Paper No. 01

Paper Title: Food Chemistry

Module-16: Protein Structure & Denaturation

The order of amino acids in a protein molecule is genetically determined. This primary sequence of amino acids must contain all the information required for the protein to assume its correct three-dimensional structure. The primary structure is composed of amino acids linked together in what are termed peptide bonds. At first glance these appear to contain only single bonds and free rotation between all such atoms would be expected.

Protein Structure and Conformation

All proteins are made up of many amino acids joined by ***peptide bonds***. Peptide bonds are strong bonds and are not easily disrupted. A ***dipeptide*** contains two amino acids joined by a peptide bond. A ***polypeptide*** contains several amino acids joined by peptide bonds. Proteins are usually much larger molecules, containing several hundred amino acids. They can be hydrolyzed, yielding smaller polypeptides, by enzymes or by acid digestion.

The sequence of amino acids joined by peptide bonds forms the backbone of a protein:

• The protein backbone consists of repeating N–C–C units.

• The amino acid side chains (R groups) project alternately from either side of the protein chain.

• The nature of the R groups determines the structure or ***conformation*** of the chain. (In other words, the shape the protein assumes in space.)

Each protein has a complex and unique conformation, which is determined by the specific amino acids and the sequence in which they occur along the chain. To understand the function of proteins in food systems and the changes that occur in proteins during processing, it is important to understand the basics of protein structure. Proteins are described as having four types of structure - primary, secondary, tertiary, and quaternary structure - and these build on each other. The primary structure determines the secondary structure and so on. The different types of protein structures are outlined below.

Primary Structure

The primary structure (***protein primary structure***) of a protein is the specific sequence of amino acids joined by peptide bonds along the protein chain. This is the simplest way of looking at protein structure. In reality, proteins do not exist simply as straight chains. However, it is the specific sequence of amino acids that determines the form or shape that a protein assumes in space. Therefore, it is essential to know the primary structure if a more detailed understanding of the structure and function of a particular protein is desired.

Secondary Structure

The secondary structure (***protein secondary structure***) of a protein refers to the three-dimensional organization of segments of the polypeptide chain. Important secondary structures include the following:

• Alpha helix: ordered structure.

• Beta pleated sheet: ordered structure.

• Random coil: disordered structure.

The ***alpha*** (α) ***helix*** is a corkscrew structure, with 3.6 amino acids per turn. It is shown in Figure 16.1. It is stabilized by intrachain hydrogen bonds; that is, the hydrogen bonds occur within a single protein chain, rather than between adjacent chains. Hydrogen bonds occur between each turn of the

helix. The oxygen and hydrogen atoms that comprise the peptide bonds are involved in hydrogen bond formation. The α-helix is a stable, organized structure. It cannot be formed if proline is present, because the bulky five-membered ring prevents formation of the helix.

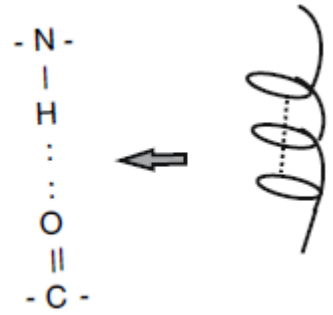


Figure 16.1. Three dimensional structure of an α-helix

The ***beta*** (β) ***pleated sheet*** is a more extended conformation than the α-helix. It can be thought of as a zigzag structure rather than a corkscrew. It is shown in Figure 16.2. The stretched protein chains combine to form β-pleated sheets. These sheets are linked together by interchain hydrogen bonds. (Interchain hydrogen bonds occur between adjacent sections of the protein chains rather than within an individual chain.) Again, the hydrogen and oxygen atoms that form the peptide bonds are involved in hydrogen bond formation. Like the α-helix, the β-pleated sheet also is an ordered structure.

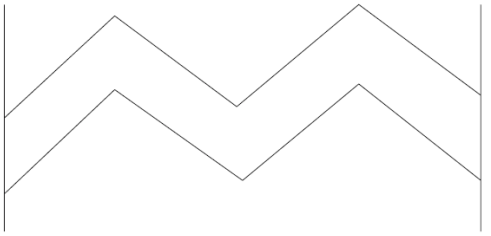


Figure 16.2. Three dimensional structure of β-pleated sheet

The ***random coil*** is a secondary structure with no regular or ordered pattern along the polypeptide chain. This is a much more flexible structure than either the α-helix or β-pleated sheet. It is formed when amino acid side chains prevent formation of the α-helix or β-sheet. This may occur if proline is present or if there are highly charged regions within the protein. A protein may contain regions of α-helix, β-sheet, and random coil at different places along the chain. How much of each type of secondary structure it contains depends on the sequence of amino acids or, in other words, on the primary structure of the protein.

Tertiary Structure

The ***tertiary structure*** of a protein refers to the three-dimensional organization of the complete protein chain. In other words, it refers to the spatial arrangement of a protein chain that contains regions of α-helix, β-sheet, and random coil. So, this structure is really an overview of a protein chain rather than a detailed look at a small section of it. Again, the tertiary structure is built on the secondary structure of a specific protein.

There are two types of protein tertiary structure:
• Fibrous proteins
• Globular proteins

Fibrous proteins include structural proteins such as collagen (connective tissue protein) or actin and myosin, which are the proteins that are responsible for muscle contraction. The protein chains are

extended, forming rods or fibers. Proteins with a fibrous tertiary structure contain a large amount of ordered secondary structure (either α-helix or β-sheet).

Globular proteins are compact molecules and are spherical or elliptical in shape, as their name suggests. These include transport proteins, such as myoglobin, which carry oxygen to the muscle. The whey proteins and the caseins, both of which are milk proteins, also are globular proteins. Globular tertiary structure is favored by proteins with a large number of hydrophobic amino acids. These orient toward the center of the molecule and interact with each other by hydrophobic interactions. Hydrophilic amino acids orient toward the outside of the molecule and interact with other molecules; for example, they may form hydrogen bonds with water. The orientation of the hydrophobic amino acids toward the center of the molecule produces the compact globular shape that is characteristic of globular proteins.

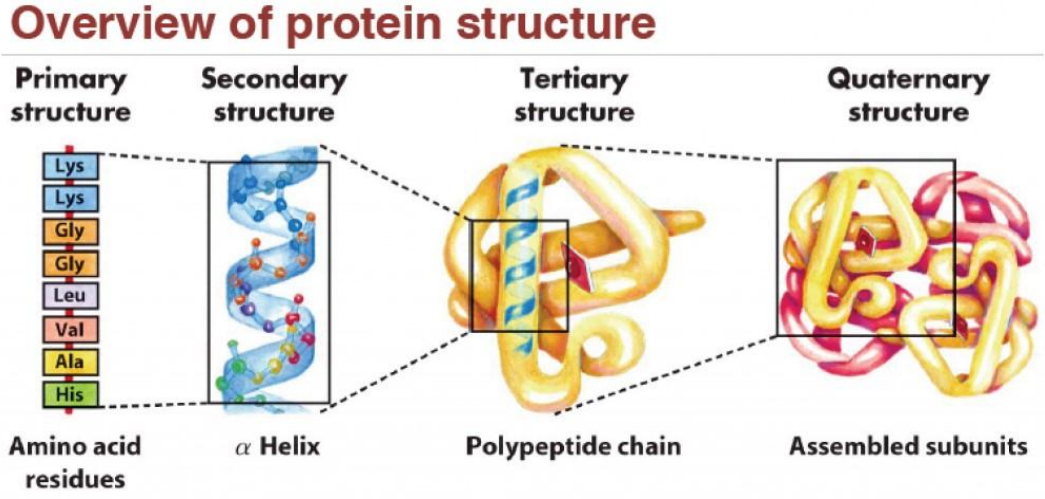
Quaternary Structure

Protein quaternary structure, or the quaternary protein structure, involves the noncovalent association of protein chains. The protein chains may or may not be identical. Examples of quaternary structure include the actomyosin system of muscles and the casein micelles of milk.

Interactions Involved in Protein Structure and Conformation

Protein primary structure involves only peptide bonds, which link the amino acids together in a specific and unique sequence. Secondary and tertiary structures may be stabilized by hydrogen bonds, disulfide bonds, hydrophobic interactions, and ionic interactions. ***Steric*** or spatial effects also are important in determining protein conformation. The space that a protein molecule occupies is determined partially by the size and shape of the individual amino acids along the protein chain. For example, bulky side chains such as proline prevent formation of the α- helix and favor random coil formation. This prevents the protein from assuming certain arrangements in space.

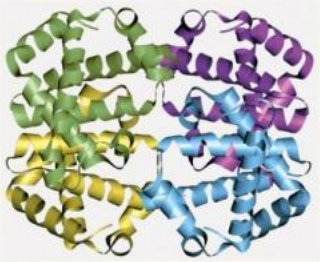
Quaternary structures are stabilized by the same interactions, with the exception of disulfide bonds. As already has been mentioned, disulfide bonds are strong, covalent bonds and so only a few disulfide bonds will have a dramatic effect on protein conformation and stability. Hydrogen bonds, on the other hand, are weak bonds, but they are important because there are so many of them.



Each protein takes on a unique native conformation in space, which almost can be considered as a "fingerprint." As already has been mentioned, the exact folding of the protein into its natural conformation is governed by the amino acids that are present in the protein and the bonds that the side chains are able to form in a protein. The amino acid sequence also is important, as the location of the amino acids along the chain determines which types of bonds will be formed and where, and thus determines how much α-helix, β-sheet, or random coil will be present in a protein. This, in

turn, determines the tertiary and quaternary structure of a protein, all of which combine to define its native conformation. Knowledge of protein conformation and stability is essential to understanding the effects of processing on food proteins.

Table.1 Summary of protein structure

Level	Description	Stabilized by	Example: Hemoglobin
Primary	The sequence of amino acids in a polypeptide	Peptide bonds	
Secondary	Formation of α-helices and β-pleated sheets in a polypeptide	Hydrogen bonding between groups along the peptide-bonded backbone	
Tertiary	Overall three-dimensional shape of a polypeptide (The model on the right shows one of hemoglobin's subunits. The black and red atoms are in the heme group that carries oxygen; they are not part of the protein itself.)	Bonds and other interactions between R-groups, or between R-groups and the peptide-bonded backbone	
Quaternary	Shape produced by combinations of polypeptides. (The model on the right shows hemoglobin, which consists of four polypeptides.)	Bonds and other interactions between R-groups, and between peptide backbones of different polypeptides	

PROTEIN DENATURATION

Measuring Protein Denaturation

Protein denaturation is commonly defined as any noncovalent change in the structure of a protein. This change may alter the secondary, tertiary or quaternary structure of the molecules. When using this definition it should be noted that what constitutes denaturation is largely dependent upon the method utilized to observe the protein molecule. Some methods can detect very slight changes in structure while other require rather large alterations in structure before changes are observed.

Loss of Solubility

One of the oldest methods utilized to follow the course of denaturation was to measure changes in solubility. Changes in solubility might be evident in simple buffers or they might exhibit themselves only after exposure to other conditions, eg. 0.25M ammonium sulfate. Proteins vary greatly in their resistance to insolubilization by a variety of procedures and some proteins that are very important in foods are insoluble in their native state. The loss of solubility is only one of the last stages in a series of changes in structure that must have occurred. As such, this is a rather crude measure of protein denaturation.

In another sense however, the loss of solubility can be related to the loss of a great number of desirable characteristics of the protein. In many cases in food systems, most structural changes other than loss of solubility are unimportant and the role of many process designs and food additives is to maintain protein solubility.

When more sophisticated techniques are utilized many changes in protein structure that eventually result in a loss of solubility can be detected. In these cases the loss of solubility is more properly

regarded as an effect of denaturation rather than as a measure of denaturation. To a consumer or a product development scientist who only observes that feathering occurs when some products are utilized to whiten coffee, loss of solubility, however, is the only event that matters. In the rest of this chapter, loss of solubility will be considered as an effect of denaturation.

Increased Proteolysis

Most native proteins are quite resistant to the action of proteolytic enzymes. During digestion, proteins are exposed to extremes of pH to alter their structures in such a way as to expose the proper groups to enzyme molecules.

For some time, it has been known that a variety of procedures that alter protein's structures make them more susceptible to proteolysis. The rate and extent of proteolysis can be utilized as an indictor of protein denaturation.

In many cases, increases in proteolysis, like decreases in solubility, are the result of many changes in protein structure. In a series of experiments on ribonuclease, researchers exposed this protein to a variety of combinations of pH and temperature. The molecule was then mixed with one of three different proteases. Under conditions of mild denaturation, they were able to observe which portions of the molecule were made susceptible to proteolysis first. Increasingly harsh treatments exposed other portions of the molecule to the action of the proteases. From these observations and knowledge of the tertiary structure of the molecule, they were able to hypothesize a pathway for the thermal denaturation of ribonuclease. This pathway was assumed to be the reverse of the pathway for protein folding, but there was no evidence for this to be the case.

Loss of Biological Activity

For those proteins that are enzymes, denaturation can be defined as the loss of enough structure to render the enzyme inactive. Changes in the rate of the reaction, the affinity for substrate, pH optimum, temperature optimum, specificity of reaction, etc., may be affected by denaturation of enzyme molecules.

Loss of enzymatic activity can be a very sensitive measure of denaturation as some assay procedures are capable of detecting very low levels of product. In some cases the loss of activity can be shown to occur only after some other changes in structure can be observed by other procedures. There may technically, then be denaturation of the protein before loss of activity occurs.

Enzymes are extremely important in the processing and preparing of food products. Processors may variously want to encourage or inhibit the activity of selected enzymes. In these cases, losses of activity may well be the only index of protein denaturation that are of interest.

A number of protein molecules may exhibit biological activities that are not enzymatic in nature. Antibodies for instance are capable of interacting with specific antigen molecules. Other proteins, like hemoglobin, may function as carriers while some, eg. ferritin, may function in the storage of specific components. The loss of any of these activities can be measured as protein denaturation.

Tritium-Hydrogen Exchange

When compounds that contain tritium are placed in water they will rapidly exchange the tritium for normal hydrogen if the groups containing the tritium are exposed to the water. Tritium may be incorporated into proteins by a number of procedures. Probably the most common in exchange experiments involves the unfolding of the protein molecule in a medium where all of the water has been replaced by tritium oxide. When the protein is removed to a normal aqueous environment, three classes of tritium are often observed. Any tritium that is on the surface of the molecule along

with any other that is not necessarily always on the surface, but that comes into contact with the surface under the conditions of study, will rapidly be lost from the molecule.

Spectroscopic Procedures

A variety of procedures have been developed that measure the interaction of electromagnetic radiation with molecules. Some of these procedures have proven to be very useful in the study of protein denaturation.

One such procedure is ultraviolet adsorption spectroscopy. This simply measures the wavelength of and the amount of ultraviolet radiation absorbed by a molecule. In proteins, both the wavelength and extent of absorption depend on the amino acids present and on their physical environments. There are a large number of such groups in a protein molecule and thus its U.V. spectrum quite often lacks detail. Under some circumstances however, these groups can absorb at a low wavelength, generally in the U.V., and then emit light at a larger wavelength. This process is known as fluorescence and is quite sensitive to the environment of the groups involved.

Both ultraviolet and fluorescence spectroscopy have been utilized to follow changes in the environments of various groups within protein molecules. Such changes in environment reflect changes in protein structure and thus denaturation.

The interaction of polarized light with protein can be measured by the techniques of circular dichroism and optical rotatory dispersion. These methods yield an indication of the extent of repeating structures present in protein and are generally utilized to give estimates of the amount of secondary structure present, eg. alpha-helix, beat sheet or coil. While these procedures do not yield very precise estimates of the exact secondary structure of proteins, they are very useful for observing changes. These methods are very sensitive and rather small changes in structure can be detected.

Causes of Protein Denaturation

Changes in the structure of proteins can be caused by a variety of factors. Some of these are encountered frequently while others are more of theoretical interests. Some of the important mechanisms of protein denaturation to food scientists will be discussed.

Thermal Denaturation

When proteins are exposed to increasing temperature, losses of solubility or enzymatic activity occurs over a fairly narrow range. Depending upon the protein studied and the severity of the heating, these changes may or may not be reversible.

As the temperature is increased, a number of bonds in the protein molecule are weakened. The first affected are the long range interactions that are necessary for the presence of tertiary structure. As these bonds are first weakened and are broken, the protein obtains a more flexible structure and the groups are exposed to solvent. If heating ceases at this stage the protein should be able to readily refold to the native structure. As heating continues, some of the cooperative hydrogen bonds that stabilize helical structure will begin to break. As these bonds are broken, water can interact with and form new hydrogen bonds with the amide nitrogen and carbonyl oxygen of the peptide bonds. The presence of water further weakens nearby hydrogen bonds by causing an increase in the effective dielectric constant near them. As the helical structure is broken, hydrophobic groups are exposed to the solvent.

The effect of exposure of new hydrogen bonding groups and of hydrophobic groups is to increase the amount of water bound by the protein molecules. The unfolding that occurs increase the hydrodynamic radius of the molecule causing the viscosity of the solution to increase. The net

result will be an attempt by the protein to minimize its free energy by burying as many hydrophobic groups while exposing as many polar groups as possible to the solvent. While this is analogous to what occurred when the protein folded originally, it is happening at a much higher temperature. This greatly weakens the short range interaction that initially direct protein folding and the structures that occur will often be vastly different from the native protein.

Upon cooling, the structures obtained by the aggregated proteins may not be those of lowest possible free energy, but kinetic barriers will prevent them from returning to the native format. Any attempt to obtain the native structure would first require that the hydrophobic bonds that caused the aggregation be broken. This would be energetically unfavorable and highly unlikely. Only when all the intermolecular hydrophobic bonds were broken, could the protein begin to refold as directed by the energy of short range interactions. The exposure of this large number of hydrophobic groups to the solvent, however, presents a large energy barrier that make such a refolding kinetically unlikely.

Exposure of most proteins to high temperatures results in irreversible denaturation. Some proteins, like caseins, however, contain little if any secondary structure and have managed to remove their hydrophobic groups from contact with the solvent without the need for extensive structure. This lack of secondary structure causes these proteins to be extremely resistant to thermal denaturation.

The increased water binding noted in the early stages of denaturation may be retained following hydrophobic aggregations. The loss of solubility that occurs will greatly reduce the viscosity to a level below that of the native proteins. The effect of thermal denaturation on the functional properties of specific proteins will be discussed in subsequent chapters.

pH Denaturation

Most proteins at physiological pH are above their isoelectric points and have a net negative charge. When the pH is adjusted to the isoelectric point of the protein, its net charge will be zero. Charge repulsions of similar molecules will be at minimum and many proteins will precipitate. Even for proteins that remain in solution at their isoelectric points, this is usually the pH of minimum solubility.

If the pH is lowered far below the isoelectric point, the protein will lose its negative and contain only positive charges. The like charges will repel each other and prevent the protein from aggregating as readily. In areas of large charge density, the intramolecular repulsion may be great enough to cause unfolding of the protein. This will have an effect similar to that of mild heat treatment on the protein structure. In some cases the unfolding may be extensive enough to expose hydrophobic groups and cause irreversible aggregation. Until this occurs such unfolding will be largely reversible.

Some proteins contain acid labile groups and even relatively mild acid treatment may cause irreversible loss of function. This generally results from the breaking of specific covalent bonds and thus should be considered separately from denaturation. Exposure to strong enough acid at elevated temperatures will first release amide nitrogen from glutamine and asparagine groups and eventually lead to hydrolysis of peptide bonds.

The effects of high pH are analogous to those of low pH. The proteins obtain a large negative charge which can cause unfolding and even aggregation. The use of high pH to solubilize and alter protein structure is very important to the formation of fibers from proteins of plant origin

A number of reactions can cause chemical modification of proteins at alkaline pH's that are

commonly encountered in protein processing. Many of these involve cysteine residues. Perhaps the most important are the base catalyzed beta eliminations of sulfur to yield dehydroalanine which can react with lysine to form lysinoalanine. This results in a loss of nutritive value of the protein and the products of the reaction may be toxic. Exposure of protein molecules to high pH should be minimized as much as is possible. Exposure to very high pH at elevated temperatures results in alkaline hydrolysis of peptide bonds.

Changes in Dielectric Constant

The addition of a solvent that is miscible with water, but that is less polar will lower the dielectric constant of the system. This will tend to increase the strength of all electrostatic interactions between molecules that were in contact with water. Many of the protein hydrogen bonds are effectively removed from the solvent and will not be affected. The presence of the less polar solvent will also have the effect of weakening the hydrophobic bonds of the proteins. These bonds depend upon an increase in the order of water when they are broken for their existence. As there is less water in the system, this becomes less important and at some level of replacement, these groups are at a lower energy level when in contact with the solvent.

The structure of the protein will be changed and hence, it will be denatured. The reversibility of the process depends to a large extent on the nature of the non-polar solvent, the extent of unfolding the temperature of the system and the rate of solvent removal. When large amounts of the solvent are present, the protein will be largely unfolded with extensive exposure of the hydrophobic groups. If the protein could be instantaneously transferred to pure water at room temperature, the protein would most likely aggregate and precipitate. The sudden exposure of the hydrophobic groups to water would cause them to try to remove themselves from the aqueous phase as soon as possible. Even before the short range interactions could redirect the folding of the protein aggregation would occur.

Denaturation at Interfaces

When proteins are exposed to either liquid-air or liquid-liquid interfaces, denaturation can occur. As a liquid-liquid interface, the protein comes into contact with a hydrophobic environment. If allowed to remain at this interface for a period of time proteins will tend to unfold and place as many of their hydrophobic groups as possible in the non-aqueous layer while maintaining as much charge as possible in the water layer.

The amount of unfolding that occurs at such an interface will depend on how rigid the three-dimensional protein structure is an on the number and location of hydrophobic groups in the molecule. A flexible, non-crosslinked protein will be able to unfold easier than will a highly structured and crosslinked one. If energy is applied to cause shear, the process will be accelerated. The shear can cause the protein to unfold, thus exposing its hydrophobic groups to the nonaqueous phase. It can also increase the interfacial area between the two phases and allow more proteins to come into contact with the nonaqueous phase.

This unfolding is essentially non-reversible because of the large energy barriers. Even if the phases should separate and the protein is forced into the aqueous phase the protein will not regain its original structure. Rather an association of hydrophobic groups will cause the protein to aggregate.

The same forces are in operation when a protein migrates to a liquid-air interface. Hydrophobic groups tend to associate in the air and the protein unfolds. The presence of shear causes to help unfold the protein and to introduce more air into the solution. Both of these effects can be minimized by keeping the temperature low (to weaken hydrophobic bonds) and by minimizing the interfacial area. If the interface is limited, then only a small amount of protein will be able to denature. The presence of this denatured protein will serve as a barrier to further denaturation.

Proteins are often utilized in food products to stabilize emulsions or to incorporate air.

Ionic Strength

Proteins are usually more soluble in dilute salt solutions than in pure water. The salts are thought to associate with oppositely charge groups in the protein. This combination of charged groups bonds more water than do the charged groups alone and protein hydration is increased. With most proteins there is little change in solubility as more salt is added until some very high salt content is reached. At very high levels of salt there is a competition between the ions and the proteins for water of hydration.

When the salt concentration is high enough, the proteins will be sufficiently dehydrated to lose solubility. Removal of the salt or dilution to a low enough concentration will usually result in the recovery of native structure.

The Effect of Protein Cross-linkers

The presence of groups that crosslink protein molecules will tend to lower the extent of protein denaturation. There are two main reasons that this is so. First, when proteins are crosslinked it is more difficult for them to unfold. As energy is added to the system and secondary bonds are weakened, the presence of crosslinkers will tend to maintain structure. This is especially true if the crosslinks are covalent as in the case of disulfide bonds. The more compact the molecule is and the greater the number of disulfide linkages present, the greater the stability of the protein. While secondary forces may be weakened and some bonds can be broken, the crosslinkers will tend to keep these groups in fairly close proximity. They also tend to prevent the exposure of large numbers of hydrophobic groups to the solvent. When conditions are returned to the native state, there is now a much greater chance for the proper secondary interaction to occur and for the protein to assume the native configuration.

A second effect has to do with the differences in entropy between the native and unfolded states. If a protein can be caused to assume a completely random coil conformation, there will be a large increase in entropy compared to the native structure. This entropy must be overcome if the protein is to refold into a native conformation. When crosslinking groups are present, a completely random coil conformation can not be assumed. These groups introduce order into the structure and there is a considerable loss in the amount of disorder that can be achieved in the most denatured state. Because of this, the entropy change between the native and denatured state is not nearly as great and there will be less of a driving force for denaturation. If the crosslinking groups are broken before denaturation and thus allowed to randomly form after denaturation, no stability will be added to the protein by the pressure of these groups.

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