5 Amino Acids, Peptides, and Proteins

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CONTENTS

5.1 INTRODUCTION

Proteins play a central role in biological systems. Although the information for evolution and biological organization of cells is contained in DNA, enzymes exclusively perform the chemical and biochemical processes that sustain the life of a cell/organism. Thousands of enzymes have been discovered. Each one of them catalyzes a highly specific biological reaction in cells. In addition to functioning as enzymes, proteins (such as collagen, keratin, elastin, etc.) also function as structural components of cells and complex organisms. The functional diversity of proteins essentially arises from their chemical make up.

Proteins are highly complex polymers, made up of 21 different amino acids. The constituents are linked via substituted amide bonds. Unlike the glycosidic and phosphodiester bonds in polysaccharides and nucleic acids, respectively, the amide linkage in proteins is a partial double bond, which further underscores the structural complexity of protein polymers. The myriad biological functions performed by proteins might not be possible but for the complexity in its composition, which gives rise to a multitude of three dimensional structural forms with different biological functions. To signify their biological importance, these macromolecules were named proteins, derived from the Greek word *proteois*, which means of the first kind.

At the elemental level, proteins contain on wt/wt basis 50–55% carbon, 6–7% hydrogen, 20–23% oxygen, 12–19% nitrogen, and 0.2–3.0% sulfur. Protein synthesis occurs in ribosomes. After synthesis, cytoplasmic enzymes modify some amino acid constituents. This changes the elemental composition of some proteins. Proteins that are not enzymatically modified in cells are called *homoproteins*, and those that are modified or complexed with nonprotein components are called *conjugated proteins* or *heteroproteins*. The nonprotein components are often referred to as *prosthetic groups*. Examples of conjugated proteins include *nucleoproteins* (ribosomes), *glycoproteins* (ovalbumin and κ-casein), *phosphoproteins* (α- and β-caseins, kinases, and phosphorylases), *lipoproteins* (proteins of egg yolk and several plasma proteins), and *metalloproteins* (hemoglobin, myoglobin and several enzymes). Glyco- and phosphoproteins contain covalently linked carbohydrate and phosphate groups, respectively, whereas the other conjugated proteins are noncovalent complexes containing nucleic acids, lipids, or metal ions. These complexes can be dissociated under appropriate conditions.

Proteins also can be classified according to their gross structural organization. Thus, *globular proteins* are those that exist in spherical or ellipsoidal shapes, resulting from folding of the polypeptide chain(s) on itself. On the other hand, *fibrous proteins* are rod-shaped molecules containing twisted linear polypeptide chains (e.g., tropomyosin, collagen, keratin, and elastin). Fibrous proteins also can be formed as a result of linear aggregation of small globular proteins, for example, actin and fibrin. A majority of enzymes are globular proteins, and fibrous proteins invariably function as *structural proteins*.

The various biological functions of proteins can be categorized as *enzyme catalysts*, *structural proteins*, *contractile proteins* (myosin, actin, and tubulin), *hormones* (insulin and growth hormone), *transfer proteins* (serum albumin, transferrin, and hemoglobin), *antibodies* (immunoglobulins), *storage proteins* (egg albumen and seed proteins), and protective proteins (toxins and allergens). Storage proteins are found mainly in eggs and plant seeds. These proteins act as sources of nitrogen and amino acids for germinating seeds and embryos. The protective proteins are a part of the defense mechanism for the survival of certain microorganisms and animals.

All proteins are essentially made up of the same primary 20 amino acids; however, some proteins do not contain all 20 amino acids. The differences in structure and function of these thousands of proteins arise from the sequence in which the amino acids are linked together via amide bonds. Literally, billions of proteins with unique properties can be synthesized by changing the amino acid sequence, the type and ratio of amino acids, and the chain length of polypeptides.

All biologically produced proteins can be used as *food proteins.* However, for practical purposes, *food proteins* may be defined as those that are easily digestible, nontoxic, nutritionally adequate,

functionally useable in food products, available in abundance, and sustainable agriculturally. Traditionally, milk, meats (including fish and poultry), eggs, cereals, legumes, and oilseeds have been the major sources of food proteins. These are mainly storage proteins in animal and plant tissues, which act as the nitrogen source for the growing embryo. However, because of the burgeoning world population, nontraditional sources of proteins for human nutrition need to be developed to meet the future demand. The suitability of such new protein sources for use in foods, however, depends on their cost and their ability to fulfill the normal role of protein ingredients in processed and domestically prepared foods.

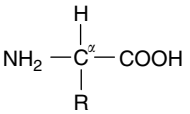
The functional properties of proteins in foods are related to their structural and other physicochemical characteristics. A fundamental understanding of the physical, chemical, nutritional, and functional properties of proteins and the changes these properties undergo during processing is essential if the performance of proteins in foods is to be improved, and if new or less costly sources of proteins are to compete with traditional food proteins.

5.2 PHYSICOCHEMICAL PROPERTIES OF AMINO ACIDS

5.2.1 General Properties

5.2.1.1 Structure and Classification

α-Amino acids are the basic structural units of proteins. These amino acids consist of a α-carbon atom covalently attached to a hydrogen atom, an amino group, a carboxyl group, and a side chain R group.



(5.1)

Natural proteins contain up to 21 different primary amino acids linked together via amide bonds. The 21st new amino acid, which has been recognized as a natural amino acid, is selenocysteine [12]. These amino acids differ only in the chemical nature of the side chain R group (Figure 5.1). The physicochemical properties, such as net charge, solubility, chemical reactivity, and hydrogen bonding potential, of the amino acids are dependent on the chemical nature of the R group.

The amino acids listed in Figure 5.1 have genetic codes, including selenocysteine. That is, each one of these amino acids has a specific *t*-RNA that translates the genetic information on *m*-RNA into an amino acid sequence during protein synthesis. Apart from the 21 primary amino acids listed in Figure 5.1, several proteins also contain other types of amino acids, which are derivatives of the primary amino acids. These *derived amino acids* are either cross-linked amino acids or simple derivatives of single amino acids. Proteins that contain derived amino acids are called *conjugated* proteins. Cystine, which is found in most proteins, is a good example of a cross-linked amino acid. Other cross-linked amino acids, such as desmosine, isodesmosine, and di- and trityrosine, are found in structural proteins such as elastin and resilin. Several simple derivatives of amino acids are found in several proteins. For example, 4-hydroxyproline and 5-hydroxylysine are found in collagen. These are the result of posttranslational modification during maturation of collagen fiber. Phosphoserine and phosphothreonine are found in several proteins, including caseins. *N*-Methyllysine is found in myosin, and γ-carboxy-glutamate is found in several blood clotting

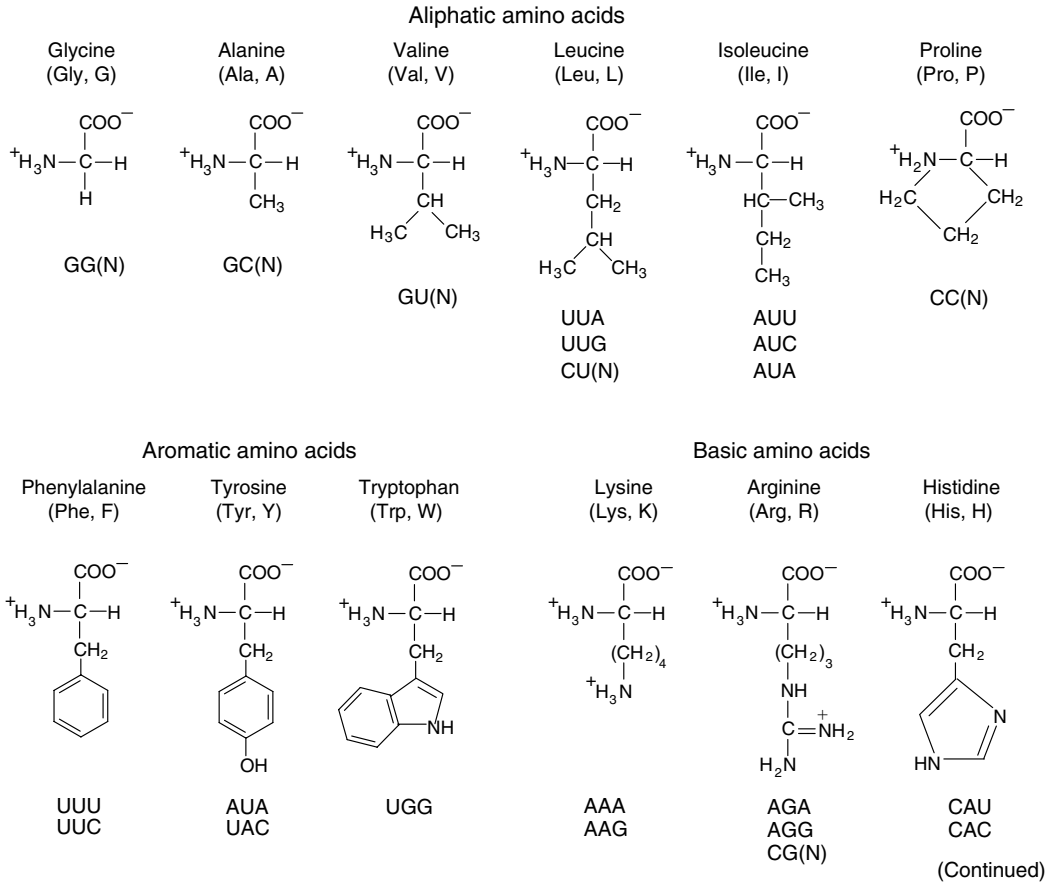
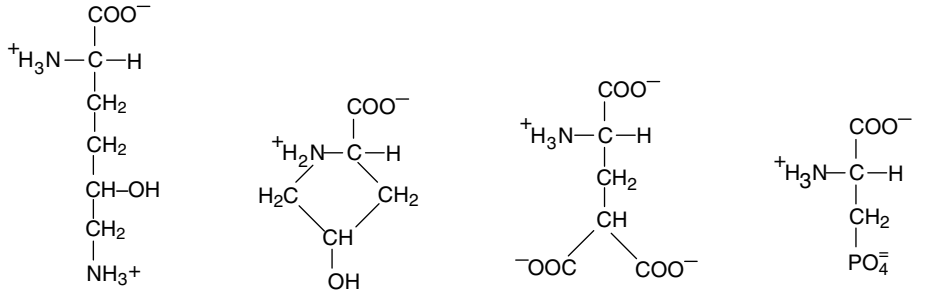


FIGURE 5.1 Primary α-amino acids that occur in proteins. The three letter and one letter codes of amino acids are shown in parenthesis. The mRNA codons for the amino acids are also shown for each amino acid.

factors and calcium binding proteins:



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5.2.1.2 Stereochemistry of Amino Acids

With the exception of Gly, the α-carbon atom of all amino acids is asymmetric, meaning that four different groups are attached to it. Because of this asymmetric center, amino acids exhibit optical

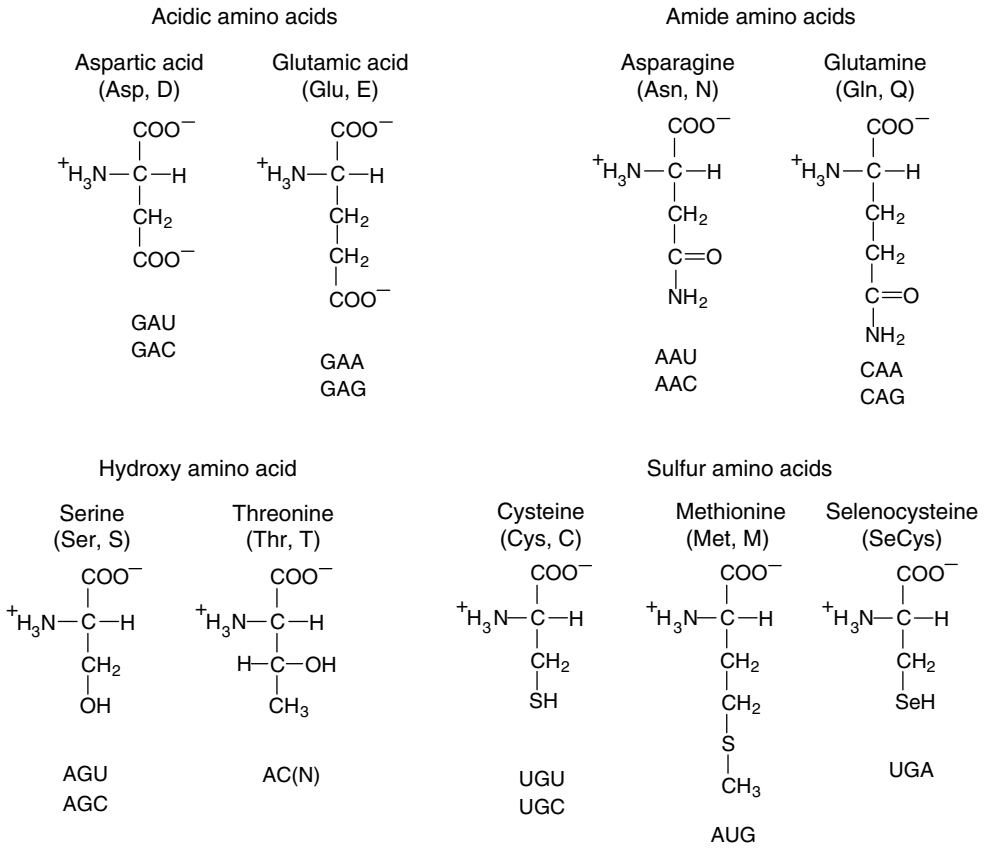
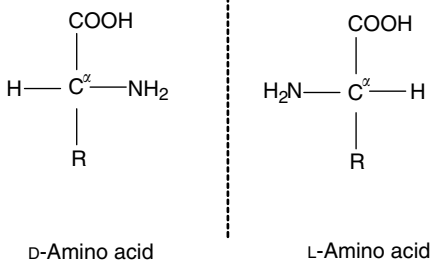


FIGURE 5.1 Continued.

activity, that is, they rotate the plane of linearly polarized light. In addition to the asymmetric α-carbon atom, the β-carbon atoms of Ile and Thr are also asymmetric, and thus both Ile and Thr can exist in four enantiomeric forms. Among the derived amino acids, hydroxyproline and hydroxylysine also contain two asymmetric carbon centers. All proteins found in nature contain only L-amino acids. Conventionally, the L- and D-enantiomers are represented as



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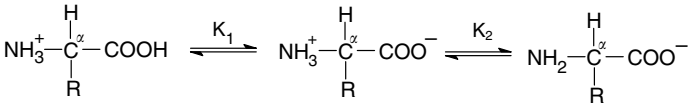
This nomenclature is based on D- and L-glyceraldehyde configurations and not on the actual direction of rotation of linearly polarized light. That is, the L-configuration does not refer to levorotation as in the case of L-glyceraldehyde. In fact most of the L-amino acids are dextrorotatory, not levorotatory.

5.2.1.3 Acid–Base Properties of Amino Acids

Since amino acids contain a carboxyl group (acidic) and an amino group (basic), they behave both as acids and bases; that is, they are *ampholytes*. For example, Gly, the simplest of all amino acids, can exist in three different ionized states, depending on the pH of the solution.



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At around neutral pH, both the α-amino and α-carboxyl groups are ionized, and the molecule is a *dipolar* or a *zwitter ion*. The pH at which the dipolar ion is electrically neutral is called the *isoelectric point* (pI). When the zwitter ion is titrated with an acid, the COO^- group becomes protonated. The pH at which the concentrations of COO^- and COOH are equal is known as pK_{a1} (i.e., negative logarithm of the acid dissociation constant K_{a1}). Similarly, when the zwitter ion is titrated with a base, the NH_3^+ group becomes deprotonated. As before, the pH at which $[NH_3^+] = [NH_2]$ is known as pK_{a2}. A typical electrometric titration curve for a dipolar amino acid is shown in Figure 5.2. In addition to the α-amino and α-carboxyl groups, the side chains of Lys, Arg, His, Asp, Glu, Cys, and Tyr also contain ionizable groups. The pK_a values of all the ionizable groups in amino acids are given in Table 5.1. The isoelectric points of amino acids can be estimated from their pK_{a1}, pK_{a2}, and pK_{a3} values, using the following expressions:

For amino acids with no charged side chain, $\quad pI = (pK_{a1} + pK_{a2})/2$

For acidic amino acids, $\quad pI = (pK_{a1} + pK_{a3})/2$, and

For basic amino acids, $\quad pI = (pK_{a2} + pK_{a3})/2$

The subscripts 1, 2, and 3 refer to α-carboxyl, α-amino, and side chain ionizable groups, respectively.

In proteins, the α-COOH of one amino acid is covalently coupled to the α-NH_2 of the next amino acid through an amide bond, thus the only ionizable groups in proteins are the N-terminus amino

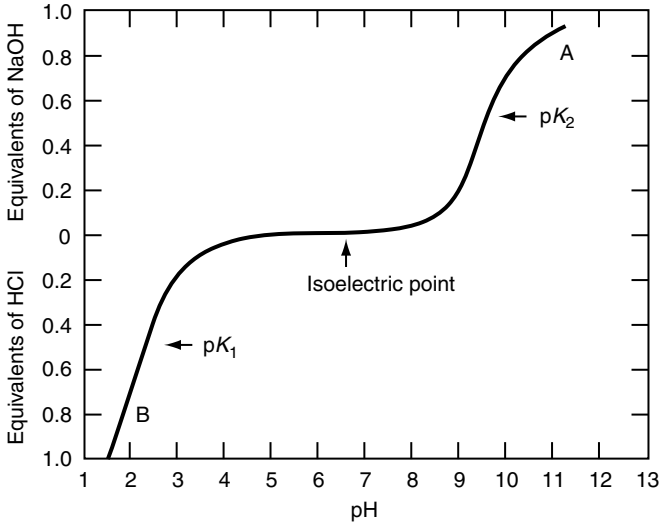


FIGURE 5.2 Titration curve of a typical amino acid.

TABLE 5.1
Properties of Ionizable Groups in Free Amino Acids at 25°C

Amino Acid	pK_{a1} •(—COOH)	pK_{a2} •($—NH_3^+$)	pK_{a3} (Side Chain)	pI
Alanine	2.34	9.69	—	6.00
Arginine	2.17	9.04	12.48	10.76
Asparagine	2.02	8.80	—	5.41
Aspartic acid	1.88	9.60	3.65	2.77
Cysteine	1.96	10.28	8.18	5.07
Glutamine	2.17	9.13	—	5.65
Glutamic acid	2.19	9.67	4.25	3.22
Glycine	2.34	9.60	—	5.98
Histidine	1.82	9.17	6.00	7.59
Isoleucine	2.36	9.68	—	6.02
Leucine	2.30	9.60	—	5.98
Lysine	2.18	8.95	10.53	9.74
Methionine	2.28	9.21	—	5.74
Phenylalanine	1.83	9.13	—	5.48
Proline	1.94	10.60	—	6.30
Serine	2.20	9.15	—	5.68
Threonine	2.21	9.15	—	5.68
Tryptophan	2.38	9.39	—	5.89
Tyrosine	2.20	9.11	10.07	5.66
Valine	2.32	9.62	—	5.96

group, the C-terminus carboxyl group, and ionizable groups on side chains. The pK_a of the ionizable groups in proteins are different from those of free amino acids (Table 5.2). The significant shift in the pK_a values in proteins as compared to free amino acids is related to altered electronic and dielectric environments of these groups in proteins. (This property is important in enzymes.)

The degree of ionization of a group at any given solution pH can be determined by using the Henderson–Hasselbach equation:

$$\text{pH} = pK_a + \log \frac{[\text{conjugated base}]}{[\text{conjugated acid}]} \tag{5.5}$$

The net charge of a protein at a given pH can be estimated by determining the degree of ionization of individual ionizable groups using this equation, and then adding up the total number of negative and positive charges.

Amino acids may be classified into several categories based on the nature of interaction of the side chains with water. Amino acids with aliphatic (Ala, Ile, Leu, Met, Pro, and Val) and aromatic side chains (Phe, Trp, and Tyr) are hydrophobic, and hence they exhibit limited solubility in water (Table 5.3). Polar (hydrophilic) amino acids are quite soluble in water and they are either charged (Arg, Asp, Glu, His, and Lys) or uncharged (Ser, Thr, Asn, Gln, and Cys). The side chains of Arg and Lys contain guanidyl and amino groups, respectively, and thus are positively charged (basic) at neutral pH. The imidazole group of His is basic in nature. However, at neutral pH its net charge is only slightly positive. The side chains of Asp and Glu acids contain a carboxyl group. These amino acids carry a net negative charge at neutral pH. Both the basic and acidic amino acids are strongly hydrophilic. The net charge of a protein at physiological conditions is dependent on the relative numbers of basic and acidic amino acids residues in the protein.

TABLE 5.2
Average pK_a Values of Ionizable Groups in Proteins

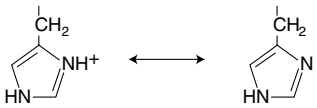
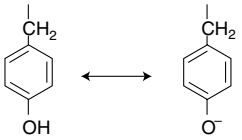
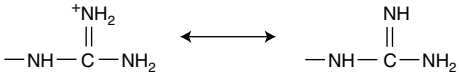
Ionizable Group	pK_a	Acid Form ⟷ Base Form
Terminal COOH	3.75	$-COOH \longleftrightarrow -COO^-$
Terminal NH_2	7.8	$-NH_3^+ \longleftrightarrow -NH_2$
Side chain COOH (Glu, Asp)	4.6	$-COOH \longleftrightarrow -COO^-$
Side chain NH_2	10.2	$-NH_3^+ \longleftrightarrow -NH_2$
Imidazole	7.0	
Sulfhydryl	8.8	$-SH \longleftrightarrow -S^-$
Phenolic	9.6	
Guanidyl	>12	

TABLE 5.3
Properties of Amino Acids at 25°C

Amino Acid	Molecular Weight	Residue Volume Δ^3	Residue Area Δ^2	Solubility (g/L)	Hydrophobicity (kcal/mol)[a] (ΔG_{tr}^0)
Ala	89.1	89	115	167.2	0.4
Arg	174.2	173	225	855.6	−1.4
Asn	132.1	111	150	28.5	−0.8
Asp	133.1	114	160	5.0	−1.1
Cys	121.1	109	135	—	2.1
Gln	146.1	144	180	7.2(37°C)	−0.3
Glu	147.1	138	190	8.5	−0.9
Gly	75.1	60	75	249.9	0
His	155.2	153	195	—	0.2
Ile	131.2	167	175	34.5	2.5
Leu	131.2	167	170	21.7	2.3
Lys	146.2	169	200	739.0	−1.4
Met	149.2	163	185	56.2	1.7
Phe	165.2	190	210	27.6	2.4
Pro	115.1	113	145	620.0	1.0
Ser	105.1	89	115	422.0	−0.1
Thr	119.1	116	140	13.2	0.4
Trp	204.2	228	255	13.6	3.1
Tyr	181.2	194	230	0.4	1.3
Val	117.1	140	155	58.1	1.7

[a] The ΔG values are relative to glycine based on the side-chain distribution coefficients (K_{eq}) between 1-octanol and water [41].

The polarities of uncharged neutral amino acids fall between those of hydrophobic and charged amino acids. The polar nature of Ser and Thr is attributed to the hydroxyl group that is able to hydrogen bond with water. Since Tyr also contains an ionizable phenolic group, which ionizes at alkaline pH, it is also considered to be a polar amino acid. However, based on its solubility characteristics at neutral pH, it should be regarded as a hydrophobic amino acid. The amide group of Asn and Gln is able to interact with water through hydrogen bonding. Upon acid or alkaline hydrolysis, the amide group of Asn and Gln is converted to a carboxyl group with release of ammonia. A majority of the Cys residues in proteins exists as cystine, which is a Cys dimer created by oxidation of thiol groups to form a disulfide cross-link.

Proline is a unique amino acid because it is the only *imino acid* in proteins. In proline, the propyl side chain is covalently linked to both the α-carbon atom and the α-amino group, forming a pyrrolidine ring structure.

5.2.1.4 Hydrophobicity of Amino Acids

One of the major factors affecting physicochemical properties, such as structure, solubility, fat-binding properties, and so forth, of proteins and peptides is the hydrophobicity of the constituent amino acid residues. Hydrophobicity can be defined as the excess free energy of a solute dissolved in water compared to that in an organic solvent under similar conditions. The most direct and simplest way to estimate hydrophobicities of amino acid side chains is experimental determination of free energy changes for dissolution of amino acid side chains in water and in an organic solvent, such as octanol or ethanol. The chemical potential of an amino acid dissolved in water can be expressed by:

$$\mu_{\mathrm{AA,w}} = \mu^{\circ}_{\mathrm{AA,w}} + RT \ln \gamma_{\mathrm{AA,w}} C_{\mathrm{AA,w}} \tag{5.6}$$

where $\mu^{\circ}_{\mathrm{AA,w}}$ is the standard chemical potential of the amino acid in the aqueous solution, γ_{AA} is the activity coefficient, C_{AA} is concentration, T is absolute temperature, and R is the gas constant. Similarly, the chemical potential of an amino acid dissolved in an organic solvent, for example, octanol, can be expressed as

$$\mu_{\mathrm{AA,oct}} = \mu^{\circ}_{\mathrm{AA,oct}} + RT \ln \gamma_{\mathrm{AA,oct}} C_{\mathrm{AA,oct}} \tag{5.7}$$

In saturated solutions, in which $C_{\mathrm{AA,w}}$ and $C_{\mathrm{AA,oct}}$ represent solubilities in water and octanol, respectively, the chemical potentials of the amino acid in water and octanol are the same, that is,

$$\mu_{\mathrm{AA,w}} = \mu_{\mathrm{AA,oct}} \tag{5.8}$$

Thus

$$\mu^{\circ}_{\mathrm{AA,oct}} + RT \ln \gamma_{\mathrm{AA,oct}} C_{\mathrm{AA,oct}} = \mu^{\circ}_{\mathrm{AA,w}} + RT \ln \gamma_{\mathrm{AA,w}} C_{\mathrm{AA,w}}. \tag{5.9}$$

The quantity $(\mu^{\circ}_{\mathrm{AA,w}} - \mu^{\circ}_{\mathrm{AA,oct}})$, which represents the difference between the standard chemical potentials arising from the interaction of the amino acid with octanol and with water, can be defined as the free energy change $(\Delta G^{0}_{\mathrm{tr,oct}\rightarrow\mathrm{w}})$ of transfer of the amino acid from octanol to water. Thus, assuming that the ratio of activity coefficients is one, the above equation can be expressed as

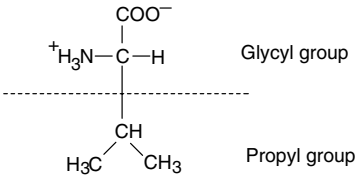
$$\Delta G^{0}_{\mathrm{tr,(oct}\rightarrow\mathrm{w)}} = -RT \ln(S_{\mathrm{AA,W}}/S_{\mathrm{AA,oct}}) \tag{5.10}$$

where $S_{\mathrm{AA,oct}}$ and $S_{\mathrm{AA,w}}$ represent solubilities of the amino acid in octanol and water, respectively.

As is true of all other thermodynamic parameters, ΔG^0_{tr} is an additive function. That is, if a molecule has two groups, A and B, covalently attached, the ΔG^0_{tr} for transfer from one solvent to another solvent is the sum of the free energy changes for transfer of groups A and B. That is,

$$\Delta G^0_{tr,AB} = \Delta G^0_{tr,A} + \Delta G^0_{tr,B} \tag{5.11}$$

The same logic can be applied to the transfer of an amino acid from octanol to water. For example, Val can be considered as a derivative of Gly with an isopropyl side chain at the α-carbon atom.



(5.12)

The free energy change of transfer of valine from octanol to water can then be considered as

$$\Delta G^0_{tr,Val} = \Delta G^0_{tr,Gly} + \Delta G^0_{tr,side\ chain} \tag{5.13}$$

or

$$\Delta G^0_{tr,side\ chain} = \Delta G^0_{tr,Val} - \Delta G^0_{tr,Gly} \tag{5.14}$$

In other words, the hydrophobicities of amino acid side chains can be determined by subtracting $\Delta G^0_{tr,Gly}$ from $\Delta G^0_{tr,Val}$.

The hydrophobicity values of amino acid side chains obtained in this manner are given in Table 5.3. Amino acid side chains with large positive ΔG^0_{tr} values are hydrophobic; they would prefer to be in an organic phase rather than in an aqueous phase. In proteins, these residues tend to locate themselves in the protein interior, where the polarity of the environment is similar to that of an organic phase. Amino acid residues with negative ΔG^0_{tr} values are hydrophilic, and these residues tend to locate themselves on the surface of protein molecules. The hydrophobicity of nonpolar residues is linearly correlated to their surface area, as shown in Figure 5.3.

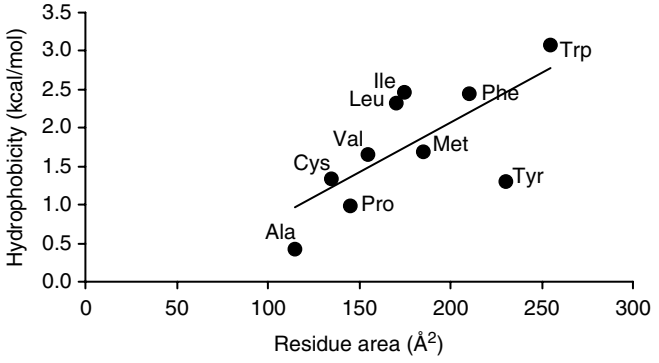


FIGURE 5.3 Correlation between surface area and hydrophobicity of nonpolar amino acid residues.

TABLE 5.4
Ultraviolet Absorbance and Fluorescence of Aromatic Amino Acids

Amino Acid	λ_{max} of Absorbance (nm)	Molar Extinction Coefficient (L mol^{-1} cm^{-1})	λ_{max} of Fluorescence (nm)
Phenylalanine	260	190	282[a]
Tryptophan	278	5500	348[b]
Tyrosine	275	1340	304[b]

[a] Excitation at 260 nm.
[b] Excitation at 280 nm.

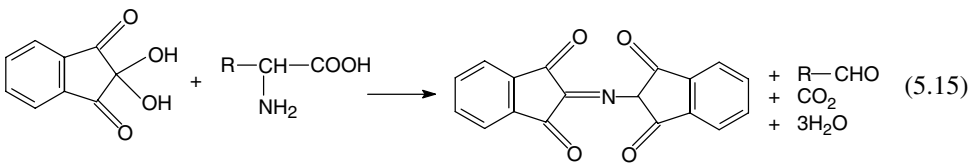
5.2.1.5 Optical Properties of Amino Acids

The aromatic amino acids Trp, Tyr, and Phe absorb light in the near-ultraviolet region (250–300 nm). In addition, Trp and Tyr also exhibit fluorescence in the ultraviolet region. The maximum wavelengths of absorption and fluorescence emission of the aromatic amino acids are given in Table 5.4. These amino acid residues are responsible for ultraviolet absorption properties of proteins in the 250–300 nm range, with maximum absorption at about 280 nm for most proteins. Since both absorption and fluorescence properties of these amino acids are influenced by the polarity of their environment, changes in the optical properties of proteins are often used as a means to monitor conformational changes in proteins.

5.2.2 Chemical Reactivity of Amino Acids

The reactive groups, such as amino, carboxyl, sulfhydryl, phenolic, hydroxyl, thioether (Met), imidazole, and guanyl, in free amino acids and proteins are capable of undergoing chemical reactions that are similar to those that would occur if they were attached to other small organic molecules. Typical reactions for various side-chain groups are presented in Table 5.5. Several of these reactions can be used to alter the hydrophilic and hydrophobic properties and the functional properties of proteins and peptides. Some of these reactions also can be used to quantify amino acids and specific amino acid residues in proteins. For example, reaction of amino acids with ninhydrin, *O*-phthaldialdehyde, or fluorescamine is regularly used in the quantification of amino acids.

Reaction with ninhydrin: The ninhydrin reaction is often used to quantify free amino acids. When an amino acid is reacted with an excess amount of ninhydrin, one mole each of ammonia, aldehyde, CO_2, and hydrindantin are formed for every mole of amino acid consumed (Equation 5.15). The liberated ammonia subsequently reacts with one mole of ninhydrin and one mole of hydrindantin, forming a purple color product known as Ruhemann's purple, which has maximum absorbance at 570 nm. Proline and hydroxyproline give a yellow color product, which has maximum absorbance at 440 nm. These color reactions provide the basis for colorimetric determination of amino acids.



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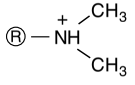
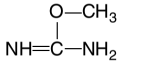
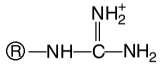
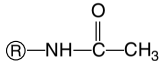
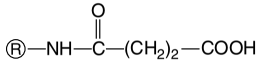
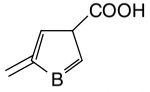
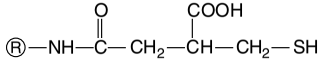
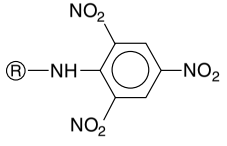
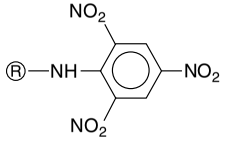
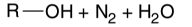
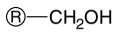
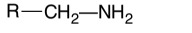
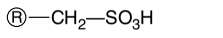
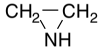
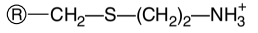
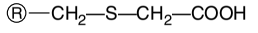
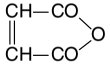
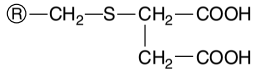
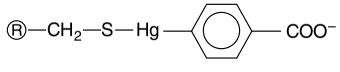
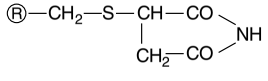
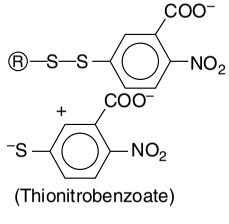
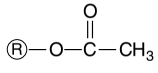
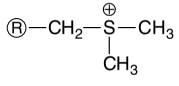
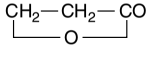
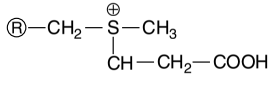
The ninhydrin reaction is usually used to determine the amino acid composition of proteins. In this case, the protein is first acid hydrolyzed to the amino acid level. The freed amino acids are then

TABLE 5.5
Chemical Reactions of Functional Groups in Amino Acids and Proteins

Type of Reaction	Reagent and Conditions	Product	Remarks
A. Amino groups			
1. Reductive alkylation	$HCHO$, $NaBH_4$ (formaldehyde)		Useful for radiolabeling proteins
2. Guanidation	 (*O*-methylisourea) pH 10.6, 4°C for 4 days		Converts lysyl side chain to homoarginine
3. Acetylation	Acetic anhydride		Eliminates the positive charge
4. Succinylation	Succinic anhydride		Introduces a negative charge at lysyl residues
5. Thiolation	 (Thioparaconic acid)		Eliminates positive charge and initiates thiol group at lysyl residues
6. Arylation	1-Fluoro-2,4-dinitrobenzene (FDNB)		Used for the determination of amino groups
	2,4,6-Trinitrobenzene sulfonic acid (TNBS)		The extinction coefficient is $1.1 \times 10^4\ M^{-1}\ cm^{-1}$ at 367 nm; used to determine reactive lysyl residues in proteins
7. Deamination	1.5 M $NaNO_2$ in acetic acid, 0°C		
B. Carboxyl groups			
1. Esterification	Acidic methanol		Hydrolysis of the ester occurs at pH > 6.0
2. Reduction	Borohydride in tetrahydrofuran, trifluoracetic acid		
3. Decarboxylation	Acid, alkali, heat treatment		Occurs only with amino acid, not with proteins

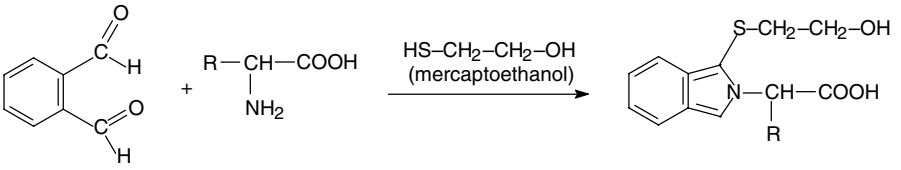
(Continued)

TABLE 5.5 (Continued)

Type of Reaction	Reagent and Conditions	Product	Remarks
C. Sulfhydryl group			
1. Oxidation	Performic acid		
2. Blocking	 (Ethyleneimine)		Introduces amino group
	Iodoacetic acid		Introduces one amino group
	 (Maleic anhydride)		Introduces two negative charges for each SH group blocked
	p-Mercuribenzoate		The extinction coefficient of this derivative at 250 nm (pH 7) is 7500 M^{-1} cm^{-1}; this reaction is used to determine SH content of proteins
	N-Ethylmaleimide		Used for blocking SH groups
	5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB)	 (Thionitrobenzoate)	One mole of thionitrobenzoate is released; the ϵ_{412} of thionitrobenzoate is 13,600 M^{-1} cm^{-1}; this reaction is used to determine SH groups in proteins
D. Serine and threonine			
1. Esterification	CH_3—COCl		
E. Methionine			
1. Alkyl halides	CH_3I		
2. β-Propiolactone			

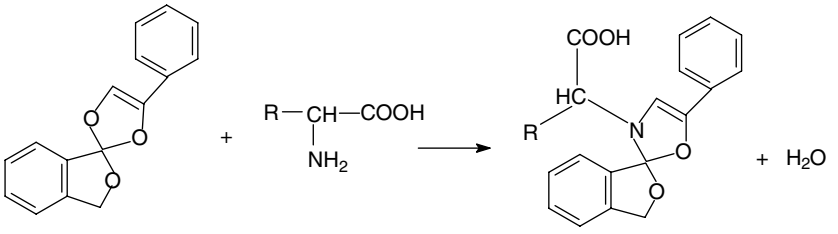
separated and identified using ion exchange/hydrophobic chromatography. The column eluates are reacted with ninhydrin and quantified by measuring absorbance at 570 and 440 nm.

Reaction with O-phthaldialdehyde: Reaction of amino acids with *O*-phthaldialdehyde (1,2-benzene dicarbonal) in the presence of 2-mercaptoethanol yields a highly fluorescent derivative that has an excitation maximum at 380 nm and a fluorescence emission maximum at 450 nm.



(5.16)

Reaction with fluorescamine: Reaction of amino acids, peptides, and proteins containing primary amines with fluorescamine yields a highly fluorescent derivative with fluorescence emission maximum at 475 nm when excited at 390 nm. This method can be used to quantify amino acids as well as proteins and peptides.



Fluorescamine

(5.17)

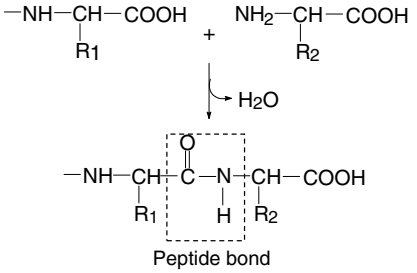
5.3 PROTEIN STRUCTURE

5.3.1 Structural Hierarchy in Proteins

Four levels of protein structure exist: *primary*, *secondary*, *tertiary*, and *quaternary*.

5.3.1.1 Primary Structure

The primary structure of a protein refers to the linear sequence in which the constituent amino acids are covalently linked through amide bonds, also known as peptide bonds. The peptide linkage results from condensation of the α-carboxyl group of ith amino acid and the α-amino group of $i+1$th amino acid with removal of a water molecule. In this linear sequence, all the amino acid residues are in the L-configuration. A protein with n amino acid residues contains $n-1$ peptide linkages.



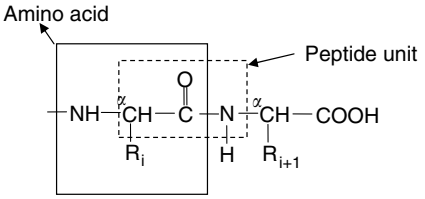
Peptide bond

(5.18)

The terminus with the free α-amino group is known as the N-terminal and that with the free α-COOH group is known as the C-terminal. By convention, N represents the beginning and C the end of the polypeptide chain when primary sequence information is indicated.

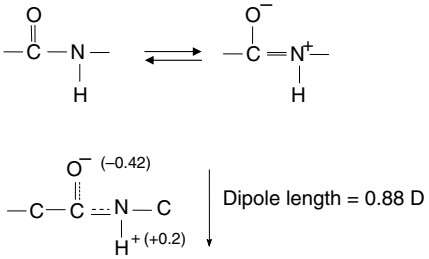
The chain length (n) and the sequence in which the n residues are linked determine the physicochemical, structural, biological properties, and functions of a protein. The amino acid sequence acts as the code for formation of secondary and tertiary structures and ultimately determines the protein's biological functionality. The molecular mass ranges from a few thousand Daltons (Da) to over a million Da. For example, titin, which is a single-chain protein found in muscle has, a molecular weight of over one million, whereas secretin has a molecular weight of about 2,300 Da. The molecular weight of most proteins is in the range of 20,000–100,000 Da.

The backbone of polypeptides can be depicted as repeating units of $-N-C-C^{\alpha}-$ or $-^{\alpha}C-C-N$. The expression $-NH-^{\alpha}CHR-CO-$ relates to an amino acid residue, whereas $-^{\alpha}CHR-CO-NH-$ represents a *peptide unit*.



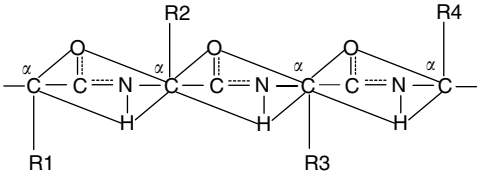
(5.19)

Although the CO—NH bond is depicted as a single covalent bond, in reality it has a partial double bond character because of the resonance structure caused by delocalization of electrons.



(5.20)

This has several important structural implications in proteins. First, the resonance structure precludes protonation of the peptide N—H group. Second, because of the partial double bond character, the rotation of the CO—NH bond is restricted to a maximum of 6°, known as ω-angle. Because of this restriction, each six-atom segment ($-C^{\alpha}-CO-NH-C^{\alpha}-$) of the peptide backbone lies in a single plane. The polypeptide backbone, in essence, can be depicted as a series of $-C^{\alpha}-CO-NH-C^{\alpha}-$ planes connected at the C_{α} atoms as shown below:

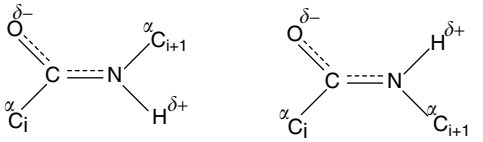


(5.21)

Since peptide bonds constitute about one-third of the total covalent bonds of the backbone, their restricted rotational freedom drastically reduces backbone flexibility. Only the $N-C^{\alpha}$ and $C^{\alpha}-C$ bonds have rotational freedoms, and these are termed ϕ (*phi*) and ψ (*psi*) dihedral angles, respectively.

These are also known as main-chain torsion angles. Third, delocalization of electrons also imparts a partial negative charge to the carbonyl oxygen atom and a partial positive charge to the hydrogen atom of the N—H group. Because of this, hydrogen bonding (*dipole–dipole interaction*) between the C═O and N—H groups of peptide backbone is possible under appropriate conditions.

Another consequence of the partial double-bond nature of the peptide bond is that the four atoms attached to the peptide bond can exist either in *cis* or *trans* configuration.



(5.22)

However, almost all protein peptide bonds exist in the *trans* configuration. This is due to the fact that the *trans* configuration is thermodynamically more stable than the *cis* configuration. Since *tran* → *cis* transformation increases the free energy of the peptide bond by 8.3 kcal/mol, isomerization of peptide bonds does not occur in proteins. One exception to this is peptide bonds involving proline residues. Since the free energy change for *trans* → *cis* transformation of peptide bonds involving proline residues is only about 1.86 kcal/mol, at high temperatures these peptide bonds sometimes do undergo *trans* → *cis* isomerization.

Although the N—C^{α} and C^{α}—C bonds are truly single bonds, and thus the N and P dihedral angles can theoretically have 360° rotational freedom, in reality their rotational freedoms are restricted by steric hindrances from side-chain atoms. These restrictions further decrease flexibility of the polypeptide chain.

5.3.1.2 Secondary Structure

Secondary structure refers to the periodic spatial arrangement of amino acid residues at certain segments of the polypeptide chain. The periodic structures arise when consecutive amino acid residues in a segment assume the same set of ϕ and ψ torsion angles. The twist of the ϕ and ψ angles is driven by near-neighbor or short-range noncovalent interactions between amino acid side chains, which leads to a decrease in local free energy. The *aperiodic* or *random* structure refers to those regions of the polypeptide chain where successive amino acid residues have different sets of ϕ and ψ torsion angles.

In general, two forms of periodic (regular) secondary structures are found in proteins. These are helical structures and extended sheet-like structures. The geometric characteristics of various regular structures found in proteins are given in Table 5.6.

Helical structures: Protein helical structures are formed when the ϕ and ψ angles of consecutive amino acid residues are twisted to a same set of values. By selecting different combinations of ϕ and ψ angles, it is theoretically possible to create several types of helical structures with different geometries. However, in proteins, only three types of helical structures, namely α-, 3_{10}-, and β-helix, are found.

Among the three helical structures, the α-helix is the major form found in proteins and it is the most stable (Figure 5.4). The pitch of this helix, that is, the increase in axial length per rotation, is 5.4 Å. Each helical rotation involves 3.6 amino acid residues, with each residue extending the axial length by 1.5 Å. The angle of rotation per residue is 100° (i.e., 360°/3.6). The amino acid side chains are oriented perpendicular to the axis of the helix.

α-Helix is stabilized by hydrogen bonding. In this structure, each backbone N–H group is hydrogen bonded to the C═O group of the fourth preceding residue. Thirteen backbone atoms

TABLE 5.6
Geometric Characteristics of Regular Polypeptide Conformations

Structure	ϕ	ψ	n	r	h (Å)	t
Right-handed α-helix	−58°	−47°	3.6	13	1.5	100°
π-Helix	−57°	−70°	4.4	16	1.15	81.8°
3_{10}-Helix	−49°	−26°	3	10	2	120°
Parallel β-sheet	−119°	+113°	2	—	3.2	—
Antiparallel β-sheet	−139°	+135°	2	—	3.4	—
Polyproline I (*cis*)	−83°	+158°	3.33	1.9	—	—
Polyproline II (*trans*)	−78°	+149°	3.00	3.12	—	—

ϕ and ψ represent dihedral angles of the N—C_α and C_α—C bonds, respectively; n is number of residues per turn; r, number of backbone atoms within a hydrogen bonded loop of helix; h, rise of helix per amino acid residue; $t = 360°/n$, twist of helix per residue.

Source: From Creighton, T. E. 1993. *Proteins: Structures and Molecular Properties*. W. H. Freeman Co., New York, pp. 158–159.

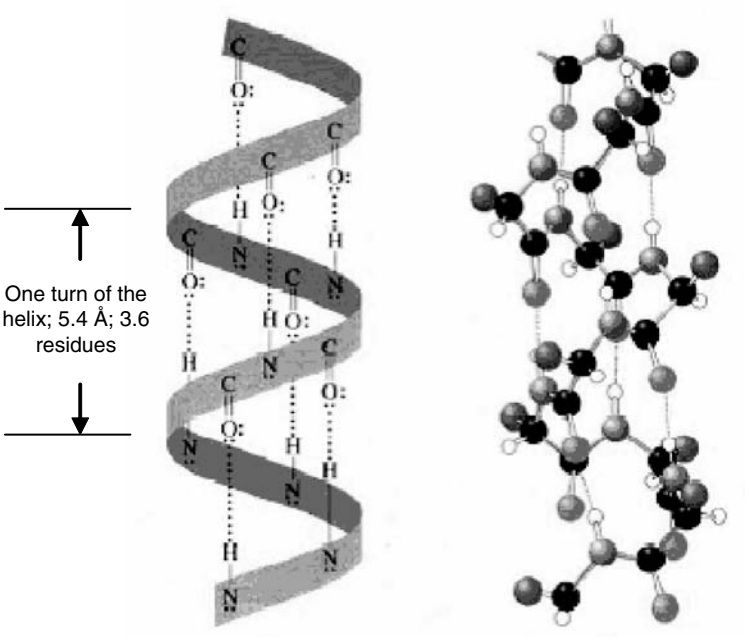


FIGURE 5.4 Spatial arrangement of polypeptides in α-helix. (From http://en.wikipedia.org/wiki/Alpha_helix)

are in this hydrogen-bonded loop, thus the α-helix is sometimes called the 3.6_{13} helix (Figure 5.4). The hydrogen bonds are oriented parallel to the helix axis, and the N, H, and O atoms of the hydrogen bond lie almost in a straight line, that is, the hydrogen bond angle is almost zero. The hydrogen bond length, that is, the N—H $\cdots$ O distance, is about 2.9 Å, and the strength of this bond is about 4.5 kcal/mol. The α-helix can exist in either a right- or left-handed orientation. However, the right-handed orientation is the more stable one.

The details for α-helix formation are embedded as a binary code in the amino acid sequence [61].The binary code is related to the arrangement of polar and nonpolar residues

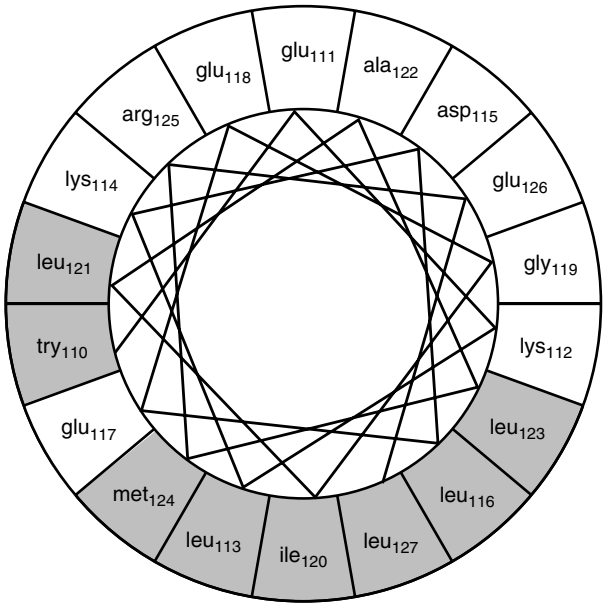


FIGURE 5.5 Cross-sectional view of the helical structure of residues 110–127 of bovine growth hormone. The top of the helical wheel (unfilled) represents the hydrophilic surface and the bottom (filled) represents the hydrophobic surface of the amphiphilic helix. (From Brems, D. N. 1990. In *Protein Folding* (Gierasch, L. M. and J. King, Eds.), American Association for the Advancement of Science, Washington, DC, p. 133. Courtesy of American Association for the Advancement of Science.)

in the sequence. Polypeptide segments with repeating seven amino acid (heptet) sequences of —P—N—P—P—N—N—P—, where P and N are polar and nonpolar residues, respectively, readily form α-helices in aqueous solutions. It is the binary code, and not the precise identities of the polar and nonpolar residues in the heptet sequence, that dictates α-helix formation. Slight variations in the binary code of the heptet are tolerated, provided other inter- or intramolecular interactions are favorable for α-helix formation. For example, tropomyosin, a muscle protein, exists entirely in a coiled-coil α-helical rod form. The repeating heptet sequence in this protein is —N—P—P—N—P—P—P—, which is slightly different from the above sequence. In spite of this variation, tropomyosin exists entirely in the α-helix form because of other stabilizing interactions in the coiled-coil rod [82].

Most of the α-helical structure found in proteins is amphiphilic in nature, that is, one-half of the helix's surface is occupied by hydrophobic residues and the other half by hydrophilic residues. This is schematically shown in the form of a helical wheel in Figure 5.5 [13]. In most proteins, the nonpolar surface of the helix faces the protein interior and is generally engaged in hydrophobic interactions with other nonpolar surfaces.

Other types of helical structures found in proteins are the β-helix and the 3_{10}-helix. The β- and 3_{10}-helices are about 0.5 kcal/mol and 1.0 kcal/mol, respectively, less stable than the α-helix. These helices exist only as short segments involving a few amino acid residues, and they are not major entities in most proteins.

In proline residues, because of the ring structure formed by covalent attachment of the propyl side chain to the amino group, rotation of the $N—C^{\alpha}$ bond is not possible, and therefore the ϕ angle has a fixed value of 70°. In addition, since there is no hydrogen at the nitrogen atom, it cannot form hydrogen bonds. Because of these two attributes, segments containing proline residues cannot form α-helices. In fact, proline is considered to be a α-helix breaker. Proteins containing high levels of proline residues tend to assume a random or aperiodic structure. For example, proline residues constitute about 17% of the total amino acid residues in β-casein, and 8.5% in α_{s1}-casein, and

because of the uniform distribution of these residues in their primary structures, α-helices are not present in these proteins and they have random structures. However, polyproline is able to form two types of helical structures, termed *polyproline I* and *polyproline II*. In polyproline I, the peptide bonds are in the *cis*-configuration, and in polyproline II they are in *trans*. Other geometric characteristics of these helices are given in Table 5.6. Collagen, which is the most abundant animal protein, exists as polyproline II-type helix. In collagen, on an average, every third residue is a glycine, which is preceded usually by a proline residue. Three polypeptide chains are entwined to form a triple helix, and the stability of the triple helix is maintained by interchain hydrogen bonds. This unique triple helix structure is responsible for the high tensile strength of collagen.

β-Sheet structure: The *β-sheet* is an extended structure with specific geometries given in Table 5.6. In this extended form, the C═O and N—H groups are oriented perpendicular to the direction of the chain, and therefore hydrogen bonding is possible only between segments (i.e., intersegment), and not within a segment (i.e., intrasegment). The β-strands are usually about 5–15 amino acid residues long. In proteins, two β-strands of the same molecule interact via hydrogen bonds, forming a sheet-like structure known as β-pleated sheet. In the sheet-like structure, the side chains are oriented perpendicular (above and below) to the plane of the sheet. Depending on the $N \rightarrow C$ directional orientations of the strands, two types of β-pleated sheet structures, namely *parallel β-sheet* and *antiparallel β-sheet*, can form (Figure 5.6). In parallel β-sheet the directions of the β-strands run parallel to each other, whereas in the other they run opposite to each other. These differences in chain directions affect the geometry of hydrogen bonds. In antiparallel β-sheets the N—H $\cdots$ O atoms lie in a straight line (zero H-bond angle), which enhances the stability of the hydrogen bond, whereas in parallel β-sheets they lie at an angle, which reduces the stability of the hydrogen bonds. Antiparallel β-sheets are, therefore, more stable than parallel β-sheets.

The binary code that specifies formation of β-sheet structures in proteins is —N—P—N—P—N—P—N—P—. Clearly, polypeptide segments containing alternating polar and nonpolar residues have

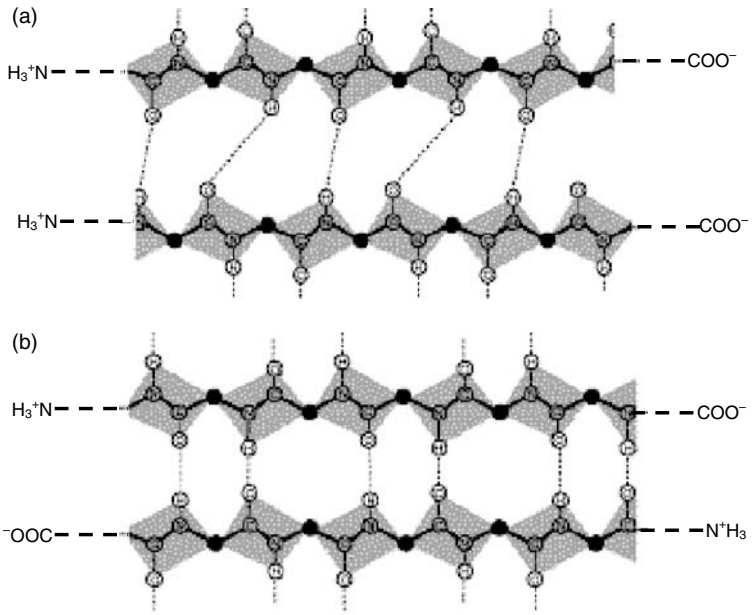


FIGURE 5.6 Parallel (a) and antiparallel (b) β-sheets. The dotted lines represent hydrogen bonds between peptide groups. The side chains at C_α atoms are oriented perpendicular (up or down) to the direction of the backbone. (From http://www.schoolscience.co.uk)

a high propensity to form β-sheet structures. Segments rich in bulky hydrophobic side chains, such as Val and Ile, also have a tendency to form a β-sheet structure. As expected, some variation in the code is tolerated.

The β-sheet structure is generally more stable than the α-helix. Proteins that contain large fractions of β-sheet structure usually exhibit high denaturation temperatures. Examples are β-lactoglobulin (51% β-sheet) and soy 11S globulin (64% β-sheet), which have thermal denaturation temperatures of 75.6 and 84.5°C, respectively. On the other hand, the denaturation temperature of bovine serum albumin, which has about 64% α-helix structure, is only about 64°C [25,27]. When solutions of α-helix-type proteins are heated and cooled, the α-helix is usually converted to β-sheet [27]. However, conversion from β-sheet to α-helix has not been observed in proteins.

Another common structural feature found in proteins is the β-bend or β-turn. This arises as a result of 180° reversal of the polypeptide chain involved in β-sheet formation. The hairpin-type bend is the result of antiparallel β-sheet formation, and the crossover bend is the result of parallel β-sheet formation. Usually, a β-bend involves a four-residue segment folding back on itself and the bend is stabilized by a hydrogen bond. The amino acid residues Asp, Cys, Asn, Gly, Tyr, and Pro are common in β-bends. The secondary structure contents of several proteins are given in Table 5.7.

5.3.1.3 Tertiary Structure

Tertiary structure refers to the spatial arrangement attained when a linear protein chain with secondary structure segments folds further into a compact three-dimensional form. The tertiary structures of β-lactoglobulin and phaseolin (the storage protein in kidney beans) are shown in Figure 5.7 [74,98].

TABLE 5.7
Secondary Structure Content of Selected Globular Proteins[a]

Protein	%α-Helix	%β-Sheet	%β-Turns	%Aperiodic
Deoxyhemoglobin	85.7	0	8.8	5.5
Bovine serum albumin	67.0	0	0	33.0
α_{s1}-Casein	15.0	12.0	19.0	54.0
β-Casein	12.0	14.0	17.0	57.0
κ-Casein	23.0	31.0	14.0	32.0
Chymotrypsinogen	11.0	49.4	21.2	18.4
Immunoglobulin G	2.5	67.2	17.8	12.5
Insulin (dimer)	60.8	14.7	10.8	15.7
Bovine trypsin inhibitor	25.9	44.8	8.8	20.5
Ribonuclease A	22.6	46.0	18.5	12.9
Egg lysozyme	45.7	19.4	22.5	12.4
Ovomucoid	26.0	46.0	10.0	18.0
Ovalbumin	49.0	13.0	14.0	24.0
Papain	27.8	29.2	24.5	18.5
α-Lactalbumin	26.0	14.0	0	60.0
β-Lactoglobulin	6.8	51.2	10.5	31.5
Soy 11S	8.5	64.5	0	27.0
Soy 7S	6.0	62.5	2.0	29.5
Phaseolin	10.5	50.5	11.5	27.5
Myoglobin	79.0	0	5.0	16.0

[a] The values represent % of total number of amino acid residues.

Source: Compiled from various sources.

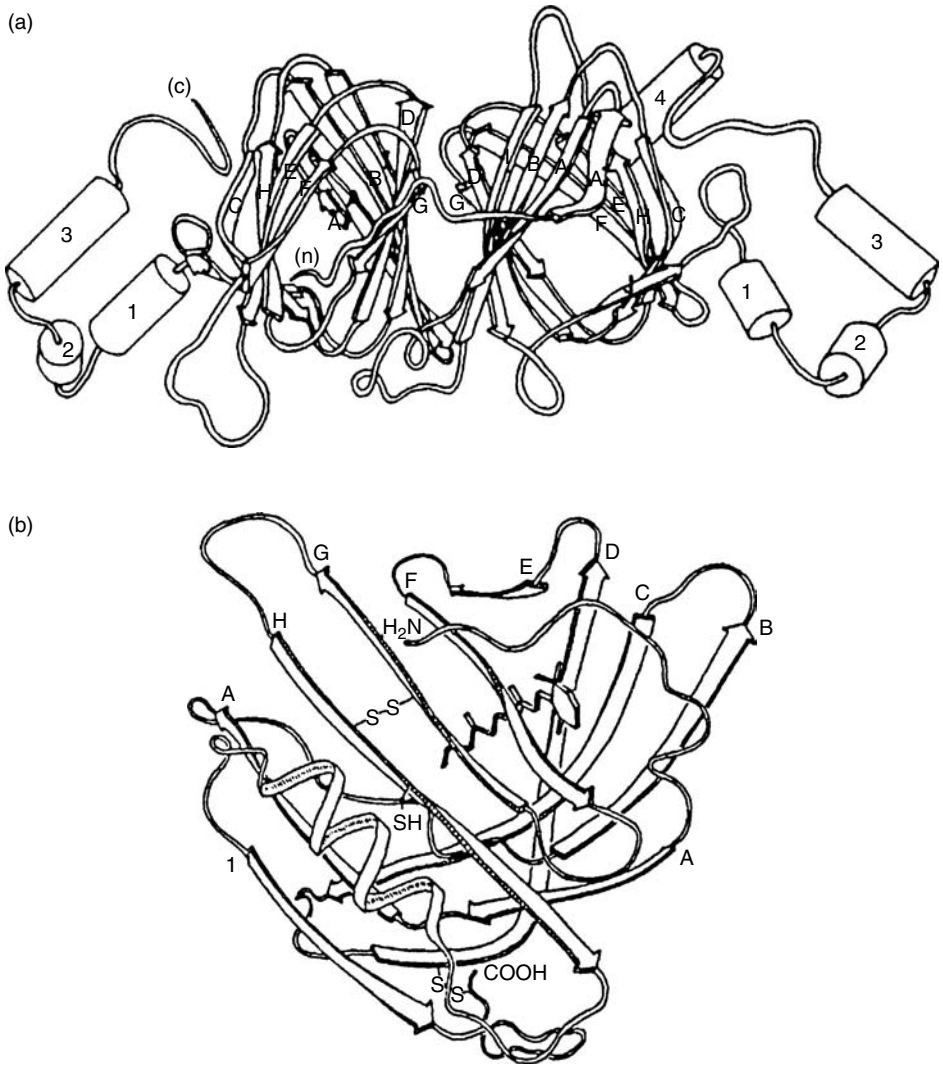


FIGURE 5.7 Tertiary structures of (a) phaseolin subunit and (b) β-lactoglobulin. The arrows indicate β-sheet strands and the cylinders indicate α-helix. (From Lawrence, M. C. et al. 1990. *EMBO J.* **9**:9–15 and Papiz, M. Z. et al. 1986. *Nature* **324**:383–385, respectively.)

Transformation of a protein from a linear configuration (primary structure) into a folded tertiary structure is a complex process. At the molecular level, the details for formation of a protein tertiary structure are present in its amino acid sequence. From a thermodynamic viewpoint, formation of tertiary structure involves optimization of various interactions (hydrophobic, electrostatic, van der Waals, and hydrogen bonding) between various groups in protein and the conformational entropy of the polypeptide chain, so that the net free energy of the molecule is reduced to the minimum value possible. The most important rearrangement that accompanies the reduction in free energy during formation of tertiary structure is the relocation of most of the hydrophobic residues at the interior of the protein structure away from the water environment and relocation of most of the hydrophilic residues, especially charged residues, at the protein–water interface. Although there is a strong general tendency for hydrophobic residues to be buried in the protein interior, this often can be accomplished only partially because of steric constraints. In fact, in most globular proteins, nonpolar residues occupy about 40–50% of the water accessible surface of protein molecules [84]. Also, some

polar groups are inevitably buried in the interior of proteins; however, these buried polar groups are invariably hydrogen bonded to other polar groups, such that their free energies are minimized in the apolar environment of the protein interior. The ratio of apolar and polar surfaces on a protein's surface enormously influences several of its physicochemical properties.

The folding of a protein from a linear structure to a folded tertiary structure is accompanied by a reduction in protein–water interfacial area. In fact, protein is forced to fold in order to mimize the protein–water interfacial area. The *accessible interfacial area* of a protein is defined as the total interfacial area of a three-dimensional space, occupied by the protein, as determined by figuratively rolling a spherical water molecule of radius 1.4 Å over the entire surface of the protein molecule. For native globular proteins, the accessible interfacial area (in $Å^2$) is a simple function of their molecular weight, M, as given by [84]:

$$A_s = 6.3M^{0.73}. \tag{5.23}$$

The total accessible interfacial area of a nascent polypeptide in its extended state (i.e., fully stretched molecule with no secondary, tertiary, or quaternary structure) is also correlated to its molecular weight by [84]:

$$A_t = 1.48M + 21 \tag{5.24}$$

The initial area of a protein that has folded during formation of a globular tertiary structure (i.e., A_b, buried area) can be estimated from Equations 5.23 and 5.24.

The fraction and distribution of hydrophilic and hydrophobic residues in the primary structure affects several physicochemical properties of the protein. For instance, the shape of a protein molecule is dictated by its amino acid sequence. If a protein contains a large number of hydrophilic residues distributed uniformly in its sequence, it will assume an elongated or rod-like shape. This is because, for a given mass, an elongated shape has a large surface-area-to-volume ratio so that more hydrophilic residues can be placed on the surface. On the other hand, if a protein contains a large number of hydrophobic residues, it will assume a globular (roughly spherical) shape. This minimizes the surface-area-to-volume ratio, enabling more hydrophobic residues to be buried in the protein interior. Among globular proteins, it is generally found that larger molecules contain larger fractions of nonpolar amino acids than do smaller molecules.

The tertiary structures of several single polypeptide proteins are made up of domains. Domains are defined as those regions of the polypeptide sequence that fold up into a tertiary form independently. These are, in essence, miniproteins within a single protein. The structural stability of each domain is largely independent of the others. In most single-chain proteins, the domains fold independently and then interact with each other to form the unique tertiary structure of the protein. In some proteins, as in the case of phaseolin (Figure 5.7), the tertiary structure may contain two or more distinct domains (structural entities) connected by a segment of the polypeptide chain. The number of domains in a protein usually depends on its molecular weight. Small proteins (e.g., lysozyme, β-lactoglobulin, and α-lactalbumin) with 100–150 amino acid residues usually form a single domain tertiary structure. Large proteins, such as immunoglobulin, contain multiple domains. The light chain of immunoglobulin G contains two domains, and the heavy chain contains four domains. The size of each of these domains is about 120 amino acid residues. Human serum albumin, which is made up of 585 amino acid residues, has three homologous domains, and each domain contains two subdomains [56].

5.3.1.4 Quaternary Structure

Quaternary structure refers to the spatial arrangement of a protein when it contains more than one polypeptide chain. Several biologically important proteins exist as dimers, trimers, tetramers, and so forth. Any of these quaternary complexes (also referred to as oligomers) can be made up of protein

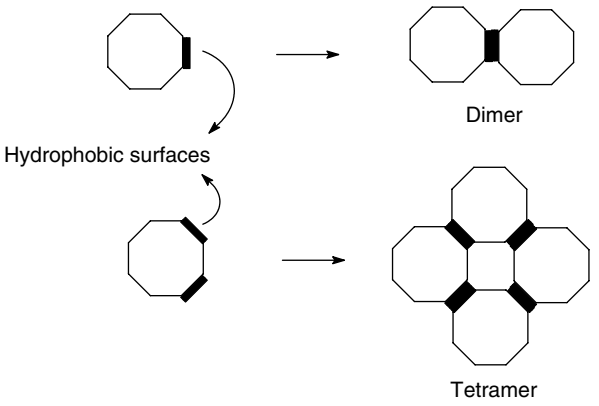


FIGURE 5.8 Schematic representation of formation of dimers and oligomers in proteins.

subunits (monomers) that are the same (homogeneous) or different (heterogeneous). For example, β-lactoglobulin exits as a dimer in the pH range 5–8, as an octomer in the pH range 3–5, and as a monomer above pH 8, and the monomeric units of these complexes are identical. On the other hand, hemoglobin is a tetramer made up of two different polypeptide chains, that is, α and β chains.

Formation of oligomeric structures is the result of specific protein–protein interactions. These are primarily driven by noncovalent interactions such as hydrogen bonding, hydrophobic and electrostatic interactions. The fraction of hydrophobic amino acids appears to influence the tendency to form oligomeric proteins. Proteins that contain >30% hydrophobic amino acid residues exhibit a greater tendency to form oligomeric structures than do those that contain fewer hydrophobic amino acid residues.

Formation of quaternary structure is primarily driven by the thermodynamic requirement to bury exposed hydrophobic surfaces of subunits. When the hydrophobic amino acid content of a protein is >30%, it is physically impossible to form a tertiary structure that will bury all of the nonpolar residues. Consequently, there is a greater likelihood of hydrophobic patches to exist on the surface, and interaction of these patches between adjacent monomers can lead to the formation of dimers, trimers, and so forth (Figure 5.8).

Many food proteins, especially cereal proteins, exist as oligomers of different polypeptides. As would be expected, these proteins typically contain more than 35% hydrophobic amino acid residues (Ile, Leu, Trp, Tyr, Val, Phe, and Pro). In addition, they also contain 6–12% proline [15]. As a consequence, cereal proteins exist in complex oligomeric structures. The major storage proteins of soybean, namely β-conglycinin and glycinin, contain about 41 and 39% hydrophobic amino acid residues, respectively. β-Conglycinin is a trimeric protein made up of three different subunits, and it exhibits complex association–dissociation phenomenon as a function of ionic strength and pH [89,123]. Glycinin is made up of 12 subunits, six of the subunits being acidic and the others basic. Each basic subunit is cross-linked to an acid subunit via a disulfide bond. The six acidic–basic pairs are held together in the oligomeric state by noncovalent interactions. Glycinin also exhibits complex association–dissociation behavior as a function of ionic strength [89].

In oligomeric proteins, the accessible surface area, A_s, is correlated to the molecular weight of the oligomer [84] by:

$$A_s = 5.3M^{0.76} \tag{5.25}$$

This relationship is different from that which applies to monomeric proteins. The surface area buried when the native oligomeric structure is formed from its constituent polypeptide subunits can

be estimated from the equation:

$$A_b = A_t - A_s = (1.48M + 21) - 5.3M_{0.76} \tag{5.26}$$

where A_t is the total accessible area of the nascent polypeptide subunits in their fully extended state.

5.3.2 Forces Involved in the Stability of Protein Structure

The process of folding of a random polypeptide chain into a unique three-dimensional structure is quite complex. As mentioned earlier, the basis for the biologically native conformation is encoded in the amino acid sequence of the protein. In the 1960s, Anfinsen and coworkers showed that when denatured ribonuclease was added to a physiological buffer solution, it refolded to its native conformation and regained almost 100% of its biological activity. Several enzymes have been subsequently shown to exhibit similar propensity. The slow but spontaneous transformation of an unfolded state to a folded state is facilitated by several intramolecular noncovalent interactions. The native conformation of a protein is a thermodynamic state in which various favorable interactions are maximized, and the unfavorable ones are minimized such that the overall free energy of the protein molecule is at the lowest possible value. The forces that contribute to protein folding may be grouped into two categories: (1) intramolecular interactions emanating from forces intrinsic to the protein molecule and (2) intramolecular interactions affected by the surrounding solvent. van der Waals and steric interactions belong to the former and hydrogen bonding, electrostatic, and hydrophobic interactions belong to the latter.

5.3.2.1 Steric Strains

Although the ϕ and ψ angles theoretically have 360° rotational freedom, their values are very much restricted because of steric hindrance from side-chain atoms. Because of this, segments of a polypeptide chain can assume only a limited number of configurations. Distortions in the planar geometry of the peptide unit, or stretching and bending of bonds, will cause an increase in the free energy of the molecule. Therefore, folding of the polypeptide chain can occur only in such a way that deformation of bond lengths and bond angles are avoided.

5.3.2.2 van der Waals Interactions

These are dipole-induced dipole and induced dipole-induced dipole interactions between neutral atoms in protein molecules. When two atoms approach each other, each atom induces a dipole in the other via polarization of the electron cloud. The interaction between these induced dipoles has an attractive as well as a repulsive component. The magnitudes of these forces are dependent on the interatomic distance. The attractive energy is inversely proportional to the sixth power of the interatomic distance, and the repulsive interaction is inversely proportional to the 12^{th} power of this distance. Therefore, at a distance r, the net interaction energy between two atoms is given by the potential energy function:

$$E_{vdW} = E_a + E_r = \frac{A}{r^6} + \frac{B}{r^{12}} \tag{5.27}$$

where A and B are constants for a given pair of atoms, and E_a and E_r are the attractive and repulsive interaction energies, respectively. van der Waals interactions are very weak, decrease rapidly with distance and become negligible beyond 6 Å. The van der Waals interaction energy for various pairs of atoms ranges from −0.04 to −0.19 kcal/mol. In proteins, however, since numerous pairs of atoms are involved in van der Waals interactions, sum of its contribution to protein folding and stability is very significant.

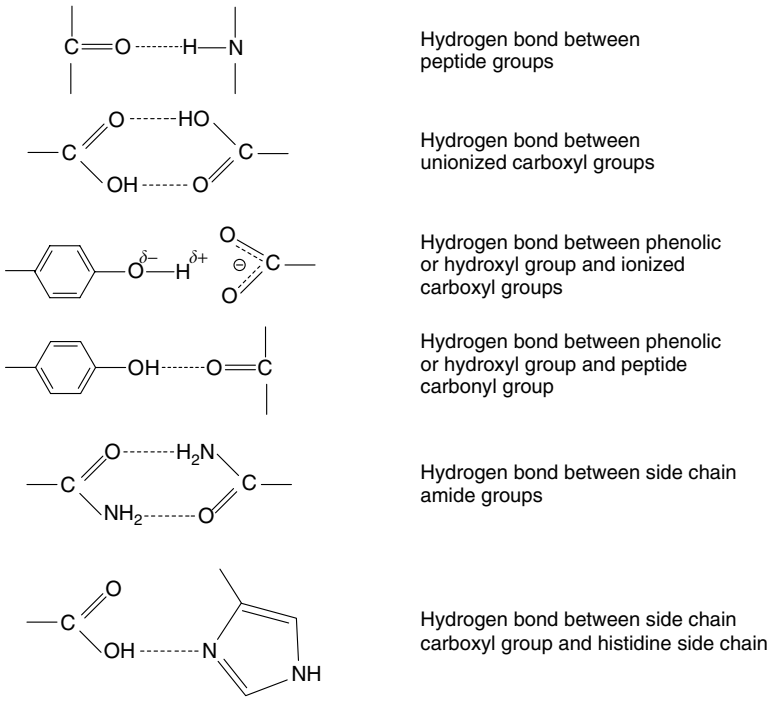


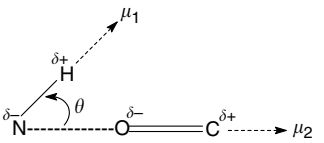
FIGURE 5.9 H-bonding groups in proteins. (From Scheraga, H. A. 1963. In *The Proteins*, 2nd edn., Vol. 1 (Neurath, H., Ed.), Academic Press, New York, pp. 478–594.)

5.3.2.3 Hydrogen Bonds

The hydrogen bond involves the interaction of a hydrogen atom that is covalently attached to an electronegative atom (such as N, O, or S) with another electronegative atom. Schematically, a hydrogen bond may be represented as D—H $\cdots$ A, where D and A are, the donor and acceptor electronegative atoms, respectively. The strength of a hydrogen bond ranges between 2 and 7.9 kcal/mol, depending on the pair of electronegative atoms involved and the bond angle.

Proteins contain several groups capable of forming hydrogen bonds. Some of the possible candidates are shown in Figure 5.9 [113]. Among these groups, the greatest number of hydrogen bonds are formed between the N—H and C═O groups of the peptide bonds in α-helix and β-sheet structures.

The peptide hydrogen bond can be considered as a strong permanent dipole–dipole interaction between the $N^{\delta-}$—$H^{\delta+}$ and $C^{\delta+}$═$O^{\delta-}$ dipoles as shown below:



(5.28)

The strength of the hydrogen bond is given by the potential energy function:

$$E_{\text{H-bond}} = \frac{\mu_1 \mu_2}{4\pi \varepsilon_0 \varepsilon r^3} \cos\theta \tag{5.29}$$

where μ_1 and μ_2 are the dipole moments, ε_0 is the permittivity of the vacuum, ε is the dielectric constant of the medium, r is the distance between the electronegative atoms, and θ is the hydrogen

bond angle. The hydrogen bond energy is directly proportional to the product of the dipole moments and to the cosine of the bond angle, and is inversely proportional to the third power of the N···O distance and to the dielectric constant of the medium. The strength of the hydrogen bond reaches a maximum when θ is zero, and it is zero when θ is 90°. The hydrogen bonds in α-helix and antiparallel β-sheet structures have a θ value very close to zero; whereas those in parallel β-sheets have larger θ values. The optimum N···O distance for maximum hydrogen bond energy is 2.9 Å. At shorter distances, the electrostatic repulsive interaction between the $N^{\delta-}$ and $O^{\delta-}$ atoms causes a significant decrease in the strength of the hydrogen bond. At longer distances weak dipole–dipole interaction between the N—H and C═O groups decreases the strength of the hydrogen bond. The strength of N—H···O═C hydrogen bonds in the interior of proteins, where the dielectric constant is close to 1, is typically about 4.5 kcal/mol. The "strength" refers to the amount of energy needed to break the bond.

The existence of hydrogen bonds in proteins is well established. Since formation of each hydrogen bond decreases the free energy of the protein by about –4.5 kcal/mol, it is commonly believed that they may act not only as the driving force for protein folding but also may contribute enormously to the stability of the native structure. However, this is not a valid assumption. Because water can compete for hydrogen bonding with N—H and C═O groups in proteins, hydrogen bonding between these groups cannot occur spontaneously, nor can formation of N—H···O═C hydrogen bonds be the driving force for formation of α-helix and β-pleated sheets in proteins. The hydrogen bond is primarily an ionic interaction. Like other ionic interactions, its stability also depends upon the dielectric constant of the environment. The stability of hydrogen bonds in secondary structures is mainly due to a low dielectric created by interaction between nonpolar residues. These bulky side chains prevent access of water to the N—H···O═C hydrogen bonds. They are only stable as long as they are protected from water.

5.3.2.4 Electrostatic Interactions

As noted earlier, proteins contain several amino acid residues with ionizable groups. At neutral pH, Asp and Glu residues are negatively charged, and Lys, Arg, and His are positively charged. At alkaline pH, Cys and Tyr residues assume a negative charge.

Depending upon the relative number of negatively and positively charged residues, proteins assume either a net negative or a net positive charge at neutral pH. The pH at which the net charge is zero is called the *isoelectric pH* (pI). The isoelectric pH is different from the *isoionic point*. Isoionic point is the pH of the protein solution in the absence of electrolytes. The isoelectric pH of a protein can be estimated from its amino acid composition and the pK_a values of the ionizable groups using the Hendersen–Hasselbach equation (Equation 5.5).

With few exceptions, almost all charged groups in proteins are distributed on the surface of the protein molecule. Since at neutral pH proteins assume either a net positive or a net negative charge, one might expect that the net repulsive interaction between like-charges would destabilize protein structure. It is also reasonable to assume that attractive interactions between oppositely charged groups at certain critical locations might contribute to the stability of the protein structure. In reality, however, the strength of these repulsive and attractive forces is minimized in aqueous solutions because of the high permittivity of water. The electrostatic interaction energy between two fixed charges q_1 and q_2 separated by distance r is given by:

$$E_{ele} = \pm \frac{q_1 q_2}{4\pi \varepsilon_0 \varepsilon r} \tag{5.30}$$

In vacuum or air ($\varepsilon = 1$), the electrostatic interaction energy between two charges at a distance of 3 to 5 Å is about ±110 to ±66 kcal/mol. In water, however, this interaction energy is reduced to ±1.4 to ±0.84 kcal/mol, which is of the order of thermal energy of the protein molecule at 37°C. Therefore, the attractive and repulsive electrostatic interactions between charges located on the protein surface

do not contribute significantly to protein stability. However, charged groups partially buried in the protein interior, where the permittivity is lower than that of water, usually form salt bridges with strong interaction energy. Thus, the electrostatic interaction energy may range between ±0.84 and ±110 kcal/mol depending on the distance and the local permittivity.

Although electrostatic interactions may not act as the primary driving force for protein folding, the penchant of charged groups to remain exposed to the aqueous environment certainly would influence the folding pattern.

5.3.2.5 Hydrophobic Interactions

It should be obvious from the foregoing discussions that, in aqueous solutions, the hydrogen bonding and electrostatic interactions between various polar groups in a polypeptide chain do not possess sufficient energy to act as driving forces for protein folding. These polar interactions in proteins are not very stable in an aqueous environment and their stabilities depend on maintenance of an apolar environment. The major force driving protein folding comes from hydrophobic interactions among nonpolar groups.

In aqueous solutions, the hydrophobic interaction between nonpolar groups is the result of thermodynamically unfavorable interaction between water and nonpolar groups. When a hydrocarbon is dissolved in water, the standard free energy change (ΔG) is positive and the volume (ΔV) and enthalpy change (ΔH) are negative. Even though ΔH is negative, meaning that there is favorable interaction between water and the hydrocarbon, ΔG is positive. Since $\Delta G = \Delta H - T\Delta S$ (where T is the temperature and ΔS is the entropy change), the positive change in ΔG must result from a large negative change in entropy, which offsets the favorable change in ΔH. The decrease in entropy is caused by formation of a clathrate or cage-like water structure around the hydrocarbon. Because of the net positive change in ΔG, interaction between water and nonpolar groups is highly restricted. Consequently, in aqueous solutions, nonpolar groups tend to aggregate, so that the area of direct contact with water is minimized (see Chapter 2). This water structure-induced interaction between nonpolar groups in aqueous solutions is known as hydrophobic interaction. In proteins, hydrophobic interaction between nonpolar side chains of amino acid residues is the major reason that proteins fold into unique tertiary structures in which a majority of the nonpolar groups are removed from the aqueous environment.

Since the hydrophobic interaction is the antithesis of solution of nonpolar groups in water, ΔG for hydrophobic interaction is negative and, ΔV, ΔH, and ΔS are positive. Unlike other noncovalent interactions, hydrophobic interactions are endothermic; that is, hydrophobic interactions are stronger at high temperatures and weaker at low temperatures (opposite to that for hydrogen bonds). The variation of hydrophobic free energy with temperature usually follows a quadratic function,

$$\Delta G_{H\phi} = a + bT + cT^2 \tag{5.31}$$

where a, b, and c are constants, and T is absolute temperature.

The hydrophobic interaction energy between two spherical nonpolar molecules can be estimated from the potential energy equation [59]:

$$E_{H\phi} = -20\frac{R_1 R_2}{R_1 + R_2} e^{-D/D_0} \text{ kcal/mol} \tag{5.32}$$

where R_1 and R_2 are the radii of the nonpolar molecules, D is the distance in nm between the molecules, and D_0 is the decay length (1 nm). Unlike electrostatic, hydrogen bonding, and van der Waals interactions, which follow a power law relationship with distance between interacting groups, the hydrophobic interaction follows an exponential relationship with distance between interacting groups. Thus, it is effective over relatively long distances (e.g., 10 nm).

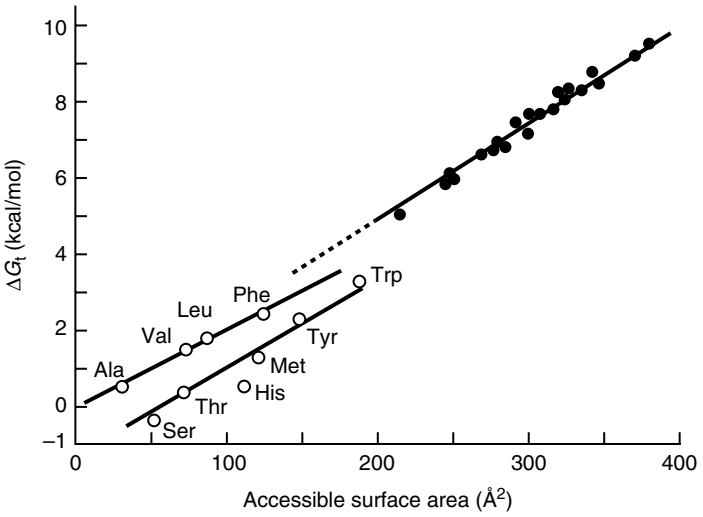


FIGURE 5.10 The relationship between hydrophobicity and accessible surface area of amino acid side chains (open circles) and hydrocarbons (filled circles). (From Richards, F. M. 1977. *Ann. Rev. Biophys. Bioeng.* **6**:151–176. Courtesy of Annual Reviews, Inc.)

The hydrophobic free energy of proteins cannot be quantified using the above equation because of involvement of several nonpolar groups. It is possible, however, to estimate the hydrophobic free energy of a protein using other empirical correlations. The hydrophobic free energy of a molecule is directly proportional to the nonpolar surface area that is accessible to water (Figure 5.10) [107]. The proportionality constant, that is, the slope, varies between 22 cal mol^{-1}Å^{-2} for Ala, Val, Leu, and Phe, and 26 cal mol^{-1}Å^{-2} for Ser, Thr, Trp, and Met side chains. On average, the hydrophobicity of amino acid side chains is about 24 cal mol^{-1}Å^{-2}. This is close to the 25 cal mol^{-1}Å^{-2} value for alkanes. This means that for the removal of every one Å^2 area of nonpolar surface from the water environment, a protein will decrease its free energy by about 24 cal/mol. Thus, the hydrophobic free energy of a protein can be estimated simply by multiplying the total buried surface area by 24 cal mol^{-1}Å^{-2}.

The buried surface area in several globular proteins and the estimated hydrophobic free energies are shown in Table 5.8 [11]. It is evident that hydrophobic free energy contributes significantly to the stability of protein structure. The average hydrophobic free energy per amino acid residue in globular proteins amounts to about 2.5 kcal/mol.

5.3.2.6 Disulfide Bonds

Disulfide bonds are the only covalent side chain cross-links found in proteins. They can occur both intramolecularly and intermolecularly. In monomeric proteins, disulfide bonds are formed as a result of protein folding. When two Cys residues are brought into proximity with proper orientation, oxidation of the sulfhydryl groups by molecular oxygen results in disulfide bond formation. Once formed, disulfide bonds help stabilize the folded structure of proteins.

Protein mixtures containing cystine and Cys residues are able to undergo sulfhydryl–disulfide interchange reactions as shown below:

(5.33)

TABLE 5.8
Accessible Surface Area (A_s), Buried Surface Area (A_b), and Hydrophobic Free Energy of Proteins

Protein	MW (Daltons)	A_s (Å^2)	A_b (Å^2)	$\Delta G_{H\phi}$ (kcal/mol)
Parvalbumin	11,450	5,930	11,037	269
Cytochrome c	11,930	5,570	12,107	294
Ribonuclease A	13,690	6,790	13,492	329
Lysozyme	14,700	6,620	15,157	369
Myoglobin	17,300	7,600	18,025	439
Retinol binding protein	20,050	9,160	20,535	500
Papain	23,270	9,140	25,320	617
Chymotrypsin	25,030	10,440	26,625	648
Subtilsin	27,540	10,390	30,390	739
Carbonic anhydrase B	28,370	11,020	30,988	755
Carboxypeptidase A	34,450	12,110	38,897	947
Thermolysin	34,500	12,650	38,431	935

A_s values are from Reference 84.
A_b was calculated from Equations 5.22 and 5.23.

This interchange reaction also can occur within a single denatured protein if it contains a free sulfhydryl group and a disulfide bond. The interchange reaction often leads to a decrease in stability of the protein molecule.

In summary, the formation of a unique three-dimensional protein structure is the net result of various repulsive and attractive noncovalent interactions and any covalent disulfide bonds.

5.3.3 Conformational Stability and Adaptability of Proteins

The stability of the native protein structure is defined as the difference in free energy between the native and denatured (or unfolded) states of the protein molecule. This is usually denoted as ΔG_D. This refers to the amount of energy needed to unfold a protein from the native state to the denatured state.

All of the noncovalent interactions discussed above, except the repulsive electrostatic interactions, contribute to the stability of the native protein structure. The stabilizing influence on the native structure of the total free energy changes attributed to these interactions amounts to hundreds of kcal/mol. However, the net ΔG_D of the majority of proteins is in the range of 5–20 kcal/mol. The major force that destabilizes the native structure is the conformational entropy of the polypeptide chain. The loss of translational, rotational, and vibrational motions that occurs when a polypeptide in a disordered state is folded into a compact structure decreases its conformational entropy. The increase in free energy resulting from this loss of conformational entropy is more than offset by favorable noncovalent interactions, resulting in a net decrease in free energy. Thus, the difference in free energy between the native and denatured states can be expressed as

$$\Delta G_{D\rightarrow N} = \Delta G_{\text{H-bond}} + \Delta G_{ele} + \Delta G_{H\phi} + \Delta G_{vdW} - T\Delta S_{conf} \tag{5.34}$$

where $\Delta G_{\text{H-bond}}$, ΔG_{ele}, $\Delta G_{H\phi}$, and ΔG_{vdW}, respectively, are free energy changes for hydrogen bonding, electrostatic, hydrophobic, van der Waals interactions, and ΔS_{conf} is the conformational entropy of the polypeptide chain. The ΔS_{conf} of a protein in the unfolded state is about 1.9 to 10 cal mol^{-1}K^{-1} per residue. Usually, an average value of 5.2 cal mol^{-1}K^{-1} per residue is assumed.

TABLE 5.9
ΔG_D Values for Selected Proteins

Protein	pH	T (°C)	ΔG_D (kcal/mol)
α-Lactalbumin	7	25	4.4
Bovine β-lactoglobulin A + B	7.2	25	7.6
Bovine β-lactoglobulin A	3.15	25	10.2
Bovine β-lactoglobulin B	3.15	25	11.9
T4 Lysozyme	3.0	37	4.6
Hen egg-white lysozyme	7.0	37	12.2
G-Actin	7.5	25	6.5
Lipase (from Aspergillus)	7.0	—	11.2
Troponin	7.0	37	4.7
Ovalbumin	7.0	25	6.0
Cytochrome C	5.0	37	7.9
Ribonuclease	7.0	37	8.1
α-Chymotrypsin	4.0	37	8.1
Trypsin	—	37	13.2
Pepsin	6.5	25	10.9
Growth hormone	8.0	25	14.2
Insulin	3.0	20	6.5
Alkaline phosphatase	7.5	30	20.3

ΔG_D represents $G_U - G_N$, where G_U and G_N are free energies of the denatured and native states, respectively, of a protein molecule.

Source: Compiled from several sources.

A protein with 100 amino acid residues at 310 K will have conformational entropy of about $5.2 \times 100 \times 310 = 161.2$ kcal/mol. This destabilizing conformational energy will reduce the net stability of the native structure resulting from noncovalent interactions.

The ΔG_D values, that is, energy required to unfold, of various proteins are presented in Table 5.9. These values clearly indicate that in spite of numerous intramolecular interactions, proteins are only marginally stable. For example, the ΔG_D values of most proteins correspond to an energy equivalent of one to three hydrogen bonds or about two to five hydrophobic interactions, suggesting that breakage of a few noncovalent interactions would destabilize the native structure of many proteins.

Conversely, it appears that proteins are not designed to be rigid molecules. They are flexible, their native state is in a metastable state, and breakage of one to three hydrogen bonds or a few hydrophobic interactions can easily cause a cooperative conformational change in proteins. Conformational adaptability to changing solution conditions is necessary to enable proteins to carry out several critical biological functions. For example, efficient binding of substrates or prosthetic ligands to enzymes invariably involves reorganization of polypeptide segments at the binding sites. On the other hand, proteins that require high structural stability to perform their physiological functions usually are stabilized by intramolecular disulfide bonds, which effectively counter the conformational entropy (i.e., the tendency of the polypeptide chain to unfold).

5.4 PROTEIN DENATURATION

The native structure of a protein is the net result of various attractive and repulsive interactions emanating from assorted intramolecular forces as well as interaction of various protein groups with surrounding solvent water. However, native structure is largely the product of the protein's environment. The native state is thermodynamically the most stable state with lowest feasible free

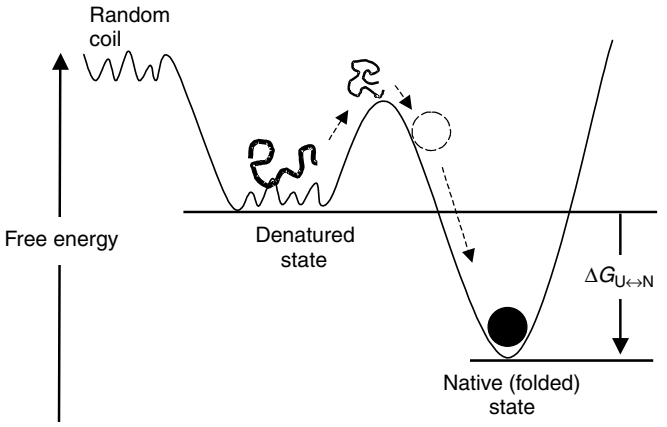


FIGURE 5.11 Schematic representation of the energy of a protein molecule as a function of its conformation. The conformation with the lowest energy is usually the native state.

energy. Any change in its environment, such as pH, ionic strength, temperature, solvent composition, and so forth, will force the molecule to assume a new equilibrium structure. Subtle changes in structure that do not drastically alter the molecular architecture of the protein are usually regarded as "conformational adaptability," whereas major changes in the secondary, tertiary, and quaternary structures without cleavage of backbone peptide bonds are regarded as "denaturation." From a structural standpoint, while the native structure of a protein is a well-defined entity with structural coordinates for each and every atom in the molecule obtainable from its crystallographic structure, it is not the case for the denatured state. Denaturation is a phenomenon wherein a well-defined initial state of a protein formed under physiological conditions is transformed into an ill-defined final state under nonphysiological conditions using a denaturing agent. It does not involve any chemical changes in the protein. In the denatured state, because of a greater degree of rotational motions of dihedral angles of the polypeptide chain, the protein can assume several conformational states differing only marginally in free energy. This is shown schematically in Figure 5.11. Some denatured states possess more residual folded structure than others. It should be noted that even in the fully denatured state, typical globular proteins, with the exception of gelatin, do not behave like a true random coil. This is because of the fact that the partial double bond character of the amide linkages and local steric restrictions caused by bulky side chains do not permit 360° rotational freedom for the polypeptide backbone.

The intrinsic viscosity ($[\eta]$) of a fully denatured protein is a function of the number of amino acid residues and is expressed by [121]:

$$[\eta] = 0.716\eta^{0.66} \tag{5.35}$$

where n is the number of amino acid residues in the protein.

Often, denaturation has a negative connotation, because it indicates loss of some properties. Many biologically active proteins lose their activity upon denaturation. In the case of food proteins, denaturation usually causes loss of solubility and some functional properties. However, from a food application standpoint, protein denaturation during processing is not always undesirable. In fact, in some cases it is highly desirable. For instance, partial denaturation of proteins at the air–water and oil–water interfaces improves their foaming and emulsifying properties, whereas excessive thermal denaturation of soy proteins diminishes their foaming and emulsifying properties. On the other hand, thermal denaturation markedly improves digestibility of legume proteins as a result of inactivation of trypsin inhibitors. In general, partially denatured proteins are more digestible than native proteins. In protein beverages, where high solubility and dispersibility of proteins is required, even partial

denaturation of protein during processing may cause flocculation and precipitation during storage and thus may adversely affect the sensory attributes of the product. Thermal denaturation is also a prerequisite for heat-induced gelation of food proteins. Thus, to develop appropriate processing strategies, it is imperative to have a basic understanding of the environmental and other factors that affect structural stability of proteins in food systems.

5.4.1 Thermodynamics of Denaturation

Denaturation is a phenomenon that involves transformation of a well-defined, folded structure of a protein, formed under physiological conditions, to an unfolded state under nonphysiological conditions. Since structure is not an easily quantifiable parameter, direct measurement of the fractions of native and denatured protein in a solution is not possible. However, conformational changes in proteins invariably affect several of its chemical and physical properties, such as ultraviolet-absorbance, fluorescence, viscosity, sedimentation coefficient, optical rotation, circular dichroism, reactivity of sulfhydryl groups, and enzyme activity. Thus, protein denaturation can be studied by monitoring changes in these physical and chemical properties.

When changes in a physical or chemical property, y, is monitored as a function of denaturant concentration or temperature, many monomeric globular proteins exhibit denaturation profiles as shown in Figure 5.12. y_N and y_D are y values for the native and denatured states, respectively, of the protein.

For most proteins, as denaturant concentration (or temperature) is increased, the value of y remains unchanged initially, and above a critical point its value changes abruptly from y_N to y_D within a narrow range of denaturant concentration or temperature. For a majority of globular proteins, this transition is very steep, indicating that protein denaturation is a cooperative process. That is, once a protein molecule begins to unfold, or once a few interactions in the protein are broken, the whole molecule completely unfolds with a further slight increase in denaturant concentration or temperature. This cooperative nature of unfolding suggests that globular proteins can exist only in the native and denatured states, that is, intermediate states are not possible. This is known as a

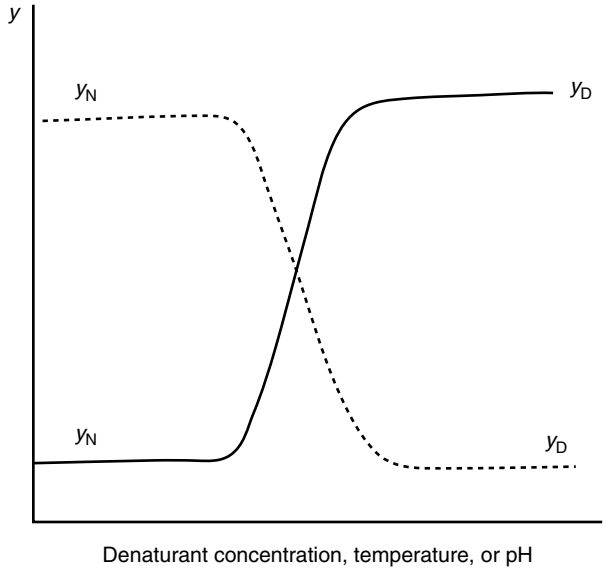


FIGURE 5.12 Typical protein denaturation curves. y represents any measurable physical or chemical property of the protein molecule that varies with protein conformation. y_N and y_D are the values of y for the native and denatured states, respectively.

"*two-state transition*" model. For this two-state model, the equilibrium between the native and the denatured state in the cooperative transition region can be expressed as

$$\begin{gathered} \mathrm{N} \overset{K_\mathrm{D}}{\rightleftharpoons} \mathrm{D} \\ K_\mathrm{D} = [\mathrm{D}]/[\mathrm{N}] \end{gathered} \tag{5.36}$$

where K_D is the equilibrium constant. Since the concentration of denatured protein molecules in the absence of a denaturant (or critical input of heat) is extremely low (about 1 in 10^9), estimation of K_D is not possible. However, in the transition region, that is, at sufficiently high denaturant concentration (or sufficiently high temperature), an increase in the population of the denatured protein molecule permits determination of the apparent equilibrium constant, $K_{\mathrm{D,app}}$. In the transition region, where both native and denatured protein molecules are present, the value of y is given by:

$$y = f_\mathrm{N} y_\mathrm{N} + f_\mathrm{D} y_\mathrm{D} \tag{5.37}$$

where f_N and f_D are the fractions of the protein in the native and denatured states, and y_N and y_D are y values for the native and denatured states, respectively. From Figure 5.12,

$$f_\mathrm{N} = \frac{(y_\mathrm{D} - y)}{(y_\mathrm{D} - y_\mathrm{N})} \tag{5.38}$$

$$f_\mathrm{D} = \frac{(y - y_\mathrm{N})}{(y_\mathrm{D} - y_\mathrm{N})} \tag{5.39}$$

The apparent equilibrium constant is given by:

$$K_\mathrm{app} = \frac{f_\mathrm{D}}{f_\mathrm{N}} = \frac{(y - y_\mathrm{N})}{(y - y_\mathrm{D})} \tag{5.40}$$

and the free energy of denaturation is given by:

$$\Delta G_\mathrm{app} = RT \ln K_{\mathrm{D,app}} \tag{5.41}$$

A plot of $-RT \ln K_{\mathrm{D,app}}$ vs. denaturant concentration is usually linear and thus the K_D and ΔG_D of the protein in pure water (or in buffer in the absence of denaturant) is obtained from the y-intercept. The enthalpy of denaturation, ΔH_D, is obtained from variation of the free energy change with temperature using the van't Hoff equation:

$$\Delta H_\mathrm{D} = -R \frac{\mathrm{d}(\ln K_\mathrm{D})}{\mathrm{d}(1/T)} \tag{5.42}$$

Monomeric proteins that contain two or more domains with different structural stabilities usually exhibit multiple transition steps in the denaturation profile. If the transition steps are well separated, the stabilities of each domain can be obtained from the transition profile by using the above two-state model. Denaturation of oligomeric proteins proceeds via dissociation of subunits, followed by denaturation of the subunits.

Protein denaturation can be reversible, especially for small monomeric proteins. When the denaturant is removed from the protein solution (or the sample is cooled), most monomeric proteins (in the absence of aggregation) refold to their native conformation under appropriate solution conditions, such as pH, ionic strength, redox potential, and protein concentration. Many proteins refold when the protein concentration is below 1 μM. Above 1 μM protein concentration, refolding

is partially inhibited because of greater intermolecular interaction at the cost of intramolecular interactions. A redox potential comparable to that of biological fluid facilitates formation of the correct pairs of disulfide bonds during refolding.

5.4.2 Denaturing Agents

5.4.2.1 Physical Agents

5.4.2.1.1 Temperature and Denaturation

Heat is the most commonly used denaturing agent in food processing and preservation. Proteins undergo varying degrees of denaturation during processing. This can affect their functional properties in foods and it is, therefore, important to understand the factors affecting protein denaturation.

When a protein solution is gradually heated above a critical temperature, it undergoes a sharp transition from the native state to the denatured state. The temperature at the transition midpoint, where the concentration ratio of native and denatured states is 1, is known either as the melting temperature T_m, or the denaturation temperature T_d. The mechanism of temperature-induced denaturation of proteins primarily involves the effect of temperature on the stability of noncovalent interactions. In this respect, the hydrogen bonding and electrostatic interactions, which are exothermic in nature, are destabilized, and hydrophobic interactions, which are endothermic, are stabilized as the temperature is increased. The strength of hydrophobic interactions reaches a maximum at about 70–80°C [22]. In addition to noncovalent interactions, temperature dependence of conformational entropy, $T\Delta S_{conf}$, also plays a major role in the stability of proteins. The conformational entropy of the chain increases as the temperature is increased, which favors an unfolded state. The net stability of a protein at a given temperature is then the sum total of these interactions. However, a careful analysis of the temperature effect on various interactions in proteins reveals the following: in globular proteins, the majority of charged groups exist on the surface of the protein molecule, fully exposed to the high dielectric aqueous medium. Because of the dielectric screening effect of water, attractive and repulsive electrostatic interactions between charged residues are greatly reduced. In addition, at physiological ionic strength, screening of charged groups in proteins by counter ions further reduces electrostatic interactions in proteins. Because of these facts, the influence of electrostatic interactions in proteins is not significant. Similarly, hydrogen bonds are unstable in an aqueous environment, and therefore their stability in proteins is dependent on hydrophobic interactions that create local low dielectric environment. This implies that so long as a nonpolar environment is maintained, the hydrogen bonds in proteins would remain intact when the temperature is increased. These facts suggest that although polar interactions are affected by temperature, they generally do not contribute to heat-induced denaturation of proteins. On the basis of these considerations, the stability of the native state of a protein can be simply regarded as the net free energy difference arising from hydrophobic interactions that tend to favor the folded state and the conformational entropy of the chain that favor the unfolded state. That is,

$$\Delta G_{N\rightarrow D} = \Delta G_{H\phi} + \Delta G_{conf} \tag{5.43}$$

The dependence of protein stability on temperature at constant pressure can be expressed as [33]

$$\frac{\partial \Delta G_{N\rightarrow D}}{\partial T} = \frac{\partial \Delta G_{H\phi}}{\partial T} + \frac{\partial \Delta G_{conf}}{\partial T} \tag{5.44}$$

Hydrophobic interactions are strengthened at higher temperatures; therefore, $\partial \Delta G_{H\phi}/\partial T > 0$. Conformational entropy increases upon unfolding of the protein; therefore, $\partial \Delta G_{conf}/\partial T < 0$. As the temperature is increased, the interplay between these opposing forces reach a point at which $\partial \Delta G_{N\rightarrow D}/\partial T \approx 0$. The temperature at which this occurs signifies the denaturation temperature (T_d)

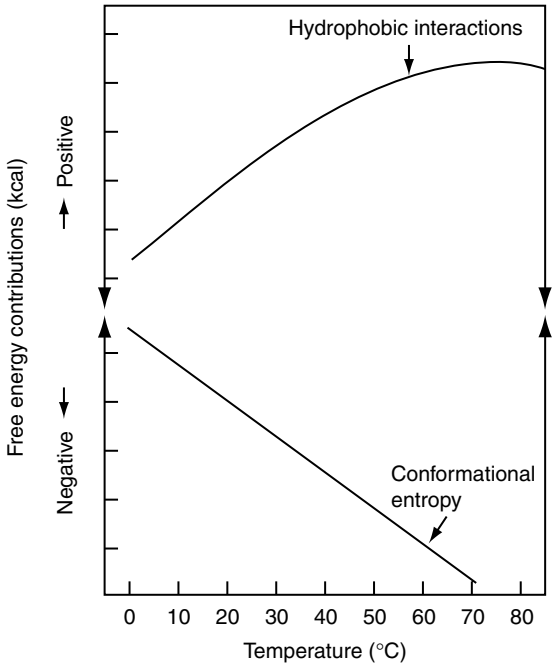


FIGURE 5.13 Relative changes in free energy contributions by hydrogen bonding, hydrophobic interactions, and conformational entropy to the stability of proteins as a function of temperature.

of the protein. The relative contributions of the major forces to stability of a protein molecule as a function of temperature are depicted in Figure 5.13. Note that the stability of hydrogen bonds in proteins is not significantly affected by the temperature. The T_d values of some proteins are listed in Table 5.10 [14].

It is often assumed that the lower the temperature, the greater will be the stability of a protein. This is not always true. Some proteins are denatured at cold temperatures [16]. For example (Figure 5.14) [18,73], the stability of lysozyme increases with lowering of temperature, whereas those of myoglobin and a mutant phage T4 lysozyme show maximum stability at about 30 and 12.5°C, respectively. Below and above these temperatures, myoglobin and phage T4 lysozyme are less stable. When stored below 0°C, these two proteins undergo cold-induced denaturation. The temperature of maximum stability (minimum free energy) depends on the relative inpact of temperature on the stabilizing and destabilizing forces in the protein. Proteins that are primarily stabilized by hydrophobic interactions are more stable at about ambient temperature than they are at refrigeration temperature. Intramolecular disulfide bonds in proteins tend to stabilize proteins at low as well as high temperatures because they counter conformational entropy of the protein chain.

Several food proteins undergo reversible dissociation and denaturation at low temperature. Glycinin, one of the storage proteins of soybean, aggregates and precipitates when stored at 2°C [68], then becomes soluble when returned to ambient temperature. When skim milk is stored at 4°C, β-casein dissociates from casein micelles, and this alters the physicochemical and rennetting properties of casein micelles. Several oligomeric enzymes, such as lactate dehydrogenase and glyceraldehyde-phosphate dehydrogenase, lose most of their enzyme activity when stored at 4°C; and this has been attributed to dissociation of the subunits. However, when warmed to and held at ambient temperature for a few hours, they reassociate and completely regain their activity [127].

The amino acid composition affects thermal stability of proteins. Proteins that contain a greater proportion of hydrophobic amino acid residues, especially Val, Ile, Leu, and Phe, tend to be more stable than the more hydrophilic proteins [137]. A strong positive correlation also exists between

TABLE 5.10
Thermal Denaturation Temperatures (T_d) and Mean Hydrophobicities of Proteins

Protein	T_d	Mean Hydrophobicity (kcal mol^{-1} $Residue^{-1}$)
Trypsinogen	55	0.89
Chymotrypsinogen	57	0.90
Elastase	57	—
Pepsinogen	60	0.97
Ribonuclease	62	0.78
Carboxypeptidase	63	—
Alcohol dehydrogenase	64	—
Bovine serum albumin	65	1.02
Hemoglobin	67	0.96
Lysozyme	72	0.90
Insulin	76	1.00
Egg albumin	76	0.97
Trypsin inhibitor	77	—
Myoglobin	79	1.05
α-Lactalbumin	83	1.03
Cytochrome C	83	1.06
β-Lactoglobulin	83	1.09
Avidin	85	0.92
Soy glycinin	92	—
Broadbean 11S protein	94	—
Sunflower 11S protein	95	—
Oat globulin	108	—

Source: Data were compiled from Bull, H. B. and K. Breese. 1973. *Arch. Biochem. Biophys*. **158**:681–686.

thermostability and the number percent of certain amino acid residues. For example, statistical analysis of fifteen different proteins has shown that thermal denaturation temperatures of these proteins are positively correlated ($r = .98$) to the sum of number percent of Asp, Cys, Glu, Lys, Leu, Arg, Trp, and Tyr residues. On the other hand, thermal denaturation temperatures of the same set of proteins are negatively correlated ($r = -.975$) to the sum of number percent of Ala, Asp, Gly, Gln, Ser, Thr, Val, and Tyr (Figure 5.15) [101]. Other amino acid residues have little influence on T_d.

Thermal stability of proteins from thermophilic and hyperthermophilic organisms, which can withstand extremely high temperatures, is also attributed to their unique amino acid composition [111]. These proteins contain lower levels of Asn and Gln residues than those from mesophilic organisms. The implication here is that because Asn and Gln are susceptible to deamidation at high temperatures, higher levels of these residues in mesophilic proteins may partly contribute to instability. The Cys, Met, and Trp contents, which can be oxidized easily at high temperatures, are also low in hyperthermostable proteins. On the other hand, thermostable proteins have high levels of Ile and Pro [117,126]. The high Ile content is believed to help in better packing of the interior core of the protein [110], which reduces buried cavities or void spaces. Absence of void spaces can reduce mobility of the polypeptide chain at high temperatures and this minimizes the increase in the configurational entropy of the polypeptide chain at high temperatures. A high content of Pro, especially in the loop regions of the protein chain, is believed to provide rigidity to the structure [75,87]. Examination of crystallographic structures of several proteins/enzymes from thermophilic

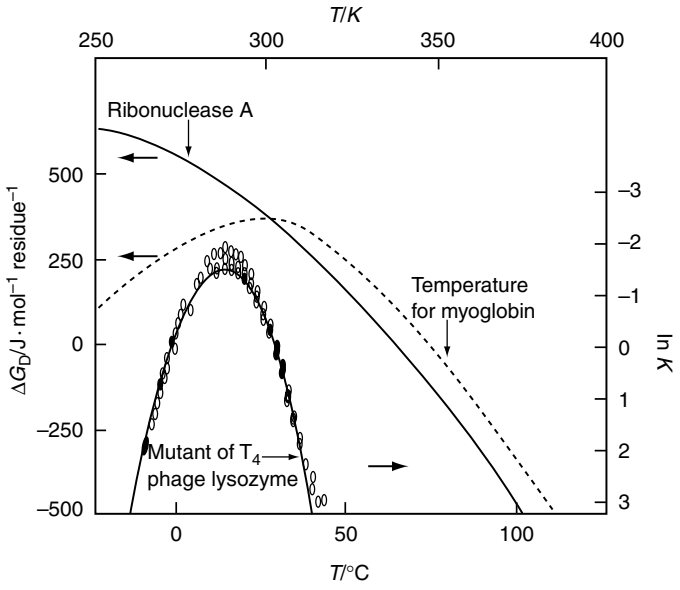


FIGURE 5.14 Variation of protein stability (ΔG_D) with temperature for myoglobin (- - - -), ribonuclease A (—), and a mutant of T4 phage lysozyme (-0-0-). K is the equilibrium constant. (Compiled from Chen, B. and J. A. Schellman. 1989. *Biochemistry* **28**:685–691 and Lapanje, S. 1978. *Physicochemical Aspects of Protein Denaturation*. Wiley-Interscience, New York.)

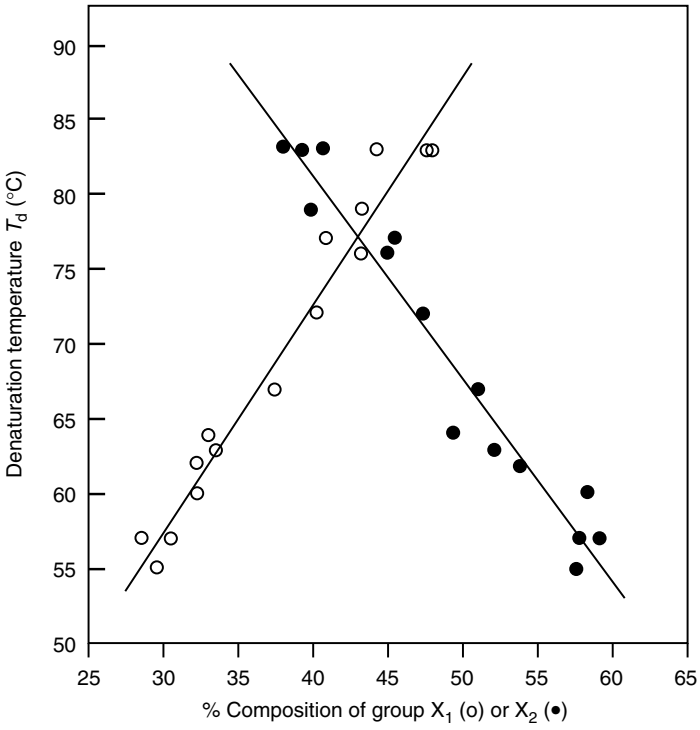


FIGURE 5.15 Group-correlations of amino acid residues to thermal stability of globular proteins. Group X_1 represents Asp, Cys, Glu, Lys, Leu, Arg, Trp, and Tyr. Group X_2 represents Ala, Asp, Gly, Gln, Ser, Thr, Val, and Tyr. (Adapted from Ponnuswamy, P. K. et al. 1982. *Int. J. Biol. Macromol*. **4**:186–190.)

organisms show that they also contain a significantly higher number of ion-pairs in crevices of proteins and a substantially higher amount of buried water molecules engaged in hydrogen bonding bridge between segments than in their mesophilic counterparts [4,132]. Taken together, it appears that polar interactions (both salt bridges and hydrogen bonding between segments) in the nonpolar protein interior are responsible for thermostability of proteins from thermophilic and hyperthermophilic organisms and such an environment is facilitated by a high content of Ile. As discussed earlier, it is conceivable that each salt bridge in the protein interior, where the dielectric constant is about 4, could increase the stability of protein structure by about 20 kcal/mol.

Thermal denaturation of monomeric globular proteins is mostly reversible. For example, when monomeric enzymes are heated above their denaturation temperatures, or even briefly held at 100°C, and then immediately cooled to room temperature, they fully regain their activities. However, thermal denaturation can become irreversible when the protein is heated at 90–100°C for a prolonged period even at neutral pH [5]. This irreversibility occurs because of several chemical changes in the protein, such as deamidation of Asn and Gln residues, cleavage of peptide bonds at Asp residues, destruction of Cys and cystine residues, and aggregation [5,124].

Water greatly facilitates thermal denaturation of proteins [46]. Dry protein powders are extremely stable to thermal denaturation. T_d decreases sharply as the water content is increased from 0 to 0.35 g water/g protein (Figure 5.16). An increase in water content from 0.35 to 0.75 g water/g protein causes only a marginal decrease in T_d. Above 0.75 g water/g protein, the T_d of the protein is same as in a dilute protein solution. The effect of hydration on thermostability is fundamentally related to protein dynamics. In the dry state, proteins have a static structure, that is, the polypeptide segments have restricted mobility. As the water content is increased, hydration and partial penetration of water into surface cavities causes swelling of the protein. This swollen state, where the protein and its water convert from an amorphous to a rubbery state, reaches a maximum value at water content of 0.3–0.4 g water/g protein at room temperature. The swelling of the protein increases chain mobility and flexibility, and the protein molecule assumes a more dynamic molten structure. When heated, this dynamic flexible structure provides greater access of water to salt bridges and peptide hydrogen bonds than is possible in the dry state, resulting in lower T_d.

Additives such as salts and sugars affect thermostability of proteins in aqueous solutions. Sugars such as sucrose, lactose, glucose, and glycerol stabilize proteins against thermal denaturation [69]. Addition of 0.5 M NaCl to proteins such as β-lactoglobulin, soy proteins, serum albumin, and oat globulin, significantly increases their T_d [25,27,54].

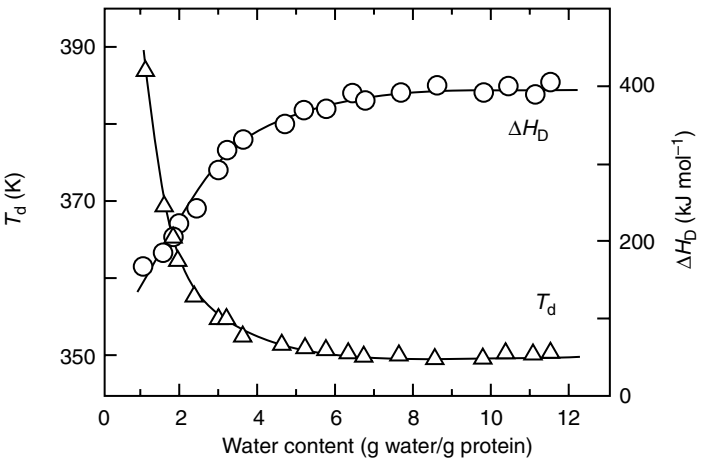


FIGURE 5.16 Influence of water content on the temperature (T_d) and enthalpy (ΔH_D) of denaturation of ovalbumin. (From Fujita, Y. and Y. Noda. 1981. *Bull. Chem. Soc. Japan* **54**:3233–3234.)

5.4.2.1.2 Hydrostatic Pressure and Denaturation

One of the thermodynamic variables that affect conformation of proteins is hydrostatic pressure. Unlike temperature-induced denaturation, which usually occurs in the range of 40–80°C at one atmospheric pressure, pressure-induced denaturation can occur at 25°C if the pressure is sufficiently great. Most proteins undergo pressure-induced denaturation in the range of 1–12 kbar as evidenced from changes in their spectral properties. The midpoint of pressure-induced transition occurs at 4–8 kbar [57].

Pressure-induced denaturation of proteins occurs mainly because proteins are flexible and compressible. Although amino acid residues are densely packed in the interior of globular proteins, some void spaces invariably exist and this leads to compressibility. The average partial specific volume of globular proteins in the hydrated state, υ^0, is about 0.74 mL/g. The partial specific volume can be considered as the sum of three components:

$$\upsilon^0 = V_C + V_{Cav} + \Delta V_{Sol} \tag{5.45}$$

where V_C is the sum of the atomic volumes, V_{Cav} is the sum of the volumes of the void spaces in the interior of the protein, and ΔV_{Sol} is the volume change due to hydration [47]. The larger the V_{Cav}, the larger is the contribution of void spaces to partial specific volume and the more unstable the protein will be when pressurized. Fibrous proteins are mostly devoid of void spaces, and hence they are more stable to hydrostatic pressure than globular proteins.

Pressure-induced denaturation of globular proteins is usually accompanied by a reduction in volume of about 30–100 mL/mol. This decrease in volume is caused by two factors: elimination of void spaces as the protein unfolds and hydration of the nonpolar amino acid residues that become exposed during unfolding. The latter event results in a decrease in volume (see Section 5.3.2). The volume change is related to the free energy change by the expression:

$$\Delta V = \frac{d(\Delta G)}{dp} \tag{5.46}$$

where p is the hydrostatic pressure.

If a globular protein completely unfolds during pressurization, the volume change should be about 2%. However, 30–100 mL/mol volume change observed in pressure-denatured proteins corresponds to only about 0.5% change in volume. This indicates that proteins only partially unfold even at hydrostatic pressure as high as 10 kbar.

Pressure-induced protein denaturation is highly reversible. Most enzymes, in dilute solutions, regain their activity once the pressure is decreased to atmospheric pressure [66]. However, regeneration of near complete activity usually takes several hours. In the case of pressure-denatured oligomeric proteins and enzymes, subunits first dissociate at 0.001–2 kbar, and then subunits denature at higher pressures [128]; when the pressure is removed, the subunits reassociate and almost complete restoration of enzyme activity occurs after several hours.

High hydrostatic pressures are being investigated as a food processing tool, for example, for microbial inactivation or gelation. Since high hydrostatic pressure (2–10 kbar) irreversibly damages cell membranes and causes dissociation of organelles in microorganisms, it will inactivate vegetative microorganisms [72]. Pressure gelation of egg white, 16% soy protein solution, or 3% actomyosin solution can be achieved by application of 1–7 kbar hydrostatic pressure for 30 min at 25°C. These pressure-induced gels are softer than thermally induced gels [94]. Also, exposure of beef muscle to 1–3 kbar hydrostatic pressure causes partial fragmentation of myofibrils, which may be useful as a means of tenderizing meat [119] and gelation of myofibrillar proteins [7]. Pressure processing, unlike thermal processing, does not harm essential amino acids, natural color and flavor, nor does it cause toxic compounds to develop. Thus, processing of foods with high hydrostatic pressure may prove advantageous (except for cost) for certain food products.

5.4.2.1.3 Shear and Denaturation

High mechanical shear generated by shaking, kneading, whipping, and so forth, can cause denaturation of proteins. Many proteins denature and precipitate when they are vigorously agitated [93]. In this circumstance, denaturation occurs because of incorporation of air bubbles and adsorption of protein molecules to the air–liquid interface. Since the energy of the air–liquid interface is greater than that of the bulk phase, proteins undergo conformational changes at the interface. The extent of conformational change depends on the flexibility of the protein. Highly flexible proteins denature more readily at an air–liquid interface than do rigid proteins. The nonpolar residues of denatured protein orient toward the gas phase and the polar residues orient toward the aqueous phase.

Several food processing operations involve high pressure, shear, and high temperature, for example, extrusion, high-speed blending, and homogenization. When a rotating blade produces a high shear rate, subsonic pulses are created and cavitation also occurs at the trailing edges of the blade. Both these events contribute to protein denaturation. The greater the shear rate, the greater is the degree of denaturation. The combination of high temperature and high shear force causes irreversible denaturation of proteins. For example, when a 10–20% whey protein solution at pH 3.5–4.5 and at 80–120°C is subjected to a shear rate of 7,500–10,000/s, it forms insoluble spherical macrocolloidal particles of about 1 μm diameter. A hydrated material produced under these conditions, "Simplesse," has a smooth, emulsion-like organoleptic character [118].

5.4.2.2 Chemical Agents

5.4.2.2.1 pH and Denaturation

Proteins are more stable against denaturation at their isoelectric point than at any other pH. At neutral pH, most proteins are negatively charged and a few are positively charged. Since the net electrostatic repulsive energy is small compared to other favorable interactions, most proteins are stable at around neutral pH. However, strong intramolecular electrostatic repulsion caused by high net charge at extreme pH values results in swelling and unfolding of the protein molecule. The degree of unfolding is greater at extreme alkaline pH values than it is at extreme acid pH values. The former behavior is attributed to ionization of partially buried carboxyl, phenolic, and sulfhydryl groups that cause unravelling of the polypeptide chain as they attempt to expose themselves to the aqueous environment. pH-induced denaturation is mostly reversible. However, in some cases, partial hydrolysis of peptide bonds, deamidation of Asn and Gln, destruction of sulfhydryl groups at alkaline pH, or aggregation can result in irreversible denaturation of proteins.

5.4.2.2.2 Organic Solvents and Denaturation

Organic solvents affect the stability of protein hydrophobic interactions, hydrogen bonding, and electrostatic interactions in different ways [52]. Since nonpolar side chains are more soluble in organic solvents than in water, organic solvents weaken hydrophobic interactions. On the other hand, since the stability and formation of peptide hydrogen bonds are enhanced in a low permittivity environment, certain organic solvents may actually strengthen or promote formation of peptide hydrogen bonds. For example, 2-chloroethanol causes an increase in α-helix content in globular proteins. The action of organic solvents on electrostatic interactions is twofold. By decreasing permittivity, they enhance electrostatic interactions between oppositely charged groups and also enhance repulsion between groups with like charge. The net effect of an organic solvent on protein structure, therefore, usually depends on the magnitude of its effect on various polar and nonpolar interactions. At low concentration, some organic solvents can stabilize several enzymes against denaturation [9]. At high concentrations, however, all organic solvents cause denaturation of proteins because of their solubilizing effect on nonpolar side chains.

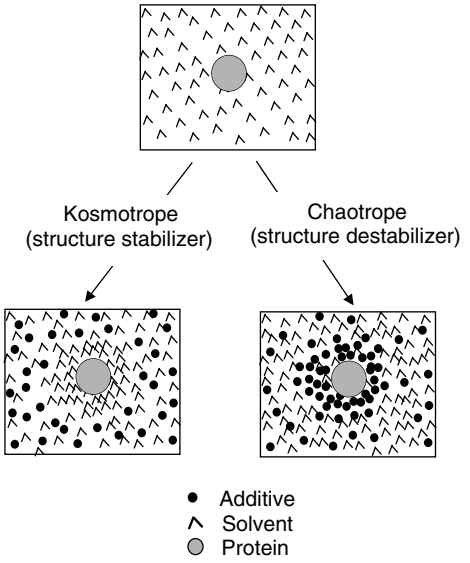


FIGURE 5.17 Schematic representation of preferential binding and preferential hydration of protein in the presence of additives. (Adapted from Creighton, T. E. 1993. *Proteins: Structures and Molecular Properties*. W. H. Freeman Co., New York, pp. 158–159.)

5.4.2.2.3 Denaturation by Small Molecular Weight Additives

Several small molecular weight solutes, such as urea, guanidine hydrochloride (GuHCl), detergents, sugars, and neutral salts affect protein stability in aqueous solutions. While urea, GuHCl, and detergents destabilize the native conformation of proteins [34], sugars tend to stabilize the native structure. In the case of neutral salts, while certain salts, such as sulfate, phosphate, and fluoride salts of sodium, termed as kosmotropes, stabilize protein structure, other salts, such as bromide, iodide, perchlorate, and thiocyanate, termed as chaotropes, destabilize protein structure.

The stabilizing or destabilizing effects of small molecular weight additives on proteins is believed to follow a universal mechanism. This is related to their preferential interaction with the aqueous phase and the protein surface. Additives that stabilize protein structure bind very weakly to the protein surface but enhance preferential hydration of the protein surface (Figure 5.17). Such additives are generally excluded from the region surrounding the protein; that is, their concentration near the protein is lower than in the bulk solution. This concentration gradient presumably creates an osmotic pressure gradient surrounding the protein molecule, sufficient enough to elevate the thermal denaturation temperature of the protein. For instance, studies on protein stabilization by glycerol have shown that lysozyme in glycerol solutions assumes a slightly compressed state compared with its state in water [51]. This might be due to creation of an exclusion zone around the protein surface for glycerol and development of an osmotic pressure gradient.

In the case of additives that destabilize protein structure, the opposite seems to be true. That is, those additives that decrease the stability of proteins preferentially bind to the protein surface and cause dehydration of the protein. In such cases, water molecules are excluded from the region surrounding the protein and the concentration of the additive in this water-excluded region is higher than in the bulk solvent. Favorable interaction of such additives with protein surface, particularly the nonpolar surface, promotes unfolding of the protein so that the buried nonpolar surfaces are further exposed for favorable interaction with the additive.

When a protein is exposed to a mixture of stabilizing and destabilizing solutes, the net effect on protein stability generally follows an additivity rule. For example, sucrose and polyols are considered to be protein structure stabilizers, whereas GuHCl is a structure destabilizer. When sucrose is mixed

with GuHCl, the concentration of GuHCl required for unfolding proteins increases with increase of sucrose concentration [122]. Furthermore, alterations in water structure caused by GuHCl and urea are countered by the addition of polyhydric compounds such as sucrose. Thus, in the presence of a polyol protein denaturation requires a higher GuHCl and urea concentration [122]. This also underscores that changes in water structure in the presence of additives is involved in some fundamental way in the transmission of the effects of additives on protein stability. The exact mechanism is still elusive, partly because "water structure" is not yet a well-defined concept.

5.4.2.2.4 Organic Solutes and Denaturation

Organic solutes, notably urea and GuHCl, cause denaturation of proteins. For many globular proteins, the midpoint of transition from the native to denatured state occurs at 4–6 M urea and at 3–4 M GuHCl at room temperature. Complete transition often occurs in 8 M urea and in about 6 M GuHCl. GuHCl is a more powerful denaturant than urea because of its ionic character. Many globular proteins do not undergo complete denaturation even in 8 M urea, whereas in 8 M GuHCl they usually exist in a random coil state (completely denatured).

Denaturation of proteins by urea and GuHCl is thought to involve two mechanisms. The first mechanism involves preferential binding of urea and GuHCl to the denatured protein. Removal of denatured protein as a protein-denaturant complex shifts the N $\longleftrightarrow$ D equilibrium to the right. As the denaturant concentration is increased, continuous conversion of the protein to protein-denaturant complex eventually results in complete denaturation of the protein. Since binding of denaturant to denatured protein is very weak, a high concentration of denaturant is needed to cause complete denaturation. The second mechanism involves solubilization of hydrophobic amino acid residues in urea and GuHCl solutions. Since urea and GuHCl have the potential to form hydrogen bonds, at high concentration these solutes breakdown the hydrogen-bonded structure of water. This destructuring of solvent water makes it a better solvent for nonpolar residues. This results in unfolding and solubilization of apolar residues from the interior of the protein molecule.

Urea- or GuHCl-induced denaturation is reversible. However, complete reversibility of urea-induced protein denaturation is sometimes difficult. This is because some urea converts to cyanate and ammonia. Cyanate reacts with amino groups and alters the charge of the protein.

5.4.2.2.5 Detergents and Denaturation

Detergents, such as sodium dodecyl sulfate (SDS), are powerful protein denaturing agents. SDS at 3–8 mM concentration denatures most globular proteins. The mechanism involves preferential binding of detergent to the denatured protein molecule. This causes a shift in equilibrium between the native and denatured states. Unlike urea and GuHCl, detergents bind strongly to denatured proteins and this is the reason for complete denaturation at a relatively low detergent concentration of 3–8 mM. Because of this strong binding, detergent-induced denaturation is irreversible. Globular proteins denatured by SDS do not exist in a random coil state; instead, they assume a α-helical rod shape in SDS solutions. This rod shape is properly regarded as denatured.

5.4.2.2.6 Chaotropic Salts and Denaturation

Salts affect protein stability in two different ways. At low concentrations, ions interact with proteins via nonspecific electrostatic interactions. This electrostatic neutralization of protein charges usually stabilizes protein structure. Complete charge neutralization by ions occurs at or below 0.2 M ionic strength and it is independent of the nature of the salt. However, at higher concentrations (>1 M), salts have ion specific effects that influence the structural stability of proteins. Salts such as Na_2SO_4 and NaF enhance, whereas NaSCN and $NaClO_4$ weaken it. Protein structure is influenced more by anions than by cations. For example, the effect of various sodium salts on the thermal denaturation temperature of β-lactoglobulin is shown in Figure 5.18. At equal ionic strength, Na_2SO_4 and NaCl increase T_d, whereas NaSCN and $NaClO_4$ decrease it. Regardless of their chemical make up and conformational differences, the structural stability of macromolecules, including DNA, is adversely

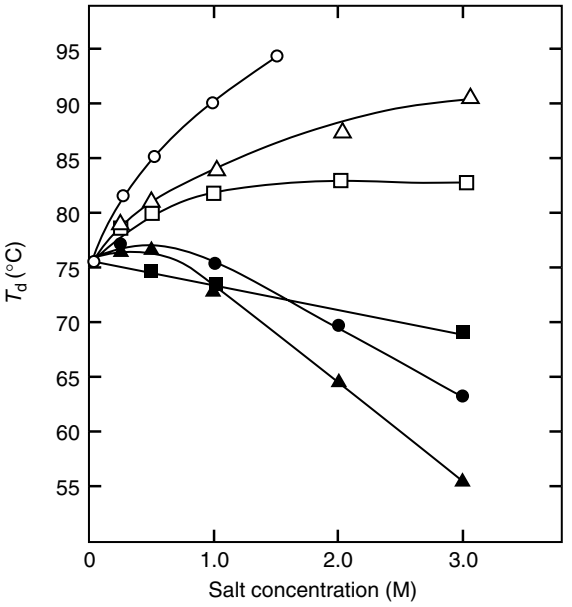


FIGURE 5.18 Effects of various sodium salts on the temperature of denaturation, T_d, of β-lactoglobulin at pH 7.0. △, NaCl; NaBr; ●, $NaClO_4$; ▲, NaSCN; ■, urea. (From Damodaran, S. 1989. *Int. J. Biol. Macromol.* **11**:2–8.)

affected by high concentrations of salts [21]. NaSCN and $NaClO_4$ are strong denaturants. The relative ability of various anions at isoionic strength to influence the structural stability of protein (and DNA) in general follows the series, $F^- < SO_4^{=} < Cl^- < Br^- < I^- < ClO_4^- < SCN^- < Cl_3CCOO^-$. This ranking is known as the Hofmeister series or chaotropic series. Floride, chloride, and sulfate salts are structure stabilizers, whereas the salts of other anions are structure destabilizers.

The mechanism of salts effects on the structural stability of proteins is related to their relative ability to bind to and alter hydration properties of proteins. Salts that stabilize proteins enhance hydration of proteins and bind weakly, whereas salts that destabilize proteins decrease protein hydration and bind strongly [8]. These effects are primarily the consequence of energy perturbations at the protein–water interface. On a more fundamental level, protein stabilization or denaturation by salts is related to their effect on bulk water structure. Salts that stabilize protein structure also enhance the hydrogen-bonded structure of water, and salts that denature proteins also breakdown bulk water structure and make it a better solvent for apolar molecules. In other words, the denaturing effect of chaotropic salts might be related to destabilization of hydrophobic interactions in proteins.

5.5 FUNCTIONAL PROPERTIES OF PROTEINS

Food preferences by human beings are based primarily on sensory attributes such as texture, flavor, color, and appearance. The sensory attributes of a food are the net effect of complex interactions among various minor and major components of the food. Proteins generally have a great influence on the sensory attributes of foods. For example, the sensory properties of bakery products are related to the viscoelastic and dough-forming properties of wheat gluten; the textural and succulence characteristics of meat products are largely dependent on muscle proteins (actin, myosin, actomyosin, and several soluble meat proteins); the textural and curd-forming properties of dairy products are due to the unique colloidal structure of casein micelles; and the structure of some cakes and the whipping properties of some desert products depend on the properties of egg-white proteins. The functional roles of various proteins in different food products are listed in Table 5.11 [64]. Functionality of food

TABLE 5.11
Functional Roles of Food Proteins in Food Systems

Function	Mechanism	Food	Protein Type
Solubility	Hydrophilicity	Beverages	Whey proteins
Viscosity	Water binding, hydrodynamic size and shape	Soups, gravies, and salad dressings, deserts	Gelatin
Water binding	Hydrogen bonding, ionic hydration	Meat sausages, cakes, and breads.	Muscle proteins, egg proteins
Gelation	Water entrapment and immobilization, network formation	Meats, gels, cakes, bakeries, cheese	Muscle proteins, egg and milk proteins
Cohesion–adhesion	Hydrophobic, ionic, and hydrogen bonding	Meats, sausages, pasta, baked goods	Muscle proteins, egg proteins, whey proteins
Elasticity	Hydrophobic bonding, disulfide crosslinks	Meats, bakery	Muscle proteins, cereal proteins
Emulsification	Adsorption and film formation at interfaces	Sausages, bologna, soup, cakes, dressings	Muscle proteins, egg proteins, milk proteins
Foaming	Interfacial adsorption and film formation	Whipped toppings, ice cream, cakes, desserts	Egg proteins, milk proteins
Fat and flavor binding	Hydrophobic bonding, entrapment	Low-fat bakery products, doughnuts	Milk proteins, egg proteins, cereal proteins

Source: Kinsella, J. E. et al. 1985. In *New Protein Foods: Seed Storage Protein*s (Altshul, A. M. and H. L. Wilcke, Eds.), Academic Press, London, pp. 107–179.

proteins refers to the physical and chemical properties that influence the performance of proteins in food systems during processing, storage, preparation, and consumption.

The sensory attributes of foods are achieved by complex interactions among various functional ingredients. For instance, the sensory attributes of a cake emanate from gelling/heat-setting, foaming, and emulsifying properties of the ingredients used. Therefore, for a protein to be useful as an ingredient in cakes and other such products, it must possess multiple functionalities. Proteins of animal origin, for example, milk (caseins), egg, and meat proteins, are widely used in fabricated foods. These proteins are mixtures of several proteins with wide ranging physicochemical properties and they are capable of performing multiple functions. For example, egg white possesses multiple functionalities such as gelation, emulsification, foaming, water binding, and heat coagulation, which make it a highly desirable protein in many foods. The multiple functionalities of egg white arise from complex interactions among its protein constituents, namely, ovalbumin, conalbumin, lysozyme, ovomucin, and other albumin-type proteins. Plant proteins (e.g., soy and other legume and oilseed proteins) and other proteins, such as whey proteins, are used to a limited extent in conventional foods. Even though these proteins are also mixtures of several proteins, they do not perform as well as animal proteins in most food products. The exact molecular properties of proteins that are responsible for the various desirable functionalities in food are poorly understood.

The physical and chemical properties that govern protein functionality include size; shape; amino acid composition and sequence; net charge and distribution of charges; hydrophobicity/hydrophilicity ratio; secondary, tertiary, and quaternary structures; molecular flexibility/rigidity; and ability to interact/react with other components. Since proteins possess a multitude of physical and chemical properties, it is difficult to delineate the role of each of these properties with respect to a given functional property.

TABLE 5.12
The Linkage Between the Physicochemical Aspects of Proteins and Their Impact on Functionalities in Foods

General Property	Functions Affected
1. Hydration	Solubility, dispersibility, wettability, swelling, thickening, water absorption, water-holding capacity
2. Surface activity	Emulsification, foaming, flavor binding, pigment binding
3. Hydrodynamic/Rheological	Elasticity, viscosity, cohesiveness, chewiness, adhesion, stickiness, gelation, dough formation, texturization

On an empirical level, the various functional properties of proteins can be viewed as manifestations of three molecular aspects of proteins: (1) hydration properties; (2) protein surface-related properties; and (3) hydrodynamic/rheological properties (Table 5.12). Although much is known about the physicochemical properties of several food proteins, prediction of functional properties from their molecular properties has not been successful. A few empirical correlations between molecular properties and certain functional properties in model protein systems have been established. However, behavior in model systems often is not the same as behavior in real food products. This is attributable, in part, to denaturation of proteins during food fabrication. The extent of denaturation depends on pH, temperature, other processing conditions, and product characteristics. In addition, in real foods, proteins interact with other food components, such as lipids, sugars, polysaccharides, salts, and minor components, and this modifies their functional behavior. Despite these inherent difficulties, considerable progress has been made toward understanding the relationship between various physicochemical properties of protein molecules and their functional properties.

5.5.1 Protein Hydration

Water is an essential constituent of foods. The rheological and textural properties of foods depend on the interaction of water with other food constituents, especially with macromolecules, such as proteins and polysaccharides. Water modifies the physicochemical properties of proteins. For example, the plasticizing effect of water on amorphous and semicrystalline food proteins changes their glass transition temperature (see Chapter 2) and T_d. The glass transition temperature refers to the conversion of a brittle amorphous solid (glass) to a flexible rubbery state, whereas the melting temperature refers to transition of a crystalline solid to a disordered structure.

Many functional properties of proteins, such as dispersibility, wettability, swelling, solubility, thickening/viscosity, water-holding capacity, gelation, coagulation, emulsification, and foaming depend on water–protein interactions. In low and intermediate moisture foods, such as bakery and comminuted meat products, the ability of proteins to bind water is critical to the acceptability of these foods. The ability of a protein to exhibit a proper balance of protein–protein and protein–water interactions is critical to their thermal gelation properties.

Water molecules bind to several groups in proteins. These include charged groups (ion–dipole interactions); backbone peptide groups; the amide groups of Asn and Gln; hydroxyl groups of Ser, Thr, and Tyr residues (all dipole–dipole interactions); and nonpolar residues (dipole–induced dipole interaction and hydrophobic hydration).

The water binding capacity of proteins is defined as grams of water bound per gram of protein when a dry protein powder is equilibrated with water vapor at 90–95% relative humidity. The water binding capacities (also sometimes called hydration capacity) of various polar and nonpolar groups of proteins are given in Table 5.13 [70]. Amino acid residues with charged groups bind about 6 moles of water per residue, the uncharged polar residues bind about 2 mol/residue, and the nonpolar groups

TABLE 5.13
Hydration Capacities of Amino Acid Residues[a]

Amino Acid Residue	Hydration (moles H_2O/mole residue)
Polar	
Asn	2
Gln	2
Pro	3
Ser, The	2
Trp	2
Asp (unionized)	2
Glu (unionized)	2
Tyr	3
Arg (unionized)	3
Lys (unionized)	4
Ionic	
Asp^-	6
Glu^-	7
Tyr^-	7
Arg^+	3
His^+	4
Lys^+	4
Nonpolar	
Ala	1
Gly	1
Phe	0
Val, Ile, Leu, Met	1

[a] Represents unfrozen water associated with amino acid residues based on nuclear magnetic resonance studies of polypeptide.

Source: Kuntz, I. D. 1971. *J. Amer. Chem. Soc.* **93**:514–516.

bind about 1 mol/residue. The hydration capacity of a protein therefore is related, in part, to its amino acid composition—the greater the number of charged residues, the greater is the hydration capacity. The hydration capacity of a protein can be calculated from its amino acid composition using the empirical equation [71]:

$$a = f_C + 0.4f_P + 0.2f_N \tag{5.47}$$

where a is g water/g protein and f_C, f_P, and f_N are the fractions of the charged, polar, and nonpolar residues, respectively, in the protein. The experimental hydration capacities of several monomeric globular proteins agree very well with those calculated from the above equation. This, however, is not true for oligomeric proteins. Since oligomeric structures involve partial burial of the protein surface at the subunit–subunit interface, calculated values are usually greater than experimental values. On the other hand, the experimental hydration capacity of casein micelles (~4 g water/g protein) is much larger than that predicted by the above equation. This is because of the enormous amount of void space within the casein micelle structure, which imbibes water through capillary action and physical entrapment.

On a macroscopic level, water binding to proteins occurs in a step-wise process. The high-affinity ionic groups are solvated first at low water activity, followed by polar and nonpolar groups. The sequence of steps involved at increasing water activity is presented in Figure 5.19 ([109]; see

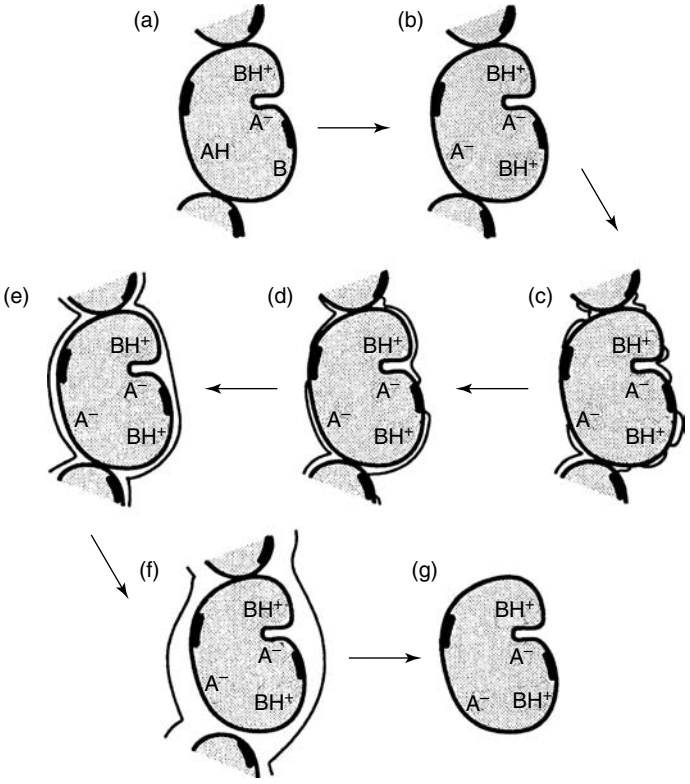


FIGURE 5.19 Sequence of steps involved in hydration of a protein. (a) Unhydrated protein. (b) Initial hydration of charged groups. (c) Water cluster formation near polar and charged sites. (d) Completion of hydration at the polar surfaces. (e) Hydrophobic hydration of nonpolar patches; completion of monolayer coverage. (f) Bridging between protein-associated water and bulk water. (g) Completion of hydrodynamic hydration. (From Rupley, J. A. et al. 1980. In *Water in Polymers* (Rowland, S. P., Ed.), ACS Symp. Ser. 127, American Chemical Society, Washington, D.C., pp. 91–139.)

also Chapter 2). Sorption isotherms of proteins, that is, the amount of water bound per gram of protein as a function of relative humidity is invariably a sigmoidal curve (see Chapter 2). For most proteins, saturated monolayer coverage of water occurs at a water activity (a_w) of about 0.7–0.8, and multilayers of water are formed at $a_w > 0.8$. The saturated monolayer coverage corresponds to about 0.3–0.5 g water/g protein. The saturated monolayer water is primarily associated with ionic, polar, and apolar groups on the surface of the protein. This water is unfreezable, does not take part as a solvent in chemical reactions, and is often referred to as "bound" water, which should be understood to mean water with "hindered" mobility. In the hydration range of 0.07–0.27 g/g, the energy required for desorption of water from the protein surface is only about 0.18 kcal/mol at 25°C. Since the thermal kinetic energy of water at 25°C is about ~1 kcal/mol, which is greater than the free energy of desorption, water molecules in the monolayer are reasonably mobile.

At $a_w = 0.9$, proteins bind about 0.3–0.5 g water/g protein (Table 5.14) [65,71]. At $a_w > 0.9$, liquid (bulk) water condenses into the clefts and crevices of protein molecules, or in the capillaries of insoluble protein systems, such as myofibrils. The properties of this water are similar to those of bulk water. This water is known as hydrodynamic water, which moves with the protein molecule.

Several environmental factors, such as pH, ionic strength, temperature, type of salts, and protein conformation influence the water-binding capacity of proteins. Proteins are least hydrated at their isoelectric pH, where enhanced protein–protein interactions results in minimal interaction with water. Above and below the isoelectric pH, because of the increase in the net charge and repulsive forces,

TABLE 5.14
Hydration Capacities of Various Proteins

Protein	g Water/g Protein
Pure proteins[a]	
Ribonuclease	0.53
Lysozyme	0.34
Myoglobin	0.44
β-Lactoglobulin	0.54
Chymotrypsinogen	0.23
Serum albumin	0.33
Hemoglobin	0.62
Collagen	0.45
Casein	0.40
Ovalbumin	0.30
Commmercial protein preparations[b]	
Whey protein concentrates	0.45–0.52
Sodium caseinate	0.38–0.92
Soy protein	0.33

[a] At 90% relative humidity.
[b] At 95% relative humidity.

Source: From Kinsella, J. E. and P. F. Fox. 1986. *CRC Crit. Rev. Food Sci. Nutr*. **24**:91–139 and Kuntz, I. D. and W. Kauzmann. 1974. *Adv. Protein Chem*. **28**:239–345.

proteins swell and bind more water. The water-binding capacity of most proteins is greater at pH 9–10 than at any other pH. This is due to ionization of sulfhydryl and tyrosine residues. Above pH 10, the loss of positively charged ε-amino groups of lysyl residues results in reduced water binding.

At low concentrations (<0.2 M), salts increase the water binding capacity of proteins. This is because hydrated salt ions, especially the anions, bind (weakly) to charged groups on proteins. At this low concentration, binding of ions to proteins does not affect the hydration shell of the charged groups on the protein, and the increase in water binding essentially comes from water associated with the bound ions. However, at high salt concentrations much of the existing water is bound by salt ions, resulting in dehydration of the protein.

The water binding capacity of proteins generally decreases as the temperature is raised, because of decreased hydrogen bonding and decreased hydration of ionic groups. The water binding capacity of a denatured protein is generally about 10% greater than that of the native protein. This is due to an increase in surface area to mass ratio with exposure of some previously buried hydrophobic groups. If denaturation leads to aggregation of the protein, then its water-binding capacity may actually decrease because of displacement of water by enhanced protein–protein interactions. Denatured food proteins generally exhibit low solubility in water. Their water binding capacities, however, are not drastically different from those in the native state. Thus, water-binding capacity cannot be used to predict the solubility characteristics of proteins. The solubility of a protein is dependent not only on water-binding capacity but also on other thermodynamic factors.

In food applications, the water-holding capacity of a protein is more important than the water binding capacity. Water-holding capacity refers to the ability of the protein to imbibe water and retain it against gravitational force within a protein matrix, such as protein gels or beef and fish muscle. This water refers to the sum of the bound water, hydrodynamic water, and the physically entrapped water. The physically entrapped water contributes more to water-holding capacity than do the bound and hydrodynamic water. However, studies have shown that the water-holding capacity of

proteins is positively correlated with water binding capacity. The ability of proteins to entrap water is associated with juiciness and tenderness of comminuted meat products and desirable textural properties of bakery and other gel-type products.

5.5.2 Solubility

The functional properties of proteins are often affected by protein solubility and those most affected are thickening, foaming, emulsifying, and gelling. Insoluble proteins have very limited uses in food.

The solubility of a protein is the thermodynamic manifestation of the equilibrium between protein–protein and protein–solvent interactions:

$$\text{Protein–Protein} + \text{Water} \rightleftarrows \text{Protein–Water} \tag{5.48}$$

The major interactions that influence the solubility characteristics of proteins are hydrophobic and ionic in nature. Hydrophobic interactions promote protein–protein interactions and result in decreased solubility, whereas ionic interactions promote protein–water interactions and result in increased solubility. Ionic residues introduce two kinds of repulsive forces between protein molecules in solution. The first involves electrostatic repulsion between protein molecules owing to a net positive or negative charge at any pH other than the isoelectric pH; the second involves repulsion between hydration shells around ionic groups.

Bigelow [11] proposed that the solubility of a protein is fundamentally related to the average hydrophobicity of the amino acid residues and the charge frequency. The average hydrophobicity is defined as

$$\Delta G = \sum \Delta g_{\text{residue}}/n \tag{5.49}$$

where $\Delta g_{\text{residue}}$ is the hydrophobicity of each amino acid side chain obtained from the free energy change for transfer from octanol to water (see Section 5.2.1.4), and n is the total number of residues in the protein. The charge frequency is defined as

$$\sigma = \frac{(n^+ + n^-)}{n} \tag{5.50}$$

where n^+ and n^- are the total number of positively and negatively charged residues, respectively, and n is the total number of residues. According to Bigelow [11], the smaller the average hydrophobicity and the larger the charge frequency, the greater will be the solubility of the protein. Although this empirical correlation is true for most proteins, it is not an absolute one. Solubility of a protein is dictated by the hydrophilicity and hydrophobicity of the protein surface that contacts with the surrounding water, rather than the average hydrophobicity and charge frequency of the molecule as a whole. Since a majority of hydrophobic residues are buried in the interior of the protein, only those nonpolar groups that are on the surface would affect the solubility. The fewer the number of surface hydrophobic patches, the greater the solubility.

Based on solubility characteristics, proteins are classified into four categories. *Albumins* are those that are soluble in water at pH 6.6 (e.g., serum albumin, ovalbumin, and α-lactalbumin), *globulins* are those that are soluble in dilute salt solutions at pH 7.0 (e.g., glycinin, phaseolin, and β-lactoglobulin), *glutelins* are those that are soluble only in acid (pH 2) and alkaline (pH 12) solutions (e.g., wheat glutelins), and *prolamines* are those soluble in 70% ethanol (e.g., zein and gliadins). Both prolamines and glutelins are highly hydrophobic proteins.

In addition to these intrinsic physicochemical properties, solubility is influenced by several solution conditions, such as pH, ionic strength, temperature, and the presence of organic solvents.

5.5.2.1 pH and Solubility

At pH values below and above the isoelectric pH, proteins carry a net positive or a net negative charge, respectively. Electrostatic repulsion and hydration of charged residues promote solubilization of the protein. When solubility is plotted against pH, most food proteins exhibit a U-shaped curve. Minimum solubility occurs at about the isoelectric pH of proteins. A majority of food proteins are acidic proteins; that is, the sum of Asp and Glu residues is greater than the sum of Lys, Arg, and His residues. Therefore, they exhibit minimum solubility at pH 4–5 (isoelectric pH), and maximum solubility at alkaline pH. The occurrence of minimum solubility near the isoelectric pH is primarily due to the lack of electrostatic repulsion, which promotes aggregation and precipitation via hydrophobic interactions. Some food proteins are highly soluble at their isoelectric pH, for example, β-lactoglobulin (pI 5.2) and bovine serum albumin (pI 5.3). This is because these proteins contain a large ratio of surface hydrophilic residues to surface nonpolar groups. It should be remembered that even though a protein is electrically neutral at its pI, it still has equal number of positive and negative charges on the surface, contributing to hydrophility of the protein. If the hydrophilicity and the hydration repulsion forces arising from these charged residues are greater than the protein–protein hydrophobic interactions, then the protein will still be soluble at the pI.

Since most proteins are highly soluble at alkaline pH 8–9, protein extraction from plant sources, such as soybean flour, is carried out at this pH. Shown in Figure 5.20 is a typical industrial process for the isolation of soy protein based on its pH-solubility behavior.

Heat denaturation changes the pH-solubility profile of proteins (Figure 5.21). Native whey protein isolate (WPI) is completely soluble in the pH range 2–9, but when heated at 70°C for 1–10 min

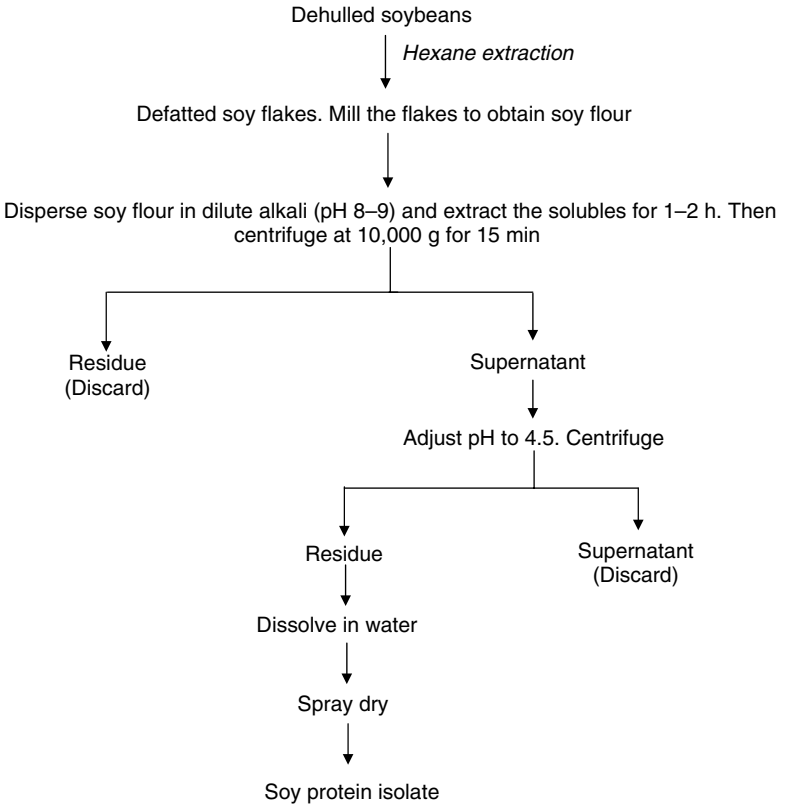


FIGURE 5.20 A typical industrial process for isolation of soy protein from defatted soy flour.

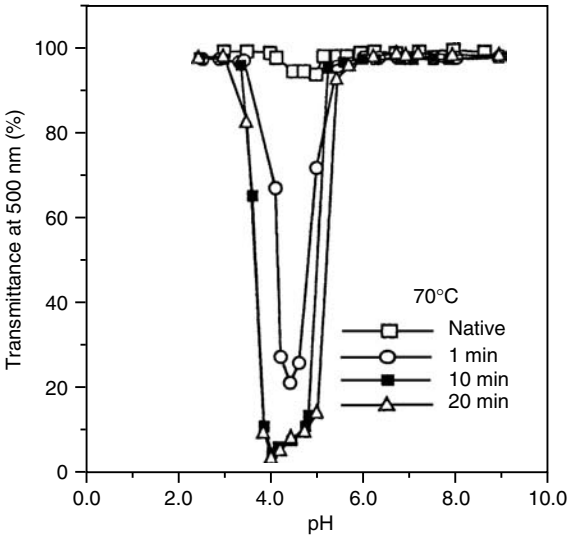


FIGURE 5.21 pH-solubility profile of whey protein isolate solutions heated at 70°C for various times. (From Zhu, H. and S. Damodaran. 1994. *J. Agric. Food Chem.* **42**:846–855.)

a typical U-shaped solubility profile develops with a solubility minimum at pH 4.5. The change in the solubility profile upon heat denaturation is due to an increase in the hydrophobicity of the protein surface as a consequence of unfolding. Unfolding alters the balance between protein–protein and protein–solvent interactions in favor of the former.

5.5.2.2 Ionic Strength and Solubility

The ionic strength of a salt solution is given by:

$$\mu = 0.5 \sum C_i Z_i^2 \tag{5.51}$$

where C_i is concentration of an ion and Z_i is its valence. At low ionic strength (<0.5), ions neutralize charges at the surface of proteins. This charge screening affects solubility in two different ways, depending on the characteristics of the protein surface. Solubility decreases for those proteins that contain a high incidence of nonpolar patches, and it increases for those that do not. The former behavior is typical for soy proteins and the latter behavior is exhibited by β-lactoglobulin. While the decrease in solubility is caused by enhanced hydrophobic interactions, the increase in solubility is caused by a decrease in the ionic activity of the protein macroion. At ionic strength >1.0, salts have ion specific effects on protein solubility. As salt concentration is increased, sulfate and fluoride salts progressively decrease solubility (salting out), whereas bromide, iodide, thiocyanate, and perchlorate salts increase solubility (salting in). At constant ionic strength, relative effectiveness of various ions on solubility follows the Hofmeister series with anions promoting solubility in the order $SO_4^= < F^- < Cl^- < Br^- < I^- < ClO_4^- < SCN^-$ and cations decreasing solubility in the order $NH_4^+ < K^+ < Na^+ < Li^+ < Mg^{2+} < Ca^{2+}$. This behavior is analogous to the effects of salts on the thermal denaturation temperature of proteins (see Section 5.4).

Generally, solubility of proteins in salt solutions follows the relation:

$$\log\left(\frac{S}{S_0}\right) = \beta - K_S C_S, \tag{5.52}$$

where S and S_0 are solubilities of the protein in the salt solution and in water, respectively, K_S is the salting out constant, C_S is molar concentration of salt, and β is a constant characteristic of only protein. K_S is positive for salting-out-type of salts and negative for salting-in-type of salts.

5.5.2.3 Temperature and Solubility

At constant pH and ionic strength, the solubility of most proteins generally increases with temperature between 0°C and 40°C. Exceptions occur with highly hydrophobic proteins, such as β-casein and some cereal proteins, which exhibit a negative relationship with temperature. Above 40°C, the increase in thermal kinetic energy causes protein unfolding (denaturation), exposure of nonpolar groups, aggregation and precipitation, that is, decreased solubility.

5.5.2.4 Organic Solvents and Solubility

Addition of organic solvents, such as ethanol or acetone, lowers the permittivity of an aqueous medium. This increases intra- and intermolecular electrostatic forces, both repulsive as well as attractive. The repulsive intramolecular electrostatic interactions cause unfolding of the protein molecule. In the unfolded state, the low permittivity of the medium promotes intermolecular hydrogen bonding between the exposed peptide groups and attractive intermolecular electrostatic interactions between oppositely charged groups. These intermolecular polar interactions lead to precipitation of the protein in organic solvents or reduced solubility in an aqueous medium. The role of hydrophobic interactions in causing precipitation in organic solvents is minimal because of the solubilizing effect of organic solvents on nonpolar residues. One exception is prolamine-type proteins. These proteins are so hydrophobic that they are soluble only in 70% ethanol.

Since solubility of proteins is intimately related to their structural states, it is often used as a measure of the extent of denaturation during extraction, isolation, and purification processes. It is also used as an index of the potential applications of proteins. Commercially prepared protein concentrates and isolates show a wide range of solubility. The solubility characteristics of these protein preparations are expressed as *protein solubility index* (PSI) or *protein dispersibility index* (PDI). Both of these terms express the percentage (%) of soluble protein present in a protein sample. The PSI of commercial protein isolates varies from 25% to 80%.

5.5.3 Interfacial Properties of Proteins

Several natural and processed foods are either foam or emulsion-type products. These types of dispersed systems are unstable unless a suitable amphiphilic substance is present at the interface between the two phases (see Chapter 13). Proteins are amphiphilic molecules and they migrate spontaneously to an air/water interface or an oil–water interface. This spontaneous migration of proteins from a bulk liquid to an interface indicates that the free energy of proteins is lower at the interface than it is in the bulk aqueous phase. Thus, when equilibrium is established, the concentration of protein in the interfacial region is always much greater than it is in the bulk aqueous phase. Unlike small molecular-weight surfactants, proteins form a highly viscoelastic film at an interface, which has the ability to withstand mechanical shocks during storage and handling. Thus, protein-stabilized foams and emulsions are more stable than those prepared with small molecule surfactants, and because of this, proteins are extensively used for these purposes.

Although all proteins are amphiphilic, they differ significantly in their surface-active properties. The differences in the surface-active properties among proteins cannot be attributed to differences in the ratio of hydrophobic to hydrophilic residues. If a large hydrophobicity/hydrophilicity ratio were the primary determinant of the surface activity of proteins, then plant proteins that contain more than 40% hydrophobic amino acid residues should be better surfactants than albumin-type proteins, such as ovalbumin and bovine serum albumin, which contain $<30\%$ hydrophobic amino

acid residues. On the contrary, ovalbumin and serum albumin are better emulsifying and foaming agents than are soy proteins and other plant proteins. Furthermore, average hydrophobicity of most proteins fall within a narrow range, yet they exhibit remarkable differences in their surface activity. It must be concluded, therefore, that differences in surface activity are related primarily to differences in protein conformation. The conformational factors of importance include stability/flexibility of the polypeptide chain, ease of adaptability to changes in the environment, and distribution pattern of hydrophilic and hydrophobic groups on the protein surface. All these conformational factors are interdependent, and they collectively have a large influence on the surface activity of proteins.

It has been shown that desirable surface-active proteins have three attributes: (1) ability to rapidly adsorb to an interface; (2) ability to rapidly unfold and reorient at an interface; and (3) an ability, once at the interface, to interact with the neighboring molecules and form a strong cohesive, viscoelastic film that can withstand thermal and mechanical motions [31].

Formation and stabilization of foams and emulsions requires the presence of a surfactant that can effectively reduce the interfacial tension between the air/oil and aqueous phases. This can be achieved by using either small surfactants, such as lecithin, monoacylglycerol, and so forth, or macromolecules, such as proteins. At equivalent concentration at an interface, proteins are generally less effective than small surfactants in decreasing the interfacial tension. Typically, most proteins decrease the tension at air–water and oil–water interfaces by about 15 mN m^{-1} at saturated monolayer coverage, compared to 30–40 mN m^{-1} for small molecule surfactants. The inability of proteins to greatly reduce the interfacial tension is related to their complex structural properties. Though proteins contain hydrophilic and hydrophobic groups in their primary structure, there are no clearly defined hydrophilic head and hydrophobic tail as found in lecithin or monoacylglycerol. These groups are randomly spread all over the primary structure of proteins, and in the tertiary folded conformation some of the hydrophobic residues exist as segregated patches on the protein surface while a majority of them are in fact buried in the interior of the protein.

The pattern of distribution of hydrophilic and hydrophobic patches on a protein surface affects its rapidity of adsorption to the air–water or oil–water interface. If the protein surface is extremely hydrophilic and contains no discernable hydrophobic patches, anchoring of the protein at the interface probably will not take place because the protein surface will have a lower free energy in the aqueous phase than at the interface. As the number of hydrophobic patches on the protein surface is increased, spontaneous adsorption to an interface becomes more probable (Figure 5.22) [26]. Single hydrophobic residues randomly distributed on the protein surface do not constitute a hydrophobic patch, nor do they possess sufficient interaction energy to strongly anchor the protein at an interface. Even though more than 40% of a typical globular protein's overall accessible surface is covered with nonpolar residues, they will not enhance protein adsorption unless they exist as segregated regions or patches. In other words, the molecular characteristics of the protein surface have an enormous influence on whether a protein will spontaneously adsorb to an interface and how effective it will be as a stabilizer of dispersions.

The mode of adsorption of proteins at an interface is different from that of small molecular-weight surfactants. In the case of small molecule surfactants, such as phospholipids and monoacylglycerols, conformational constraints for adsorption and orientation do not exist because hydrophilic and hydrophobic moieties are present at the opposite ends of the molecule. In the case of proteins, however, the distribution pattern of hydrophobic and hydrophilic patches on the surface and the structural rigidity of the molecule cause constraints to adsorption and orientation. Because of the bulky, folded nature of proteins, once adsorbed, a large portion of the molecule remains in the bulk phase and only a small portion is anchored at the interface (Figure 5.23). The tenacity with which this small portion of the protein molecule remains attached at the interface depends on the number of peptide segments anchored to the interface and the energetics of interaction between these segments and the interface. The protein will be retained at the interface only when the sum of negative free energy changes of segment interactions is much greater than the thermal kinetic energy of the protein molecule. The number of peptide segments anchored at the interface depends, in part, on the conformational

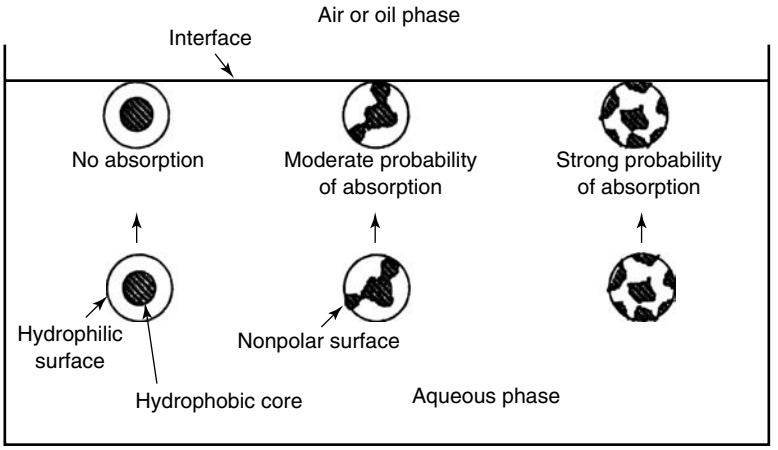


FIGURE 5.22 Schematic representation of the role of surface hydrophobic patches on the probability of adsorption of proteins at the air–water interface. (From Damodaran, S. 1990. *Adv. Food Nutr. Res*. **34**:1–79.)

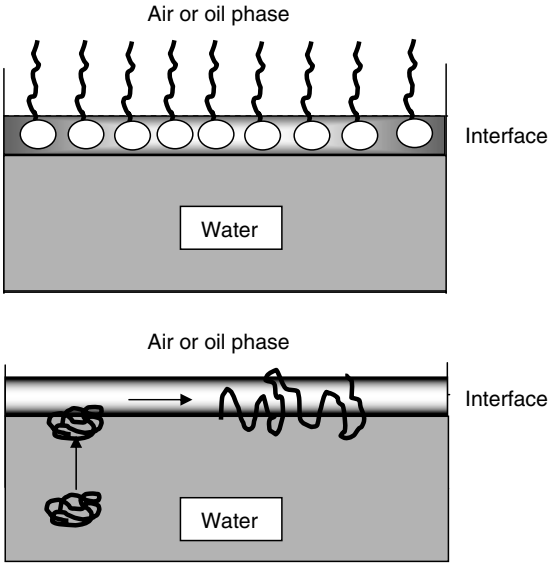


FIGURE 5.23 Difference in the mode of adsorption of a small molecule surfactant and a protein at the air–water or oil–water interface.

flexibility of the molecule. Highly flexible molecules, such as caseins, can undergo rapid conformational changes once they are adsorbed at the interface, enabling additional polypeptide segments to bind to the interface. On the other hand, rigid globular proteins such as lysozyme and soy protein cannot undergo extensive conformational changes at the interface.

At interfaces, polypeptide chains assume three distinct configurations: trains, loops, and tails (Figure 5.24) [26]. The trains are segments that are in direct contact with the interface, loops are segments of the polypeptide that are suspended in the aqueous phase, and tails are N- and C-terminal segments of the protein that are usually located in the aqueous phase. The relative distribution of these three configurations depends on the conformational characteristics of the protein. The greater the proportion of polypeptide segments in a train configuration, the stronger is the binding, and the lower is the interfacial tension.

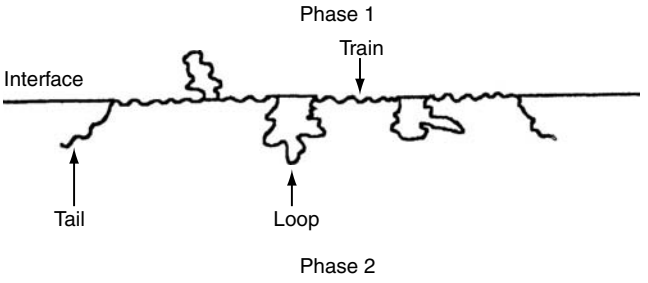


FIGURE 5.24 The various configurations of a flexible polypeptide at an interface. (From Damodaran, S. 1990. *Adv. Food Nutr. Res*. **34**:1–79.)

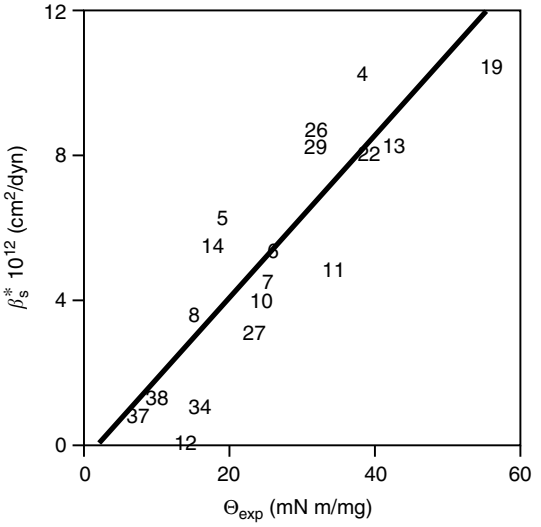


FIGURE 5.25 Relationship between adiabatic compressibility and surface activity of proteins. The numbers in the plot refer to the identities of proteins (see Reference 106 for further details.)

The single most important molecular property that impacts surface activity of proteins is molecular flexibility. This relates to a protein's innate ability to undergo rapid conformational change when it is transferred from one environment to another, for example, from a bulk aqueous phase to an interface. Adiabatic compressibility of proteins is often used as a measure of their molecular flexibility. Investigations on several unrelated proteins have shown that the dynamic surface activity of proteins, that is, the reduction in surface tension caused by one milligram of protein per cm^2 during adsorption from the bulk phase to the air–water interface, is positively and linearly correlated to the adiabatic compressibility (viz., flexibility) of proteins (Figure 5.25) [106]. Rapid conformation change at an interface is essential for the protein to reorient its hydrophobic and hydrophilic residues toward the oil and the aqueous phases and also to maximize the exposure and partitioning of these residues toward the two phases. This will ensure a rapid reduction in the interfacial tension, especially during the initial stages of formation of an emulsion.

The mechanical strength of a protein film at an interface depends on cohesive intermolecular interactions. These include attractive electrostatic interactions, hydrogen bonding, and hydrophobic interactions. Interfacial polymerization of adsorbed proteins via disulfide–sulfhydryl interchange reactions also increases their viscoelastic properties. The concentration of protein in the interfacial film is about 20–25% (w/v), and the protein exists in almost a gel-like state. The balance of various noncovalent interactions is crucial to the stability and viscoelastic properties of this gel-like film.

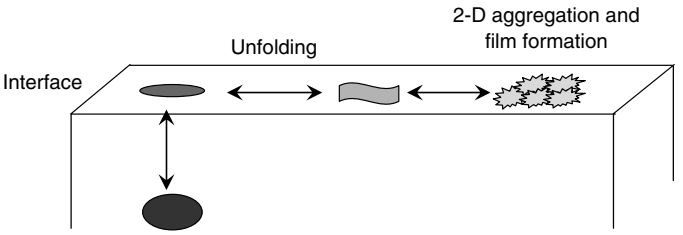


FIGURE 5.26 Schematic illustration of various molecular processes occurring in protein films at interfaces.

For example, if hydrophobic interactions are too strong, this can lead to interfacial aggregation, coagulation, and eventual precipitation of the protein, which will be detrimental to film integrity. If repulsive electrostatic forces are much stronger than attractive interactions, this may prevent formation of a thick, cohesive film. Therefore, a proper balance of attractive, repulsive, and hydration interactions is required to form a stable viscoelastic film. The various molecular processes that occur during adsorption and formation of protein films at interfaces are summarized in Figure 5.26.

The basic principles involved in formation and stability of emulsions and foams are very similar. However, since the energetics of these interfaces is different, the molecular requirements for protein functionality at these interfaces are not the same. In other words, a protein that is a good emulsifier may not be a good foaming agent.

It should now be clear that the behavior of proteins at interfaces is very complex and not well understood. Therefore, the following discussion of the emulsifying and foaming properties of food proteins will be largely qualitative in nature.

5.5.3.1 Emulsifying Properties

The physical chemistry of emulsion formation and the factors affecting creaming, flocculation, coalescence, and stability were reviewed in Chapter 13.

Several natural and processed foods, such as milk, egg yolk, coconut milk, soy milk, butter, margarine, mayonnaise, spreads, salad dressings, frozen desserts, frankfurter, sausage, and cakes are emulsion-type products, where proteins play an important role as an emulsifier. In natural milk, a membrane composed of lipoproteins stabilizes the fat globules. When milk is homogenized, a protein film comprised of casein micelles and whey proteins replaces the lipoprotein membrane. Homogenized milk is more stable against creaming than natural milk is because the casein micelle-whey protein film is stronger than the natural lipoprotein membrane.

5.5.3.1.1 Methods for Determining the Emulsifying Properties of Proteins

The emulsifying properties of food proteins are evaluated by several methods, such as size distribution of oil droplets formed, emulsifying activity, emulsion capacity (EC), and emulsion stability.

Emulsifying activity index: The physical and sensory properties of a protein-stabilized emulsion depend on the size of the droplets formed and the total interfacial area created. The average droplet size of emulsions can be determined by several methods, such as light microscopy (not very reliable), electron microscopy, light scattering (photon correlation spectroscopy), or use of a Coulter counter. Knowing mean droplet size, total interfacial area can be obtained from the relation:

$$A = \frac{3\phi}{R} \tag{5.53}$$

where ϕ is the volume fraction of the dispersed phase (oil) and R is the mean radius of the emulsion particles. If m is the mass of the protein, then the Emulsifying Activity Index (EAI), that is, the

interfacial area created per unit mass of protein is

$$\text{EAI} = \frac{3\phi}{Rm}. \tag{5.54}$$

Another simple and more practical method to determine EAI of proteins is the turbidimetric method [99]. The turbidity of an emulsion is given by:

$$T = \frac{2.303A}{l} \tag{5.55}$$

where A is absorbance and l is path length. According to Mie theory of light scattering, the interfacial area of an emulsion is twice its turbidity. If ϕ is the volume fraction of the oil, and C is the weight of protein per unit volume of the aqueous phase, then the EAI of the protein is given by:

$$\text{EAI} = \frac{2T}{(1-\phi)C}. \tag{5.56}$$

It should be mentioned that in the original article [99], ϕ, instead of $(1 - \phi)$ was used in the denominator of the above equation. The above expression is the correct one because ϕ is defined as the oil volume fraction, and thus $(1 - \phi)C$ is the total mass of protein in a unit volume of the emulsion [17]. Although this method is simple and practical, the main drawback is that it is based on measurement of turbidity at one single wavelength, 500 nm. Since the turbidity of food emulsions is wavelength dependent, the interfacial area obtained from turbidity at 500 nm is not very accurate. Therefore, use of the above equation to estimate mean particle diameter or the number of emulsion particles present in the emulsion gives results that are not very reliable. However, the method can be used for qualitative comparison of emulsifying activities of different proteins, or changes in the emulsifying activity of a protein after various treatments.

Protein load: The amount of protein adsorbed at the oil–water interface of an emulsion has a bearing on its stability. To determine the amount of protein adsorbed, the emulsion is centrifuged, the aqueous phase is separated, and the cream phase is repeatedly washed and centrifuged to remove any loosely adsorbed proteins. The amount of protein adsorbed to the emulsion particles is determined from the difference between the total protein initially present in the emulsion and the amount present in the wash fluid from the cream phase. Knowing the total interfacial area of the emulsion particles, the amount of protein adsorbed/m^2 of the interfacial area can be calculated. Generally, the protein load is in the range of about 1–3 mg/m^2 of interfacial area. As the volume fraction of the oil phase is increased, the protein load decreases at constant protein content in the total emulsion. For high-fat emulsions and small-sized droplets, more protein is obviously needed to adequately coat the interfacial area and stabilize the emulsion.

Emulsion capacity (EC): EC is the volume (mL) of oil that can be emulsified per gram of protein before phase inversion (a change from oil-in-water emulsion to water-in-oil) occurs. This method involves addition of oil or melted fat at a constant rate and temperature to an aqueous protein solution that is continuously agitated in a food blender. Phase inversion is detected by an abrupt change in viscosity or color (usually a dye is added to the oil), or by an increase in electrical resistance. For a protein-stabilized emulsion, phase inversion usually occurs when ϕ is about 0.65–0.85. Inversion is not instantaneous, but is preceded by formation of a water-in-oil-in-water double emulsion. Since EC is expressed as volume of oil emulsified per gram protein at phase inversion, it decreases with increasing protein concentration once a point is reached where unadsorbed protein accumulates in the aqueous phase. Therefore, to compare emulsion capacities of different proteins, EC vs. protein concentration profiles should be used instead of EC at a specific protein concentration.

Emulsion stability: Protein stabilized emulsions are often stable for days. Thus, a detectable amount of creaming or phase separation is usually not observed in a reasonable amount of time when samples are stored at atmospheric conditions. Therefore, drastic conditions, such as storage at elevated temperature or separation under centrifugal force is often used to evaluate emulsion stability. If centrifugation is used, stability is then expressed as percent decrease in interfacial area (i.e., turbidity) of the emulsion, or percent volume of cream separated, or as the fat content of the cream layer. More often, however, emulsion stability is expressed as

$$\text{ES} = \frac{\text{volume of cream layer}}{\text{total volume of emulsion}} \times 100 \tag{5.57}$$

where the volume of the cream layer is measured after a standardized centrifugation treatment. A common centrifugation technique involves centrifugation of a known volume of emulsion in a graduated centrifuge tube at 1300 g for 5 min. The volume of the separated cream phase is then measured and expressed as percentage of the total volume. Sometimes centrifugation at a relatively low gravitational force (180 g) for a longer time (15 min) is used to avoid coalescence of droplets.

The turbidimetric method (see above) can also be used to evaluate emulsion stability. In this case stability is expressed as Emulsion Stability Index (ESI), which is defined as the time to achieve a turbidity of the emulsion that is one-half of the original value.

The methods used to determine emulsion stability are very empirical. The most fundamental quantity related to stability is the change in interfacial area with time, but this is difficult to measure directly.

5.5.3.1.2 Factors Influencing Emulsification

The properties of protein-stabilized emulsions are affected by several factors. These include intrinsic factors, such as pH, ionic strength, temperature, presence of low molecular-weight surfactants, sugars, oil phase volume, type of protein, and the melting point of the oil used; and extrinsic factors such as type of equipment, rate of energy input, and rate of shear. Standardized methods for systematically evaluating the emulsifying properties of proteins have not emerged. Therefore, results among laboratories cannot be accurately compared and this has hampered the understanding of the molecular factors that affect emulsifying properties of proteins.

The general forces involved in the formation and stabilization of emulsion were discussed in Chapter 13. Therefore, only the molecular factors that affect protein-stabilized emulsions need be discussed here.

Solubility plays a role in emulsifying properties, but 100% solubility is not an absolute requirement. While highly insoluble proteins do not perform well as emulsifiers, no reliable relationship exists between solubility and emulsifying properties in the 25–80% solubility range [76]. However, since the stability of a protein film at the oil–water interface is dependent on favorable interactions with both the oil and aqueous phases, some degree of solubility is likely to be necessary. The minimum solubility requirement for good performance may vary among proteins. In meat emulsions, such as in sausage and frankfurter, solubilization of myofibrillar proteins in 0.5 M NaCl enhances their emulsifying properties. Some commercial soy protein isolates that are isolated by thermal processing have poor emulsifying properties because of their very low solubility.

The formation and stability of protein-stabilized emulsions are affected by pH. Several mechanisms are involved. Generally, proteins that have high solubility at the isoelectric pH (e.g., serum albumin, gelatin, and egg-white proteins) show maximum emulsifying activity and EC at that pH. The lack of net charge and electrostatic repulsive interactions at the isoelectric pH helps maximize protein load at the interface and promotes formation of a highly viscoelastic film, both of which contribute to emulsion stability. However, the lack of electrostatic repulsive interactions among emulsion particles can, in some instances promote flocculation, coalescence, and thus decrease emulsion stability. On the other hand, if the protein is highly hydrated at the isoelectric pH (unusual), then

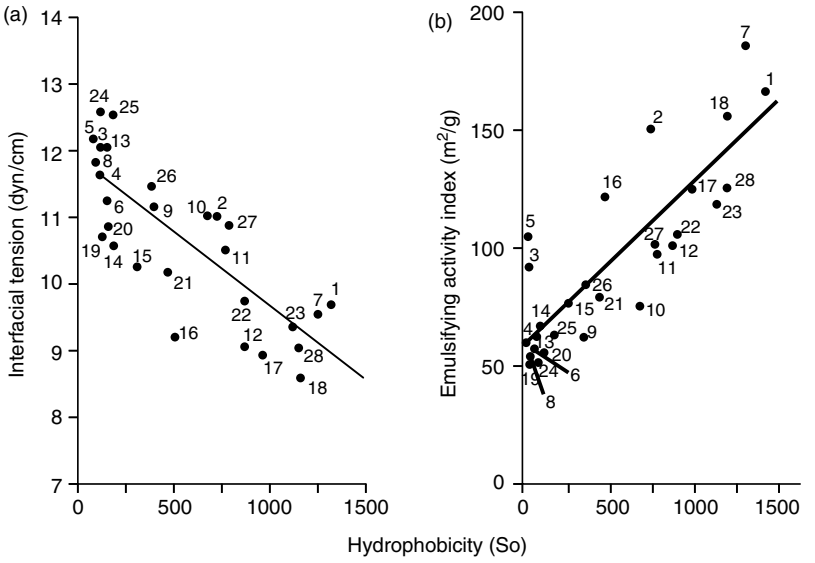


FIGURE 5.27 Correlations of surface hydrophobicity of various proteins with (a) oil–water interfacial tension and (b) EAI. Surface hydrophobicity was determined from the amount of hydrophobic fluorescent probe bound per unit weight of protein. The numbers in the plots represent (1) bovine serum albumin; (2) β-lactoglobulin; (3) trypsin; (4) ovalbumin; (5) conalbumin; (6) lysozyme; (7) κ-casein; (8–12) ovalbumin denatured by heating at 85°C for 1, 2, 3, 4, or 5 min, respectively; (13–18) lysozyme denatured by heating at 85°C for 1, 2, 3, 4, 5, or 6 min, respectively; (19–23) ovalbumin bound to 0.2, 0.3, 1.7, 5.7, or 7.9 mole dodecyl sulfate per mol protein, respectively; (24–28) ovalbumin bound to 0.3, 0.9, 3.1, 4.8, or 8.2 mol linoleate per mole protein, respectively. (From Kato, A. and S. Nakai. 1980. *Biochim. Biophys. Acta* **624**:13–20.)

hydration repulsion forces between emulsion particles may prevent flocculation and coalescence, and thus stabilize the emulsion. Because most food proteins (caseins, commercial whey proteins, meat proteins, and soy proteins) at their isoelectric pH are sparingly soluble, poorly hydrated and lack electrostatic repulsive forces, they are generally poor emulsifiers at this pH. These proteins may, however, be effective emulsifiers when moved away from their isoelectric pH.

The emulsifying properties of proteins show a weak positive correlation with surface hydrophobicity, but not with mean residue hydrophobicity (i.e., kcal mol^{-1} $residue^{-1}$). The ability of various proteins to decrease interfacial tension at the oil–water interface and to increase the EAI is related to their surface hydrophobicity values (Figure 5.27). However, this relationship is by no means perfect. The emulsifying properties of several proteins, such as β-lactoglobulin, α-lactalbumin, and soy proteins, do not show a strong correlation with surface hydrophobicity.

The surface hydrophobicity of proteins is usually determined by measuring the amount of a hydrophobic fluorescent probe, such as *cis*-parinaric acid, that can bind to the protein [62]. Although this method provides some information on the hydrophobicity of the protein surface, it is questionable whether the measured value truly reflects the "hydrophobicity" of the protein surface. The true definition of surface hydrophobicity is that portion of the nonpolar surface of the protein that makes contact with the surrounding bulk water. However, *cis*-parinaric acid is capable of binding only to hydrophobic cavities. These protein cavities are accessible to nonpolar ligands, but they are not accessible to water and may not be accessible to either phase in an oil–water emulsion, unless the protein is able to undergo rapid conformational rearrangement at the interface. The poor correlation of surface hydrophobicity (as measured by *cis*-parinaric acid binding) with the emulsifying properties of some proteins may be related to the fact that *cis*-parinaric acid provides no indication of molecular flexibility. Molecular flexibility at the oil–water interface may be the most important determinant of the emulsifying properties of proteins.

Partial denaturation of proteins prior to emulsification, which does not result in insolublization, usually improves their emulsifying properties. This is due to increased molecular flexibility and surface hydrophobicity. In the unfolded state, proteins containing free sulfhydryl groups and disulfide bonds undergo slow polymerization via disulfide–sulfhydryl interchange reaction [32]. This leads to formation of a highly viscoelastic film at the oil–water interface. Excessive heat denaturation may impair the emulsifying properties by rendering the protein insoluble.

Small molecule emulsifiers, such as phospholipids, which are generally found in foods, compete with proteins for adsorption at the oil–water interface [24,38,67]. Since small molecule surfactants can diffuse rapidly to the interface and lack conformational constraints for reorientation at the interface, they can effectively inhibit adsorption of proteins at high concentrations. If small molecule emulsifiers are added to a protein-stabilized emulsion, they can displace the protein from the interface and cause instability in the emulsion.

Another factor that affects protein-stabilized emulsions is the protein composition. Food proteins in general are mixtures of several protein components. For instance, egg protein is a mixture of five major proteins and several minor protein components. Likewise, whey protein is a mixture of α-lactalbumin, β-lactoglobulin, and several other minor proteins. Seed storage proteins, such as soy protein isolate, contain at least two major protein fractions, viz., legumins and vicillins. During emulsification, the protein components of the mixture compete with each other for adsorption to the interface. The composition of the protein film formed at the interface is dependent on relative surface activities of various protein components of the mixture. For instance, when a 1:1 mixture of α- and β-caseins are allowed to adsorb to the oil–water interface, the amount of α-casein in the protein film at equilibrium is almost twice that of β-casein [30]. At the air–water interface, however, an exactly opposite behavior is observed [6]. Variations in the protein composition of the bulk phase would affect protein composition of the adsorbed film and possibly the stability of the emulsion.

At high concentration, protein mixtures generally exhibit incompatibility of mixing in solution [100]. In mixed protein films at the oil–water interface, where the local protein concentration is in the range of 15–30%, it is likely that two-dimensional phase separation of the proteins can occur with storage time. Evidence for this at the air–water [105,114] and oil–water [30] interfaces has been reported. If distinct phase separation of proteins occurs in mixed protein films around oil droplets, it is conceivable that the interface of such phase-separated regions might act as source of instability in emulsions. However, a direct correlation between thermodynamic incompatibility of mixing of proteins in mixed protein films at the oil–water interface and the kinetic stability of emulsions made of protein mixtures is yet to be determined.

5.5.3.2 Foaming Properties

Foams consist of an aqueous continuous phase and a gaseous (air) dispersed phase. Many processed foods are foam-type products. These include whipped cream, ice cream, cakes, meringue, bread, souffles, mousses, and marshmallow. The unique textural properties and mouthfeel of these products stem from the dispersed tiny air bubbles. In most of these products, proteins are the main surface active agents that help in the formation and stabilization of the dispersed gas phase.

Generally, bubbling, whipping, or shaking a protein solution creates protein-stabilized foams. The foaming property of a protein refers to its ability to form a thin tenacious film at gas–liquid interfaces so that large quantities of gas bubbles can be incorporated and stabilized. Foaming properties are evaluated by several means. The *foamability* or *foaming capacity* of a protein refers to the amount of interfacial area that can be created by the protein. It can be expressed in several ways, such as *overrun* (or steady state foam volume) or *foaming power* (or foam expansion). Overrun is defined as

$$\text{Overrun} = \frac{\text{Volume of foam}}{\text{Volume of initial liquid}} \times 100 \tag{5.58}$$

TABLE 5.15
Comparative Foaming Power of Protein Solutions

Protein Type	Foaming Power[a] at 0.5% Protein Conc. (w/v) (%)
Bovine serum albumin	280
Whey protein isolate	600
Egg albumen	240
Ovalbumin	40
Bovine plasma	260
β-Lactoglobulin	480
Fibrinogen	360
Soy protein (enzyme hydrolyzed)	500
Gelatin (acid-processed pigskin)	760

[a] Calculated according to Equation 5.56.

Source: From Poole, S. et al. 1984. *J. Sci. Food Agric.* **35**:701–711.

The foaming power (FP), is expressed as

$$\text{FP} = \frac{\text{Volume of gas incorporated}}{\text{Volume of liquid}} \times 100 \tag{5.59}$$

Foaming power generally increases with protein concentration until a maximum value is attained. It is also affected by the method used for foam formation. FP at a given protein concentration is often used as a basis for comparing the foaming properties of various proteins. The FPs of various proteins at pH 8.0 are given in Table 5.15 [82].

Foam stability refers to the ability of protein to stabilize foam against gravitational and mechanical stresses. Foam stability is often expressed as the time required for 50% of the liquid to drain from foam or for a 50% reduction in foam volume. These are very empirical methods, and they do not provide fundamental information about the factors that affect foam stability. The most direct measure of foam stability is the reduction in foam interfacial area as a function of time. This can be done as follows. According to the Laplace principle, the internal pressure of a bubble is greater than the external (atmospheric) pressure, and under stable conditions the pressure difference, ΔP, is

$$\Delta P = p_{\text{i}} - p_{\text{o}} = \frac{4\gamma}{r} \tag{5.60}$$

where p_{i} and p_{o} are the internal and external pressures, respectively, r is radius of the foam bubble, and γ is surface tension. According to the above equation, the pressure inside a closed vessel containing foam will increase when the foam collapses. The net change in the pressure is [92]

$$\Delta P = \frac{-2\gamma \Delta A}{3V} \tag{5.61}$$

where V is the total volume of the system, ΔP is the pressure change, and ΔA is the net change in interfacial area resulting from the fraction of collapsed foam. The initial interfacial area of the foam is given by:

$$A_0 = \frac{3V\Delta P_\infty}{2\gamma} \tag{5.62}$$

where ΔP_∞ is the net pressure change when the entire foam is collapsed. The A_0 value is a measure of foamability, and the rate of decrease of A with time can be used as a measure of foam stability. This approach has been used to study the foaming properties of food proteins [133,135].

The *strength* or *stiffness* of the foam refers to the maximum weight a column of foam can withstand before it collapses. Measuring foam viscosity also assesses this property.

5.5.3.2.1 Environmental Factors Influencing Foam Formation and Stability

pH: Several studies have shown that protein-stabilized foams are more stable at the isoelectric pH of the protein than at any other pH, provided there is no insolublization of the protein at pI. At or near the isoelectric pH region, the lack of repulsive interactions promotes favorable protein–protein interactions and formation of a viscous film at the interface. In addition, an increased amount of protein is adsorbed to the interface at the pI because of lack of repulsion between the interface and the adsorbing molecules. These two factors improve both foamability and foam stability. If the protein is sparingly soluble at pI, as most food proteins are, then only the soluble protein fraction will be involved in foam formation. Since the concentration of this soluble fraction is very low, the amount of foam formed will be less, but the stability will be high. Although the insoluble fraction does not contribute to foamability, adsorption of these insoluble protein particles may stabilize the foam, probably by increasing cohesive forces in the protein film. Generally, adsorption of hydrophobic particles increases the stability of foams. At pH other than pI, foamability of proteins is often good, but foam stability is poor. Egg-white proteins exhibit good foaming properties at pH 8–9 and at their isoelectric pH 4–5.

Salts: The effects of salts on the foaming properties of proteins depend on the type of salt and the solubility characteristics of the protein in that salt solution. The foamability and foam stability of most globular proteins, such as bovine serum albumin, egg albumin, gluten, and soy proteins increase with increasing concentration of NaCl. This behavior is usually attributed to neutralization of charges by salt ions. However, some proteins, such as whey proteins, exhibit the opposite effect: both foamability and foam stability decrease with increasing concentration of NaCl (Table 5.16) [136]. This is attributed to salting-in of whey proteins, especially β-lactoglobulin. Proteins that are salted-out in a given salt solution generally exhibit improved foaming properties; whereas, those that are salted-in generally exhibit poor foaming properties. Divalent cations, such as Ca^{2+} and Mg^{2+}, dramatically improve both foamability and foam stability at 0.02–0.4 M concentration. This is primarily due to cross-linking of protein molecules and creation of films with better viscoelastic properties [134].

TABLE 5.16
Effect of NaCl on Foamability and Foam Stability of Whey Protein Isolate

NaCl Concentration (M)	Total Interfacial Area (cm^2/ml of foam)	Time for 50% Collapse of Initial Area (s)
0.00	333	510
0.02	317	324
0.04	308	288
0.06	307	180
0.08	305	165
0.10	287	120
0.15	281	120

Source: Compiled from Zhu, H. and S. Damodaran. 1994. *J. Food Sci*. **59**:554–560.

Sugars: Addition of sucrose, lactose, and other sugars to protein solutions often impairs foamability, but improves foam stability. The positive effect of sugars on foam stability is due to increased bulk phase viscosity, which reduces the rate of drainage of the lamella fluid. The depression in foam overrun is mainly due to enhanced stability of protein structure in sugar solutions. Because of this, the protein molecule is less able to unfold upon adsorption at the interface. This decreases the ability of the protein to reduce interfacial tension, produce large interfacial areas and large foam volume during whipping. In sugar containing, foam-type dessert products, such as meringues, souffles, and cakes, it is preferable to add sugar after whipping when possible. This will enable the protein to adsorb, unfold, and form a stable film, and then the added sugar will increase foam stability by increasing the viscosity of the lamella fluid.

Lipids: Lipids, especially phospholipids, when present at concentrations >0.5%, markedly impair the foaming properties of proteins. Because lipids are more surface-active than proteins, they readily adsorb at the air–water interface and inhibit adsorption of proteins during foam formation. Since lipid films lack the cohesive and viscoelastic properties necessary to withstand the internal pressure of the foam bubbles, the bubbles rapidly expand, then collapse during whipping. Thus, lipid-free whey protein concentrates (WPC) and isolates, soy proteins, and egg proteins without egg yolk display better foaming properties than do lipid-contaminated preparations.

Protein concentration: Several properties of foams are influenced by protein concentration. The greater the protein concentration, the stiffer is the foam. Foam stiffness results from small bubble size and high viscosity. The stability of the foam is enhanced by greater protein concentrations because this increases viscosity and facilitates formation of a multilayer, cohesive, protein film at the interface. Foamability generally reaches a maximum value at some point during increase of protein concentration. Some proteins, for example, serum albumin, are able to form relatively stable foams at 1% protein concentration, whereas proteins such as WPI and soy proteins require a minimum of 2–5% to form relatively stable foam. Generally, most proteins display maximum foamability at 2–8% concentration. The interfacial concentration of proteins in foams is about 2–3 mg/m^2.

Partial heat denaturation improves the foaming properties of proteins. For instance, heating of WPI at 70°C for 1 min improves, whereas heating at 90°C for 5 min decreases foaming properties even though the heated proteins remain soluble in both instances [135]. The decrease in foaming properties of WPI heated at 90°C is due to extensive polymerization of the protein via disulfide-sulfhydryl interchange reactions. The highly cross-linked and polymerized protein is unable to adsorb to the air–water interface during foaming.

The method of foam generation influences the foaming properties of proteins. Air introduction by bubbling or sparging usually results in "wet" foam with a relatively large bubble size. Whipping at moderate speed generally results in foam with small-sized bubbles because the shearing action results in partial denaturation of the protein before adsorption occurs. However, whipping at high shear rate or "overbeating" can decrease foaming power because of extensive denaturation, aggregation, and precipitation of proteins.

Some foam-type food products, such as marshmallow, cakes, and bread, are heated after the foam is formed. During heating, expansion of air and decreased viscosity can cause bubble rupture and collapse of the foam. In these instances, the integrity of the foam depends on gelation of the protein film at the interface so sufficient mechanical strength is developed to stabilize the foam. Gelatin, gluten, and egg white, which display good foaming and gelling properties, are highly suitable for this purpose.

5.5.3.2.2 Molecular Properties Influencing Foam Formation and Stability

For a protein to perform effectively as a foaming agent or an emulsifier it must meet the following basic requirements: (1) it must be able to rapidly adsorb to the air–water interface, (2) it must readily

unfold and rearrange at the interface, and (3) it should be able to form a viscous cohesive film through intermolecular interactions. The molecular properties that affect foaming properties are molecular flexibility, charge density and distribution, and hydrophobicity.

The free energy of the air–water interface is significantly greater than that of the oil–water interface. Therefore, to stabilize the air–water interface, the protein must have the ability to rapidly adsorb to the freshly created interface, and instantaneously decrease the interfacial tension to a low value. The lowering of interfacial tension is dependent on the ability of the protein to rapidly unfold, rearrange, and expose hydrophobic groups at the interface. Random-coil-type proteins, such as β-casein, perform well in this manner. On the other hand, tightly folded globular proteins, such as lysozyme, adsorb very slowly, only partially unfold, and reduce the surface tension only slightly [130]. Lysozyme is, therefore, a poor foaming agent. Thus, molecular flexibility at the interface is quintessential for good performance as a foaming agent.

Apart from molecular flexibility, hydrophobicity also plays a role in foamability of proteins. The foaming power of proteins is positively correlated with the *mean* hydrophobicity. However, the foaming power of proteins varies curvilinearly with *surface* hydrophobicity, and a significant correlation does not exist between these two properties at hydrophobicity values of greater than 1000 [63]. This indicates that a surface hydrophobicity of at least 1000 is needed for initial adsorption of proteins at the air–water interface, whereas, once adsorbed, the ability of the protein to create more interfacial area during foam formation depends on the mean hydrophobicity of the protein.

A protein that displays good foamability need not be a good foam stabilizer. For example, although β-casein exhibits excellent foamability, the stability of the foam is poor. On the other hand, lysozyme exhibits poor foamability, but its foams are very stable. Generally, proteins that possess good foaming power do not have the ability to stabilize foam, and proteins that produce stable foams often exhibit poor foaming power. It appears that foamability and stability are influenced by two different sets of molecular properties of proteins that are often antagonistic. Whereas foamability is affected by rate of adsorption, flexibility, and hydrophobicity, stability depends on the rheological properties of the protein film. The rheological properties of films depend on hydration, thickness, protein concentration, and favorable intermolecular interactions. Proteins that only partially unfold and retain some degree of folded structure usually form thicker, denser films, and more stable foams (e.g., lysozyme and serum albumin) than do those that completely unfold (e.g., β-casein) at the air–water interface. In the former case, the folded structure extends into the subsurface in the form of loops. Noncovalent interactions, and possibly disulfide cross-linking, between these loops promote formation of a gel network, which has excellent viscoelastic and mechanical properties. For a protein to possess good foamability and foam stability it should have an appropriate balance between flexibility and rigidity, should easily undergo unfolding, and should engage in abundant cohesive interactions at the interface. However, what extent of unfolding is desirable for a given protein is difficult, if not impossible, to predict. In addition to these factors, foam stability usually exhibits an inverse relationship with the charge density of proteins. High charge density apparently interferes with formation of a cohesive film.

Most food proteins are mixtures of various proteins, and therefore their foaming properties are influenced by interaction between the protein components at the interface. The excellent whipping properties of egg white are attributed to interactions between its protein components, such as ovalbumin, conalbumin, and lysozyme. Several studies have indicated that the foaming properties of acidic proteins can be improved by mixing them with basic proteins, such as lysozyme and clupeine [102]. This enhancing effect seems to be related to the formation of an electrostatic complex between the acidic and basic proteins.

Limited enzymatic hydrolysis of proteins generally improves their foaming properties. This is because of increased molecular flexibility and greater exposure of hydrophobic groups. However, extensive hydrolysis impairs foamability because low molecular weight peptides cannot form a cohesive film at the interface.

5.5.4 Flavor Binding

Proteins themselves are odorless. However, they can bind flavor compounds, and thus affect the sensory properties of foods. Several proteins, especially oilseed proteins and WPCs, carry undesirable flavors, which limits their usefulness in food applications. These off-flavors are mainly due to aldehydes, ketones, and alcohols generated by oxidation of unsaturated fatty acids. Upon formation, these carbonyl compounds bind to proteins and impart characteristic off-flavors. For example, the beany and grassy flavor of soy protein preparations is attributed to the presence of hexanal. The binding affinity of some of these carbonyls is so strong that they resist even solvent extraction. A basic understanding of the mechanism of binding of off-flavors to proteins is needed so that appropriate methods can be developed for their removal.

The flavor-binding property of proteins also has desirable aspects, because they can be used as flavor carriers or flavor modifiers in fabricated foods. This is particularly useful in meat analogues containing plant proteins, where successful simulation of a meat-like flavor is essential for consumer acceptance. In order for a protein to function as a good flavor carrier, it should bind flavors tightly, retain them during processing, and release them during mastication of food in the mouth. However, proteins do not bind all flavor compounds with equal affinity. This leads to uneven and disproportionate retention of some flavors and undesirable losses during processing. Because protein-bound flavorants do not contribute to taste and aroma unless they are released readily in the mouth, knowledge of the mechanisms of interaction and binding affinity of various flavorants is essential if effective strategies for producing flavor-protein products or for removing off-flavors from protein isolates are to be devised.

5.5.4.1 Thermodynamics of Protein–Flavor Interactions

In water-flavor model systems, addition of proteins causes a reduction in the headspace concentration of flavor compounds. This is due to binding of flavors to proteins. The mechanism of flavor binding to proteins depends upon the moisture content of the protein sample, but interactions are normally noncovalent. Dry protein powders bind flavors mainly via van der Waals, hydrogen bonding, and electrostatic interactions. Physical entrapment within capillaries and crevices of dry protein powders may also contribute to flavor properties of dry protein powders. In liquid or high moisture foods, the mechanism of flavor binding by proteins primarily involves interaction of the nonpolar flavor compounds (ligands) with hydrophobic patches/cavities on the protein surface. In addition to hydrophobic interactions, flavor compounds with polar head groups, such as hydroxyl and carboxyl groups may also interact with proteins via hydrogen bonding and electrostatic interactions. After binding to the surface hydrophobic regions, aldehydes and ketones may be able to diffuse into the hydrophobic interior of the protein molecule.

Flavor–protein interaction is usually completely reversible. However, aldehydes, can covalently bind to the amino group of lysine side chains and this interaction is nonreversible. However, only the noncovalently bound fraction can contribute to aroma and taste of the protein product.

The extent of flavor binding by hydrated proteins depends on the number of hydrophobic binding regions available on the protein surface [28]. The binding sites are usually made up of groups of hydrophobic residues segregated in the form of a well-defined cavity. Single nonpolar residues on the protein surface are less likely to act as binding sites. Under equilibrium conditions, the reversible noncovalent binding of a flavor compound with proteins follows the Scatchard equation:

$$\frac{\upsilon}{[L]} = nK - \upsilon K \tag{5.63}$$

where υ is moles of ligand bound per mole of protein, n is the total number of binding sites per mole of protein, $[L]$ is the free ligand concentration at equilibrium, and K is the equilibrium binding constant (M^{-1}). According to this equation, a plot of $\upsilon/[L]$ vs. υ will be a straight line; the values

TABLE 5.17
Thermodynamic Constants for Binding of Carbonyl Compounds to Proteins

Protein	Carbonyl Compound	n (moles/mole)	K (M^{-1})	ΔG (kcal/mol)
Serum albumin	2-Nonanone	6	1800	−4.4
	2-Heptanone	6	270	−3.3
β-Lactoglobulin	2-Heptanone	2	150	−3.0
	2-Octanone	2	480	−3.7
	2-Nonanone	2	2440	−4.7
Soy Protein				
Native	2-Heptanone	4	110	−2.8
	2-Octanone	4	310	−3.4
	2-Nonanone	4	930	−4.1
	5-Nonanone	4	541	−3.8
	Nonanal	4	1094	−4.2
Partially denatured	2-Nonanone	4	1240	−4.3
Succinylated	2-Nonanone	2	850	−4.0

n, number of binding sites in native state; K, equilibrium binding constant.

Source: Compiled from Damodaran, S. and J. E. Kinsella. 1980. *J. Agric. Food Chem.* **28**:567–571; Damodaran, S. and J. E. Kinsella. 1981. *J. Agric. Food Chem.* **29**:1249–1253; and O'Neill, T. E. and J. E. Kinsella. 1987. *J. Agric. Food Chem.* **35**:770–774.

of K and n can be obtained from the slope and the intercept, respectively. The free energy change for binding of ligand to protein is obtained from the equation

$$\Delta G = -RT \ln K,$$

where R is the gas constant and T is absolute temperature. The thermodynamic constants for the binding of carbonyl compounds to various proteins are presented in Table 5.17 [28,29,95]. The binding constant increases by about threefold for each methylene group increment in chain length, with a corresponding free energy change of −0.55 kcal/mol per CH_2 group. This indicates that the binding is hydrophobic in nature.

It is assumed in the Scatchard relationship that all ligand-binding sites in a protein have the same affinity, and that no conformational changes occur upon binding of the ligand to these sites. Contrary to the latter assumption, proteins generally do undergo a modest conformational change upon binding of flavor compounds. Diffusion of flavor compounds into the interior of the protein may disrupt hydrophobic interactions between protein segments, and thus destabilize the protein structure. Flavor ligands with reactive groups, such as aldehydes, can covalently bind to the ε-amino groups of lysine residues, change the net charge of the protein, and thus cause protein unfolding. Unfolding generally results in exposure of new hydrophobic sites for ligand binding. Because of these structural changes, Scatchard plots for protein are generally curvilinear. In the case of oligomeric proteins, such as soy proteins, conformational changes may involve both dissociation and unfolding of subunits. Denatured proteins generally exhibit a large number of binding sites with weak association constants. Methods for measuring flavor binding can be found in References 28 and 29.

5.5.4.2 Factors Influencing Flavor Binding

Since volatile flavors interact with hydrated proteins mainly via hydrophobic interactions, any factor that affects hydrophobic interactions or surface hydrophobicity of proteins will influence flavor

binding. Temperature has very little effect on flavor binding, unless there is significant thermal unfolding of the protein occurs. This is because the association process is primarily entropy driven, not enthalpy driven. Thermally denatured proteins exhibit increased ability to bind flavors; however, the binding constant is usually low compared to that of native proteins. The effects of salts on flavor binding are related to their salting-in and salting-out properties. Salting-in-type salts, which destabilize hydrophobic interactions, decrease flavor binding, whereas salting-out-type salts increase flavor binding.

The effect of pH on flavor binding is generally related to pH-induced conformational changes in proteins. Flavor binding is usually enhanced more at alkaline pH than at acid pH; this is because proteins tend to denature more extensively at alkaline pH than at acid pH. Breakage of protein disulfide bonds, which occurs at alkaline pH and causes unfolding of proteins, usually increases flavor binding. Proteolysis, which disrupts and decreases the number of hydrophobic regions in proteins, decreases flavor binding. This can be used as a way of removing off-flavors from oilseed proteins.

5.5.5 Viscosity

The consumer acceptability of several liquid and semisolid-type foods (e.g., gravies, soups, beverages, etc.) depends on the viscosity or consistency of the product. The viscosity of a solution relates to its resistance to flow under an applied force (or shear stress). For an ideal solution, the shear stress (i.e., force per unit area, F/A) is directly proportional to the shear rate (i.e., the velocity gradient between the layers of the liquid, $\mathrm{d}v/\mathrm{d}r$). This is expressed as

$$\frac{F}{A} = \eta \frac{\mathrm{d}v}{\mathrm{d}r} \tag{5.64}$$

The proportionality constant η is known as the viscosity coefficient. Fluids that obey the above expression are called Newtonian fluids.

The flow behavior of solutions is greatly influenced by solute type. Large molecular weight soluble polymers greatly increase viscosity even at very low concentrations. This again depends on several molecular properties such as size, shape, flexibility, and hydration. Solutions of randomly coiled macromolecules display greater viscosity than do solutions of compact folded macromolecules of same molecular weight.

Most macromolecular solutions, including protein solutions, do not display Newtonian behavior, especially at high protein concentrations. For these systems, the viscosity coefficient decreases when the shear rate increases. This behavior is known as *pseudoplastic* or *shear-thinning*, and follows the relationship:

$$\frac{F}{A} = m\left(\frac{\mathrm{d}v}{\mathrm{d}r}\right)^n \tag{5.65}$$

where m is the consistency coefficient and n is an exponent known as the "flow behavior index." The pseudoplastic behavior of protein solutions arises because of the tendency of protein molecules to orient their major axes in the direction of flow. Dissociation of weakly held dimers and oligomers into monomers also contribute to shear-thinning. When shearing or flow is stopped, the viscosity may or may not return to the original value depending on the rate of relaxation of the protein molecules to random orientation. Solutions of fibrous proteins, for example, gelatin and actomyosin, usually remain oriented, and thus do not quickly regain their original viscosity. On the other hand, solutions of globular proteins, for example, soy proteins and whey proteins, rapidly regain their viscosity when flow is stopped. Such solutions are called *thixotropic*.

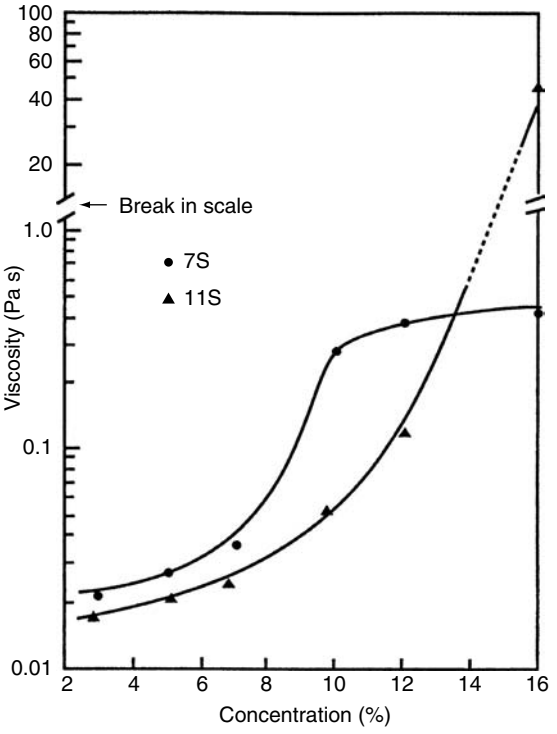


FIGURE 5.28 Effect of concentration on viscosity (or consistency index) of 7S and 11S soy protein solutions at 20°C. (From Rao, M. A. et al. 1986. In *Food Engineering and Process Applications* (Le Maguer, M. and P. Jelen, Eds.), Elsevier Applied Sci., New York, pp. 39–48.)

The viscosity (or consistency) coefficient of most protein solutions follows an exponential relationship with protein concentration because of both protein–protein interactions and interactions between the hydration spheres of protein molecules. An example involving soy protein fractions is shown in Figure 5.28 [104]. At high protein concentrations or in protein gels, where protein–protein interactions are numerous and strong, proteins display plastic viscoelastic behavior. In these cases, a specific amount of force, known as "yield stress," is required to initiate flow.

The viscosity behavior of proteins is a manifestation of complex interactions among several variables, including size, shape, protein–solvent interactions, hydrodynamic volume, and molecular flexibility in the hydrated state. When dissolved in water, proteins absorb water and swell. The volume of the hydrated molecules is much larger than their unhydrated volume. The protein-associated water induces long-range effects on the flow behavior of the solvent. The dependence of viscosity on shape and size of protein molecules follows the relationship:

$$\eta_{sp} = \beta C(\bar{\upsilon}_2 + \delta_1 \bar{\upsilon}_1) \tag{5.66}$$

where η_{sp} is specific viscosity, β is the shape factor, C is concentration, $\bar{\upsilon}_2$ and $\bar{\upsilon}_1$ are specific volumes of unhydrated protein and solvent, respectively, and δ_1 is grams of water bound per gram of protein. Here, $\bar{\upsilon}_2$ is also related to molecular flexibility; the greater the specific volume of the protein, the greater is its flexibility.

The viscosity of dilute protein solutions is expressed in several ways. *Relative viscosity* η_{rel} refers to the ratio of viscosity of the protein solution to that of the solvent. It is measured in an

Ostwald–Fenske type capillary viscometer, and is expressed as

$$\eta_{\text{rel}} = \frac{\eta}{\eta_0} = \frac{\rho t}{\rho_0 t_0} \tag{5.67}$$

where ρ and ρ_0 are densities of protein solution and solvent, respectively, and t and t_0 are times of flow for a given volume of protein solution and solvent, respectively, through the capillary. Other forms of expressing viscosity can be obtained from the relative viscosity. *Specific viscosity* is defined as

$$\eta_{\text{sp}} = \eta_{\text{rel}} - 1 \tag{5.68}$$

Reduced viscosity is

$$\eta_{\text{red}} = \frac{\eta_{\text{sp}}}{C} \tag{5.69}$$

where C is the protein concentration, and *intrinsic viscosity* is

$$[\eta] = \text{Lim}\frac{\eta_{\text{sp}}}{C} \tag{5.70}$$

The intrinsic viscosity, $[\eta]$, is obtained by extrapolating a plot of reduced viscosity vs. protein concentration to zero protein concentration (Lim). Since protein–protein interactions are nonexistent at infinite dilution, intrinsic viscosity accurately depicts the effects of shape and size on the flow behavior of individual protein molecules. Changes in the hydrodynamic shape of proteins that result from heat and pH treatments can be studied by measuring their intrinsic viscosities.

5.5.6 Gelation

A gel is an intermediate phase between a solid and a liquid. Technically, it is defined as "a substantially diluted system that exhibits no steady state flow" [43]. It is made up of polymers cross-linked via either covalent or noncovalent bonds to form a network that is capable of entrapping water and other small molecular-weight substances (see Chapter 13).

Protein gelation refers to transformation of a protein from the "sol" state to a "gel-like" state. Heat, enzymes, or divalent cations under appropriate conditions facilitate this transformation. All these agents induce formation of network structure; however, the types of covalent and noncovalent interactions involved, and the mechanism of network formation can differ considerably.

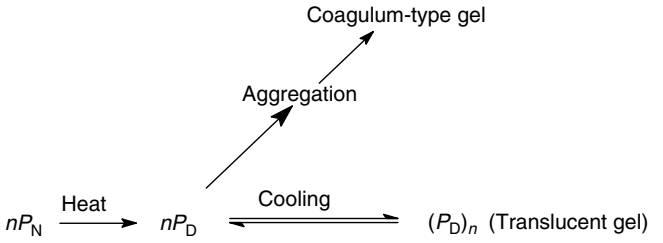
Most food protein gels are prepared by heating a moderately concentrated protein solution. In this mode of gelation, the protein in a "sol" state is first transformed into a "progel" state by denaturation. In the "sol" state, the number of noncovalent bonding groups available in proteins for network structure formation is limited. The progel state however is usually a viscous liquid state in which some degree of protein denaturation and polymerization has already occurred. Also, in the progel state, a critical number of functional groups, such as hydrogen bonding and hydrophobic groups that can form intermolecular noncovalent bonds, become exposed so that the second stage, formation of a protein network, can occur. The conversion of sol to progel is irreversible because many protein–protein interactions occur between the unfolded molecules. When the progel is cooled to ambient or refrigeration temperature, the decrease in the thermal kinetic energy facilitates formation of stable noncovalent bonds among exposed functional groups of the various molecules and this constitutes gelation.

The interactions involved in network formation are primarily hydrogen bonds, and hydrophobic and electrostatic interactions. The relative contributions of these forces vary with the type of protein, heating conditions, the extent of denaturation, and environmental conditions. Hydrogen bonding

and hydrophobic interactions contribute more than electrostatic interactions to network formation except when multivalent ions are involved in cross-linking. Since proteins generally carry a net charge, electrostatic repulsion occurs among protein molecules and this is not usually conducive to network formation. However, charged groups are essential for maintaining protein–water interactions and water-holding capacity of gels.

Gel networks that are sustained primarily by noncovalent interactions are thermally reversible; that is, upon reheating they will melt to a progel state, as is commonly observed with gelatin gels. This is especially true when hydrogen bonds are the major contributors to the network. Since hydrophobic interactions are strong at elevated temperatures, gel networks formed primarily by hydrophobic interactions are thermally irreversible, for example, egg white gels. Proteins that contain both cysteine and cystine groups can undergo polymerization via sulfhydryl–disulfide interchange reactions during heating and form a continuous covalent network upon cooling. Such gels are usually thermally irreversible. Examples of gels of this type are ovalbumin, β-lactoglobulin, and whey protein gels.

Proteins form two types of gels, that is, coagulum (opaque) gels and translucent gels. The type of gel formed by a protein is dictated by its molecular properties and solution conditions. Proteins containing large amounts of nonpolar amino acid residues undergo hydrophobic aggregation upon denaturation.



(5.71)

P_N is native state, P_D is unfolded state, and n is the number of protein molecules taking part in cross-linking.

These insoluble aggregates then randomly associate and set into an irreversible coagulum-type gel. Since the rate of aggregation and network formation is faster than the rate of denaturation, proteins of this type readily set into a gel network even while being heated. The opaqueness of these gels is due to light scattering caused by the unordered (isotropic) network of insoluble protein aggregates. Coagulum-type gels are generally weak and are prone to syneresis.

Proteins that contain small amounts of nonpolar amino acid residues form soluble complexes upon denaturation. Since the rate of association of these soluble complexes is slower than the rate of denaturation, and the gel network is predominantly formed by hydrogen bonding interactions, they often do not set into a gel until heating followed by cooling has occurred (typically 8–12% protein concentration is used). Upon cooling, the slow rate of association of the soluble complexes facilitates formation of an ordered translucent gel network.

At the molecular level, coagulum-type gels tend to form when the sum of Val, Pro, Leu, Ile, Phe, and Trp residues of the protein exceeds 31.5 mol% [116]. Those that contain <31.5 mol% of the above hydrophobic residues usually form translucent gels when water is the solvent. However, this dictum is not obeyed when salt solutions are used as the solvents. For example, the hydrophobic amino acid content of β-lactoglobulin is 32 mol%, yet it forms a translucent gel in water. However, when NaCl is included, it forms a coagulum-type gel even when the salt concentration is as low as 50 mM. This occurs because of charge neutralization by NaCl, which promotes hydrophobic aggregation upon heating. Thus, the balance between attractive hydrophobic interactions and repulsive electrostatic interactions fundamentally controls gelation mechanism and the gel appearance. These two forces in effect control the balance between protein–protein and protein–solvent interactions in a gelling

system. If the former is much greater than the latter, a precipitate is likely to form. If protein–solvent interactions predominate, the system may not gel. A coagulum gel or a translucent gel results when the magnitude of hydrophobic and hydrophilic forces are somewhere in-between these two extremes.

Protein gels are highly hydrated systems, containing up to 98% water in some cases. The water entrapped in these gels has chemical potential (activity) similar to that in dilute aqueous solutions, but lacks fluidity and cannot be easily expressed out. The mechanism by which liquid water can be held in a semisolid state in gels is not well understood. However, the fact that translucent gels, formed primarily by hydrogen bonding interactions, hold more water than coagulum-type gels and are less prone to syneresis, suggests that much of the water is hydrogen bonded to C═O and N—H groups of the peptide bonds, is associated with charged groups in the form of hydration shells, and/or exists in extensively hydrogen-bonded ice-like water–water networks. It is also possible that within the restricted environment of the microstructure of the gel network, water may exist as a hydrogen-bonding cross-linker between C═O and N—H groups of peptide segments (see Chapter 2). This may restrict the flowability of water within each cell, the more so as the cell size decreases. It is also likely that some water may be held as capillary water in the pores of the gel structure, especially in coagulum gels.

The stability of a gel network against thermal and mechanical forces is dependent on the number and types of cross-links formed per monomer chain. Thermodynamically, a gel network would be stable only when the sum of the interaction energies of a monomer in the gel network is greater than its thermal kinetic energy. This is dependent on several intrinsic (such as the size, net charge, etc.) and extrinsic factors (such as pH, temperature, ionic strength, etc.). The square root of the hardness of protein gels exhibits a linear relationship with molecular weight [125]. Globular proteins with molecular weight $<$23,000 Da cannot form a heat-induced gel at any reasonable protein concentration, unless they contain at least one free sulfhydryl group or a disulfide bond. The sulfhydryl groups and disulfide bonds facilitate polymerization, and thus increase the effective molecular weight of polypeptides to $>$23,000 Da. Gelatin preparations with effective molecular weights of $<$20,000 Da cannot form a gel.

Another critical factor is protein concentration. To form a self-standing gel network, a minimum protein concentration, known as least concentration endpoint (LCE), is required [50]. The LCE is 8% for soy proteins, 3% for egg albumin, and about 0.6% for gelatin. Above this minimum concentration, the relationship between gel strength, G, and protein concentration, C, usually follows a power law:

$$G \propto (C - C_0)^n \tag{5.72}$$

where C_0 is the LCE. For proteins, the value of n varies from 1 to 2.

Several environmental factors, such as pH, salts, and other additives also affect gelation of proteins. At or near isoelectric pH, proteins usually form coagulum-type gels. At extremes of pH, weak gels are formed because of strong electrostatic repulsion. The optimum pH for gel formation is about 7–8 for most proteins.

Formation of protein gels can sometimes be facilitated by limited proteolysis. A well-known example is cheese. Addition of chymosin (rennin) to casein micelles in milk results in the formation of a coagulum-type gel. This is achieved by cleavage of κ-casein, a micelle component, causing release of a hydrophilic portion, known as the glycomacropeptide. The remaining so-called para-casein micelles possess a highly hydrophobic surface that facilitates formation of a weak gel network.

Enzymic cross-linking of proteins at room temperature can also result in formation of a gel network. Transglutaminase is the enzyme often used to prepare these gels. This enzyme catalyses formation of ε-(γ-glutamyl)lysyl cross-links between the glutamine and lysyl groups of protein molecules [91]. Using this enzymic cross-linking method, highly elastic and irreversible gels can be formed even at low protein concentration.

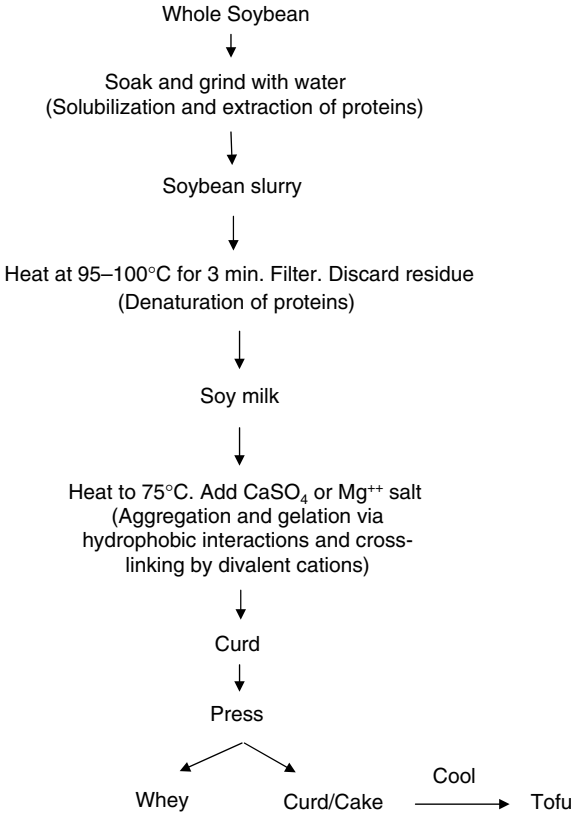


FIGURE 5.29 A typical commercial process for tofu manufacture.

Divalent cations, such as Ca^{2+} and Mg^{2+}, can also be used to form protein gels. These ions form cross-links between negatively charged groups of protein molecules. A good example of this type of gel is tofu from soy proteins. Alginate gels also can be formed in this manner. A general method for making tofu is outlined in Figure 5.29.

5.5.7 Texturization

Texturization connotes transformation of a protein from a globular state to a fibrous physical structure that has meat-like mouthfeel characteristics. The various functional properties that texturized protein products are expected to possess include chewiness, elasticity, softness, and juiciness. Vegetable proteins are often the preferred protein source for texturization, primarily because they lack other desirable functional properties that proteins of animal origin display. Textured vegetable proteins are manufactured using two different processes, namely *spun-fiber texturization* and *extrusion texturization*.

5.5.7.1 Spun-Fiber Texturization

In this process, a highly concentrated (~20% w/v) soy protein isolate solution is adjusted to pH 12–13 and aged until the viscosity of the solution increases to 50,000–100,000 centipoise as a result of protein denaturation and certain alkali-induced cross-linking reactions. This highly viscous "dope" is then pumped through a spinneret, a device with a plate containing thousands of micron-size holes. The fibrous extrudate is passed through a bath containing phosphoric acid and salt at pH 2.5. The

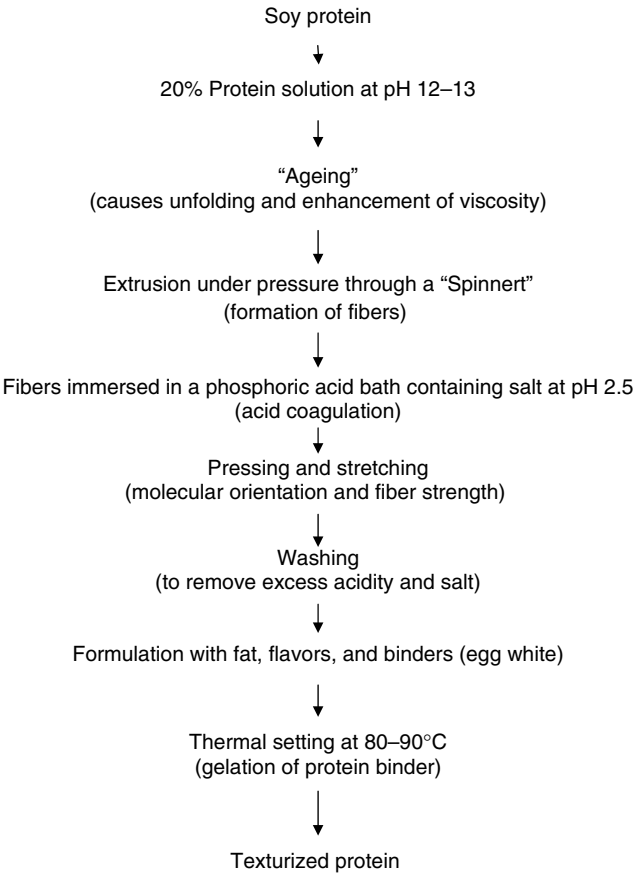


FIGURE 5.30 A typical spun-fiber process for texturization of soy proteins.

protein coagulates instantaneously in this bath and becomes a fibrous mass. The fiber is then "towed" through steel rolls where it is compressed and stretched to enhance its strength. The fiber is then passed through a washing bath where excess acidity and salt are removed. The washed fibers are then passed through a series of tanks containing fat, flavors, colors, and binders depending on the final product. The fiber is then heated at 80–90°C to induce gelation of the binder protein. Egg white is often used as a binder because of its excellent heat coagulation properties. The final product is dried and sized. A process flow chart for the spun-fiber texturization process is outlined in Figure 5.30.

5.5.7.2 Extrusion Texturization

In this process, defatted soy flour or soy protein concentrate with high protein solubility index (PSI) is conditioned with steam and the moisture content is adjusted to 20–25%. This solid mass is then fed to an extruder, which is mainly a rotating screw housed in a tapered cylindrical barrel in which the space between the screw and the barrel decreases progressively along the screw axis. As the protein mass advances through the screw, it is rapidly heated to 150–180°C. This high temperature and the progressive build up of pressure as the mass moves down the screw results in pressure-cooking, and as a result the protein mass melts and the proteins are denatured. In technical terms this is known as thermoplastic melt. The denatured proteins become aligned in fiber form as the mass moves through the screw. When the mass exits the die, sudden release of pressure evaporates water and puffs the product. Adjusting the pressure and temperature can control puffing. If a dense product is desired, the mass is

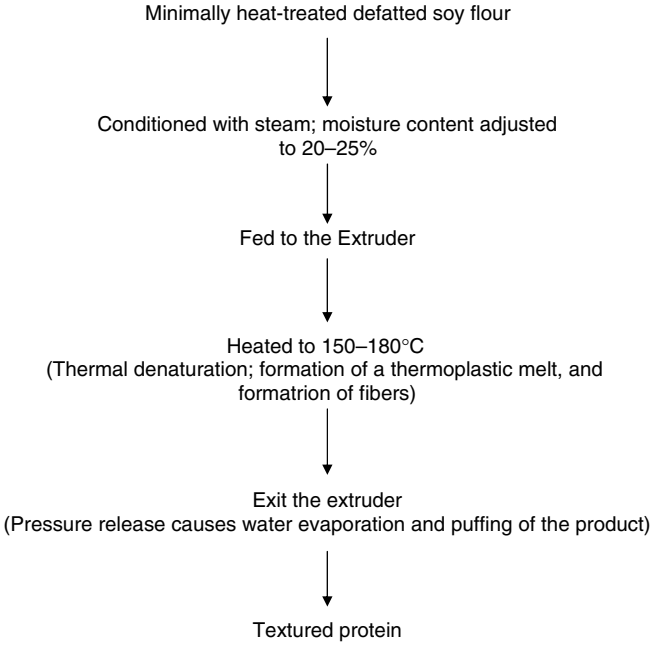


FIGURE 5.31 Extrusion texturization of soy flour.

cooled before it exits the die. The extrudate is then cut to pieces and processed further depending on its use. A general process flow chart for extrusion texturization of proteins is shown in Figure 5.31.

The general principles involved in both these methods are thermal or alkaline denaturation of proteins, realignment of the denatured proteins in the form of a fibrous network, binding of the fibers using a protein binder, and flavoring of the final product. Texturized vegetable proteins are increasingly being used as meat extenders in comminuted meat products (meat patties, sauces, burgers, etc.) and as meat analogs or "imitation meat."

5.5.8 Dough Formation [79,80,115]

When a mixture of wheat flour and water (about 3:1 ratio) is kneaded, it forms a viscoelastic dough suitable for making bread and other bakery products. These unusual dough characteristics are mainly attributable to the proteins in wheat flour.

Wheat flour contains several soluble and insoluble protein fractions. The soluble proteins, comprising about 20% of the total proteins, are primarily albumin and globulin type enzymes and certain minor glycoproteins. These proteins do not contribute to the dough-forming properties of wheat flour. The major storage protein of wheat is gluten. Gluten is a heterogeneous mixture of proteins, mainly gliadins and glutenins, with limited solubility in water. When mixed with water, gluten forms viscoelastic dough capable of entrapping gas during fermentation.

Gluten has a unique amino acid composition, with Gln and Pro accounting for more than 40% of the amino acid residues (Table 5.18). The low water solubility of gluten is attributable to its low content of Lys, Arg, Glu, and Asp residues, which together amount to $<10\%$ of the total amino acid residues. About 30% of gluten's amino acid residues are hydrophobic, and the residues contribute greatly to its ability to form protein aggregates by hydrophobic interactions, and to bind lipids and other nonpolar substances. The high glutamine and hydroxyl amino acid ($\sim 10\%$) contents of gluten are responsible for its water binding properties. In addition, hydrogen bonding between glutamine and hydroxyl residues of gluten polypeptides contributes to its cohesion–adhesion properties. Cysteine and cystine residues account for 2–3 mol% of gluten's total amino acid residues. During formation of

TABLE 5.18
Amino Acid Composition of Glutenin and Gliadin

Amino Acid	Glutenin (mol%)	Gliadin (mol%)
Cys	2.6	3.3
Met	1.4	1.2
Asp	3.7	2.8
Thr	3.4	2.4
Ser	6.9	6.1
Glx[a]	28.9	4.6
Pro	11.9	16.2
Gly	7.5	3.1
Ala	4.4	3.3
Val	4.8	4.8
Ile	3.7	4.3
Leu	6.5	6.9
Tyr	2.5	1.8
Phe	3.6	4.3
Lys	2.0	0.6
His	1.9	1.9
Arg	3.0	2.0
Trp	1.3	0.4

[a] Glx corresponds to mixture of Glu and Gln. Most of Glx in wheat protein is in the form of Gln (37).

the dough, these residues undergo sulfhydryl–disulfide interchange reactions, resulting in extensive polymerization of gluten proteins [115].

Several physical and chemical transformations occur during mixing and kneading of a mixture of wheat flour and water. Under the applied shear and tensile forces, gluten proteins absorb water and partially unfold. The partial unfolding of protein molecules facilitates hydrophobic interactions and sulfhydryl–disulfide interchange reactions, which result in formation of thread-like polymers. These linear polymers in turn are believed to interact with each other, presumably via hydrogen bonding, hydrophobic associations, and disulfide cross-linking, to form a sheet-like film capable of entrapping gas. Because of these transformations in gluten, the resistance of the dough increases with time until a maximum is reached and this is followed by a decrease in resistance indicative of a breakdown in the network structure. The breakdown involves alignment of polymers in the direction of shear and some scission of disulfide cross-links, which reduces the polymer size. The time it takes to reach maximum dough strength (R_{max}) during kneading is used as a measure of wheat quality for bread making—a longer time indicating better quality.

The viscoelasticity of wheat dough is related to the extent of sulfhydryl–disulfide interchange reactions. This view is supported by the fact that when reductants, such as cysteine, or sulfhydryl blocking agents, such as *N*-ethylmaleimide, are added to dough, viscoelasticity decreases greatly. On the other hand, addition of oxidizing agents, such as iodates and bromates, increase the elasticity of the dough. This implies that wheat gluten rich in SH and S—S groups might possess superior bread making qualities, but this relationship is unreliable. Thus, interactions other than disulfide cross-links, such as hydrogen bonding and hydrophobic interactions, also play a vital role in viscoelasticity of wheat dough.

Differences in bread-making qualities of different wheat cultivars may be related to differences in the composition of gluten itself. As mentioned earlier, gluten is made up of gliadins and glutenins. Gliadins are comprised of four groups, namely α-, β-, γ-, and ω-gliadins. In gluten these exist as single polypeptides with molecular weights ranging from 30,000 to 80,000 Da. Gliadins contain even number of cysteine residues. They exist as intramolecular disulfide bonds. The disulfide bonds are

buried in the interior of the protein so that they do not take part in sulfhydryl–disulfide interchange reactions with other proteins. The disulfide bonds appear to remain as intramolecular disulfides during dough making. Thus, dough made from isolated gliadins and starch is viscous but not viscoelastic.

Glutenins, on the other hand, are heterogeneous polypeptides with molecular weights ranging from 12,000 to 130,000 Da. These are further classified into high molecular weight (MW > 90,000, HMW) and low molecular weight (MW < 90,000, LMW) glutenins. In gluten, these glutenin polypeptides are present as polymers joined by disulfide cross-links, with molecular weights ranging into the millions. Because of their ability to polymerize extensively via sulfhydryl–disulfide interchange reactions, glutenins contribute greatly to the elasticity of dough. Some studies have shown a significant positive correlation between HMW glutenin content and bread making quality in some wheat varieties [10]. Available information indicates that a specific pattern of disulfide cross-linked association between LMW and HMW glutenins in gluten structure may be far more important to bread quality than the amount of HMW protein. For example, association/polymerization among LMW glutenins gives rise to a structure similar to that formed by HMW gliadin. This type of structure contributes to viscosity of the dough, but not to elasticity. In contrast, the dough elasticity increases when LMW glutenins cross-link to HMW glutenins via disulfide cross-links (in gluten). It is possible that in good quality wheat varieties, more of the LMW glutenins may polymerize to HMW, whereas in poor quality wheat varieties, most of the LMW glutenins may polymerize among themselves. These differences in associated states of glutenins in gluten of various wheat varieties may be related to differences in their conformational properties, such as surface hydrophobicity, and reactivity of sulfhydryl and disulfide groups.

In summary, hydrogen bonding among amide and hydroxyl groups, hydrophobic interactions, and sulfhydryl–disulfide interchange reactions all contribute to the development of the unique viscoelastic properties of wheat dough. However, culmination of these interactions into good dough making properties may depend on the structural properties of each protein and the proteins with which it associates in the overall gluten structure.

Because polypeptides of gluten, especially the glutenins, are rich in proline, they have very little ordered secondary structure. Whatever ordered structure initially exists in gliadins and glutenins is lost during mixing and kneading. Therefore, no additional unfolding occurs during baking.

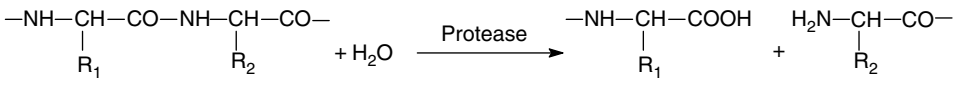
Supplementation of wheat flour with albumin and globulin type proteins, for example, whey proteins and soy proteins, adversely affects the viscoelastic properties and baking quality of the dough. These proteins decrease bread volume by interfering with formation of the gluten network. Addition of phospholipids or other surfactants to dough counters the adverse effects of foreign proteins on loaf volume. In this case, the surfactant/protein film compensates for the impaired gluten film. Although this approach results in acceptable loaf volume, the textural and sensory qualities of the bread are less desirable than normal.

Isolated gluten is sometimes used as a protein ingredient in nonbakery products. Its cohesion–adhesion properties make it an effective binder in comminuted meat and surimi-type products.

5.6 PROTEIN HYDROLYSATES

Partial hydrolysis of proteins using proteolytic enzymes is one of the strategies for improving the functional properties. Functional properties such as solubility, dispersibility, foaming, and emulsification can be potentially improved by limited proteolysis of proteins. Protein hydrolysates have many uses in speciality foods such as geriatric foods, nonallergenic infant formula, sports drinks, and diet foods. Because protein hydrolysates can be readily digested, they are particularly useful in infant formula and geriatric foods.

Proteolysis denotes enzymatic hydrolysis of peptide bonds in proteins.



(5.73)

In this reaction, for every peptide bond cleaved by the enzyme, one mole each of carboxyl group and amino group is liberated. When the reaction is allowed to completion, the final product is a mixture of all constituent amino acids of the protein. Incomplete proteolysis results in liberation of a mixture of polypeptides from the original protein. The functional properties of the protein hydrolysate is dependent upon the degree of hydrolysis (DH) and the physicochemical properties, that is, size, solubility, and so forth, of the polypeptides in the hydrolysate.

The DH is defined as the fraction of peptide bonds cleaved and it is often expressed as percentage:

$$\%\mathrm{DH} = \frac{n}{n_{\mathrm{T}}} \times 100 \tag{5.74}$$

where n_{T} is the total number of moles of peptide bonds present in one mole of protein and n is the number of moles of peptide bonds cleaved per of mole of protein. When molar mass of a protein is not known or the protein sample is a mixture of various proteins, n and n_{T} are expressed as the number of peptide bonds per gram of protein.

The DH is generally monitored using the pH-Stat method. The principle behind this method is that when a peptide bond is hydrolyzed, the newly formed carboxyl group completely ionizes at pH > 7, which releases H^+ ion. As a result, the pH of the protein solution progressively decreases with time of hydrolysis. In the pH range 7–8, the number of moles of H^+ ion released is equivalent to the number of moles of peptide bonds hydrolyzed. In the pH-Stat method, the pH of the protein solution is maintained at a constant pH by titrating with NaOH. The number of moles of NaOH consumed during proteolysis is equivalent to the number of moles of peptide bonds cleaved.

Several proteases can be potentially used for preparing protein hydrolysates. Some of these proteases are site-specific enzymes (Table 5.19). Because of their specificity, the types of polypeptide fragments released in the hydrolysate differ between proteases. Alcalase from *Bacillus licheniformis* is a major commercial enzyme used in the manufacture of protein hydrolysate. This enzyme belongs to a family of subtilisins, which are serine proteases.

5.6.1 Functional Properties

The functional properties of protein hydrolysates depend on the type of enzymes used in their preparation. This is primarily because of differences in the size and other physicochemical properties of the polypeptides released during hydrolysis. Generally, solubility of most proteins improves after hydrolysis regardless of the enzyme used. The greater the DH, the higher is the solubility. However,

TABLE 5.19
Specificity of Various Proteases

Protease	Type	Specificity
Elastase	Endoproteinase	Ala—aa; Gly—aa
Bromelain	Endoproteinase	Ala—aa; Tyr—aa
Trypsin	Endoproteinase	Lys—aa; Arg—aa
Chymotrypsin	Endoproteinase	Phe—aa; Trp—aa; Tyr—aa
Pepsin	Endoproteinase	Leu—aa; Phe—aa
V-8 protease	Endoproteinase	Asp—aa; Glu—aa
Thermolysin	Endoproteinase	aa—Phe; aa—Leu
Alcalase	Endoproteinase	Nonspecific
Papain	Endoproteinase	Lys—aa; Arg—aa; Phe—aa; Gly—aa
Prolylendopeptidase	Endoproteinase	Pro—aa
Subtilisin A	Endoproteinase	Nonspecific

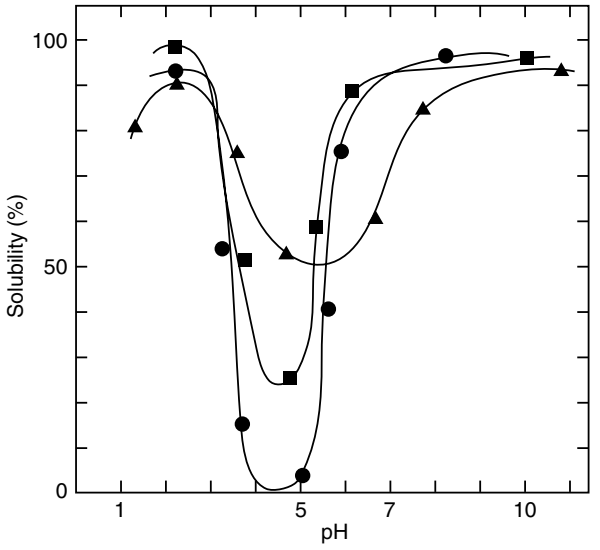


FIGURE 5.32 pH-solubility profiles of native casein and of *Staphylococcus aureus* V-8 protease-modified casein. The solubility was expressed as percent of total protein in solution. ●, native casein; ■, 2% DH; ▲, 6.7% DH. (From Adler-Nissen, J. 1979. *J. Agric. Food Chem.* **27**:1256–1260.)

the net increase in solubility depends on the type of enzyme used. Shown in Figure 5.32 [2] is the pH-solubility profile of casein before and after hydrolysis with V-8 protease. It should be noted that the solubility of casein at its isoelectric pH is significantly increased after partial hydrolysis. This type of behavior is also observed with other proteins. Higher protein solubility is particularly important in acidic protein drinks in which precipitation and sedimentation is undesirable.

Since solubility of a protein is essential for its foaming and emulsifying properties, partially hydrolyzed proteins generally show improved foaming and emulsifying properties. However, this improvement is dependent on the type of enzyme used and the DH. Generally, the foaming and emulsifying capacity improve up to DH $< 10\%$ and decrease at DH $> 10\%$. On the other hand, the stabilities of foams and emulsions made with protein hydrolysates are generally lower than that of the intact protein. One of the reasons for this is the inability of the short polypeptides to form a cohesive viscoelastic film at the air–water and oil–water interfaces.

Protein hydrolysates generally do not form heat-induced gels. One exception is gelatin. Gelatin is produced from collagen by acid or alkaline hydrolysis. Gelatin is a heterogeneous mixture of polypeptides. The weight-average-molecular weight of polypeptides in a gelatin sample depends on the DH. This profoundly affects their gel strength. The higher the weight-average-molecular weight, the higher is the gel strength. Gelatin samples with weight-average-molecular weight $<$20,000 Da do not form gels at all gelatin concentration [43]. The gelling properties of commercial gelatin products are expressed in terms of bloom rating measured using a bloom gelometer. The bloom rating is defined as the weight in grams required for driving a plunger of a gelometer 4 cm into a 6.67% (w/v) gelatin gel that has been incubated for 17 h in a water bath at 10°C. Table 5.20 shows the bloom rating requirements for various types of gelatin-based food products.

5.6.2 Allergenicity

Several food proteins, including cow's milk, soy proteins, gluten, egg proteins, and peanut proteins, cause severe allergic reactions in children and adults. Among the population who are allergic to milk proteins, about 60% are allergic to caseins, 60–80% to β-lactoglobulin, and 50% to α-lactalbumin [1]. However, hydrolysates of these proteins possess lower allergenicity than their native counterparts.

TABLE 5.20
Bloom Rating Requirements for Some Gelatin-Based Food Products

Product	Bloom Rating (g)	Concentration Used in Food (%)
Jelly beans	220	7–8
Fruit jelly	100–120	10–12
Marshmallow	220	2–3
Lozenges	50–100	1

Allergenicity of intact proteins arises because of the presence of antigenic sites (epitopes) that bind to immunoglobulin E (IgE). In protein hydrolysates, the epitopes are destroyed by proteolytic cleavage. For instance, hydrolysis of casein up to 55% DH using pancreatin (mixture of pancreatic enzymes) decreases its allergenicity by about 50% [81]. Similarly, whey protein hydrolysates with 12.9–16.1% DH fail to produce allergenic reaction when tested in guinea pigs sensitized with intact whey proteins [97]. Thus, protein hydrolysates are the preferred source of protein/amino acid for infants and children who are predisposed or at high risk of developing allergic reaction to food proteins.

The net reduction in allergenicity of protein hydrolysates depends on the type of protease used. Nonspecific proteases or a mixture of proteases are more effective than a site-specific protease in reducing the allergenicity of proteins. The DH also plays a role: the higher the DH, the greater is the reduction of allergenicity. For these reasons, the efficacy of proteases in reducing allergenicity of a protein is often expressed as allergenicity reduction index (ARI). ARI is defined as the ratio of % reduction in allergenicity to %DH.

5.6.3 Bitter Peptides

One of the most undesirable properties of protein hydrolysates is their bitter flavor. The bitterness emanates from certain peptides released during hydrolysis. There is ample evidence that bitterness of peptides is related to hydrophobicity. Peptides with a mean residue hydrophobicity of <1.3 kcal/mol are not bitter (see Chapter 10). On the other hand, peptides with a mean residue hydrophobicity of >1.4 kcal/mol are bitter [3]. In this case, often the mean residue hydrophobicity of the peptides is calculated using the free energies of transfer of amino acid residues from ethanol to water (see Table 10.1). Formation of bitter peptides in protein hydrolysates depends on the amino acid composition and sequence, and the type of enzymes used. Hydrolysates of highly hydrophobic proteins such as casein, soy proteins, and corn protein (zein) are very bitter, whereas hydrolysates of hydrophilic proteins such as gelatin are less bitter. Caseins and soy proteins hydrolyzed with several commercial proteases produce several bitter peptides. The bitterness can be reduced or eliminated by using a mixture of endo- and exopeptidases, which further breakdown bitter peptides into fragments that have <1.3 kcal/mol mean residue hydrophobicity.

5.7 NUTRITIONAL PROPERTIES OF PROTEINS

Proteins differ in their nutritive value. Several factors, such as essential amino acids content and digestibility, contribute to these differences. The daily protein requirement, therefore, depends on the type and composition of proteins in a diet.

5.7.1 Protein Quality

The "quality" of a protein is related mainly to its essential amino acids content and digestibility. High quality proteins are those that contain all the essential amino acids at levels greater than the FAO/WHO/UNU [40] reference levels, and a digestibility comparable to or better than those of egg white or milk proteins. Animal proteins are of better "quality" than plant proteins.

Proteins of major cereals and legumes are often deficient in at least one of the essential amino acids. While proteins of cereals, such as rice, wheat, barley, and maize are very low in lysine and rich in methionine, those of legumes and oilseeds are deficient in methionine and rich or adequate in lysine. Some oilseed proteins, such as peanut protein, are deficient in both methionine and lysine contents. The essential amino acids whose concentrations in a protein are below the levels of a reference protein are termed *limiting amino acids.* Adults consuming only cereal proteins or legume proteins have difficulty maintaining their health; children below 12 years of age on diets containing only one of these protein sources cannot maintain a normal rate of growth. The essential amino acid contents of various food proteins are listed in Table 5.21 [35,40].

Both animal and plant proteins generally contain adequate or more than adequate amounts of His, Ile, Leu, Phe + Tyr, and Val. These amino acids usually are not limiting in staple foods. More often, Lys, Thr, Trp, and the sulfur containing amino acids are the limiting amino acids. The nutritional quality of a protein that is deficient in an essential amino acid can be improved by mixing it with another protein that is rich in that essential amino acid. For example, mixing of cereal proteins with legume proteins provides a complete and balanced level of essential amino acids. Thus, diets containing appropriate amounts of cereals and legumes (pulses) and otherwise nutritionally complete are often adequate to support growth and maintenance. A poor quality protein also can be nutritionally improved by supplementing it with essential free amino acids that are under-represented. Supplementation of legumes with Met and cereals with Lys usually improves their quality.

The nutritional quality of a protein or protein mixture is ideal when it contains all of the essential amino acids in proportions that produce optimum rates of growth and/or optimum maintenance capability. The ideal essential amino acid patterns for children and adults are given in Table 5.22 [108]. However, because actual essential amino acid requirements of individuals in a given population vary depending on their nutritional and physiological status, the essential amino acid requirements of preschool children (age 2–5) are generally recommended as a safe level for all age groups [39].

Overconsumption of any particular amino acid can lead to "amino acid antagonism" or toxicity. Excessive intake of one amino acid often results in an increased requirement for other essential amino acids. This is due to competition among amino acids for absorption sites on the intestinal mucosa. For example, high levels of Leu decrease absorption of Ile, Val, and Tyr even if the dietary levels of these amino acids are adequate. This leads to an increased dietary requirement for the latter three amino acids. Overconsumption of other essential amino acids also can inhibit growth and induce pathological conditions.

5.7.2 Digestibility

Although the content of essential amino acids is the primary indicator of protein quality, true quality also depends on the extent to which these amino acids are utilized in the body. Thus, digestibility (bioavailability) of amino acids can affect the quality of proteins. Digestibilities of various proteins in humans are listed in Table 5.23 [40]. Food proteins of animal origin are more completely digested than those of plant origin. Several factors affect digestibility of proteins.

5.7.2.1 Protein Conformation

The structural state of a protein influences its hydrolysis by proteases. Native proteins are generally less completely hydrolyzed than partially denatured ones. For example, treatment of phaseolin (a protein from kidney beans) with a mixture of proteases results only in limited cleavage of the protein resulting in liberation of a 22,000 Da polypeptide as the main product. When heat-denatured phaseolin is treated under similar conditions, it is completely hydrolyzed to amino acids and dipeptides. Generally, insoluble fibrous proteins and extensively denatured globular proteins are difficult to hydrolyze.

TABLE 5.21
Essential Amino Acid Contents and Nutritional Value of Proteins from Various Sources (mg/g Protein)

	Protein Source												
Property (mg/g Protein)	**Egg Milk**	**Cow's**	**Beef**	**Fish**	**Wheat**	**Rice**	**Maize**	**Barley**	**Soybean**	**Field (Boiled)**	**Pea**	**Peanut Bean**	**French**
Amino acid concentration (mg/g protein)													
His	22	27	34	35	21	21	27	20	30	26	26	27	30
Ile	54	47	48	48	34	40	34	35	51	41	41	40	45
Leu	86	95	81	77	69	77	127	67	82	71	70	74	78
Lys	70	78	89	91	23[a]	34[a]	25[a]	32[a]	68	63	71	39[a]	65
Met + Cys	57	33	40	40	36	49	41	37	33	22[b]	24[b]	32	26
Phe + Tyr	93	102	80	76	77	94	85	79	95	69	76	100	83
Thr	47	44	46	46	28	34	32[b]	29[b]	41	33	36	29[b]	40
Trp	17	14	12	11	10	11	6[b]	11	14	8[a]	9[a]	11	11
Val	66	64	50	61	38	54	45	46	52	46	41	48	52
Total essential amino acids	512	504	480	485	336	414	422	356	466	379	394	400	430
Protein content (%)	12	3.5	18	19	12	7.5	—	—	40	32	28	30	30
Chemical score (%) (based on FAO/WHO, 1985 pattern)	100	100	100	100	40	59	43	55	100	73	82	67	—
PER	3.9	3.1	3.0	3.5	1.5	2.0	—	—	2.3	—	2.65	—	—
BV (on rats)	94	84	74	76	65	73	—	—	73	—	—	—	—
NPU	94	82	67	79	40	70	—	—	61	—	—	—	—

[a] Primary limiting acid.
[b] Second limiting acid.
PER, protein efficiency ratio; BV, biological value; NPU, net protein utilization.

Source: Eggum, B. O. and R. M. Beames. 1983. In *Seed Proteins* (Gottschalk, W. and H. P. Muller, Eds.), Nijhoff/Junk, The Hague, pp. 499–531 and FAO/WHO/UNU. 1985. Energy and protein requirements, Report of a joint FAO/WHO/UNU Expert Consultation. World Health Organization Technical Rep. Ser. 724, WHO, Geneva.

TABLE 5.22
Recommended Essential Amino Acid Pattern for Food Proteins

	Recommended Pattern (mg/g Protein)			
Amino Acid	**Infant**	**Preschool Child (2–5 years)**	**Preschool Child (10–12 years)**	**Adult**
Histidine	26	19	19	16
Isoleucine	46	28	28	13
Leucine	93	66	44	19
Lysine	66	58	44	16
Met + Cys	42	25	22	17
Phe + Tyr	72	63	22	19
Threonine	43	34	28	9
Tryptophan	17	11	9	5
Valine	55	35	25	13
Total	434	320	222	111

Source: From FAO/WHO/UNU. 1985. Energy and protein requirements, Report of a joint FAO/WHO/UNU Expert Consultation. World Health Organization Technical Rep. Ser. 724, WHO, Geneva.

TABLE 5.23
Digestibility of Various Food Proteins in Humans

Protein Source	**Digestibility (%)**	**Protein Source**	**Digestibility (%)**
Egg	97	Millet	79
Milk, cheese	95	Peas	88
Meat, fish	94	Peanut	94
Maize	85	Soy flour	86
Rice (polished)	88	Soy protein isolate	95
Wheat, whole	86	Beans	78
Wheat flour, white	96	Corn, cereal	70
Wheat gluten	99	Wheat, cereal	77
Oatmeal	86	Rice cereal	75

Source: From FAO/WHO/UNU. 1985. Energy and protein requirements, Report of a joint FAO/WHO/UNU Expert Consultation. World Health Organization Technical Rep. Ser. 724, WHO, Geneva.

5.7.2.2 Antinutritional Factors

Most plant protein isolates and concentrates contain trypsin and chymotrypsin inhibitors (Kunitz type and Bowman-Birk type) and lectins. These inhibitors impair complete hydrolysis of legume and oilseed proteins by pancreatic proteases. Lectins, which are glycoproteins, bind to intestinal mucosa cells and interfere with absorption of amino acids. Lectins and Kunitz-type protease inhibitors are thermolabile, whereas the Bowman-Birk type inhibitor is stable under normal thermal processing conditions. Thus, heat-treated legume and oilseed proteins are generally more digestible than native protein isolates (despite some residual Bowman-Birk type inhibitor). Plant proteins also contain other antinutritional factors, such as tannins and phytate. Tannins, which are condensation products of polyphenols, covalently react with ε-amino groups of lysine residues. This inhibits trypsin-catalyzed cleavage of the polypeptides at lysine sites.

5.7.2.3 Processing

Interaction of proteins with polysaccharides and dietary fiber also reduces the rate and completeness of hydrolysis. This is particularly important in extruded food products where high temperature and pressure is often used. Proteins undergo several chemical alterations involving lysine residues when exposed to high temperatures and alkaline pH. Such alterations reduce their digestibility. Reaction of reducing sugars with ε-amino groups also decreases digestibility of lysine.

5.7.3 Evaluation of Protein Nutritive Value

Since the nutritional quality of proteins can vary greatly and is affected by many factors, it is important to have procedures for evaluating quality. Quality estimates are useful for: (a) determining the amount required to provide a safe level of essential amino acids for growth and maintenance and (b) monitoring changes in the nutritive value of proteins during food processing, so that processing conditions that minimize quality loss can be devised. The nutritive quality of proteins can be evaluated by several biological, chemical, and enzymatic methods.

5.7.3.1 Biological Methods

Biological methods are based on weight gain or nitrogen retention in test animals when fed with a protein-containing diet. A protein-free diet is used as the control. The protocol recommended by FAO/WHO [39] is generally used for evaluating protein quality. Rats are the usual test animals, although humans are sometimes used. A diet containing about 10% protein on a dry weight basis is used to ensure that the protein intake is below daily requirements. Adequate energy is supplied in the diet. Under these conditions, protein in the diet is utilized to the maximum possible extent for growth. The number of test animals used must be sufficient to assure results that are statistically reliable. A test period of 9 days is common. During each day of the test period, the amount (g) of diet consumed is tabulated for each animal, and the feces and urine are collected for nitrogen analysis.

The data from animal feeding studies are used in several ways to evaluate protein quality. The *protein efficiency ratio* (PER) is the weight (in grams) gained per gram protein consumed. This is a simple and commonly used expression. Another useful expression is *net protein ratio* (NPR). This is calculated as follows:

$$\text{NPR} = \frac{(\text{weight gain}) - (\text{weight loss of protein} - \text{free group})}{\text{protein ingested}} \tag{5.75}$$

NPR values provide information on the ability of proteins to support both maintenance and growth. Since rats grow much faster than humans, and a larger percentage of protein is used for maintenance in growing children than in rats, it is often questioned whether PER and NPR values derived from rat studies are useful for estimating human needs [108]. Although this argument is a valid one, appropriate correction procedures are available.

Another approach to evaluating protein quality involves measuring nitrogen uptake and nitrogen loss. This allows calculation of two useful protein quality parameters. *Apparent protein digestibility* or *coefficient of protein digestibility* is obtained from the difference between the amount of nitrogen ingested and the amount of nitrogen excreted in the feces. However, since total fecal nitrogen also includes metabolic or endogenous nitrogen, correction should be made to obtain *true protein digestibility*. True digestibility (TD) can be calculated in the following manner:

$$\text{TD} = \frac{I - (F_{\text{N}} - F_{\text{k,N}})}{I} \times 100 \tag{5.76}$$

where I is nitrogen ingested, F_{N} is total fecal nitrogen, and $F_{\text{k,N}}$ is endogenous fecal nitrogen. $F_{\text{k,N}}$ is obtained by feeding a protein-free diet.

True digestibility gives information on the percentage of nitrogen intake absorbed by the body. However, it does not provide information on how much of the absorbed nitrogen is actually retained or utilized by the body.

Biological value, BV, is calculated as follows:

$$\text{BV} = \frac{I - (F_{\text{N}} - F_{\text{k,N}}) - (U_{\text{N}} - U_{\text{k,N}})}{I - (F_{\text{N}} - F_{\text{k,N}})} \times 100 \tag{5.77}$$

where U_{N} and $U_{\text{k,N}}$ are the total and endogenous nitrogen losses, respectively, in the urine.

Net protein utilization (NPU), that is, the percentage of nitrogen intake retained as body nitrogen, is obtained from the product of TD and BV. Thus,

$$\text{NPU} = \text{TD} \times \text{BV} = \frac{I - (F_{\text{N}} - F_{\text{k,N}}) - (U_{\text{N}} - U_{\text{k,N}})}{I} \times 100 \tag{5.78}$$

The PER, BVs, and NPUs of several food proteins are presented in Table 5.21.

Other bioassays that are occasionally used to evaluate protein quality include assays for: enzyme activity, changes in the essential amino acid content of plasma, levels of urea in the plasma and urine, and rate of repletion of plasma proteins or gain in body weight of animals previously fed a protein-free diet.

5.7.3.2 Chemical Methods

Biological methods are expensive and time consuming. Determining its content of amino acids and comparing this with the essential amino acid pattern of an ideal reference protein can obtain quick assessment of a protein's nutritive value. The ideal pattern of essential amino acids in proteins (reference protein) for preschool children (2–5 years) is given in Table 5.22 [40] and this pattern is used as the standard for all age groups except infants. Each essential amino acid in a test protein is given a *chemical score*, which is defined as

$$\frac{\text{mg amino acid per g test protein}}{\text{mg same amino acid per g reference protein}} \times 100 \tag{5.79}$$

The essential amino acid that shows the lowest score is the most limiting amino acid in the test protein. The chemical score of this limiting amino acid provides the chemical score for the test protein. As mentioned earlier, Lys, Thr, Trp, and sulfur amino acids are often the limiting amino acids in food proteins. Therefore, the chemical scores of these amino acids are often sufficient to evaluate the nutritive value of proteins. The chemical score enables estimation of the amount of a test protein or protein mix needed to meet the daily requirement of the limiting amino acid. This can be calculated as follows:

$$\text{Required intake of protein} = \frac{\text{Recommended intake of egg or milk protein}}{\text{Chemical score of protein}} \times 100 \tag{5.80}$$

One of the advantages of the chemical score method is that it is simple and allows one to determine the complementary effects of proteins in a diet. This also allows one to develop high quality protein diets by mixing various proteins suitable for various feeding programs. There are, however, several drawbacks to use the chemical score method. An assumption underlying chemical score is that all test proteins are fully or equally digestible and that all essential amino acids are fully absorbed. Because this assumption is often violated, correlation between results from bioassays and chemical scores is often not good. However, the correlation improves when chemical scores are corrected for protein digestibility. The apparent digestibility of proteins can be rapidly determined *in vitro* using

a combination of three or four enzymes, such as trypsin, chymotrypsin, peptidase, and bacterial protease.

Another shortcoming of chemical score is that it does not distinguish between D- and L-amino acids. Since only L-amino acids are usable in animals, the chemical score overestimates the nutritive value of a protein, especially in proteins exposed to high pH, which cause racemization. The chemical score method is also incapable of predicting the negative effects of high concentrations of one essential amino acid on the bioavailability of other essential amino acids, and it also does not account for the effect of antinutritional factors, such as protease inhibitors and lectins, that might be present in the diet. Despite these major drawbacks, recent findings indicate that chemical scores when corrected for protein digestibility correlate well with biological assays for those proteins having BVs above 40%; when the BV is below 40%, the correlation is poor [39].

5.7.3.3 Enzymic and Microbial Methods

In vitro enzymic methods are sometimes used to measure the digestibility and release of essential amino acids. In one method, test proteins are first digested with pepsin and then with pancreatin (freeze-dried powder of pancreatic extract) [83]. In another method, proteins are digested with three enzymes, namely, pancreatic trypsin, chymotrypsin, and porcine intestinal peptidase, under standard assay conditions [39]. These methods, in addition to providing information on innate digestibility of proteins, are useful for detecting processing-induced changes in protein quality.

Growth of several microorganisms, such as *Streptococcus zymogenes*, *Streptococcus faecalis*, *Leuconostoc mesenteroides*, *Clostridium perfringens*, and *Tetrahymena pyriformis* (a protozoan) also have been used to determine the nutritional value of proteins [44]. Of these microorganisms, *T. pyriformis* is particularly useful, because its amino acid requirements are similar to those of rats and humans.

5.8 PROCESSING-INDUCED PHYSICAL, CHEMICAL, AND NUTRITIONAL CHANGES IN PROTEINS

Commercial processing of foods can involve heating, cooling, drying, application of chemicals, fermentation, irradiation, or various other treatments. Of these, heating is most common. This is commonly done to inactivate microorganisms, to inactivate endogenous enzymes that cause oxidative and hydrolytic changes in foods during storage, and to transform an unappealing blend of raw food ingredients into a wholesome and organoleptically appealing food. In addition, proteins such as bovine β-lactoglobulin, α-lactalbumin and soy protein, that sometimes cause allergenic or hypersensitive responses, can sometimes be rendered innocuous by thermal denaturation. Unfortunately, the beneficial effects achieved by heating proteinaceous foods are generally accompanied by changes that can adversely affect the nutritive value and functional properties of proteins. In this section, both desirable and undesirable effects of food processing on proteins will be discussed.

5.8.1 Changes in Nutritional Quality and Formation of Toxic Compounds

5.8.1.1 Effect of Moderate Heat Treatments

Most food proteins are denatured when exposed to moderate heat treatments (60–90°C, 1 h or less). Extensive denaturation of proteins often results in insolublization, which may impair those functional properties that are dependent on solubility. From a nutritional standpoint, partial denaturation of proteins often improves the digestibility and biological availability of essential amino acids.

Several purified plant proteins and egg protein preparations, even though free of protease inhibitors, exhibit poor *in vitro* and *in vivo* digestibility. Moderate heating improves their digestibility without developing toxic derivatives.

In addition to improving digestibility, moderate heat treatment also inactivates several enzymes, such as proteases, lipases, lipoxygenases, amylases, polyphenoloxidase, and other oxidative and hydrolytic enzymes. Failure to inactivate these enzymes can result in development of off-flavors, rancidity, textural changes, and discoloration of foods during storage. For instance, oilseeds and legumes are rich in lipoxygenase. During crushing or cracking of these beans for extraction of oil or protein isolates, this enzyme, in the presence of molecular oxygen, catalyzes oxidation of polyunsaturated fatty acids to initially yield hydroperoxides. These hydroperoxides subsequently decompose and liberate aldehydes and ketones, which impart off-flavor to soy flour and soy protein isolates and concentrates. To avoid off-flavor formation, it is necessary to thermally inactivate lipoxygenase prior to crushing.

Moderate heat treatment is particularly beneficial for plant proteins, because they usually contain proteinaceous antinutritional factors. Legume and oilseed proteins contain several trypsin and chymotrypsin inhibitors. These inhibitors impair efficient digestion of proteins, and thus reduce their biological availability. Furthermore, inactivation and complexation of trypsin and chymotrypsin by these inhibitors induces over production and secretion of these enzymes by the pancreas, which can lead to pancreatic hypertropy (enlargement of the pancreas) and pancreatic adenoma. Legume and oilseed proteins also contain lectins, which are glycoproteins. These are also known as phytohemagglutinins because they cause agglutination of red blood cells. Lectins exhibit a high binding affinity for carbohydrates. When consumed by humans, lectins impair protein digestion [103] and cause intestinal malabsorption of other nutrients. The latter consequence results from binding of lectins to membrane glycoproteins of intestinal mucosa cells, which alters their morphology and transport properties [96]. Both protease inhibitors and lectins found in plant proteins are thermolabile. Toasting of legumes and oilseeds or moist heat treatment of soy flour inactivates both lectins and protease inhibitors, improves the digestibility and PER of these proteins (Figure 5.33) [45], and prevents pancreatic hypertrophy [53]. These antinutritional factors do not pose problems in home-cooked or

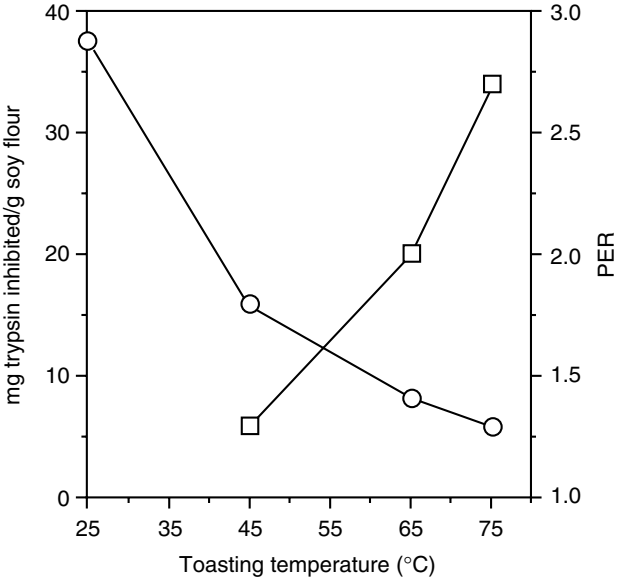


FIGURE 5.33 Effect of toasting on trypsin inhibitory activity and PER of soy flour. (Adapted from Friedman, M. and M. R. Gumbmann. 1986. *Adv. Exp. Med. Biol.* **199**:357–390.)

industrially processed legumes and flour-based products when heating conditions are adequate to inactivate them.

Milk and egg proteins also contain several protease inhibitors. Ovomucoid, which possesses antitryptic activity, constitutes about 11% of egg albumen. Ovoinhibitor, which inhibits trypsin, chymotrypsin, and some fungal proteases, is present at a 0.1% level in egg albumen. Milk contains several protease inhibitors, such as plasminogen activator inhibitor (PAI) and plasmin inhibitor (PI), derived from blood. All of these inhibitors lose their activity when subjected to moderate heat treatment in the presence of water.

The beneficial effects of heat treatment also include inactivation of protein toxins, such as botulinum toxin from *Clostridium botulinum* (inactivated by heating at 100°C) and enterotoxin from *Staphylococcus aureus*.

5.8.1.2 Compositional Changes During Extraction and Fractionation

Preparation of protein isolates from biological sources involves several unit operations, such as extraction, isoelectric precipitation, salt precipitation, thermocoagulation, and ultrafiltration/diafiltration. It is very likely that some of the proteins in the crude extract might be lost during some of these operations. For example, during isoelectric precipitation, some sulfur-rich albumin-type proteins, which are usually soluble at isoelectric pH, might be lost in the supernatant fluid. Such losses can alter the amino acid composition and nutritional value of protein isolates compared to those of crude extracts. For instance, WPC prepared by ultrafiltration/diafiltration and ion exchange methods undergo marked changes in their proteose–peptone contents. This markedly affects their foaming properties.

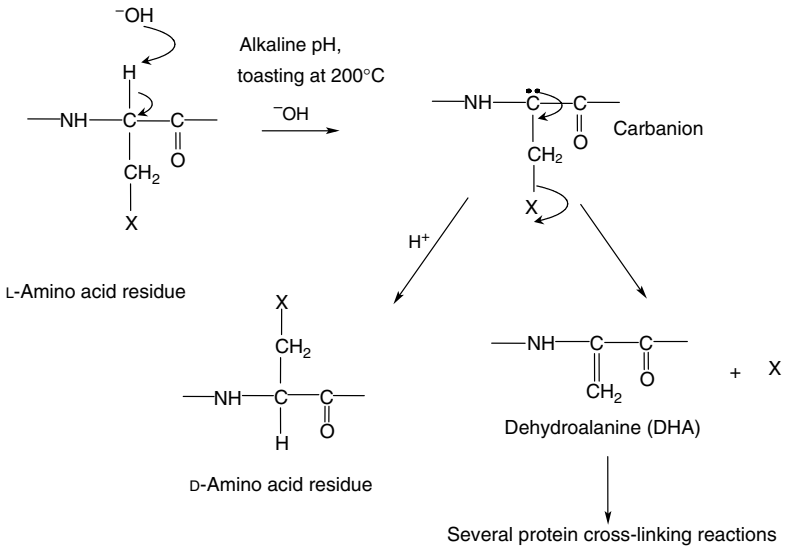
5.8.1.3 Chemical Alteration of Amino Acids

Proteins undergo several chemical changes when processed at high temperatures. These changes include racemization, hydrolysis, desulfuration, and deamidation. Most of these chemical changes are irreversible, and some of these reactions result in formation of modified amino acid types that are potentially toxic.

5.8.1.3.1 Racemization

Thermal processing of proteins at alkaline pH, as is done to prepare texturized foods, invariably leads to partial racemization of L-amino acid residues to D-amino acids [77]. Acid hydrolysis of proteins also causes some racemization of amino acids [42] as does roasting of proteins or protein containing foods above 200°C [55]. The mechanism at alkaline pH involves initial abstraction of the proton from the α-carbon atom by a hydroxyl ion. The resulting carbanion loses its tetrahedral asymmetry. Subsequent addition of a proton from solution can occur either from the top or bottom of the carbanion. This equal probability results in racemization of the amino acid residue (Equation 5.81) [77]. The rate of racemization of a residue is affected by the electron withdrawing power of the side chain. Thus, residues such as Asp, Ser, Cys, Glu, Phe, Asn, and Thr are racemized at a faster rate than are other amino acid residues [78]. The rate of racemization is also dependent on hydroxyl ion concentration, but is independent of protein concentration. Interestingly, the rate of racemization is about ten times faster in proteins than in free amino acids [78], suggesting that intramolecular forces in a protein reduce the activation energy of racemization. In addition to racemization, the carbanion formed under alkaline pH also can undergo β-elimination reaction to yield a reactive intermediate dehydroalanine. Cysteine and phosphoserine residues display greater propensity for this route than other amino acid residues. This is one of the reasons why a significant amount of D-cysteine is not

found in alkali treated proteins.

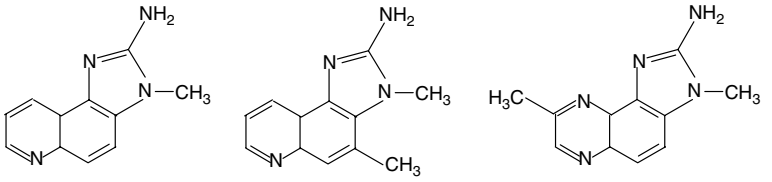


(5.81)

Racemization of amino acid residues causes a reduction in protein digestibility because the peptide bonds involving D-amino acid residues are less efficiently hydrolyzed by gastric and pancreatic proteases. This leads to loss of essential amino acids that have racemized, and impairs the nutritional value of the protein. D-Amino acids are also less efficiently absorbed through intestinal mucosa cells and even if absorbed, they cannot be utilized in *in vivo* protein synthesis. Moreover, some D-amino acids, for example, D-proline, have been found to be neurotoxic in chickens [20].

In addition to racemization and β-elimination reactions, heating of proteins at alkaline pH destroys several amino acid residues, such as Arg, Ser, Thr, and Lys. Arg decomposes to ornithine.

When proteins are heated above 200°C, as is commonly encountered on food surfaces during broiling, baking, and grilling, amino acid residues undergo decomposition and pyrolysis. Several of the pyrolysis products have been isolated and identified from broiled and grilled meat, and they are highly mutagenic as determined by the Ames test. The most carcinogenic/mutagenic products are formed from pyrolysis of Trp and Glu residues [19]. Pyrolysis of Trp residues gives rise to formation of carbolines and their derivatives. Mutagenic compounds are also produced in meats at moderate temperatures (190–200°C). These are known as IQ (imidazo quinolines) compounds, which are condensation products of creatine, sugars, and certain amino acids, such as Gly, Thr, Ala, and Lys [60]. The three most potent mutagens formed in broiled fish are shown below.



2-Amino-3-methylimidazo-[4,5-*f*]quinoline (IQ)

2-Amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ)

2-Amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx)

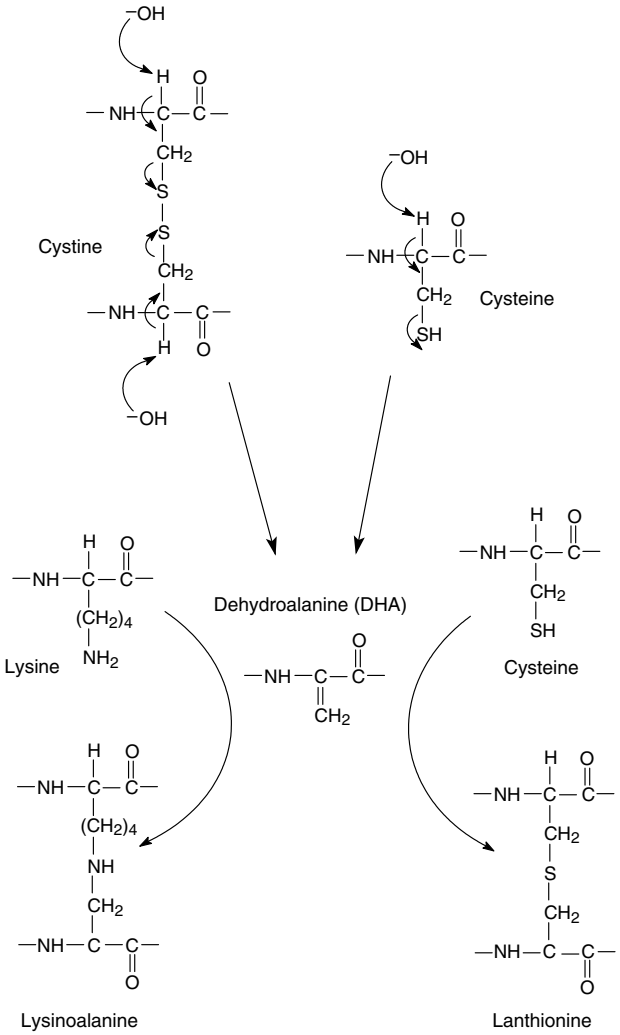
(5.82)

Following heating of foods according to recommended procedures, IQ compounds are generally present only at very low concentrations (μg amounts).

5.8.1.3.2 Protein Cross-linking

Several food proteins contain both intra- and intermolecular cross-links, such as disulfide bonds in globular proteins, desmosine, and isodesmosine, and di- and trityrosine type cross-links in fibrous proteins such as keratin, elastin, resilin, and collagen. Collagen also contains ε-*N*-(γ-glutamyl)lysyl and/or ε-*N*-(γ-aspartyl)lysyl cross-links. One of the functions of these cross-links in native proteins is to minimize proteolysis *in vivo*. Processing of food proteins, especially at alkaline pH, also induces cross-link formation. Such unnatural covalent bonds between polypeptide chains reduce digestibility and biological availability of essential amino acids that are involved in, or near, the cross-link.

As discussed in the previous section, heating at alkaline pH or heating above 200°C at neutral pH, results in abstraction of the proton from the α-carbon atom resulting in formation of a carbanion, which leads to formation of dehydroalanine (DHA) residue. DHA formation can also occur via a one-step mechanism without the carbanion intermediate. Once formed, the highly reactive DHA residues react with nucleophilic groups, such as the ε-amino group of a lysyl residue, the thiol group of Cys residue, the δ-amino group of ornithine (formed by decomposition of arginine), or a histidyl residue, resulting in formation of lysinoalanine, lanthionine, ornithoalanine, and histidinylalanine cross-links, respectively, in proteins. Lysinoalanine is the major cross-link commonly found in alkali treated proteins because of the abundance of readily accessible lysyl residues (Equation 5.83).



(5.83)

The formation of protein–protein cross-links in alkali-treated proteins decreases their digestibility and biological value. The decrease in digestibility is related to the inability of trypsin to cleave the peptide bond in the lysinoalanine cross-link. Moreover, the steric constraints imposed by the cross-links also prevent hydrolysis of other peptide bonds in the neighborhood of the lysinoalanine and similar cross-links. Evidence suggests that free lysinoalanine is absorbed in the intestine, but the body does not utilize it and most of it is excreted in the urine. Some lysinoalanine is metabolized in the kidney. The inability of the body to cleave the lysinoalanine covalent bond reduces the bioavailability of lysine in alkali-treated proteins.

Rats fed 100 ppm of pure lysinoalanine or 3000 ppm of protein-bound lysinoalanine develop nephrocytomegaly (i.e., kidney disorder). However, such nephrotoxic effects have not been observed in other animal species, such as quails, mice, hamsters, and monkeys. This has been attributed to differences in the types of metabolites formed in rats vs other animals. At levels encountered in foods, protein-bound lysinoalanine apparently does not cause nephrotoxicity in humans. Nevertheless, minimization of lysinoalanine formation during alkali processing of proteins is a desirable goal.

The lysinoalanine contents of several commercial foods are listed in Table 5.24 [120]. The extent of formation of lysinoalanine is dependent on pH and temperature. The higher the pH, the greater is the extent of lysinoalanine formation. High temperature heat treatment of foods, such as milk, causes a significant amount of lysinoalanine to form even at neutral pH. Lysinoalanine formation in proteins can be minimized or inhibited by adding small molecular-weight nucleophilic compounds, such as cysteine, ammonia, or sulfites. The effectiveness of cysteine results because the nucleophilic

TABLE 5.24
Lysinoalanine (LAL) Content of Processed Foods

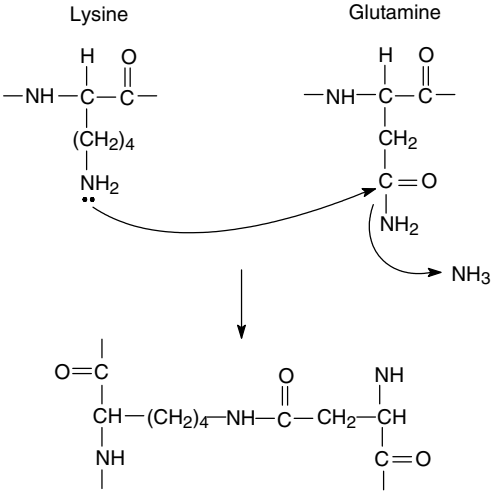
Food	LAL (μg/g Protein)
Corn chips	390
Pretzels	500
Hominy	560
Tortillas	200
Taco shells	170
Milk, infant formula	150–640
Milk, evaporated	590–860
Milk, condensed	360–540
Milk, UHT	160–370
Milk, HTST	260–1,030
Milk, spray-dried powder	0
Skim milk, evaporated	520
Simulated cheese	1,070
Egg white solids, dried	160–1,820
Calcium caseinate	370–1,000
Sodium caseinate	430–6,900
Acid casein	70–190
Hydrolyzed vegetable protein	40–500
Whipping agent	6,500–50,000
Soy protein isolate	0–370
Yeast extract	120

Source: Swaisgood, H. E. and G. L. Catignani. 1991. *Adv. Food Nutr. Res*. **35**:185–236.

SH group reacts more than 1000 times faster than the ε-amino group of lysine. Sodium sulfite and ammonia exert their inhibitory effect by competing with the ε-amino group of lysine for DHA. Blocking of ε-amino groups of lysine residues by reaction with acid anhydrides prior to alkali-treatment also decreases the formation of lysinoalanine. However, this approach results in loss of lysine and may be unsuitable for food applications.

Under normal conditions used for processing of several foods, only small amounts of lysinoalanine are formed. Thus, toxicity of lysinoalanine in alkali-treated foods is not believed to be a major concern. However, reduction in digestibility, loss of bioavailability of lysine, and racemization of amino acids (some of which are toxic) are all undesirable outcomes in alkali-treated foods such as texturized vegetable proteins.

Excessive heating of pure protein solutions or proteinaceous foods low in carbohydrate content also results in formation of ε-*N*-(γ-glutamyl)lysyl and ε-*N*-(γ-aspartyl)lysyl cross-links. These involve a transamidation reaction between Lys and Gln or Asn residues (Equation 5.84). The resulting cross-links are termed isopeptide bonds because they are foreign to native proteins. Isopeptides resist enzymatic hydrolysis in the gut and these cross-linkages therefore impair digestibility of proteins, and bioavailability of lysine.



(5.84)

ε-*N*-(γ-Glutamyl)lysine cross-link

Ionizing radiation of foods results in the formation of hydrogen peroxide through radiolysis of water in the presence of oxygen, and this, in turn, causes oxidative changes in, and polymerization of, proteins. Ionizing radiation also may directly produce free radicals via ionization of water.

$$H_2O \rightarrow H_2O^+ + e^- \tag{5.85}$$

$$H_2O^+ + H_2O \rightarrow H_3O^+ + {}^{\bullet}OH \tag{5.86}$$

The hydroxyl free radical can induce formation of protein free radicals, which in turn may cause polymerization of proteins.

$$P + {}^{\bullet}OH \rightarrow P^{\bullet} + H_2O \tag{5.87}$$

$$\begin{aligned} P^{\bullet} + P^{\bullet} &\rightarrow P - P \\ P^{\bullet} + P &\rightarrow P - P^{\bullet} \end{aligned} \tag{5.88}$$

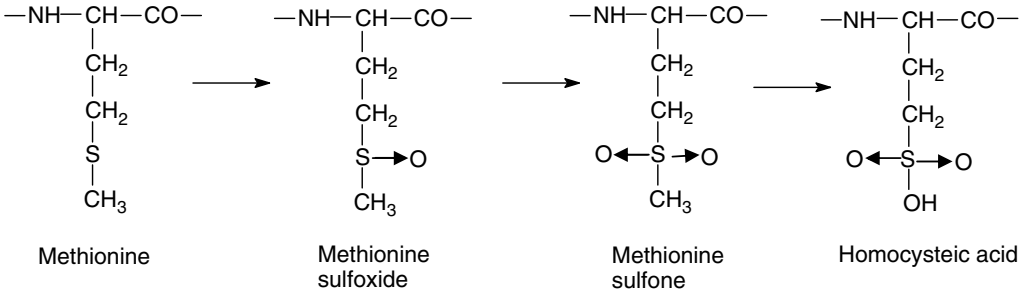
Heating of protein solutions at 70–90°C and at neutral pH generally leads to sulfhydryl–disulfide interchange reactions (if these groups are present), resulting in polymerization of proteins. However, this type of heat-induced cross-link generally does not have an adverse effect on the digestibility of proteins and bioavailability of essential amino acids because these bonds can be broken *in vivo*.

5.8.1.4 Effects of Oxidizing Agents

Oxidizing agents such as hydrogen peroxide and benzoyl peroxide are used as bactericidal agents in milk, as bleaching agents in cereal flours, protein isolates, and fish protein concentrate, and for detoxification of oilseed meals. Sodium hypochlorite is also commonly used as a bactericidal and detoxifying agent in flours and meals. In addition to oxidizing agents that are sometimes added to foods, several oxidative compounds are endogenously produced in foods during processing. These include free radicals formed during irradiation of foods, during peroxidation of lipids, during photooxidation of compounds such as riboflavin and chlorophyll, and during nonenzymatic browning of foods. In addition, polyphenols present in several plant protein isolates can be oxidized by molecular oxygen to quinones at neutral to alkaline pH, and this will lead ultimately to peroxides. These highly reactive oxidizing agents cause oxidation of several amino acid residues and polymerization of proteins. The amino acid residues most susceptible to oxidation are Met, Cys, Trp, and His, and to a lesser extent Tyr.

5.8.1.4.1 Oxidation of Methionine

Methionine is easily oxidized to methionine sulfoxide by various peroxides. Incubation of protein bound methionine or free methionine with hydrogen peroxide (0.1 M) at elevated temperature for 30 min results in complete conversion of methionine to methionine sulfoxide [23]. Under strong oxidizing conditions, methionine sulfoxide is further oxidized to methionine sulfone, and in some cases to homocysteic acid.

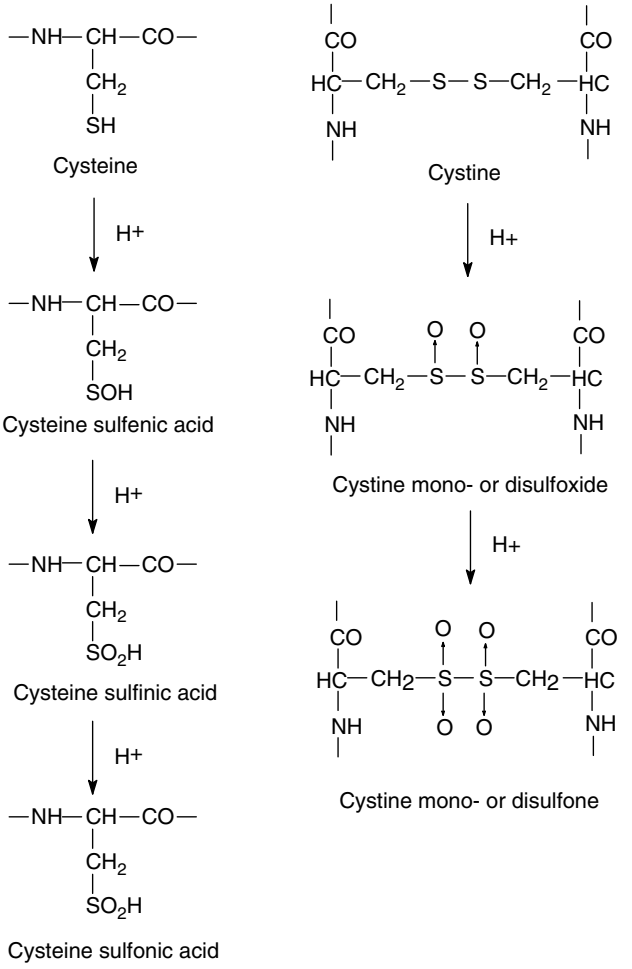


(5.89)

Methionine becomes biologically unavailable once it is oxidized to methionine sulfone or homocysteic acid. Methionine sulfoxide, on the other hand, is reconverted to Met under acidic conditions in the stomach. Further, evidence suggests that any methionine sulfoxide passing through the intestine is absorbed and reduced *in vivo* to methionine. However, *in vivo* reduction of methionine sulfoxide to methionine is slow. The PER or NPU of casein oxidized with 0.1 M hydrogen peroxide (which completely transforms methionine to methionine sulfoxide) is about 10% less than that of control casein.

5.8.1.4.2 Oxidation of Cysteine and Cystine

Under alkaline conditions, cysteine and cystine follow the β-elimination reaction pathway to produce DHA. However, at acidic pH, oxidation of cysteine and cystine in simple systems results in formation of several intermediate oxidation products. Some of these derivatives are unstable.



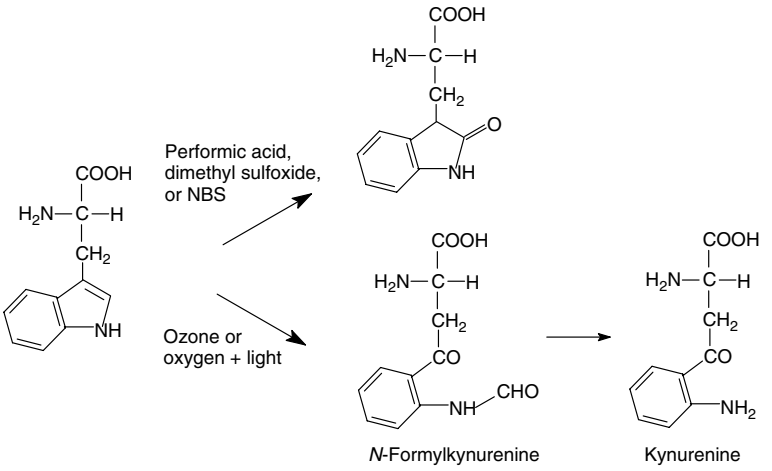
(5.90)

Mono- and disulfoxides of L-cystine are biologically available, presumably because they are reduced back to L-cystine in the body. However, mono- and disulfone derivatives of L-cystine are biologically unavailable. Similarly, while cysteine sulfenic acid is biologically available, cysteine sulfinic acid and cysteic acid are not. The rate and extent of formation of these oxidation products in acidic foods are not well documented.

5.8.1.4.3 Oxidation of Tryptophan

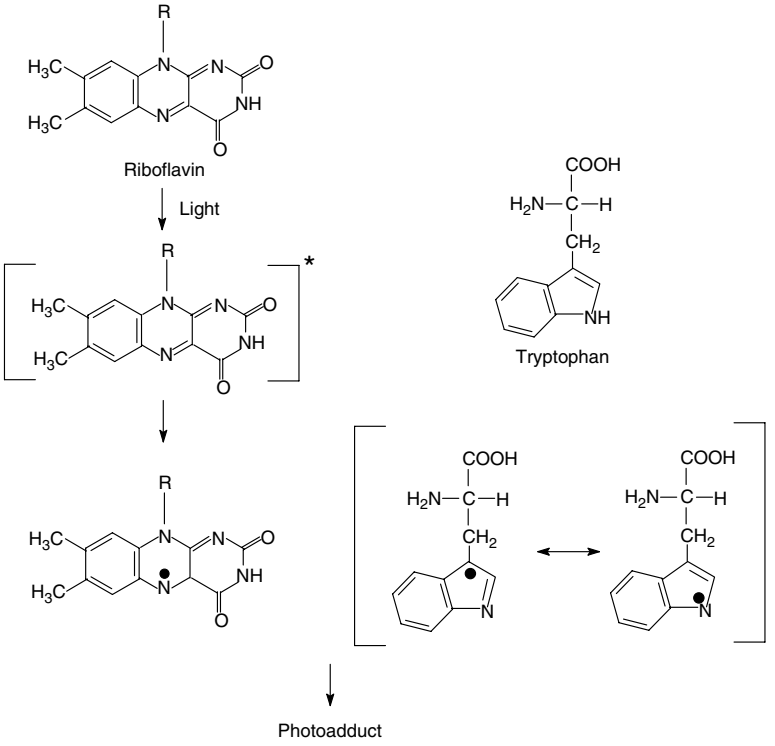
Among the essential amino acids, Trp is exceptional because of its role in several biological functions. Therefore, its stability in processed foods is of major concern. Under acidic, mild, oxidizing conditions, such as in the presence of performic acid, dimethylsulfoxide, or *N*-bromosuccinimide (NBS), Trp is oxidized mainly to β-oxyindolylalanine. Under acidic, severe, oxidizing conditions, such as in the presence of ozone, hydrogen peroxide or peroxidizing lipids, Trp is oxidized to

N-formylkynurenine, kynurenine, and other unidentified products.



(5.91)

Exposure of Trp to light in the presence of oxygen and a photosensitizer, such as riboflavin or chlorophyll, leads to formation of *N*-formylkynurenine and kynurenine as major products and several other minor ones. Depending upon the pH of the solution, other derivatives, such as 5-hydroxyformylkynurenine (pH > 7.0) and a tricyclic hydroperoxide (pH 3.6–7.1), are also formed [86]. In addition to the photooxidative products, Trp forms a photoadduct with riboflavin.



(5.92)

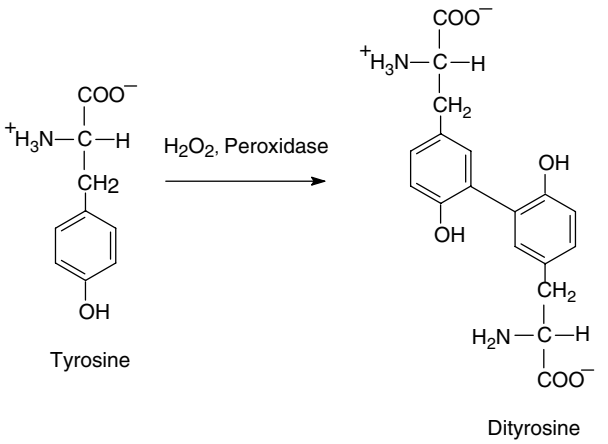
Both protein-bound and free tryptophan is capable of forming this adduct. The extent of formation of this photoadduct is dependent on availability of oxygen, being greater under anaerobic conditions [112].

The oxidation products of Trp are biologically active. In addition, kynurenines are carcinogenic in animals, and all other Trp photo-oxidation products as well as the carbolines formed during broiling/grilling of meat products exhibit mutagenic activities and inhibit growth of mammalian cells in tissue cultures. The tryptophan–riboflavin photoadduct shows cytotoxic effects on mammalian cells, and exerts hepatic dysfunctions during parenteral nutrition. These undesirable products are normally present in extremely low concentration in foods unless an oxidation environment is purposely created.

Among the amino acid side chains, only those of Cys, His, Met, Trp, and Tyr are susceptible to sensitized-photooxidation. In the case of Cys, cysteic acid is the end product. Met is photooxidized first to methionine sulfoxide, and finally to methionine sulfone and homocysteic acid. Photooxidation of histidine leads to the formation of aspartate and urea. The photooxidation products of tyrosine are not known. Since foods contain endogenous as well as supplemented riboflavin (vitamin B2), and usually are exposed to light and air, some degree of sensitized-photooxidation of the above amino acid residues would be expected to occur. In milk, free methionine is converted to methional by light-activated oxidation, which imparts a characteristic flavor to the milk. At equimolar concentrations, the rates of oxidation of the sulfur amino acids and Trp are likely to follow the order Met > Cys > Trp.

5.8.1.4.4 Oxidation of Tyrosine

Exposure of tyrosine solutions to peroxidase and hydrogen peroxide results in oxidation of tyrosine to dityrosine. Occurrence of this type of cross-link has been found in natural proteins, such as resilin, elastin, keratin, and collagen, and more recently in doughs.



(5.93)

5.8.1.5 Carbonyl–Amine Reactions

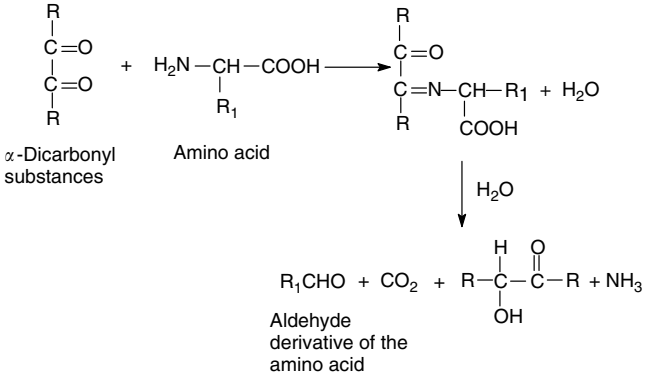
Among the various processing-induced chemical changes in proteins, the Maillard reaction (nonenzymatic browning) has the greatest impact on its sensory and nutritional properties. The Maillard reaction refers to a complex set of reactions initiated by reaction between amines and carbonyl compounds, which, at elevated temperature, decompose and eventually condense into insoluble brown product known as melanoidins (see Chapter 14). This reaction occurs not only in foods during processing, but also in biological systems. In both instances, proteins and amino acids typically provide the amino component, and reducing sugars (aldoses and ketoses), ascorbic acid, and carbonyl compounds generated from lipid oxidation provide the carbonyl component.

Some of the carbonyl derivatives from the nonenzymatic browning sequence react readily with free amino acids. This results in degradation of the amino acids to aldehydes, ammonia, and carbon dioxide and the reaction is known as *Strecker degradation.* The aldehydes contribute to aroma development during the browning reaction. Strecker degradation of each amino acid produces a

TABLE 5.25
Characteristic Flavor Notes of Aldehydes Produced by Strecker Degradation of Amino Acids

Amino Acid	Typical Flavor
Phe, Gly	Caramel-like
Leu, Arg, His	Bread-like, toasted
Ala	Nutty
Pro	Bakery, cracker
Gln, Lys	Buttery
Met	Broth, beany
Cys, Gly	Smokey, burnt
α-Amino butyric acid	Walnut
Arg	Popcorn-like

specific aldehyde with a distinctive aroma (Table 5.25).



(5.94)

The Maillard reaction impairs protein nutritional value. Some of the products are antioxidants and some may be toxic; but the toxic products probably are not hazardous at concentrations encountered in foods. Because the ε-amino group of lysine is the major source of primary amines in proteins, it is frequently involved in the carbonyl–amine reaction and typically suffers a major loss in bioavailability when this reaction occurs. The extent of Lys loss depends on the stage of the browning reaction. Lysine involved in the early stages of browning, including the Schiff's base, is biologically available. These early derivatives are hydrolyzed to lysine and sugar in the acidic conditions of the stomach. However, beyond the stage of ketosamine (Amadori product) or aldosamine (Heyns product), lysine is no longer biologically available. This is primarily because of poor absorption of these derivatives in the intestine [36]. It is important to note that no color has developed at this stage. Although sulfite inhibits formation of brown pigments [129], it cannot prevent loss of lysine availability, because it cannot prevent formation of Amadori or Heyns products.

Biological activity of lysine at various stages of the Maillard reaction can be determined chemically by addition of 1-fluoro-2,4-dinitrobenzene (FDNB), followed by acid hydrolysis of the derivatized protein. FDNB reacts with available ε-amino groups of lysyl residues. The hydrolysate is then extracted with ethyl ether to remove unreacted FDNB, and the concentration of ε-dinitrophenyl-lysine (ε-DNP-lysine) in the aqueous phase is determined by measuring absorbance at 435 nm. Available lysine also can be determined by reacting 2,4,6-trinitrobenzene sufonic acid (TNBS) with the ε-amino group. In this case, the concentration of ε-trinitrophenyl-lysine (ε-TNP-lysine) derivative is determined from absorbance at 346 nm.

Nonenzymatic browning not only causes major losses of lysine, but reactive unsaturated carbonyls and free radicals formed during the browning reaction cause oxidation of several other essential amino acids, especially Met, Tyr, His, and Trp. Cross-linking of proteins by dicarbonyl compounds produced during browning decreases protein solubility and impairs digestibility of proteins.

Some of Maillard brown products are suspected mutagens. Although mutagenic compounds are not necessarily carcinogenic, all known carcinogens are mutagens. Therefore, the formation of mutagenic Maillard compounds in foods is of concern. Studies with mixtures of glucose and amino acids have shown that the Maillard products of Lys and Cys are mutagenic, whereas those of Trp, Tyr, Asp, Asn, and Glu are not, as determined by the Ames test. It should be pointed out that pyrolysis products of Trp and Glu (in grilled and broiled meat) also are mutagenic (Ames test). As discussed earlier, heating of sugar and amino acids in the presence of creatine produces the most potent IQ-type mutagens (see Equation 5.82). Although results based on model systems cannot be reliably applied to foods, it is possible that interaction of Maillard products with other small molecular-weight constituents in foods may produce mutagenic and/or carcinogenic substances.

On a positive note, some Maillard reaction products, especially the reductones, do have antioxidative activity [88]. This is due to their reducing power and their ability to chelate metals, such as Cu and Fe that are prooxidants. The amino reductones formed from the reaction of triose reductones with amino acids such as Gly, Met, and Val show excellent antioxidative activity.

Besides reducing sugars, other aldehydes and ketones present in foods can also take part in the carbonyl–amine reaction. Notably, gossypol (in cotton seed), glutaraldehyde (added to protein meals to control deamination in the rumen of ruminants), and aldehydes (especially malonaldehyde) generated from the oxidation of lipids may react with amino groups of proteins. Bifunctional aldehydes, such as malonaldehyde can cross-link and polymerize proteins. This may result in insolublization, loss of digestibility and bioavailability of lysine, and loss of functional properties of proteins. Formaldehyde also reacts with the ε-amino group of lysine residues; the toughening of cod-type fish muscle during frozen storage is believed to be due to reactions of formaldehyde with fish proteins.

$$\underset{\text{Protein amino group}}{\text{P}{-}\text{NH}_2} + \underset{\text{Malondialdehyde}}{\text{OHC}{-}\text{CH}_2{-}\text{CHO}} \rightarrow \underset{\text{Protein–Protein cross-linkage}}{\text{P}{-}\text{N}{=}\text{CH}{-}\text{CH}_2{-}\text{CH}{=}\text{N}{-}\text{P}} \tag{5.95}$$

5.8.1.6 Other Reactions of Proteins in Foods

5.8.1.6.1 Reactions with Lipids

Oxidation of unsaturated lipids leads to formation of alkoxy and peroxy free radicals. These free radicals in turn react with proteins, forming lipid–protein free radicals. These lipid–protein conjugated free radicals can undergo polymerization cross-linking of proteins leading to a variety of cross-linked products.

$$\text{LH} + \text{O}_2 \rightarrow \text{LOO}^{\bullet} \tag{5.96}$$

$$\text{LOO}^{*} + \text{LH} \rightarrow \text{LOOH} + \text{L}^{\bullet} \tag{5.97}$$

$$\text{LOOH} \rightarrow \text{LO}^{\bullet} + \text{HO}^{\bullet} \tag{5.98}$$

$$\text{LO}^{\bullet} + \text{PH} \rightarrow \text{LOP} \tag{5.99}$$

$$\text{LOP} + \text{LO}^{\bullet} \rightarrow {}^{\bullet}\text{LOP} + \text{LOH} \tag{5.100}$$

$${}^{\bullet}\text{LOP} + {}^{\bullet}\text{LOP} \rightarrow \text{POLLOP} \tag{5.101}$$

or

$$LOO^{\bullet} + PH \rightarrow LOOP \quad (5.102)$$

$$LOOP + LOO^{\bullet} \rightarrow {}^{\bullet}LOOP + LOOH \quad (5.103)$$

$${}^{\bullet}LOOP + {}^{\bullet}LOOP \rightarrow POOLLOOP \quad (5.104)$$

$${}^{\bullet}LOOP + {}^{\bullet}LOP \rightarrow POLLOOP \quad (5.105)$$

In addition, the lipid free radicals can also induce formation of protein free radicals at cysteine and histidine side chains, which may then undergo cross-linking and polymerization reactions.

$$LOO^{*} + PH \rightarrow LOOH + P^{*} \quad (5.106)$$

$$LO^{\bullet} + PH \rightarrow LOH + P^{\bullet} \quad (5.107)$$

$$P^{\bullet} + P^{\bullet} \rightarrow P - P^{\bullet} \quad (5.108)$$

$$P - P^{\bullet} + PH \rightarrow P - P - P^{\bullet} \quad (5.109)$$

$$P - P - P^{\bullet} + P^{\bullet} \rightarrow P - P - P - P \quad (5.110)$$

Lipid hydroperoxides (LOOH) in foods can decompose, resulting in liberation of aldehydes and ketones, notably malonaldehyde. These carbonyl compounds react with amino groups of proteins via carbonyl–amine reaction and Schiff's base formation. As discussed earlier, reaction of malonaldehye with lysyl side chains leads to cross-linking and polymerization of proteins. The reaction of peroxidizing lipids with proteins generally has deleterious effects on nutritional value of proteins. Noncovalent binding of carbonyl compounds to proteins also imparts off-flavors.

5.8.1.6.2 Reactions with Polyphenols

Phenolic compounds, such as *p*-hydroxybenzoic acid, catechol, caffeic acid, gossypol, and quercein, are found in all plant tissues. During maceration of plant tissues, these phenolic compounds can be oxidized by molecular oxygen at alkaline pH to quinones. This can also occur by the action of polyphenoloxidase, which is commonly present in plant tissues. These highly reactive quinones can irreversibly react with the sulfhydryl and amino groups of proteins. Reaction of quinones with SH and α-amino groups (N-terminal) is much faster than it is with ε-amino groups. In addition, quinones can also undergo condensation reactions, resulting in formation of high molecular weight brown color pigments. These brown products remain highly reactive and readily combine with SH and amino groups of proteins. Quinone–amino group reactions decrease the digestibility and bioavailability of protein-bound lysine and cysteine.

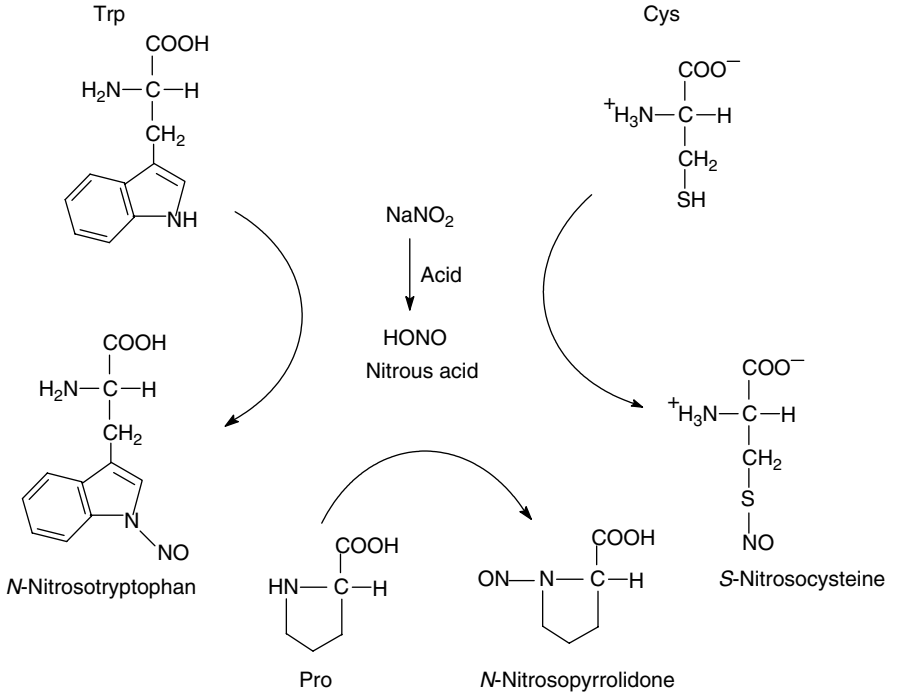
5.8.1.6.3 Reactions with Halogenated Solvents

Halogenated organic solvent are often used to extract oil and some antinutritive factors from oilseed products, such as soybean and cottonseed meals. Extraction with trichloroethylene results in formation of a small amount of *S*-dichlorovinyl-L-cysteine, which is toxic. On the other hand, the solvents dichloromethane and tetrachloroethylene do not seem to react with proteins. 1,2-Dichloroethane reacts with Cys, His, and Met residues in proteins. Certain fumigants, such as methyl bromide, can alkylate Lys, His, Cys, and Met residues. All of these reactions decrease the nutritional value of proteins and some are of concern from a safety standpoint.

5.8.1.6.4 Reactions with Nitrites

Reaction of nitrites with secondary amines, and to some extent with primary and tertiary amines, results in formation of *N*-nitrosoamine, which is one of the most carcinogenic compounds formed in foods. Nitrites are usually added to meat products to improve color and to prevent bacterial growth.

The amino acids (or residues) primarily involved in this reaction are Pro, His, and Trp. Arg, Tyr, and Cys also can react with nitrites. The reaction occurs mainly under acidic conditions and at elevated temperatures.

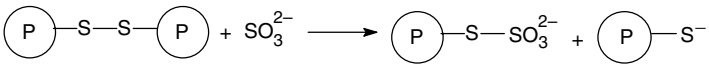


(5.111)

The secondary amines produced during the Maillard reaction, such as Amadori and Heyns products, also can react with nitrites. Formation of *N*-nitrosamines during cooking, grilling, and broiling of meat has been a major concern, but additives, such as ascorbic acid and erythorbate, are effective in curtailing this reaction.

5.8.1.6.5 Reaction with Sulfites

Sulfites reduce disulfide bonds in proteins to yield *S*-sulfonate derivatives. They do not react with cysteine residues.



(5.112)

In the presence of reducing agents, such as cysteine or mecaptoethanol, the *S*-sulfonate derivatives are converted back to cysteine residues. *S*-Sulfonates decompose under acidic (as in stomach) and alkaline pH to disulfides. The *S*-sulfonation does not decrease the bioavailability of cysteine. The increase in electronegativity and the breakage of disulfide bonds in proteins upon *S*-sulfonation causes unfolding of protein molecules, which affects their functional properties.

5.8.2 Changes in the Functional Properties of Proteins

The methods or processes used to isolate proteins can affect their functional properties. Minimum denaturation during various isolation steps is generally desired because this helps to retain acceptable protein solubility, which is often a prerequisite to functionality of these proteins in food products.

In some instances, controlled or partial denaturation of proteins can improve certain functional properties.

Proteins are often isolated using isoelectric precipitation. The secondary, tertiary, and quaternary structures of most globular proteins are stable at their isoelectric pH, and the proteins readily become soluble again when dispersed at neutral pH. On the other hand, protein entities such as casein micelles are irreversibly destabilized by isoelectric precipitation. The collapse of micellar structure in isoelectrically precipitated casein is due to several factors, including solubilization of colloidal calcium phosphate and the change in the balance of hydrophobic and electrostatic interactions among the various casein types. The compositions of isoelectrically precipitated proteins are usually altered from those of the raw materials. This is because some minor proteins fractions are reasonably soluble at the isoelectric pH of the major component and are therefore do not precipitate. This change in composition affects the functional properties of the protein isolate.

Ultrafiltration (UF) is widely used to prepare WPCs. Both protein and nonprotein composition of WPC are affected by removal of small solutes during UF. Partial removal of lactose and ash, strongly influence the functional properties of WPC. Furthermore, increased protein–protein interactions occur in the UF concentrate during exposure to moderate temperatures (50–55°C) and this decreases solubility and stability of the ultrafiltered protein, which in turn changes its water binding capacity and alters its properties with respect to gelation, foaming, and emulsification. Among the ash constituents, variations in calcium and phosphate content significantly affect the gelling properties of WPC. Whey protein isolates prepared by ion exchange contain little ash, and because of this they have functional properties that are superior to those of isolates obtained by ultrafiltration/diafiltration.

Calcium ions often induce aggregation of proteins. This is attributable to formation of ionic bridges involving Ca^{2+} ions and the carboxyl groups. The extent of aggregation depends on calcium ion concentration. Most proteins show maximum aggregation at 40–50 mM Ca^{2+} ion concentration. With some proteins, such as caseins and soy proteins, calcium aggregation leads to precipitation, whereas, in the case of whey protein isolate, a stable colloidal aggregate forms (Figure 5.34).

Exposure of proteins to alkaline pH, particularly at elevated temperatures, causes irreversible conformational changes. This is partly because of deamidation of Asn and Gln residues, and

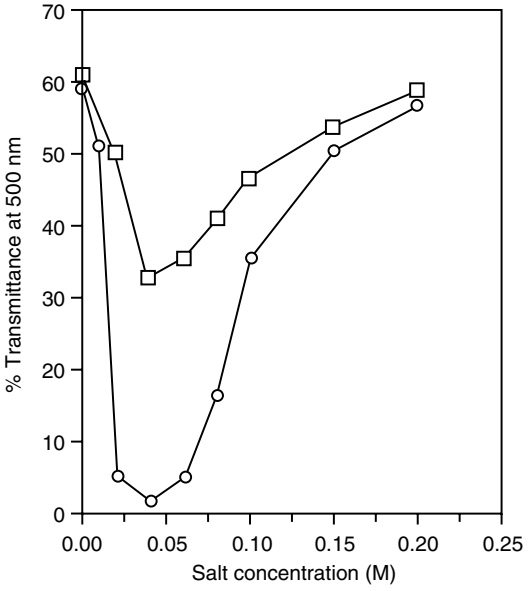


FIGURE 5.34 Salt concentration vs. turbidity of whey protein isolate (5%) in $CaCl_2$ (○) and $MgCl_2$ (□) solutions after incubating for 24 h at ambient temperature. (From Zhu, H. and S. Damodaran. 1994. *J. Agric. Food Chem.* **42**:856–862.)

β-elimination of cystine residues. The resulting increase in the electronegativity and breakage of disulfide bonds causes gross structural changes in proteins exposed to alkali. Generally, alkali-treated proteins are more soluble, and possess improved emulsification and foaming properties.

Hexane is often used to extract oil from oilseeds, such as soybean and cottonseed. This treatment invariably causes denaturation of proteins in the meal, and thus impairs their solubility and other functional properties.

The effects of heat treatments on chemical changes in, and functional properties of, proteins are described in Section 5.6. Scission of peptide bonds involving aspartyl residues during severe heating of protein solutions liberates small molecular-weight peptides. Severe heating under alkaline and acid pH conditions also causes partial hydrolysis of proteins. The amount of small molecular-weight peptides in protein isolates can affect their functional properties.

5.9 CHEMICAL AND ENZYMATIC MODIFICATION OF PROTEINS

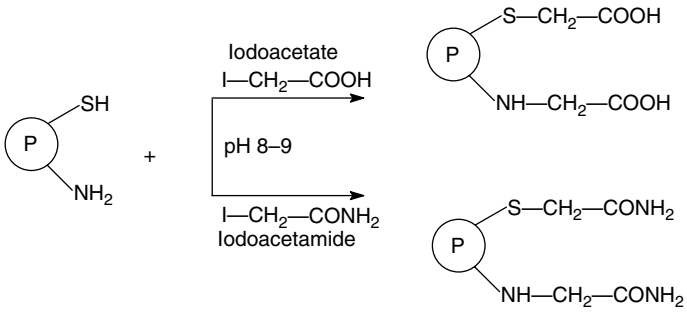
5.9.1 Chemical Modifications

The primary structure of proteins contains several reactive side chains. The physicochemical properties of proteins can be altered and their functional properties can be improved by chemically modifying the side chains. However, it should be cautioned that although chemical derivatization of amino acid side chains can improve functional properties of proteins, it can also impair nutritional value, create some amino acid derivatives that are toxic, and pose regulatory problems although similar reactions may occur *in vivo* or *in situ*.

Since proteins contain several reactive side chains, numerous chemical modifications can be achieved. Some of these reactions are listed in Table 5.5. However, only a few of these reactions may be suitable for modification of food proteins. The ε-amino groups of lysyl residues and the SH group of cysteine are the most reactive nucleophilic groups in proteins. The majority of chemical modification procedures involve these groups.

5.9.1.1 Alkylation

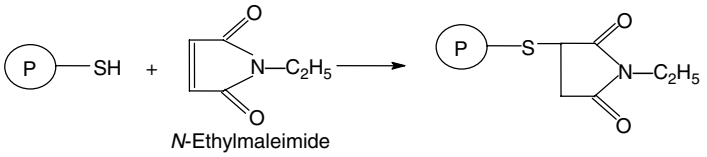
The SH and amino groups can be alkylated by reacting with iodoacetate or iodoacetamide. Reaction with iodoacetate results in elimination of the positive charge of the lysyl residue and introduction of negative charges at both lysyl and cysteine residues.



(5.113)

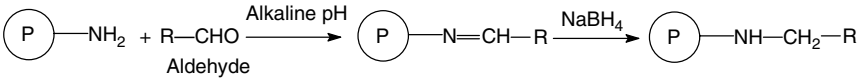
The increase in the electronegativity of the protein may alter the pH-solubility profile of proteins, and may also cause unfolding. On the other hand, reaction with iodoacetamide results only in elimination of positive charges. This will also cause a local increase in electronegativity, but the number of negatively charged groups in proteins will remain unchanged. Reaction with iodoacetamide effectively blocks sulfhydryl groups so disulfide-induced protein polymerization cannot occur.

Sulfhydryl groups also can be blocked by reaction with *N*-ethylmaleimide (NEM).



(5.114)

Amino groups can also be reductively alkylated with aldehydes and ketones in the presence of reductants, such as sodium borohydride ($NaBH_4$) or sodium cyanoborohydride ($NaCNBH_3$). In this case, the Schiff base formed by reaction of the carbonyl group with the amino group is subsequently reduced by the reductant. Aliphatic aldehydes and ketones or reducing sugars can be used in this reaction. Reduction of the Schiff base prevents progression of the Maillard reaction, resulting in a glycoprotein as the end product (reductive glycosylation).

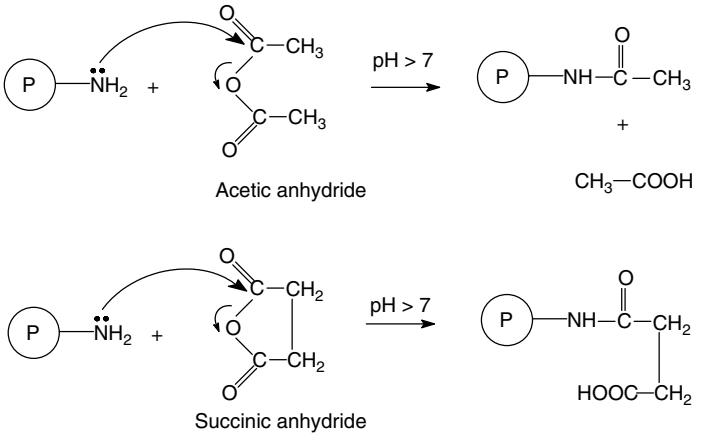


(5.115)

The physicochemical properties of the modified protein will be affected by the reactant used. Hydrophobicity of the protein can be increased if an aliphatic aldehyde or ketone is selected for the reaction and changing the chain length of the aliphatic group can vary the degree of hydrophobicity. On the other hand, if a reducing sugar is selected as the reactant, then the protein will become more hydrophilic. Since glycoproteins exhibit superior foaming and emulsifying properties (as in the case of ovalbumin), reductive glycosylation of proteins should improve solubility and interfacial properties of proteins.

5.9.1.2 Acylation

The amino groups can be acylated by reacting with several acid anhydrides. The most common acylating agents are acetic anhydride and succinic anhydride. Reaction of protein with acetic anhydride results in elimination of the positive charges of lysyl residues, and a corresponding increase in electronegativity. Acylation with succinic or other dicarboxylic anhydrides results in replacement of positive change with a negative change at lysyl residues. This increases the electronegativity of proteins and unfolding of the protein if extensive reaction is allowed to occur.

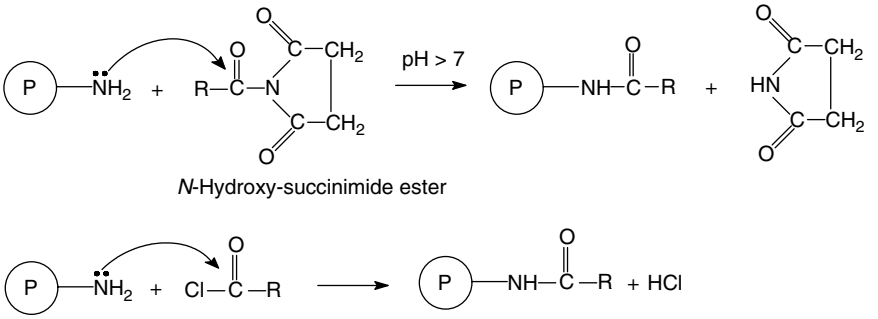


(5.116)

Acylated proteins are generally more soluble than native proteins. In fact, the solubility of caseins and other less soluble proteins can be increased by acylation with succinic anhydride. However, succinylation, depending on the extent of modification, usually impairs other functional properties. For example, succinylated proteins exhibit poor heat-gelling properties, because of the strong electrostatic repulsive forces. The high affinity of succinylated proteins for water also lessens their adsorptivity at oil–water and air–water interfaces, thus impairing their foaming and emulsifying properties. Also, because several carboxyl groups are introduced, succinylated proteins are more sensitive to calcium induced precipitation than is the parent protein.

Acetylation and succinylation reactions are irreversible. The succinyl–lysine isopeptide bond is resistant to cleavage catalyzed by pancreatic digestive enzymes. Furthermore, the intestinal mucosa cells poorly absorb succinyl–lysine. Thus, succinylation and acetylation greatly reduce the nutritional value of proteins.

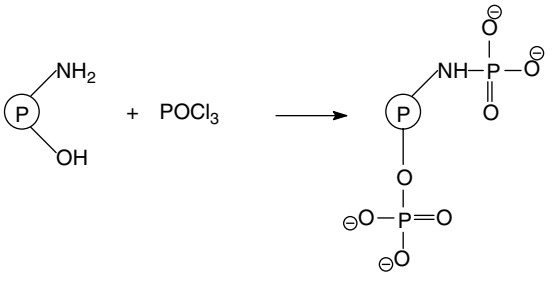
Attaching long chain fatty acids to the ε-amino group of lysyl residues can increase the amphiphilicity of proteins. This can be accomplished by reacting a fatty acylchloride or *N*-hydroxy-succinimide ester of a fatty acid with a protein. This type of modification can enhance lipophilicity and fat binding capacity of proteins; and can also facilitate formation of novel micellar structures and other types of protein aggregates.



(5.117)

5.9.1.3 Phosphorylation

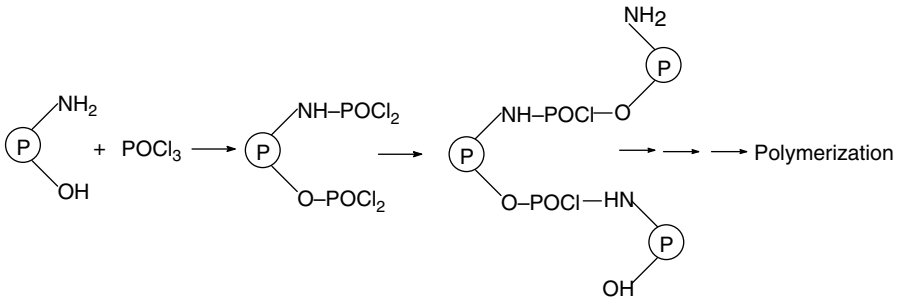
Several natural food proteins, such as caseins, are phosphoproteins. Phosphorylated proteins are highly sensitive to calcium-ion-induced coagulation, which may be desirable in simulated cheese-type products. Proteins can be phosphorylated by reacting them with phosphorus oxychloride, $POCl_3$. Phosphorylation occurs mainly at the hydroxyl group of serine and threonine residues and at the amino group of lysyl residues. Phosphorylation greatly increases protein electronegativity.



(5.118)

Phosphorylation of amino groups results in addition of two negative charges for each positive charge eliminated by the modification. Under certain reaction conditions, especially at high protein concentration, phosphorylation with $POCl_3$ can lead to polymerization of proteins, as shown below. Such

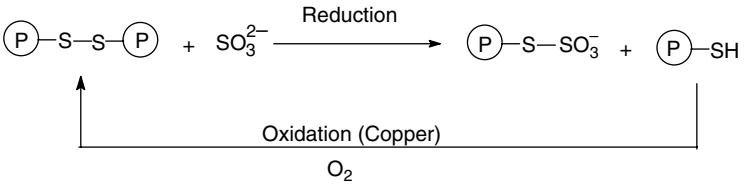
polymerization reactions tend to minimize the increase in electronegativity and calcium sensitivity of the modified protein. The N—P bond is acid labile. Thus, under the conditions prevailing in the stomach, the *N*-phosphorylated proteins would be expected to undergo dephosphorylation and regeneration of lysyl residues. Thus, the digestibility of lysine is probably not significantly impaired by chemical phosphorylation.



(5.119)

5.9.1.4 Sulfitolysis

Sulfitolysis refers to conversion of disulfide bonds in proteins to *S*-sulfonate derivative using a reduction–oxidation system involving sulfite and copper (Cu^{II}) or other oxidants. The mechanism is shown below:



(5.120)

Addition of sulfite to protein initially cleaves the disulfide bond, resulting in the formation of one $S—SO_3^-$ and one free thiol group. This is a reversible reaction, and the equilibrium constant is small. In the presence of an oxidizing agent, such as copper(II), the newly liberated SH groups are oxidized back to either intra- or intermolecular disulfide bonds, and these, in turn, are again cleaved by sulfite ions present in the reaction mixture. The reduction–oxidation cycle repeats itself until all of the disulfide bonds and sulfhydryl groups are converted to *S*-sulfonate derivative [49].

Both cleavage of disulfide bonds and incorporation of SO_3^- groups cause conformational changes in proteins, which affect their functional properties. For example, sulfitolysis of proteins in cheese whey dramatically changes their pH-solubility profiles (Figure 5.35) [48].

5.9.1.5 Esterification

Carboxyl groups of Asp and Glu residues in proteins are not highly reactive. However, under acidic conditions, these residues can be esterified with alcohols. These esters are stable at acid pH, but are readily hydrolysed at alkaline pH.

5.9.2 Enzymatic Modification

Several enzymatic modifications of proteins/enzymes are known to occur in biological systems. These modifications can be grouped into six general categories, namely, glycosylation, hydroxylation, phosphorylation, methylation, acylation, and cross-linking. Such enzymatic modifications of proteins *in vitro* can be used to improve their functional properties. Although numerous enzymatic

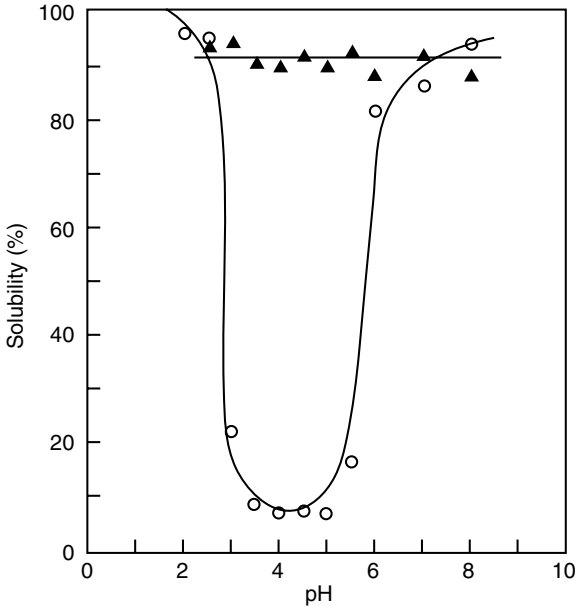


FIGURE 5.35 The pH vs. protein solubility profile of (▲) raw sweet whey and (○) sulfonated sweet whey (From Gonzalez, J. M. and S. Damodaran. 1990. *J. Food Sci*. **55**:1559–1563.)

modifications of proteins are possible, only a few of them are practical for modifying proteins intended for food use.

5.9.2.1 Enzymatic Hydrolysis

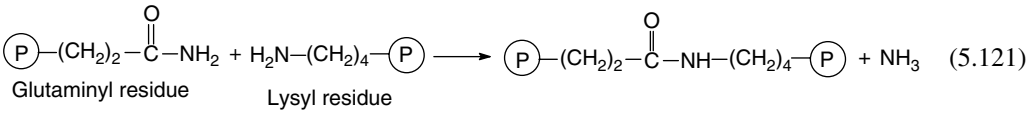
Hydrolysis of food proteins using proteases, such as pepsin, trypsin, chymotrypsin, papain, and thermolysin, alters their functional properties. Extensive hydrolysis by nonspecific proteases, such as papain, causes solubilization of even poorly soluble proteins. Such hydrolysates usually contain low molecular-weight peptides of the order of two to four amino acid residues. Extensive hydrolysis damages several functional properties, such as gelation, foaming, and emulsifying properties (see Section 5.6 for more details).

5.9.2.2 Plastein Reaction

The plastein reaction refers to a set of reactions involving initial proteolysis, followed by resynthesis of peptide bonds by a protease (usually papain or chymotrypsin). The protein substrate, at low concentration, is first partially hydrolysed by papain. When the hydrolysate containing the enzyme is concentrated to 30–35% solids and incubated, the enzyme randomly recombines the peptides, generating new peptide bonds. The plastein reaction also can be performed in a one-step process, in which a 30–35% protein solution (or a paste) is incubated with papain in the presence of L-cysteine [131]. In both cases, however, the molecular weight of the polypeptides formed is typically smaller than the original protein. Thus, the enzyme, especially papain and chymotrypsin, acts both as a protease and an esterase under certain conditions. Since the structure and amino acid sequence of plastein products are different from those of the original protein, they often display altered functional properties. When L-methionine is included in the reaction mixture, it is covalently incorporated into the newly formed polypeptides. Thus, the plastein reaction can be exploited to improve the nutritional quality of methionine or lysine deficient food proteins.

5.9.2.3 Protein Cross-Linking

Transglutaminase catalyses an acyl-transfer reaction that involves reaction between the ε-amino group of lysyl residues (acyl acceptor) and the amide group of glutamine residues (acyl donor), resulting in the formation of an isopeptide cross-link.



(5.121)

This reaction can be used to cross-link different proteins, and to produce new forms of food proteins that might have improved functional properties. At high protein concentration, transglutaminase-catalyzed cross-linking leads to formation of protein gels and protein films at room temperature [85,90,91]. This reaction also can be used to improve nutritional quality of proteins by cross-linking lysine and/or methionine at the glutamine residues (Table 5.23) [58].

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