chapter 24

Protein Separation and Characterization Procedures

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24.1 INTRODUCTION

Many protein separation techniques are available to food scientists. Several of the separation techniques described in this chapter are used commercially for the production of food or food ingredients, whereas others are used to purify a protein from a food for further study in the research laboratory. Although not the primary focus of this chapter, many methods have been developed to rapidly purify recombinant proteins. In general, separation techniques exploit the biochemical differences in protein solubility, size, charge, adsorption characteristics, and biological affinities for other molecules. These physical characteristics then are used to purify individual proteins from complex mixtures.

The biochemical, nutritional, and functional properties of food proteins can be characterized in a variety of ways. This chapter describes methods of amino acid analysis and several methods for protein nutritional quality analysis. Finally, protein solubility, emulsification, and foaming tests are described, along with gelation and dough formation, for the characterization of protein functional properties. A list of abbreviations is included to summarize terms used throughout this chapter.

24.2 METHODS OF PROTEIN SEPARATION

24.2.1 Initial Considerations

Usually, several separation techniques are used in sequence to purify a protein from a food. In general, the purity of a protein preparation can be improved as more separation steps are added, although this will usually result in a lower recovery or yield. Food ingredients such as protein concentrates may be prepared using only one separation step when high purity is not necessary. Three or more separation steps are often used in sequence to prepare a pure protein for laboratory study. For example, a very common purification procedure for protein includes precipitation by ammonium sulfate, followed by hydrophobic interaction chromatography, ion-exchange chromatography, and lastly gel filtration.

Before starting a separation sequence, it is necessary to learn as much as possible about the biochemical properties of a protein to determine any distinctive characteristics that will make separation easier, such as molecular mass, isoelectric point (pI), solubility properties, denaturation temperature, metal ion binding, and specific ligand recognition. The first separation step should be one that can easily be used with large quantities of material. This is often a technique that utilizes the differential solubility properties of a protein. Each succeeding step in a purification sequence will use a different mode of separation. Some of the most common methods of purification are described in this section and include precipitation, ion-exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, and size-exclusion chromatography. A summary of separation methods described in this chapter can be found in Table 24.1. More detailed information about the various purification techniques can be found in several published sources [1–3].

24.2.2 Separation by Fractional Precipitation

24.2.2.1 *Principle*

Separation by fractional precipitation exploits the differential solubility properties of proteins in solution. Fractional precipitation is one of the simplest methods for separating a protein of interest from other proteins and contaminants in a mixture. Proteins are polyelectrolytes; thus, solubility characteristics are determined by the type and charge of amino acids in the molecule. Proteins can be selectively precipitated or solubilized by changing **buffer pH, ionic strength, dielectric constant,** or **temperature**. These separation techniques are advantageous when working with large quantities of material, are relatively quick, and are not usually influenced by other food components. Precipitation techniques are used most commonly during early stages of a purification sequence.

24.2.2.2 *Procedures*

24.2.2.2.1 Salting Out

Proteins have unique solubility profiles in neutral salt solutions. Low concentrations of neutral salts usually increase the solubility of proteins; however, proteins are precipitated from solution as ionic strength is increased. This property can be used to precipitate a protein from a complex mixture. **Ammonium sulfate** [$(NH_4)_2SO_4$] is commonly used because it is highly soluble, although other neutral salts such as NaC1 or KCl may be used to salt out proteins. Generally a two-step procedure is used to maximize separation efficiency. In the first step, $(NH_4)_2SO_4$ is added at a concentration just below that necessary to precipitate the protein of interest. When the solution is centrifuged, less soluble proteins are precipitated while the protein of interest remains in solution. The second step is performed at an $(NH_4)_2SO_4$ concentration just above that necessary to precipitate the protein of interest. When the solution is centrifuged, the protein is precipitated, while more soluble proteins remain in the supernatant. One disadvantage of this method is that large quantities of salt contaminate the precipitated protein and often must be removed before the protein is resolubilized in buffer. Tables and formulas are available in many biochemistry books and online (type "ammonium sulfate calculator" into your web browser) to determine the proper amount of $(NH_4)_2SO_4$ to achieve a specific concentration.

24.1 table Summary of protein separation methods

Method	*Basis of separation*	*Principle*
Addition of ammonium sulfate	Precipitation	Proteins are precipitated from solution as ionic strength increases, using a neutral salt, such as ammonium sulfate
Isoelectric precipitation	Precipitation	Protein has no net charge at pI, so it aggregates and precipitates from solution
Solvent fractionation	Precipitation	Water miscible organic solvents decrease the dielectric constant of an aqueous solution and decrease the solubility of most proteins, so proteins precipitate from solution
Protein denaturation	Precipitation	Proteins heated to high temperature or adjusted to extremes of pH precipitate from solution
Ion-exchange chromatography	Adsorption (based on charge)	Charged protein molecules in solution are reversibly adsorbed to a charged solid support matrix via electrostatic interactions. Bound proteins are eluted from a column by gradually changing the ionic strength or pH of the eluting solution
Affinity chromatography	Adsorption (based on specific biochemical characteristics)	Protein is adsorbed to chromatographic matrix that contains a ligand covalently bound to a solid support; the ligand used has reversible, specific, and unique binding affinity for the protein of interest. Protein that binds to ligand can be unbound by changing the pH, temperature, or concentration of salt or ligand in the eluting buffer
Dialysis	Size	Semipermeable membrane permits passage of small molecules but not larger molecules. Pore sizes of membranes are specified as molecular weight cutoff
Membrane processes (e.g., microfiltration, ultrafiltration, nanofiltration, reverse osmosis)	Size	Pressure is applied to solution sitting on semipermeable membrane with certain molecular weight cutoff. Small molecules pass through, and large ones are retained
Size-exclusion chromatography	Size	Proteins in solution flow through a column packed with beads that have different average pore sizes. Molecules larger than the pores are excluded and pass through the column quickly. Smaller molecules enter the pores of the beads, so elute more slowly from the column, at a rate dependent on their size
SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis)	Size	Proteins bind SDS to become negatively charged; under a constant voltage they move through an acrylamide gel matrix at a rate based on size alone as all molecules are highly negatively charged
IEF (isoelectric focusing)	Charge	Proteins are separated by charge in an electric field on a gel matrix in which a pH gradient has been generated using ampholytes. Under constant voltage, proteins migrate to the location on the gradient at which pH equals the pI of the protein. Size is not a factor

24.2.2.2.2 Isoelectric Precipitation

The **isoelectric point** (pI) is defined as the pH at which a protein has no net charge in solution. This is determined by the ionizable acidic and basic amino acids making up the protein. Proteins usually aggregate and precipitate at their pI because there is no electrostatic repulsion between molecules. Proteins have different pIs; thus, they can be separated from each other by adjusting solution pH. When the pH of a solution is adjusted to the pI of a protein, the protein precipitates, while proteins with different pIs remain in solution. The precipitated protein can be resolubilized in another solution of different pH.

24.2.2.2.3 *Solvent Fractionation*

Protein solubility at a fixed pH and ionic strength is a function of the **dielectric constant** of a solution. Thus, proteins can be separated based on solubility differences in **organic solvent-water** mixtures. The addition of water-miscible organic solvents, such as **ethanol** or **acetone**, decreases the dielectric constant of an aqueous solution and decreases the solubility of most proteins. Organic solvents decrease ionization of charged amino acids, resulting in protein aggregation and precipitation. The optimum quantity of organic solvent to precipitate a protein varies from 5% to 60%. Solvent fractionation is usually performed at 0 °C or below to prevent protein denaturation caused by temperature increases that occur when organic solvents are mixed with water.

24.2.2.2.4 *Denaturation of Contaminating Proteins*

Many proteins are **denatured** and precipitated from solution when heated above a certain temperature or by adjusting a solution to highly acid or basic pHs. Proteins that are stable at high temperatures or at extremes of pH may be separated by this technique because many contaminating proteins will precipitate while the protein of interest remains in solution.

24.2.2.3 *Applications*

All of the above techniques are commonly used to fractionate proteins. The differential solubility of selected muscle proteins in $(NH_4)_2SO_4$ and acetone and temperature stability at 55 °C are illustrated in Table 24.2. These three techniques can be combined in sequence to prepare muscle proteins of high purity.

24.2 table Conditions for fractionating water-soluble muscle proteins using differential solubility techniques

	Precipitation range		
Enzyme	*$(NH_4)_2SO_4$, pH 5.5, 10 °C (Percent saturation)*	*Acetone, pH 6.5, −5 °C (Percent vol/vol)*	*Stability[a], pH 5.5, 55 °C*
Phosphorylase	30–40	18–30	U
Pyruvate kinase	55–65	25–40	S
Aldolase	45–55	30–40	S
Lactate dehydrogenase	50–60	25–35	S
Enolase	60–75	35–45	U
Creatine kinase	60–80	35–45	U
Phosphoglycerate kinase	60–75	45–60	S
Myoglobin	70–90	45–60	U

Adapted from Scopes [4] with permission of the University of Wisconsin Press. From Briskey, E.J., R.G. Cassens, and B.B. Marsh. The Physiology and Biochemistry of Muscle as Food. Copyright 1970

[a] *U* unstable, *S* stable at heating temperature

One of the best examples of the commercial use of differential solubility to separate proteins is in the production of protein concentrates. Soy protein concentrate can be prepared from defatted soybean flakes or flour using several methods. Soy proteins can be precipitated from other soluble constituents in the flakes or flour using a 60–80% aqueous alcohol solution, by isoelectric precipitation at pH 4.5 (which is the pI of many soy proteins), or by denaturation with moist heat. These methods have been used to produce concentrates containing greater than 65% protein. Two or three separation techniques can be combined in sequence to produce soy protein isolates with protein concentrations above 90%.

24.2.3 Separation by Liquid Chromatography

24.2.3.1 *Principle*

In addition to fractional precipitation methods, purification of a protein usually includes one or more types of liquid chromatography. Chromatographic separations are based on the differential affinity of a mixture of proteins in solution (mobile phase) for the stationary phase. These chromatographic methods can be performed in columns at atmospheric pressure (by gravity flow) or under applied pressures using centrifugation or **high-performance liquid chromatography** (HPLC) (Chap. 13). Ultra-performance liquid chromatography (UPLC) (Chap. 13, Sect. 13.2.3.3), fast protein liquid chromatography (FPLC), and fast-performance liquid chromatography are variations of HPLC, the primary difference being the pressures applied within the system and the properties of the stationary phase. Ion-exchange chromatography, hydrophobic interaction chromatography, and affinity chromatography are commonly used for protein purification and will be described briefly.

24.2.3.2 *Procedures*

24.2.3.2.1 *Ion-Exchange Chromatography*

Ion-exchange chromatography is defined as the reversible adsorption between charged molecules and ions in solution and a charged solid support matrix (Chap. 12, Sect. 12.4.4). Ion-exchange chromatography is the most commonly used protein separation technique and results in an average eightfold purification. A positively charged matrix is called an **anion exchanger** because it binds negatively charged ions or molecules in solution. A negatively charged matrix is called a **cation exchanger** because it binds positively charged ions or molecules. The most commonly used exchangers for protein purification are anionic diethylaminoethyl-derivatized supports, followed by carboxymethyl and phospho-cation exchangers.

The protein of interest is first adsorbed to the ion exchanger under buffer conditions (ionic strength and pH) that maximize the affinity of the protein for the

matrix. Contaminating proteins of different charges pass through the exchanger unabsorbed. Proteins bound to the exchangers are selectively eluted from the column by gradually changing the ionic strength or pH of the eluting solution. As the composition of the eluting buffer changes, the charges of the proteins change and their affinity for the ion-exchange matrix is decreased. Protein purification by ion-exchange chromatography can be performed in a column at atmospheric pressure or under applied high pressures. In addition, proteins can be purified using ion-exchange resins in a batch extraction.

24.2.3.2.2 Affinity Chromatography

Affinity chromatography is a type of adsorption chromatography in which a protein is separated in a chromatographic matrix containing a **ligand** covalently bound to a solid support (Chap. 12, Sect. 12.4.5). A ligand can be defined as a substance (molecule or metal) that has a reversible, specific, and unique binding affinity for a receptor site on a protein (Table 24.3). Ligands may be biospecific, such as enzyme inhibitors, enzyme substrates, coenzymes, or antibodies. Other types of ligands include certain dyes and metal ions. Thus, the protein is separated from a complex mixture due to its affinity or specific binding interaction with a ligand immobilized on a solid support.

The protein is passed through a column containing the ligand bound to a solid support, under buffer conditions (pH, ionic strength, temperature, and protein concentration) that maximize binding of the protein to the ligand. Contaminating proteins and molecules that do not bind the ligand are eluted. The bound protein is then desorbed or eluted from the column under conditions that decrease the affinity of the protein for the bound ligand, by changing the pH, temperature, or concentration of salt or ligand in the eluting buffer.

Recombinant proteins are often purified by immobilized metal ion affinity chromatography followed by size-exclusion chromatography. Recombinant proteins can be engineered to contain multiple histidine residues (six to ten molecules of histidine). The proteins containing this polyhistidine tag will bind to an affinity column containing immobilized divalent metal ions, such as nickel. Once contaminants are washed from the column, the tagged protein is eluted using a gradient of imidazole counter ligands (the amino acid histidine contains an imidazole functional group).

Affinity chromatography is a very powerful technique and is a commonly used protein purification procedure. The average purification achieved by affinity chromatography is approximately 100-fold, although 1,000-fold increases in purification have been reported. This technique is more powerful than size exclusion and ion exchange which usually achieve less than a 12-fold purification. Many covalently bound ligands and associated buffers are commercially available as kits. Preactivated solid supports used for covalent bonding of various ligands are also commercially available.

24.3 table Examples of common biological interactions used to separate proteins via affinity chromatography

Protein target	*Ligand*
Enzyme	Substrate, inhibitor, cofactor
Antibody	Antigen
Glycoprotein	Lectin, polysaccharide
Hormone	Hormone receptor
Proteins with histidine, cysteine, or tryptophan residues on the surface	Metal ions

24.2.3.2.3 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography is another type of chromatography often used in protein purification schemes (Chap. 12, Sect. 12.4.3; Chap. 13, Sect. 13.3.3). In this technique, proteins are separated due to their reversible interaction with a hydrophobic stationary phase. Hydrophobic interactions are increased at high ionic strength, so this type of chromatography is often used after ammonium sulfate precipitation or after using a salt gradient to elute a protein from an ion-exchange column. The bound protein is usually eluted by decreasing the ionic strength of the mobile phase using a stepwise or gradient elution.

24.2.3.3 *Applications*

Ion-exchange chromatography is commonly used to separate proteins in the laboratory and can be used for separation and quantification of amino acids as described in Sect. 24.3.1. Ion-exchange chromatography is used to isolate proteins while removing lactose, minerals, and fat from sweet dairy whey. Whey protein isolates (containing greater than 90 % protein) and several protein fractions, such as alpha-lactalbumin, lactoperoxidase, and lactoferrin, are purified from sweet dairy whey using cation-exchange chromatography [5]. Whey protein isolates are used as supplements in nutrition bars and beverages, since the high-quality protein is soluble and digestible.

Affinity chromatography is commonly used for protein purification in the research laboratory and may be used for commercial preparation of proteins by chemical suppliers. It is not generally used for commercial production of food protein ingredients due to the high costs involved, although this technique is used to purify some high-value bioactive peptides and proteins for nutritional applications. Glycoproteins, commonly purified by affinity chromatography, can be separated from other proteins in a complex mixture by utilization of the high carbohydrate-binding affinity of lectins. Lectins, such as concanavalin A, are carbohydrate-binding proteins that can be bound to a

solid support and used to bind the carbohydrate moiety of glycoproteins that are applied to the column. Once the glycoproteins are bound to the column, they can be desorbed using an eluting buffer containing an excess of lectin. The glycoproteins bind preferentially to the free lectins and elute from the column.

24.2.4 Separation by Size

24.2.4.1 *Principle*

Protein molecular masses range from about 10,000 to over 1,000,000 Da; thus, size is a logical parameter to exploit for separations. Actual separation occurs based on the **Stokes radius** of the protein, not on the molecular mass. Stokes radius is the average radius of the protein in solution and is determined by protein conformation. For example, a globular protein may have an actual radius very similar to its Stokes radius, whereas a fibrous or rod-shaped protein of the same molecular mass may have a Stokes radius that is much larger than that of the globular protein. Thus, one limitation of these methods is that two proteins of the same molecular mass may occasionally separate differently.

24.2.4.2 *Procedures*

24.2.4.2.1 Dialysis

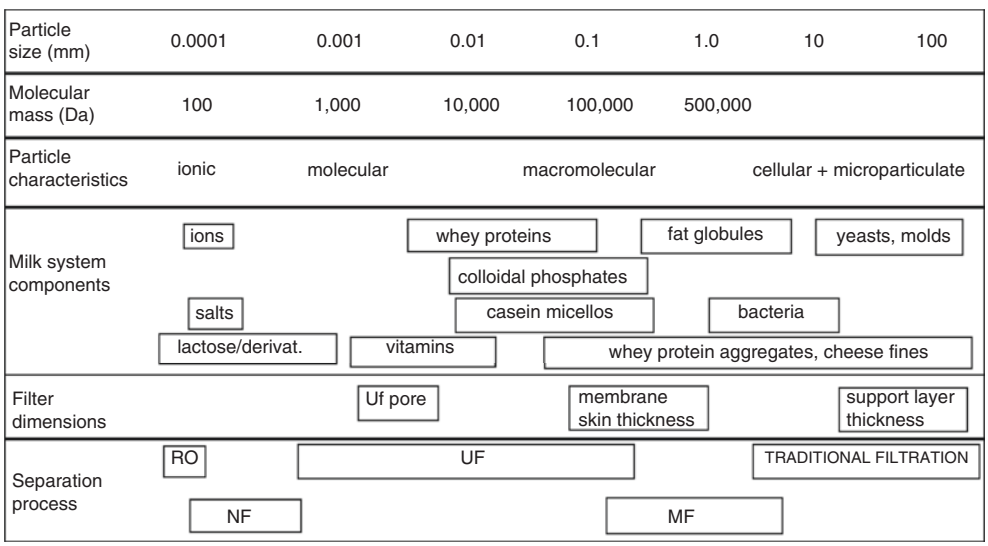
Dialysis is used to separate molecules in solution by the use of semipermeable membranes that permit passage of small molecules but not larger molecules. To perform dialysis, a protein solution is placed into dialysis tubing that has been tied or clamped at one end. The other end of the tubing is sealed, and the bag is placed in a large volume of water or buffer (usually 500–1,000 times greater than the sample volume inside the dialysis tubing) which is slowly stirred. Solutes of low molecular mass diffuse from the bag, while buffer diffuses into the bag. Dialysis is simple; however, it is a relatively slow method, usually requiring at least 12 h and one or more changes of buffer. The protein solution inside the bag is often diluted during dialysis, due to osmotic strength differences between the solution and dialysis buffer. Dialysis can be used to change buffer composition or pH and to remove salt and other impurities of low molecular mass between purification steps or to adjust the buffer composition of a final protein preparation.

24.2.4.2.2 Membrane Processes

Microfiltration, ultrafiltration, nanofiltration, and reverse osmosis all are processes that use a semipermeable membrane for the separation of solutes on the basis of size under an applied pressure. These methods are similar to dialysis but are much faster and are applicable to both small- and large-scale separations. Molecules larger than the membrane cutoff are retained and become part of the retentate, while smaller molecules pass through the membrane and become part of the filtrate.

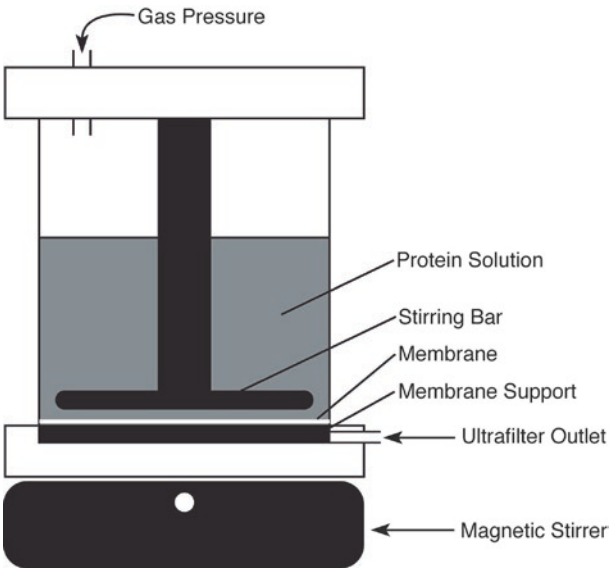
These membrane processes differ mainly in the porosity of the membranes and in the operating pressure used. The porosity of the membrane (membrane pore diameter) sequentially decreases and the pressure used sequentially increases for microfiltration, ultrafiltration, nanofiltration, and reverse osmosis. The approximate pore size of each membrane process relative to the different components present in milk is shown in Fig. 24.1.

Ultrafiltration is commonly used in protein research laboratories, with various commercial units available. A stirred cell ultrafiltration unit is illustrated



24.1 figure

Range of particle sizes used for various membrane filtration techniques compared to particle sizes of milk components and microorganisms. *RO* reverse osmosis, *NF* nanofiltration, *UF* ultrafiltration, *MF* microfiltration (Adapted from Jelen [6], with permission of the International Dairy Federation)



24.2 figure Schematic diagram of a stirred cell ultrafiltration unit

in Fig. 24.2. The protein solution in the stirred cell is filtered through the semipermeable membrane by gas pressure, leaving a concentrated solution of proteins larger than the membrane cutoff point inside the cell. Disposable centrifugal filtering units are available for small sample volumes with membrane cutoff values ranging from 3,000 to 100,000 Da. Solvents and molecules smaller than the membrane pore size are forced through the membrane by centrifugation resulting in concentration and purification of the protein in the retentate.

24.2.4.2.3 Size-Exclusion Chromatography

Size-exclusion chromatography, also known as gel filtration or gel permeation chromatography, is a column technique that can be used to separate proteins on the basis of size (Chap. 12, Sect. 12.4.6). This technique can also be used for buffer exchange, desalting, and removal of low molecular weight impurities. A protein solution flows through a column packed with a solid support of porous beads made of a cross-linked polymeric material such as agarose or dextran. Beads of different average pore sizes that allow for efficient fractionation of proteins of different molecular masses are commercially available. Molecules larger than the pores in the beads are excluded, moving quickly through the column and eluting from the column in the shortest times. Small molecules enter the pores of the beads and are retarded, thus moving very slowly through the column. Molecules of intermediate sizes partially interact with the porous beads and elute at intermediate times. Consequently, molecules are eluted from the column in order of decreasing size.

Molecular mass can be calculated by chromatographing the unknown protein and several proteins of known molecular mass. Standards of known molecular mass are commercially available and can be used to prepare a standard curve. A plot of the **elution volume** (V_e) of each protein versus **log of the molecular mass** yields a straight line. Size-exclusion techniques generally can be used to estimate molecular mass within ±10 %; however, errors can occur if the Stokes radii of the unknown protein and standards are quite different.

24.2.4.3 *Applications*

Microfiltration can be used to remove particles and microorganisms and has been applied to wastewater treatment and to remove the bacteria from milk and beer. Ultrafiltration and nanofiltration are used to concentrate a protein solution, remove salts, exchange buffer, or fractionate proteins on the basis of size. Ultrafiltration is used to concentrate milk for cheesemaking and to manufacture whey protein products, whereas nanofiltration has been used to remove monovalent ions from salt whey. Ultrafiltration is used to concentrate whole liquid egg and liquid egg white prior to spray drying. Reverse osmosis is often used to purify water and to remove aqueous salts, metal ions, simple sugars, and other small impurities with molecular mass below 2,000. The various membrane systems can be used in combination, for example, ultrafiltration and reverse osmosis in sequence are used to concentrate and fractionate whey proteins, then remove salts and lactose.

Dialysis and size-exclusion chromatography are primarily used in the analytical laboratory in a protein separation sequence. Dialysis may be used to change the buffer to one of the appropriate pH and ionic strength during purification or prior to electrophoresis of a protein sample. Dialysis is used after $(NH_4)_2SO_4$ precipitation of a protein to remove excess salt and other small molecules and to solubilize protein in a new buffer. Size-exclusion chromatography is used to remove salts, change buffers, fractionate proteins, and estimate protein molecular mass.

24.2.5 Separation by Electrophoresis

24.2.5.1 *Polyacrylamide Gel Electrophoresis*

24.2.5.1.1 Principle

Electrophoresis is defined as the migration of charged molecules in a solution through an electrical field. The most common type of electrophoresis performed with proteins is zonal electrophoresis in which proteins are separated from a complex mixture into bands by migration in aqueous buffers through a solid polymer matrix called a gel. **Polyacrylamide gels** are the most common matrix for zonal electrophoresis of proteins, although other matrices such as starch and agarose

may be used. Gel matrices can be formed in glass tubes or as slabs between two glass plates.

Separation depends on the friction of the protein within the matrix and the charge of the protein molecule as described by the following equation:

$$\text{Mobility} = \frac{(\text{Applied voltage})(\text{Net charge on molecule})}{\text{Friction of the molecule}} \tag{24.1}$$

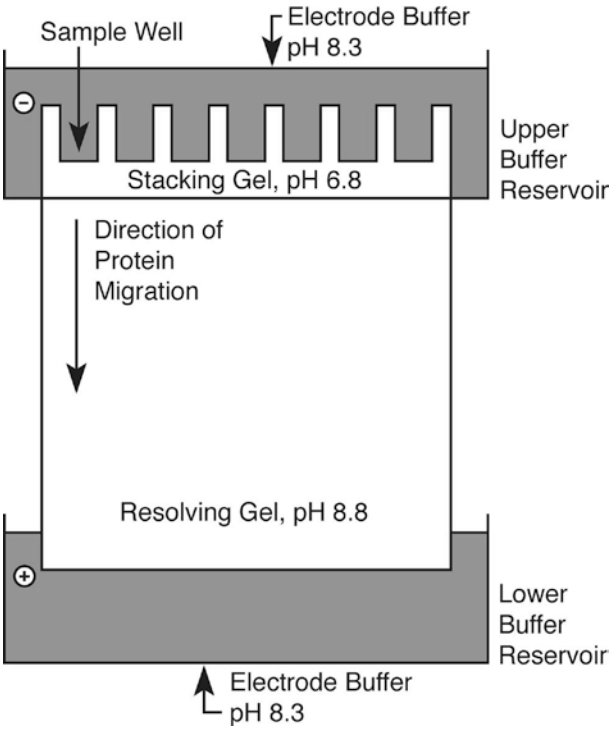
Proteins are positively or negatively charged, depending on solution pH and their isoelectric point (pI). A protein is negatively charged if solution pH is above its pI, whereas a protein is positively charged if solution pH is below its pI. The magnitude of the charge and applied voltage will determine how far a protein will migrate in an electrical field. The higher the voltage and stronger the charge on the protein, the greater the migration within the electrical field. Molecular size and shape, which determine the Stokes radius of a protein, also determine migration distance within the gel matrix. Mobility of proteins decreases as molecular friction increases due to an increase in Stokes radius; thus, smaller proteins tend to migrate faster through the gel matrix. Similarly, a decrease in pore size of the gel matrix will decrease mobility.

In nondenaturing or **native electrophoresis**, proteins are separated in their native form based on charge, size, and shape of the molecule. Another form of electrophoresis commonly used for separating proteins is denaturing electrophoresis. **Polyacrylamide gel electrophoresis** (PAGE) with an anionic detergent, **sodium dodecyl sulfate** (SDS), is used to separate protein subunits by size. Proteins are solubilized and dissociated into subunits in a buffer containing SDS and a reducing agent. **Reducing agents**, such as mercaptoethanol or dithiothreitol, are used to reduce disulfide bonds within a protein subunit or between subunits. Proteins bind SDS, become negatively charged, and are separated based primarily on size alone.

24.2.5.1.2 Procedures

A power supply and electrophoresis apparatus containing the polyacrylamide gel matrix and two buffer reservoirs are necessary to perform a separation. A representative slab gel and electrophoresis unit is shown in Fig. 24.3. The power supply is used to generate the electric field by providing a source of constant current, voltage, or power. The electrode buffer controls the pH to maintain the proper charge on the protein and conducts the current through the polyacrylamide gel. Commonly used buffer systems include an anionic tris-(hydroxymethyl)aminomethane buffer with a resolving gel at pH 8.8 and a cationic acetate buffer at pH 4.3.

The polyacrylamide gel matrix is formed by polymerizing **acrylamide** and a small quantity (usually 5% or less) of the **cross-linking reagent**, **N**,**N**′-**methylenebisacrylamide**, in the presence of a **catalyst**, **tetramethylethylenediamine** (TEMED), and **source of free radicals**, **ammonium persulfate**. Gels can be made in the laboratory or purchased precast.



24.3 figure Schematic diagram of a slab gel electrophoresis unit indicating the pHs of the stacking and resolving gels and the electrode buffer in an anionic discontinuous buffer system

A discontinuous gel matrix is usually used to improve resolution of proteins within a complex mixture. The discontinuous matrix consists of a **stacking gel** with a large pore size (usually 3–4% acrylamide) and a **resolving gel** of a smaller pore size. The stacking gel, as its name implies, is used to stack or concentrate the proteins into very narrow bands prior to their entry into the resolving gel. At pH 6.8, a voltage gradient is formed between the chloride (high negative charge) and glycine ions (low negative charge) in the electrode buffer, which serves to stack the proteins into narrow bands between the ions. Migration into the resolving gel of a different pH disrupts this voltage gradient and allows separation of the proteins into discrete bands.

The pore size of the resolving gel is selected based on the molecular mass of the proteins of interest and is varied by altering the concentration of acrylamide in solution. Proteins are usually separated on resolving gels that contain 4–15% acrylamide. Acrylamide concentrations of 15% may be used to separate proteins with molecular mass below 50,000 Da. Proteins greater than 500,000 Da are often separated on gels with

acrylamide concentrations below 7%. A **gradient gel** in which the acrylamide concentration increases from the top to the bottom of the gel is often used to separate a mixture of proteins with a large molecular mass range.

To perform a separation, proteins in a buffer of the appropriate pH are loaded on top of the stacking gel. **Bromophenol blue tracking dye** is added to the protein solution. This dye is a small molecule that migrates ahead of the proteins and is used to monitor the progress of a separation. After an electrophoresis run, the separated protein bands in the gels can be visualized using a nonspecific **protein stain** such as **Coomassie Brilliant Blue stain**, **silver stain**, or a **fluorescent gel stain**. Specific enzyme stains or antibodies can be used to detect a particular protein or enzyme.

The electrophoretic or **relative mobility** (R_m) of each protein band is calculated as

$$R_m = \frac{\text{Distance protein migrated from start of resolving gel}}{\text{Distance between start of running gel and tracking dye}} \quad (24.2)$$

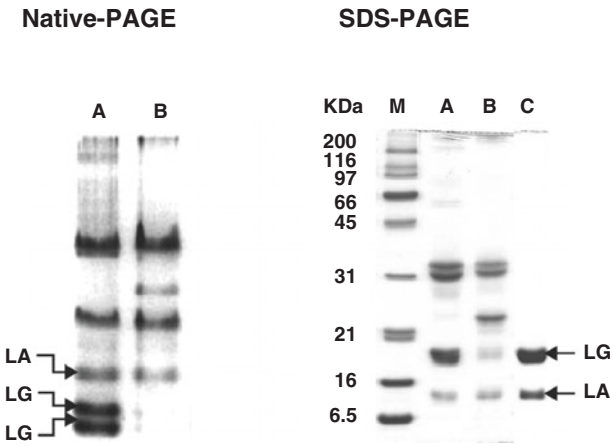
Additional procedural details can be found in several sources [1–3].

24.2.5.1.3 Applications

Electrophoretic techniques can be used as one step in a purification process or to aid in the biochemical characterization of a protein. Electrophoresis can be used to help determine the purity of a protein extract. Commercially available preparative electrophoresis units are used to purify large quantities of protein. Small quantities of protein can be eluted and collected from electrophoresis gels using electroelution techniques. Alternatively, proteins can be transferred from electrophoresis gels to a membrane and then stained with antibodies specific to a target protein in a process called electroblotting or western blotting. Western blotting is discussed in more detail in Chap. 27.

Electrophoresis is often used to determine the protein composition of a food product. For example, differences in the protein composition of soy protein concentrates and whey protein concentrates produced by different separation techniques can be detected. The lanes on the left side of Fig. 24.4 illustrate the protein patterns observed when extracts of unheated and heated whey proteins are separated by native PAGE.

SDS-PAGE is used in characterization protocols to determine subunit composition of a protein and to estimate subunit molecular mass. Molecular mass can usually be estimated within an error of ±5%, although highly charged proteins or glycoproteins may be subject to a larger error. Molecular mass is determined by comparing R_m of the protein subunit with R_m of protein standards of known molecular mass (Fig. 24.5). Commercially prepared protein standards are available in several molecular mass ranges. To prepare a standard curve, logarithms of protein standard molecular mass are plotted against their corresponding R_m values. The molecular mass of the unknown protein is determined from its R_m value using the standard curve. Electropherograms of unheated and heated whey proteins separated by SDS-PAGE are also illustrated in Fig. 24.4, right side.



24.4 figure

Electrophoresis of whey proteins fractionated from raw and heated milk. *Left panel*: native-polyacrylamide gel electrophoresis (PAGE). Lane A = raw milk; lane B = heated milk. *Right panel*: sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lane M = molecular weight markers; lane A = raw milk; lane B = heated milk; lane C = β-lactoglobulin (LG) standard and α-lactalbumin (LA) standard. Ten micrograms of protein were loaded onto each lane. Note a decrease in two isoforms of LG after heating (Reprinted from Chen et al. [7] with permission)

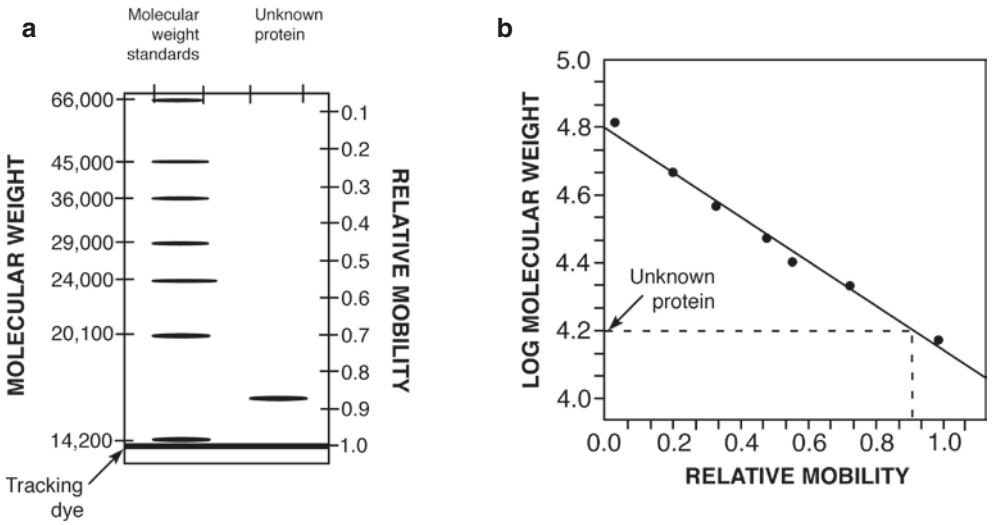
24.2.5.2 Isoelectric Focusing

24.2.5.2.1 Principle

Isoelectric focusing, also termed electrofocusing, is a modification of electrophoresis, in which proteins are separated by charge in an electric field on a gel matrix in which a pH gradient has been generated using ampholytes. Proteins are focused or migrate to the location in the gradient at which pH equals the pI of the protein. At this point, the protein has no net charge. Resolution is among the highest of any protein separation technique and can be used to separate proteins with pIs that vary less than 0.02 of a pH unit.

24.2.5.2.2 Procedure

A pH gradient is formed using **ampholytes**, which are small polymers (molecular masses of less than 1,000 Da) containing both positively and negatively charged groups. An ampholyte mixture is composed



24.5 figure

Use of SDS-PAGE to determine the molecular mass of a protein. (**a**) Separation of molecular mass standards and the unknown protein. (**b**) Standard curve for estimating protein molecular mass

of thousands of polymers that exhibit a range of pH values. Ampholyte mixtures are available that cover a narrow pH range (e.g., 1–2 pH units) or a broad range (e.g., 4–6 pH units) and should be selected for use based on properties of the proteins to be separated.

Ampholytes are added to the gel solution prior to polymerization. Once the gel is formed and a current applied, the ampholytes migrate to produce a linear pH gradient; negatively charged ampholytes migrate toward the anode, while positively charged ampholytes migrate toward the cathode. Proteins migrate within this pH gradient depending on their charge until they reach the pH at which they have no net charge (at their pI).

24.2.5.2.3 Applications

Isoelectric focusing is the method of choice for determining the isoelectric point of a protein by comparison to standard marker proteins of known pI. This technique is an excellent method for determining the purity of a protein preparation. Isoelectric focusing can also be used to detect changes in protein composition due to posttranslational modifications such as glycosylation or phosphorylation. Genetic variants of many plant and animal proteins are visualized using isoelectric focusing. Isoelectric focusing is used to differentiate closely related animal and fish species based on protein patterns. The US Food and Drug Administration publishes the Regulatory Fish Encyclopedia [8] which contains isoelectric focusing patterns of about 1,700 finfish and shellfish species. This guide helps state and federal officials to identify species substitution and thus detect product adulteration and economic fraud.

Isoelectric focusing and SDS-PAGE can be combined to produce a two-dimensional electrophoretogram that is extremely useful for separating very complex mixtures of proteins. This technique is called **two-dimensional electrophoresis**. Proteins are first separated in tube gels by isoelectric focusing. The tube gel containing the separated proteins is then placed on top of an SDS-PAGE slab gel, and proteins are separated. Thus, proteins are separated first on the basis of charge and then according to size and shape. Over 1,000 proteins in a complex mixture have been resolved using this technique. This method is used to verify the genetic purity of hybrid seeds as well as evaluating the up- or downregulation of proteins in various biological processes and disease states in humans and animals. Two-dimensional electrophoresis gels have been used to determine the differences in muscle proteins extracted from raw and cooked pork and goose meat [9].

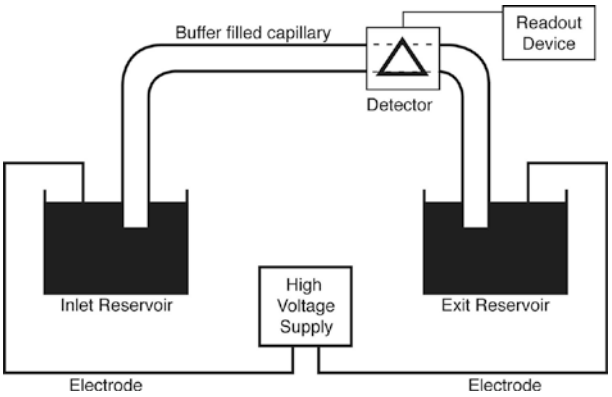
24.2.5.3 *Capillary Electrophoresis*

24.2.5.3.1 Principle

Capillary electrophoresis has been described as a hybrid technique incorporating aspects of traditional slab gel electrophoresis and liquid chromatography. Similar principles apply for the separation of proteins by both capillary and conventional electrophoretic techniques; proteins can be separated on the basis of charge or size in an electric field. The primary difference between capillary electrophoresis and conventional electrophoresis (described previously) is that **capillary tubing** is used in place of acrylamide gels cast in tubes or slabs. As the separated proteins migrate along the capillary tubing, they are detected using detectors originally developed for chromatography.

24.2.5.3.2 Procedure

A schematic diagram of a capillary electrophoresis system is shown in Fig. 24.6. A capillary electrophoresis



24.6 figure Schematic diagram of a capillary electrophoresis system

system is comprised of a capillary column, power supply, detector, and two buffer reservoirs. The sample is introduced into the inlet side of the capillary tube by simply replacing the inlet buffer reservoir with the sample solution and applying low pressure or voltage across the capillary until the desired volume of sample has been loaded onto the column. Capillaries are composed of fused silica with internal diameters that commonly range from 10 to 100 µm. Column length varies from a few centimeters to 100 cm. High electric fields (5–30kV/cm) can be used as the narrow columns dissipate heat very effectively, allowing for short run times of 10–30 min.

Protein bands are not visualized by staining as in conventional electrophoresis. Instead, protein peaks are detected on the column as they migrate past a detector, similar to those used in liquid chromatography. Ultraviolet (UV)-visible detectors are most common, although fluorescence and conductivity detectors are available. The data obtained from a capillary electrophoresis run look like a typical chromatogram from a high-performance liquid chromatograph or gas chromatograph (see Chaps. 13 and 14). Proteins can be labeled with a fluorescent derivative to increase sensitivity when a fluorescent detector is used.

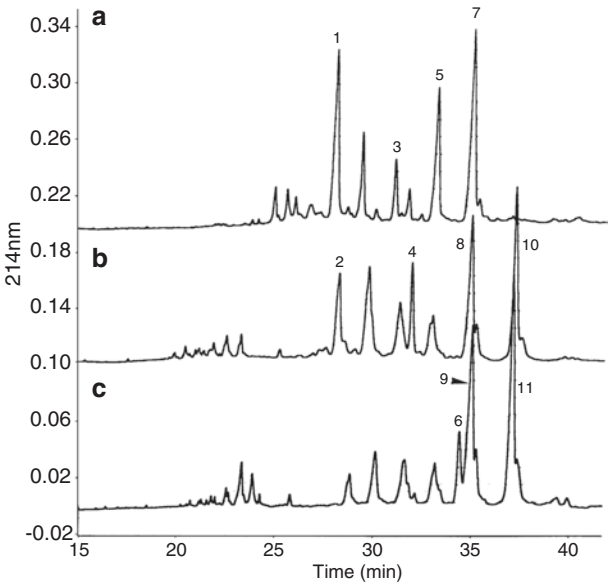
There are three variations of capillary electrophoresis commonly used for protein separations. **Capillary zone electrophoresis** or **free-solution electrophoresis** is the most commonly used form of capillary electrophoresis. It is much like native PAGE, except proteins are separated in free solution inside capillary tubes filled with buffer of the desired pH. Diffusion is prevented within the narrow diameter of the capillaries eliminating the need for a gel matrix. In capillary zone electrophoresis, electroosmotic flow also influences the separation of proteins within capillary tubes. The negatively charged fused silica capillary wall [containing silanol groups (SiO^-)] attracts positively charged ions (cations) from the buffer to form a double-ion layer at the interface between the capillary column wall and the buffer. When the electric field is applied, the cations forming the double layer are attracted toward the cathode and "pull" other molecules (independent of charge) in the same direction. Thus, in free-solution capillary electrophoresis, cations, anions, and uncharged molecules can be separated in a single run. Electroosmotic flow can be controlled by changing the pH or ionic strength of the buffer to alter the charge on the capillary wall and change the rate of protein migration.

SDS capillary gel electrophoresis techniques can be used to separate proteins by size and to determine molecular mass. In this technique proteins are denatured and dissociated in the presence of SDS and a reducing agent, then fractionation occurs in polyacrylamide gel-filled capillaries of specific pore sizes. Alternatively, linear polymers, such as methyl cellulose, dextrans, or polyethylene glycol, are added to the buffer within the capillary in a technique called dynamic sieving capillary electrophoresis. These entangled polymers act like the pores of the polyacrylamide gel to slow migration of the larger proteins and allow separation by size.

Proteins also can be separated on the basis of their isoelectric points, in a technique called **capillary isoelectric focusing**. Ampholytes (described in Sect. 24.2.5.2.2) are used to form a pH gradient within the capillary tube. A gel matrix is not needed. In this technique, electroosmotic flow is minimized by coating the capillary walls with buffer additives to prevent undesirable effects caused by surface charge.

24.2.5.3.3 Applications

Capillary electrophoresis is used primarily in analytical labs, although the use for routine quality control purposes is increasing. Capillary zone electrophoresis is used for a variety of applications, including the fractionation of milk, cereal, soybean, and muscle proteins [10]. Figure 24.7 illustrates the results obtained by capillary zone electrophoresis when used to fractionate the casein protein fraction of three mammalian milks. Microchip electrophoresis, a miniaturized version of capillary electrophoresis using small volume microchannels, has been developed recently [12]. Electrophoretic separations on microchips can be completed in only a few minutes. This technique has been used to identify 15 different wheat varieties based on variations in the subunit composition of a major wheat protein, glutenin [13]. Results were comparable to those obtained using SDS-PAGE, but with an assay time of less than one minute per analysis.



24.7 figure

Electropherograms illustrating differences in casein proteins isolated from (**a**) cow, (**b**) ewe, and (**c**) goat milks when separated by capillary zone electrophoresis. The peaks were identified as (*1*) bovine αS1-casein; (*2*) ovine αS1-casein; (*3*) bovine κ-casein; (*4*) ovine κ-casein; (*5*) bovine β-casein A1; (*6*) caprine κ-casein; (*7*) bovine β-casein A2; (*8*) ovine β2-casein; (*9*) caprine β2-casein; (*10*) ovine β1-casein; and (*11*) caprine β1-casein. Peaks eluting from the capillary column were detected by UV absorption (Reprinted from Molina et al. [11] with permission)

24.3 PROTEIN CHARACTERIZATION PROCEDURES

24.3.1 Amino Acid Analysis

24.3.1.1 *Principle*

Amino acid analysis is used to quantitatively determine the amino acid composition of a relatively pure protein. Amino acid analysis is divided into three steps. First, the protein sample is hydrolyzed to release the amino acids. Amino acids are then separated using chromatographic techniques. Finally, the separated amino acids are detected and quantified. To date, there are no published official standard methods for amino acid analysis [14] in the United States, although some AOAC methods [15] are available describing protein hydrolysis procedures in specific foods. Many new methods are currently under development. Ion-exchange chromatography and reversed-phase liquid chromatography with pre- or post-column derivatization for detection are in widespread use and will be described in this section.

24.3.1.2 *Procedures*

In the most commonly used procedure, a protein sample is **hydrolyzed** in constant boiling 6 *N* HC1 at 110 °C for 24 h to release amino acids prior to chromatography. Accurate quantification of some amino acids is difficult because they may be destroyed or converted into other reaction products during hydrolysis. Consequently, special hydrolysis procedures must be used to prevent errors. Research is ongoing to shorten the hydrolysis time, automate the process, and optimize the recovery of all amino acids after hydrolysis.

Tryptophan is completely destroyed by acid hydrolysis. Methionine, cysteine, threonine, and serine are progressively destroyed during hydrolysis; thus, the duration of hydrolysis will influence results. Asparagine and glutamine are quantitatively converted to aspartic and glutamic acid, respectively, and cannot be measured. Isoleucine and valine are hydrolyzed more slowly in 6 *N* HCl than other amino acids, while tyrosine may be oxidized.

In general, losses of threonine and serine can be estimated by hydrolysis of samples for three periods of time (i.e., 24, 48, and 72 h) followed by amino acid analysis. Compensation for amino acid destruction may be made by calculation to zero time assuming first-order kinetics. Valine and isoleucine are often estimated from a 72 h hydrolysate. Cysteine and cystine can be converted to the more stable compound, cysteic acid, by hydrolysis in performic acid and then hydrolyzed in 6 *M* HCl and chromatographed. Tryptophan can be separated chromatographically after a basic hydrolysis or analyzed using a method other than amino acid analysis.

In the original methods developed in the 1950s, amino acids were separated by **cation-exchange chromatography** using a stepwise elution with three buffers of increasing **pH** and **ionic strength**. Variations of this method are still commonly used today and may include the use of gradient elution protocols. In a procedure called **post-column derivatization**, amino acids eluting from the column are derivatized and quantified by reaction with **ninhydrin** (reacts with primary amino group of amino acids) to produce a colored product that was measured spectrophotometrically. The method was automated in the late 1970s and adapted for use with high-performance liquid chromatographs in the 1980s as new ion-exchange resins were developed that could withstand high pressures. Amino acids eluting from the column also may also be derivatized with ***o*-phthalaldehyde** (OPA) (reacts with the primary amino group of amino acids), then measured with a fluorescence detector. (Note: The ninhydrin and OPA methods can be used not only for amino acid analysis but also to monitor **hydrolysis of proteins** and to assay for **protease activity**, since these result in an increase of free primary amino groups.)

Other methods were developed in the 1980s using **pre-column derivatization** of the amino acids followed by reversed-phase HPLC. The hydrolyzed amino acids are derivatized prior to chromatography with **phenyl isothiocyanate** (reacts with primary amino group of amino acids), OPA, 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC), or other compounds, separated by reversed-phase HPLC, and quantified by UV or fluorescence spectroscopy. Methods using pre-column derivatizations may be more sensitive and can detect about 0.5–1.5 uM quantities of amino acids. Chromatographic runs usually take 30 min or less.

The quantity of each amino acid in a peak is determined by spiking the sample with a known quantity of internal standard. The **internal standard** is often an amino acid, such as norleucine, not commonly found in a food product. Results are usually expressed as mole percent. This quantity is calculated by dividing the mass of each amino acid (determined from the chromatogram) by its molecular mass, summing the values for all amino acids, dividing each by the total moles, and multiplying the result by 100.

Many new methods of amino acid analysis are currently under development, including procedures using liquid chromatography coupled with mass spectrometry [14], but these are not yet in widespread use.

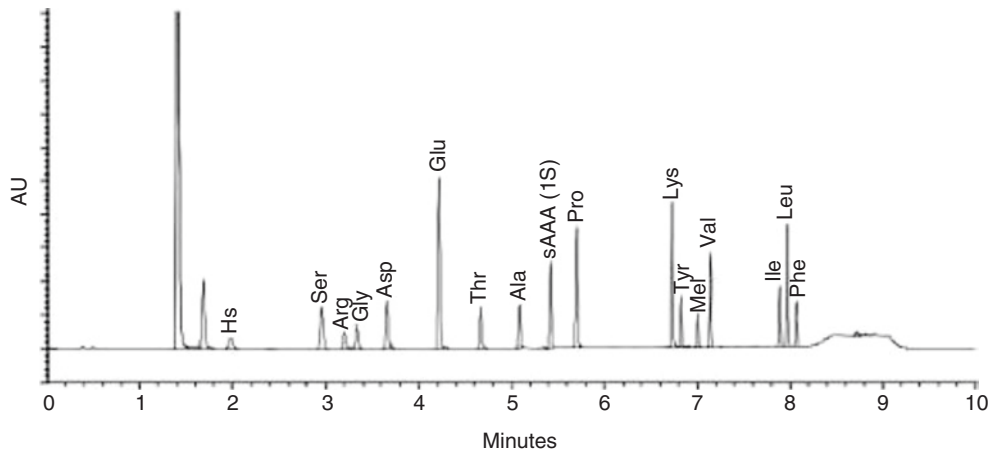
24.3.1.3 *Applications*

Amino acid analysis is used to determine the amino acid composition of a protein, determine quantities of essential amino acids to evaluate protein quality, identify proteins based on the amino acid profile, detect uncommon amino acids, and corroborate synthetic or recombinant protein structures. Amino acid analysis provides information for estimating the molecular mass of a protein. Amino acid analysis is also used to meet FDA nutritional labeling regulations for protein. In addition, the Food and Agriculture Organization (FAO) of the United Nations [16] recommends that amino acid analysis be used in place of the Kjeldahl method (N determination) for total protein, although they note that amino acid analysis requires the use of more sophisticated instrumentation and better trained technicians that are not always readily available in some countries. Proteins used in animal diets, infant formulas, sports nutrition products, and therapeutic human diets are often analyzed for protein quality to ensure adequate quantities of essential amino acids. A chromatogram illustrating the separation of amino acids from a casein hydrolysate on a reversed-phase column in under 10 min is shown in Fig. 24.8. The amino acids were derivatized prior to separation (pre-column) using AQC and fractionated by UPLC using an internal standard such as alpha-aminobutyric acid [17].

24.3.2 Protein Nutritional Quality

24.3.2.1 *Introduction*

The nutritional quality of a protein is determined by the **amino acid composition** and the **digestibility** of that protein. **Antinutritional factors** can affect the nutritional quality of a protein. However, foods that contain heat-labile antinutritional factors (e.g., trypsin inhibitors) are usually cooked prior to consumption, thereby inactivating the inhibitor that might reduce protein digestibility. Some foods contain heat-stable antinutritional factors (e.g., tannins) that can decrease the nutritive value of a protein.



24.8 figure

High-performance liquid chromatographic analysis of amino acids from a casein hydrolysate using pre-column derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) and separated on a reversed-phase column using an Acquity UPLC system (Waters Corp.) with a 2,996 photodiode array detection system at 260 nm. A BEH C18 column (100 × 2.1 mm i.d., 1.7 um) at a flow rate of 0.7 mL/min and column temperature of 55 °C was used. The injection volume was 1 uL. Gradient elution was performed using eluent (**a**) AccQ·Tag$_{ultra}$ eluent A concentrate (5%, v/v) and water (95%, v/v) and eluent (**b**) AccQ·Tag$_{ultra}$ (Adapted from Boogers et al. [17] with permission)

Many protein quality assessment methods utilize information about the essential amino acid content of a food. **Essential amino acids** are those that cannot be synthesized in the body and must be present in the diet. Although there are some special cases due to age and medical status of an individual, the amino acids generally categorized as essential (or indispensable) include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. Requirements for these amino acids have been determined for various age groups of humans (Table 24.4). The **first-limiting amino acid** of a human food is defined as the essential amino acid present in the lowest amount compared to a reference protein or to human requirements.

A food scientist's concerns regarding protein nutritional quality include meeting the requirements of nutrition labeling, formulating products of high protein quality, and testing the effects of food processing on protein digestibility. The development of valid methods to measure protein quality of foods for humans has been the focus of extensive research efforts over the past 50–60 years [19]. Protein nutritional quality assays may utilize animals in biological tests (*in vivo* assays), chemical or biochemical assays (*in vitro* assays), and/or simply calculations. Because of the time and expense of *in vivo* methods, *in vitro* assays and calculations based on amino acid content often are used to estimate protein quality. This section

24.4 table Amino acid requirements of infants, preschool children, adolescents, and adults (males and females combined)

		His	*Ile*	*Leu*	*Lys*	*SAA*	*AAA*	*Thr*	*Trp*	*Val*
Age (years)		Amino acid requirements (mg/kg per day)								
0.5	(Infants)	22	36	73	64	31	59	34	9.5	49
1–2	(Preschool children)	15	27	54	45	22	40	23	6.4	36
11–14	(Adolescent)	12	22	44	35	17	30	18	4.8	29
>18	(Adult)	10	20	39	30	15	25	15	4.0	26
		Scoring pattern (mg/g protein requirement)								
0.5	(Infants)	20	32	66	57	28	52	31	8.5	43
1–2	(Preschool children)	18	31	63	52	26	46	27	7.4	42
11–14	(Adolescent)	16	30	60	48	23	41	25	6.5	40
>18	(Adult)	15	30	59	45	22	38	23	6.0	39

His histidine, *Ile* isoleucine, *Leu* leucine, *Lys* lysine, *SAA* sulfur amino acids, *AAA* aromatic amino acids, *Thr* threonine, *Trp* tryptophan, *Val* valine
Adapted from Food and Agriculture Organization [18]

24.5 table Summary of methods used to measure protein nutritional quality

Method	*What is measured*	*Application*
Protein digestibility-corrected amino acid score (PDCAAS)	Amino acid content of first-limiting amino acid compared to requirements of preschool age children and true digestibility based on rat feeding experiment	Nutrition labeling for all but infant foods (to express g protein as % Daily Value)
Protein efficiency ratio (PER)	Weight gain of rats per g protein consumed	Nutrition labeling for infant foods (to express g protein as % Daily Value)
pH shift in vitro digestibility	Change in pH due to enzymatic digestion of protein under standard conditions	Rapid test of protein digestibility
DNFB (1-fluoro-2,3-dinitrobenzene) method of available lysine	Amount of lysine that has not already reacted with other food constituents and thus becomes unavailable as an essential amino acid	Determine effect of heat treatment during food processing on available lysine
Amino acid score (AAS)	Amino acid content of first-limiting amino acid, compared to requirements of preschool age children	Part of PDCAAS assay
Essential amino acid index (EAAI)	Amino acid content of each of nine essential amino acids, compared to their content in a reference protein	Rapid calculation to determine optimum amounts of various proteins in food formulation

of the chapter covers the tests and calculations required for nutrition labeling and mentions briefly several other protein quality methods for specialized applications. Please refer to Table 24.5 which summarizes methods for measuring protein nutritional quality.

24.3.2.2 *Protein Digestibility-Corrected Amino Acid Score*

24.3.2.2.1 Principle

The **protein digestibility-corrected amino acid score** (PDCAAS method) is used to estimate protein nutritional quality by combining information from (1) a calculation that compares the amount of the first-limiting amino acid in a protein to the amount of that amino acid in a reference protein, and (2) an *in vivo* assay measuring the digestibility of the protein by rats. For nutrition labeling, the PDCAAS must be determined by methods described in 21 CFR 101.9 [20].

24.3.2.2.2 Procedure

1. Determine the amino acid composition of the food.
2. Calculate the **amino acid score** for the first-limiting amino acid, using the requirements of preschool age children as a reference pattern.

$$\text{Amino acid score} = \frac{\text{mg of amino acid in 1 g of test protein}}{\text{mg of amino acid in 1 g of reference protein}} \quad (24.3)$$

3. Feed male weanling rats standardized diets with 10 % test protein or with no protein, following the procedure for true protein digestibility (AOAC Method 991.29) [15]. **True digestibility** is calculated based on nitrogen ingested and feed intake, corrected for metabolic losses in the feces. If available, published values of true digestibility for the test protein can be used.
4. Calculate PDCAAS:

$$\text{Amino acid score} \times \%\ \text{True digestibility} \quad (24.4)$$

5. For nutritional labeling: (50 g = Daily Value for protein)

$$\%\ \text{Daily Value} = \frac{100 \times (\text{g protein/serving} \times \text{PDCAAS value})}{50\,\text{g protein}} \quad (24.5)$$

24.3.2.2.3 Applications

The Nutrition Labeling and Education Act (NLEA) requires that the percent Daily Value used on nutrition labels must be determined using the PDCAAS method, except for foods intended for consumption by infants (see also Chap. 3, Sect. 3.2.1.7). Because of the time and cost associated with the PDCAAS method, protein on the nutrition label is often expressed only as amount and not as a percent of the Daily Value. However, if a food label includes any claim regarding the protein, the nutrition label must include protein expressed as a percent of the Daily Value [20] [21CFR 101.9 (c) (7)].

The PDCAAS method generally is thought to better estimate protein quality for humans than the **protein efficiency ratio** (PER) method, which measures rat growth [19]. Rat growth is not comparable to that of adult humans, but it is more comparable to that of human infants. Therefore, the PER method is used to estimate protein quality of only infant foods. The actual digestion of protein by rats is thought to be fairly comparable to that by humans, so the protein digestibility portion of the PDCAAS method utilizes true digestibility as determined with rats. The PDCAAS method includes information on both amino acid composition and protein digestibility, since these are the factors that determine protein nutritional quality. However, perhaps as a limitation to the PDCAAS method, the amino acid score portion of the PDCAAS method includes only information about the first-limiting amino acid and not other essential amino acids. There is no differentiation in amino acid score between two proteins limiting to the same extent in one amino acid, but with the one protein only limiting in that amino acid and another protein limiting in many amino acids.

The continued use of the PDCAAS method is currently under debate by the Food and Agriculture Organization (FAO) of the United Nations [18]. A new protein quality measure, the **digestible indispensable amino acid score** (DIAAS), has been recommended to replace the PDCAAS as the method of choice for dietary protein quality assessment for regulatory purposes. DIAAS is defined as:

$$\text{DIAAS}\,(\%) = 100 \times \frac{(\text{mg of digestible dietary indispensable amino acid in 1g of the dietary protein})}{(\text{mg of the same dietary indispensable amino acid in 1g of the reference protein})}$$

This approach treats each essential amino acid as an individual nutrient, instead of only using the first-limiting amino acid as done in the PDCAAS method.

FAO is also recommending standardization of amino acid analysis methodologies. The DIAAS method is not yet approved by the FDA for nutritional labeling in the United States.

24.3.2.3 *Protein Efficiency Ratio*

24.3.2.3.1 Principle

The PER method (AOAC Method 960.48) [15] estimates protein nutritional quality in an *in vivo* assay by measuring rat growth as weight gain per gram of protein fed.

24.3.2.3.2 Procedure

1. Determine the nitrogen content of the test protein-containing sample and calculate the protein content.
2. Formulate a standardized test protein diet and a casein control diet to each contain 10% protein.
3. Feed groups of male weanling rats the diet and water ad libitum for 28 days.
4. Record the weight of each animal at the beginning of the assay, at least every 7 days during the assay, and at the end of the 28 days.
5. Record the food intake of each animal during the 28-day feeding trial.
6. Calculate the PER using the average total weight gain and average total protein intake for each diet group at day 28:

$$\text{PER} = \text{Total weight gain of test group (g)} / \text{Total protein consumed (g)} \quad (24.6)$$

7. Normalize the PER value for the test protein (i.e., compare the quality of the test protein to that of casein) by assigning casein a PER of 2.5.

$$\text{Adjusted or corrected PER} = \text{PER of test protein} / \text{PER of casein control} \quad (24.7)$$

24.3.2.3.3 Application

The Food and Drug Administration (FDA) requires that the PER method be used when determining protein as a percent of the Daily Value on the nutrition label of foods intended for consumption by infants [20] (21 CFR 101) (see also Chap. 3, Sect. 3.2.1.7). The PER method is limited to this application because the essential amino acid requirements for young rats are similar to those of human infants, but not those of other age groups. The PER method is time consuming, and it does not give any value to a protein for its ability to simply maintain body weight (i.e., a protein that produces no weight gain in the assay has a PER of zero).

24.3.2.4 *Other Protein Nutritional Quality Tests*

24.3.2.4.1 Essential Amino Acid Index

Essential amino acid index (EAAI) estimates protein nutritional quality based on the content of all essential amino acids compared to a reference protein (or human requirements). The EAAI is a rapid method to evaluate and optimize the amino acid content of food formulations. Unlike the amino acid score component of the PDCAAS method that considers only the first-limiting amino acid, the EAAI method accounts for all essential amino acids. However, EAAI does not include any estimate of protein digestibility, which could be affected by processing method. The essential amino acid content of the test protein (determined by amino acid analysis or from literature values) is compared to that of a reference protein (e.g., casein or human requirements) as follows: (Note: Use values for methionine plus cystine, and phenylalanine plus tyrosine, because they can substitute for one another as essential amino acids.)

$$\text{Essential amino acid index} = \sqrt[9]{\left(\frac{\text{mg of lysine in 1g of test protein}}{\text{mg of lysine in 1g reference protein}}\right) \times (\text{etc. for other 8 essential amino acids})} \quad (24.8)$$

24.3.2.4.2 In Vitro Protein Digestibility

The **pH shift method** is an *in vitro* protein digestibility assay used to estimate the digestibility of a protein by measuring the extent of protein hydrolysis upon reaction under standardized conditions with commercial digestive enzymes. The digestion procedure is designed to simulate human digestion of protein by using the enzymes trypsin, chymotrypsin, peptidase, and a bacterial protease. The pH of the protein solution drops when proteases break peptide bonds, release carboxyl groups, and liberate hydrogen ions. The pH at the end of the digestion period is used to calculate protein digestibility.

The pH shift method is the enzyme digestibility part of the **protein efficiency ratio-calculation method** (C-PER) (AOAC Method 982.30) [15], which combines a calculation of the essential amino acid composition with a calculation based on the *in vitro* assay of digestibility. The C-PER assay is intended for routine quality control screening of foods and food ingredients, to estimate the PER as it would be determined with the rat bioassay method.

The *in vitro* digestibility assay can provide a rapid and inexpensive means to determine and compare the protein digestibility of food products. This assay could

be used to determine the effect of processing conditions on the protein digestibility of food products with the same formulation.

24.3.2.5 *Lysine Availability*

If the nutritional quality of a heat-processed product is lower than expected from its amino acid composition, one should assess the availability of lysine in the product. The **free ε-amino group** of the essential amino acid lysine can react with many food constituents during processing and storage to form biologically unavailable complexes with reduced nutritional quality. Lysine can readily complex with reducing sugars in the Maillard browning reaction, oxidized polyphenols, and oxidized lipids. Such reactions are accelerated upon heating and under alkaline conditions. **Lysinoalanine**, often found in alkali-treated proteins, decreases both the digestibility of the protein and the availability of lysine as an essential amino acid. The most commonly used method to measure **available lysine** is a spectrophotometric method that utilizes the reagent 1-fluoro-2,3-dinitrobenzene (DNFB, also referred to as FDNB). The DNFB method, detailed in AOAC Method 975.44 [15], can be used to determine if food processing operations have reduced the availability of lysine in a food.

24.3.3 Assessment of Protein Functional Properties

Protein functionality has been defined as the physical and chemical properties of protein molecules that affect their behavior in food products during processing, storage, and consumption. The functional properties of proteins contribute to the quality attributes, organoleptic properties, and processing yields of food. It is often desirable to characterize the functional properties of food proteins to optimize their use in a food product. Three of the most important protein functional properties in foods include **solubility**, **emulsification**, and **foaming**. This chapter will highlight only a few of the many methods available to measure these three functional properties. Two other functional properties of proteins, **gelation** and **dough formation**, are closely related to viscosity. It should be pointed out that there is no single functional property test that is applicable to all food systems, so careful test selection is imperative.

24.3.3.1 *Solubility*

24.3.3.1.1 Principle

One of the most popular tests of protein functionality is solubility. Proteins usually need to be soluble under the conditions of use for optimal functionality in food systems. Many other important functional attributes of proteins are influenced by protein solubility, such as thickening (viscosity effects), foaming, emulsification, water binding, and gelation properties.

Solubility is dependent on the balance of hydrophobic and hydrophilic amino acids that make up the protein, especially those amino acids on the surface of the molecule. Protein solubility also is dependent on the thermodynamic interactions between the protein and the solvent. Protein solubility is influenced by solvent polarity, pH, ionic strength, ion composition, and interactions with other food components, such as lipids or carbohydrates. Common food processing operations, such as heating, freezing, drying, and shearing, may all influence the solubility of proteins in a food system. Proteolysis by endogenous proteases also may alter protein solubility.

24.3.3.1.2 Procedures

There are many standardized methods to measure protein solubility including those published by the American Oil Chemists' Society [21] and the AACC International [22]. Other names synonymous with protein solubility include protein dispersibility index and nitrogen solubility index.

In a typical solubility procedure, a protein is dispersed in water or buffer at a specified pH, and the dispersion is centrifuged using defined conditions. Protein insoluble under the test conditions precipitates while soluble protein remains in the supernatant. Buffer and test conditions must be carefully controlled as these parameters have a large influence on results. Total protein and protein in the supernatant are measured, usually by the Kjeldahl method or a colorimetric procedure, such as the Bradford assay or the bicinchoninic acid (BCA) assay (Chap. 18, Sects. 18.4.1.2 and 18.4.2.3). Percentage protein solubility is measured by dividing the protein in the supernatant (soluble protein) by the total protein and multiplying by 100.

24.3.3.2 *Emulsification*

24.3.3.2.1 Principle

Food **emulsions** include margarine, butter, milk, cream, infant formulas, mayonnaise, processed cheese, salad dressings, ice cream, and some highly comminuted meat products, such as bologna. Emulsions are mixtures of two or more immiscible liquids, one of which is dispersed as droplets in the other. Oil and water are the two most common immiscible liquids found in food emulsions, although many other food components are usually present. The droplets are collectively called the **discontinuous** or **dispersed phase**, whereas the liquid surrounding the droplets is the **continuous phase**. Energy, in the form of homogenization, blending, or shaking, is used to disperse one immiscible liquid into the other.

Proteins are used as emulsifiers to lower the interfacial energy between phases to facilitate emulsion formation and to improve the stability of emulsions. Proteins migrate to the surface of a droplet during emulsion formation to form a protective layer or membrane on the surface, thus reducing interactions between the two immiscible phases. Emulsions are inherently unstable. The quality of an emulsion is dictated by many factors including droplet size, droplet size distribution, density differences between the two phases, viscosity of the two phases, electrostatic and steric interactions between molecules at the interface, and thickness and viscosity of the adsorbed protein layer. Additional information can be found in textbooks devoted to the topic of emulsification [23].

24.3.3.2.2 Procedures

Food emulsions usually are highly complex systems with multiple ingredients, so industrial scientists often choose to investigate the properties of emulsions in simplified model systems containing only a few of the most important ingredients. For a protein-based emulsion, this model system may contain only water or buffer, oil, and protein. It must be remembered that pH, salt concentration, temperature, type and amount of oil, protein concentration, energy input, and temperature during emulsion formation have a large effect on the properties of the final emulsion. These parameters must be selected prior to establishing a procedure.

The droplet size of the dispersed phase in an emulsion has a large influence on the emulsion quality. Droplet size can influence appearance, stability, and rheological properties of an emulsion, and hence the quality of a food product. Smaller droplets of more uniform size indicate a better emulsion. Droplet size can be determined by turbidimetric techniques, microscopy, laser diffraction, and electrical pulse counting [23].

An efficient emulsifier can prevent the breakdown or phase separation of an emulsion during storage. Emulsions can be stable for a long period of time (months to years), so often test protocols include a destabilization step involving physical or chemical stress. **Emulsion stability** can be tested by centrifugation or agitation of an emulsion at a given speed and time to determine the amount of creaming or oil separation that occurs. This is a fairly rapid test, but may not adequately represent the breakdown of the emulsion during normal storage conditions. Another method involves measuring the change in particle size distribution of the dispersed phase over time [24] and can be performed by laser diffraction or using the LUMiSizer®.

Many other more sophisticated techniques are available to measure the properties of food emulsions and include measures of interfacial properties, measurement of the dispersed phase volume fraction, characterization of emulsion rheology, and investigations into droplet charge [23].

24.3.3.3 *Foaming*

24.3.3.3.1 Principle

Foams are coarse dispersions of gas bubbles in a liquid or semisolid continuous phase. Like emulsions, foams require energy input during formation and are inherently unstable. Whipping, shaking, and sparging (gas injection) are three common methods of foam formation. Proteins or other large macromolecules in the continuous phase lower the surface tension between the two phases during foam formation and impart stability to films formed around the gas bubbles. Foams are found in cakes, breads, marshmallows, whipped cream, meringues, ice cream, soufflés, mousses, and beer.

24.3.3.3.2 Procedures

Foam volume and foam stability are two important parameters used to evaluate foams. **Foam volume** is dependent on the ability of a protein to lower surface tension between the aqueous phase and gas bubbles during foam formation. The volume of foam generated during a standardized foaming process is recorded and can be compared to other foams made under identical conditions. Often the foam is formed in a blender and then transferred into a graduated cylinder for measurement. Another common approach is to measure foam overrun or foam expansion (i.e., important in ice cream making). This is an indirect measure of the amount of air incorporated into the foam. Percent **overrun** can be calculated as follows:

$$\text{Overrun (\%)} = \frac{(\text{wt. 100 mL liquid} - \text{wt. 100 mL foam})}{(\text{wt. 100 mL foam})} \times 100 \tag{24.9}$$

Foam stability depends on the properties of the protein film formed around the gas droplets. Free liquid is released as a foam breaks down. A more stable foam usually takes a longer time to collapse. Foam stability is often expressed as a half-life. A sample is whipped for a fixed time under standardized conditions. In one of the simplest methods, the foam is placed in a funnel over a graduated cylinder. The time for half of the original weight or volume of the foam to drain away from the foam is recorded. The greater the half-life, the more stable the foam.

Foam volume and stability are influenced by energy input, pH, temperature, heat treatment, and

the type and concentration of ions, sugars, lipids, and proteins in the foam. Hence, all of these variables, except the one under test, must be standardized when designing a procedure to measure foam properties. Other methods can be used to better understand the molecular properties of the foam including: (1) determination of interfacial properties such as surface pressure and film thickness, (2) characterization of the viscoelastic properties of the film, and (3) characterization of the bubble size and distribution.

24.3.3.4 *Applications of Testing for Solubility, Emulsification, and Foaming*

Proteins perform a variety of functions in food systems. The quality of many foods is dependent on the successful manipulation of protein functional properties during processing. The solubility of a protein under a given set of conditions must be known for optimizing the use of that protein in a food product. For example, proteins must be soluble for optimal function in most beverages. Slight formulation modifications may change product pH and subsequently protein solubility.

Many proteins become insoluble and less functional once they are denatured. Thus, solubility often is used as an index of protein denaturation that may occur during processing. In general, proteins must be soluble to migrate to surfaces during foaming and emulsification. Any change in a process or ingredient that affects solubility also may alter the foaming and emulsification properties of that protein. Freezing, heating, shearing, and other processes can influence the functional properties of proteins in food systems. Thus, it may be necessary to reevaluate solubility, emulsion, and foaming properties of proteins if any processing changes are made.

Food product developers often need to understand the effect of ingredient substitutions on emulsion or foaming properties and subsequent product quality. Variations in raw materials can lead to differences in protein functionality and ultimately product quality. Protein functional tests can be used to compare two ingredients from different manufacturers or to verify the quality of protein ingredients in each batch purchased from a manufacturer. For example, the solubility of different commercial whey and soy protein concentrates may vary, leading to differences in other functional responses and, ultimately, product quality. Also, if a product developer is trying to substitute soy protein for egg protein in a formulation, it might be important to know how the functional attributes of each ingredient are affected by environmental conditions, such as pH or salt concentration. It often is desirable to know how long an emulsion or foam will be stable under a certain set of storage conditions. For example, emulsion stability tests are often used to evaluate the long-term stability of infant formulas and beverages.

24.3.3.5 *Gelation and Dough Formation*

Two other protein functional properties, gelation and dough formation, are closely related to viscosity and are described here only briefly.

24.3.3.5.1 Gelation

Protein gels are made by treating a protein solution with heat, enzymes, or divalent cations under appropriate conditions. While most food **protein gels** are made by heating protein solutions, some can be made by limited enzymatic proteolysis (e.g., chymosin action on casein micelle to form cheese curd), and some are made by addition of the divalent cations Ca^{2+} or Mg^{2+} (e.g., tofu from soy proteins). Proteins are transformed from the "soluble" state to a cross-linked "gel-like" state in protein gelation. The continuous, cross-linked network structure formed may involve covalent (i.e., disulfide bond) and/or noncovalent interactions (i.e., hydrogen bonds, hydrophobic interactions, electrostatic interactions). Some protein gel networks made by heating a protein solution are thermally reversible (e.g., gelatin gel that is formed by heating then cooling gelatin, but reverts to a liquid when reheated), while some protein gels are thermally irreversible. The stability of a protein gel is affected by a variety of factors, such as the nature and concentration of the protein, temperature, rates of heating and cooling, pH, ionic strength, and the presence of other food constituents. Therefore, these variables must be standardized and controlled to measure and compare gelation properties of proteins. Techniques used to measure rheological properties of foods such as compression, extension, and torsion analysis are applied to determine the properties of protein gels and are described in more detail in Chap. 29. In addition, many empirical tests have been developed to measure gel properties of specific proteins, such as the **Bloom test** designed to measure the strength of gelatin gels and the **folding test** to measure the elasticity of surimi gels.

24.3.3.5.2 Dough Formation

Wheat protein is unique in its ability to form a **viscoelastic dough** suitable for making bread and other bakery products. Gluten, the major storage protein of wheat, is a heterogeneous mixture of the proteins gliadins and glutenins. The unique amino acid composition of these proteins makes possible the formation of a viscoelastic dough, which can entrap carbon dioxide gas during yeast fermentation. Bread-making quality of wheat varieties is commonly tested by measuring

dough strength, viscosity, and extensibility. The effect of dough ingredients (e.g., whey or soy proteins, phospholipids, surfactants) can be tested in the same way.

Dough strength is measured under standardized conditions with a variety of commercially available instruments. The **Mixograph**® is used to test the mixing properties of flour to ensure the dough will have the proper consistency, which is essential for automated manufacture of baked products. A **Farinograph**® is a torque meter to determine the water absorption of flour and the mixing properties of dough. The Extensograph®, often used in conjunction with the Farinograph, measures the extensibility of dough and thus can predict baking behavior. The **Rapid Visco Analyser**® (RVA) is a cooking viscometer to rapidly test starch-pasting properties. The RVA incorporates heating, cooling, and variable shear, to test the viscosity of starches, cereals, and other foods. The **Alveograph**® is used to measure gluten strength and extensibility by measuring the force required to blow and break a bubble of dough. Other tests of wheat quality include the **Glutomatic**® which measures the wet gluten content of flour.

24.4 SUMMARY

There are a variety of techniques used to separate and characterize proteins. Separation techniques rely on the differences in the solubility, size, charge, and adsorption characteristics of protein molecules. Ion-exchange chromatography is used to separate proteins on the basis of charge. Affinity chromatography utilizes ligands, such as enzyme inhibitors, coenzymes, or antibodies, to specifically bind proteins to a solid support. Proteins can be separated by size using dialysis, ultrafiltration, and size-exclusion chromatography. Electrophoresis can be used to separate proteins from complex mixtures on the basis of size and charge. SDS-PAGE also can be used to determine the molecular mass and subunit composition of a protein. Isoelectric focusing can be used to determine the isoelectric point of a protein. Capillary electrophoresis is an adaptation of conventional electrophoresis in which proteins are separated in capillary tubes. Chromatographic techniques are used in amino acid analysis to determine the amino acid composition of a protein. The nutritional quality of a protein is determined by the amino acid composition and protein digestibility. The PDCAAS is thought to be a better method than the PER method. The functional properties of proteins are used to characterize a protein for a particular application in a food. Common tests of protein functionality include solubility, emulsification, foaming, and gelation. No single test is applicable to all food systems.

Abbreviations

$(NH_4)_2SO_4$	Ammonium sulfate
AOAC	Association of Official Analytical Chemists International
AQC	6-Aminoquinolyl-N-ydroxysuccinimidyl carbamate
C-PER	Protein efficiency ratio-calculation method
DIAAS	Digestible indispensable amino acid score
EAAI	Essential amino acid index
FAO	Food and Agriculture Organization
FPLC	Fast protein liquid chromatography
HPLC	High-performance liquid chromatography
NFDM	Nonfat dry milk
OPA	o-Phthalaldehyde
PAGE	Polyacrylamide gel electrophoresis
PDCAAS	Protein digestibility-corrected amino acid score
PER	Protein efficiency ratio
pI	Isoelectric point
R_m	Relative mobility
SDS	Sodium dodecyl sulfate
TEMED	Tetramethylethylenediamine
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet
V_e	Elution volume

24.5 STUDY QUESTIONS

1. You have a protein system with the following characteristics:

Protein	*Solubility in $(NH_4)_2SO_4$ (%)*	*Solubility in ethanol (%)*	*pI*	*Denaturation temperature (°C)*
1	10–20	5–10	4.6	80
2	70–80	10–20	6.4	40
3	60–75	10–20	4.6	40
4	50–70	5–10	6.4	70

   Describe how you would separate protein 4 from the others.
2. Compare and contrast the principles and procedures of SDS-PAGE vs. isoelectric focusing to separate proteins. Include in your explanation how and why it is possible to separate proteins by each method and what you can learn about the protein by running it on each type of system.
3. Explain how capillary electrophoresis differs from SDS-PAGE.
4. Briefly describe what each of the following tells you about the characteristics of the proteins of interest described in the statement (Note: The protein is not the same one in each statement):

(a) When subjected to dialysis using tubing with a molecular mass cutoff of 3,000 Da, a protein of interest is found in the retentate (i.e., not in the filtrate).
(b) When subjected to ultrafiltration using a membrane with a molecular mass cutoff of 10,000 Da, a protein of interest is found in the filtrate (i.e., not in the retentate).
(c) When the protein was subjected to ion-exchange chromatography using an anion-exchange column and a buffer of pH 8.0, a protein of interest is bound to the column.
(d) When a protein of interest was subjected to isoelectric focusing, the protein migrated to a position of approximately pH 7.2 in the pH gradient of the gel.
(e) When a protein of interest was subjected to SDS-PAGE in both the presence and absence of mercaptoethanol, the protein appeared as three bands at molecular mass 42,000, 45,000, and 48,000 Da.
(f) When a solution with various proteins was heated to 60 °C, the protein of interest was found in the precipitate obtained upon centrifugation of the solution.

5. You are submitting a soy protein sample to a testing laboratory with an amino acid analyzer (ion-exchange chromatography) so that you can obtain the amino acid composition. Explain how (a) the sample will be treated initially and (b) the amino acids will be quantified as they elute from the ion-exchange column. Describe the procedures. (Note: You want to quantify all the amino acids.)

   (a) How will samples be treated initially?
   (b) How will amino acids be quantified?

6. In amino acid analysis, a protein sample hydrolyzed to individual amino acids is applied to a cation-exchange column. The amino acids are eluted by gradually increasing the pH of the mobile phase.

   (a) Describe the principles of ion-exchange chromatography.
   (b) Differentiate anion vs. cation exchangers.
   (c) Explain why changing the pH allows different amino acids to elute from the column at different times.

7. Briefly describe the differences between the following assay procedures:

   (a) Amino acid score vs. essential amino acid index
   (b) PDCAAS vs. amino acid score

8. You are helping to develop a new process for making a high-protein snack food from cereal grains and soy. You want to determine the protein quality of the snack food under various processing (toasting and drying) conditions. Considering the number of samples to be tested, you cannot afford an expensive *in vivo* assay, and you cannot wait more than a few days to get the results.

   (a) What method would you use to compare the protein quality of the snack food made under different processing conditions? Include an explanation of the principles involved.
   (b) You suspect that certain time-temperature combinations lead to over-processed products. Your testing from (7a) shows that these samples have a lower nutritional quality. What amino acid(s) in the snack food would you suspect to be the most adversely affected by thermal abuse?
   (c) What test(s) could you use to confirm that amino acid(s) have become nutritionally unavailable by the over-processing? How are these tests conducted?

9. Define "protein functionality" and list three important functional properties of foods.
10. Describe a functional test used to measure:

    (a) Protein solubility
    (b) Emulsion stability
    (c) Foam volume

11. You need to reconstitute nonfat dry milk (NFDM) powder for use in a yogurt formula. You have experienced a large amount of variation in the hydration times required for different lots of NFDM. As the quality control manager, you need to develop a test to measure the solubility and hydration characteristics of each lot of NFDM before it is used (in hopes of avoiding future rehydration problems).

    (a) How would you measure solubility and what precautions would you take with the method you develop?
    (b) Assume that you did the following for the test you developed: (1) mix 20 g of NFDM with 200 g water at 60 °C, (2) blend for 2 min, (3) transfer 50 mL to a centrifuge tube, (4) centrifuge at 10,000 × g for 5 min, and (5) measure the protein content of the supernatant after centrifugation. If the supernatant contains 2.95% protein and the starting NFDM contained 36.4% protein, what is the percent protein solubility?

12. You work for a fluid milk manufacturer that sells milk used in steamed coffee products.

With this application, the milk needs to form large amounts of stable foam. Periodically, you have been receiving complaints that the milk does not produce adequate foam when steamed. As a result, you need to design a quality control method to measure foam characteristics of each lot of milk before it is shipped. Describe the test(s) you would perform.

24.6 PRACTICE PROBLEMS

1. Using the data provided in the table below:
   (a) Calculate the EAAI for defatted soy flour.
   (b) Determine the amino acid score for the soy flour.
   (c) Calculate the PDCAAS, using the true digestibility value of 87% for defatted soy flour.

Amino acid	*Soy[a] (mg/g protein)*	*Reference pattern[b] (mg/g protein)*
Histidine	26	18
Isoleucine	46	31
Leucine	78	63
Lysine	64	52
Methionine/ cystine	26	26
Phenylalanine/ tyrosine	88	46
Threonine	39	27
Tryptophan	14	7.4
Valine	46	42

2. You work for a manufacturer of protein supplements sold to body builders. You need to screen several proteins that may be used in a new protein supplement. You have three samples (A, B, and C) to evaluate. The amino acid profiles of these three samples and the reference profile (i.e., amino acid requirements of preschool age children) are shown below.

Amino acid	*Reference profile*	*Sample A*	*Sample B*	*Sample C*
Histidine	18	26	35	24
Isoleucine	31	50	55	35
Leucine	63	65	46	32
Lysine	52	80	92	80
Methionine/ cysteine	26	70	48	50
Phenylalanine/ tyrosine	46	70	90	85
Threonine	27	51	40	39
Tryptophan	7.4	16	22	25
Valine	42	60	64	42

   (a) Calculate the PDCAAS for each supplement. (Assume that the true digestibilities of Samples A, B, and C are 87%, 93%, and 64%, respectively.)
   (b) Which sample would you use if Sample A costs $1.25/lb, Sample B costs $3.25/lb, and Sample C costs $1.15/lb?

Answers

1. (a) Essential amino acid index

$$= \sqrt[9]{\frac{(26/18)(46/31)(78/63)(64/52)(26/26)}{(88/46)(39/27)(14/7.4)(46/42)}}$$

$$= \sqrt[9]{\frac{(1.44)(1.48)(1.24)(1.23)(1.00)(1.91)(1.44)}{(1.89)(1.10)}}$$

$$= \sqrt[9]{18.5866}$$

$$= 1.38$$

   (b) Amino acid score = 26/26 = 1.00; lowest ratio represents the limiting amino acid, methionine/cystine.
   (c) PDCAAS = amino acid score x true digestibility = 1.00 (0.87) = 0.87

2. (a) The first-limiting amino acid for all three samples is leucine. (Identify by determining the ratio of each amino acid compared to reference profile.)

PDCAAS = amino acid score for first-limiting amino acid x true digestibility:

$$\text{Sample A} = (65/63) \times .87 = .90$$
$$\text{Sample B} = (46/63) \times .93 = .68$$
$$\text{Sample C} = (32/63) \times .64 = .33$$

   (b) The cost-to-protein quality ratio for each sample is as follows:

$$\text{Sample A} = (\$1.25/.90) = \$1.39$$
$$\text{Sample B} = (\$3.25/.68) = \$4.78$$
$$\text{Sample C} = (\$1.15/.33) = \$3.48$$

Sample A provides the highest amount of usable protein per dollar, suggesting it would be best to use.

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