Molecular and Cellular Biochemistry **87**: 5–30, 1989.

Review

Caseins of various origins and biologically active casein peptides and oligosaccharides: Structural and physiological aspects

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Received 7 June 1988; accepted 29 November 1988

Key words: caseins, biologically active casein peptides, casein oligosaccharides, casein structures, immunomodulators, platelet function

Summary

The first part of the present review is focused on structural aspects concerning the so far studied casein fractions of various origins: they are compared to the four classical major bovine caseins (α_{s1}-, α_{s2}-, β- and κ). The calcium-sensitive casein fractions are always phosphorylated whereas κ-caseins are glycosylated. The study of the casein genes showed that the calcium-sensitive caseins diverged from a common ancestral gene and during the evolution, intergenic and intragenic duplications occurred. The considerable conservation of the phosphorylation sites emphasizes the importance of phosphorylated residues for the function of caseins, i.e. the formation of micelles and the binding of Ca^{2+}. In κ-caseins all the prosthetic sugar groups are linked by O-glycosidic linkages: their number varies from 0 to 5 in bovine κ-casein and up to 10 in human κ-casein. The structures of the known κ-casein carbohydate moieties are described. Finally the milk clotting process (interaction κ-casein/chymosin) is compared to the blood clotting process (interaction fibrinogen/thrombin): a large number of similarities could be noted between both clotting phenomena.

The second part of the review is devoted to the study of short casein peptides endowed with various biological activities. Some of them behaved as immunomodulators or casomorphins or angiotensin I converting enzyme inhibitors; others demonstrated an effect on platelet functions. A 'strategic zone' containing immunostimulating and opioid peptides could be located in cow and human β-caseins. Furthermore bitter peptides, emulsifying peptides, calcium absorption enhancing peptides, chymosin-inhibiting peptides, have also been described and several further properties have been attributed to the κ-caseinoglycopeptide; two tetrasaccharides isolated from the latter possess blood group activities.

In conclusion caseins, the main milk proteins, should not only be considered as a nutriment but as a possible source of biologically active components.

If, in the future, some of the discussed active peptides cannot be characterized *in vivo*, they can all, nevertheless, be synthesized and used either as food additives or in pharmacology.

Introduction

Bovine milk proteins have been extensively studied (for review articles, [1–7]) whereas proteins of other milks have by far been less investigated. Even human milk, essential until relatively recent times to the survival of the human species, has been submitted only to a limited number of studies.

There are only a few major classes of milk proteins which are synthesized in the mammary gland as larger precursors: they are split by a protease and, after possible post-translational modification(s) in the Golgi apparatus, are then excreted.

Casein is the most abundant milk protein and is secreted almost exclusively as large calcium-dependent aggregates termed micelles [8]. Casein is defined as the protein fraction precipitated from raw skim milk by acidification at pH 4.6 and 20 °C. It is not a single entity but is in fact a heterogeneous group of phosphoproteins secreted during lactation in response to the lactogenic hormones, prolactin and hydrocortisone [9].

Four major bovine caseins (α_{s1}, α_{s2}, β and κ) have been identified. The α_s-caseins are precipitated by a very small concentration of calcium, β-casein by moderate concentrations of calcium, whereas κ-casein is calcium insensitive: it is essential for micelle formation and is also glycosylated. κ-Casein is the sole substrate of chymosin during the primary phase of milk coagulation.

The present review is focused on a series of achievements realized in the research field of caseins from different origins. It is dealing in a first part with their protein and gene structures and their post-translational modifications (phosphorylation and prosthetic sugar groups). The milk clotting process (interaction κ-casein/chymosin) will then be compared to the blood clotting process (interaction fibrinogen/thrombin) and numerous points common to both coagulation phenomena will be discussed. Finally we want to point out that caseins should not only be considered as a nutriment but as a source of biologically active peptides playing important physiological roles: the latter will be extensively discussed.

Sequence data (proteins and genes)

Amino acid sequences of caseins established by chemical techniques (manual or automated Edman degradation)

The complete amino acid sequences of cow α_{s1}-, α_{s2}-, β- and κ-caseins have been established before 1980. For human casein, only the β- and κ-casein sequences are known. In general κ-casein was the most studied casein fraction: four κ-caseins of different origins (cow, goat, ewe and human) and seven κ-caseinoglycopeptides (the C-terminal third of κ-casein) have been investigated. All these fractions are constituted by single polypeptide chains.

α_{s1}-Casein (bovine origin) (Fig. 1)

For this protein, five variants A, B, C, D, E have been characterized. The complete amino acid sequence of bovine α_{s1}-casein B was established by Mercier *et al.* [10]. The salient features of this protein of 199 amino acids are a higher number of acidic than of basic residues; a high amount (8.5%) with an uniform distribution of prolyl residues; an absence of cysteinyl residues; three hydrophobic (residues 1→44, 90→113 and 132→199) and two hydrophilic regions (residues 45→89 and 114→131). The phosphorylated sites have also been identified: 8–9 phosphate groups are linked to hydroxyamino acid residues (Fig. 1). The region from residues 62 to 70 with its four phosphoseryls is similar to the region from residues 13 to 21 in bovine β-casein. The C and D variants differ from the B variant by the substitutions of Gly/Glu in position 192 and of the P Thr/Ala in position 53, respectively. The E variant differs from the B variant by two substitutions: Lys/Gln in position 59 and Gly/Glu in position 192. The A variant is characterized by a deletion of 13 amino acid residues (from residues 14 to 26). Bovine α_{s0}-casein has been shown to differ from α_{s1}-casein B only with respect to its phosphate content.

α_{s2}-Casein (bovine origin) (Fig. 2)

A polymorphism of α_{s2}-casein was detected by gel electrophoresis [11]. Brignon *et al.* [12] described the complete amino acid sequence of bovine α_{s2}-casein-Cn A. The latter contains 2 cysteinyl residues and a fairly high number [11] of phosphate groups. The peptide chain contains 207 amino acids and carries a large number of positive charges located all along the chain especially in the 47-amino acid long C-terminal fragment. The phosphate groups have been located in three regions (residues 8→16, 56→61 and 129→143). An internal sequence homology was ob-

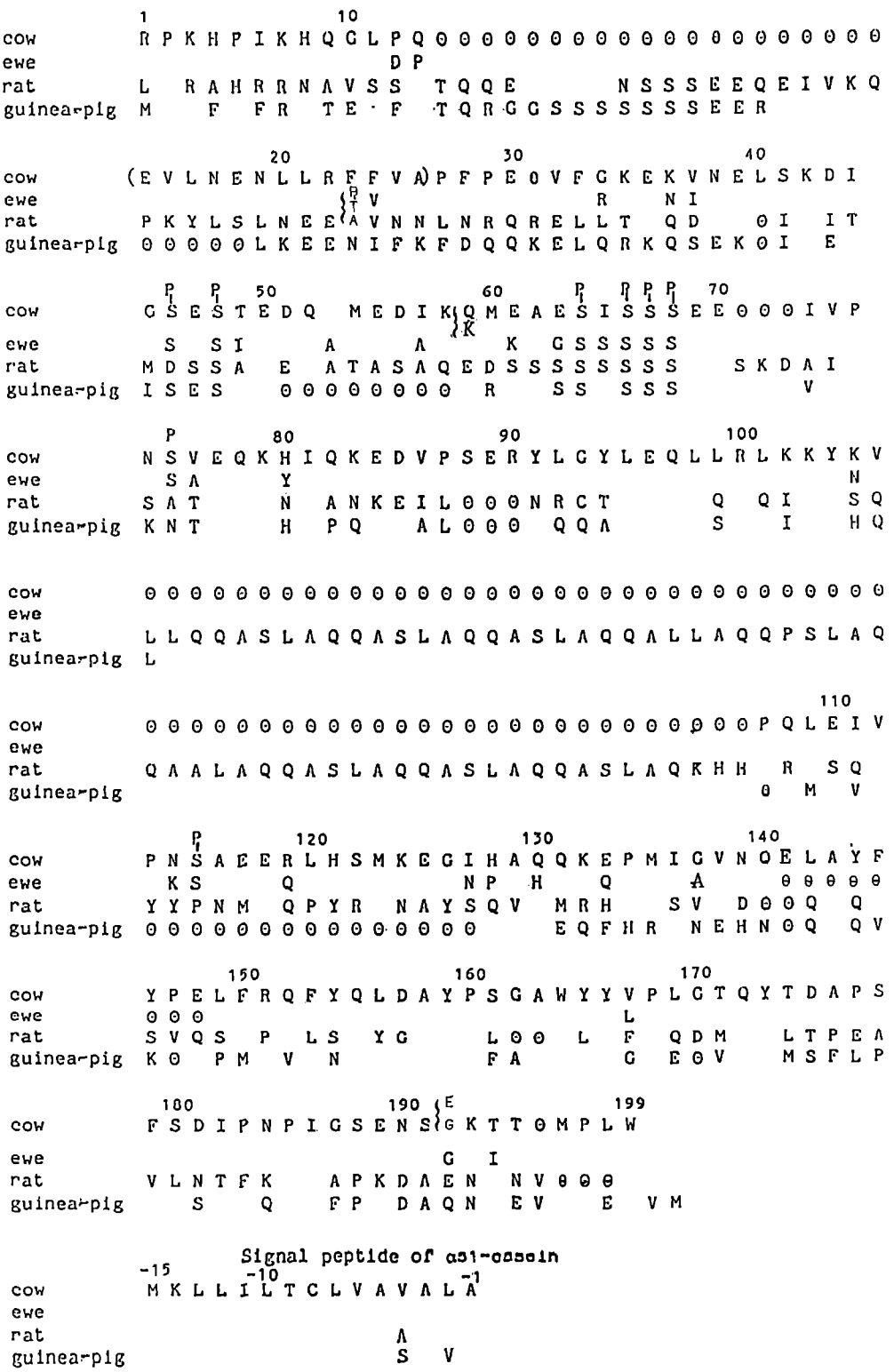


Fig. 1. Amino acid sequence of cow α_{s1}-casein: comparison with ewe α_{s1}-casein, rat α-casein and guinea pig B-casein [10, 38, 31, 35].

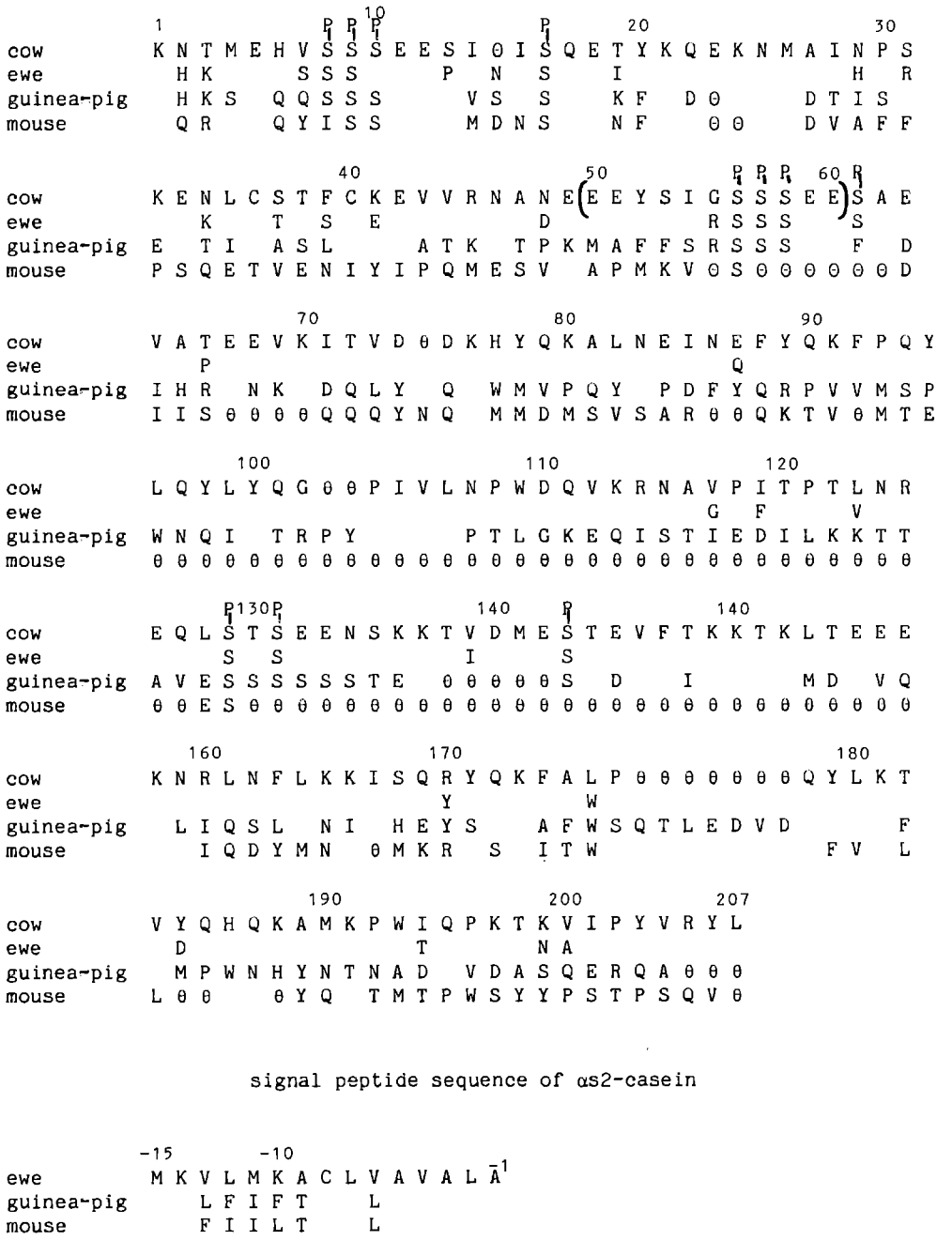


Fig. 2. Amino acid sequence of cow α_{s2}-casein: comparison with ewe α_{s2}-casein, guinea-pig A-casein and mouse ϵ-casein [12, 39, 34, 36].

served between two segments of 76 residues: they contain 38% identical residues; thus α_{s2}-casein originates from the duplication of a primitive gene. The primary structure of the variant α_{s2}-Cn D differs from that of the reference variant α_{s2}-Cn A by the deletion of a 9 amino acid long peptide situated between residues 50–60.

α_{s2}-, α_{s3}-, α_{s4}- and α_{s6}-caseins have identical peptide chains and seem to differ only by their phosphate content (10–13 phosphate groups/mol) and are now all called α_{s2}-casein.

β-Casein

β-Casein (from ruminants) (Fig. 3)
Seven variants of β-casein (A^1, A^2, A^3, B, C, D, E) have been discovered by Grosclaude *et al.* [13]. The sequence of bovine β-casein A^2, established by Ribadeau-Dumas *et al.* [14], is constituted by 209 amino acids including five phosphoseryls. β-Casein can be ranged among the most hydrophobic proteins and has a high proline content (16.7%). The fragment containing the first 50 residues displays a high negative net charge. Minor caseins have previously been isolated and constitute fragments of β-casein. Compared to βA^2, the three variants βA^1, βB and βC differ all by a Pro/His substitution (residue 67). In addition, in βB Ser (residue 122) is replaced by Arg and in βC Glu (residue 37) by Lys whereas Ser (residue 35) is no more phosphorylated. Compared to βA^2, in the variant βA^3 His (residue 106) is replaced by Gln, in the variant D $^{P}_{Ser}$ (residue 18) by Lys and in the variant E Glu (residue 36) by Lys. The sequence of water buffalo β-casein (15) has been partially described and is quite related to bovine β-casein. Only five amino acid substitutions have so far been observed.

β-Casein (human origin) (Fig. 3)
The primary structure of human β-casein has been determined by Greenberg *et al.* [16]. For each form of this phosphorylated protein (0–5 P/mol), phosphorylated sites at specific seryl and threonyl residues have been identified and located near the amino terminus within the first 10 residues of this 212-amino acid long molecule. Sequence comparison of human β-casein with the bovine protein reveals 47% identity. The location of prolyl and the charged residues is generally conserved. The sequence data indicate the existence of a genetic polymorphism involving uncharged residues in human β-casein.

κ-Casein

During the primary phase of the milk clotting process, κ-casein is the unique casein fraction affected by chymosin. Jollès *et al.* [3, 17, 18] established that at the beginning of the enzymic reaction, a Phe-Met linkage (residues 105→106) was specifically split in cow as well as in sheep κ-caseins. An insoluble part, para-κ-casein (N-terminal moiety of κ-casein) was formed and a soluble κ-caseinoglycopeptide (C-terminal moiety of κ-casein) was released. κ-Casein also plays a major role in the stabilization of the casein micelles in their natural environment. The heterogeneity of cow κ-casein is due to the genetic variants A and B as well as to the prosthetic sugar groups because κ-casein is the sole glycosylated component among the casein fractions.

κ-Casein (bovine origin) (Fig. 4)
The sequence of bovine κ-casein has been established by Jollès *et al.* [19] and Mercier *et al.* [20]. Bovine κ B-casein is made up of a single polypeptide chain containing 169 amino acids with an N-terminal pyroglutamic acid residue. Para-κ-casein is a very hydrophobic molecule whereas κ-caseinoglycopeptide is very hydrophilic: thus κ-casein is an amphipatic protein.

κ-Casein (human origin) (Fig. 4)
The 158-residues long sequence of human κ-casein has been established by Brignon *et al.* [21]. Only 22% of the residues are identical in homologous positions with goat, ewe, cow and rat κ-caseins (Fig. 4). The rate of divergence of the 93-amino acid long N-terminal segment (para-κ-casein) appears to be higher than that of the remaining part of the molecule.

κ-Casein (other origins) (Fig. 4)
The primary structures of sheep κ A-casein and goat κ-casein [22–24] have been established. When compared to cow κA-casein (Fig. 4), sheep κA- and goat caseins present two insertions and 26 or 28 replacements, respectively [22–24].

κ-Caseinoglycopeptides (different origins) (Fig. 4)
The preparation of a κ-caseinoglycopeptide is easier than that of κ-casein. When milk is treated with a low amount of chymosin (EC 3.4.23.4), κ-casein is digested: para-κ-casein precipitates and the κ-caseinoglycopeptide remains soluble. The amino acid sequences of the κ-caseinoglycopeptide from seven species (cow, zebu, water buffalo, goat, sheep, pig and human) have been established [23–27, 29]. During the course of the evolution, κ-casein, the casein component stabilizing the micelles, under-

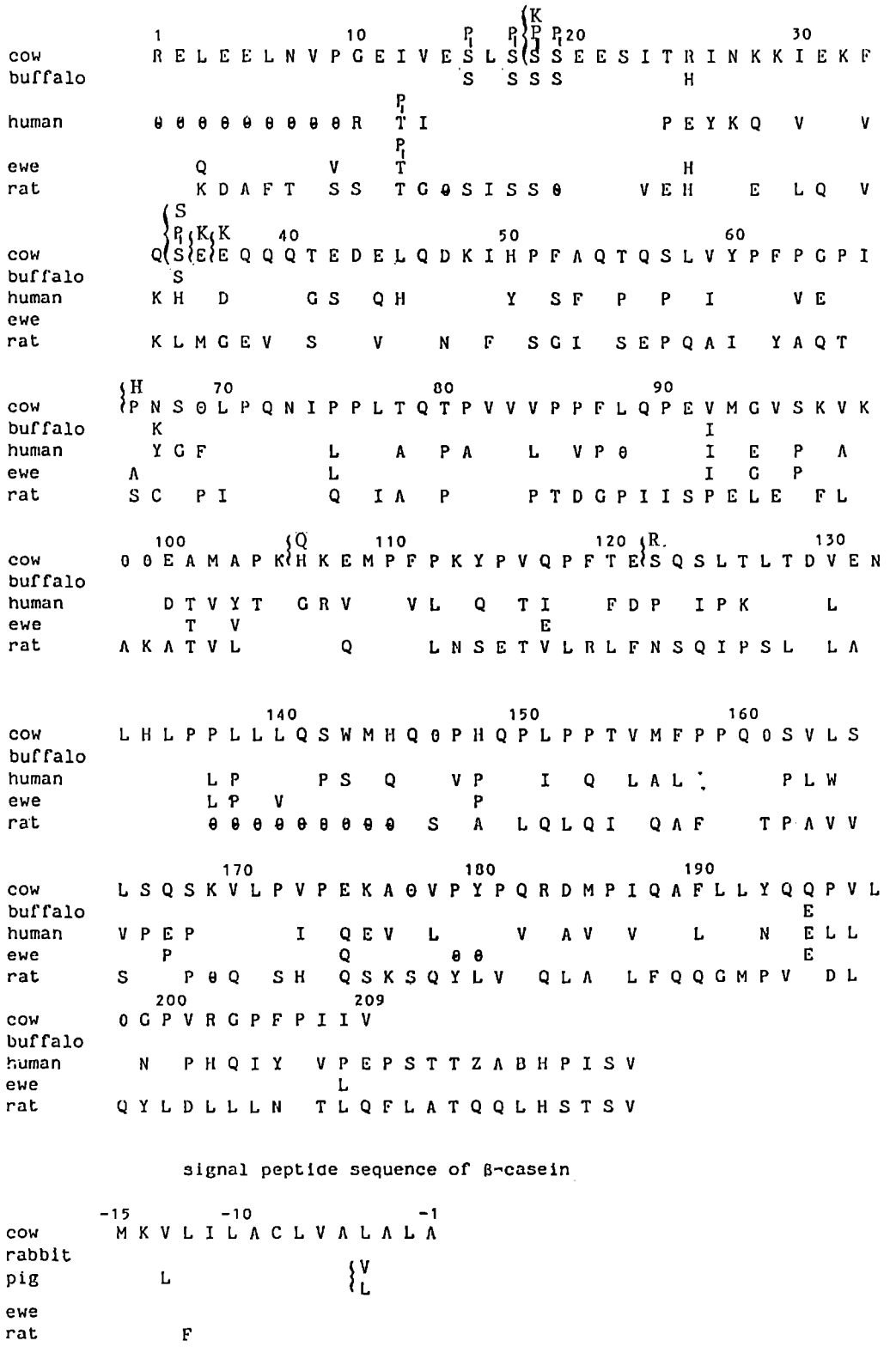
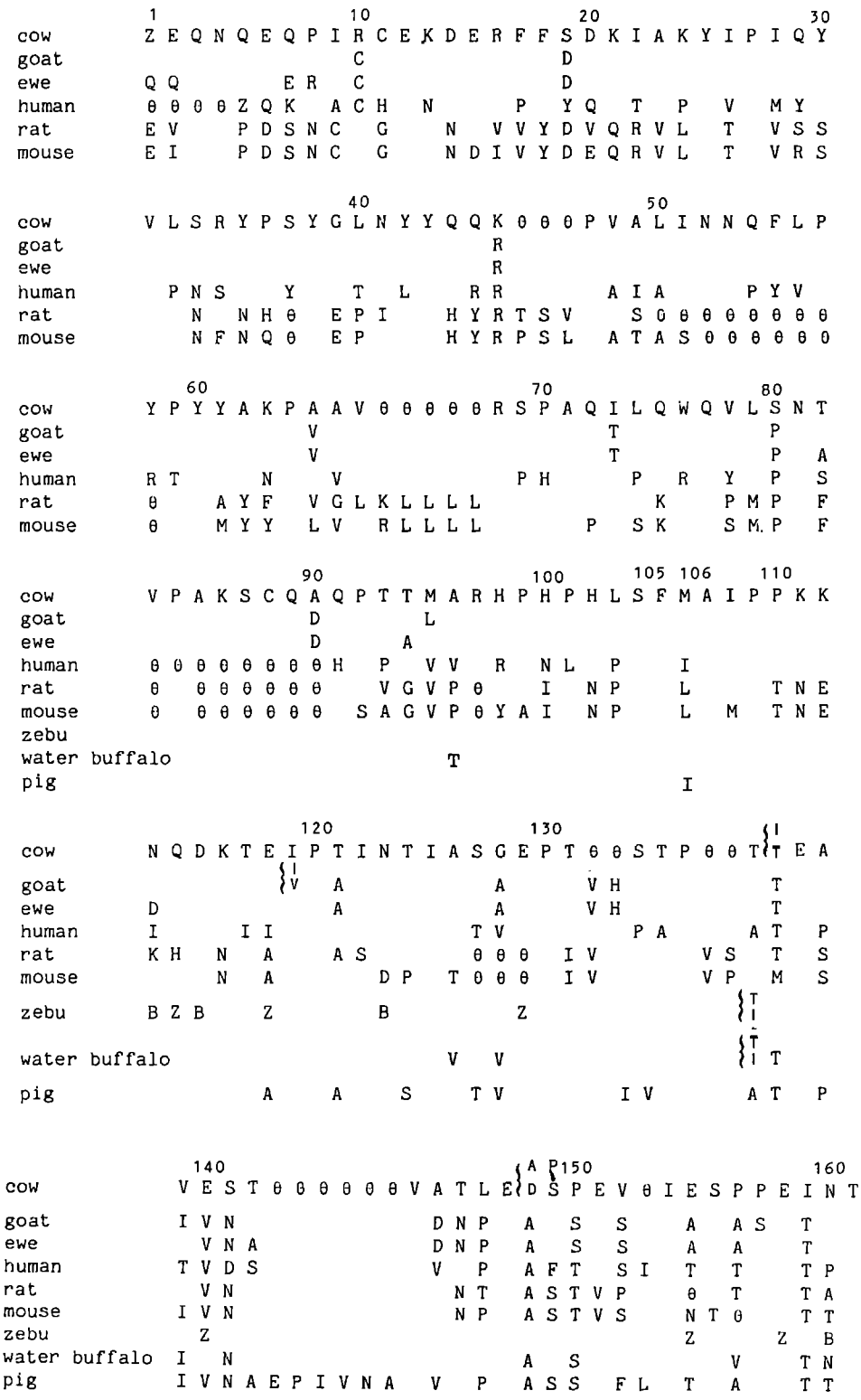


Fig. 3. Amino acid sequence of cow β-casein: comparison with water buffalo, human, ewe and rat β-caseins [14–16, 38, 32].


1 10 20 30
cow Z E Q N Q E Q P I R C E K D E R F F S D K I A K Y I P I Q Y
goat C D
ewe Q Q E R C D
human θ θ θ θ Z Q K A C H N P Y Q T P V M Y
rat E V P D S N C G N V V Y D V Q R V L T V S S
mouse E I P D S N C G N D I V Y D E Q R V L T V R S
40 50
cow V L S R Y P S Y G L N Y Y Q Q K θ θ θ P V A L I N N Q F L P
goat R
ewe R
human P N S Y T L R R A I A P Y V
rat N N H θ E P I H Y R T S V S θ θ θ θ θ θ θ θ
mouse N F N Q θ E P H Y R P S L A T A S θ θ θ θ θ θ
60 70 80
cow Y P Y Y A K P A A V θ θ θ θ θ R S P A Q I L Q W Q V L S N T
goat V T P
ewe V T P A
human R T N V P H P R Y P S
rat θ A Y F V G L K L L L L K P M P F
mouse θ M Y Y L V R L L L L P S K S M P F
90 100 105 106 110
cow V P A K S C Q A Q P T T M A R H P H P H L S F M A I P P K K
goat D L
ewe D A
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zebu
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cow N Q D K T E I P T I N T I A S G E P T θ θ S T P θ θ T T E A
goat V A A V H T
ewe D A A V H T
human I I I T V P A A T P
rat K H N A A S θ θ θ I V V S T S
mouse N A D P T θ θ θ I V V P M S
zebu B Z B Z B Z T I
water buffalo V V T I T
pig A A S T V I V A T P
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cow V E S T θ θ θ θ θ θ V A T L E D S P E V θ I E S P P E I N T
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ewe V N A D N P A S S A A T
human T V D S V P A F T S I T T T P
rat V N N T A S T V P θ T T A
mouse I V N N P A S T V S N T θ T T
zebu Z Z Z B
water buffalo I N A S V T N
pig I V N A E P I V N A V P A S S F L T A T T

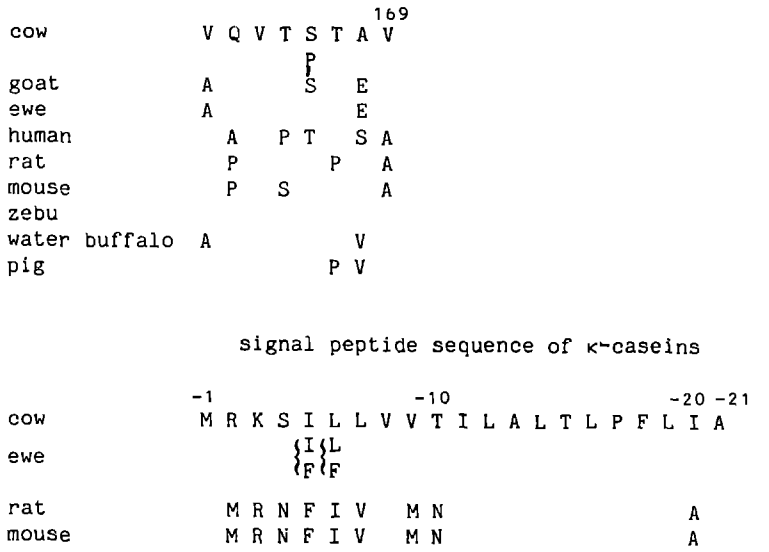


Fig. 4. Amino acid sequence of cow κ-casein: comparison with goat, ewe, human, rat and mouse κ-caseins and with zebu, water buffalo and pig κ-caseinoglycopeptides [19–25, 33, 37].

went extensive sequence changes including insertions or deletions. The rate of change of the κ-caseinoglycopeptides is among the most rapid of all so far studied protein families as previously noted for the fibrinopeptides. Nevertheless the overall acidic character of the κ-caseinoglycopeptides is preserved, suggesting that negative electrical charges are involved in the stabilizing properties of κ-casein. The striking conservation of the primary structure around the protease-sensitive peptide bond 105→106, whose unusual lability depends on the integrity of the nearby sequence, stresses the biological importance of the release of the κ-caseinoglycopeptide and the subsequent clotting of micelles which normally occurs in the digestive tract.

Signal peptides (Figs. 1–4)

α_{s1}-, α_{s2}-, β- and κ-caseins are synthesized as preproteins with hydrophobic transient amino terminal extensions that are selectively removed during the transfer of these proteins through the endoplasmic reticulum membranes according to the mechanism outlined in the 'signal' concept [30]. The fact that the sequences of the casein signal peptides have been highly conserved in the course of evolution suggests that the integrity of the overall structure of this transient extension is necessary to allow an efficient transfer of these caseins [30].

Amino acid sequences of caseins deduced from nucleotide sequences by molecular biology techniques

The recombinant DNA technology allowed to deduce further casein sequences, especially from non-ruminants. In rodents and humans, the casein types differ from the cow components and the nomenclature used for the bovine casein fractions cannot always be used. In rat, four caseins (α, β, γ, κ) have been characterized but rat γ-casein does not correspond to any of the bovine caseins. In guinea-pig, three caseins (one casein A and two casein B variants) have been isolated. In rabbit, α_s- and κ-casein homologs have been identified as well as a third component termed χ-casein. In mouse, eight caseins have been identified: two α-caseins, two β-caseins, one γ-casein, one δ-casein, one ε-casein and one κ-casein. Figure 5 indicates the correspondance between the bovine casein fractions and other casein components whose amino acid sequences have been established. In human only a β-casein and a κ-casein have

	Bovine casein fractions				
Origin	αs1	αs2	β	κ	
Ewe	αs1	αs2	β	κ	
Goat				κ	
Buffalo			β		
Human			β	κ	
Rat	α		β	κ	γ
Guinea-pig	B	A			
Mouse		ε		κ	

Fig. 5. Relationship between the classical bovine casein fractions and the caseins of various origins whose sequences have so far been established.

so far been characterized.

cDNA clones corresponding to mRNAs encoding specific milk proteins have been isolated from rat, mouse, rabbit, guinea-pig, human and bovine mammary specific cDNA libraries and many of them have been completely sequenced.

Rodent caseins

Rat casein

First, results concerning rat caseins are reported as the molecular biology study in this field is the most advanced.

The complete sequence of rat α-casein mRNA has been determined by Hobbs and Rosen [31]. The 1402 nucleotide α-casein mRNA encodes a 15-amino acid long signal peptide and the mature protein (269 residues). Significant homology was detected between rat α- and bovine α_{s1}-caseins (Fig. 1). A unique feature of the rat α-casein sequence is an insertion in the coding region containing 10 repeated elements of 18 nucleotides each.

The complete sequence of a 1072 nucleotide β-casein cDNA insertion in the hybrid plasmid pC β 23 has been determined by Blackburn *et al.* [32]. The deduced 216-amino acid long sequence compared to β-casein sequences from several species shows that approximately 38% of the amino acids have been conserved in the rat, ovine, bovine and human sequences: these conserved amino acids occur in clusters throughout the protein (Fig. 3). One such cluster containing the majority of the potential casein phosphorylation sites is located near the amino terminus. 14 out of 15 amino acids in the signal peptide sequence of the precasein are identical between the rat and bovine β-precaseins.

The complete sequence of rat γ-casein mRNA has been determined by Hobbs and Rosen [31]. The 864 nucleotide γ-casein mRNA encodes a 15-amino acid long signal peptide and a mature protein of 164 residues (Fig. 6). No significant homology appears between rat γ-casein and other caseins. However comparison of the rat γ-casein sequence with the ovine α_{s1}-, α_{s2}-caseins revealed two regions of homology corresponding to the signal peptide and the phosphorylation sites [31].

An 820-nucleotide long cDNA clone for the κ-casein from rat mammary gland was isolated and its nucleotide sequence determined by Nakhasi *et al.* [33]. The deduced amino acid sequence revealed a 21-amino acid long signal peptide and a mature protein of 157 amino acids. The signal peptide of rat κ-casein was highly homologous to that of the preprotein of ovine κ-casein. A low homology (49%) was apparent when κ-casein from mature cow milk was compared with rat κ-casein (Fig. 4).

Guinea-pig casein

The nucleotide sequence (1036 bases) of guinea-pig casein A mRNA has been determined by Hall *et al.* [34]. The deduced 223-amino acid long sequence of guinea-pig precasein A exhibited 30% homology with bovine α_{s2}-casein (Fig. 2): the most striking similarities include the location of potential phosphorylation sites and similar positions for the two cysteinyl residues.

Nucleotide sequence analysis of cloned guinea-pig casein B cDNA established by Hall *et al.* [35] allowed to identify two casein variants related to the bovine and rat α_{s1}-caseins (Fig. 1). The deduced

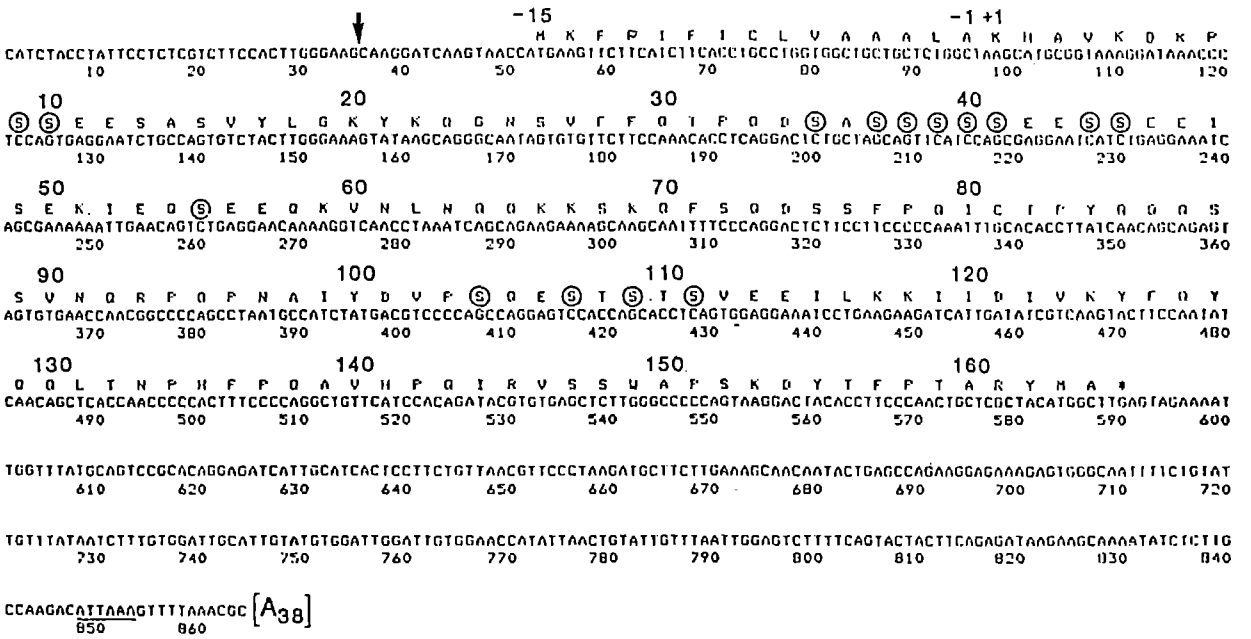


Fig. 6. The complete nucleotide and the encoded amino acid sequence of the rat pre-γ-casein mRNA [31].

amino acid sequences comprised a 15-amino acid long hydrophobic signal peptide followed by the 150 or 165 amino acids of the mature secreted proteins. Amino acid homology was restricted to the bovine or rat phosphorylation sites and to the signal precursor sequences.

Mouse casein

Hennighausen *et al.* [36] isolated cDNA clones which correspond to the mRNA coding for the smallest of the mouse caseins, named ε-casein. The deduced amino acid sequence revealed a 15-amino acid long signal peptide and a mature protein of 128 amino acids. Mouse ε- and cow α_{s2}-caseins show amino acid sequence homologies situated in the N-terminal regions of the mature proteins (Fig. 2). The signal peptide of mouse ε-casein is homologous to the signal peptides of the calcium precipitable caseins of other species. Mouse ε-casein contains a Ser-Ser-Glu-Glu sequence which is a site for potential multiple phosphorylations.

A cDNA clone for κ-casein mRNA from the lactating mouse mammary gland was isolated and its nucleotide sequence determined by Thompson *et al.* [37]. Analysis of the deduced amino acid sequence revealed a precursor protein with a 21-amino acid long signal sequence and a mature protein of 160 amino acids, the mature mouse protein being 3 amino acids longer than the rat κ-casein (Fig. 4). Comparison of the deduced amino acid sequence of mouse κ-casein with that of the rat showed 85% homology. However, when the amino acid sequences of rat and mouse κ-caseins were compared with ovine κ-casein, only a 45% homology was observed.

Ruminant caseins

Ovine casein

The primary structure of the mRNA coding for ovine α_{s1}-casein was determined by Mercier *et al.* [38]. The deduced 206-amino acid long sequence of ovine pre-α_{s1}-casein differed from that of its bovine counterpart (genetic variant B) by 24 amino acid substitutions and a deletion of 8 amino acid residues occurring in the polypeptide chain of the mature protein (Fig. 1).

The nucleotide sequence of α_{s2}-casein mRNA has been established by Boisnard and Petrissant [39]. A heterogeneity was evidenced in the mRNA population of α_{s2}-casein. The amino acid sequence

deduced from the nucleotide sequence included 15 amino acids for the signal peptide and 208 amino acids for the mature protein. The homology between bovine α_{s2}-casein and ovine α_{s2}-casein was 88% (Fig. 2).

Bovine casein

The nucleotide sequences corresponding to bovine α_{s1}- and κ-casein mRNAs were reported by Stewart *et al.* [40], that corresponding to bovine β-casein by Jimenez-Flores *et al.* [41] and those corresponding to bovine α_{s2}- and β-caseins by Stewart *et al.* [42].

Casein genes

The four types of cow caseins are under control of four autosomal genes [43]. The genes encoding the milk proteins seem to have evolved rapidly as illustrated by interspecies comparison of homologous amino acid sequences and apparent lack of specific milk proteins in some species.

Mercier *et al.* [10] have suggested that the calcium-sensitive caseins (bovine α_{s1}-, α_{s2}- and β-caseins) diverged from a common ancestral gene based upon the very limited amino acid sequence homology of the phosphorylation sites, the conservation of the signal peptides and genetic analysis indicating their presence as a gene cluster [43] and location on a single chromosome [44].

A part of the rat casein genome is known [45, 46]. The 7.2 kb rat β-casein gene has been isolated and characterized by Jones *et al.* [45]. The β-casein gene contains 9 exons ranging in size from 21 to 525 bp. The γ-casein gene is approximately 15 kb in length and contains a minimum of 9 small exons separated by large intervening sequences [46]. The γ-casein gene is an unusually large and complex gene containing only 540 nucleotides of amino acid encoding sequences. The γ-casein gene has an intron/exon ratio of 16:1. In addition, the γ-casein gene contains several families of highly repeated sequences interspersed throughout the intervening and flanking regions.

A hypothetical model of the evolution of the calcium-sensitive casein gene family was proposed by Jones *et al.* [45]. Based upon the rates of divergence of the casein signal peptides, a calcium sensitive casein-like gene appears to have originated 300 million years ago at the time of the appearance of primitive mammals. This casein-like gene may have been the result of exon recruitment bringing together the basic elements of the modern casein: a 5′ noncoding region, a signal peptide exon, an exon with a minor phosphorylation site at its 3′ end, and a hydrophobic domain [45]. Between 300 million years ago and the mammalian radiation, 75 million years ago, two types of duplication appear to have occurred. The phosphorylation exon was duplicated intragenically to create the series of small exons observed in the rat β-casein gene. Intergenic duplications also occurred creating the individual members of the calcium-sensitive caseins. It is unlikely that κ-casein, a calcium insensitive component with a distinct signal peptide sequence is a product of these intragenic duplications.

Phosphorylation sites of casein

Phosphorylation of caseins is a post-translational event and Mercier [47] suggested that the process may occur during the transfer of the completed polypeptide chains from the smooth endoplasmic reticulum to the Golgi apparatus where most of phosphate incorporation presumably occurs.

Mercier [47] has shown that the around fifty phosphorylated hydroxy-amino acid residues hitherto investigated in caseins from different species always occur in tripeptide sequences Ser/Thr-X-A when X represents any amino acid and A an acidic one. The occurrence of the above tripeptide sequence is a necessary but not sufficient condition for the phosphorylation of the caseins. Possible factors of constraint are involved such as different intrinsic properties of both phosphate acceptor residues and acidic determinants, the characteristics of the local environment in terms of overall charge and hydrophilicity, secondary structure, steric hindrance and insufficient available pool of casein kinase(s).

The considerable conservation of the phosphorylation sites in all the caseins emphasizes the importance of the phosphorylated residues for the function of the caseins, i.e. the formation of micelles and the binding of Ca^{2+}.

Prosthetic sugar moieties in κ-caseins

Location of the attachment sites of the prosthetic sugar parts in κ-caseins

κ-Casein is the unique casein fraction containing sugars.

Bovine κ-casein

In the κ-caseinoglycopeptide which contains all the carbohydrates of κ-casein, the code sequence Asn-X-Thr/Ser is not present: indeed κ-casein is devoid of N-glycosidic linkages. Since 1966 Malpress *et al.* [48] and Fiat *et al.* [49] demonstrated the presence of O-glycosidic linkages in the cow κ-caseinoglycopeptide.

The isolation of glycopeptides obtained after enzymic digestions of the κ-caseinoglycopeptide allowed to localize the prosthetic sugar groups. Jollès *et al.* [50] and Fiat *et al.* [51] characterized in mature cow milk κ-casein one glycopeptide Gly-Glu-Pro-Thr-Ser-Thr-Pro-Thr (residues 128→135) whose prosthetic sugar group was linked to Thr 131 or 133. Kanamori *et al.* [52, 53] described four short glycopeptides corresponding to residues 128→139, 127→132, 135→139 and 128→141. The carbohydrate chains were attached to threonines 131, 133, 135 (or 136) and to serine 141. In bovine colostrum κ-casein Fiat *et al.* [51] isolated two glycopeptides corresponding to residues 128→135 and 141→145: threonines 131 and 142 were linked to the sugars. Doi *et al.* [54] reported the isolation of one glycopeptide (residues 127→139) with three threonines (131, 133 and 135) bound to the carbohydrate chains. In conclusion, the carbohydrates are attached to the κ-caseinoglycopeptide through five or six O-glycosidic linkages between N-acetylgalactosamine and threonine or serine residues (Fig. 7).

More recently Zevaco and Ribadeau-Dumas [55] studied the carbohydrate binding sites of bovine κ-casein by HPLC techniques and showed that the number of potential carbohydrate binding sites is situated between 3 and 5. By high-performance gel-permeation chromatography Vreeman *et al.* [56] identified at least ten components in bovine κ-casein each differing in N-acetylneuraminic acid and/or phosphorus content. He found that in cow κ-casein the carbohydrate-free fraction turns around 34%, the sum of the fractions with one and two NeuAc

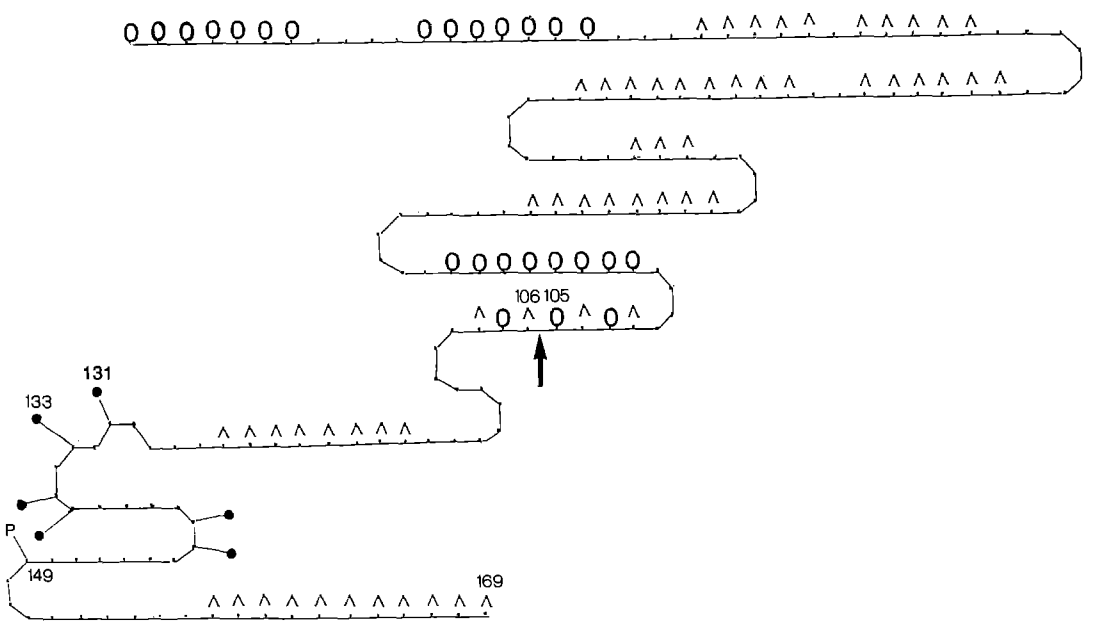


Fig. 7. Schematic diagram of the predicted structure of cow κA-casein. Residues are represented in their respective conformational state: helical (o), β-sheet (Λ), coil (—), β-turn (⌐¬). ↑, carbohydrate moiety; the arrow between residues 105 and 106 indicates the chymosin-sensitive bond; Ser 149 is phosphorylated [58].

groups (but only one GalNAc): 30%, the fraction with three or four NeuAc groups (but two GalNAc): 18%, with five and six NeuAc groups (but three GalNAc): 6%. He could suggest that glycosylation of cow κ-casein occurs by a random mechanism.

The secondary structure of cow κ-casein was established according to the predictive rules of Chou and Fasman [57] by Loucheux-Lefebvre *et al.* [58]: the glycosylation and phosphorylation sites are all located in β-turns (Fig. 7).

κ-Caseins from other species

In sheep κ-casein, Jollès *et al.* [50] suggested that the sugar part might be situated in the same areas as in cow κ-casein.

The study of glycopeptides obtained after enzymic digestion of the human κ-caseinoglycopeptide demonstrated the presence of several (up to 10) prosthetic sugar groups distributed throughout the peptide chain [59]. The predicted secondary structure was in accordance with the high number of sugar groups situated all but one in β-turns [59] (Fig. 8).

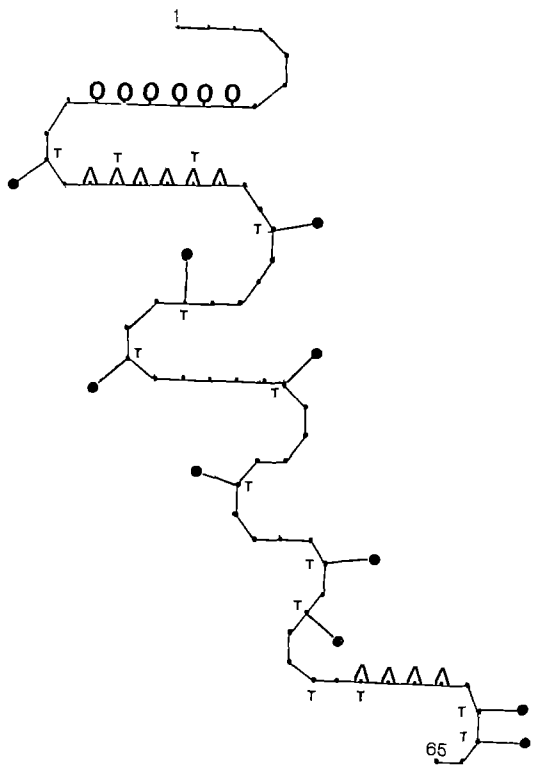


Fig. 8. Schematic diagram of the predicted secondary structure of human κ-caseinoglycopeptide: helical (o), β-sheet (Λ), coil (—), β-turn ( ); , carbohydrate moiety; T represents a threonine residue.

Structure of the κ-casein carbohydrate moieties

Cow κ-casein

Mature milk κ-casein. Jollès *et al.* [60] described the glycan part of cow κ-casein which is constituted by three different monosaccharides: galactose (Gal), N-acetylgalactosamine (GalNAc) and N-acetylneuraminic acid (NeuAc). The carbohydrate chains exhibit a large structural microheterogeneity. The structures of four different oligosaccharides have been established by mass spectrum techniques by Fournet *et al.* [61, 62] and by NMR by van Halbeek *et al.* [63] (Table 1). They all contain Gal β (1→3) GalNAc-ol which is the core for mucin-type glycoproteins.

Colostrum κ-casein. Colostrum κ-casein contains about twice as much carbohydrate as does κ-casein from mature milk with two additional monosaccharides: N-acetylglucosamine and fucose. Moreover, 66 h after calving, the sugar content of colostrum κ-caseinoglycopeptide decreases to the normal level with the disappearance of N-acetylglucosamine [64]. Nine different oligosaccharides from cow colostrum κ-casein obtained between 15 min and 66 h after calving have been isolated and their structures established by Saito *et al.*, van Halbeek *et al.*

Table 1. Structure of the carbohydrate moieties of mature cow milk κ-casein [62, 63].

Disaccharide

1. Gal β 1,3 GalNAc ol

Trisaccharides

2. NeuAc α 2,3 Gal β 1,3 GalNAc ol
3. Gal β 1,3 — GalNAc ol
   NeuAc α 2,6 —

Tetrasaccharide

4. NeuAc α 2,3 Gal β 1,3 — GalNAc ol
   NeuAc α 2,6 —

and Fiat *et al.* (Table 2) [65–69]. Oligosaccharides 1, 3 and 5 are identical to 1, 2 and 4 from mature cow milk κ-casein whereas the remaining sugars contain GlcNAc.

The variability of neutral oligosaccharides in cow κ-casein as a function of time after calving is quite important. Fiat *et al.* [69] reported that the disaccharide Gal β (1→3) GalNAc-ol is present in cow colostrum κ-casein (42% of the total sugars) 15 min after parturition: its amount decreases then very quickly and it reappears after 66 h as the unique neutral sugar. The tetrasaccharide Gal β (1→3) [Gal β (1→4) GlcNAc β (1→6)] GalNAc-ol is always present in cow colostrum κ-casein but its amount depends on the time after parturition (44.3% 15 min after parturition and 89% 18 h later). Small amounts of the trisaccharide Gal β (1→3) [GlcNAc β (1→6)] GalNAc-ol (10%) [69] and of a pentasaccharide with fucose (4%) were continuously identified.

Table 2. Structure of the carbohydrate moieties of cow colostrum κ-casein [65–69].

Disaccharide

1. Gal β 1,3 GalNAc ol

Trisaccharides

2. Gal β 1,3 → GalNAc ol
   GlcNAc β 1,6 →
3. NeuAc α 2,3 Gal β 1,3 GalNAc ol

Tetrasaccharides

4. Gal β 1,3 → GalNAc ol
   Gal β 1,4 GlcNAc β 1,6 →
5. NeuAc α 2,3 Gal β 1,3 → GalNAc ol
   NeuAc α 2,6 →
6. GlcNAc β 1,3 Gal β 1,3 → GalNAc ol
   NeuAc α 2,6 →

Pentasaccharides

7. NeuAc α 2,3 Gal β 1,3 → GalNAc ol
   Gal β 1,4 GlcNAc β 1,6 →
8. Gal β 1,3 → GalNAc ol
   Gal β 1,4 GlcNAc β 1,6 →
   Fuc α 1,3

Hexasaccharide

9. NeuAc α 2,3 Gal β 1,3 → GalNAc ol
   NeuAc α 2,3 Gal β 1,4 GlcNAc β 1,6 →

κ-Caseins from other species

Ovine milk. Three asialyl components from ovine κ-casein have been isolated and their structures established by Soulier *et al.* [70]. The disaccharide Gal β (1→3) GalNAc-ol and the tetrasaccharide Gal β (1→3) [Gal β (1→4) GlcNAc β (1→6)] GalNAc-ol are identical to those of bovine κ-casein whereas the pentasaccharide ([3], Table 3) has not yet been described [70]. The tetra- and pentasaccharides were isolated only from the colostrum.

Human milk. The carbohydrate content of human κ-caseinoglycopeptide is around 55% whereas cow κ-casein contains only 10% sugars. Not only Gal, GalNAc and NeuAc but also Fuc and GlcNAc are constituent monosaccharides. Van Halbeek *et al.* [71] isolated nine oligosaccharides from desialylated human κ-caseinoglycopeptide and established their structures by ^{1}H-NMR techniques (table 4). The disaccharide GlcNac β (1→6) GalNAc-ol and the trisaccharide Gal β (1→4) GlcNAc β (1→6) GalNAc-ol represent novel types of core structures for mucin-type carbohydrate chains. Recently Saito *et al.* [72] isolated seven neutral oligosaccharides (sugars 5 and 9 to 14, Table 4) from human κ-caseinoglycopeptide and determined their chemical structure by methylation and ^{13}C-NMR analyses. Two types of elongation pathways (sugars 5, 9, 11, 13 and 5, 10, 12, 14, Table 4) are suggested in the enzymic pathways.

Table 3. Structure of the asialyl oligosaccharide chains from ovine κ-caseins [70].

Disaccharide

1. Gal β 1,3 GalNAc ol

Tetrasaccharide

from colostrum only

2. Gal 1,3 → GalNAc ol
   Gal β 1,4 GlcNAc β 1,6 →

Pentasaccharide

3. Gal 1,3 → GalNAc ol
   Gal 1,3 Gal β 1,4 GlcNAc β 1,6 →

Table 4. Structure of the carbohydrate moieties of human κ-casein [71, 72].

Monosaccharides

1. GalNAc ol
2. GlcNAc ol

Disaccharides

3. GlcNAc β 1,6 GalNAc ol
4. Gal β 1,3 GlcNAc ol
5. Gal β 1,3 GalNAc ol
6. Gal β 1,4 GlcNAc ol

Trisaccharides

7. Gal β 1,4 GlcNAc β 1,6 GalNAc ol
8. Gal β 1,3 / GlcNAc β 1,6 → GalNAc ol

Tetrasaccharides

9. Gal β 1,3 / Gal β 1,4 GlcNAc β 1,6 → GalNAc ol
10. Gal β 1,3 / Fuc α 1,4 GlcNAc β 1,6 → GalNAc ol

Pentasaccharides

11. GlcNAc β 1,3 Gal β 1,3 (6) / Gal β 1,4 GlcNAc β 1,6 → GalNAc ol
12. GlcNAc β 1,3 Gal β 1,3 (6) / Fuc α 1,4 GlcNAc β 1,6 → GalNAc ol

Hexasaccharides

13. Fuc α 1,4 GlcNAc 1,3 Gal β 1,3 (6) / Gal β 1,4 GlcNAc β 1,6 → GalNAc ol
14. Fuc α 1,4 GlcNAc β 1,3 Gal β 1,3 (6) / Fuc α 1,4 GlcNAc β 1,6 → GalNAc ol

Role of the sugars in κ-casein

The role of the sugars in κ-casein has been the subject of many controversies

Relationship between the micelle size and the sugars

A relationship between the micelle size and the rate of glycosylation of κ-casein was described by Slattery [73]. The functional role of the carbohydrate portion of κ-casein for micelle formation is still not clear, but it is noteworthy that the beginning of micelle formation in milk (2 days after calving) coincides with the decrease of the κ-casein sugar content to a normal level (66 h after calving) [64]. Carroll and Farrell [74] using double-antibody and electron microscopic analyses have pointed out that there is much difference of localization of κ-casein between the large and small micelles. Their results have supported Slattery's micelles model. An inverse relationship between the size of casein micelles and the κ-casein sugar content has been described [73] although it was also claimed by Creamer *et al.* [75] that the largest native casein micelles contain a higher proportion of glycosylated κ-casein than the smaller ones. More recently Dalgleish [76] pointed out that there is no change in micellar size with the presence of glycosylated or non-glycosylated forms of κ-casein. The location of the glycosylated part of κ-casein in bovine casein micelles was investigated by lectin-labelled gold markers. Horisberger and Rouvet-Vauthey [77] reported that glycosylated κ-casein seems to be distributed uniformly throughout most micelles of all sizes.

Role of the sugars in the primary phase of milk clotting

During the primary phase of milk clotting [3, 17, 18] cow κ-casein is digested by chymosin: a Phe-Met linkage between residues 105→106 is split and a κ-caseinoglycopeptide which contains all the sugars of κ-casein is released. It was claimed [78–81] that the rate of κ-caseinoglycopeptide release decreases with increasing carbohydrate content. But Vreeman *et al.* [56] reported that the kcat/Km found for the cleavage of whole κ-casein at pH 6.6 was of the same magnitude as the kcat/Km found for the carbohydrate-free fraction.

Conclusion: Evolutionary aspects. Comparison between blood (fibrinogen/thrombin) and milk (casein/chymosin) coagulation processes

The caseins are among the most rapidly evolving protein families so far studied. In many species only three regions of the casein mRNAs are conserved: the 5′ non coding region, the signal peptide-coding region and the region encoding the phosphorylation sites. These regions correspond exactly to the nutritional role of the caseins which must perform three functions: 1) to be secreted; 2) to form protein aggregates termed micelles and 3) to be phosphorylated to allow Ca^{2+} binding and transport. The conserved 5′ non coding region and the signal peptide allow casein protein secretion [45]. Casein proteins aggregate and form micelles by the interaction of their carboxyl-terminal hydrophobic domains and the conserved phosphorylation sites allow to bind Ca^{2+}.

The clotting of blood and the clotting of milk are two physiologically important coagulation processes and share the following features described by Jollès and Henschen [82]: a) limited proteolysis: thrombin cleaves only two Arg-Gly bonds, one in the Aα- and one in the Bβ-chain of fibrinogen, similarly to chymosin which cleaves a unique Phe-Met linkage in κ-casein; b) during both coagulation processes, short soluble peptides are released, the fibrinopeptides A and B and the κ-caseinoglycopeptide; c) the structures of both peptides are highly variable from one species to another; d) some amino acids have never been found in any fibrinopeptides and κ-caseinoglycopeptides i.e. Cys and Trp; e) all fibrinopeptides carry a substantial negative charge and the κ-caseinoglycopeptides are also acidic.

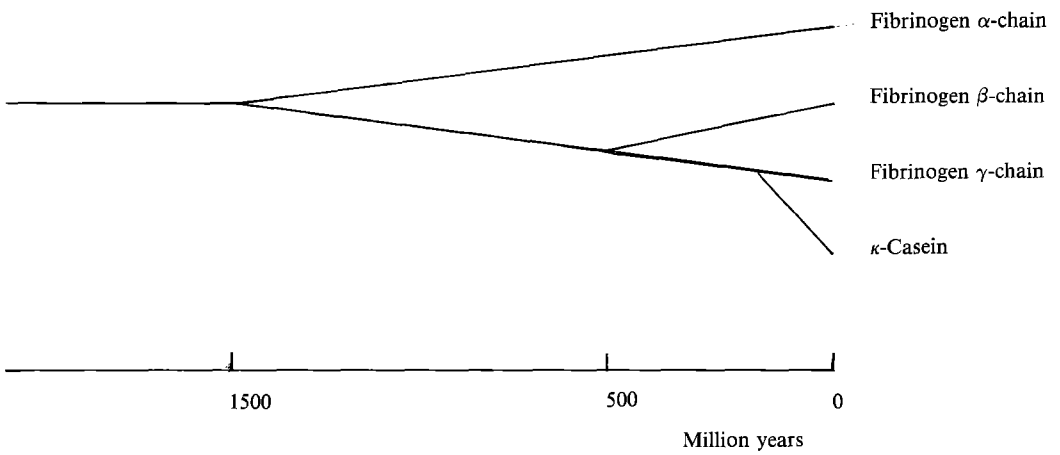
κ-Casein, involved in the milk clotting process and human fibrinogen γ-chain, involved in the blood clotting process, show structural similarities. Several long κ-casein sections, corresponding to 80% of the whole protein molecule, have their counterparts in the N-terminal part (2/3 of the molecule) of the fibrinogen γ-chain in that 31–42% of the amino acid residues occupy identical positions [83]. The section of κ-casein which contains the chymosin-sensitive bond has a counterpart not only in the γ- but also in the Bβ-chain of fibrinogen. Amino acid sequences of κ-caseins from rat, mouse and sheep were 36–53% homologous with rat and human γ-fibrinogen. The extent of homology was similar (32%) when nucleotide sequences of corresponding cDNAs were compared [37]. When nucleotide sequences were compared, mouse κ-casein cDNA showed homology only with the second half of the rat γ-fibrinogen cDNA. The homology with the human γ-fibrinogen cDNA spanned over two regions, one between nucleotides 1–328 and the second between nucleotides 591–726 [37].

Jollès *et al.* [83] hypothesized that κ-casein and γ-fibrinogen may have evolved from a common ancestor. The strong homology between the B β- and γ-chain of fibrinogen indicates that they have a common ancestor which then should have existed more than 450 million years ago. As the κ-caseins have appeared much later during evolution than the fibrinogens, it seems reasonable that they could have evolved from only one of the fibrinogen chains, i.e. the γ-chain (Table 5).

Biologically active casein peptides and oligosaccharides

During a long time, milk proteins and particularly caseins have been considered only as food proteins for young mammals. Casein is the principal source of amino acids for the young animal. But beside a nutritional role, casein has also a physiological importance. Since 1979 many authors described peptides isolated from casein which have a biological ac-

Table 5. Tentative evolutionary relationship between fibrinogen and κ-casein [82,83].



tivity. Figure 9 shows the location of these various peptides in casein.

Opioid peptides

The first and the most studied biologically active casein peptides were the opioid peptides.

β-Casomorphins

In 1979 Brantl *et al.* [84] reported that a material which displayed opioid activity in the guinea-pig ileum longitudinal muscle myenteric plexus preparation arose from an enzymatic casein digest. In fact the responsible substance was a set of peptides isolated from bovine β-casein and named β-casomorphins. Several β-casomorphins have been isolated and corresponded to fragments of bovine β-casein inside the sequence 60→70 (Fig. 10).

β-Casomorphins have been demonstrated to behave like μ-type opioid agonists in opioid receptor binding studies [85, 86] or in isolated organ preparations [85, 86] and have been shown to elicit opioid analgesia in rats [85, 87]. The pentapeptide is the most potent one. β-Casomorphins, although known to be highly resistant to proteolytic enzymes, are demonstrated to be rapidly degraded in bovine and rat plasma [88]; it could be suggested that these peptides can survive in the blood and reach their putative brain receptors in the peptidase resistant precursor form, i.e., procasomorphin [88–90]. β-Casomorphins have also been studied *in vivo*. After intracarotid injection of [^{3}H] β-casomorphin-5 in rats, Ermisch *et al.* [91] concluded that β-casomorphin-5 is accumulated in the blood-brain barrier-free areas to a relatively high degree. *In vivo* considerable amounts of β-casomorphin-7 immunoreactive materials have been detected in blood from new-born calves, probably under the form of a precursor [91] and also in small intestine contents after ingestion of cow's milk in humans [92].

Derivatives of β-casomorphins (i.e. morphiceptin, valmuceptin, depilorphin...) modified by substituting the natural L-amino acids by their D-analogs or by D-pipecolic acid as well as by amidation of their C-terminal amino acids are more active than β-casomorphins [93, 94].

Exorphins

Peptides isolated from pepsin digests of α-casein are called exorphins because of their exogenous origin and morphin-like activity. Analysis of these peptides shows that they correspond to the sequences 90→96 (Arg-Tyr-Leu-Gly-Tyr-Leu-Glu) and 90→95 of cow α_{s1}-casein. The heptapeptide is the most potent. These peptides are relatively resistant to proteolytic enzymes [95, 96].

Casoxin

The peptide Ser-Thr-Tyr-Pro-Ser-Tyr-OCH_3 cor-

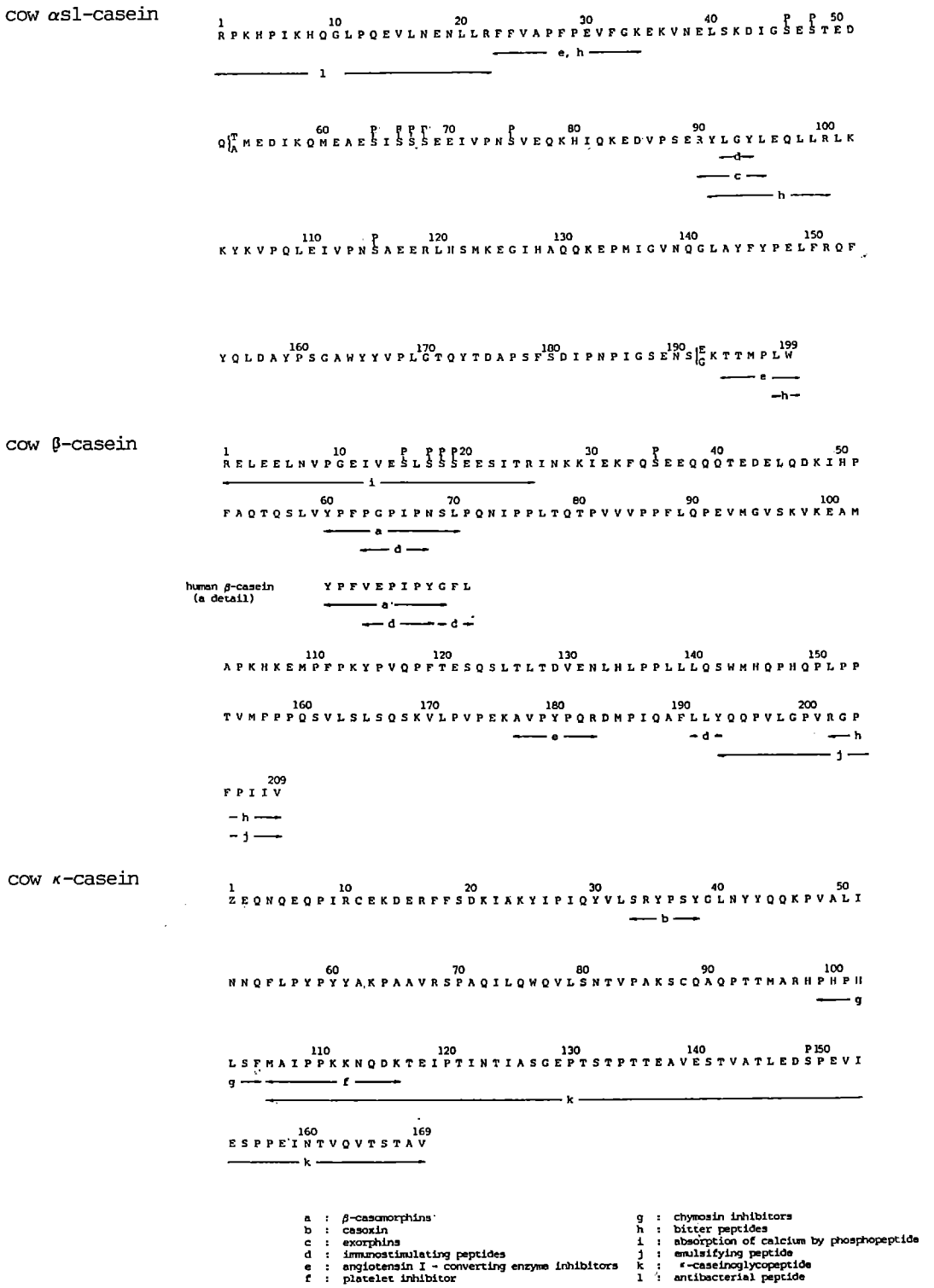


Fig. 9. Biologically active casein peptides: their localisation in various casein fractions.

	Bovine β-casein	Human β-casein
	60 70	51 63
'strategic zone'	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu	Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro-Tyr-Gly-Phe-Leu
Immunostimulating peptides		
hexapeptide	Pro-Gly-Pro-Ile-Pro-Asn	Val-Glu-Pro-Ile-Pro-Tyr
tripeptide		Gly-Phe-Leu
β-casomorphins		
1 → 4	Tyr-Pro-Phe-Pro	Tyr-Pro-Phe-Val
	Tyr-Pro-Phe-Pro-$CONH_2$ (morphiceptin)	Tyr-Pro-Phe-Val-$CONH_2$ (valmuceptin)
		Tyr-Pro-Phe-$_D$Val-$CONH_2$ (devalmuceptin)
1 → 5	Tyr-Pro-Phe-Pro-Gly	Tyr-Pro-Phe-Val-Glu
1 → 7	Tyr-Pro-Phe-Pro-Gly-Pro-Ile (β-casomorphin)	Tyr-Pro-Phe-Val-Glu-Pro-Ile
1 → 8	Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro	Tyr-Pro-Phe-Val-Glu-Pro-Ile-Pro

Fig. 10. Localisation of immunostimulating peptides and β-casomorphins of human and bovine β-caseins: characterization of a 'strategic zone' [102].

responding to the sequence 33→38 of cow κ-casein and called casoxin is an antagonist selective for the μ-and κ- types of opioid receptors [97]. This peptide suppressed the opioid agonist activity of enkephalin.

Casein peptides implicated in immunomodulation

Opioid peptides

Opioid peptides have also been demonstrated to exert *in vitro* and *in vivo* immunomodulating activities [98, 99]: they enhance lymphocyte proliferative responses.

As opiate receptors have been demonstrated on T lymphocytes [100] and human phagocytic leukocytes [101], Migliore-Samour and Jollès [102] suggested that opioid casein peptides, β-casomorphins or exorphins, with a great affinity for μ opiate receptors have an endorphin-like activity on neonate immune cells, particularly with respect to the development of T cell functions and cellular immunity.

Immunostimulating casein peptides

The presence of some immunostimulating short casein peptides has been detected by Migliore-Samour and Jollès [102] in human as well as in cow milk caseins. Two peptides, an hexapeptide Val-Glu-Pro-Ile-Pro-Tyr (residues 54→59 of human β-casein) [103] and a tripeptide Gly-Leu-Phe, not yet located in the known sequences of human caseins [104] stimulated phagocytosis of mouse macrophages at a concentration as low as 0.1 μM and exerted in mice a protective effect against *Klebsiella pneumoniae* infection (*in vivo* test) when injected intravenously at 0.3 and 1 mg/kg, 24 h before lethal infectious challenge. An analogue of the tripeptide Gly-Phe-Leu (residues 60→63 of human β-casein, just following the hexapeptide) exhibited weaker but significant activities. These peptides also stimulated human macrophages to phagocytize human senescent red blood cells [105].

Migliore-Samour and Jollès [102] noted that in the human β-casein the hexapeptide (residues 54→59) is situated at the C-terminal part of human β-casomorphin (residues 51→57) and is followed by the tripeptide Gly-Phe-Leu (residues 60→63). The bovine analogue of the human hexapeptide Pro-Gly-Pro-Ile-Pro-Asn also stimulated phagocytosis of opsonized sheep red blood cells (SRBC) by murine macrophages. This part of the molecule (residues 51 → 63 of human and residues 60 → 70 of bovine β-caseins) seems to play a biological role and could be considered as a 'strategic zone' of β-casein (Fig. 10).

Inhibitors of angiotensin I-converting enzyme

Angiotensin I-converting enzyme (ACE, EC 3.4.15.1) catalyzes both the production of the vasoconstrictor angiotensin II and the inactivation of the vasodilator bradykinin and of the enkephalins in the guinea-pig ileum [106]. Inhibitors of this en-

zyme might increase bradykinin and enkephalin activities. Bradykinin, known as a mediator of the acute inflammatory process, is able to stimulate macrophages, to enhance lymphocyte migration and to induce secretion of lymphokinines from lymphocyte cultures [107].

Maruyama *et al.* [108, 109] and Henriques *et al.* [110] described peptide inhibitors of ACE: CEI_{12}, Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys (residues 23→34 of cow α_{s1}-casein B variant), CEI_5, Phe-Phe-Val-Ala-Pro (residues 23→27 of cow α_{s1}-casein B variant), $CEI_{\beta 7}$, Ala-Val-Pro-Tyr-Pro-Gln-Arg (residues 177→183 of cow β-casein), $CEI_{\alpha c6}$, Thr-Thr-Met-Pro-Leu-Trp (residues 194→199 of cow α_{s1}-casein).

Casein peptides involved in platelet function

Fibrinogen has a bifunctional role: it participates in both platelet aggregation and fibrin formation. Its role in platelet aggregation is mediated by its binding to platelet receptors that become available by stimuli such as ADP. The C-terminal sequence (residues 400→411) of human fibrinogen γ-chain interacts with a recognition site located on stimulated platelets. The dodecapeptide itself inhibits platelet aggregation and ^{125}I-fibrinogen binding to platelets [111]. Homologies have been found between the sequence of cow κ-casein and the γ-chain of human fibrinogen. So Jollès *et al.* [112] found that the natural or synthetic undecapeptide Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys (residues 106 → 116 of cow κ-casein) inhibited both aggregation of ADP-treated platelets and binding of ^{125}I-fibrinogen to ADP-treated platelets. Two smaller tryptic peptides contained in the undecapeptide (residues 106→112 and 113→116) as well as another longer peptide situated around the chymosin-sensitive bond (residues 103→111 of cow κ-casein) and containing a part of the undecapeptide have a much lower effect on platelet aggregation and do not inhibit fibrinogen binding at the same concentrations [112].

Chymosin inhibitors

Gly-Pro-Arg which is the N-terminal region of human fibrin α-chain inhibits the thrombin action on fibrinogen and can prevent the polymerization of fibrin. Because of the analogy casein/fibrinogen, six peptides corresponding to a more or less long C-terminal sequence of cow para-κ-casein were found to inhibit the chymosin action (Table 6): the pentapeptide Pro-His-Leu-Ser-Phe (residues 101→105 of cow κ-casein) was the most efficient.

Bitter peptides

In recent years, many people have described various bitter peptides. Some have been isolated from natural food-stuffs as cheese. In addition some workers have reported that hydrolysis of proteins with proteolytic enzymes is usually accompanied by forma-

Table 6. Inhibition (%) of the chymosin digestion of cow κ-casein in the presence of various synthetic peptides derived from κ-casein.

Peptide	Inhibition (%) of the amount of cow κ-caseinoglycopeptide liberated when cow κ-casein was digested by chymosin in the presence of synthetic peptides	
	3 mg peptide	4 mg peptide
Without	0	0
From cow κ-casein		
Pro-His-Pro-His-Leu-Ser-Phe	30	+
Ac-Pro-His-Leu-Ser-Phe	31	44
Pro-His-Leu-Ser-Phe	47	94
Ac-His-Leu-Ser-Phe	45	+
His-Leu-Ser-Phe	16	32
Ac-Leu-Ser-Phe	41	38
Met-Ala-Ile-Pro-Pro-Lys	0	0
Asn-Gln-Asp-Lys	0	0
Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys	0	0
Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys	<10	
From fibrinogen γ-chain		
Gly-Pro-Arg-Pro	0	0

\+ : Determination not possible as the peptide was not soluble in this concentration.
Ac : Acetyl.

tion of bitter taste. Minamiura *et al.* [113, 114] isolated bitter peptides from a casein hydrolyzate obtained by alkaline proteinases of *Bacillus subtilis.* One of these peptides corresponds to the sequence situated near the C-terminal side of cow β-casein (Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val residue 202 → 209). Matoba *et al.* [115] characterized also bitter peptides from a tryptic digest of whole casein. One peptide Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys corresponds to the sequence 23→34 of cow α_{s1}-casein and another, Gly-Pro-Phe-Pro-Ile-Ile-Val, to the C-terminal sequence (residues 203→209) of cow β-casein. Van Leeuwen [116] described the peptide which includes residues 91→100 of bovine α_{s1}-casein as the major contributor to the bitterness of cheese. (Tyr-Leu-Gly-Tyr-Leu-Glu-Gln-Leu-Leu-Arg).

Absorption of calcium by casein phosphopeptide

Casein phosphopeptide (CPP) corresponding to the first 25 amino acid residues of bovine β-casein injected into a ligated loop of rat small intestine enhanced absorption of calcium and augmented the deposition of calcium in the femur [117]. Furthermore CPP inhibited the precipitation of calcium phosphate *in vitro* suggesting that this peptide enhances calcium absorption from the small intestinal lumen by increasing the concentration of soluble calcium. CPP is an important factor in raising the availability of calcium in milk.

Emulsifying properties of a casein peptide

A hydrophobic peptide of 17 residues Tyr-Gln-Gln-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile-Val (residues 193 → 209 cow β-casein) had little emulsifying activity at a neutral pH; however when mixed with the hydrophilic cow κ-caseinoglycopeptide, its emulsifying activity increased greatly [118].

Casein peptides with physiological activities in the stomach of the calf

In the stomach of the calf during the first phase of the digestion of the casein diet, Yvon and Pelissier [119] showed that the main peptides released were the κ-caseinoglycopeptide (residues 106→169 of cow κ-casein), fragments 1→23 and 165→199 of cow α_{s1}-casein and fragment 193→209 of cow β-casein. κ-Caseinoglycopeptide is almost the only one to be released during the first hour. Stan and Chernikov [120] reported that κ-caseinoglycopeptide could inhibit gastrin and then the acid secretion in stomach. This macropeptide could be involved in the liberation of gastrointestinal hormones. Consequently secretion of pancreatic proteinases would be maintained for a certain time at a low level [119]. Lahav [121] described that a mild casein hydrolysate had anti-bacterial properties and suggested that fragment 1→23 of α_{s1}-casein was responsible for this property.

κ-Caseinoglycopeptide stimulating the growth of Lactobacillus bifidus

A glycopeptide fraction isolated from human milk casein stimulated the growth of *Lactobacillus bifidus* subspecies *pennsylvanicus.* It was most likely the κ-caseinoglycopeptide. Bezkorovainy *et al.* [122] proposed that human milk casein may serve a dual function: that serving the nutritional needs of the breast-fed infant and that stimulating the growth of *L. bifidus.* Azuma *et al.* [123] reported that human κ-caseinoglycopeptide released by the action of chymosin or pepsin had strong bifidus growth-promoting activity for *B. infantis.*

Biological activities of some oligosaccharides isolated from κ-casein

Fiat *et al.* [69, 124] showed that two tetrasaccharides isolated from cow κ-casein possess blood group activities. Cross antigenic reactivity was observed between the N-blood group substances and the cow and sheep κ-caseinoglycopeptides by the hemagglu-

tination inhibition test using an anti-N rabbit immune serum or anti-N phytohemagglutinin from *Vicia graminea.*

The tetrasaccharide NeuAc α (2→3) Gal β (1→3) [NeuAc α (2→6)] GalNAc is responsible of this activity [124].

The tetrasaccharide Gal β (1→3) [Gal β (1→4) GlcNAc β (1→6)] GalNAc and the cow colostrum κ-caseinoglycopeptide which also contains this sugar part inhibit the hemagglutination of blood group I human erythrocytes [69].

Conclusion

It was somewhat surprising to realize gradually that the sequences of a large number of peptides endowed with various biological activities occur in different bovine and human caseins. Do these active peptides exist *in vivo*?

The physiological occurrence and the possible role of these peptides, particularly in the newborn, remain an open question. In the case of human neonates for at least three weeks after birth, both gastric secretion and pepsin activity are weak [125]; the stomach enzymes are almost completely inactivated by the high pH levels, 5.1 to 6.4, two hours after the start of breast-feeding [126]. The major part of the food taken by the newborn leaves the stomach after minimal protein digestion [127]: hydrolysis of the human milk proteins occurs mainly in the proximal small intestine through the action of various enzymes such as trypsin or chymotrypsin. These physiological data are not incompatible with the possible appearance of the β-casein 'strategic zone' peptides (Fig. 10) when their preparation procedure is considered [103, 128]. Their uptake into the blood is possible owing to the permeability of the newborn gut for proteins and antigens [129, 130]: the presence of human β-casomorphin 1→8 material (Fig. 10) was observed in the plasma of nursing mothers by immunoenzymatic assays [131].

In the calf stomach, during the first phase of the digestion, the main peptides released from bovine casein [119] were fragment 106→169 of κ-casein, fragments 1→23 and 165→199 of α_{s1}-casein and fragment 193→209 of β-casein which all are endowed with biological activities. After a 7 h digestion, β-casomorphin (1→7, Fig. 10) or larger fragments were not detected; they may be produced after the intestinal digestive process as suggested by the existence of β-casomorphin in the plasma of newborn calves after milk intake, demonstrated by radioimmunoassay [132] and even in the juice of the small intestine of adult humans after ingestion of bovine milk [92]. Some peptides may also occur under the form of precursors (as procasomorphin) [91] and become active only later.

Finally even if some of the above discussed peptides cannot, in the future, be characterized *in vivo*, they can call be synthesized and possibly used either as food additives or in pharmacology.

Acknowledgement

Skilful preparation of the manuscript by Mrs. J. Rolland was very much appreciated.

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