

Protein Denaturation and Hydrolysis

The processing and handling of the milk will change the physico-chemical environment which would result in the denaturation of the protein molecule.

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 non-physiological conditions.

Change in the structure of proteins can be caused by a variety of factors (**heat, acid, alkali** or other agents such as **sound waves, surface forces, pressure, UV radiation and ionizing radiations**) that results in loss of solubility and coagulation which is otherwise known as denaturation. Treatment with organic solvents (**alcohol, acetone and solutes like urea, guanidine and ionic detergents**) would also result in protein denaturation. Denatured proteins lose their biological activity (e.g. enzymes), but not their nutritional value. Indeed, their digestibility is improved compared to the native structures, which are relatively resistant to enzymatic hydrolysis.

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 type and 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 hydrogen bonds that stabilize helical structure will begin to break. As these bonds are broken, water can interact and form new hydrogen bonds with the amide nitrogen and carbonyl oxygen of 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 hydrophobic groups 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.

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 form. 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. Only when all the intermolecular hydrophobic bonds were broken, could the protein begin to refold as directed by the energy of short range interactions.

Effect of pH on protein 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.

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 readily aggregating.

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.

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.

A number of reactions can cause chemical modification of proteins at alkaline pH that are commonly encountered in protein processing. Many of these involve cysteine residues. This result in a loss of nutritive value of the protein and the products of there action 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.

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.

Protein unfolds at hydrophobic interfaces because tertiary structure of a protein is not rigid. Any change in conformation that yields a higher energy state will spontaneously go back to the state of lowest energy. As a part of this process, hydrophobic groups will occasionally be positioned so that they have increased contact with the aqueous phase. When this occurs, these groups will assume the configuration of lowest free energy and will be removed from the water. If a hydrophobic group is exposed while a protein is in contact with a polar solvent, these groups will find a state of lower energy exists if they enter into the solvent phase. This will continue to occur until random fluctuations in protein structure can no longer yield a configuration of lower free energy.

The amount of unfolding that occurs at such an interface will depend on how rigid the three dimensional protein structure is on the number and location of hydrophobic groups in the molecule. A flexible, non-cross linked protein will be able to unfold easier than will a highly structured and cross linked 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 non-aqueous phase. It can also increase the interfacial area between the two phases and allow more proteins to come into contact with the non-aqueous phase. Even if the phases should separate and the protein is forced into the aqueous phase the protein will not regain its original structure.

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 helps to 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.

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 binds more water than do the charged groups alone and protein hydration is increased.

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.

Effect of protein cross linkers:

The presence of groups that cross link protein molecules will tend to lower the extent of protein denaturation. There are two main reasons for this type of behavior. When proteins are cross linked it is more difficult for them to unfold. As energy is added to the system and secondary bonds are weakened, the presence of cross linkers will tend to maintain structure. This is especially true if the cross links 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 are weakened and some bonds can be broken, the cross linkers 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 greater chance for the proper secondary interaction to occur and for the protein to assume the native configuration.

Hydrolysis of proteins:

Proteins are polymers of amino acids which are held by the polypeptide bonds between them.

When these bonds are broken the protein splits in to smaller peptide and proceed to release for amino acids or smaller peptides.

For these reactions, the presence of a suitable agent, or enzymes are required.

The solution containing these smaller peptides and even amino acids is called hydrolysate solution. This process will help the digestion of the proteins and also study of the various amino acids present in the protein.

Fat Globule Membrane Proteins their Properties and Role

Milk-fat globules originate near the basal region of the secretory cells as small droplets of fat. They migrate through the cytoplasm, gradually increasing in size, as the synthesis of triacylglycerol proceeds. The milk fat globules are secreted from the apical surface of the cell, surrounded by a membrane thin bilayer, the Milk Fat Globule Membrane (MFGM). MFGM are formed by a unique and quantitatively small subcategory of milk proteins (approx. 2-4% of total protein in human milk).

Milk Fat Globule Membrane Protein:

MFGM proteins are having the nutraceutical and biological importance.

MFGM is a rich source of membrane proteins.

MFGM glycoproteins seem to contribute to the prevention of pathogenic organisms infections, being able to act as specific bacterial and viral ligands in the stomach of newborns, to prevent the attack of the intestinal mucosa. The diversity of the glycans found in MFGM is thought to enable the glycoproteins to perform this function in the acidic environment of the stomach.

Some forms of gastric diseases such as peptic ulcer, chronic type B gastritis and gastric cancer can be attributed to the colonization of gastric mucosa by *Helicobacter pylori*. Nondefatted and defatted MFGM preparations, given orally, caused equal healing effect on *H. pylori* infection of gastric mucosa in BALB/CA mice.

Composition of Fat Globule Membrane Protein:

The fat globule membrane contains approximately 50% protein and accounts for about 1% of the total protein of the milk.

Several enzymes have been associated with this membrane. At least 25 different enzymes have been reported to be isolated from the fat globule membrane which includes the 5'nucleotidase, alkaline phosphatase, acid phosphatase, aldolase, xanthine oxidase.

Apart from the enzymes, the proteins of the fat globule membrane protein exists as polypeptides of varying molecular weight.

These micro lipid droplets are encircled by a special membrane composed of lipid bilayer and proteins. This membrane has been designated the milk fat/lipid globule membrane. The MFGM originates from the plasma membrane of the mammary gland secretory cells by extrusion of lipid droplet from the cell.

Because each lipid droplet in milk has its own MFGM, the lipid droplets are present in milk in a dispersed form and, therefore, do not aggregate with each other.

Only after destruction of the structure of the MFGM through eg., a mechanical force like churning, the lipid droplets aggregate and subsequently form large fat clumps.

Milk fat globule membrane is composed of proteins and lipids in a 1:1 weight ratio.

The main protein of the MFGM is the **glycoprotein butyrophilin** (about 40% of the total proteins of the MFGM) and second representative protein of the MFGM is **xanthine oxidase**, which comprises 12-13% of the total proteins. Other proteins are present in MFGM each at 5% or less.

These proteins are an integral part of the plasma membrane of secretory mammary gland cell.

Role of Fat Globule Membrane Proteins:

Among the health beneficial components of the MFGM proteins are cholesterolemia lowering factor, inhibitors of cancer cell growth, vitamin binders, inhibitor of *Helicobacter pylori*, xanthine oxidase as a bactericidal agent, butyrophilin as a possible suppressor of multiple sclerosis, and phospholipids as agents against colon cancer, gastrointestinal pathogens, Alzheimer's disease, depression, and stress. So, bovine MFGM act as a potential nutraceutical.

Quantification of Proteins in Milk

The proteins in milk are estimated by using either their component amino acids or by their chemical reactions or by their physical forms.

Physical Methods:

Direct weighing: Since the ultimate objective of protein analyses is to determine the weight of protein in a given quantity of material, it might seem that separation of the protein and direct weighing would be the simplest method to use. Since removing lipids, salts, other solutes, and water completely is difficult, it is not generally employed for routine analyses. Obviously, however, direct weighing is the ultimate standard for all other methods.

Volume measurements: Methods based on precipitating protein, centrifuging in a calibrated tube, and measuring its volume, have often been suggested. Eg., The Hart method for casein. Fat is extracted with chloroform and the casein precipitated with acetic acid, centrifuged, and measured. Methods based on volume measurements of proteins are not used to any extent in the dairy industry at present except for assessing the amount of insoluble material in milk powders (solubility index).

Turbidimetric methods:

It is sometimes convenient to form a suspension of insoluble protein and to estimate the protein content by optical methods. Either light transmittance or light scattering can be measured.

Methods employing the former principle are called turbidimetric, those using the latter, nephelometric.

In recent years a turbidimetric method devised by Harland and Ashworth has been used to a considerable extent for determining the concentration of undenatured serum proteins in heated and dry skim milks. In this method the casein and denatured serum proteins are precipitated by saturation with sodium chloride. The resulting filtrate is then diluted and acidified and its light transmittance determined. The protein content is determined from a calibration curve in which % transmittance is plotted against protein nitrogen content of the filtrate (determined by Kjeldahl).

Methods of this kind are rapid, require little sample, and are conveniently adaptable to photometers and colorimeters found in most laboratories.

Nevertheless, turbidimetric methods are not of the highest accuracy and precision because light absorption in turbid systems depends not only on the amount of dispersed material present but also on its degree of dispersion. The degree of dispersion depends on time of standing after development of turbidity, pH, salt concentration, method and rate of precipitation, and concentration of material.

Refractive index measurements:

Most proteins have a refractive index increment of about 0.0018, means that 1 g of protein dissolved in 100 ml of solution increases the refractive index by that amount.

With an accurate refractometer such as the Zeiss dipping refractometer, which has a sensitivity of ± 0.00003 , it is possible to determine protein concentrations satisfactorily.

Temperature must be carefully controlled since most proteins exhibit a considerable temperature coefficient for refractive index increment.

The casein is isolated by acid precipitation, washed and dissolved in alkali, and the refractive index is determined.

Absorption of ultraviolet radiation:

The absorption of UV radiation at wavelengths of 280 nm, due to tyrosine, tryptophan, and phenylalanine residues in the protein, can be used as a method for determining protein content.

This method is particularly valuable for a known pure protein whose extinction coefficient is known. It may also be useful as an approximate measure of the total protein content of a mixture if an average extinction coefficient can be assumed to apply. Obviously the extinction coefficients of proteins vary with their contents of tyrosine, tryptophan and phenylalanine.

The presence of materials other than proteins that absorb at this wavelength would seriously limit the method, but the common salts and other solutes do not absorb radiation at 280 nm.

Chemical Methods:

Determination of nitrogen:

Determining protein content is the Kjeldahl procedure for nitrogen, as nitrogen is a characteristic element in proteins. This method involves the oxidation of the sample with sulfuric acid and a catalyst. Carbon and hydrogen are oxidized to CO_2 and H_2O , and reduced forms of nitrogen (such as $-\text{NH}_2$ and $>\text{NH}$) are retained in the digest as ammonium ions. The digest may be made alkaline and the ammonia distilled, and titrated, or it may be determined colorimetrically directly in the digest by means of Nessler's reagent.

Kjeldahl method has disadvantages such as there is the problem of separating protein from other nitrogenous materials. In milk about 5% of the total nitrogen is in the form of low molecular weight non-protein nitrogenous materials.

The protein can be separated from these by precipitation with trichloroacetic acid. This precipitation, however, does not separate protein from nitrogen-containing lipids. Since the amount of lipid nitrogen is small, comprising only about 0.270 of the total milk nitrogen, it is usually neglected (i.e., included as protein nitrogen).

A second difficulty with nitrogen methods for determining protein arises from variation in the nitrogen content of various proteins. Proteins vary in nitrogen content from 14-19% and thus a single universal conversion factor cannot be used. Fortunately the principal milk proteins exhibit much less variation ranging from 15.3-16.0%. An average factor of 6.38 (corresponding to 15.65% nitrogen) is commonly used for milk proteins to convert nitrogen to protein. For individual milk protein, the correct factor for that protein should be used.

A third difficulty concerned with the Kjeldahl method is the problem of proper digestion and no loss of nitrogen. A great many different modifications of the original Kjeldahl procedure have been suggested to accelerate the digestion while still attaining complete digestion. Copper, mercury, or selenium are used as catalysts and frequently Na_2SO_4 or K_2SO_4 is added to elevate the boiling point during digestion. The use of boric acid to receive the ammonia as it is distilled off is an advantage in that only one reagent, the standard acid for titrating, need to be standardized and measured accurately.

Formol Titration:

Considerable use has been made of titration with formaldehyde as a means of determining protein content in milk and milk fractions. Such titrations have been of particular interest as rapid methods of determining the casein content of milk for cheese making.

As per Bureau of Indian Standards, for the estimation of total milk protein a factor of 1.7 is used and while for casein the factor 1.38 is used.

All such methods involve titration of a sample of milk to the end point of an indicator such as phenolphthalein, or adding a solution of formaldehyde, and titrating the acid liberated to the same end point. The amount of alkali used in the second titration is a measure of the amino groups that were originally present and combined with the formaldehyde.

Instrumental Methods:

Several instrumental methods have been developed for the estimation of protein content in milk and milk products.

A major advantages of these instrumental methods over the other techniques are that these are nondestructive, require little or no sample preparation, and measurements are rapid and precise.

For quality control purposes, it is often more useful to have rapid and simple measurements of protein content and therefore these techniques are most suitable.

For fundamental studies in the laboratory, where pure proteins are often analyzed, **UV-visible spectroscopic techniques** are often preferred because they give rapid and reliable measurements, and are sensitive to low concentrations of protein.