Core course 7

Unit 11: Milk-- Protein and Enzymes

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Proteins are defined as high molecular weight polymers of α-amino acids that are formed by living organisms. The primary structure of proteins consists of a polypeptide chain of amino acids residues joined through peptide bonds. Milk contains a heterogenous mixture of proteins. Normal cow and buffalo milk contains about 3.5% proteins. The concentration changes significantly during lactation, especially during first few days of post-partum. The greatest change occurs in the whey protein fraction. The natural function of the milk protein is to supply the young mammals with the essential amino acids required for the development of muscular and other protein containing tissues and with a number of biologically active proteins i.e., immunoglobulin, vitamin-binding-metal-binding proteins and various hormones. The young of different species are born at different states of maturity, and consequently have different nutritional and physiological requirements. These differences are reflected in the protein content in the milk of the species, which ranges from less than 1 % to about 24%. The protein content in milk is directly related to the growth rate of the young one of that species. In addition, the properties of many dairy products depend on the properties of milk proteins since the proteins particularly the casein contributes significantly to the body and texture of the products although the fat, lactose, and especially the salts exert significant modifying influences.

In the present study the proteins and enzymes of milk are covered under following sections:

1.Proteins

- General structure, amphoteric nature
- Difference between casein and serum protein
- Different types of casein (Acid and Rennet)
- Uses of casein
- Fractionation of protein

2.Enzymes

- Catalase

Alkaline phosphatase
Lipases and
proteases

Classification of milk proteins:
The broad classification of the milk proteins in summarized in Table-1

When milk is acidified to pH 4.6 (the isoelectric point) at room temperature, about 80% of the total protein in cow and buffalo milk precipitates out of solution. This fraction is called casein and the protein which remains in the soluble in the solution is referred as whey or serum protein. The solution also contains small amounts of non protein nitrogenous compounds also. The ratio of casein: whey protein varies between the species. In human milk it is 40:60 while in cow, buffalo, sheep, and goat milk it is 80:20.

Casein in fresh raw milk is present in micellar form mostly 0.02 to 0.30 μ m in diameter, comprised of some 20 to 150000 casein molecules. These micelles also contain inorganic matter mainly calcium phosphate, about 8 g per 100g casein. The casein micelles are voluminous and hold more water than individual casein molecule. The casein can be fractionated into α_{s1}casein, α_{s2} casein, β casein and κ- casein.

The caseins, especially α_{s2} casein, are rich in lysine, an essential amino acid in which many plant proteins are deficient. Hence, casein and skim milk powder are very good nutritional supplements for cereal proteins which are deficient in lysine. But due to high lysine content, casein and products containing casein undergoes extensive non-enzymatic Maillard browning on heating in presence of reducing sugars i.e., lactose.

The existence and properties of casein micelles have several consequences in the use of milk. The stability of milk products during heating, concentrating, and holding is largely determined by these micelles. The rheological properties of sour milk and concentrated milk are largely determined by the changes occurring in these micelles. The interaction of these micelles with air- water and oil- water interfaces is important in homogenization. The casein micelles also influence some of the properties of the homogenized products.

Amphoteric property: In chemistry, an amphoteric compound is a molecule or ion that can react both as an acid as well as a base. The amphoteric property of proteins is due to presence of free carboxylic and free amino groups at the end of proteins molecule. All amino acid is contain atleast two ionizable groups, the α amino groups and α-carboxylic group which are responsible for the acid – base-behavior. Because of the amphoteric nature, the proteins act sometimes as acids and sometimes as bases depending upon the pH of their medium.

Whey Proteins

Whey proteins constitute about 20% of the total protein of cow and buffalo milk. Sometimes this fraction of protein is also referred and serum proteins or non-casein nitrogen. It has two well defined groups of proteins called lactoglobulin and lactoalbumin. Lactoglobulin fraction consists mainly of immunoglobulins especially IgG_1 with lesser amounts of IgG_2, IgA and IgM. The lactoalbumin fractions contain three main proteins β- lactoblobulin, α- lactoglobulin and blood serum albumin.

Major differences between the caseins and whey proteins.

1. In contrast to the caseins, whey proteins do not precipitate from the solution when the pH of the milk adjusted to pH 4.6. This difference in the properties of the two milk proteins is exploited in the preparation of industrial casein and varities of cheese (i.e. cottage cheese, quarg and cream cheese).
2. Chymosin and some other proteinases (known as rennet) produce slight but very specific changes in casein, resulting in its coagulation in the presence of Ca^{2+}. Whey proteins do not undergo such alterations. The coagulation property of casein through the action of rennet is exploited in the manufacture of most cheese varieties and rennet casein.
3. Casein is very stale to high temperatures. Milk may be heated at natural pH (around 6.7) at 100^0C for long time without coagulation and it withstands the milk sterilizing temperature. The whey proteins on the other hand are heat labile, being completely denatured by heating at 90^0C for 10 min.
4. Caseins are phosphoproteins, containing on an average of 0.85% phosphorus while the whey proteins does not contain phosphorus the phosphate groups are responsible for many of the important characteristics of casein. It helps in binding calcium and hence making it nutritionally rich protein, particularly for young ones. The phosphate is also responsible for very highheat stability of milk.
5. Casein is low in Sulphur content (0.8%) while the whey proteins are rich source of Sulphur (about 1.7%).

6. The whey proteins are molecularly dispersed in solution or have simple quaternary structures, whereas casein have a complicated quaternary structure and exist in milk as colloidal form.
7. Casein is synthesized in mammary glands and is found nowhere in nature. Some of the whey proteins such as β-Lactoglobulin and α-Lactoglobulin are also synthesized in mammary gland while others such as bovine serum albumin and immunoglobulins are derived from the blood.

Fractionation of proteins:

The two major milk proteins can be separated by acidifying the milk to isoelectric point i,e. pH of 4.6, where the casein gets precipitated and the whey proteins will be in the fractionation of milk proteins as shown in fig 1 and fraction of casein into casein fractions as shown in fig 2.
The fractionation of proteins particularly into casein and whey proteins is of economic importance. The whey proteins have many physiological functions such as providing immunity of minor components to the target area in the body. For example, lactoglobulin can bind retinol and protect from oxidation and transporting it through the stomach to small intestine. The whey proteins are also used in many pharmaceutical formulations.The casein fractions are used in pharmaceuticals.

Production of caseins

There are two main methods of production of casein on an industrial scale. They are 1) isoelectric precipitation method i.e, acid casein and
2) enzymatic method i.e., rennet coagulation.
Two types of acid casein are being made commercially: lactic and hydrochloric caseins.

Acid casein manufacture:

Lactic acid casein
For the manufacture of lactic acid casein, skim milk (pH 6.6) is first pasteurized (72^0C for 15 s). It is then cooled to setting temperature (around 30^0C) and inoculated with lactic acid-producing bacteria, known as starters such as *Lactococcus lactis* sub-species *cremoris*, *Streptococcus thermophilus* at the rate of 0.1- 0.2% of milk taken). The milk is incubated, without agitation for a period of 14-16 h. During this period, some of the lactose in the milk is converted to lactic acid by the starter bacteria and the pH is reduced to about 4.6, causing coagulation of the casein (and the milk). After the pH of the milk has reached 4.6-4.7, the

coagulum is broken and cooked *i.e.* heated to a temperature of 50-55^{0}C. After a brief period of residence in a cooking under acidic conditions, the casein gets precipitated. After cooking, the whey is drained and the curd particles are washed thoroughly with water, pressed to remove excess water. Finally, minced and dried in hot air oven or using fluidized bed drier. The dried casein is then milled to get particles of desired particle size. Acid casein is insoluble in water. But soluble caseinate can be prepared by dispersing the casein in water by adjusting the pH to 6.5 – 7.0 with NaOH to produce sodium caseinate. Sometimes KOH is used to obtain potassium caseinates. The caseinates are usually spray dried.

Mineral acid casein

For the precipitation of mineral acid casein, pasteurised skim milk at a pH of 6.6 is mixed thoroughly with dilute (0.25 mol L-1) acid at a temperature of about 30^{0}C to a pH of approximately 4.6. In this case, because of the very vigorous agitation and the short mixing time, the casein is precipitated as very fine, individual particles in a liquid serum (whey), unlike the gel/coagulum formed in lactic acid casein manufacture. The acidified milk mixture is then cooked and processed similar to as described for the production of lactic acid casein.

Rennet casein manufacture

When rennet casein is made, the skim milk is not acidified. Hence, the pH remains at 6.6 throughout the manufacturing process. Following pasteurization of the skim milk, it is cooled to a setting temperature of about 30^{0}C, and microbial rennet is added at the rate of 1.5 to 2.0grams for every 100 litres of skim milk. The rennet added milk is kept undisturbed till a firm gel is formed. The renneting process completes in40 min. The clotted milk is then cut into one square inch cubes using wire knives and cooked and the casein processed in a manner similar to that described for lactic acid casein. Most of the rennet casein is used in the manufacture of cheese analogues. The rennet casein is generally prepared from good quality fresh skim milk and hence they are used in food and pharmaceuticals

Yield

The yield of commercial casein is about 3 kg/100 kg skim milk.

Uses of casein products in foods

Casein is generally not consumed as a food on its own. Casein products are used mainly as ingredients in foods for modifying the physical properties of that food or to provide nutritional supplementation. As a consequence, they usually form a relatively minor proportion of the food. The function and various usage of casein in the different food products are listed below;

a) As Edible applications of casein products

Bakery Cheese products
Coffee whiteners and creamers, Confectionery
Cultured milk products, yoghurt *etc.* High fat powders, shortenings and spreads
Ice cream and frozen desserts Infant foods
Instant breakfast and beverages Meat products
Nutritional food bars Pasta
Pharmaceuticals Soups and gravies
Sports drinks, Whipped toppings

b) As Protein supplements

The casein molecule unique ability to form a gel or clot in the stomach makes it very efficient in nutrient supply. The clot provides a sustained slow release of amino acids into the blood stream, sometimes lasting for several hours. Casein is available as *hydrolyzed casein*, where it is hydrolyzed by a protease such as trypsin. Hydrolyzed forms are noted to taste bitter and such supplements are often refused by infants and lab animals in favour of intact casein.

c) As medical and dental uses

Casein-derived compounds are used in tooth remineralization products to stabilize amorphous calcium phosphate (ACP) and release the ACP onto tooth surfaces, where it can facilitate remineralization

d) As non-food uses of casein products

Adhesive for wood, *e.g.* plywood;
Adhesive for foil laminates and paper
Coatings for paper and cardboard
Horticultural spreaders
Joint cements in wallboard
Leather tanning
Paints
Photo-resist
Stock foods
Synthetic fibres

Textile sizing

Enzymes in milk

Enzymes are organic catalysts elaborated by living cells that catalyze many of the reactions. Apart from their specificity in catalyzing the reaction, they are protein in general. Milk also contains several indigenous enzymes as constituent of the milk. Consequently, some of the enzymes will be entering the milk which has not been utilized during the biosynthesis of the milk. Similarly some enzymes get incorporated directly as a measure to protect the constituents after the production of milk. As such the enzymes naturally found in milk play a role not only during the processing but also during their storage. As many as 60 indigenous enzymes have been detected in cow and buffalo milk.

Many of the enzymes indigenous to milk are technologically important due to following reasons:

1. Deterioration: The most important commercially important enzyme, proteinase, acid phosphatase and xanthine oxidas e.g enzyme lipase or for preservation of milk quality. e.g sulphydral oxidase, superoxide dismutase.
2. Thermal history of milk- e.g are alkaline phosphatase, γ-glutamyl transpeptidase, lactoperoxidase.
3. Indication of mastitis- N-acetyle-β-D-glucosaminidase, acid phasophatase. The concentration of several enzymes increases due to mastitic infection.
4. Amtimicrobial activity- lysozyme, lactoperoxidase.

Lipase:

Lipase catalyses the hydrolysis of milk fat, causing hydrolytic rancidity in milk and fat rich milk products. Cow milk contains about 1 – 2mg milk lipoprotiein lipase per litre. Some lipase is in the cream fraction, however, the native enzymes is in the skim milk fraction, where about 90% is bound to casein micelles. The lipolytic system in most milk becomes active only when the milk fat globule membrane is damaged by agitation, homogenization, or temperature fluctuation.

<u>Significance of lipase enzyme</u>: The effect of lipolysis is the rancid flavour which becomes detectable when the acid degree value exceeds mEq/litre. Lipolysis may also produce varieties of other effects. One of the most noticeable is the lowering of surface tension. The liberated fatty acids, especially their salts, mono and diglycerides are responsible for depressing the surface tension of milk. On the other hand the lipolysis plays a positive role in ripening of cheese. The fatty acids particularly the low chain fatty acids as a result of lipolysis during ripening of

cheese are responsible for the development of desirable cheesy flavour in the ripened varieties of cheeses. In addition to the fatty acids, the esters and other compounds of fatty acids collectives contributes to the cheese flavour.

Milk alkaline phosphatase

Milk contains several phosphatases, the principal ones being acid and alkaline phosphatases. Among the two phosphatases, alkaline phosphatase is of technological importance. Alkaline phosphatase is concentrated in fat globule, hence cream contains more amount of this enzyme than in the milk.
Milk alkaline phosphatase is used as the method of preference for determining whether the milk has been pasteurized adequately. Inactivation of alkaline phosphatase by pasteurization is an index of destruction of *Mycobacterium tuberculosis.* The inactivation of phosphatase enzyme by easy and simple chemical methods can be determined.

<u>Reactivation of alkaline phosphatase</u>: the UHT treated and freshly pasteurized milk (pasteurized by high temperature short time method) shows phosphatase test negative indicating adequate heat treatment given to milk during processing. When the milk is stored at 30^0C for 6 hours, the pasteurized or UHT treated milk answers positive for phosphatase test. Reactivation of alkaline phosphatase is of considerable practical significance since regulatory tests for pasteurization assume the absence of phosphatase activity. The sulphydral group released by the whey proteins during heat treatment is responsible for reactivation of the enzyme during storage.

Catalase

This enzyme catalyzes the decomposition of H_2O_2 to H_2O and nascent oxygen ([o]).Catalase activity is higher in mastitis milk and colostrum than in normal milk. It increases with the multiplication of bacteria. Crystalline catalase has been prepared from several sources, but as small amount is present in milk it's not fully purified. Milk catalase has a molecular weight of about 210,000, its isoelectricpH ≈ 5.5, and it contains heme iron. Catalase activities of 300 μmol per min per liter have been reported for milk.

Hydrogen peroxide is a very effective chemical sterile agent, though it causes some damage to the physic-chemical properties and nutritional value of milk protein. Mainly damage is by oxidizing methionine. It is used as a milk preservative in US for treatment of cheese milk(but not permitted in our country). Excess hydrogen peroxide present in milk can be reduced to water and oxygen by treating with exogenous catalase enzyme. This enzyme is used to detect the presence of hydrogen peroxide in milk which is added as preservative in milk.

Proteases:

Milk contains two main proteinases, plasmin (alkaline milk proteinase) and cathepsin D (acid milk proteinase), and several other proteinases. In terms of technological significance, plasmin is the most important among indigenous proteinases. Plasmin accompany the casein micelles on the rennet coagulation of milk and are concentrated in cheese wherein plasmin contributes to the primary proteolysis of casein in cheeses.

The exogenous proteinases are of great importance in cheese making. The use of rennet (rennin and pepsin) is the principal application in proteinases in food processing. Rennet is next to amylases among industrial application of enzymes. Protein hydrolysates which are obtained by partial hydrolysis of proteins are used as food flavorings in soups and gravies and in dietetic foods. The functional properties of milk proteins may be improved by limited proteolysis under controlled conditions by using proteinases. Acid soluble caseins are suitable for beverages and other foods can be produced by partial hydrolysis of caseins. Controlled proteolysis improves the meltability of cheeses but excess proteolysis causes bitterness in the product. Limited hydrolysis of whey proteins reduces its emulsifying capacity but increases its specific foam volume.

On the other hand the heat stable proteinases enzymes produced by psychrotrophic bacteria causes age gelation defect in the ultra high temperature treated milk during storage. i.e. though the milk is sterilized by UHT treatment, the heat stable proteinases gels the milk proteins and milk will have gel like structure which is not accepted by the consumers.

Table-1. Broad classification of milk proteins

Protein	Concentration in milk
α –Casein	15-19 g/L
β - Casein	9– 11 g/L
κ -Casein	2-4 g/L
Whey proteins Lactoglobulins Immunoglobulins	5-7 g/L

IgGimmunoglobulins IgMimmunoglobulins IgA immunoglobulins Lactalbumins	0.6-1.7g/L 0.5-1.8 g/L 2-4 g/L
serum albumin	0.2-0.4 g/L

Fig. 1

Fractionation of proteins:

Skim milk

↓

Cool the milk to $6^{0}C$

↓

Acidify with dilute Hydrochloric acid to pH 4.6

↓

Holding for 30 minute

↓

Filter

↓

Whey → Add saturated magnesium sulphate → Filter

- Precipitate → Lactoglobulin
- solution → Lactalbumin

solid mass → Press → Casein

Fig 2.

Fractionation of casein