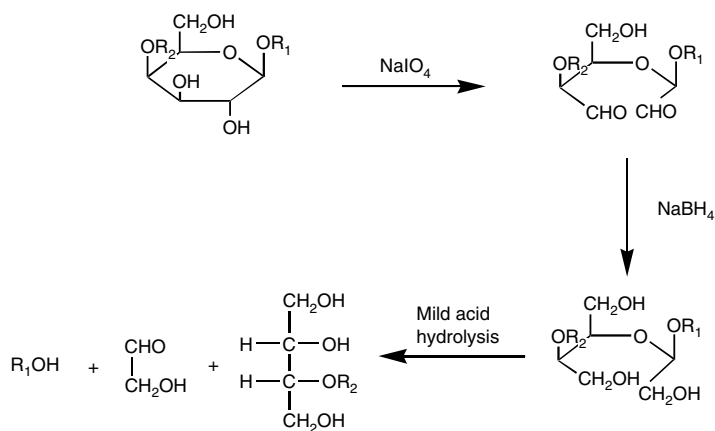
FIGURE 7.13 Periodate oxidation of oligosaccharide cleaves two adjacent glycol groups to dialdehydes, which are then reduced by sodium borohydride, followed by mild acid hydrolysis.

Working Procedure

1. Add 50 mg oligosaccharide or polysaccharide in 50 ml 0.02 M sodium periodate (pH 4.5) to a flask and incubate reaction mixture at 4°C for 18 h.
2. Destroy excess periodate with a few milliliters of ethylene glycol and dialyze the reaction mixture with water to remove low molecular weight substances.
   (**Note:** in the case of oligosaccharide, dialysis membrane of low molecular weight cut-off should be used to keep the sample inside the membrane.)
3. Freeze dry the oxidized oligosaccharide or polysaccharide.
4. Dissolve the sample in 10 ml of water and add 5 mg of sodium borohydride. Incubate the reaction mixture at room temperature for 24 h.
5. Dialyze the sample and freeze dry.
6. Dissolve the sample in 0.5 ml of 0.02 M HCl in a screw-capped tube and incubate the reaction mixture in a boiling water bath for 20 min. For comparison, hydrolyze also 2 mg native oligosaccharide or polysaccharide in 0.1 ml 0.1 M HCl in a boiling water bath for 3 h.
7. Analyze both hydrolysates by paper chromatography using solvent system of n-butanol/pyridine/water (6:4:3 by volume).
8. Stain the paper with silver nitrate and sodium hydroxide reagents.

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Index

Biological Sciences

PRINCIPLES *and* REACTIONS *of* PROTEIN EXTRACTION, PURIFICATION, *and* CHARACTERIZATION

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