4 Milk proteins

4.1 Introduction

Normal bovine milk contains about 3.5% protein. The concentration changes significantly during lactation, especially during the first few days post-partum (Figure 4.1); the greatest change occurs in the whey protein fraction (Figure 4.2). The natural function of milk proteins is to supply 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, e.g. immunoglobulins, vitamin-binding, metal-binding proteins and various protein hormones. The young of different species are born at very different states of maturity, and, consequently, have different nutritional and physiological requirements. These differences are reflected in the protein content of the milk of the species, which ranges

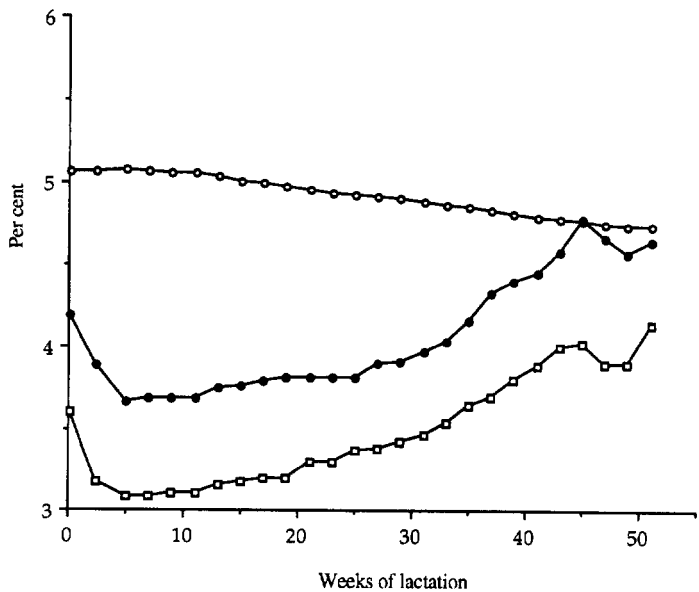


Figure 4.1 Changes in the concentrations of lactose (○), fat (●) and protein (□) in bovine milk during lactation.

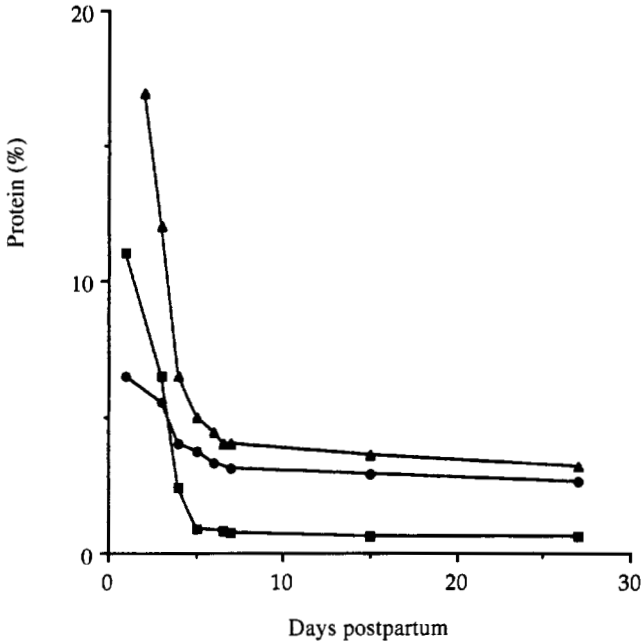


Figure 4.2 Changes in the concentration of total protein (▲) and of casein (●) and whey proteins (■) in bovine milk during the early stage of lactation.

from *c.* 1 to *c.* 24% (Table 4.1). The protein content of milk is directly related to the growth rate of the young of that species (Figure 4.3), reflecting the requirements of protein for growth.

The properties of many dairy products, in fact their very existence, depend on the properties of milk proteins, although the fat, lactose and especially the salts, exert very significant modifying influences. Casein products are almost exclusively milk protein while the production of most cheese varieties is initiated through the specific modification of proteins by proteolytic enzymes or isoelectric precipitation. The high heat treatments to which many milk products are subjected are possible only because of the exceptionally high heat stability of the principal milk proteins, the caseins.

Traditionally, milk was paid for mainly on the basis of its fat content but milk payments are now usually based on the content of fat plus protein. Specifications for many dairy products include a value for protein content. Changes in protein characteristics, e.g. insolubility as a result of heat denaturation in milk powders or the increasing solubility of cheese proteins during ripening, are industrially important features of these products.

It is assumed that the reader is familiar with the structure of proteins; for convenience, the structures of the amino acids found in milk are given in

Table 4.1 Protein content (%) in the milks of some species

Species	Casein	Whey protein	Total
Bison	3.7	0.8	4.5
Black bear	8.8	5.7	14.5
Camel (bactrian)	2.9	1.0	3.9
Cat	–	–	11.1
Cow	2.8	0.6	3.4
Domestic rabbit	9.3	4.6	13.9
Donkey	1.0	1.0	2.0
Echidna	7.3	5.2	12.5
Goat	2.5	0.4	2.9
Grey seal	–	–	11.2
Guinea-pig	6.6	1.5	8.1
Hare	–	–	19.5
Horse	1.3	1.2	2.5
House mouse	7.0	2.0	9.0
Human	0.4	0.6	1.0
Indian elephant	1.9	3.0	4.9
Pig	2.8	2.0	4.8
Polar bear	7.1	3.8	10.9
Red kangaroo	2.3	2.3	4.6
Reindeer	8.6	1.5	10.1
Rhesus monkey	1.1	0.5	1.6
Sheep	4.6	0.9	5.5
White-tailed jack rabbit	19.7	4.0	23.7

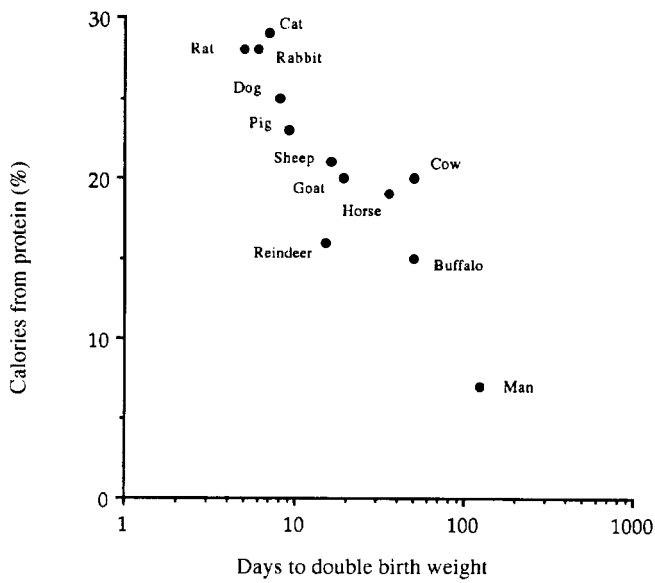


Figure 4.3 Relationship between the growth rate (days to double birth weight) of the young of some species of mammal and the protein content (expressed as % of total calories derived from protein) of the milk of that species (from Bernhart, 1961).

Appendix 4A. We have retained the term cystine to indicate two disulphide-linked cysteines.

4.2 Heterogeneity of milk proteins

Initially, it was believed that milk contained only one type of protein but about 100 years ago it was shown that the proteins in milk could be fractionated into two well-defined groups. On acidification to pH 4.6 (the isoelectric pH) at around 30°C, about 80% of the total protein in bovine milk precipitates out of solution; this fraction is now called **casein**. The protein which remains soluble under these conditions is referred to as **whey** or **serum protein** or **non-casein nitrogen**. The pioneering work in this area was done by the German scientist, Hammarsten, and consequently isoelectric (acid) casein is sometimes referred to as **casein nach Hammarsten**.

The ratio of casein : whey proteins shows large interspecies differences; in human milk, the ratio is c. 40 : 60, in equine (mare's) milk it is 50 : 50 while in the milks of the cow, goat, sheep and buffalo it is *c.* 80 : 20. Presumably, these differences reflect the nutritional and physiological requirements of the young of these species.

There are several major differences between the caseins and whey proteins, of which the following are probably the most significant, especially from an industrial or technological viewpoint:

1. In contrast to the caseins, the whey proteins do not precipitate from solution when the pH of milk is adjusted to 4.6. This characteristic is used as the usual **operational definition** of casein. This difference in the properties of the two milk protein groups is exploited in the preparation of industrial casein and certain varieties of cheese (e.g. cottage, quarg and cream cheese). Only the casein fraction of milk protein is normally incorporated into these products, the whey proteins being lost in the whey.
2. Chymosin and some other proteinases (known as rennets) produce a very slight, specific change in casein, resulting in its coagulation in the presence of Ca^{2+}. Whey proteins undergo no such alteration. The coagulability of casein through the action of rennets is exploited in the manufacture of most cheese varieties and rennet casein; the whey proteins are lost in the whey. The rennet coagulation of milk is discussed in Chapter 10.
3. Casein is very stable to high temperatures; milk may be heated at its natural pH (*c.* 6.7) at 100°C for 24 h without coagulation and it withstands heating at 140°C for up to 20 min. Such severe heat treatments cause many changes in milk, e.g. production of acids from lactose resulting in a decrease in pH and changes in the salt balance, which eventually cause the precipitation of casein. The whey proteins, on the

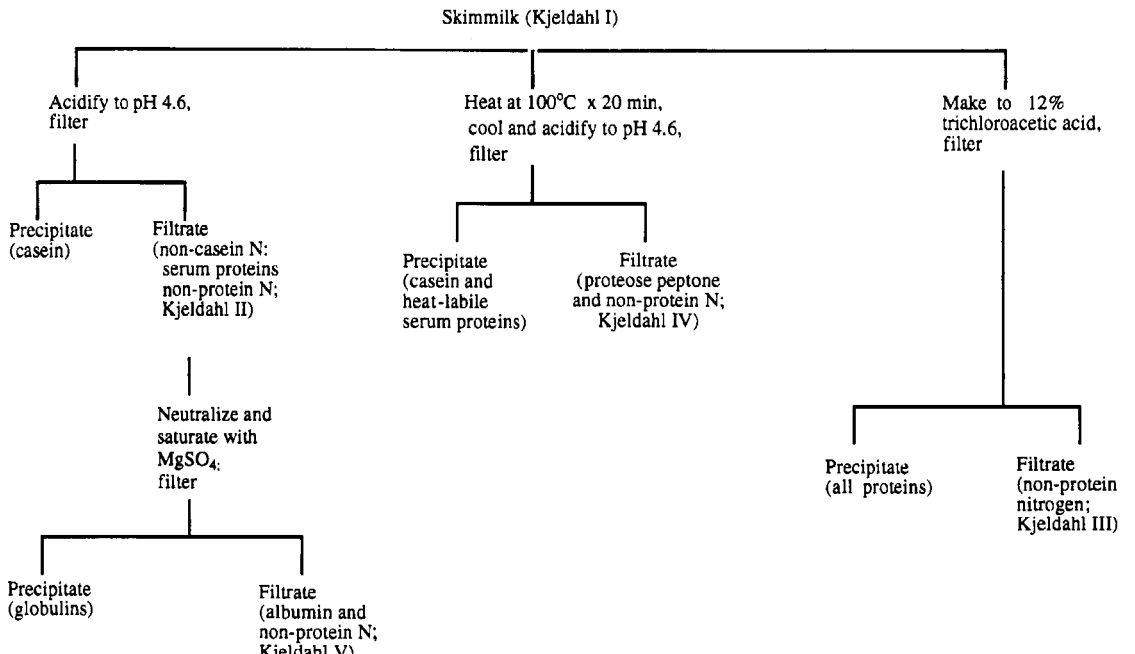
other hand, are relatively heat labile, being completely denatured by heating at 90°C for 10 min. Heat-induced changes in milk are discussed in Chapter 9.

4. Caseins are phosphoproteins, containing, on average, 0.85% phosphorus, while the whey proteins contain no phosphorus. The phosphate groups are responsible for many of the important characteristics of casein, especially its ability to bind relatively large amounts of calcium, making it a very nutritionally valuable protein, especially for young animals. The phosphate, which is esterified to the protein via the hydroxyl group of serine, is generally referred to as **organic phosphate**. Part of the inorganic phosphorus in milk is also associated with the casein in the form of **colloidal calcium phosphate** (*c.* 57% of the inorganic phosphorus) (Chapter 5).

   The phosphate of casein is an important contributor to its remarkably high heat stability and to the calcium-induced coagulation of rennet-altered casein (although many other factors are involved in both cases).
5. Casein is low in sulphur (0.8%) while the whey proteins are relatively rich (1.7%). Differences in sulphur content become more apparent if one considers the levels of individual sulphur-containing amino acids. The sulphur of casein is present mainly in methionine, with low concentrations of cysteine and cystine; in fact the principal caseins contain *only* methionine. The whey proteins contain significant amounts of both cysteine and cystine in addition to methionine and these amino acids are responsible, in part, for many of the changes which occur in milk on heating, e.g. cooked flavour, increased rennet coagulation time (due to interaction between β-lactoglobulin and κ-casein) and improved heat stability of milk pre-heated prior to sterilization.
6. Casein is synthesized in the mammary gland and is found nowhere else in nature. Some of the whey proteins (β-lactoglobulin and α-lactalbumin) are also synthesized in the mammary gland, while others (e.g. bovine serum albumin and the immunoglobulins) are derived from the blood.
7. The whey proteins are molecularly dispersed in solution or have simple quaternary structures, whereas the caseins have a complicated quaternary structure and exist in milk as large colloidal aggregates, referred to as micelles, with particle masses of 10^6-10^9 Da.
8. Both the casein and whey protein groups are heterogeneous, each containing several different proteins.

4.2.1 Other protein fractions

In addition to the caseins and whey proteins, milk contains two other groups of proteins or protein-like material, i.e. the proteose–peptone fraction and the non-protein nitrogen (NPN) fraction. These fractions were recognized as early as 1938 by Rowland but until recently very little was



Total nitrogen = Kjeldahl I
Casein = Kjeldahl I – Kjeldahl II
Non-protein nitrogen = Kjeldahl III

Proteose peptone N = Kjeldahl IV – Kjeldahl III
Serum protein = Kjeldahl II – Kjeldahl IV

Figure 4.4 Scheme for quantifying the principal protein fractions in milk.

known about them. Rowland observed that when milk was heated to 95°C for 10 min, 80% of the nitrogenous compounds in whey were denatured and co-precipitated with the casein when the pH of the heated milk was adjusted subsequently to 4.6. He considered that the heat-denaturable whey proteins represented the lactoglobulin and lactalbumin fractions and designated the remaining 20% 'proteose–peptone'. The proteose–peptone fraction was precipitated by 12% trichloracetic acid (TCA) but some nitrogenous compounds remained soluble in 12% TCA and were designated as nonprotein nitrogen.

A scheme for the fractionation of the principal groups of milk proteins, based on that of Rowland, is shown in Figure 4.4.

4.3 Preparation of casein and whey proteins

Skim milk prepared by mechanical separation (see Chapter 3) is used as the starting material for the preparation of casein and whey proteins.

4.3.1 Acid (isoelectric) precipitation

Acidification of milk to about pH 4.6 induces coagulation of the casein. Aggregation occurs at all temperatures, but below about 6°C the aggregates are very fine and remain in suspension, although they can be sedimented by low-speed centrifugation. At higher temperatures (30–40°C), the aggregates are quite coarse and precipitate readily from solution. At temperatures above about 50°C, the precipitate tends to be stringy and difficult to handle.

For laboratory-scale production of casein, HCl is usually used for acidification; acetic or lactic acids are used less frequently. Industrially, HCl is also usually used; H_2SO_4 is used occasionally but the resulting whey is not suitable for animal feeding ($MgSO_4$ is a laxative). Lactic acid produced *in situ* by a culture of lactic acid bacteria is also widely used, especially in New Zealand, the principal producer of casein.

The inorganic colloidal calcium phosphate associated with casein in normal milk dissolves on acidification of milk to pH 4.6 so that if sufficient time is allowed for solution, isoelectric casein is essentially free of calcium phosphate. In the laboratory, best results are obtained by acidifying skim milk to pH 4.6 at 2°C, holding for about 30 min and then warming to 30–35°C. The fine precipitate formed at 2°C allows time for the colloidal calcium phosphate to dissolve (Chapter 5). A moderately dilute acid (1 M) is preferred, since concentrated acid may cause localized coagulation. Acid production by a bacterial culture occurs slowly and allows time for colloidal calcium phosphate to dissolve. The casein is recovered by filtration or centrifugation and washed repeatedly with water to free the casein of lactose and salts. Thorough removal of lactose is essential since even traces of

lactose will interact with casein on heating via the Maillard browning reaction, with undesirable consequences.

The procedure used for the industrial production of acid (isoelectric) casein is essentially the same as that used on a laboratory scale, except for many technological differences (section 4.15.1).The whey proteins may be recovered from the whey by salting out, dialysis or ultrafiltration.

4.3.2 Centrifugation

Because they occur as large aggregates, micelles, most (90–95%) of the casein in milk is sedimented by centrifugation at 100 000 g for 1 h. Sedimentation is more complete at higher (30–37°C) than at low (2°C) temperature, at which some of the casein components dissociate from the micelles and are non-sedimentable. Casein prepared by centrifugation contains its original level of colloidal calcium phosphate and can be redispersed as micelles with properties essentially similar to the original micelles.

4.3.3 Centrifugation of calcium-supplemented milk

Addition of $CaCl_2$ to about 0.2 M causes aggregation of the casein such that it can be readily removed by low-speed centrifugation. If calcium is added at 90°C, the casein forms coarse aggregates which precipitate readily. This principle is used in the commercial production of some 'casein co-precipitates' in which the whey proteins, denatured on heating milk at 90°C for 10 min, co-precipitate with the casein. Such products have a very high ash content.

4.3.4 Salting-out methods

Casein can be precipitated from solution by any of several salts. Addition of $(NH_4)_2SO_4$ to milk to a concentration of 260 g l^{-1} causes complete precipitation of the casein together with some whey proteins (immunoglobulins, Ig). $MgSO_4$ may also be used. Saturation of milk with NaCl at 37°C precipitates the casein and Igs while the major whey proteins remain soluble, provided they are undenatured. This characteristic is the basis of a commercial test used for the heat classification of milk powders which contain variable levels of denatured whey proteins.

4.3.5 Ultrafiltration

The casein micelles are retained by fine-pore filters. Filtration through large-pore ceramic membranes is used to purify and concentrate casein on a laboratory scale. Ultrafiltration (UF) membranes retain both the caseins

and whey proteins while lactose and soluble salts are permeable; total milk protein may be produced by this method. The casein micelles permeate the membranes used in microfiltration (pore size $\sim 0.05-10\,\mu m$) but bacteria are retained by membranes with pores of less than $0.5\,\mu m$, thus providing a method for removing more than 99.9% of the bacteria in milk without heat treatment; microfiltration is being used increasingly in several sectors of the dairy industry.

Industrially, whey proteins are prepared by ultrafiltration or diafiltration of whey (to remove lactose and salts), followed by spray drying; these products, referred to as whey protein concentrates, contain 30–80% protein.

4.3.6 Gel filtration (gel permeation chromatography)

Filtration through cross-linked dextrans (e.g. Sephadex, Pharmacia, Uppsala, Sweden) makes it possible to fractionate molecules, including proteins, on a commercial scale. It is possible to separate the casein and whey proteins by gel filtration but the process is uneconomical on an industrial scale.

4.3.7 Precipitation with ethanol

The caseins may be precipitated from milk by *c.* 40% ethanol while the whey proteins remain soluble; lower concentrations of ethanol may be used at lower pH values.

4.3.8 Cryoprecipitation

Casein, in a mainly micellar form, is destabilized and precipitated by freezing milk or, preferably, concentrated milk, at about $-10°C$; casein prepared by this method has some interesting properties but is not produced commercially at present.

4.3.9 Rennet coagulation

Casein may be coagulated and recovered as rennet casein by treatment of milk with selected proteinases (rennets). However, one of the caseins, κ-casein, is hydrolysed during renneting and therefore the properties of rennet casein differ fundamentally from those of acid casein. Rennet casein, which contains the colloidal calcium phosphate of milk, is insoluble in water at pH 7 but can be dissolved by adding calcium sequestering agents, usually citrates or polyphosphates. It has desirable functional properties for certain food applications, e.g. in the production of cheese analogues.

4.3.10 Other methods for the preparation of whey proteins

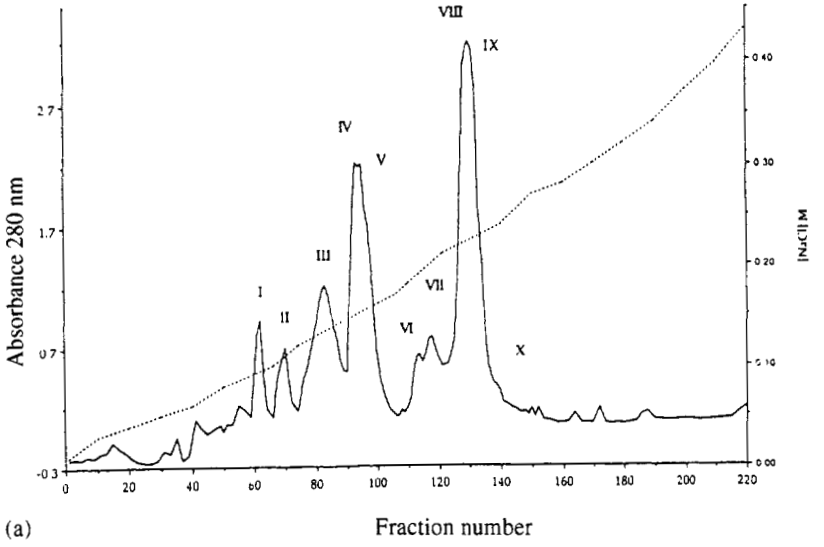
Highly purified whey protein preparations, referred to as whey protein isolates (containing 90–95% protein), are prepared industrially from whey by ion exchange chromatography. Denatured (insoluble) whey proteins, referred to as lactalbumin, may be prepared by heating whey to 95°C for 10–20 min at about pH 6.0; the coagulated whey proteins are recovered by centrifugation. The whey proteins may also be precipitated using $FeCl_3$ or polyphosphates (section 4.15.6).

4.4 Heterogeneity and fractionation of casein

Initially, casein was considered to be a homogeneous protein. Heterogeneity was first demonstrated in the 1920s by Linderstrøm-Lang and co-workers, using fractionation with ethanol-HCl, and confirmed in 1936 by Pedersen, using analytical ultracentrifugation, and in 1939 by Mellander, using free boundary electrophoresis. Three components were demonstrated and named α-, β- and γ-casein in order of decreasing electrophoretic mobility and represented 75, 22 and 3%, respectively, of whole casein. These caseins were successfully fractionated in 1952 by Hipp and collaborators based on differential solubilities in urea at *c.* pH 4.6 or in ethanol/water mixtures; the former is widely used although the possibility of forming artefacts through interaction of casein with cyanate produced from urea is of concern.

In 1956, Waugh and von Hippel showed that the α-casein fraction of Hipp *et al.* contained two proteins, one of which was precipitated by low concentrations of Ca^{2+} and was called α_s-casein (s = sensitive) while the other, which was insensitive to Ca^{2+}, was called κ-casein. α_s-Casein was later shown to contain two proteins which are now called α_{s1}- and α_{s2}-caseins. Thus, bovine casein contains four distinct gene products, designated α_{s1}-, α_{s2}-, β- and κ-caseins which represent approximately 37, 10, 35 and 12% of whole casein, respectively.

Various chemical methods were developed to fractionate the caseins but none gives homogeneous preparations. Fractionation is now usually achieved by ion-exchange chromatography on, for example, DEAE-cellulose, using urea-containing buffers; quite large (e.g. 10 g) amounts of caseinate can be fractionated by this method, with excellent results (Figure 4.5a, b). Good results are also obtained by ion-exchange chromatography using urea-free buffers at 2–4°C. High performance ion-exchange chromatography (e.g. Pharmacia FPLC™ on Mono Q or Mono S) gives excellent results for small amounts of sample (Figure 4.5c, d). Reversed-phase HPLC or hydrophobic interaction chromatography may also be used but are less effective than ion-exchange chromatography.



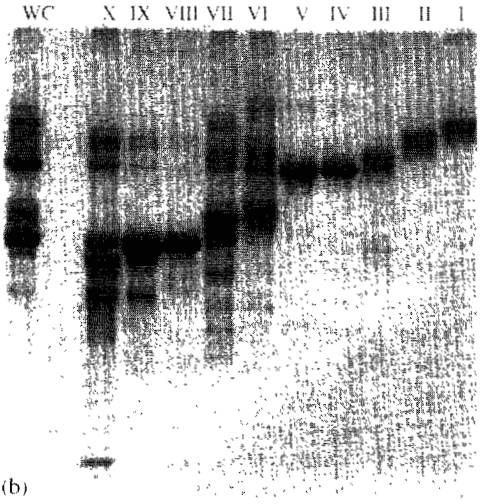
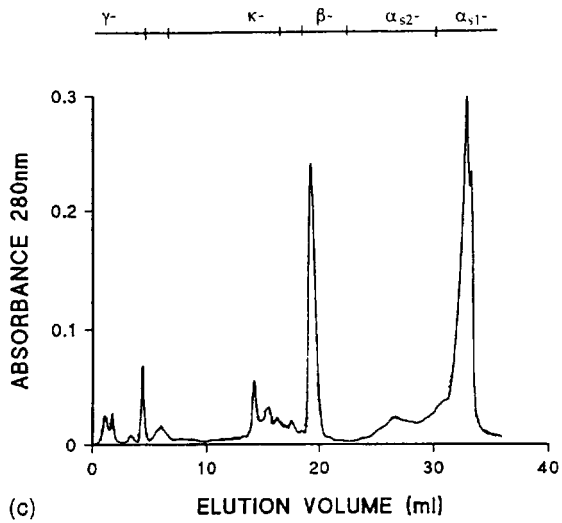
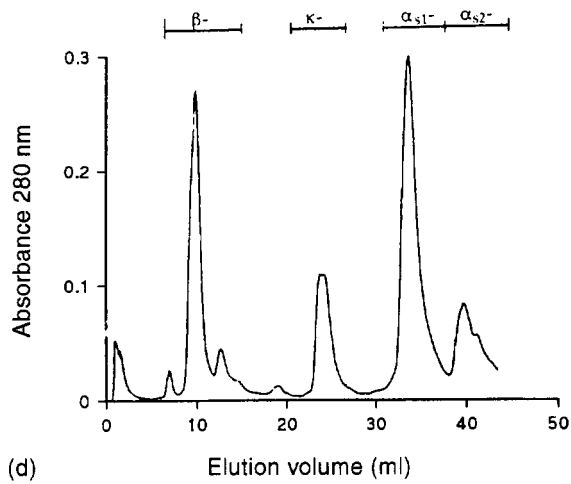


Figure 4.5 (a) Chromatogram of sodium caseinate on an open column of DEAE cellulose anion exchanger. Buffer: 5 M urea in imidazole–HCl buffer, pH 7.0; gradient: 0–0.5 M NaCl. (b) Urea polyacrylamide gel electrophoretograms of the fractions from (a). (c) Chromatogram of sodium caseinate on a Pharmacia Mono Q HR5/5 anion exchange column. Buffer: 6 M urea in 5 mM bis-tris-propane/7 mM HCl, pH 7; gradient: 0–0.5 M NaCl. (d) Chromatogram of sodium caseinate on a Pharmacia Mono S HR5/5 cation exchange column. Buffer: 8 M urea in 20 mM acetate buffer, pH 5; gradient: 0–1.0 M NaCl.



(c)



(d)

Figure 4.5 (*Continued*)

The caseins may be quantified by densitometrically scanning polyacrylamide gel electrophoretograms (section 4.4.1) but more quantitative results are obtained by ion-exchange chromatography using urea-containing buffers. However, it should be realized that the specific absorbance of the individual caseins differs greatly (Table 4.2).

Table 4.2 Properties of some milk proteins (modified from Walstra and Jenness, 1984)

	Caseins				Whey proteins		
Property	α_{s1}-B 8P	α_{s2}-A 11P	β-A^2 5P	κ-B 1P	α-la-B	β-lg-B	Serum albumin
Molecular weight	23 614	25 230	23 983	19 023[a]	14 176	18 363	66 267
Residues/molecule							
Amino acids	199	207	209	169	123	162	582
Proline	17	10	35	20	2	8	34
Cysteine	0	2	0	2	8	5	35
Intramolecular disulphide bonds	0	0	0	0	4	2	17
Phosphate	8	11	5	1	0	0	0
Carbohydrate	0	0	0	[b]	[c]	[d]	0
Hydrophobicity (kJ/residue)	4.9	4.7	5.6	5.1	4.7	5.1	4.3
Charge							
mol % residues	34	36	23	21	28	30	34
Net charge/residue	−0.10	−0.07	−0.06	−0.02[e]	−0.02	−0.04	−0.02
Distribution	Uneven	Uneven	Very uneven	Very uneven	Even	Even	
A_{280}	10.1	14.0[f]	4.5	10.5	20.9	9.5	6.6

[a]Exclusive of carbohydrate residues.
[b]Variable, see text.
[c]A small fraction of the molecules.
[d]0, except for a rare variant (Dr).
[e]Average.
[f]A_{290}.

4.4.1 Resolution of caseins by electrophoresis

Zonal electrophoresis in starch gels containing 7 M urea was used by Wake and Baldwin in 1961 to resolve casein into about 20 bands (zones); the two principal bands were α_{s1}- and β-caseins. Incorporation of urea was necessary to dissociate extensive intermolecular hydrophobic bonding. Electrophoresis in polyacrylamide gels (PAGE), containing urea or sodium dodecyl sulphate (SDS), was introduced in 1963; resolution was similar to starch gels (SGE) but since it is easier to use, PAGE has become the standard technique for analysis of caseins; a schematic representation of a urea–PAGE electrophoretogram of whole casein is shown in Figure 4.6. Owing to the presence of intermolecular disulphide bonds, κ-casein resolves poorly on

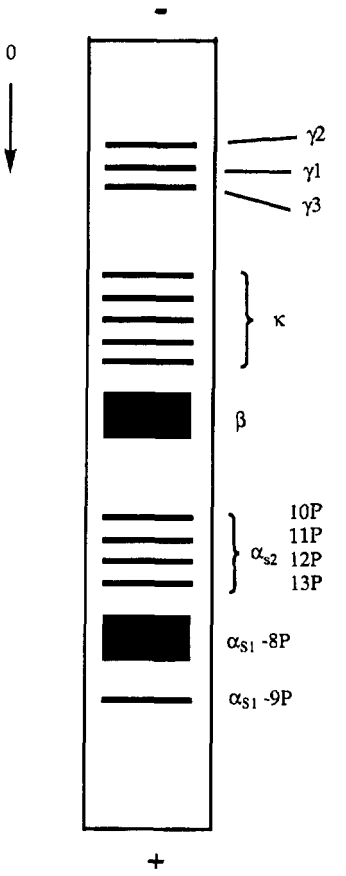


Figure 4.6 Schematic diagram of an electrophoretogram of sodium caseinate in a polyacrylamide gel containing 5 M urea in tris-hydroxymethylamine buffer, pH 8.9. 0 indicates origin.

SGE or PAGE unless it is reduced, usually by 2-mercaptoethanol ($HSCH_2CH_2OH$), or alkylated. Electrophoretic techniques for the analysis of casein were reviewed by Swaisgood (1975).

4.4.2 Microheterogeneity of the caseins

Each of the four caseins, α_{s1}, α_{s2}, β and κ, exhibits variability, which we will refer to as **microheterogeneity**, arising from five causes:

Variability in the degree of phosphorylation. Each of the four caseins is phosphorylated to a characteristic but variable level:

Casein	Number of phosphate residues
α_{s1}	8, occasionally 9
α_{s2}	10, 11, 12 or 13
β	5, occasionally 4
κ	1, occasionally 2 or perhaps 3

The number of phosphate groups in the molecule is indicated as α_{s1}-CN 8P or α_{s1}-CN 9P, etc. (CN = casein).

Disulphide bonding. The two principal caseins, α_{s1} and β, contain no cysteine or cystine but the two minor caseins, α_{s2} and κ, each contains two cysteines per mole which normally exist as intermolecular disulphide bonds. Under non-reducing conditions, α_{s2}-casein exists as a disulphide-linked dimer (previously known as α_{s5} casein) while κ-casein exists as a series of disulphide-linked molecules ranging from dimers to decamers.

Hydrolysis of primary caseins by plasmin. In 1969, Groves and coworkers showed that the γ-casein fraction, as isolated by Hipp *et al.*, is very heterogeneous, containing at least four distinct proteins: γ-casein, temperature-sensitive casein (TS, which is soluble in the cold but precipitates above 20°C), R-casein and S-casein. These four proteins were shown to be C-terminal fragments of β-casein. In 1976, the nomenclature of the γ-casein group was revised, as shown in Figure 4.7 and Table 4.3.

γ-Caseins are produced from β-casein by proteolysis by plasmin, an indigenous proteinase in milk (Chapter 8). The corresponding N-terminal fragments are the principal components of the proteose–peptone (PP) fraction, i.e. PP5 (β-CN f1–105/107), PP8 slow (β-CN f29–105/107) and PP8 fast (β-CN f1–28). Normally, the γ-caseins represent only about 3% of whole casein but levels may be very much higher (up to 10%) in late lactation and mastitic milks. Because of its high isoelectric point (6), some γ-casein may be lost on isoelectric precipitation. γ-Caseins can be readily

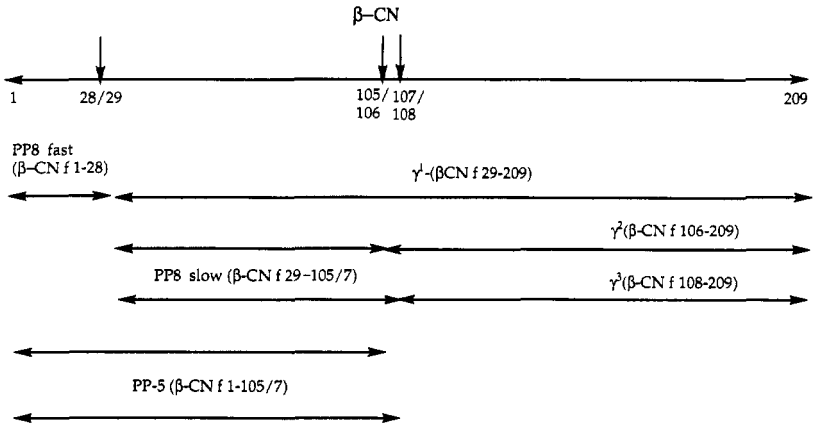


Figure 4.7 Principal products produced from β-casein by plasmin.

Table 4.3 Old and revised nomenclature for γ-caseins

Old	Trivial	Recommended nomenclature (β-casein sequence)
γ	γ-A^1, γ_1-A^2, γ_1-A^3, γ_1-B	β-CN A^1, A^2, A^3, B (f29–209)
TS-A^2	γ^2-A^2	β-CN A^2, (f106–209)
S	γ_2-B	β-CN B, (f106–209)
R	γ_3-A	β-CN A^2, (f108–209)
TS-B	γ_3-B	β-CN B (f108–209)

A and B indicate genetic variants, see p. 162.

prepared by chromatography on DEAE-cellulose since they do not adsorb even at low ionic strength (0.02 M) at pH 6.5; γ^1-casein adsorbs at pH 8.5 but γ^2- and γ^3-caseins do not.

Isolated α_{s2}-casein in solution is also very susceptible to plasmin; eight peptide bonds are hydrolysed with the production of 14 peptides. Plasmin also hydrolyses α_{s2}-casein in milk but the peptides formed have not been identified, although at least some are included in the proteose–peptone fraction.

Although less susceptible than β- and α_{s2}-caseins, isolated α_{s1}-casein in solution is also readily hydrolysed by plasmin. It has been suggested that a minor ill-defined fraction of casein, called λ-casein, consists of plasmin-produced fragments of α_{s1}-casein, but the situation is unclear.

Variations in the degree of glycosylation. κ-Casein is the only one of the principal milk proteins which is normally glycosylated but, as discussed on

p. 173, the level of glycosylation varies, resulting in 10 molecular forms of κ-casein.

Genetic polymorphism. In 1956, Aschaffenburg and Drewry discovered that the whey protein, β-lactoglobulin (β-lg), exists in two forms, A and B, which differ from each other by only a few amino acids. The milk of any individual animal may contain β-lg A or B or both, and the milk is indicated as AA, BB or AB with respect to β-lg. This phenomenon was referred to as **genetic polymorphism** and has since been shown to occur in all milk proteins; a total of about 30 variants have been demonstrated by PAGE. Since PAGE differentiates on the basis of charge, only polymorphs which differ in charge, i.e. in which a charged residue is replaced by an uncharged one or vice versa, will be detected; therefore, it is very likely that many more than 30 polymorphs exist.

The genetic variant present is indicated by a Latin letter, e.g. α_{s1}-CN A-8P, α_{s1}-CN B-8P, α_{s1}-CN B-9P, etc.

The frequency with which certain genetic variants occurs is breed-specific, and hence genetic polymorphism has been useful in the phylogenetic classification of cattle and other species. Various technologically important properties of the milk proteins, e.g. cheesemaking properties and the concentration of protein in milk, are correlated (linked) with specific polymorphs and significant research is ongoing on this subject. The genetic polymorphism of milk proteins has been comprehensively reviewed by Ng-Kwai-Hang and Grosclaude (1992) and Jakob and Puhan (1992).

4.4.3 Nomenclature of the caseins

During studies on casein fractionation, especially during the 1960s, various names were assigned to isolated fractions. To rationalize the nomenclature of milk proteins, the American Dairy Science Association established a Nomenclature Committee which published its first report in 1956 (Jenness *et al.*, 1956); the report has been revised regularly (Brunner *et al.*, 1960; Thompson *et al.*, 1965; Rose *et al.*, 1970; Whitney *et al.*, 1976; Eigel et al., 1984). An example of the recommended nomenclature is α_{s1}-CN A-8P, where α_{s1}-CN is the gene product, A is the genetic variant and 8P is the number of phosphate residues. The Committee recommends that in situations where confusion may arise through the use of a Greek letter alone, the relative electrophoretic mobility be given in brackets, thus α_{s2}-CN A-12P (1.00). The heterogeneity and nomenclature of the caseins in bovine milk is summarized in Figure 4.8.

In addition to simplifying and standardizing the nomenclature of the milk proteins, the characteristics of the various caseins and whey proteins are summarized in the above articles, which are very valuable references.

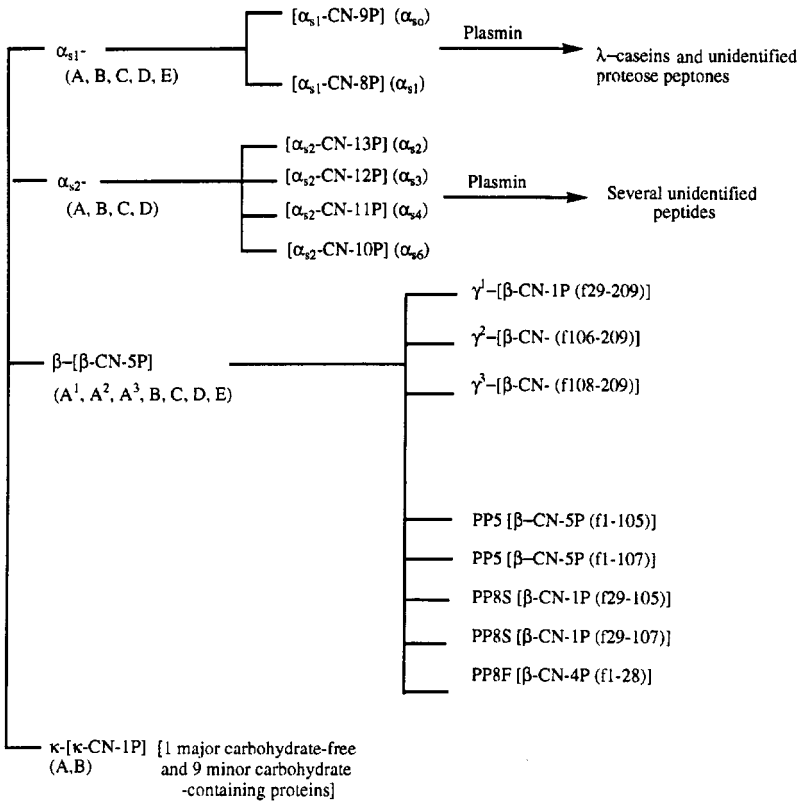


Figure 4.8 Heterogeneity of bovine casein.

4.5 Some important properties of the caseins

4.5.1 Chemical composition

The principal chemical and physicochemical properties of the principal milk proteins are summarized in Table 4.2. Some of the properties of the caseins are discussed in more detail below (see Swaisgood, 1992, for a review).

Amino acid composition. The approximate amino acid composition of the main caseins is shown in Table 4.4. Amino acid substitutions in the principal genetic variants can be deduced from the primary structures (Figures 4.9–4.12). Four features of the amino acid profile are noteworthy:

1. All the caseins have a high content (35–45%) of apolar amino acids (Val, Leu, Ile, Phe, Tyr, Pro) and would be expected to be poorly soluble in aqueous systems, but the high content of phosphate groups, low level of

Table 4.4 Amino acid composition of the major proteins occurring in the milk of western cattle (Swaisgood, 1982)

Acid	α_{s1}-Casein B	α_{s2}-Casein A	κ-Casein B	β-Casein A[2]	γ_1-Casein A[2]	γ_2-Casein A[2]	γ_3-Casein A	β-Lacto-globulin A	α-Lact-albumin B
Asp	7	4	4	4	4	2	2	11	9
Asn	8	14	7	5	3	1	1	5	12
Thr	5	15	14	9	8	4	4	8	7
Ser	8	6	12	11	10	7	7	7	7
SerP	8	11	1	5	1	0	0	0	0
Glu	24	25	12	18	11	4	4	16	8
Gln	15	15	14	21	21	11	11	9	5
Pro	17	10	20	35	34	21	21	8	2
Gly	9	2	2	5	4	2	2	3	6
Ala	9	8	15	5	5	2	2	14	3
$\frac{1}{2}$ Cys	0	2	2	0	0	0	0	5	8
Val	11	14	11	19	17	10	10	10	6
Met	5	4	2	6	6	4	4	4	1
Ile	11	11	13	10	7	3	3	10	8
Leu	17	13	8	22	19	14	14	22	13
Tyr	10	12	9	4	4	3	3	4	4
Phe	8	6	4	9	9	5	5	4	4
Trp	2	2	1	1	1	1	1	2	4
Lys	14	24	9	11	10	4	3	15	12
His	5	3	3	5	5	4	3	2	3
Arg	6	6	5	4	2	2	2	3	1
PyroGlu	0	0	1	0	0	0	0	0	0
Total residues	199	207	169	209	181	104	102	162	123
Molecular weight	23 612	25 228	19 005	23 980	20 520	11 822	11 557	18 362	14 174
$H\Phi_{ave}$ (kJ/residue)	4.89	4.64	5.12	5.58	5.85	6.23	6.29	5.03	4.68

1 Glu-Val-Leu-Asn-Glu-Asn-Leu-
H. Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln-Gly-Leu-Pro-Gln-.....
21
Leu-Arg-Phe-Phe-Val-Ala-(Variants B, C, D, E)
-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu-Lys-Val-Asn-Glu-Leu
.....(Variant A)

41 Ala (Variants A, B, C, E) Gln (Variants A, B,
Ser-Lys-Asp-Ile-Gly-*SerP*-Glu-*SerP*-Thr-Glu-Asp-Gln- -Met-Glu-Asp-Ile-Lys- -Met
ThrP (Variant D) Glu (Variant E)
61
Glu-Ala-Glu-*SerP*-Ile-*SerP*-*SerP*-*SerP*-Glu-Glu-Ile-Val-Pro-Asn-*SerP*-Val-Glu-Gln-Lys-His-

81
Ile-Gln-Lys-Glu-Asp-Val-Pro-Ser-Glu-Arg-Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg-

101
Leu-Lys-Lys-Tyr-Lys-Val-Pro-Gln-Leu-Glu-Ile-Val-Pro-Asn-*SerP*-Ala-Glu-Glu-Arg-Leu-

121
His-Ser-Met-Lys-Glu-Gly-Ile-His-Ala-Gln-Gln-Lys-Glu-Pro-Met-Ile-Gly-Val-Asn-Gln-

141
Glu-Leu-Ala-Tyr-Phe-Tyr-Pro-Glu-Leu-Phe-Arg-Gln-Phe-Tyr-Gln-Leu-Asp-Ala-Tyr-Pro-

161
Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp-Ala-Pro-Ser-Phe-Ser-

181 Glu (Variant A, B, D) 199
Asp-Ile-Pro-Asn-Pro-Ile-Gly-Ser-Glu-Asn-Ser- -Lys-Thr-Thr-Met-Pro-Leu-Trp. OH
Gly (Variant C, E)

Figure 4.9 Amino acid sequence of bovine α_{s1}-casein, showing the amino acid substitutions or deletions in the principal genetic variants (from Swaisgood, 1992).

sulphur-containing amino acids and high carbohydrate content in the case of κ-casein offset the influence of apolar amino acids. The caseins are, in fact, quite soluble: solutions containing up to 20% protein can be prepared in water at 80–90°C. High temperatures are necessary to offset high viscosity, which is the limiting factor in preparing casein solutions. The high viscosity is a reflection of the high water binding capacity (WBC) of casein, i.e. about 2.5 g $H_2O\,g^{-1}$ protein. Such high WBC gives casein very desirable functional properties for incorporation into various foods, e.g. sausage and other comminuted meat products, instant desserts, synthetic whipping creams, etc., and large quantities of casein are used commercially for these purposes.

2. All the caseins have a very high proline content: 17, 10, 35 and 20 Pro residues per mole of α_{s1}-, α_{s2}-, β- and κ-caseins, respectively (out of a total of 199, 207, 209 and 169 residues, respectively). Such high levels of proline

1
H. Lys-Asn-Thr-Met-Glu-His-Val-*SerP*-*SerP*-*SerP*-Glu-Glu-Ser-Ile-Ile-*SerP*-Gln-Glu-Thr-Tyr-

21
Lys-Gln-Glu-Lys-Asn-Met-Ala-Ile-Asn-Pro-Ser-Lys-Glu-Asn-Leu-Cys-Ser-Thr-Phe-Cys-

41
Lys-Glu-Val-Val-Arg-Asn-Ala-Asn-Glu-Glu-Glu-Tyr-Ser-Ile-Gly-*SerP*-*SerP*-*SerP*-Glu-Glu-

61
SerP-Ala-Glu-Val-Ala-Thr-Glu-Glu-Val-Lys-Ile-Thr-Val-Asp-Asp-Lys-His-Tyr-Gln-Lys-

81
Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe-Tyr-Gln-Lys-Phe-Pro-Gln-Tyr-Leu-Gln-Tyr-Leu-Tyr-

101
Gln-Gly-Pro-Ile-Val-Leu-Asn-Pro-Trp-Asp-Gln-Val-Lys-Arg-Asn-Ala-Val-Pro-Ile-Thr-

121
Pro-Thr-Leu-Asn-Arg-Glu-Gln-Leu-*SerP*-Thr-*SerP*-Glu-Glu-Asn-Ser-Lys-Lys-Thr-Val-Asp-

141
Met-Glu-*SerP*-Thr-Glu-Val-Phe-Thr-Lys-Lys-Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg-

161
Leu-Asn-Phe-Leu-Lys-Lys-Ile-Ser-Gln-Arg-Tyr-Gln-Lys-Phe-Ala-Leu-Pro-Gln-Tyr-Leu-

181
Lys-Thr-Val-Tyr-Gln-His-Gln-Lys-Ala-Met-Lys-Pro-Trp-Ile-Gln-Pro-Lys-Thr-Lys-Val-
(Leu)
201 207
Ile-Pro-Tyr-Val-Arg-Tyr-Leu. OH

Figure 4.10 Amino acid sequence of bovine α_{s2}-casein A, showing nine of the 10–13 phosphorylation sites (from Swaisgood, 1992).

result in a very low content of α-helix or β-sheet structures in the caseins. The caseins are, therefore, readily susceptible to proteolysis without prior denaturation by, for example, acid or heat. Perhaps this is an important characteristic in neonatal nutrition.

3. As a group, the caseins are deficient in sulphur amino acids which limits their biological value (80; egg albumen = 100). α_{s1}- and β-caseins contain no cysteine or cystine while α_{s2}- and κ-caseins have two cysteine residues per mole, which normally exist as intermolecular disulphides.

   The principal sulphydryl-containing protein in milk is the whey protein β-lactoglobulin (β-lg), which contains one sulphydryl group; normally, this sulphydryl group is buried within the molecule and is unreactive. Following denaturation, e.g. by heat above *c.* 75°C, the —SH group of β-lg becomes exposed and reactive and undergoes a sulphydryl–disulphide interchange with κ-casein (and possibly with α_{s2}-casein and α-lactalbumin also) with very significant effects on some of the technologically important physicochemical properties of milk, e.g. heat stability and rennet coagulability (Chapters 9 and 10).

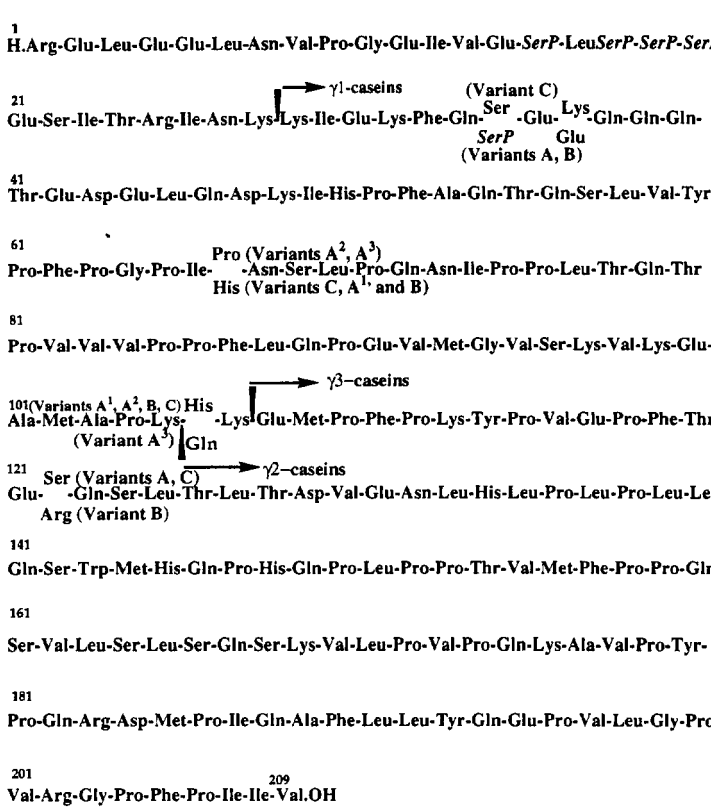


Figure 4.11 Amino acid sequence of bovine β-casein, showing the amino acid substitutions in the genetic variants and the principal plasmin cleavage sites (▼) (from Swaisgood, 1992).

4. The caseins, especially α_{s2}-casein, are rich in lysine, an essential amino acid in which many plant proteins are deficient. Consequently, casein and skim-milk powder are very good nutritional supplements for cereal proteins which are deficient in lysine. Owing to the high lysine content, casein and products containing it may undergo extensive non-enzymatic Maillard browning on heating in the presence of reducing sugars (Chapter 2).

   At pH values on the acid side of their isoelectric point, proteins carry a net positive charge and react with anionic dyes (e.g. amido black or orange G), forming an insoluble protein–dye complex. This is the principle of the rapid dye-binding methods for quantifying proteins in milk and milk products and for visualizing protein bands in gel electrophoretograms; dye-binding is normally performed at pH 2.5–3.5.

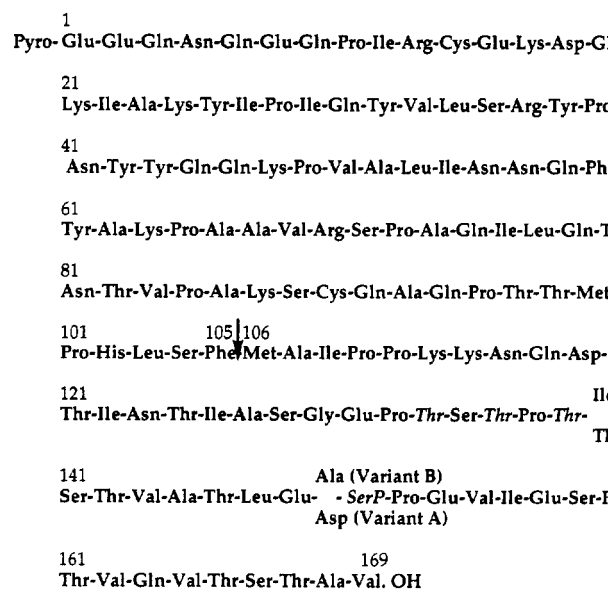


Figure 4.12 Amino acid sequence of bovine κ-casein, showing the amino acid substitutions in genetic polymorphs A and B and the chymosin cleavage site, ↓. Sites of post-translational phosphorylation or glycosylation are italicized (from Swaisgood, 1992).

Lysine is the principal cationic residue in caseins, with lesser amounts of arginine and histidine ($pK_a \sim 6$).

Since the caseins differ in lysine content (14, 24, 11 and 9 residues for α_{s1}-, α_{s2}-, β- and κ-caseins, respectively) they have different dye-binding capacities. This feature may be of some commercial significance in connection with dye-binding methods for protein analysis if the ratio of the caseins in the milks of individual animals varies (as it probably does). It should also be considered when calculating the protein concentration of zones on electrophoretograms stained with these dyes.

The absorbance of 1% solutions of α_{s1}-, α_{s2}-, β- and κ-caseins at 280 nm in a 1 cm light path is 10.1, 14.0, 4.4 and 10.5, respectively. Since the protein concentration in eluates from chromatography columns is usually monitored by absorbance at 280 nm, cognisance should be taken of the differences in specific absorbance when calculating the concentrations of individual caseins in samples.

Primary structure. The primary structures of the four caseins of bovine milk are shown in Figures 4.9–4.12. The sequences of some non-bovine caseins have been established also.

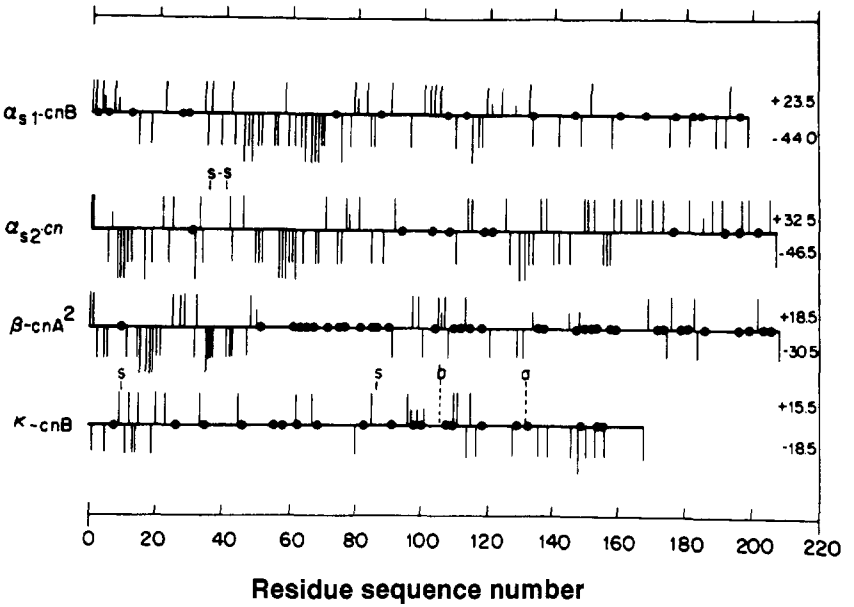


Figure 4.13 Distribution of charged residues (pH 6–7), proline (●) and cysteine (S) in α_{s1}-, α_{s2}-, β- and κ-caseins. a, Location of oligosaccharide moieties; and b, chymosin cleavage site in κ-casein (from Walstra and Jenness, 1984).

An interesting feature of the primary structures of all caseins is that polar and apolar residues are not uniformly distributed but occur in clusters, giving hydrophobic and hydrophilic regions (Figures 4.13–4.15). This feature makes the caseins good emulsifiers. The organic phosphates, which are attached to serines, occur in clusters due to the mechanism by which phosphorylation occurs (see below and section 4.14.4). The phosphate clusters bind Ca^{2+} strongly. The proline residues are fairly uniformly distributed, giving the caseins a type of poly-proline helix. β-Casein is the most hydrophobic of the caseins and α_{s2}-casein is the most hydrophilic. The C-terminal region of κ-casein is strongly hydrophilic due to a high content of sugars (in some cases), few apolar residues and no aromatic residues, while the N terminus is strongly hydrophobic; this detergent-like structure is probably important in micelle stabilization. The hydrophilic segment of κ-casein is cleaved off during rennet action, rendering the residual caseins coagulable by Ca^{2+} (Chapter 10).

The caseins are one of the most evolutionarly divergent families of mammalian proteins. Since their function is nutritional, minor amino acid substitutions or deletions are not critical. Holt and Sawyer (1993), who aligned the published sequences of α_{s1}-, β- and κ-caseins from various

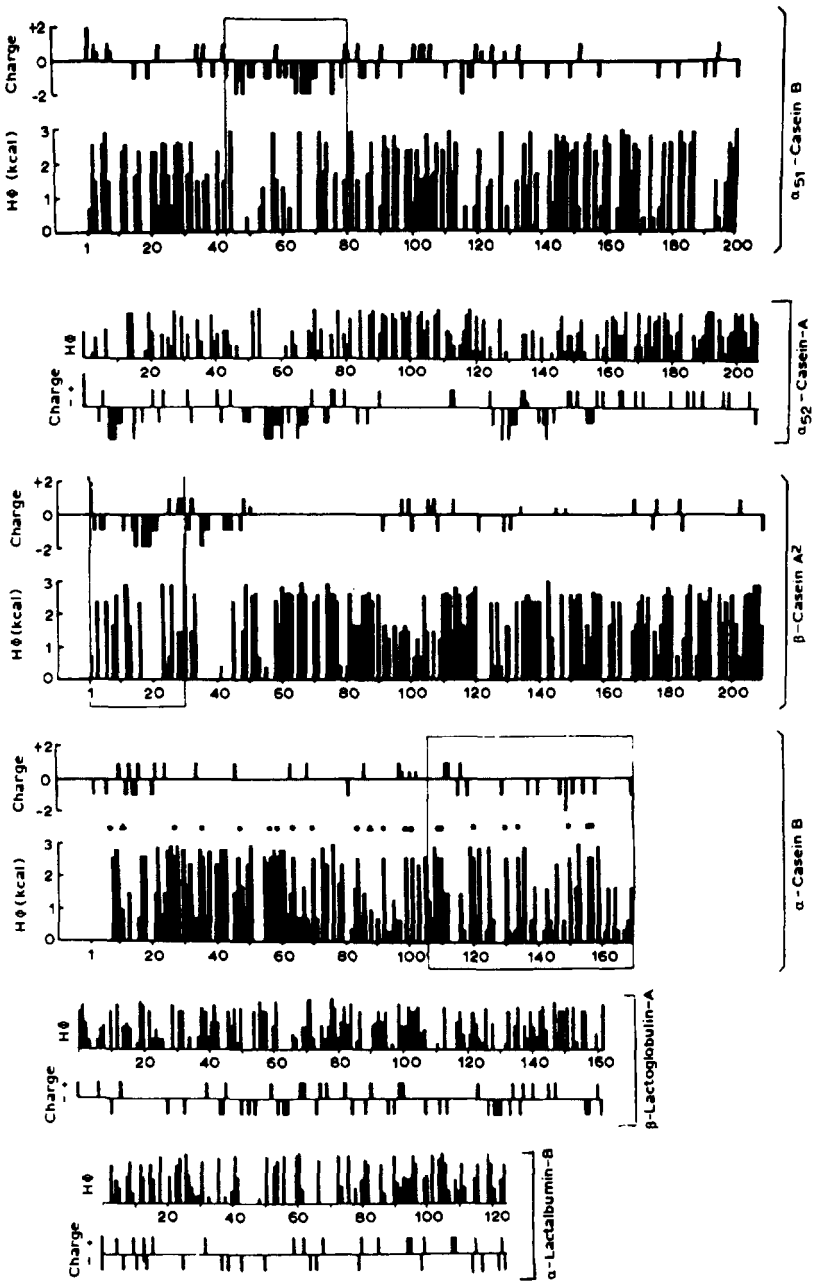


Figure 4.14 Schematic representation of the distribution of hydrophobic and charged residues in the principal milk proteins (from Swaisgood, 1992).

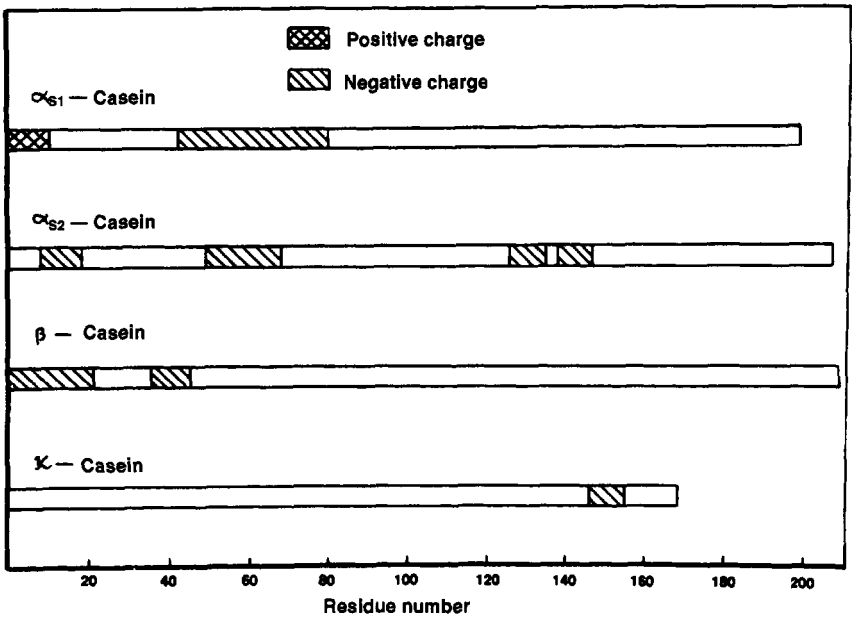


Figure 4.15 Ten residue sequences of bovine caseins with a charge density of 0.5 or greater at pH 6.6 (from Swaisgood, 1992).

species, found very little homology. Although the sequences of β-caseins from cow, sheep, mouse, rat, rabbit and human could be aligned readily, very little homology was evident between all six species (Figure 4.16): the only long homologous sequence was the signal peptide, the two N-terminal residues of the mature protein and the sequence SSEE (residues 18–21 of the mature protein, which is the principal phosphorylation site). The sequence of the signal peptides of α_{s1}- and κ-caseins also show a high degree of interspecies homology but several long insertions were required to obtain even a moderate degree of alignment of the sequences of the mature proteins.

Casein phosphorus. Milk contains about 900 mg phosphorus l^{-1}, which occurs in five types of phosphate-containing compounds, as will be discussed in Chapter 5:

- inorganic: soluble and colloidal phosphates;
- organic: phospholipids, casein and sugar phosphates, nucleotides (ATP, UTP, etc.).

Whole casein contains about 0.85% phosphorus; α_{s1}-, β- and κ-caseins contain 1.1, 0.6 and 0.16% P, respectively; on a molar basis, α_{s1}-, α_{s2}-, β- and

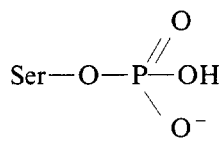
```
                10    ↓   20        30        40        50        60        70        80        90        100
COW   MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRIN-KKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPG--PIP-NSLPQNIPPLTQTP
SHE   MKVLILACLVALALAREQEELNVVGETVESLSSSEESITHIN-KKIEKFQSEEQQQTEDELQDKIHPFAQAQSLVYPFTG--PIP-NSLPQNILPLTQTP
MOU   MKVFILACLVALALARE-TTFTVSSET-DSI-SSEESVEHINEQKLQKVNLMGQLQAEDVLQAKVHSSIQSQPQAFPYAQAQTISCNPVPQNIQPIAQPP
RAT   MKVFILACLVALALAREKDAFTVSSET-GSI-SSEESVEHINE-KLQKVKLMGQVQSEDVLQNKFHSGIQSEPKAIPYAQ--TISCSPIPQNIQPIAQPP
RAB   MKVLILACLVALALAREKEQLSVPTEAVGSVSSSEE-ITHINKQKLETIKHVEQLLREEKLQDKILPFIQS---LFPFAE--RIPYPTLPQNILNLAQLD
MAN   MKVLILACLVALALARE---------TIESLSSSEESITEYK-QKVEKVKHEDQQQGEDEHQDKIYPSFQPQPLIYPFVE--PIPYGFLPQNILPLAQPA
      ***+**************           *+ **** ++ ++  *+++++   • + *+ +* *+ +  *       *+    +*+    **** +++*

                110       120       130       140       150       160       170       180       190       200
COW   VVV--PPFLQPEVMGVSKVKEAMAPKHKEMPFPKYP-VEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVM-FPPQSVLSLSQSKVLPVPQKA
SHE   VVV--PPFLQPEIMGVPKVKETMVPKHKEMPFPKYP-VEPFTESQSLTLTDVEKLHLPLPLVQSWMKQPPQPLPPTVM-FPPQSVLSLSQPKVLPVPQKA
MOU   VVPSLGPVISPELESFLKAKATILPKHKQMPLLNSETVLRLINSQIPSLASLANLHLPQSLVQ-LLAQVVQAFPQTHL-VSSQTQLSLPQSKVLYFLQQV
RAT   VVPTDGPIISPELESFLKAKATVLPKHKQMPFLNSETVLRLFNSQIPSLD-LANLHLPQSPAQ-LQAQIVQAFPQTPAVVSSQPQLSHPQSKSQYLVQQL
RAB   MLL---PLLQPEIMEDPKAKETIIPKHKLMPFLKSPKTVPFVDSQILNLREMKNQHLLLPQLLPFMHQVFQPFPQTPI-PYPQALLSLPQSKFMPIVPQV
MAN   VVL---PVPQPEIMEVPKAKDTVYTKGRVMPVLKSP-TIPFFDPQIPKVTDLENLQLPLPLLQPLMQQVPQPIPQTLA-LPPQPLWSVPQPKVLPIPQQV
      ++    *+ +**++   *+* ++ +*++ **++++ + ++  +*++ +  + +++*+++  +    *+ *++*+*     +*  +* +*+*  ++ ++

                210       220       230       240
COW   VPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV
SHE   VP--QRDMPIQAFLLYQEPVLGPVRGPFPILV
MOU   APFLPQDMSVQDLLQYLE-LLNPTVQ-FPATPQHSVSV
RAT   APLFQQGMPVQDLLQYLDLLLNPTLQ-FLATQQLHSTSV
RAB   VPYPQRDMPIQALQLFQELLF-PTHQGYPVVQPIAPVNV
MAN   VPYPQRAVPVQALLLNQELLLNPTHQIYPVTQPLAPVHNPISV
      +*  ++ +++*++++ ++ ++ *+ + ++
```

Figure 4.16 Homology of β-casein from a selection of species; * indicates residues identical at the same position for all species; + indicates similar residues, - an inserted space. ↓ indicates the N terminus of the mature protein (from Holt and Sawyer, 1993).

κ-caseins contain 8(9), 10–13, 5(4) and 1(2,3) moles P per mole. The phosphorus is very important:

- nutritionally, *per se*, and because it can bind large amounts of Ca^{2+}, Zn^{2+} and probably other polyvalent metals;
- it increases the solubility of caseins;
- it probably contributes to the high heat stability of casein; and
- it is significant in the coagulation of rennet-altered casein during the secondary phase of rennet action (Chapter 10).

The phosphorus is covalently bound to the protein and is removed only by very severe heat treatments, high pH or some phosphatases. The phosphate is esterified mainly to serine (possibly a little to threonine) as a monoester:



Phosphorylation occurs in the Golgi membranes of the mammary cell, catalysed by two serine-specific casein kinases. Only certain serines are phosphorylated; the principal recognition site is Ser/Thr.X.Y, where Y is a glutamyl and occasionally an aspartyl residue; once a serine residue has been phosphorylated, SerP can serve as a recognition site. X may be any amino acid but a basic or a very bulky residue may reduce the extent of phosphorylation. However, not all serine residues in a suitable sequence are phosphorylated, suggesting that there may be a further topological requirement, e.g. a surface location in the protein conformation.

Casein carbohydrate. α_{s1}-, α_{s2}- and β-caseins contain no carbohydrate but κ-casein contains about 5%, consisting of *N*-acetylneuraminic acid (sialic acid), galactose and *N*-acetylgalactosamine. The carbohydrate exists as tri- or tetrasaccharides, located toward the C-terminal of the molecule, attached through an *O*-threonyl linkage, mainly to Thr_{131} of κ-casein (Figure 4.17). The number of oligosaccharides per κ-casein molecule varies from 0 to 4. The variability of glycosylation results in at least nine, and probably 10, molecular forms of κ-casein (Table 4.5). The κ-casein in colostrum is even more highly glycosylated; more sugars are present and the structures are more complex and uncertain.

The carbohydrate is attached to the (glyco)macropeptides which are produced from κ-caseins on hydrolysis by rennets. The carbohydrate bestows on κ-casein quite high solubility and hydrophilicity. It is also

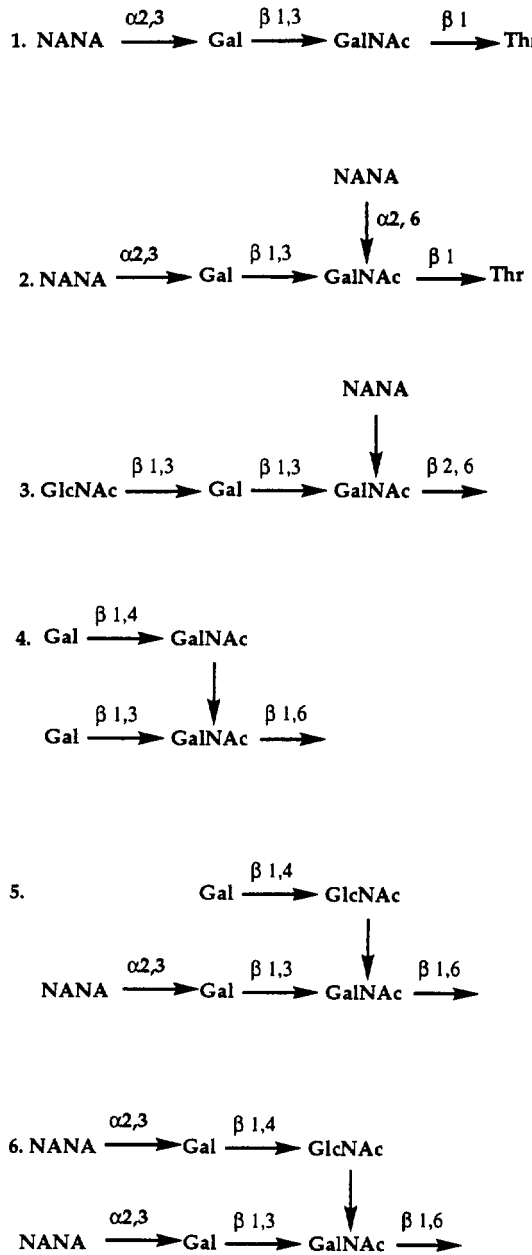


Figure 4.17 Oligosaccharides attached to casein isolated from bovine milk (1–2) or colostrum (1–6) (from Eigel *et al.*, 1984).

Table 4.5 Variability of bovine κ-casein with respect to sugars and phosphate

Fraction	Galactose	*N*-acetyl-galactosamine	*N*-acetyl-neuraminic acid	Phosphate
B-1	0	0	0	1
B-2	1	1	1	1
B-3	1	1	2	1
B-4	0	0	0	2
B-5	2	2	3	1
B-6	0	0	0(4)	3(1)
B-7	3	3	6	1
B-8	4	4	8	1
B-9	5	5	10	1

responsible for the solubility of the glycomacropeptides in 12% TCA (see Chapter 10). Although the sugars increase the hydrophilicity of casein, they are not responsible for the micelle-stabilizing properties of κ-casein, the carbohydrate-free form being as effective in this respect as the glycosylated forms.

4.5.2 Secondary and tertiary structures

Physical methods, such as optical rotary dispersion and circular dichroism, indicate that the caseins have relatively little secondary or tertiary structure, probably due to the presence of high levels of proline residues, especially in β-casein, which disrupt α-helices and β-sheets. However, theoretical calculations (Kumosinski, Brown and Farrell, 1993a, b; Kumosinski and Farrell, 1994) indicate that while α_{s1}-casein has little α-helix, it probably contains some β-sheets and β-turns. The C-terminal half of α_{s2}-casein probably has a globular conformation (i.e. a compact structure containing some α-helix and β-sheet) while the N-terminal region probably forms a randomly structured hydrophilic tail. Theoretical calculations suggest that β-casein could have 10% of its residues in α-helices, 17% in β-sheets and 70% in unordered structures. κ-Casein appears to be the most highly structured of the caseins, perhaps with 23% of its residues in α-helices, 31% in β-sheets and 24% in β-turns. Energy-minimized models of α_{s1}-, β- and κ-caseins are shown in Figure 4.18a–c. Holt and Sawyer (1993) coined the term 'rheomorphic' to describe the caseins as proteins with an open, flexible, mobile conformation in order to avoid using the 'demeaning' term, 'random coil'.

The lack of secondary and tertiary structures is probably significant for the following reasons:

1. The caseins are readily susceptible to proteolysis, in contrast to globular proteins, e.g. whey proteins, which are usually very resistant in their

native state. This has obvious advantages for the digestibility of the caseins, the natural function of which is presumably nutritional and hence easy digestibility in the 'native' state is important. The caseins are also readily hydrolysed in cheese, which is important for the development of cheese flavour and texture (Chapter 10). However, casein hydrolysates may be bitter due to a high content of hydrophobic amino acids (small hydrophobic peptides tend to be bitter). The caseins are readily hydrolysed by proteinases secreted by spoilage micro-organisms.

2. The caseins adsorb readily at air–water and oil–water interfaces due to their open structure, relatively high content of apolar amino acid residues and the uneven distribution of amino acids. This gives the caseins very good emulsifying and foaming properties, which are widely exploited in the food industry.
3. The lack of higher structures probably explains the high stability of the caseins to denaturing agents, including heat.

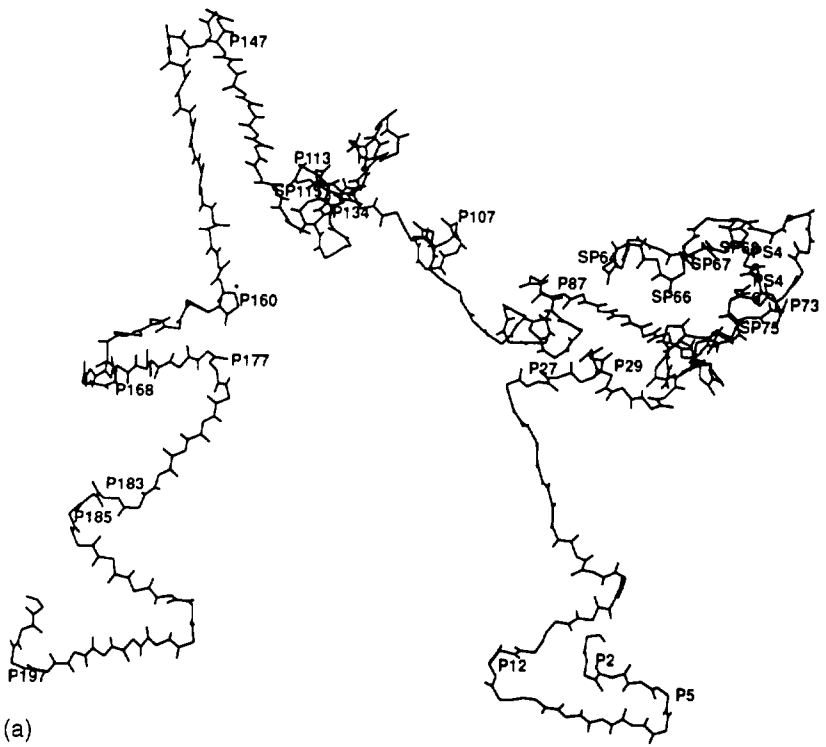


Figure 4.18 Energy-minimized models of the tertiary structures of bovine α_{s1}- (a), β- (b) and κ- (c) caseins (from Kumosinski, Brown and Farrell, 1993a, b; Kumosinski and Farrell, 1994)

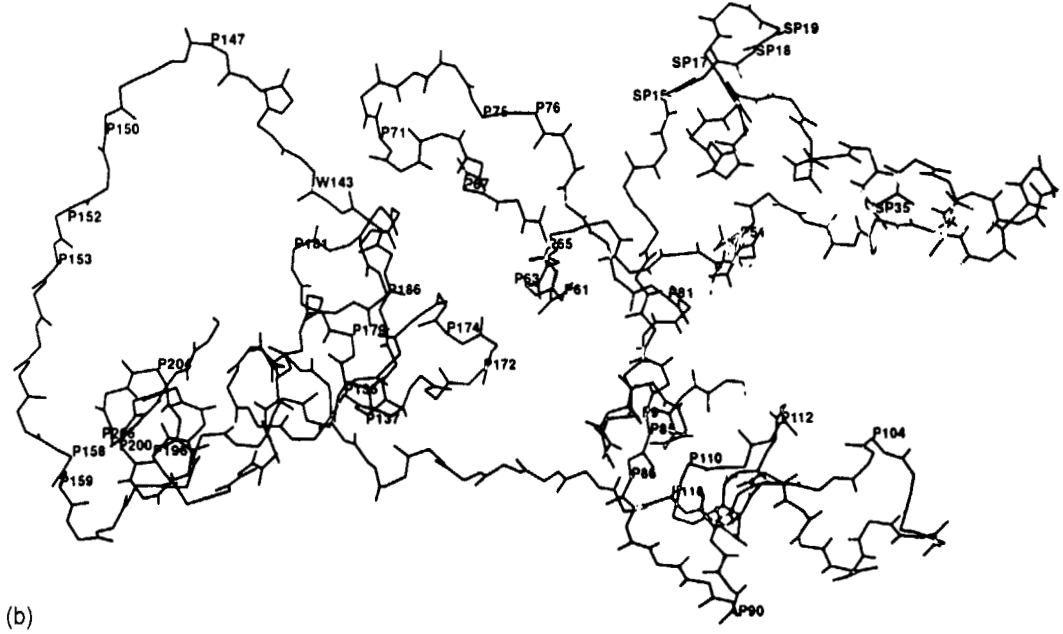


Figure 4.18 (*Continued*).

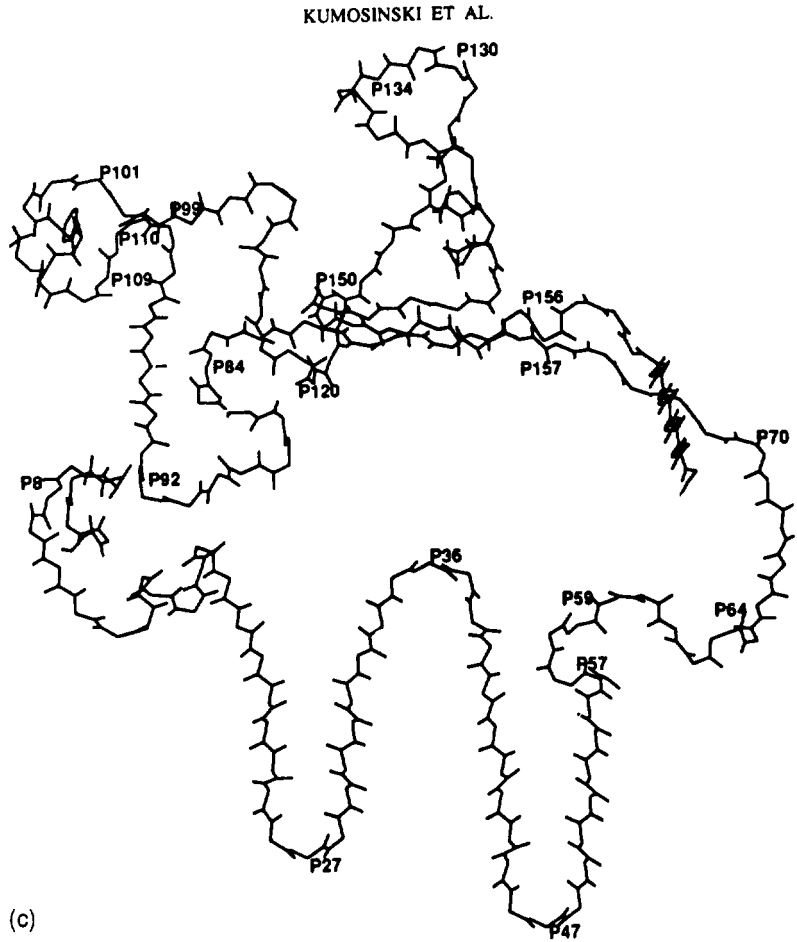


Figure 4.18 (*Continued*).

4.5.3 Molecular size

All the caseins are relatively small molecules, ranging in molecular weight from about 20 to 25 kDa (Table 4.2).

4.5.4 Hydrophobicity

The caseins are often considered to be rather hydrophobic molecules. However, consideration of the amino acid composition indicates that they are not particularly so; in fact, some are more hydrophilic than the whey protein, β-lactoglobulin (Table 4.2). However, the caseins do have high

surface hydrophobicity, in contrast to the globular whey proteins. In globular proteins, the hydrophobic residues are buried, as far as possible, within the molecule, with most of the hydrophilic residues exposed on the surface. Owing to the relative lack of secondary and tertiary structures in the caseins, such an arrangement is not possible, and hence the hydrophobic residues are rather exposed.

Thus, the caseins are relatively small, relatively hydrophobic, amphipathic, randomly or flexibly structured molecules, with relatively low levels of secondary and tertiary structures.

4.5.5 Influence of Ca^{2+} on caseins

At all temperatures, α_{s1}-CN B and C are insoluble in calcium-containing solutions and form a coarse precipitate at Ca^{2+} concentrations greater than about 4 mM. α_{s1}-CN A, from which the very hydrophobic sequence, residues 13–26, is deleted, is soluble at $[Ca^{2+}]$ up to 0.4 M in the temperature range 1–33°C. Above 33°C, it precipitates but redissolves on cooling to 28°C. The presence of α_{s1}-CN A modifies the behaviour of α_{s1}-CN B so that an equimolar mixture of the two is soluble in 0.4 M Ca^{2+} at 1°C; α_{s1}-CN B precipitates from the mixture at 18°C and both α_{s1}-CN A and B precipitate at 33°C. α_{s1}-CN A does not form normal micelles with κ-casein. Since α_{s1}-CN A occurs at very low frequency, these abnormalities are of little consequence in dairy processing but may become important if the frequency of α_{s1}-CN A increases as a result of breeding practices.

The α_{s2}-caseins are also insoluble in Ca^{2+} (above about 4 mM) at all temperatures, but their behaviour has not been studied in detail.

β-Casein is soluble at high concentrations of Ca^{2+} (0.4 M) at temperatures below 18°C, but above 18°C β-casein is very insoluble, even in the presence of low concentrations of Ca^{2+} (4 mM). Ca-precipitated β-casein redissolves readily on cooling to below 18°C. About 20°C is also the critical temperature for the temperature-dependent polymerization of β-casein and the two phenomena may be related.

κ-Casein is soluble in Ca^{2+} at all concentrations up to those at which general salting-out occurs. Solubility is independent of temperature and pH (outside the pH range at which isoelectric precipitation occurs). Not only is κ-casein soluble in the presence of Ca^{2+} but it is capable of stabilizing α_{s1}-, α_{s2}- and β-caseins against precipitation by Ca^{2+} (section 4.5.8).

4.5.6 Action of rennets on casein

This subject is dealt with in Chapter 10. Suffice it to say here that κ-casein is the only major casein hydrolysed by rennets during the primary phase of milk coagulation, which is the first step in the manufacture of most cheese varieties.

4.5.7 Casein association

All the major caseins associate with themselves and with each other. In unreduced form, κ-casein is present largely as disulphide-linked polymers. κ-Casein also forms hydrogen and hydrophobic bonds with itself and other caseins but these secondary associations have not been studied in detail.

At 4°C, β-casein exists in solution as monomers of molecular mass 25 kDa. As the temperature is increased, the monomers polymerize to form long thread-like chains of about 20 units at 8.5°C and to still larger aggregates at higher temperatures. The degree of association is dependent on protein concentration. The ability to form thread-like polymers may be important in micelle structure. β-Casein also undergoes a temperature-dependent conformational change in which the content of poly-L-proline helix decreases with increasing temperature. The transition temperature is about 20°C, i.e. very close to the temperature at which β-casein becomes insoluble in Ca^{2+}.

α_{s1}-Casein polymerizes to form tetramers of molecular mass 113 kDa; the degree of polymerization increases with increasing protein concentration and increasing temperature.

The major caseins interact with each other and, in the presence of Ca^{2+}, these associations lead to the formation of casein micelles.

4.5.8 Casein micelle structure

Composition and general features. About 95% of the casein exists in milk as large colloidal particles, known as micelles. On a dry matter basis, casein micelles contain *c.* 94% protein and 6% low molecular weight species referred to as colloidal calcium phosphate, consisting of calcium, magnesium, phosphate and citrate. The micelles are highly hydrated, binding about 2.0 g $H_2O\,g^{-1}$ protein. Some of the principal properties of casein micelles are summarized in Table 4.6.

Electron microscopy shows that casein micelles are generally spherical in shape, with diameters ranging from 50 to 500 nm (average *c.* 120 nm) and masses ranging from 10^6 to 10^9 Da (average about 10^8 Da). There are very many small micelles but these represent only a small proportion of the volume or mass (Figure 4.19). There are 10^{14}–10^{16} micelles ml^{-1} milk; they are roughly two micelle diameters (240 nm) apart, i.e. they are quite tightly packed. The surface (interfacial) area of the micelles is very large, $5 \times 10^4\,cm^2\,ml^{-1}$; hence, the surface properties of the micelles are critical to their behaviour.

Since the micelles are of colloidal dimensions, they are capable of scattering light and the white colour of milk is due largely to light scattering by the casein micelles; the white colour is lost if the micelles are disrupted, e.g. by removing colloidal calcium phosphate (by citrate, ethylene

Table 4.6 Average characteristics of casein micelles (modified from McMahon and Brown, 1984)

Characteristic	Value
Diameter	120 nm (range: 50–500 nm)
Surface area	8×10^{-10} cm^2
Volume	2.1×10^{-15} cm^3
Density (hydrated)	1.0632 g cm^{-3}
Mass	2.2×10^{-15} g
Water content	63%
Hydration	3.7 g H_2O g^{-1} protein
Voluminosity	4.4 cm^3 g^{-1}
Molecular weight (hydrated)	1.3×10^9 Da
Molecular weight (dehydrated)	5×10^8 Da
Number of peptide chains	10^4
Number of paticles per ml milk	10^{14}–10^{16}
Surface of micelles per ml milk	5×10^4 cm^3
Mean free distance	240 nm

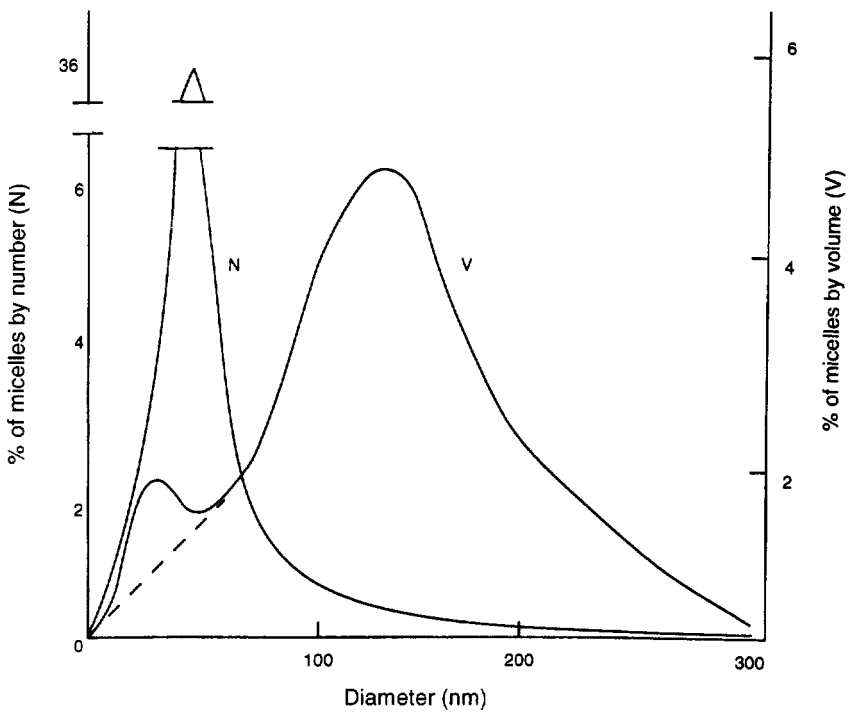


Figure 4.19 Number and volume frequency distribution of casein micelles in bovine milk (from Walstra and Jenness, 1984).

diaminetetraacetic acid (EDTA) or oxalate), by increasing pH (to greater than 9), or by the addition of urea or SDS.

Stability

1. The micelles are stable to the principal processes to which milk is normally subjected (except those in which it is intended to destabilize the micelles, e.g. rennet- and acid-induced coagulation). They are very stable at high temperatures, coagulating only after heating at 140°C for 15–20 min at the normal pH of milk. Such coagulation is not due to denaturation in the narrow sense of the word but to major changes which occur in milk exposed to such high heat treatments, including a decrease in pH due to the pyrolysis of lactose to various acids, dephosphorylation of the casein, cleavage of κ-casein, denaturation of the whey proteins and their attachment to the casein micelles, precipitation of soluble calcium phosphate on the micelles and a decrease in hydration (Chapter 9).
2. They are stable to compaction, e.g. they can be sedimented by ultracentrifugation and redispersed readily by mild agitation.
3. They are stable to commercial homogenization but are changed slightly at very high pressures (500 MPa).
4. They are stable to high $[Ca^{2+}]$, up to at least 200 mM at temperatures up to 50°C.
5. They aggregate and precipitate from solution when the pH is adjusted to the isoelectric point of caseins (*c.* pH4.6). Precipitation at this pH, which is temperature-dependent (i.e. does not occur at temperatures below 5-8°C and occurs over a wide pH range, perhaps 3.0–5.5, at higher temperatures, e.g. 70°C), occurs owing to the loss of net positive or negative charge as the pH approaches 4.6.
6. As the pH of milk is reduced, the colloidal calcium phosphate (CCP) dissolves and is completely soluble at pH 4.9 (Chapter 5). pH adjustment, followed by dialysis against bulk milk, is a convenient and widely used technique for varying the CCP content of milk. As the concentration of CCP is reduced, the properties of the micelles are altered but they retain some of their structure even after removing 70% of the CCP. Removal of more than 70% of the CCP results in disintegration of the micelles into smaller particles (aggregates).
7. Many proteinases catalyse the hydrolysis of a specific bond in κ-casein, as a consequence of which the micelles aggregate or gel in the presence of Ca^{2+} or other divalent ions. This is the key step in the manufacture of most cheese varieties (Chapter 10).
8. The micelles are destabilized by *c.* 40% ethanol at pH 6.7 and by lower concentrations if the pH is reduced.
9. They are destabilized by freezing (cryodestabilization) due to a decrease in pH and an increase in the $[Ca^{2+}]$ in the unfrozen phase of milk (Chapters 2 and 5).

Principal micelle characteristics. The structure of the casein micelles has attracted the attention of scientists for a considerable time. Knowledge of micelle structure is important because the stability and behaviour of the micelles are central to many dairy processing operations, e.g. cheese manufacture, stability of sterilized, sweetened-condensed and reconstituted milks and frozen products. Without knowledge of the structure and properties of the casein micelle, attempts to solve many technological problems faced by the dairy industry will be empirical and not generally applicable. From the academic viewpoint, the casein micelle presents an interesting and complex problem in protein quaternary structure.

Since the pioneering work of Waugh in 1958, a considerable amount of research effort has been devoted to elucidating the structure of the casein micelle, and several models have been proposed. This work has been reviewed in the references cited in the next section. The principal properties of the casein micelles are listed below and the models which best meet these requirements discussed briefly in the next section.

1. κ-Casein, which represents about 15% of total casein, is a critical feature of micelle structure and stability and must be located so as to be able to stabilize the calcium-sensitive α_{s1}-, α_{s2}- and β-caseins, which represent about 85% of total casein.
2. The κ-casein content of casein micelles is inversely proportional to their size, while the content of colloidal calcium phosphate is directly related to size.
3. Ultracentrifugally sedimented micelles have a hydration of 1.6–2.7 g $H_2O\,g^{-1}$ protein but voluminosities of 3–7 ml g^{-1} have been found by viscosity measurements and calculation of specific hydrodynamic volumes. These values suggest that the micelle has a porous structure in which the protein occupies about 25% of the total volume.
4. Chymosin and similar proteinases, which are relatively large molecules (*c.* 36 kDa), very rapidly and specifically hydrolyse most of the micellar κ-casein.
5. When heated in the presence of whey proteins, as in normal milk, κ-casein and β-lactoglobulin interact to form a disulphide-linked complex which modifies many properties of the micelles, including rennet coagulability and heat stability.
6. Removal of colloidal calcium phosphate (CCP) results in disintegration of the micelles into particles of mass $\sim 3 \times 10^6$ Da. The properties of the CCP-free system are very different from those of the normal milk system, e.g. it is sensitive to and precipitated by relatively low concentrations of Ca^{2+}, it is more stable to high temperatures, e.g. 140°C, and is not coagulable by rennets. Many of these properties can be restored, at least partially, by increased concentrations of calcium.
7. The micelles can be dispersed (dissociated) by urea or SDS, suggesting the involvement of hydrogen and hydrophobic bonds in micelle integrity.

8. The micelles can be destabilized by alcohols, acetone and similar solvents, suggesting an important role for electrostatic interactions in micelle structure.
9. As the temperature is lowered, caseins, especially β-casein, dissociate from the micelles; depending on the method of measurement, 10–50% of β-casein is non-micellar at 4°C.
10. Electron microscopy shows that the interior of the micelles are not uniformly electron dense.
11. The micelles have a surface (zeta) potential of about −20 mV at pH 6.7.

Micelle structure. Various models of casein micelle structure have been proposed and refined over the past 40 years. Progress has been reviewed regularly, including Schmidt (1982), McMahon and Brown (1984), Farrell (1988), Holt (1992, 1994), Rollema (1992) and Visser (1992).

The proposed models fall into three general categories, although there is some overlap:

1. core-coat;
2. internal structure;
3. subunit (submicelles); in many of the models in this category, it is proposed that the submicelles have a core-coat structure.

For many years there has been strong support for the view that the micelles are composed of submicelles of mass $\sim 10^6$ Da and diameter 10-15 nm. This model was introduced in 1967 by Morr who proposed that the submicelles are linked together by CCP, giving the micelle an open porous structure. On removal of CCP, e.g. by acidification/dialysis, EDTA, citrate or oxalate, the micelles disintegrate. Disintegration may also be achieved by treatment with urea, SDS or at pH greater than 9; presumably,

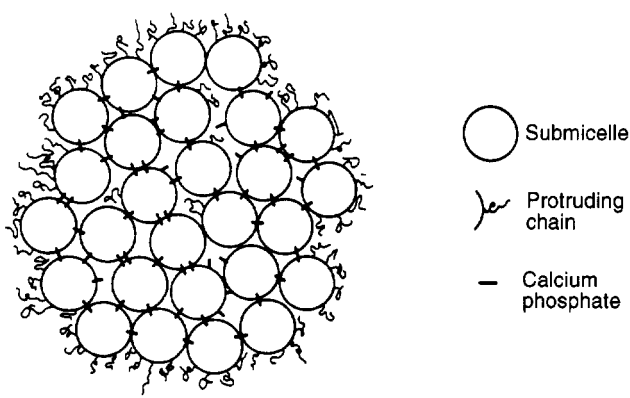


Figure 4.20 Submicelle model of the casein micelle (from Walstra and Jenness, 1984).

these treatments do not solubilize CCP, suggesting that other forces, e.g. hydrophobic and hydrogen bonds, contribute to micelle structure.

The submicellar model has undergone several refinements (see Schmidt, 1982; Walstra and Jenness, 1984; Ono and Obata, 1989). The current view is that the κ-casein content of the submicelles varies and that the κ-casein-deficient submicelles are located in the interior of the micelles with the κ-casein-rich submicelles concentrated at the surface, giving the micelles a κ-casein-rich layer but with some α_{s1}-, α_{s2}- and β-caseins also exposed on the surface. It is proposed that the hydrophilic C-terminal region of κ-casein protrudes from the surface, forming a layer 5–10 nm thick and giving the micelles a hairy appearance (Figure 4.20). This hairy layer is responsible for micelle stability through a major contribution to zeta potential (−20 mV) and steric stabilization. If the hairy layer is removed, e.g. specific hydrolysis of κ-casein, or collapsed, e.g. by ethanol, the colloidal stability of the micelles is destroyed and they coagulate or precipitate.

Although the submicellar model of the casein micelle readily explains many of the principal features and physicochemical reactions undergone by the micelles and has been widely supported, it has never enjoyed unanimous support and two alternative models have been proposed recently. Visser (1992) proposed that the micelles are spherical conglomerates of individual casein molecules randomly aggregated and held together partly by salt bridges in the form of amorphous calcium phosphate and partly by other forces, e.g. hydrophobic bonds, with a surface layer of κ-casein. Holt (1992, 1994) depicted the casein micelle as a tangled web of flexible casein

Figure 4.21 Model of the casein micelle (modified from Holt, 1994).

molecules forming a gel-like structure in which microgranules of colloidal calcium phosphate are an integral feature and from the surface of which the C-terminal region of κ-casein extends, forming a hairy layer (Figure 4.21). These models retain two of the central features of the submicellar model, i.e. the cementing role of CCP and the predominantly surface location of κ-casein.

Holt (1992, 1994) also proposed that, in addition to supplying amino acids, caseins should be considered to have a biological function, i.e. to enable a high concentration of calcium to be carried in stable form in milk; without the stabilizing effect of casein, calcium phosphate would precipitate in the mammary cells, resulting in ectopic mineralization, which might lead to the death of the mammary gland or of the whole animal. A similar situation occurs with kidney stones, gallstones and calcified synovial and salivary fluid.

Since the micelles are closely packed, intermicellar collisions are frequent; however, the micelles do not normally remain together after collisions. The micelles are stabilized by two principal factors: (1) a surface (zeta) potential of *c.* −20 mV at pH 6.7, which, alone, is probably too small for colloidal stability, and (2) steric stabilization due to the protruding κ-casein hairs.

4.6 Whey proteins

About 20% of the total protein of bovine milk belongs to a group of proteins generally referred to as **whey** or **serum proteins** or **non-casein nitrogen**. Acid and rennet wheys also contain casein-derived peptides; both contain proteose–peptones, produced by plasmin, mainly from β-casein, and the latter also contains (glyco)macropeptides produced by rennets from κ-casein. These peptides are excluded from the present discussion.

4.6.1 Preparation

The whey proteins, as a group, are readily prepared from milk by any of the methods described in section 4.3, i.e.

1. the proteins remaining soluble at pH 4.6;
2. soluble in saturated NaCl;
3. soluble after rennet coagulation of the caseins;
4. by gel permeation chromatography;
5. by ultracentrifugation, with or without added Ca^{2+}.

The whey prepared by any of the above methods, except 4, contains lactose and soluble salts. Total whey proteins may be prepared from the

wheys by dialysis and drying the retentate. The products prepared by these various methods differ: acid whey contains some γ-casein and proteose–peptones; immunoglobulins are co-precipitated with the caseins by saturated NaCl; rennet whey contains the κ-CN macropeptides produced by rennet action, plus, perhaps, very small amounts of other caseins; small casein micelles remain in the ultracentrifugal supernatant, especially if Ca is not added. The salt composition of the serum differs very considerably in wheys produced by various methods.

On a commercial scale, whey protein-rich products are prepared by:

1. Ultrafiltration/diafiltration of acid or rennet whey to remove varying amounts of lactose, and spray-drying to produce **whey protein concentrates** (30–80% protein).
2. Ion-exchange chromatography: proteins are adsorbed on an ion exchanger, washed free of lactose and salts and then eluted by pH adjustment. The eluate is freed of salts by ultrafiltration and spray-dried to yield **whey protein isolate**, containing about 95% protein.
3. Demineralization by electrodialysis and/or ion exchange, thermal evaporation of water and crystallization of lactose.
4. Thermal denaturation, recovery of precipitated protein by filtration/centrifugation and spray-drying, to yield **lactalbumin** which has very low solubility and limited functionality.

Several other methods are available for the removal of whey proteins from whey but are not used commercially. Several methods for the purification of the major and minor whey proteins on a commercial scale have also been developed and will be discussed briefly in sections 4.15.6 and 4.16.

4.6.2 Heterogeneity of whey proteins

It was recognized 60 years ago that whey prepared by any of the above methods contained two well-defined groups of proteins which could be fractionated by saturated $MgSO_4$ or half saturated $(NH_4)_2SO_4$; the precipitate (roughly 20% of total N) was referred to as **lactoglobulin** and the soluble protein as **lactalbumin**.

The lactoglobulin fraction consists mainly of immunoglobulins (Ig), especially IgG_1, with lesser amounts of IgG_2, IgA and IgM (section 4.10). The lactalbumin fraction of bovine milk contains three main proteins, β-lactoglobulin (β-lg), α-lactalbumin (α-la) and blood serum albumin (BSA), which represent approximately 50, 20 and 10% of total whey protein, respectively, and trace amounts of several other proteins, notably lactotransferrin, serotransferrin and several enzymes. The whey proteins of sheep, goat

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