# Chemistry of the Caseins

4

T. Huppertz

## 4.1 Introduction

Milk protein constitutes an important part of the human diet. For the neonate, milk or infant formula is the only type of food consumed; however, whereas milk does not constitute a major part of the diet after the neonatal stage of most other mammals, the human diet in many parts of the world continues to include high levels of dairy products. The popularity of milk proteins in the human diet is undoubtedly a result of the combination of their excellent nutritional value and high level of functionality. The relative ease of isolation of proteins from milk has led not only to the creation of a wide variety of functional and nutritional milk protein ingredients, but also to milk proteins being the best characterized of all food proteins. The primary structures of all milk proteins have been determined and for all the major whey proteins, three-dimensional structures have been elucidated. Because of the fact that attempts to crystallize caseins have thus far remained unsuccessful, the full secondary and tertiary structure of the caseins remains to be elucidated. Although caseins have higher flexibility than typical globular proteins, e.g. whey proteins, the previous classification of caseins as random coil or natively denatured proteins appears inaccurate as a definite degree secondary and tertiary structure has been identified for the caseins.

The aim of this chapter is to provide the current state-of-the-art with respect to the chemistry of caseins. As with previous reviews on this topic by Swaisgood (1982, 1992, 2003), casein composition and nomenclature, chemical composition and primary structure of the caseins, post-translational modification, secondary structures as well as physicochemical properties of caseins, such as self-association and the interactions with calcium, will be covered. For studies on the isolation of casein, the reader is referred to Swaisgood (2003). Higher order structures of caseins and the structure and stability of the association colloids in which caseins naturally exist, i.e. casein micelles, are outside the scope of this chapter and are covered in Chaps. 5 and 6, respectively. The focus in this chapter will be on the caseins in bovine milk; interspecies variability in casein composition is covered in Chap. 13.

## 4.2 Casein Composition and Nomenclature

The American Dairy Science Association Committee on the Nomenclature, Classification and Methodology of Milk Proteins originally defined the bovine caseins as those phosphoproteins that precipitate from raw milk by

T. Huppertz (✉)
NIZO food research, P.O. Box 20,
6710 BA, Ede, The Netherlands
e-mail: thom.huppertz@nizo.com

P.L.H. McSweeney and P.F. Fox (eds.), *Advanced Dairy Chemistry: Volume 1A: Proteins: Basic Aspects, 4th Edition*, DOI 10.1007/978-1-4614-4714-6_1, 

**Table 4.1** Current and former nomenclature of caseins and major peptides derived therefrom. Reference proteins are printed in italics

| Current | Former |
|---|---|
| $\alpha_{s1}$-CN | |
| $\alpha_{s1}$-CN A-8P | $\alpha_{s1}$-CN A |
| *$\alpha_{s1}$-CN B-8P* | *$\alpha_{s1}$-CN B* |
| $\alpha_{s1}$-CN B-9P | $\alpha_{s0}$-CN |
| $\alpha_{s1}$-CN C-8P | $\alpha_{s1}$-CN C |
| $\alpha_{s1}$-CN D-9P | $\alpha_{s1}$-CN D |
| $\alpha_{s1}$-CN E-8P | $\alpha_{s1}$-CN E |
| $\alpha_{s2}$-CN | |
| $\alpha_{s2}$-CN A-10P | $\alpha_{s6}$-CN A |
| *$\alpha_{s2}$-CN A-11P* | *$\alpha_{s4}$-CN A* |
| $\alpha_{s2}$-CN A-12P | $\alpha_{s3}$-CN A |
| $\alpha_{s2}$-CN A-13P | $\alpha_{s2}$-CN A |
| β-CN | |
| β-CN $A^1$-5P | β-CN $A^1$ |
| *β-CN $A^2$-5P* | *β-CN $A^2$* |
| β-CN $A^3$-5P | β-CN $A^3$ |
| β-CN C-4P | β-CN C |
| β-CN D-4P | β-CN D |
| β-CN E-5P | β-CN E |
| κ-CN | |
| *κ-CN A-1P* | *κ-CN A* |
| κ-CN B-1P | κ-CN B |

acidification to pH 4.6 at 20°C (Jenness *et al.*, 1956). Subsequent reports by the committee recommended that the caseins could be differentiated according to their relative electrophoretic mobility in alkaline polyacrylamide or starch gels containing urea, with or without β-mercaptoethanol (Whitney *et al.*, 1976) or, more recently, according to their primary amino acid sequences (Eigel *et al.*, 1984; Farrell Jr *et al.*, 2004). Accordingly, four gene products can be identified: $\alpha_{s1}$-casein ($\alpha_{s1}$-CN), $\alpha_{s2}$-casein ($\alpha_{s2}$-CN), β-casein (β-CN) and κ-casein (κ-CN). Typical concentrations of $\alpha_{s1}$-CN, $\alpha_{s2}$-CN, β-CN and κ-CN in bovine milk are 12–15, 3–4, 9–11 and 2–4 $g\,L^{-1}$, respectively, and the caseins account for ~75–80% of total milk protein. For all caseins, various genetic variants have been identified. In addition, all caseins show considerable micro-heterogeneity, arising from post-translational modification; all caseins are phosphorylated, whereas glycosylation has been shown only for κ-CN. As discussed in further detail later, $\alpha_{s1}$-CN, $\alpha_{s2}$-CN and β-CN are classified as the calcium-sensitive caseins, whereas κ-CN is calcium insensitive. In addition to the aforementioned gene products, γ-caseins and λ-caseins have been identified, which arise from the hydrolysis of β-CN and $\alpha_{s1}$-CN, respectively, by the indigenous milk proteinase, plasmin. Enzymatic hydrolysis of milk proteins by plasmin is outside the scope of this chapter and is dealt with in detail in Chap. 12.

Current nomenclature of caseins and some casein fractions, as well as former classifications by which they were known, is shown in Table 4.1. In such nomenclature, a Latin letter indicates the generic variant of the proteins, whereas differences in the degree of post-translational modification are indicated by an Arabic number, followed by the letter P to indicate that the post-translational variation arises from phosphorylation. For example, $\alpha_{s1}$-CN B-8P refers to genetic variant B of $\alpha_{s1}$-CN containing eight phosphorylated amino acid residues. For each of the caseins, one of the variants outlined in Table 4.1 is considered to be the reference protein; these reference proteins are $\alpha_{s1}$-CN B-8P, $\alpha_{s2}$-CN A-11P, β-CN $A^2$-5P and κ-CN A-1P.

## 4.3 $\alpha_{s1}$-Casein

### 4.3.1 Primary Structure of $\alpha_{s1}$-Casein

The $\alpha_{s1}$-CN family represents ~40% of total casein in bovine milk. The reference protein for the $\alpha_{s1}$-CN family is $\alpha_{s1}$-CN B-8P, with ExPASy entry name and file number of CAS1_Bovin and P02662, respectively. The amino acid sequence of $\alpha_{s1}$-CN B-8P, which predominates in the milk of *Bos taurus* and was first established by Mercier *et al.* (1971) and Grosclaude *et al.* (1973), is shown in Fig. 4.1. The protein consists of 199 amino acid residues, with 8 of the 16 Ser residues in the protein being phosphorylated, i.e. $Ser_{45}$, $Ser_{47}$, $Ser_{64}$, $Ser_{66}$, $Ser_{67}$, $Ser_{68}$, $Ser_{75}$ and $Ser_{115}$ (Mercier *et al.*, 1971). In $\alpha_{s1}$-CN B-9P, previously denoted $\alpha_{s0}$-CN, $Ser_{41}$ is also phosphorylated

| 1 | | | | | | | 10 | | | | | | | | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Arg- | Pro- | Lys- | His- | Pro- | Ile- | Lys- | His- | Gln- | Gly- | Leu- | Pro- | Gln- | Glu- | Val- | Leu- | Asn- | Glu- | Ans- | Leu- |
| 21 | | | | | | | 30 | | | | | | | | 40 |
| Leu- | Arg- | Phe- | Phe- | Val- | Ala- | Pro- | Phe- | Pro- | Glu- | Val- | Phe- | Gly- | Lys- | Glu- | Lys- | Val- | Asn- | Glu- | Leu- |
| 41 | | | | | | | 50 | | | | | | | | 60 |
| Ser- | Lys- | Asp- | Ile- | Gly- | SerP- | Glu- | SerP- | Thr- | Glu- | Asp- | Gln- | Ala- | Met- | Glu- | Asp- | Ile- | Lys- | Gln- | Met- |
| 61 | | | | | | | 70 | | | | | | | | 80 |
| Glu- | Ala- | Glu- | SerP- | Ile- | SerP- | SerP- | SerP- | Glu- | Glu- | Ile- | Val- | Pro- | Asn- | SerP- | Val- | Glu- | Gln- | Lys- | His- |
| 81 | | | | | | | 90 | | | | | | | | 100 |
| Ile- | Gln- | Lys- | Glu- | Asp- | Val- | Pro- | Ser- | Glu- | Arg- | Tyr- | Leu- | Gly- | Tyr- | Leu- | Glu- | Gln- | Leu- | Leu- | Arg- |
| 101 | | | | | | | 110 | | | | | | | | 120 |
| Leu- | Lys- | Lys- | Tyr- | Lys- | Val- | Pro- | Gln- | Leu- | Glu- | Ile- | Val- | Pro- | Asn- | SerP- | Ala- | Glu- | Glu- | Arg- | Leu- |
| 121 | | | | | | | 130 | | | | | | | | 140 |
| His- | Ser- | Met- | Lys- | Glu- | Gly- | Ile- | His- | Ala- | Gln- | Gln- | Lys- | Glu- | Pro- | Met- | Ile- | Gly- | Val- | Asn- | Gln- |
| 141 | | | | | | | 150 | | | | | | | | 160 |
| Glu- | Leu- | Ala- | Tyr- | Phe- | Tyr- | Pro- | Glu- | Leu- | Phe- | Arg- | Gln- | Phe- | Tyr- | Gln- | Leu- | Asp- | Ala- | Tyr- | Pro- |
| 161 | | | | | | | 170 | | | | | | | | 180 |
| Ser- | Gly- | Ala- | Trp- | Tyr- | Tyr- | Val- | Pro- | Leu- | Gly- | Thr- | Gln- | Tyr- | Thr- | Asp- | Ala- | Pro- | Ser- | Phe- | Ser- |
| 181 | | | | | | | 190 | | | | | | | | 200 |
| Asp- | Ile- | Pro- | Asn- | Pro- | Ile- | Gly- | Ser- | Glu- | Asn- | Ser- | Glu- | Lys- | Thr- | Thr- | Met- | Pro- | Leu- | Trp | |

**Fig. 4.1** Amino acid sequence of bovine $\alpha_{s1}$-CN B-8P

(Manson *et al.*, 1977). De Kruif and Holt (2003) identified two centres of phosphorylation in $\alpha_{s1}$-CN, i.e. f41–51, containing $Ser_{41}$ (only in the 9P variant), $Ser_{45}$ and $Ser_{47}$, and f61-70, containing residues $Ser_{64}$, $Ser_{66}$, $Ser_{67}$ and $Ser_{68}$. These centres of phosphorylation are crucial in the stabilization of the calcium phosphate nanoclusters in the casein micelles (De Kruif and Holt, 2003).

The amino acid composition and properties of $\alpha_{s1}$-CN B-8P are shown in Table 4.2. Based on amino acid composition, the molecular mass of the protein prior to post-translational modification is estimated at ~23.0 kDa, which increases to ~23.6 kDa as a result of the phosphorylation of eight Ser residues. Based on the primary sequence, a pI of ~4.9 would be expected for $\alpha_{s1}$-CN, but this decreases by ~0.5 pH units through the phosphorylation of the eight Ser residues. Such values are in line with reported pI of $\alpha_{s1}$-CN varying from 4.4 to 4.8 (Trieu-Cuot and Gripon, 1981; Eigel *et al.*, 1984). The aliphatic index, grand average hydropathicity (GRAVY) and hydrophobicity all suggest a moderately hydrophobic protein. $\alpha_{s1}$-CN B-8P contains 25 amino acid residues capable of carrying a positive charge and 40 capable of carrying a negative charge. A distribution of the charge over the polypeptide chain is shown in Fig. 4.2, which clearly highlights a positively charged N-terminus and a high concentration of negative charges, including the two clusters of phosphorylation, between residues 30 and 80. A moderate and even distribution of positive and negative charges is found between residues 81 and 150, whereas the remainder of the protein, with the exception of the 10 amino acid C-terminus, is largely unchanged. Distribution of hydrophobicity, according to the scale of Tanford (1962), of $\alpha_{s1}$-CN B is also shown in Fig. 4.2. In this scale, positive values represent a hydrophobic character whereas negative values represent a hydrophilic character. Some distinct patches of significant hydrophobicity can be observed, i.e. residues 20–35 and 160–175.

**Table 4.2** Amino acid composition and properties of $\alpha_{s1}$-CN B-8P

| Amino acid | $\alpha_{s1}$-CN B-8P | | |
|---|---|---|---|
| Ala | 9 | Total residues | 199 |
| Arg | 6 | Positively charged residues (Lys/Arg/His) | 25 |
| Asn | 8 | Negatively charged residues (Glu/Asp/SerP) | 40 |
| Asp | 7 | Aromatic residues (Tyr/Phe/Thr) | 20 |
| Cys | 0 | | |
| Gln | 14 | Molecular mass | |
| Glu | 25 | Based on primary structure | 22,975 Da |
| Gly | 9 | Including phosphorylation | 23,599 Da |
| His | 5 | | |
| Ile | 11 | pI | |
| Leu | 17 | Based on primary structure | 4.91 |
| Lys | 14 | Including phosphorylation | 4.42 |
| Met | 5 | | |
| Phe | 8 | Extinction coefficient at 280 nm[a] | 25900 $M^{-1}$ $cm^{-1}$ |
| Pro | 17 | | |
| Ser | 16 | Absorbance at 1 g $L^{-1}$ at 280 nm[a] | 1.127 |
| Thr | 5 | | |
| Trp | 2 | Aliphatic index[a] | 75.43 |
| Tyr | 10 | | |
| Val | 11 | Grand average of hydropathicity (GRAVY)[a] | −0.704 |
| | | $H\Phi_{ave}$ (kJ/residue)[a] | 4.89 |

[a]Values are based on the primary structures of the protein and do not take into account post-translational modification of the structures

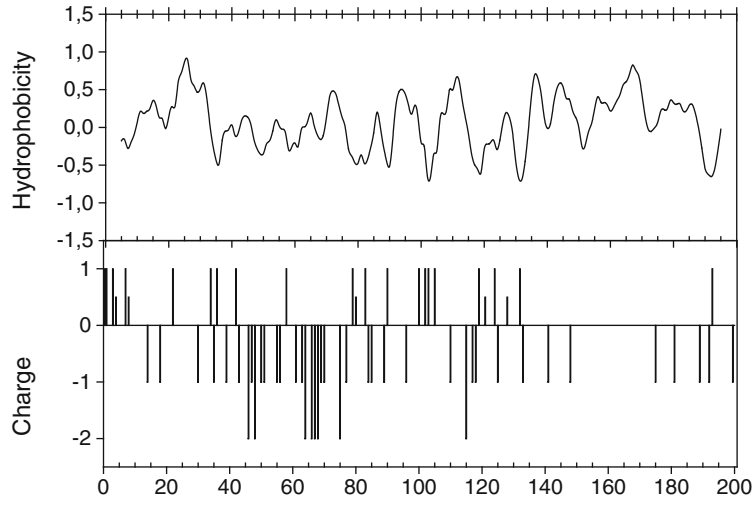


**Fig. 4.2** Distribution of hydrophobicity (*top*) and charged residues (*bottom*) along the amino acid chain of $\alpha_{s1}$-CN B-8P. Hydrophobicity was calculated using the scale of Tanford (1962) with values representing the average on a 7 amino acid window with the relative weight of each amino acid in the window being 1.0 for the centre amino acid and 0.75, 0.50 and 0.25 for the amino acids located 1, 2 or 3 positions from the centre of the window. Hydrophobicity was calculated based on the primary amino acid sequence in the absence of post-translational modification. Charged amino acid residues include Lys (+1), Arg (+1), His (+0.5), Glu (-1), Asp (-1), SerP (-2), the N-terminus (+1) and the C-terminus (-1)

### 4.3.2 Genetic Variation of $\alpha_{s1}$-Casein

In addition to the B-variant of $\alpha_{s1}$-CN, a number of other genetic variants have been identified, an overview of which is shown in Table 4.3. In $\alpha_{s1}$-CN A, the amino acid residues 14–26 are missing as a result of exon skipping (Grosclaude *et al.*, 1970); this variant has been found in Holstein Friesians, Red Holsteins and German Red cattle (Ng-Kwai-Hang *et al.*, 1984; Grosclaude, 1988; Erhardt, 1993). Variant $\alpha_{s1}$-CN C predominates in the milk of *Bos indicus* and *Bos grunniens* (Eigel *et al.*, 1984) and contains Gly instead of Glu at position 192 (Grosclaude *et al.*, 1969). In $\alpha_{s1}$-CN D, which has been found in various breeds in France (Grosclaude, 1988) and Italy (Mariani and Russo, 1975) as well as in Jerseys in the Netherlands (Corradini, 1969), the Ala residue at position 53 is replaced by a phosphorylated Thr residue (Grosclaude *et al.*, 1972). A replacement at position 59 of Gln by Lys and at position 192 of Glu by Gly yields $\alpha_{s1}$-CN E, which is found in *Bos grunniens* (Grosclaude *et al.*, 1976), whereas $\alpha_{s1}$-CN F contains Leu instead of SerP at position 66 and is found in German Black and White cattle (Erhardt, 1993). Finally, $\alpha_{s1}$-CN G was discovered in Italian Brown cows (Mariani *et al.*, 1995), but no amino acid sequence has been reported for this variant to date, whereas $\alpha_{s1}$-CN H arises from an eight amino acid deletion at positions 51–58 (Mahe *et al.*, 1999).

**Table 4.3** Differences in the amino acid sequence of genetic variants of αs1-casein compared to $\alpha_{s1}$-CN B-8P

| | Position | | | | | |
|---|---|---|---|---|---|---|
| Variant | 14–26 | 51–58 | 53 | 59 | 66 | 192 |
| A | Deleted | | | | | |
| *B* | | | *Ala* | *Gln* | *SerP* | *Glu* |
| C | | | | | | Gly |
| D | | | ThrP | | | |
| E | | | | Lys | | Gly |
| F | | | | | Leu | |
| G | | | | | | |
| H | | Deleted | | | | |

### 4.3.3 Secondary Structure of $\alpha_{s1}$-Casein

The secondary structure of $\alpha_{s1}$-CN has been studied using a number of different approaches. While Fourier transform infrared (FTIR) spectroscopy studies by Byler and Susi (1986) found no secondary structure in $\alpha_{s1}$-CN, other studies have reported varying degrees of secondary structure elements in $\alpha_{s1}$-CN. The percentage of α-helix in $\alpha_{s1}$-CN has been estimated as 5–15% (Herskovits, 1966), 8–13% (Byler *et al.*, 1988), 20% (Creamer *et al.*, 1981) or 13–15% (Malin *et al.*, 2005). For β-sheet, values of 17–20% were reported (Byler *et al.*, 1988; Creamer *et al.*, 1981), whereas Malin *et al.* (2005) reported 34–46% extended β-sheet-like structures in $\alpha_{s1}$-CN. In addition, 29–35% β-turn structures have been reported for $\alpha_{s1}$-CN (Byler *et al.*, 1988). In addition, the presence of polyproline II structures in $\alpha_{s1}$-CN is evident from Raman optical activity spectra (Smyth *et al.*, 2001). Higher order structures of caseins are described in further detail in Chap. 5.

### 4.3.4 Self Association of $\alpha_{s1}$-Casein

Self-association of $\alpha_{s1}$-CN is characterized by progressive strongly pH- and ionic strength-dependent consecutive self-association to dimers, tetramers, hexamers, etc. (Ho and Waugh, 1965; Payens and Schmidt, 1965, 1966; Schmidt and van Markwijk, 1968; Swaisgood and Timasheff, 1968; Schmidt, 1970a, b). At pH 6.6 and ionic strength >0.003, the monomers exist in a rapidly equilibrating equilibrium with oligomers; increasing ionic strength results in increasing association constants and the appearance of larger oligomers (Ho and Waugh, 1965; Schmidt and van Markwijk, 1968; Schmidt, 1970b). The free energy for formation of the various oligomers is comparable; hence, all species exist at appreciable concentrations, but they occur to different extents. At an ionic strength of 0.003, only monomers are present, whereas at an ionic strength of 0.01, a monomer–dimer equilibrium exists; at an

ionic strength of 0.2, dimers and tetramers are favoured, while the formation of larger oligomers becomes progressively less favourable (Ho and Waugh, 1965; Schmidt, 1970b). Likewise, as the pH is increased above 6.6, the electrostatic repulsive free energy increases, resulting in smaller association constants yielding a lowered degree of association (Swaisgood and Timasheff, 1968). The larger association constants, and resulting much stronger association, of $\alpha_{s1}$-CN C compared to $\alpha_{s1}$-CN B can be explained by the change in electrostatic free energy (Schmidt, 1970a) due to its smaller net charge. However, $\alpha_{s1}$-CN D behaves identically to $\alpha_{s1}$-CN B (Schmidt, 1970a) although its net charge is greater than that of $\alpha_{s1}$-CN B. It should be noted that the $\alpha_{s1}$-CN B to $\alpha_{s1}$-CN D substitution, at position 53 (Table 4.3) occurs in the polar domain, whereas the $\alpha_{s1}$-CN B to $\alpha_{s1}$-CN C substitution, at position 192 (Table 4.3), occurs in the hydrophobic domain which is more likely to be in the association contact surface. Enzymatic deimination of five of the six Arg residues of $\alpha_{s1}$-CN reduces the susceptibility of the protein to self-association (Azuma *et al.*, 1991).

### 4.3.5 Interactions of $\alpha_{s1}$-Casein with Calcium

When considering the interactions of $\alpha_{s1}$-CN, or any of the other caseins, with calcium, or other cations, two aspects should be considered, i.e. the binding of calcium by the protein and the calcium-induced precipitation of the protein by calcium. $\alpha_{s1}$-CN is one of the calcium-sensitive caseins; precipitation of $\alpha_{s1}$-CN occurs in the range of 3–8 mM $CaCl_2$ (Schmidt, 1969; Bingham *et al.*, 1972; Toma and Nakai, 1973; Dalgleish and Parker, 1980; Aoki *et al.*, 1985; Farrell Jr *et al.*, 1988) and occurs more readily for $\alpha_{s1}$-CN B than for $\alpha_{s1}$-CN A (Farrell Jr *et al.*, 1988). When $CaCl_2$ concentration exceeds ~0.1 mM, the solubility of $\alpha_{s1}$-CN increases again, due to the salting-in effect (Farrell Jr *et al.*, 1988). Calcium-induced precipitates of $\alpha_{s1}$-CN are readily solubilized in 4 M urea, suggesting that no calcium-induced cross-linkage of proteins occurred and that the driving forces behind the calcium-induced association are driven by hydrogen bonding and hydrophobic interactions in the absence of electrostatic repulsion (Aoki *et al.*, 1985).

An extensive investigation into the calcium-binding and calcium-induced precipitation of $\alpha_{s1}$-CN by Dalgleish and Parker (1980) highlighted that the binding of calcium by the protein decreases considerably with increasing ionic strength. In addition, the concentration of calcium required to induce precipitation of $\alpha_{s1}$-CN also increases with increasing ionic strength, but not proportionally to calcium binding, i.e. the degree of calcium binding which is required to induce precipitation of $\alpha_{s1}$-CN decreases with increasing ionic strength (Dalgleish and Parker, 1980). Calcium binding by $\alpha_{s1}$-CN decreases when pH decreases below 7.0, but decreasing pH increases the concentration of calcium required to induce precipitation of $\alpha_{s1}$-CN (Dalgleish and Parker, 1980). Calcium-induced aggregation of $\alpha_{s1}$-CN was described as a monomer–octamer equilibrium, followed by Smoluchowski aggregation in which only the octamers participate (Dalgleish et al., 1981). Dephosphorylation reduces the number of calcium-binding sites on the protein and also reduces the stability of $\alpha_{s1}$-CN to calcium-induced precipitation (Yamuachi *et al.*, 1967; Bingham *et al.*, 1972; Aoki *et al.*, 1985). Deimination of Arg residues in $\alpha_{s1}$-CN enhances calcium binding, as well as the stability of the protein to calcium-induced precipitation (Azuma *et al.*, 1991).

Detailed analyses of the effects of calcium binding on $\alpha_{s1}$-CN have indicated several equilibria. The addition of up to 1 mM $CaCl_2$ to $\alpha_{s1}$-CN induces an exothermic process, possibly hydrogen-bond formation (Holt *et al.*, 1975), binding of calcium only to phosphorylated Ser residues (Ono *et al.*, 1976) and the transfer of Tyr and Trp residues from an aqueous to an apolar environment (Ono *et al.*, 1976). As the concentration of $CaCl_2$ is increased from 1 to 3 mM, the aforementioned exothermic phase is followed by an increasingly endothermic reaction, possibly hydrophobic interactions (Holt *et al.*, 1975); the burying of the aromatic chromophores is abated (Ono *et al.*, 1976), whereas calcium binding by both phosphorylated Ser and carboxylate-containing residues occurs (Ono *et al.*, 1976);

turbidity increases slightly to a plateau level (Holt *et al*., 1975) and increasing numbers of bent-chain polymers are observed (Dosaka *et al*., 1980). Finally, between 3 and 5 mM calcium chloride, the reaction becomes very endothermic (Holt *et al*., 1975); binding of calcium, primarily to carboxylate-containing residues, continues (Ono *et al*., 1976); the turbidity increases dramatically (Holt *et al*., 1975) and precipitation eventually occurs. These results suggest that binding of $Ca^{2+}$ to high-affinity phosphoseryl clusters in the polar domain alters its interaction with the hydrophobic domain, bringing about a conformational change in that domain which allows some association to occur. Further binding to carboxyl residues throughout the structure reduces the electrostatic repulsion and, consequently, interaction of the hydrophobic domains leads to the formation of large aggregates.

## 4.4 $\alpha_{s2}$-Casein

### 4.4.1 Primary Structure of $\alpha_{s2}$-casein

The $\alpha_{s2}$-CN family constitutes up to 10% of the total casein fraction in bovine milk and consists of two major and several minor components, and exhibits varying levels of phosphorylation (Swaisgood, 1992; Farrell Jr *et al*., 2009) and intermolecular disulfide bonding (Rasmussen *et al*., 1992, 1994). The reference protein for this family is $\alpha_{s2}$-CN A-11P, a single-chain polypeptide with an internal disulfide bond with ExPASy entry name and file number CAS2_Bovin and P02663, respectively. The primary structure of $\alpha_{s2}$-CN A-11P (Fig. 4.3), reported by Brignon *et al*. (1977), has been changed to Gln rather than Glu at position 87, as indicated by cDNA sequencing (Stewart *et al*., 1987) and DNA sequencing (Groenen *et al*., 1993). In addition to the aforementioned 11P variant of $\alpha_{s2}$-CN A, 10P, 12P and 13P forms of this protein have also been observed (Brignon *et al*., 1976). Three centres of phosphorylation have been identified, i.e. f8–16, which contains the phosphorylated residues Ser8, Ser9, Ser10 and Ser16; f56–63, which contains the phosphorylated residues Ser56, Ser57, Ser58 and Ser61; and f126–133, which contains the phosphorylated residues Ser129 and Ser131 (De Kruif and Holt, 2003).

The primary sequence of $\alpha_{s2}$-CN A-11P, as outlined in Fig. 4.3, contains two Cys residues, i.e. Cys36 and Cys40, which occur in intra- and intermolecular disulphide bonds. In $\alpha_{s2}$-CN isolated from bovine milk, >85% of the protein is

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | | | | | | | | | **10** | | | | | | | | | | **20** |
| Lys- | Asn- | Thr- | Met- | Glu- | His- | Val- | SerP- | SerP- | SerP- | Glu- | Glu- | Ser- | Ile- | Ile- | SerP- | Gln- | Glu- | Thr- | Tyr- |
| **21** | | | | | | | | | **30** | | | | | | | | | | **40** |
| Lys- | Gln- | Glu- | Lys- | Asn- | Met- | Ala- | Ile- | Asn- | Pro- | Ser- | Lys- | Glu- | Asn- | Leu- | Cys- | Ser- | Thr- | Phe- | Cys- |
| **41** | | | | | | | | | **50** | | | | | | | | | | **60** |
| Lys- | Glu- | Val- | Val- | Arg- | Asn- | Ala- | Asn- | Glu- | Glu- | Glu- | Tyr- | Ser- | Ile- | Gly- | SerP- | SerP- | SerP- | Glu- | Glu- |
| **61** | | | | | | | | | **70** | | | | | | | | | | **80** |
| SerP- | Ala- | Glu- | Val- | Ala- | Thr- | Glu- | Glu- | Val- | Lys- | Ile- | Thr- | Val- | Asp- | Asp- | Lys- | His- | Tyr- | Gln- | Lys- |
| **81** | | | | | | | | | **90** | | | | | | | | | | **100** |
| Ala- | Leu- | Asn- | Glu- | Ile- | Asn- | Gln- | Phe- | Tyr- | Gln- | Lys- | Phe- | Pro- | Gln- | Tyr- | Leu- | Gln- | Tyr- | Leu- | Tyr- |
| **101** | | | | | | | | | **110** | | | | | | | | | | **120** |
| Gln- | Gly- | Pro- | Ile- | Val- | Leu- | Asn- | Pro- | Trp- | Asn- | Gln- | Val- | Lys- | Arg- | Asn- | Ala- | Val- | Pro- | Ile- | Thr- |
| **121** | | | | | | | | | **130** | | | | | | | | | | **140** |
| Pro- | Thr- | Leu- | Asn- | Arg- | Glu- | Gln- | Leu- | SerP- | Thr- | SerP- | Glu- | Glu- | Asn- | Ser- | Lys- | Lys- | Thr- | Val- | Asp- |
| **141** | | | | | | | | | **150** | | | | | | | | | | **160** |
| Met- | Glu- | Ser- | Thr- | Glu- | Val- | Phe- | Thr- | Lys- | Lys- | Thr- | Lys- | Leu- | Thr- | Glu- | Glu- | Glu- | Lys- | Asn- | Arg- |
| **161** | | | | | | | | | **170** | | | | | | | | | | **180** |
| Leu- | Asn- | Phe- | Leu- | Lys- | Lys- | Ile- | Ser- | Gln- | Arg- | Tyr- | Gln- | Lys- | Phe- | Ala- | Leu- | Pro- | Gln- | Tyr- | Leu- |
| **181** | | | | | | | | | **190** | | | | | | | | | | **200** |
| Lys- | Thr- | Val- | Tyr- | Gln- | His- | Gln- | Lys- | Ala- | Met- | Lys- | Pro- | Trp- | Ile- | Gln- | Pro- | Lys- | Thr- | Lys- | Val- |
| **201** | | | | | | | | | **210** | | | | | | | | | | |
| Ile- | Pro- | Tyr- | Val- | Arg- | Tyr- | Leu | | | | | | | | | | | | | |

**Fig. 4.3** Amino acid sequence of $\alpha_{s2}$-CN A-11P

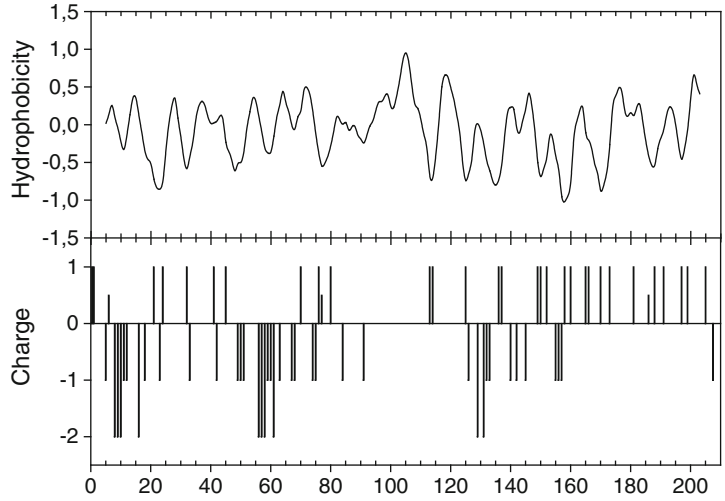


**Fig. 4.4** Distribution of hydrophobicity (*top*) and charged residues (*bottom*) along the amino acid chain of $\alpha_{s2}$-CN A-11P. Hydrophobicity was calculated using the scale of Tanford (1962) with values representing the average on a 7 amino acid window with the relative weight of each amino acid in the window being 1.0 for the centre amino acid and 0.75, 0.50 and 0.25 for the amino acids located 1, 2 or 3 positions from the centre of the window. Hydrophobicity was calculated based on the primary amino acid sequence in the absence of post-translational modification. Charged amino acid residues include Lys (+1), Arg (+1), His (+0.5), Glu (-1), Asp (-1), SerP (-2), the N-terminus (+1) and the C-terminus (-1)

in monomeric form containing the intramolecular disulphide bond, with the remaining fraction of $\alpha_{s2}$-CN consisting of dimers, which can be oriented parallel or antiparallel (Rasmussen *et al.*, 1992, 1994). Brignon *et al.* (1977) pointed out that two very large segments of $\alpha_{s2}$-CN, of ~80 residues, show very high sequence homology with each other and may arise from gene duplication. Sequence alignment by Farrell Jr *et al.* (2009) showed that the best homologous alignment was for residues 42–122 and 124–207. According to Farrell Jr *et al.*, (2009), the $\alpha_{s2}$-CN molecule can be divided into five distinct regions. Residues 1–41 and 42–80 form typical casein phosphopeptide regions with high charge and low hydrophobicity, whereas residues 81–125 form a slightly positively charged region of high hydrophobicity and residues 126–170 form the so-called phosphopeptide analogue, with high negative charge but low phosphate content; finally, residues 171–207 have high positive charge and high hydrophobicity (Farrell Jr *et al.*, 2009). Similar trends are available from the hydrophobicity and charge distribution in Fig. 4.4.

Some properties of $\alpha_{s2}$-CN are given in Table 4.4. The 207 amino acids yield a molecular mass of ~24.3 kDa, which further increases to 25.2 kDa as a result of the phosphorylation of 11 Ser residues. For the non-phosphorylated polypeptide chain of $\alpha_{s2}$-CN, a pI of ~8.3 is predicted, but the aforementioned phosphorylation of 11 Ser residues decreases pI considerably to ~4.9. Because of the high level of charged residues, i.e. 33 residues able to carry a positive charge and 39 capable of carrying a negative charge, $\alpha_{s2}$-CN is generally regarded as the most hydrophilic of the caseins.

### 4.4.2 Genetic Polymorphism of $\alpha_{s2}$-Casein

The A variant of $\alpha_{s2}$-CN is most frequently observed in Western breeds. The B variant was observed with low frequencies in Zebu cattle in South Africa, but a specific site of mutation for $\alpha_{s2}$-CN B has not been identified to date. Variant $\alpha_{s2}$-CN C was observed in yaks in the Nepalese

**Table 4.4** Amino acid composition and properties of $\alpha_{s2}$-CN A-11P

| Amino acid | $\alpha_{s2}$-CN A-11P | | |
|---|---|---|---|
| Ala | 8 | Total residues | 207 |
| Arg | 6 | Positively charged residues (Lys/Arg/His) | 33 |
| Asn | 14 | Negatively charged residues (Glu/Asp/SerP) | 39 |
| Asp | 4 | Aromatic residues (Tyr/Phe/Thr) | 20 |
| Cys | 2 | | |
| Gln | 16 | Molecular mass | |
| Glu | 24 | Based on primary structure | 24,348 Da |
| Gly | 2 | Including phosphorylation | 25,206 Da |
| His | 3 | | |
| Ile | 11 | pI | |
| Leu | 13 | Based on primary structure | 8.34 |
| Lys | 24 | Including phosphorylation | 4.95 |
| Met | 4 | | |
| Phe | 6 | Extinction coefficient at 280 nm[a] | 29,005 $M^{-1}$ $cm^{-1}$ |
| Pro | 10 | | |
| Ser | 17 | Absorbance at 1 g $L^{-1}$ at 280 nm[a] | 1.191 |
| Thr | 15 | | |
| Trp | 2 | Aliphatic index[a] | 68.7 |
| Tyr | 12 | | |
| Val | 14 | Grand average of hydropathicity (GRAVY)[a] | −0.918 |
| | | $H\Phi_{ave}$ (kJ/residue)[a] | 4.64 |

[a]Values are based on the primary structures of the protein and do not take into account post-translational modification of the structures

**Table 4.5** Differences in the amino acid sequence of genetic variants of $\alpha_{s2}$-casein compared to $\alpha_{s2}$-CN A-11P

| | Position | | | |
|---|---|---|---|---|
| Variant | 33 | 47 | 51–59 | 130 |
| *A* | *Glu* | *Ala* | | *Thr* |
| B | | | | |
| C | Gly | Thr | | Ile |
| D | | | Deleted | |

valley and the Republic of Mongolia (Grosclaude *et al.*, 1976, 1982). As shown in Table 4.5, the C variant differs from the A variant at positions 33, 47 and 130, where Gly, Thr and Ile replace Glu, Ala and Thr, respectively (Mahe and Grosclaude, 1982). Variant $\alpha_{s2}$-CN D was observed in Vosgienne and Montbeliarde breeds (Grosclaude *et al.*, 1978) and in three Spanish breeds (Osta *et al.*, 1995). The D variant differs from $\alpha_{s2}$-CN A by the deletion of nine amino acid residues from positions 51–59 (Grosclaude *et al.*, 1978), which is caused by the skipping of exon VIII, a 27-nucleotide sequence that encodes amino acid residues 51–59 (Bouniol *et al.*, 1993).

### 4.4.3 Secondary Structure of $\alpha_{s2}$-Casein

Estimates of the secondary structure of $\alpha_{s2}$-CN have been obtained using a variety of techniques and show considerable differences. Garnier *et al.* (1978) suggested 54% α-helix, 15% β-sheet, 19% turns and 13% unspecified structure, whereas Hoagland *et al.* (2001) suggested 24–32% α-helix, 27–37% β-sheet, 24–31% turns and 9–22% unspecified structure. Furthermore, Tauzin *et al.* (2003) suggested 45% α-helix, 6% β-sheet and 49% unspecified structure, whereas 15% polyproline II structure was suggested by Adzhubei and Sernberg (1993). Most recently, Farrell *et al.* (2009) suggested 46% α-helix, 9% β-sheet, 12% turns, 7% polyproline II, 19% non-continuous α-helix or β-sheet and 7% unspecified

secondary structure. Higher order structures of caseins are described in further detail in Chap. 5.

### 4.4.4 Association Properties of $\alpha_{s2}$-Casein

Given the aforementioned amphipathic and highly charged structure of $\alpha_{s2}$-CN, it is not surprising that its self-association properties strongly depend on ionic strength (Snoeren *et al.*, 1980). $\alpha_{s2}$-CN associates less extensively than $\alpha_{s1}$-CN, but it does exhibit consecutive self-associations, the extent of which at 20°C reaches a maximum at an ionic strength of 0.2–0.3, but decreases at higher ionic strength (Snoeren *et al.*, 1980). This perhaps unexpected decrease in association at higher ionic strengths may be due to ionic suppression of electrostatic interactions between the N-terminal and the C-terminal domains (Snoeren *et al.*, 1980). Snoeren *et al.* (1980) assumed that $\alpha_{s2}$-CN particles under such conditions are spherical, which is indeed apparent from the electron micrographs reported by Thorn *et al.* (2008). However, when $\alpha_{s2}$-CN is incubated at higher temperatures, e.g. 37 or 50°C, ribbon-like fibrils with a diameter of ~12 nm and length >1 μm, which occasionally form loop structures, are observed (Thorn *et al.*, 2008). The formation of such fibrillar structures is optimal at pH 6.5–6.7 and more extensive at higher temperature. The presence of $\alpha_{s1}$-CN inhibits fibril formation by $\alpha_{s2}$-CN, whereas the presence of β-CN has little effect on $\alpha_{s2}$-CN fibril formation. Fibril formation is also reduced when the intra- and intermolecular disulphide bonds in $\alpha_{s2}$-CN are disrupted by the reducing agent, dithiothreitol (Thorn *et al.*, 2008).

### 4.4.5 Interactions of $\alpha_{s2}$-Casein with Calcium

Of the caseins, $\alpha_{s2}$-casein has the highest number of phosphorylated residues and is also the most sensitive to calcium-induced precipitation. Calcium-induced precipitation of $\alpha_{s2}$-CN occurs at calcium concentrations less than 2 mM (Toma and Nakai, 1973; Aoki *et al.*, 1985). As for $\alpha_{s1}$-CN precipitates, calcium-induced precipitates of $\alpha_{s2}$-CN are readily solubilized in 4 M urea, suggesting that no calcium-induced cross-linkage of proteins occurs and that the driving forces behind the calcium-induced interaction are driven by hydrogen bonding and hydrophobic interactions in the absence of electrostatic repulsion (Aoki *et al.*, 1985). This is further substantiated by the fact that dephosphorylation of $\alpha_{s2}$-CN renders the protein insoluble at neutral pH, probably due to the low net charge on the protein at these conditions (Aoki *et al.*, 1985; Table 4.4).

## 4.5 β-Casein

### 4.5.1 Primary Structure of β-Casein

The β-CN family constitutes up to 35% of the casein of bovine milk. The reference protein for this family, β-CN $A^2$-5P, contains 209 residues and its ExPASy entry name and file number are CASB_Bovin and P02666, respectively. The protein was chemically sequenced by Ribadeau-Dumas *et al.* (1972), sequenced from its cDNA by Jimenez-Flores *et al.* (1987) and Stewart *et al.* (1987) and from its gene by Bonsing *et al.* (1988). The sequence for β-CN $A^2$-5P is shown in Fig. 4.5. This sequence was corrected from the original sequences by Yan and Wold (1984) and Carles *et al.* (1988) and differs from the original sequences at four positions: Glu for Gln at positions 117, 175 and 195 and reversal of Pro137 and Leu138. The changes at residues 117 and 175 were confirmed by both groups and by gene sequencing, whereas the reversal of residues 137 and 138 is not in agreement with cDNA-sequencing data (Jimenez-Flores et al., 1987), which is in accordance with the original data. However, the Leu-Pro substitution is a one-base change, and mutations could occur and not be observed by HPLC-mass spectroscopy (MS) of peptides or by electrophoresis of the proteins. Preference is, however, given to the two aforementioned independent protein-sequencing reports. In a similar fashion, the change at position 195 is not in agreement with the cDNA

```
 1                                  10                                  20
Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val- Pro-Gly-Glu- Ile- Val-Glu-SerP-Leu-SerP-SerP-SerP-Glu-
 21                                 30                                  40
Glu- Ser- Ile- Thr-Arg- Ile- Asn-Lys-Lys- Ile- Glu-Lys-Phe-Gln-SerP-Glu- Glu- Gln- Gln-Gln-
 41                                 50                                  60
Thr-Glu-Asp-Glu-Leu-Gln-Asp-Lys- Ile- His-Pro-Phe-Ala-Gln- Thr- Gln- Ser- Leu- Val- Tyr-
 61                                 70                                  80
Pro-Phe-Pro-Gly-Pro- Ile- Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile- Pro- Pro- Leu- Thr- Gln- Thr-
 81                                 90                                  100
Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro-Glu-Val-Met-Gly-Val- Ser- Lys- Val- Lys-Glu-
101                                110                                 120
Ala-Met-Ala-Pro-Lys-His-Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro- Val- Glu- Pro- Phe-Thr-
121                                130                                 140
Glu-Ser-Gln-Ser-Leu-Thr-Leu-Thr-Asp-Val-Glu-Asn-Leu-His-Leu-Pro-Leu-Pro-Leu-Leu-
141                                150                                 160
Gln-Ser-Trp-Met-His-Gln-Pro-His-Gln-Pro-Leu-Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln-
161                                170                                 180
Ser-Val-Leu-Ser-Leu-Ser-Gln-Ser-Lys-Val-Leu-Pro-Val-Pro-Gln-Lys-Ala-Val-Pro-Tyr-
181                                190                                 200
Pro-Gln-Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-
201
Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val
```

**Fig. 4.5** Amino acid sequence of β-CN $A^2$-5P

results, but, in this case, three other lines of evidence support the occurrence of only Glu at residue 195, i.e. the two protein-sequencing corrections noted previously, the invariance on electrophoresis of β-CN (f108–209) from the $A^1$, $A^2$ and $A^3$ genetic variants (Groves, 1969); and the purification from cheese of a bitter peptide *β*-CN (f193–209), the sequence of which is identical to the chemically corrected sequences (Gouldsworthy *et al.*, 1996).

Some features of β-CN $A^2$-5P are shown in Table 4.6, whereas the distribution of charge and hydrophobicity over the molecule is shown in Fig. 4.6. This 209 amino acid protein has a molecular mass which is increased from 23.6 kDa for the primary structure to 24.0 kDa following phosphorylation of the aforementioned five Ser residues. The pI of the non-phosphorylated amino acid is estimated at 5.1, which decreases to ~4.7 as a result of phosphorylation, which is somewhat lower than experimental values of 4.8–5.0 observed by Trieu-Cuot and Gripon (1981). Some of the unique properties of β-CN are derived from the fact that it is strongly amphipathic. The N-terminus of β-CN, residues 1–40, contains essentially all the net charge of the molecule and has a low hydrophobicity and contains only two Pro residues. This section also contains the five phosphorylated Ser residues, i.e. $Ser_{15}$, $Ser_{17}$, $Ser_{18}$, $Ser_{19}$ and $Ser_{35}$, of which the first four form a centre of phosphorylation (De Kruif and Holt, 2003). The middle section of β-CN, i.e. residues 41–135, contains little charge and moderate hydrophobicity, whereas the C-terminal, section 136–209, contains many of the apolar residues and is characterized by little charge and high hydrophobicity.

### 4.5.2 Genetic Polymorphism of β-Casein

In addition to the aforementioned $A^2$ variant of β-CN, a number of other genetic variants have been observed. The amino acid substitutions giving rise to all variants of β-CN are given in Table 4.7. In addition, Chung *et al.* (1995) identified variant $A^4$ in native Korean cattle using electrophoresis only; its substitutions compared to the $A^2$ reference protein are thus far unknown. The $A^1$ variant of β-CN differs from the $A^2$ variant only by the substitution at position 67 of His for Pro (Bonsing *et al.*, 1988), whereas the $A^3$ variant contains Gln instead of His at position 106 (Ribadeau-Dumas *et al.*, 1970). In addition, β-CN B contains the aforementioned mutation for

**Table 4.6** Amino acid composition and properties of β-CN $A^2$-5p

| Amino acid | β-CN $A^2$-5P | | |
|---|---|---|---|
| Ala | 5 | Total residues | 209 |
| Arg | 4 | Positively charged residues (Lys/Arg/His) | 20 |
| Asn | 5 | Negatively charged residues (Glu/Asp/SerP) | 28 |
| Asp | 4 | Aromatic residues (Tyr/Phe/Thr) | 14 |
| Cys | 0 | | |
| Gln | 20 | Molecular mass | |
| Glu | 19 | Based on primary sequence | 23,583 Da |
| Gly | 5 | Including phosphorylation | 23,973 Da |
| His | 5 | | |
| Ile | 10 | pI | |
| Leu | 22 | Based on primary sequence | 5.13 |
| Lys | 11 | Including phosphorylation | 4.65 |
| Met | 6 | | |
| Phe | 9 | Extinction coefficient at 280 nm[a] | 11,460 $M^{-1}$ $cm^{-1}$ |
| Pro | 35 | | |
| Ser | 16 | Absorbance at 1 g $L^{-1}$ at 280 nm[a] | 0.486 |
| Thr | 9 | | |
| Trp | 1 | Aliphatic index[a] | 88.5 |
| Tyr | 4 | | |
| Val | 19 | Grand average of hydropathicity (GRAVY)[a] | −0.355 |
| | | $H\Phi_{ave}$ (kJ/residue)[a] | 5.58 |

[a]Values are based on the primary structures of the protein and do not take into account post-translational modification of the structures

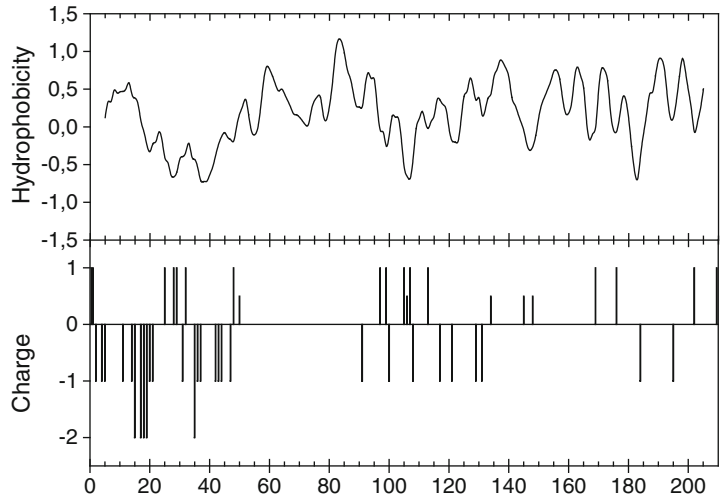


**Fig. 4.6** Distribution of hydrophobicity (*top*) and charged residues (*bottom*) along the amino acid chain of β-CN $A^2$-5P. Hydrophobicity was calculated using the scale of Tanford (1962) with values representing the average on a 7 amino acid window with the relative weight of each amino acid in the window being 1.0 for the centre amino acid and 0.75, 0.50 and 0.25 for the amino acids located 1, 2 or 3 positions from the centre of the window. Hydrophobicity was calculated based on the primary amino acid sequence in the absence of post-translational modification. Charged amino acid residues include Lys (+1), Arg (+1), His (+0.5), Glu (-1), Asp (-1), SerP (-2), the N-terminus (+1) and the C-terminus (-1)

**Table 4.7** Differences in the amino acid sequence of genetic variants of β-casein compared to β-CN $A^2$-5P

| | Position | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variant | 18 | 25 | 35 | 36 | 37 | 67 | 72 | 88 | 93 | 106 | 122 | 137/138 | 152 | ? |
| $A^1$ | | | | | | His | | | | | | | | |
| $A^2$ | *SerP* | *Arg* | *SerP* | *Glu* | *Glu* | *Pro* | *Gln* | *Leu* | *Met* | *His* | *Ser* | *Leu/Pro* | *Pro* | *Gln* |
| $A^3$ | | | | | | | | | | Gln | | | | |
| B | | | | | | His | | | | | Arg | | | |
| C | | | Ser | | Lys | His | | | | | | | | |
| D | Lys | | | | | | | | | | | | | |
| E | | | | Lys | | | | | | | | | | |
| F | | | | | | His | | | | | | | Leu | |
| G | | | | | | His | | | | | | Leu | | |
| $H^1$ | | Cys | | | | | | Ile | | | | | | |
| $H^2$ | | | | | | | Glu | | Leu | | | | | Glu |
| I | | | | | | | | | Leu | | | | | |

the $A^1$ variant, as well as Arg for Ser at position 122 (Grosclaude *et al.*, 1974a). Likewise, β-CN C is also a variant of β-CN $A^1$, which is not phosphorylated at $Ser_{35}$ and contains Lys instead of Glu at position 37. β-CN D differs from β-CN $A^2$ only at position 18, whereas it contains Lys instead of a phosphorylated Ser residue, whereas β-CN E contains Lys instead of Glu at position 36 (Grosclaude *et al.*, 1974b). Visser *et al.* (1995) identified β-CN F, which contains the $A^1$ substitution in addition to Leu for Pro at residue 152. Dong and Ng-Kwai-Hang (1998) identified β-CN G-5P, which is similar to β-CN $A^1$ and F but contains a Leu in place of Pro at either position 137 or 138, depending on the sequence assigned, as the Pro-Leu reversal, as outlined above, is controversial. Han *et al.* (2000) showed that β-CN $H^1$ represents two substitutions relative to the corrected reference β-CN $A^2$, i.e. Arg to Cys at position 25 and Leu to Ile at position 88. A genetic variant, discovered by Senocq *et al.* (2002), was termed β-CN $H^2$, which differs from the $A^2$ variant at two known positions, i.e. Leu instead of Met at position 93 and Glu instead of Gln at position 72; in addition, a substitution of Gln to Glu occurs somewhere between residues 114 and 169 but was not located (Senocq *et al.*, 2002). Finally, the I variant of β-CN was described by Jann *et al.* (2002) and contains only the Leu for Met substitution of the $H^2$ variant at position 93.

### 4.5.3 Secondary Structure of β-Casein

Originally, β-CN was predicted to have little or no secondary structure and, with the exception of 10% α-helix, was predicted to occur as a random coil (Herskovits, 1966; Noelken and Reibstein, 1968), which was further supported by the results of Caessens *et al.* (1999). The presence of α-helix structure in β-CN was further shown by Creamer *et al.* (1981), Graham *et al.* (1984), Farrell Jr *et al.* (2001) and Qi *et al.* (2004, 2005), with values ranging from 7 to 25%. However, 15–33% β-sheet structure was also reported to be present in β-CN, as well as 20–30% turns (Creamer *et al.*, 1981; Graham *et al.*, 1984; Farrell Jr *et al.*, 2001; Qi *et al.*, 2004, 2005). Using optical rotary dispersion analysis, Garnier (1966) suggests that polyproline II could be an important feature in β-casein structure. Subsequent studies have indeed confirmed the presence of 20–25% polyproline II structure in β-CN (Farrell Jr *et al.*, 2001; Syme *et al.*, 2002; Qi *et al.*, 2004). Higher order structures of caseins are dealt with in detail in Chap. 5.

### 4.5.4 Association Properties of β-Casein

The presence of distinct polar and hydrophobic domains in β-CN clearly manifests itself in the extremely temperature-dependent self-association behaviour of β-CN. At 0–4°C, primarily monomers of β-CN are observed (Payens and Van Markwijk, 1963), but even under these conditions, polymeric structure is not entirely absent (Farrell Jr *et al*., 2001). The hydrodynamic behaviour of β-CN under these conditions approaches that of a random coil, with the Stokes radius of 3.7 nm, determined by gel chromatography (Schmidt and Payens, 1972), agreeing well with values obtained by sedimentation and viscosity, and is also consistent with the 4–5-nm size of spherical particles observed by electron microscopy (Andrews *et al*., 1979). Small angle X-ray scattering indicates a radius of gyration of 4.6 nm (Schmidt and Payens, 1972, Andrews *et al*., 1979).

As the temperature is increased above 4–5°C, β-CN undergoes a highly cooperative, reversible, rapidly equilibrating discrete self-association, yielding large polymers with a narrow size distribution (Payens and Van Markwijk, 1963; Payens and Heremans, 1969; Payens *et al*., 1969; Schmidt and Payens, 1972; Niki *et al*., 1977; Andrews *et al*., 1979; Arima *et al*., 1979; Buchheim and Schmidt, 1979; Evans and Phillips, 1979; Takase *et al*., 1980; Schmidt, 1982; Thurn *et al*., 1987; Kajiwara *et al*., 1988; Leclerc and Calmettes, 1997a, b, 1998; Farrell Jr *et al*., 2001; De Kruif and Grinberg, 2002; O'Connell *et al*., 2003; Qi *et al*., 2004, 2005; Gagnard *et al*., 2007). The properties for this monomer–polymer equilibrium can be treated using a shell model for the polymer micelle with a continuous distribution of intermediates between the monomer and largest polymer micelle (Tai and Kegeles, 1984; De Kuif and Grinberg, 2002; O'Connell *et al*., 2003; Mikheeva *et al*., 2003). There appears to be a critical concentration above which micelles are formed, ranging from less than 0.5 mg/mL to about 2 mg/mL (Schmidt and Payens, 1972; Niki *et al*., 1977; Evans *et al*., 1979), which depends on the temperature, ionic strength and pH. The size of the polymer micelle has been characterized by the number of monomers in the polymer, estimates of which have been shown to vary from 15 to 60 (Schmidt and Payens, 1972; Buchheim and Schmidt, 1979; Takase *et al*., 1980; Thurn *et al*., 1987; Kajiwara *et al*., 1988; Farrell Jr *et al*., 2001); the radius of gyration, with varying estimates of 7.3–13.5 nm (Andrews *et al*., 1979; Thurn *et al*., 1987; Kajiwara *et al*., 1988); the Stokes radius of ~15 nm (Niki *et al*., 1977; Thurn *et al*., 1987) and the radius observed by electron microscopy of 8–17 nm (Arima *et al*., 1979; Buchheim and Schmidt, 1979). Increasing ionic strength shifts the equilibrium towards the polymer micelle but affects the number of monomers in the micelle only slightly (Schmidt and Payens, 1972; Takase *et al*., 1980), whereas increasing the temperature shifts the equilibrium position and increases the number of monomers in the micelle (Takase *et al*., 1980). In the theoretical ratio, radius of gyration/Stokes radius is 0.775 for a hard sphere (Thurn *et al*., 1987; Kajiwara *et al*., 1988), while that observed for the β-CN polymer micelle is less than 0.6, suggesting the immobilization of water in a soft outer layer surrounding a more dense core (Kajiwara *et al*., 1988).

Removal of the C-terminal three hydrophobic residues, Ile-Ile-Val, greatly reduces the association (Thompson *et al*., 1967; Evans and Phillips, 1979), as does removal of the C-terminal 17 amino acids (Qi *et al*., 2005). Removal of these 17 amino acids (Qi *et al*., 2005) or the 20 C-terminal amino acids (Berry and Creamer, 1975) renders β-CN virtually incapable of binding the hydrophobic surface probe ANS. The importance of hydrophobic interactions in the micellization of β-CN is further exemplified by the enhanced micellization when $H_2O$ is replaced by $D_2O$ (Evans and Phillips, 1979) or when ethanol is added (Mikheeva *et al*., 2003) and by the reduced micellization of β-CN in the presence of urea (Mikheeva *et al*., 2003). The importance of charges on the N-terminus on the micellization of β-CN is strongly impaired by the absence of post-translational phosphorylation but this loss of micellization is partially restored by duplication of the 6 N-terminal amino acids of β-CN in expression (Gagnard *et al*., 2007).

### 4.5.5 Interactions of β-Casein with Calcium and Other Cations

Compared to $\alpha_{s1}$-CN and $\alpha_{s2}$-CN, β-CN is less sensitive to calcium-induced precipitation. At 37°C, β-CN precipitates in the range of 8–15 mM $Ca^{2+}$ at 37°C (Schmidt, 1969; Parker and Dalgleish, 1981; Farrell Jr *et al*., 1988). However, at 1°C, β-CN remains in solution at concentrations up to 400 mM $CaCl_2$ (Farrell Jr *et al*., 1988). Under physiological conditions, β-CN is capable of binding approximately seven calcium ions per molecule (Parker and Dalgleish, 1981; Baumy and Brule, 1988). Binding of calcium by β-CN increases with increasing temperature, whereas an increase in ionic strength reduces the binding of calcium by β-CN (Parker and Dalgleish, 1981; Baumy and Brule, 1988). In addition, the binding of calcium by β-CN decreases with decreasing pH (Baumy and Brule, 1988). The binding of other di- and trivalent cations has also been studied; binding of magnesium, zinc and manganese shows comparable dependence on pH and ionic strength to the binding of calcium, whereas the binding of iron and copper by β-CN is virtually independent of pH and ionic strength (Baumy and Brule, 1988). The amount of calcium required to induce precipitation of β-CN decreases strongly with increasing temperature, whereas decreases in the amount of calcium bound by β-CN at the point of precipitation are also observed (Parker and Dalgleish, 1981). Both dephosphorylation and glycation of β-casein have been shown to improve the stability of β-casein to calcium-induced precipitation (Darewicz *et al*., 1999).

## 4.6 κ-Casein

### 4.6.1 Primary Structure of κ-Casein

Within the caseins, κ-CN displays some rather unique features. It is the smallest of the caseins, is has a low level of phosphorylation, has a low sensitivity to calcium and is the only one of the caseins to occur in glycosylated form. The primary sequence of the 169 amino acid κ-CN A 1P, which is the parent protein of the κ-CN family and has the ExPASy entry name CASK_Bovin and file accession number P02668, is shown in Fig. 4.7. Like for the other caseins, variable degrees of phosphorylation have also been found for κ-CN. The monophosphorylated form of κ-CN appears to be phosphorylated exclusively at $Ser_{149}$, whereas the diphosphorylated form of κ-CN is phosphorylated at $Ser_{149}$ and $Ser_{121}$ (Mercier, 1981; Minkiewicz *et al*., 1996; Talbo *et al*., 2001; Holland *et al*., 2006). For the triphosphorylated form of κ-CN,

| | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | | | | | | | | | **10** | | | | | | | | | | **20** |
| Gln- | Glu- | Gln- | Asn- | Gln- | Glu- | Gln- | Pro- | Ile- | Arg- | Cys- | Glu- | Lys- | Asp- | Glu- | Arg- | Phe- | Phe- | Ser- | Asp- |
| **21** | | | | | | | | | **30** | | | | | | | | | | **40** |
| Lys- | Ile- | Ala- | Lys- | Tyr- | Ile- | Pro- | Ile- | Gln- | Tyr- | Val- | Leu- | Ser- | Arg- | Tyr- | Pro- | Ser- | Tyr- | Gly- | Leu- |
| **41** | | | | | | | | | **50** | | | | | | | | | | **60** |
| Asn- | Tyr- | Tyr- | Gln- | Gln- | Lys- | Pro- | Val- | Ala- | Leu | Ile- | Asn- | Asn- | Gln- | Phe- | Leu- | Pro- | Tyr- | Pro- | Tyr- |
| **61** | | | | | | | | | **70** | | | | | | | | | | **80** |
| Tyr- | Ala- | Lys- | Pro- | Ala- | Ala- | Val- | Arg- | Ser- | Pro | Ala- | Gln- | Ile- | Leu- | Gln- | Trp- | Gln- | Val- | Leu- | Ser- |
| **81** | | | | | | | | | **90** | | | | | | | | | | **100** |
| Asn- | Thr- | Val- | Pro- | Ala- | Lys- | Ser- | Cys- | Gln- | Ala | Gln- | Pro- | Thr- | Thr- | Met- | Ala- | Arg- | His- | Pro- | His- |
| **101** | | | | | | | | | **110** | | | | | | | | | | **120** |
| Pro- | His- | Leu- | Ser- | Phe- | Met- | Ala- | Ile- | Pro- | Pro | Lys- | Lys- | Asn- | Gln- | Asp- | Lys- | Thr- | Glu- | Ile- | Pro- |
| **121** | | | | | | | | | **130** | | | | | | | | | | **140** |
| Thr- | Ile- | Asn- | Thr- | Ile- | Ala- | Ser- | Gly- | Glu- | Pro | Thr- | Ser- | Thr- | Pro- | Thr- | Thr- | Glu- | Ala- | Val- | Glu- |
| **141** | | | | | | | | | **150** | | | | | | | | | | **160** |
| Ser- | Thr- | Val- | Ala- | Thr- | Leu- | Glu- | Asp- | SerP- | Pro | Glu- | Val- | Ile- | Glu- | Ser- | Pro- | Pro- | Glu- | Ile- | Asn- |
| **161** | | | | | | | | | | | | | | | | | | | |
| Thr- | Val- | Gln- | Val- | Thr- | Ser- | Thr- | Ala- | Val | | | | | | | | | | | |

**Fig. 4.7** Primary amino acid sequence of κ-CN A-1P

**Table 4.8** Amino acid composition and properties of κ-CN A-1P

| Amino acid | κ-CN A-1P | | |
|---|---|---|---|
| Ala | 14 | Total residues | 169 |
| Arg | 5 | Positively charged residues (Lys/Arg/His) | 17 |
| Asn | 8 | Negatively charged residues (Glu/Asp/SerP) | 28 |
| Asp | 4 | Aromatic residues (Tyr/Phe/Thr) | 14 |
| Cys | 2 | | |
| Gln | 15 | Molecular mass | |
| Glu | 12 | Based in primary sequence | 18,974 Da |
| Gly | 2 | Including phosphorylation | 19,052 Da |
| His | 3 | | |
| Ile | 12 | pI | |
| Leu | 8 | Based on primary sequence | 5.93 |
| Lys | 9 | Including phosphorylation | 5.60 |
| Met | 2 | | |
| Phe | 4 | Extinction coefficient at 280 nm[a] | 19035 $M^{-1}$ $cm^{-1}$ |
| Pro | 20 | | |
| Ser | 13 | Absorbance at 1 g $L^{-1}$ at 280 nm[a] | 1.003 |
| Thr | 15 | | |
| Trp | 1 | Aliphatic index[a] | 73.3 |
| Tyr | 9 | | |
| Val | 11 | Grand average of hydropathicity[a] | –0.557 |
| | | $H\Phi_{ave}$ (kJ/residue)[a] | 5.12 |

[a]Values are based on the primary structures of the protein and do not take into account post-translational modification of the structures

Holland *et al*. (2006) recently reported that the additional amino acid residue to be phosphorylated is not a Ser residue, but Thr145.

Some features of κ-CN A-1P are shown in Table 4.8, whereas the distribution of hydrophobicity and charge over the protein chain are shown in Fig. 4.8. Based on the amino acid sequence, it can be deduced that of the 169 amino acids, 17 can be positively charged, whereas 28 can be negatively charged and there are a further 14 aromatic residues. Both hydrophobicity and charge are distributed unevenly throughout the protein (Fig. 4.8). Negative charges are found only in the N-terminal fragment 1–20 and the C-terminal fragment 115–169; the intermittent fragment 21–114 is devoid of negatively charged residues. Additional negative charges arising from phosphorylation are also in the C-terminal segment 115–169, as would be negative charges arising from glycosylation, which, as discussed later, can occur on six Thr residues in this segment. Positive charges can be found in the N-terminal segment 1–116, but not in the C-terminal segment 117–169. Hydrophobicity distributions highlight, as for charges, an uneven distribution of hydrophobicity throughout κ-CN. Segment 1–20 shows predominantly hydrophilic behaviour, whereas segment 21–110 contains some strongly hydrophobic patches, which is in agreement with the absence of negatively charged and a low number of positively charged residues in this segment. Segment 110–120 is strongly hydrophilic, whereas the remainder, i.e. segment 121–169 shows some hydrophilic and hydrophobic areas. It should be noted that post-translational phosphorylation and glycosylation occurring in this part of the protein will reduce hydrophobicity considerably.

Not taking into account post-translational modification, the molecular mass of κ-CN A was reported as 19.0 kDa. Increases in mass arise from post-translational phosphorylation and glycosylation. Based on the amino acid sequence, a pI for κ-CN A of ~5.9 can be expected. However,

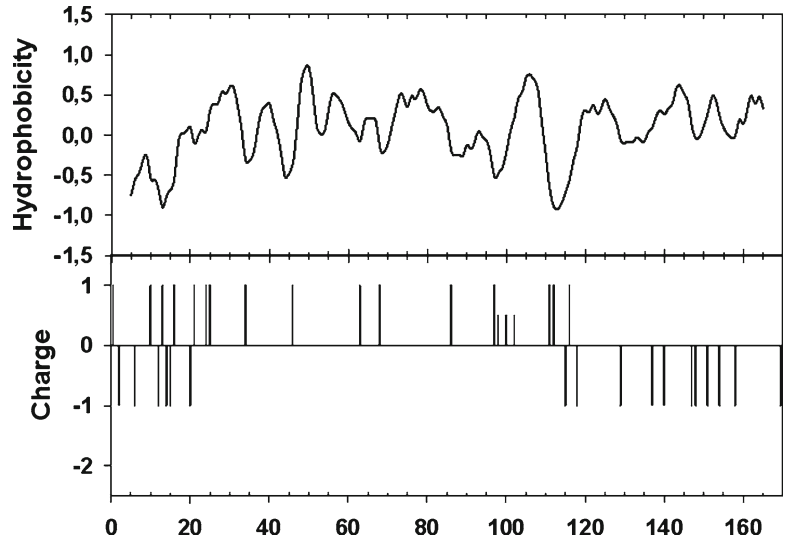


**Fig. 4.8** Distribution of hydrophobicity (*top*) and charged residues (*bottom*) along the amino acid chain of κ-CN A-1P. Hydrophobicity was calculated using the scale of Tanford (1962) with values representing the average on a 7 amino acid window with the relative weight of each amino acid in the window being 1.0 for the centre amino acid and 0.75, 0.50 and 0.25 for the amino acids located 1, 2 or 3 positions from the centre of the window. Hydrophobicity was calculated based on the primary amino acid sequence in the absence of post-translational modification. Charged amino acid residues include Lys (+1), Arg (+1), His (+0.5), Glu (-1), Asp (-1), SerP (-2), the N-terminus (+1) and the C-terminus (-1)

experimental observations have shown considerably lower values for the isoelectric point of κ-CN, as low as pH 3.5 (Holland *et al*., 2006), which is due to increased negative charges on the protein arising from post-translational phosphorylation and glycosylation. For the non-glycosylated monophosphorylated variants of κ-CN A and B, pI values of 5.56 and 5.81 were found by two-dimensional electrophoresis, with consistent reductions in pI apparent with increasing degree of phosphorylation and glycosylation (Holland *et al*., 2004).

### 4.6.2 Genetic Variation of κ-Casein

κ-CN A predominates in Western breeds, with the exception of Jerseys (Thompson and Farrell Jr, 1974; Bech and Kristiansen, 1990; Ng-Kwai-Hang and Grosclaude, 2003). In addition, a number of other variants of κ-CN have also been identified (Table 4.9). The major other variant of κ-CN is κ-CN B, which differs from κ-CN A by substitution at position 136 of Ile for Thr and at position 148 of Ala for Asp (Mercier *et al*., 1973). The C variant of κ-CN differs from κ-CN A by substitution of His for Arg at position 97 (Miranda *et al*., 1993). The E variant of κ-CN arises from a substitution at position 155, i.e. Gly for Ser (Miranda *et al*., 1993). κ-CN $F^1$ was discovered in both Zebu and Black and White hybrid cattle and contains Val instead of Asp at position 148 (Sulimova *et al*., 1992). κ-CN $F^2$ was reported to be a variant of κ-CN B, containing His instead of Arg at position ten (Prinzenberg *et al*., 1996). Erhardt *et al*. (1996) reported the occurrence of κ-CN $G^1$ in alpine breeds, which, in addition to the substitutions occurring for κ-CN B, also contains Cys instead of Arg at position 97. κ-CN $G^2$ was shown to occur in the milk of *Bos grunniens* and was shown to contain Ala instead of Asp at position 148. In Pinzgauer cattle, Prinzenberg *et al*. (1999) identified κ-CN H, which differed from κ-CN A by an Ile for Thr substitution at position 135. In another study, Prinzenberg *et al*. (1999) described κ-CN I,

**Table 4.9** Differences in the amino acid sequence of genetic variants of κ-casein compared to κ-CN A-1P

| | Position | | | | | | |
|---|---|---|---|---|---|---|---|
| Variant | 10 | 97 | 104 | 135 | 136 | 148 | 155 |
| *A* | *Arg* | *Arg* | *Ser* | *Thr* | *Thr* | *Asp* | *Ser* |
| B | | | | | Ile | Ala | |
| C | | His | | | | | |
| E | | | | | | | Gly |
| $F^1$ | | | | | | Val | |
| $F^2$ | His | | | | Ile | Ala | |
| $G^1$ | | Cys | | | Ile | Ala | |
| $G^2$ | | | | | | Ala | |
| H | | | | | Ile | | |
| I | | | Ala | | | | |
| J | | | | | Ile | Ala | Arg |

which differs from κ-CN A by Ala for Ser substitution at position 104. Finally, Mahe *et al*. (1999) described the occurrence of κ-CN J, which seems to have arisen from an Arg for Ser mutation at position 155 in *Bos taurus* cattle on the Ivory Coast. As outlined previously, however, κ-CN A and B predominate strongly in Western breeds of cattle.

From a technological perspective, the Phe105-Met106 bond in κ-CN is extremely important, as it is the hydrolysis of this bond by chymosin, or proteinases with comparable specificity, that initiates the gelation of milk, which will ultimately be processed into a cheese curd and a ripened or unripened cheese. The N-terminal segment 1–105 arising from the chymosin-induced hydrolysis of κ-CN is called *para*-κ-CN, whereas the C-terminal fragment 106–169 is called the caseinomacropeptide (CMP); when CMP is glycosylated, it is often referred to as glycomacropeptide (GMP). From Table 4.9, it is apparent that this sequence is conserved in all genetic variants of κ-CN. However, for κ-CN I, the adjoining Ser104 residue is replaced by the considerably more hydrophobic Ala residue. It is also worthwhile noticing that, as outlined further in later stages, all post-translational modifications of κ-CN occur in the CMP segment of the molecule.

### 4.6.3 Glycosylation of κ-Casein

Of the caseins, κ-CN is the only one for which post-translational glycosylation has been shown to occur. Vreeman *et al*. (1986) observed that ~40% of κ-CN is non-glycosylated, whereas the remainder can contain up to six glycans. Glycosylation sites in κ-CN were found to be the Thr residues at positions 121, 131, 133, 142, 145 and 165 (Pisano *et al*., 1994; Molle and Leonil, 1995; Minkiewicz *et al*., 1996). Holland *et al*. (2004, 2005, 2006) showed that the different glycoforms of κ-CN can be separated readily by 2D electrophoresis on the basis of isoelectric point and molecular mass, yielding up to 16 different spots for κ-CN with isoelectric points down to ~3.5. Such separations have laid the basis for the recent elucidation of the glycosylation pattern of κ-CN. Using tandem MS sequencing of chemically tagged peptides, it was observed that the mono-glycoform of κ-CN was glycosylated exclusively at Thr131, the di-glycoform exclusively at Thr131 and Thr142 and the tri-glycoform at Thr131, Thr133 and Thr142 (Holland *et al*., 2005). The tetra-glycoform of κ-CN B was shown to be glycosylated at Thr145, in addition to the three already-mentioned glycosylation sites, Thr131, Thr133 and Thr142 (Holland *et al*., 2006). The remaining two glycosylation sites of κ-CN were not confirmed by Holland *et al*. (2006) but are most likely, as proposed by Pisano *et al*. (1994) and Minkewicz *et al*. (1996), to be Thr121 and Thr165. In general, κ-CN B appears to be more heavily glycosylated than κ-CN A, also displaying a more complex and variable glycosylation pattern (Coolbear *et al*., 1996).

A variety of glycans have been shown to be attached to κ-CN, all of which have been shown to be attached to Thr residues. These glycans consist of galactose (Gal), *N*-acetylglucosamine (GalNAc) and *N*-acetyl neuraminic acid (NANA). The monosaccharide GalNac, the disaccharide Galβ(1–3)GalNac, the trisaccharides NANAcα(2–3)Galβ(1–3)GalNAc and Galβ(1–3)

[NANAcα(2–6)]GalNac and the tetrasaccharide NANAcα(2–3)Gal1β(1–3)[NANAcα(2–6)] GalNac have been identified attached to κ-CN. Saito and Itoh (1992) estimated the presence of 56.0% tetrasaccharide, 18.5% branched trisaccharide, 18.4% linear trisaccharide, 6.3% disaccharide and 0.8% monosaccharide.

### 4.6.4 Disulphide-Bonding Patterns of κ-Casein

The presence of the two Cys residues in κ-CN, i.e. Cys11 and Cys88, creates a complex disulphide-bonding pattern between κ-CN molecules in bovine milk. Swaisgood *et al*. (1964) showed that κ-CN obtained without reduction was apparently randomly cross-linked by intermolecular disulphide bonds, to give oligomers, with the smallest detectable oligomer having a mass of ~60 kDa, corresponding to a trimer. The existence of disulphide-cross-linked oligomers has since been substantiated (Talbot and Waugh, 1970; Farrell Jr *et al*., 1988; Groves *et al*., 1992), with the further suggestion that, during biosynthesis, reduced monomers first interact with the calcium-sensitive caseins to form micelles, followed by random cross-linking by oxidation (Pepper and Farrell Jr, 1982). In κ-CN isolated from bovine milk, only ~10% of total κ-CN appears to be in the monomeric form (Farrell Jr *et al*., 1996).

Both disulphide-cross-linked oligomers and reduced κ-CN are capable of forming polymer micelles and stabilizing calcium-sensitive caseins (Talbot and Waugh, 1970; Vreeman, 1979). In the monomeric form of κ-CN, Cys11 and Cys88 form an intramolecular disulphide bond. However, κ-CN complexes arising to octamers and larger have also been found in bovine milk. These complexes contain an apparently random distribution of disulphides, i.e. Cys11 to Cys88, Cys11 to Cys11, Cys88 to Cys11 and Cys88 to Cys88. Whether these patterns remain after isolation of the κ-CN from milk is strongly dependent on the physicochemical conditions of isolation. Particularly the presence of reducing agents such as β-mercaptoethanol and dithiothreitol will significantly impact the oligomeric distributions of κ-CN. As outlined in Table 4.9, κ-CN $G^1$ even contains a third Cys residue, i.e. Cys97. The impact hereof on the disulphide-bonding pattern has, however, not been studied to date.

### 4.6.5 Secondary Structure of κ-Casein

The secondary structure of κ-CN has been studied using a number of methods. NMR studies by Rollema *et al*. (1988) suggest a high degree of flexibility, particularly in the macropeptide part of κ-CN. Some structure, however, has been detected for κ-CN using spectroscopic methods such as FTIR and CD. Estimates suggest that κ-CN may contain 10–20% α-helix, 20–30% β-structure and 15–25% turns (Byler and Susi, 1986; Griffin *et al*., 1986; Ono *et al*., 1987; Kumosinski *et al*., 1991, 1993; Sawyer and Holt, 1993; Farrell Jr *et al*., 1996, 2003). The degree of estimated α-helical structure in κ-CN increases with increasing temperature (10–70°C), while the proportion of β-structure and turns decreases with temperature (Farrell *et al*., 2003). In addition, analysis in the presence of alcohols also results in a higher degree of α-helix in κ-CN. Several structural motifs have also been suggested, including possible antiparallel and parallel β-sheets or βαβ structure in the hydrophobic domain (Raap *et al*., 1983) and a β-turn-β-strand-β-turn motif centred on the chymosin-sensitive $Phe_{105}$-$Met_{106}$ region (Creamer *et al*., 1998). The latter motif appears to be conserved in κ-CN from various species, as would be expected for specific sensitivity to aspartyl proteinases (Holt and Sawyer, 1988). Using a Raman optical activity study, Syme *et al*. (2002) identified the presence of polyproline II helical confirmation in κ-CN. Some of the predicted structure occurs in the polar macropeptide domain but the stability of ordered structure in a region of such high net charge and apparent hydration would seem questionable and contradicts the great deals of flexibility; this part of the molecule was found to exhibit in the NMR studies by Rollema *et al*. (1988).

### 4.6.6 Association Behavior of κ-Casein

When isolated from milk, κ-CN occurs in the form of multimeric complexes. Analysis by analytical ultracentrifugation suggests that the weight average molecular weight of these complexes is ~1,180 kDa at 25°C and ~1,550 kDa at 37°C (Groves *et al*., 1998). Electron microscopy studies have shown a radius of 5.0–7.5 nm, 9–10 nm (Parry and Carroll, 1969) or 8.9 nm (Farrell Jr *et al*., 1996). Similar values have been observed by gel permeation chromatography (9.4 nm; Pepper and Farrell Jr, 1982), dynamic light scattering (9.6 nm; Farrell Jr *et al*., 1996) and small angle neutron scattering (SANS), for which values for values of a radius of 7.4 nm (Thurn *et al*., 1987) and 8 nm (De Kruif *et al*., 2002) have been reported. Micelle size, structure and interaction radius were found to be independent of protein concentration (De Kruif *et al*., 2002). Both calcium and iron have been found to be present in isolated κ-CN, and their chelation by EDTA has been reported to result in disruption of the κ-CN particle, with subsequent aggregation into particles with a considerably broader size distribution (Farrell Jr *et al*., 1996).

Reduction of the disulphide bridges in aforementioned κ-CN particles leads to amphipathic monomers which can, like β-CN, associate into micellar structures; unlike the micellization of β-CN, micellization of reduced κ-CN shows no strong temperature dependence (Swaisgood *et al*., 1964; Vreeman *et al*., 1981). This suggests that micellization of reduced κ-CN is less dominated by hydrophobic interactions than micellization of β-CN. For the monomer–polymer micelle equilibrium of reduced κ-CN, the critical micelle concentration varies from 0.53 at an ionic strength of 0.1–0.24 mg/mL at an ionic strength of 1.0 (Vreeman, 1979; Vreeman *et al*., 1977, 1981). The degree of polymerization has been estimated at ~30 κ-CN molecules per micelle, yielding a molecular mass of ~570–600 kDa (Vreeman, 1979; Vreeman *et al*., 1981, 1986) and an estimated diameter of 23 nm (Vreeman *et al*., 1981). Such results are in agreement with values derived from SANS measurements on reduced κ-CN micelles; such measurements led De Kruif and May (1991) to conclude that reduced κ-CN micelles are spherical and consist of a dense core of ~6–7 nm, surrounded by a more open outer layer, protruding up to 14.7 nm from the centre of the core. The interactions between micelles of reduced κ-CN can be described as that of the so-called hard spheres (De Kruif and May, 1991).

When reduced and carboxymethylated κ-CN was incubated at 37°C, it was observed in addition to spherical particles, there was also a high proportion of fibrillar structures present (Farrell Jr *et al*., 2003). The formation of such fibrillar structures, with a diameter of 10–12 nm and lengths up to 600 nm, was subsequently shown to occur for native, reduced and carboxymethylated κ-CN (Thorn *et al*., 2005; Ecroyd *et al*., 2008, 2010; Leonil *et al*., 2008). When native κ-CN is used, it is the dissociated form that is involved in fibril formation (Ecroyd *et al*., 2010). Fibril formation, which has been shown to result in an increased proportion of β-sheet structure (Ecroyd *et al*., 2008; Leonil *et al*., 2008), is more extensive at higher temperature (Thorn *et al*., 2005) and is more extensive for non-glycosylated κ-CN than for its glycosylated counterpart (Leonil *et al*., 2008). The presence of $\alpha_s$-CNs or β-CN inhibits fibril formation (Thorn *et al*., 2005; Leonil *et al*., 2008), whereas BSA does not inhibit fibril formation (Thorn *et al*., 2005). Segment Tyr25-Lys86 of κ-CN appears to be incorporated into the protease-resistant core of the fibrils (Ecroyd *et al*., 2008) whereas fragment 106–169, i.e., the macropeptide, in either glycosylated or non-glycosylated form, does not form fibrils under comparable circumstances (Leonil *et al*., 2008).

### 4.6.7 Interactions of κ-Casein with Calcium

Compared to the other caseins, interactions of calcium with κ-CN have studied far less. This is probably due to the fact that κ-CN is, unlike $\alpha_{s1}$-CN, $\alpha_{s2}$-CN and β-CN, the so-called calcium insensitive, i.e., it is not precipitated in the pres-

ence of excess calcium. Ono *et al*. (1980), studying the binding of calcium to κ-CN, observed that binding of calcium to phosphorylated Ser residues reached a plateau at 1 mM $CaCl_2$, whereas binding of calcium by carboxyl groups increased linearly up to 3 mM $CaCl_2$ and more slowly at higher concentrations. Spectra obtained from circular dichroism and UV analysis indicate that the binding of calcium to κ-CN does not induce changes in the secondary structure of the protein (Ono *et al*., 1980). Given the aforementioned potential role of calcium in stabilizing κ-CN particles isolated from milk (Farrell *et al*., 1996), further study on the interactions of calcium with κ-casein appears warranted.

## References


Adzhubei, A.A. and Sternberg, M.J.E. (1993). Left-handed polyproline II helices commonly occur in globular proteins. *J. Mol. Biol.* **229**, 472–493.

Andrews, A.L., Atkinson, D., Evans, M.T.A., Finer, E.G., Green, J.P., Phillips, M.C. and Robertson, R.N. (1979). The conformation and aggregation of bovine β-casein A. I. Molecular aspects of thermal aggregation. *Biopolymers* **18**, 1105–1121.

Aoki, T., Toyooka, K. and Kako, Y. (1985). Role of phosphate groups in the calcium sensitivity of $\alpha_{s2}$-casein. *J. Dairy Sci.* **68**, 1624–1629.

Arima, S., Niki, R. and Takase, K. (1979). Structure of β-casein. *J. Dairy Res.* **46**, 281–282.

Azuma, N., Nara, K. and Kanno, C. (1991). Enzymic modification of $\alpha_{s1}$-casein with peptidylarginine deiminase: preparation of less acid-coagulable and less calcium-sensitive casein. *J. Dairy Res.* **58**, 421–429.

Baumy, J.J. and Brule, G. (1988). Effect of pH and ionic strength on the binding of bivalent cations to β-casein. *Lait* **68**, 409–418.

Bech, A.M. and Kristiansen, K.R. (1990). Milk protein polymorphism in Danish dairy cattle and the influence of genetic variants on milk yield. *J. Dairy Res.* **57**, 53–62.

Berry, G.P. and Creamer, L.K. (1975). The association of bovine β-casein. The importance of the C-terminal region. *Biochem.* **14**, 3542–3545.

Bingham, E.W., Farrell, H.M., and Carroll, R.J., Jr. (1972). Properties of dephosphorylated αs1-casein. Precipitation by calcium ions and micelle formation. *Biochem*. **11**, 2450–2454.

Bonsing, J., Ring, J.M., Stewart, A.F. and MacKinlay, A.G. (1988). Complete nucleotide sequence of the bovine beta-casein gene. *Aust. J. Biol. Sci.* **41**, 527–537

Bouniol, C., Printz, C. and Mercier J.-C. (1993). Bovine $\alpha_{S2}$-casein D is generated by exon VIII skipping. *Gene* **128**, 289–293.

Brignon, G., Ribadeau-Dumas, B. and Mercier, J.-C. (1976). Premiers elements de structure primaire des caseines $\alpha_{s2}$ bovines. *FEBS Lett*. **71**, 111–116.

Brignon, G.B., Ribadeau-Dumas, B., Mercier, J.-C., Pelissier, J.-P. and Das, B.C. (1977). The complete amino acid sequence of bovine $\alpha_{S2}$-casein. *FEBS Lett.* **76**, 274–279.

Buchheim, W. and Schmidt, D.G. (1979). On the size of monomers and polymers of β-casein. *J. Dairy Res.* **46**, 277–280.

Byler, D.M. and Susi, H. (1986). Examination of the secondary structure of proteins by deconvoluted FTIR spectra. *Biopolymers* **25**, 469–487.

Byler, D.M., Farrell, H.M., Jr. and Susi, H. (1988). Raman spectroscopic study of casein structure. *J. Dairy Sci*. **71**, 2622–2629.

Caessens, P.W.J.R., De Jongh, H.H.J., Noide, W. and Gruppen, H. (1999). The absorption-induced secondary structure of β-casein and of distinct parts of its sequence. *Biochim. Biophys. Acta* **1430**, 73–83.

Carles, C., Huet, J.-C. and Ribadeau-Dumas, B. (1988). A new strategy for primary structure determination of proteins: application to β-casein. *FEBS Lett.* **229**, 265–272.

Chung, E.R., Han, S.K. and Rhim, T.J. (1995). Milk protein polymorphisms as genetic markers in Korean native cattle. *Asian Austral. J. Anim. Sci.* **8**, 187–194.

Coolbear, K.P., Elgar, D.F. and Ayers, J.S. (1996). Profiling of genetic variants of bovine κ-casein macropeptide by electrophoretic and chromatographic techniques. *Int. Dairy J.* **6**, 1055–1068.

Corradini, C. (1969). Distribution of the genetic variants $\alpha_{s1}$-, β-, and κ-caseins in milk from Jersey cows in The Netherlands. *Neth. Milk Dairy J.* **23**, 79–82.

Creamer, L.K., Richardson, T. and Parry, D.A.D. (1981). Secondary structure of bovine $\alpha_{s1}$- and β-casein in solution. *Arch. Biochem. Biophys.* **211**, 689–696.

Creamer, L.K., Plowman, J.E., Liddell, M.J., Smith, M.H. and Hill. J.P. (1998). Micelle stability: κ-casein structure and function. *J. Dairy Sci.* **81**, 3004–3012.

Dalgleish, D.G. and Parker, T.G. (1980). Binding of calcium ions to bovine $\alpha_{s1}$-casein and precipitability of the protein-calcium ion complexes. *J. Dairy Res.* **47**, 113–122.

Dalgleish, D.G., Paterson, E. and Horne, D.S. (1981). Kinetics of aggregation of $\alpha_{s1}$-casein/$Ca^{2+}$ mixtures: charge and temperature effects. *Biophys. Chem.* **13**, 307–314.

Darewicz, M., Dziuba, J., Mioduszewska, H. and Minkiewicz, P. (1999). Modulation of physico-chemical properties of bovine β-casein by non-enzymatic glycation associated with enzymatic dephosphorylation. *Acta Aliment.* **28**, 339–354.

De Kruif, C.G. and Grinberg, V.Y. (2002). Micellisation of β-casein. *Colloid. Surface. A* **210**, 183–190.

De Kruif, C.G. and Holt, C. (2003). Casein micelle structure, functions and interactions, in *Advanced Dairy Chemistry 1: Proteins*, 3rd edn. P.F. Fox and P.L.H. McSweeney, eds., Kluwer Academic/Plenum Publishers, New York. pp. 233–276.

De Kruif, C.G. and May, R.P. (1991). κ-Casein micelles: structure, interaction and gelling studied by small-angle neutron scattering. *Eur. J. Biochem.* **200**, 431–436.

De Kruif, C.G., Tuinier, R., Holt, C., Timmins, P.A. and Rollema, H.S. (2002). Physicochemical study of κ- and β-casein dispersion and the effect of cross-linking by transglutaminase. *Langmuir* **18**, 4885–4891.

Dong, C. and Ng-Kwai-Hang, K.F. (1998). Characterization of a nonelectrophoretic genetic variant of β-casein by peptide mapping and mass spectroscopic analysis. *Int. Dairy J.* **8**, 967–972.

Dosaka, S., Kimura, T., Taneya, S., Sone, T., Kaminogawa, S. and Yamauchi, K. (1980). Polymerization of $\alpha_{s1}$-casein by calcium ions. *Agric. Biol. Chem.* **44**, 2443–2448.

Ecroyd, H., Koudelka, T., Thorn, D.C., Williams, D.M., Devlin, G., Hoffman, P. and Carver, J.A. (2008). Dissociation from the oligomeric state is the rate-limiting step in fibril formation by κ-casein. *J. Biol. Chem.* **283**, 9012–9022.

Ecroyd, H., Thorn, D.C., Lui, Y. and Carver, J.A. (2010). The dissociated form of κ-casein is the precursor to its amyloid fibril formation. *Biochem. J.* **429**, 251–260.

Eigel, W.N., Butler, J.E., Ernstrom, C.A., Farrell, H.M., Jr., Harwalkar, V.R., Jenness, R. and Whitney, R.M. (1984). Nomenclature of proteins of cow's milk: fifth revision. *J. Dairy Sci.* **67**, 1599–1631.

Erhardt, G. (1993). A new $\alpha_{s1}$-casein allele in bovine milk and its occurrence in different breeds. *Anim. Genet.* **24**, 65–66.

Erhardt, G. (1996). Detection of a new κ-casein variant in the milk of Pinzgauer cattle. *Anim. Genet.* **27**, 105–107.

Evans, M.T.A. and Phillips, M.C. (1979). The conformation and aggregation of bovine beta-casein A. II. Thermodynamics of thermal association and the effects of changes in polar and apolar interactions on micellization. *Biopolymers* **18**, 1123–1140.

Farrell, H.M. Jr., Kumosinski, T.F., Thompson, M.P. and Pulaski, P. (1988). Calcium induced associations of the caseins: a thermodynamic linkage approach to precipitation and resolubilization. *Arch. Biochem. Biophys.* **265**, 146–158.

Farrell, H.M. Jr., Kumosinski, T.F., Cooke, P.H., King, G., Hoagland, P.D., Wickham, E.D., Dower, H.J. and Groves, M.L. (1996). Particle size of purified κ-casein: metal effect and correspondence with predicted three-dimensional molecular models. *J. Prot. Chem.* **15**, 435–445.

Farrell, H.M. Jr., Wickham, E.D., Unruh, J.J., Qi, P.X. and Hoagland, P.D. (2001). Secondary structure studies of bovine caseins: temperature dependence of β-casein structure as analyzed by circular dichroism and FTIR spectroscopy and correlation with micellization. *Food Hydrocolloid* **15**, 341–354.

Farrell, H.M. Jr., Cooke, P.H., Wickham, E.D., Piotrowski, E.G. and Hoagland, P.D. (2003). Environmental influences of bovine κ-casein: reduction and conversion to fibrillar (amyloid) structures. *J. Prot. Chem.* **22**, 259–273.

Farrell, H.M., Jr., Jimenez-Flores, R., Bleck, G.T., Brown, E.M., Butler, J.E., Creamer, L.K., Hicks, C.L., Hollar, C.M., Ng-Kwai-Hang, K.F. and Swaisgood, H.E. (2004). Nomenclature of the proteins of cows' milk: sixth revision. *J. Dairy Sci.* **87**, 1641–1674.

Farrell, H.M. Jr., Malin, E.L., Brown, E.M. and Mora-Gutierrez, A. (2009). Review of the chemistry of $\alpha_{s2}$-casein and the generation of a homolgous molecular model to explain its properties. *J. Dairy Sci.* **92**, 1338–1353.

Gagnard, S., Zuev, Y., Gaudin, J.-C., Fedotov, V., Choiset, Y., Axelos, M.A.V., Chobert, J.-M. and Haertle, T. (2007). Modifications of the charges at the N-terminus of bovine β-casein: consequences on its structure and its micellisation. *Food Hydrocolloid* **21**, 180–190.

Garnier, J. (1966). Conformation of β-casein in solution. Analysis of a thermal transition between 5 and 40°C. *J. Mol. Biol.* **19**, 586–590.

Garnier, J., Osguthorpe, D.J. and Robinson, B. (1978). Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* **120**, 97–120.

Gouldsworthy, A.M., Leaver, J. and Banks, J.M. (1996). Application of a mass spectrometry sequencing technique for identifying peptides present in Cheddar cheese. *Int. Dairy J.* **6**, 781–791.

Graham, E.R.B., Malcolm, G.N. and McKenzie, H.A. (1984). On the isolation and conformation of bovine β-casein A1. *Int. J. Biol. Macromol.* **6**, 155–161.

Griffin, M.C.A., Price, J.C. and Martin, S.R. (1986). Effect of alcohols on the structure of caseins: circular dichroism studies of κ-casein A. *Int. J. Biol. Macromol.* **8**, 367–371.

Groenen, M.A.M., Dijkhof, R.E.M., Verstege, A.J.M. and Van der Poel, J.J. (1993). The complete sequence of the gene encoding bovine $\alpha_{S2}$-casein. *Gene* **123**, 187–193.

Grosclaude, F. (1988). Le polymorphisme genetique des principales actoproteines bovines. Relations avec la quantite, la composition et les aptitudes fromageres du lait. *INRA Prod. Anim.* **1**, 5–17.

Grosclaude, F., Mercier, J.-C. and Ribadeau-Dumas, B. (1969). Sur la localisation, dans la séquence COOH-terminale de la caseine alpha-S1 bovine, de la substitution GLU-GLY différenciant les variants génétiques B et C. *CR Acad. Sci. D: Sci. Natur.* **268**, 3133–3136.

Grosclaude, F., Mahe, M.-F., Mercier, J.-C. and Ribadeua-Dumas, B. (1970). Localisation, dans la partie NH2-terminale de la caséine $\alpha_{s1}$ bovine, d'une délétion de 13 acides aminés différenciant le variant a des variants B et C. *FEBS Lett.* **11**, 109–112.

Grosclaude, F., Mahe, M.-F., Mercier, J.-C. and Ribadeau-Dumas, B. (1972). Caracterisation des variants genetiques des caseines $\alpha_{s1}$ et β bovines. *Eur. J. Biochem.* **26**, 328–337.

Grosclaude, F., Mahe, M.-F. and Ribadeau-Dumas, B. (1973). Structure primaire de la caseine $\alpha_{s1}$- et de la caseine β-bovine. *Eur. J. Biochem.* **40**, 323–324.

Grosclaude, F., Mahe, M.-F. and Mercier, J.-C. (1974a). Comparison du polymorphisme genetique des lacto-

proteines du zebu et des bovines. *Ann. Genet. Sel. Anim.* **6**, 305–329.

Grosclaude, F., Mahe, M.-F. and Voglino, G.-F. (1974b). Le variant βE et le code de phosphorylation des caseins bovines. *FEBS Lett.* **45**, 3–5.

Grosclaude, F., Mahe, M.-F., Mercier, J.-C., Bonnemaire, J. and Teissier, J.H. (1976). Polymorphisme des lactoproteines de bovines Nepalais. Polymorphisme des caseine $\alpha_{s2}$-mineurs. *Ann. Genet. Sel. Evol.* **8**, 461–479.

Grosclaude F., Joudrier, P. and Mahe, M.-F. (1978). Polymorphisme de la caseine αS2 bovine: Etroite liaison du locus αS2-Cn avec les loci αs1-Cn, β-Cn et κ-Cn; mise en evidence d'une deletion dans le variant αS2-Cn D. *Ann. Genet. Sel. Anim.* **10**, 313–327.

Grosclaude, F., Mahe, M.-F. and Accolas, J.P. (1982). Note sur le polymorphisme genetique des lactoproteines de bovins et de yaks Mongols. *Ann. Genet. Sel. Anim.* **14**, 545–550.

Groves, M.L. (1969). Some minor components of casein and other phosphoproteins in milk. A review. *J. Dairy Sci.* **52**, 1155–1165.

Groves, M.L., Dower, H.J. and Farrell, H.M. Jr. (1992). Reexamination of the polymeric distributions of κ-casein isolated from bovine milk. *J. Prot. Chem.* **11**, 21–27.

Groves, M.L., Wickham, E.D. and Farrell, H.M. Jr. (1998). Environmental effects on disulphide bonding patterns of bovine κ-casein. *J. Prot. Chem.* **17**, 73–84.

Han, S.K., Shin, Y.C. and Byun, H.D. (2000). Biochemical, molecular and physiological characterization of a new β-casein variant detected in Korean cattle. *Anim. Genet.* **31**, 49–51.

Herskovits, T.T. (1966). On the conformation of casein. ORD properties. *Biochem.* **5**, 1018–1026.

Ho, C. and Waugh, D.F. (1965). Interactions of bovine αs-casein with small ions. *J. Am. Chem. Soc.* **87**, 110–117.

Hoagland, P.D., Unruh, J.J., Wickham, E.D. and Farrell, H.M. Jr. (2001). Secondary structure of bovine $\alpha_{s2}$-casein: theoretical and experimental approaches. *J. Dairy Sci.* **84**, 1944–1949.

Holland, J.W., Deeth, H.C. and Alewood, P.F. (2004). Proteomic analysis of κ-casein microheterogeniety. *Proteomics* **4**, 743–752.

Holland, J.W., Deeth, H.C. and Alewood, P.F. (2005). Analysis of *O*-glycosylation site occupancy in bovine κ-casein glycoforms separated by two-dimensional gel electrophoresis. *Proteomics* **5**, 990–1002.

Holland, J.W., Deeth, H.C. and Alewood, P.F. (2006). Resolution and characterization of multiple isoforms of bovine κ-casein by 2-DE followed by reversible cysteine-tagging enrichment strategy. *Proteomics* **6**, 3087–3095.

Holt, C. and Sawyer, L. (1988). Primary and predicted secondary structures of the caseins in relation to their biological functions. *Protein Eng.* **2**, 251–259.

Holt, C., Parker, T.G. and Dalgleish, D.G. (1975). The thermochemistry of reactions between $\alpha_{s1}$-casein and calcium chloride. *Biochim. Biophys. Acta* **379**, 638–644.

Jann, O., Ceriotti, G., Caroli, A. and Erhardt, G. (2002). A new variant in exon VII of the bovine β-casein gene (CSN2) and its distribution among European cattle breeds. *J. Anim. Breed. Genet.* **119**, 65–68.

Jenness, R., Larson, B.L., McMeekin, T.L., Swanson, A.M., Whitnah, C.H. and Whitney, R.M. (1956). Nomenclature of the proteins of bovine milk. *J. Dairy Sci.* **39**, 536–541.

Jimenez-Flores, R., Kang, Y.C. and Richardson, T. (1987). Cloning and sequence analysis of bovine β-casein cDNA. *Biochem. Biophys. Res. Comm.* **142**, 617–621.

Kajiwara, K., Niki, R., Urakawa, H., Hiragi, Y., Donkai, N. and Nagura, M. (1988). Micellar structure of β-casein observed by small-angle X-ray scattering. *Biochim. Biophys. Acta* **955**, 128–134.

Kumosinski, T.F., Brown, E.M. and Farrell, H.M. Jr. (1991). Three-dimensional molecular modeling of bovine caseins: κ-casein. *J. Dairy Sci.* **74**, 2879–2887.

Kumosinski, T.F., Brown, E.M. and Farrell, H.M. Jr. (1993). Refined molecular modeling of bovine caseins: a refined energy-minimized κ-casein structure. *J. Dairy Sci.* **76**, 2507–2520.

Leclerc, E. and Calmettes, P. (1997a). Interactions in micellar solutions of β-casein. *Physica B* **234–236**, 207–209.

Leclerc, E. and Calmettes, P. (1997b). Interactions in micellar solutions of β-casein. *Phys. Rev. Lett.* **78**, 150–153.

Leclerc, E. and Calmettes, P. (1998). Structure of β-casein micelles. *Physica B* **241–243**, 1141–1143.

Leonil, J., Henry, G., Jouanneau, D., Delage, M.-M., Forge, V. and Putaux, J.-L. (2008). Kinetics of fibril formation of bovine κ-casein indicate a conformational reaarangement as a critical step in the process. *J. Mol. Biol.* **381**, 1267–1280.

Mahe, M.-F. and Grosclaude, F. (1982). Polymorphisme de la caseine $\alpha_{S2}$ des bovines: characterization du variant C du yak (*Bos grunniens*). *Ann. Genet. Sel. Anim.* **14**, 401–416.

Mahe, M.-F., Miranda, G., Queral, R., Bado, A., Souvenir-Zafidrajaona, P. and Grosclaude, F. (1999). Genetic polymorphism of milk proteins in African *Bos taurus* and *Bos indicus* populations characterization of variants $\alpha_{S1}$-Cn H and κ-Cn. *J. Genet. Sel. Evol.* **31**, 239–253.

Malin, E.L., Brown, E.M., Wickham, E.D. and Farrell, H.M. Jr. (2005). Contributions of terminal peptides to associative behavior of αs1-casein. *J. Dairy Sci.* **88**, 2318–2328.

Manson, W., Carolan, T. and Annan, W.D. (1977). Bovine $\alpha_{s0}$-casein; a phosphorylated homologue of $\alpha_{s1}$-casein. *Eur. J. Biochem.* **78**, 411–417.

Mariani, P. and Russo, V. (1975). Varianti genetiche delle α protein del latte nella razza. *Rendena. Riv. Zootec. Vet.* **3**, 345–348.

Mariani, P., Summer, A., Anghinetti, A., Senese, C., Di Gregorio, P., Rando, P. and Serventi, P. (1995). Effects

of the $\alpha_{s1}$-CN G allele on the percentage distribution of caseins $\alpha_{s1}$-, $\alpha_{s2}$-, β-, and κ- in Italian Brown cows. *Ind. Latte* **31**, 3-13.

Mercier, J.-C. (1981). Phosphorylation of the caseins, present evidence for an amino acid triplet code posttranslationally recognized by specific kinases. *Biochimie* **63**, 1–17.

Mercier, J.-C., Grosclaude, F. and Ribadeau-Dumas, B. (1971). Structure primaire de la caseine $\alpha_{s1}$ bovine. Sequence complete. *Eur. J. Biochem.* **23**, 41–51.

Mercier, J.-C., Brignon, G. and Ribadeau-Dumas, B. (1973). Structure primarie de la caseine κ-bovine B. Sequence complete. *Eur. J. Biochem.* **35**, 222–235.

Mikheeva, L.M., Grinberg, N.V., Grinberg, V.Ya., Khokhlov, A.R. and De Kruif, C.G. (2003). Thermodynamics of micellization of bovine β-casein by high-sensitivity differential scanning calorimetry. *Langmuir* **19**, 2913–2921.

Minkiewicz, P., Slangen, C.J., Lagerwerf, F.M., Haverkamp, J., Rollema, H.S. and Visser, S. (1996). Reversed-phase high-performance liquid chromatographic separation of bovine κ-casein macropeptide and characterization of isolated fractions. *J. Chrom. A* **743**, 123–135.

Miranda, G., Anglade, P., Mahe, M.-F. and Erhardt, G. (1993). Biochemical characterization of the bovine genetic kappa-casein C and E variants. *Anim. Genet.* **4**, 27–31.

Molle, D. and Leonil, J. (1995). Heterogeneity of the bovine κ-casein caseino-macropeptide, resolved by liquid chromatography on-line with electrospray ionization mass spectrometry. *J. Chrom. A* **708**, 223–230.

Ng-Kwai-Hang, K.F. and Grosclaude, F. (2003). Genetic polymorphism of milk proteins, in *Advanced Dairy Chemistry 1: proteins*, 3rd edn., P.F Fox and P.L.H. McSweeney, eds., Kluwer Acedemic/Plenum Publishers, New York. pp. 739-816.

Ng-Kwai-Hang, K.F., Hayes, J.F., Moxley, J.E. and Monardes, H.G. (1984). Association of genetic variants of casein and milk serum proteins with milk, fat, and protein production by dairy cattle. *J. Dairy Sci.* **67**, 835–840.

Niki, V.R., Takase, K. and Arima, S. (1977). Über die gestalt and größe des temperaturabhängigen assoziats von β-casein. *Milchwissenschaft* **32**, 577–582.

Noelken, M. and Reibstein, M. (1968). The conformation of β-casein B. *Arch. Biochem. Biophys*. **123**, 397.

O'Connell, J.E., Grinberg, V.Ya. and De Kruif, C.G. (2003). Association behavior of β-casein. *J. Colloid Interf. Sci.* **258**, 33–39.

Ono, T., Kaminogawa, S., Odagiri, S. and Yamauchi, K. (1976). A study on the binding of calcium ions to $\alpha_{s1}$-casein. *Agric. Biol. Chem.* **40**, 1717–1723.

Ono, T., Yahagi, M. and Odagiri, S. (1980). The binding of calcium to κ-casein and *para*-κ-casein. *Agric. Biol. Chem.* **44**, 1499–1503.

Ono T., Yada, R., Yunati, K. and Nakai, S. (1987). Comparison of the comformations of κ-casein, *para*-κ-casein and glycomacropeptide. *Biochim. Biophys. Acta.* **911**, 318–325.

Osta, R., Marcos, S. and Rodellar, C. (1995). A *Mnl*I polymorphism at the bovine $\alpha_{s2}$-casein gene. *Anim. Genet.* **26**, 213.

Parker, T.G. and Dalgleish, D.G. (1981). Binding of calcium ions to bovine β-casein. *J. Dairy Res.* **48**, 71–76.

Parry, R.M. and Carroll, R.J. (1969). Location of κ-casein in milk micelles. *Biochim. Biophys. Acta* **194**, 138–150.

Payens, T.A.J. and Heremans, K. (1969). Effect of pressure on the temperature-dependent association of β-casein. *Biopolymers* **8**, 335–345.

Payens, T.A.J. and Schmidt, P.G. (1965). The thermodynamic parameters of the association of $\alpha_{s1}$-casein C. *Biochim. Biophys. Acta* **109**, 214–222.

Payens, T.A.J. and Schmidt, D.G. (1966). Boundary spreading of rapidly polymerizing αs1-casein B and C during sedimentation. *Arch. Biochem. Biophys.* **115**, 136–145.

Payens, T.A.J. and Van Markwijk, B.W. (1963). Some features of the association of β-casein. *Biochim. Biophys. Acta* **71**, 517–530.

Payens, T.A.J., Brinkhuis, J.A. and Van Markwijk, B.W. (1969). Self-association in non-ideal systems. Combined light scattering and sedimentation measurements in β-casein solutions. *Biochim. Biophys. Acta* **175**, 434–437.

Pepper, L. and Farrell, H.M. Jr. (1982). Interactions leading to formation of casein submicelles. *J. Dairy Sci.* **65**, 2259–2266.

Pisano, A., Packer, N.H., Redmond, J.W., Williams, K.L. and Gooley, A.A. (1994). Characterization of O-linked glycosylation motifs in the glycopeptides domain of bovine κ-casein. *Glycobiology* **4**, 837–844.

Prinzenberg, E.-M., Hiendleder, S., Ikonen, T. and Erhardt, G. (1996). Molecular genetic characterization of new bovine κ-casein alleles CSN3-F and CSN3-G and genotyping by PCR-RFLP. *Anim. Genet.* **27**, 347–349.

Prinzenberg, E.-M., Krause, I. and Erhardt, G. (1999). SCCP analysis of the bovine CSN3 locus discriminates six alleles corresponding to known protein variants (A, B, C, E, F, G) and three new DNA polymorphism (H, I, A1). *Anim. Biotechnol.* **10**, 49–62.

Qi, P.X., Wickham, E.D. and Farrell, H.M. Jr. (2004). Thermal and alkaline denaturation of bovine β-casein. *Prot. J.* **23**, 389–402.

Qi, P.X., Wickham, E.D., Piotrowski, E.G., Fagerquist, C.K. and Farrell, H.M. Jr. (2005). Implication of C-terminal deletion on the structure and stability of bovine β-casein. *Prot. J.* **24**, 431–444.

Raap, J., Kerling, K.E.T., Vreeman, H.J. and Visser, S. (1983). Peptide substrates for chymosin (rennin): conformational studies of κ-casein-related oligopeptides by circular dichroism and secondary structure prediction. *Arch. Biochem. Biophys.* **221**, 117–124.

Rasmussen, L.K., Hojrup, P. and Petersen, T.E. (1992). Localization of two interchain disulfide bridges in dimers of bovine $\alpha_{S2}$-casein. *Eur. J. Biochem.* **203**, 381–386.

Rasmussen, L.K., Hojrup, P. and Petersen, T.E. (1994). Disulphide arrangement in bovine caseins: localization of intrachain disulphide bridges in monomers of κ- and $\alpha_{s2}$-casein from bovine milk. *J. Dairy Res.* **61**, 485–493.

Ribadeau-Dumas, B., Grosclaude, F. and Mercier, J.-C. (1970). Localization in the peptide chain of bovine beta casein of the His-Gln substitution differentiating the A2 and A3 genetic variants. *CR Acad. Sci. D: Sci. Natur.* **270**, 2369–2372.

Ribadeau-Dumas, B., Brignon, G., Grosclaude, F. and Mercier, J.-C. (1972). Structure primaire de la casein β bovine. *Eur. J. Biochem.* **25**, 505–514

Rollema, H.S., Brinkhuis, J.A. and Vreeman, H.J. (1988) 1H-NMR studies of bovine kappa-casein and casein micelles. *Neth. Milk Dairy J.* **42**, 233–248.

Saito, T. and Itoh, T. (1992). Variations and distributions of *O*-glycosidically linked sugar chains in bovine κ-casein. *J. Dairy Sci.* **75**, 1768–1774.

Sawyer, L. and Holt, C. (1993). The secondary structure of milk proteins and their biological function. *J. Dairy Sci.* **76**, 3062–3078.

Schmidt, D.G. (1969). *On the Association of $\alpha_{s1}$-Casein*, Ph.D. Thesis, University of Utrecht.

Schmidt, D.G. (1970a). Differences between the association of the genetic variants B, C and D of αs1-casein. *Biochim. Biophys. Acta* **221**, 140–142.

Schmidt, D.G. (1970b). The association of $\alpha_{s1}$-casein B at pH 6.6. *Biochim. Biophys. Acta* **207**,130–138.

Schmidt, D.G. (1982). Association of caseins and casein micelle structure, in, *Developments in Dairy Chemistry* 1, P.F. Fox, ed., Elsevier Applied Science, London. pp. 61–86.

Schmidt, D.G. and Payens, T.A.J. (1972). The evaluation of positive and negative contributions to the second virial coefficient of some milk proteins. *J. Coll. Interface Sci.* **39**, 655–662.

Schmidt, D.G. and Van Markwijk, B.W. (1968). Further studies on the associating subunit of $\alpha_{s1}$-casein. *Biochim. Biophys. Acta* **154**, 613–614.

Senocq, D., Molle, D., Pochet, S., Leonil, J., Dupont, D. and Levieux, D. (2002). A new bovine β-casein genetic variant characterized by a Met93→Leu93 substitution in the sequence A2. *Lait* **82**, 171–180.

Smyth, E., Syme, C.D., Blanch, E.W., Hecht, L., Vasak, M. and Barron, L.D. (2001). Solution structure of native proteins with irregular folds from Raman optical activity. *Biopolymers* **58**, 138–151.

Snoeren, T.H.M., Van Markwijk, B. and Van Montfort, R. (1980). Some physicochemical properties of bovine $\alpha_{s2}$-casein. *Biochim. Biophys. Acta* **622**, 266–276.

Stewart, A.F., Bonsing, J., Beattie, C.W., Shah, F., Willis, I.M. and Mackinlay, A.G. (1987). Complete nucleotide sequences of bovine $\alpha_{s2}$- and β-casein cDNAs: comparisons with related sequences in other species. *Mol. Biol. Evol.* **4**, 231–241.

Sulimova, G.E., Sokolova, S.S., Semikozova, O.P., Nguet, L.M. and Berberov, E.M. (1992). Analysis of DNA polymorphisms of clustered genes in cattle: casein genes and genes of the major histocompatibility complex (BOLA). *Tsitol. I Genetika* **26**, 18–26.

Swaisgood, H. E. (1982). Chemistry of milk proteins, in, *Developments in Dairy Chemistry 1: Proteins*. P.F. Fox, ed., Applied Science Publishers, New York. pp. 1–59.

Swaisgood, H.E. (1992). Chemistry of the caseins, in, *Advanced Dairy Chemistry 1: Proteins*, 2nd edn. P.F. Fox, ed., Elsevier Applied Science, New York. pp. 63–110.

Swaisgood, H.E. (2003). Chemistry of caseins, in, *Advanced Dairy Chemistry 1: Proteins*, 3rd edn. P.F. Fox and P.L.H. McSweeney, eds., Kluwer Academic/Plenum Publishers, New York. pp. 139–201.

Swaisgood, H.E. and Timasheff, S.N. (1968). Association of $\alpha_{s1}$-casein C in the alkaline pH range. *Arch. Biochem. Biophys.* **125**, 344–361.

Swaisgood, H.E., Brunner, J.R. and Lillevik, H.A. (1964). Physical parameters of κ-casein from cow's milk. *Biochem.* **3**, 1616–23.

Syme, C.D., Blanch, E.W., Holt, C., Jakes, R., Goedert, M., Hecht, L. and Barron, L.D. (2002). A Raman optical activity study of rheomorphism in casein, synucleins and tau. New insight into the structure and behavior of natively unfolded proteins. *Eur. J. Biochem.* **269**, 148–156.

Tai, M.-S. and Kegeles, G. (1984). A micelle model for the sedimentation behavior of bovine β-casein. *Biophys. Chem.* **20**, 81–87.

Takase, K., Niki, R. and Arima, S. (1980). A sedimentation equilibrium study of the temperature-dependent association of bovine β-casein. *Biochim. Biophys. Acta* **622**, 1–8.

Talbo, G.H., Suckau, D., Malkoski, M. and Reynolds, E.C. (2001). MALDI-PSD-MS analysis of the phosphorylation sites of caseinomacropeptide. *Peptides* **22**, 1093–1098.

Talbot, B. and Waugh, D.F. (1970). Micelle-forming characteristics of monomeric and covalent polymeric κ-caseins. *Biochem.* **9**, 2807–2813.

Tanford C. (1962). Contribution of hydrophobic interactions to the stability of the globular conformation of proteins. *J. Am. Chem. Soc.* **84**, 4240–4274.

Tauzin, J., Miclo, L., Roth, S., Molle, D. and Gaillard, J.-L. (2003). Tryptic hydrolysis of bovine $\alpha_{s2}$-casein: identification and release kinetics of peptides. *Int. Dairy J.* **13**, 15–27.

Thompson, M.P. and Farrell, H.M. Jr. (1974). Genetic variants of the milk proteins, in, *Lactation*, Vol. III, B.L. Larson and V.R. Smith, eds., Academic, New York. pp. 109–134.

Thompson, M.P., Kalan, E.B. and Greenberg, R. (1967). Properties of caseins modified by treatment with carboxypeptidase A. *J. Dairy Sci.* **50**,767–769.

Thorn, D.C., Meehan, S., Sunde, M., Rekas, A., Gras, S.L., MacPhee, C.E., Dobson, C.M., Wilson, M.R. and Carver, J.A. (2005). Amyloid fibril formation by bovine milk κ-casein and its inhibition by the

molecular chaperones $\alpha_s$- and β-casein. *Biochem* **44**, 17027–17036.

Thorn, D.C., Ecroyd, H., Sunde, M., Poon, S. and Carver, J.A. (2008). Amyloid fibril formation by bovine milk $\alpha_{s2}$-casein occurs under physiological conditions yet is prevented by its natural counterpart, αs1-casein. *Biochem.* **47**, 3926–3936.

Thurn, A., Burchard, W. and Niki, R. (1987). Structure of casein micelles. I. Small angle neutron scattering and light scattering from β-casein and κ-casein. *Colloid Polymer Sci.* **265**, 653–666.

Toma, S.J. and Nakai, S. (1973). Calcium sensitivity and molecular-weight of casein-$\alpha_{s5}$. *J. Dairy Sci.* **56**, 1559–1562.

Trieu-Cuot, P. and Gripon J.-C. (1981). Electrofocusing and two-dimensional electrophoresis of bovine caseins. *J. Dairy Res.* **48**, 303–310.

Visser, S., Slangen, C.J., Lagerwerf, F.M., Van Dongen, W.S. and Haverkamp, J. (1995). Identification of a new variant of bovine beta-casein using reversed-phase high-performance liquid-chromatography and mass-spectrometric analysis. *J. Chrom. A* **711**, 141–150.

Vreeman, H.J. (1979). The association of bovine SH-κ-casein at pH 7.0. *J. Dairy Res.* **46**, 271–276.

Vreeman, H.J., Both, P., Brinkhuis, J.A. and Van der Spek, C. (1977). Purification and some physicochemical properties of bovine κ-casein. *Biochim. Biophys. Acta* **491**, 93–103.

Vreeman, H.J., Brinkhuis, J.A. and Van der Spek, C. (1981). Some association properties of bovine SH-κ-casein. *Biophys. Chem.* **14**, 185–193.

Vreeman, H.J., Visser, S., Slangen, C.J. and Van Riel, J.A.M. (1986). Characterization of bovine κ-casein fraction and the kinetics of chymosin-induced macropeptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance gel-permeation chromatography. *Biochem. J.* **240**, 87–97.

Whitney, R.M., Brunner, J.R., Ebner, K.E., Farrell, H.M. Jr., Josephson, R.V., Morr, C.V. and Swaisgood, H.E. (1976). Nomenclature of the proteins of cow's milk: fourth revision. *J. Dairy Sci.* **59**, 795–815.

Yamuachi, K., Takemoto, S. and Tsugo, T. (1967). Calcium-binding property of dephosphorylated casein. *Agric. Biol. Chem.* **31**, 54–63.

Yan, S.-C.B. and Wold, F. (1984). Neoglycoproteins: in vitro introduction of glycosyl units at glutamines in β-casein using transglutaminase. *Biochem.* **23**, 3759–3765.