Primary structure of κ-casein isolated from mares' milk

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Summary. In this work the purification and the complete primary structure of κ-casein from equine milk are reported for the first time. Mares' milk casein was separated by RP-HPLC into four fractions. Complete primary sequence was obtained by sequence analysis of the protein in the fastest eluting peak isolated by chromatography. This sequence was 95% identical to that reported for the C-terminal portion of the zebras' κ-casein and showed high similarity with κ-caseins from sources other than *Equidae*, confirming that this protein was indeed κ-casein in equine milk. The presence of post-translational modifications in equine κ-casein was investigated by mass spectroscopy, after enzymic dephosphorylation. Two main components were found, the smaller component being more abundant. Equine κ-casein was recognized by a lectin specific for one of the glucosidic bonds in the saccharide moiety of bovine κ-casein. Sequence comparison with prevision studies showed that the distribution of charged and hydrophobic regions in equine κ-casein was similar, but not identical, to that found in the bovine protein; these regions are associated with the role of κ-casein in the formation and stabilization of the micellar structure of casein in milk.

Keywords: κ-Casein, mares' milk, *Equidae*, protein sequence.

Literature on milk from equine species is not abundant, in spite of increasing interest for equine milk in the field of cosmetics and of health-conscious nutrition (Marconi & Panfili, 1998). Mares' milk has a composition very different from that of cows' milk. It is much more similar to human milk, in particular the low nitrogen content, the low casein/whey proteins ratio, and the high content of lactose (Kalliala *et al.* 1951; Schryver *et al.* 1986). Several characteristics of mares' milk, such as high levels of polyunsaturated fatty acids and a low cholesterol content (Solaroli *et al.* 1993) suggest that it could be of interest for use in human nutrition. The whey proteins in mares' milk have a much lower thermal sensitivity than those in bovine milk, making mares' milk less sensitive to thermal sanitation processes (Bonomi *et al.* 1994).

Detailed information is available on the sequence of major whey proteins in donkeys' and mares' milk (Conti *et al.* 1984*a*, *b*; Jollès *et al.* 1984; Kaminogawa

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et al. 1984; Godovac-Zimmerman *et al*. 1985, 1987). However, limited information is available on the casein fraction (Jennes & Sloan, 1970; Visser *et al*. 1982; Ono *et al*. 1989). Casein fractions homologous to bovine β-caseins have been identified in equine milk (Visser *et al*. 1982), although no sequence information is available. The presence of κ-casein in mares' milk is still debated (Kotts & Jennes, 1976; Visser *et al*. 1982). Among the different *Equidae*, only the C-terminal sequence of κ-casein in zebra has been determined (Gatesy *et al*. 1996).

In this work we report the purification and some properties of κ-casein in mares' milk. The complete sequence of the protein was determined, and similarities with extensively characterized bovine κ-casein are discussed.

materials and methods

Raw milk from five mares, at a comparable lactation state, from the same herd was pooled and used in the present study. Milk samples were collected, pooled, frozen immediately, and stored at −20 °C until required. Frozen milk samples were thawed at room temperature and defatted by centrifugation at 5000 ***g*** at 4 °C for 15 min. The whole casein fraction was separated from thawed and defatted milk by ultracentrifugation, according to Visser *et al*. (1982) as modified by Iametti *et al*. (1998). Precipitated casein was washed twice by resuspension in water, followed by low-speed centrifugation, and lyophilized. SDS-PAGE was performed on samples reduced with 0·1 % (v/v) 2-mercaptoethanol in a Mini-Prot 12 % monomer gel (Bio-Rad Laboratories, Hercules, CA 94547, USA).

Reversed-phase (RP) HPLC of caseins was carried out with minor modifications of the procedure of Jaubert & Martin (1992), adapted to separation of equine caseins (Iametti *et al*. 1998). Typically, 1–2 mg of lyophilized casein was washed with 50 mM-acetate buffer, pH 4·6, and with water, before being dissolved in 25 mM-acetate buffer, pH 5·5, containing 6 M-urea and 30 mM-DTT, and being applied on a Vydac C_4 column (0·46 × 25 cm; Resolution Systems, Holland, MI 49423, USA) connected to a Waters 625 HPLC system (Waters Corporation, Milford, MA 01757, USA) with a Waters 490 detector set at 280 nm. The column was equilibrated in 0·1 % (v/v) trifluoroacetic acid (TFA) in water containing 1 mM-DTT (buffer A). Elution of caseins was achieved by continuous gradient of buffer A to buffer B (0·1 % TFA in 80 % (v/v) acetonitrile, containing 1 mM-DTT). The gradient was run from 0 to 37 % B in 5 min, and then to 55 % B in another 34 min at a flow rate of 0·8 ml/min. Fractions from five identical runs were collected manually, pooled, and evaporated to dryness in a Savant SpeedVac system (Savant Instruments, Holbrook, NY 11471, USA).

Sequence determinations were carried out on the protein corresponding to the fastest-eluting component from RP-HPLC without prior treatments. Reduction of the protein was performed in 8 M-urea, 0·4 M-NH_4HCO_3 using 7·5 mM-dithiothreitol. The mixture was incubated at 50 °C for 15 min. Cysteine derivatization was achieved by adding 14 mM-iodoacetamide after cooling to room temperature. After 15 min of incubation the mixture was diluted 1:3 with water and the resulting material was digested with endoprotease Lys-C (weight ratio protein/protease 20/1; Roche Molecular Biochemicals, Mannheim D-68305, Germany) and with endoprotease Asp-N (ratio protein/protease 100/1; Roche Molecular Biochemicals), at 37 °C overnight. In both cases the peptides were separated directly by RP-HPLC using a Jasco instrument (Japan Spectroscopy Co., Tokyo 192–8537, Japan) equipped with an

Uvidec-100 V detector set at 220 nm and an Aquapore C-8 RP-300 column (7 × 250 mm; Waters Corporation). The chromatographic conditions used were: buffer A 0·1% TFA, buffer B acetonitrile + 0·075% TFA, flow rate 2 ml/min. Gradient was from 0 to 60% B in 85 min (Negri *et al.* 1994). Peptide sequence analysis was performed using an Applied Biosystems automated Protein Sequencer (Model 477/A; Perkin-Elmer Applied Biosystems, Foster City, CA 94404, USA) based on the Edman degradation chemistry. Database searches, sequence similarities studies and the elaboration of hydropathy/charge profiles were performed by accessing the Swiss Prot Data Bank and the European Bioinformatics Institute at the EMBL Outstation.

Electrospray mass spectrometry (ES-MS) analysis was performed using a Platform single-quadrupole mass spectrometer (VG-Biotech, Altrincham WA14 5RZ, UK). Dephosphorylation of protein samples was carried out by treatment with a potato alkaline phosphatase according to published procedures (Ferranti *et al.* 1997). Different durations of the treatment with phosphatase were used. Dephosphorylated protein samples (10 μl, 50 pmol) were injected into the ion source at a flow rate of 10 μl/min; the spectra were scanned from 1400 to 600 at 10 s/scan. Mass scale calibration was carried out using the multiple-charged ions of a separate introduction of myoglobin. Mass values are reported as average masses. Quantitative analysis of individual components was performed by integrating the signals from the multiple charged ions of the single species (Ferranti *et al.* 1995).

Identification of glycosylated proteins was performed by dot-blotting of casein fractions on nitrocellulose membranes, followed by recognition of the glycosylated proteins by a biotin-conjugated lectin from *Arachis hypogaea* or by biotin-labelled concanavalin A (L6135 and C2272, respectively, Sigma Chemical Co., St. Louis, MO 63178, USA). Protein-bound lectins were detected *in situ* by peroxidase-conjugate streptavidin (S5512; Sigma), followed by colour development with 1-chloronaphthol and hydrogen peroxide as the peroxidase substrates (Hawkes, 1982). Bovine κ-casein (C0406, Sigma) was used as a reference protein in these experiments.

RESULTS

In order to separate individual casein components, milk was ultracentrifuged, allowing complete recovery of the whole casein fraction from mares' milk, as indicated by SDS-PAGE analysis (not shown). The same technique also showed that, after washing, the compact casein pellet obtained by ultracentrifugation did not contain residual whey proteins, confirming previous reports (Iametti *et al.* 1998).

The various casein fractions were separated by RP-HPLC, as shown in Fig. 1. Four major chromatographic fractions were obtained (numbered progressively in their elution order). Fraction 1 and 3 represented the least abundant casein fractions. In these chromatographic conditions, bovine, ovine and caprine κ-casein are always present in the fastest moving component (Visser *et al.* 1986; Jaubert & Martin, 1992).

Fig. 2 shows the primary structure of the protein separated as fraction 1 in the chromatography of whole casein. The protein sequence was determined after two enzymic digestions of fraction 1 without further treatment, and by sequence overlapping of the hydrolytic peptides. The data reported here are deposited in the Swiss Protein Data Bank under the accession number P82187. The molecular weight calculated from the sequence was 18567.

As shown in Fig. 3, the sequence of the protein in fraction 1 has a 97% similarity (95% identity) to that reported for the C-terminal portion of the zebras' κ-casein (CASK-EQUGR, accession number Q28400 in the Swiss Prot Data Bank), and

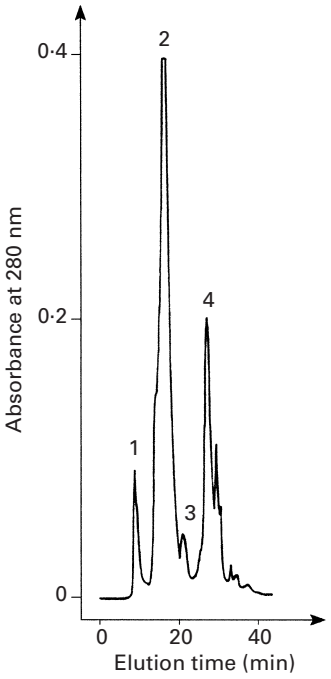

Fig. 1. RP-HPLC chromatograms of caseins from equine milk. Letters on each peak indicate the elution order.

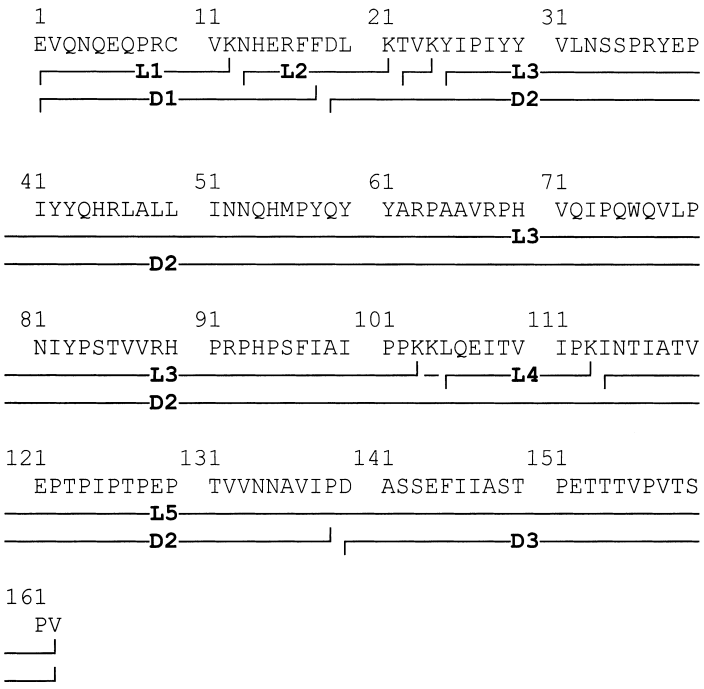

Fig. 2. Amino acid sequence of the protein corresponding to fraction 1 from RP-HPLC. Labels L and D indicate sequences that were derived from Lys-C or Asp-N cleavage, respectively.

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Horse             EVQNQEQPRCVKNHERFFDLKTVKYIPIYYVLNSSPRYEPIYYQHRLALLINNQHMPYQY 60
Zebra       (97)  --------------------------IYYVLNSSPRYEPIYYQHRLAVLINNQHMPYQY 33
Dromedary   (63)  EVQNQEQPTCFEKVERLLNEKTVKYFPIQFVQSRYPSYGINYYQHRLAVPINNQFIPYPN 60
Human       (62)  EVQNQKQPACHENDERPFYQKTAPYVPMYYVPNSYPYYGTNLYQRRPAIAINNPYVPRTY 60
Pig         (60)  EEQNQEKLTRCESDKRLFNEEKVKYIPIYYMLNRFPSYG-FFYQHRSAVSPNRQFIPYPY 59
Sheep       (56)  QEQNQEQRICCEKDERFFDDKIAKYIPIQYVLSRYPSYGLNYYQQRPVALINNQFLPYPY 60
Goat        (54)  QEQNQEQPICCEKDERFFDDKIAKYIPIQYVLSRYPSYGLNYYQQRPVALINNQFLPYPY 60
Cow         (54)  QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPY 60
                     **          *..      *:*: :: .  * *    **:: .   *. .:*

Horse             YARPAAVRPHVQIPQWQVLP--------NIYPSTVVRHPRPHPSFIAIPPKKLQEITVIP 112
Zebra             YARPAAVRPHVQIPQWQVLP--------NIYPSTVVRHPRPHPSFIAIPPKKLQEKTVIP 85
Dromedary         YAKPVAIRLHAQIPQCQALP--------NIDPPTVERRPRPRPSFIAIPPKKTQDKTVNP 112
Human             YANPAVVRPHAQIPQRQYLP--------NSHPPTVVRRPNLHPSFIAIPPKKIQDKIIIP 112
Pig               YARPVVAGPHAQKPQWQDQP--------NVYPPTVARRPRPHASFIAIPPKKNQDKTAIP 111
Sheep             YAKPVAVRSPAQTLQWQVLPNAVPAKSCQDQPTAMARHPHPHLSFMAIPPKKDQDKTEIP 120
Goat              YAKPVAVRSPAQTLQWQVLPNTVPAKSCQDQPTTLARHPHPHLSFMAIPPKKDQDKTEVP 120
Cow               YAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIP 120
                  **.*..    .*  * *  .        :  *.:: *:*. : **:*****  *:    *

Horse             KINTIATVEP--TPIPTPEPTVVNN-----AVIPDASSEFIIASTPETTTVPVTSPV- 162
Zebra             KINTIATVEP--TPIPTPEPTVNN------AVIPDASSEFIIASTPETTTVPVTSPVV 135
Dromedary         TINTIATVEP--TPAPATEPTVDS------VVTPEAFSESIITSTPETTTVAVTPPTA 162
Human             AINTVATVEP--PVIPTAEPAVNT------VVIAEASSEFITTSTPETTTVQITSTEI 162
Pig               AINSIATVEP--TIVPATEPIVNAEPIVNAVVTPEASSEFLITSAPETTTVQVTSPVV 167
Sheep             AINTIASAEPTVHSTPTTEAVVNA-------VDNPEASSESIASAPETNTAQVTSTEV 171
Goat              AINTIASAEPTVHSTPTTEAIVNT-------VDNPEASSESIASASETNTAQVTSTEV 171
Cow               TINTIASGEP--TSTPTTEAVEST-------VATLEDSPEVIESPPEINTVQVTSTAV 169
                   **::*: **     *:.*.            *     *     *..* .*. :*..
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Fig. 3. Alignment of the sequences of κ-casein from different sources. For clarity, only 8 out of 47 sequences described in the Swiss Prot Data Bank are reported in the figure. ''Horse'' indicates the sequence reported in Fig. 2. Other sequences are listed in the order of decreasing homology, as calculated by the program CLUSTALW. Percent homology to horse κ-casein is given in parentheses at the beginning of each sequence. Symbols indicate: (*), conserved residues; (:), conservative substitutions; (.), semi-conservative substitutions.

presents a similarity higher than 50% with κ-caseins from sources other than *Equidae*. This strongly suggests that this protein represents κ-casein from mares' milk.

Mass spectrometry analysis was carried out on the protein component in fraction 1 from RP-HPLC after dephosphorylation. As for other caseins, treatment with phosphatase was found necessary to facilitate ES-MS analysis and to minimize peak broadening (Ferranti *et al.* 1997). After dephosphorylation for four hours, ES-MS mass analysis gave a single broad peak with four main components, having masses of 19400 ± 2, 18833 ± 3, 18567 ± 2, and 18538 ± 2. The peak corresponding to the mass calculated from sequence studies (i.e. 18567) was the most abundant. Two main components were present in the single peak after overnight dephosphorylation, representing about 90% of the total protein, with masses of 18538 ± 2 and 19400 ± 2. The smaller component was present in a 9/1 molar excess over the larger one. The unit mass difference of 29 between the most abundant component in the protein after extensive dephosphorylation and the mass calculated from sequence analysis suggests that extensive dephosphorylation may lead to some artifactual degradation of the protein, as observed with other caseins.

The difference of 833 mass units found between the mass calculated from sequence studies and that of the heaviest component detected by ES-MS may suggest glycosylation, possibly along with other post-translational modifications. For instance, the 266 mass units difference between the 18833 and 18567 components

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could stem from three phosphorylated residues (plus some additional counter-ions). Components with different mass have been found in variable ratios in bovine κ-caseins, where the lower-abundance high molecular-weight forms stem from glycosylation of a non-glycosylated one, which in turn may present different phosphorylation sites (Swaisgood, 1992).

Of all individual chromatographic fractions from the RP-HPLC separation in Fig. 1, fraction 1 was unique in being recognized in dot-blot experiments by a lectin from *Arachis hypogea*, specific for the presence of a Gal-(β-1,3)GalNAc link. None of the RP-HPLC mares' casein fractions nor bovine κ-casein were recognized by concanavalin A, a lectin that is used for recognition of glycosylation patterns containing α-mannosidic or α-glucosidic moieties.

DISCUSSION

The sequence data presented here show that the purified fraction 1 from RP-HPLC fractionation of mare's milk casein was κ-casein. The presence of κ-casein in mares' milk has been long questioned, especially in view of the unusual size and properties of the casein micelles in mares' milk. To understand the possible role of κ-casein in mares' milk, we compared some of its properties – as deduced from the sequence – with those of bovine κ-casein.

As shown by the charge profiles in Fig. 4, which do not take into account any post-translational phosphorylation, both the bovine and the equine κ-caseins show a similar charge distribution at pH 6·5. The N-terminal region is close to electrical neutrality in both proteins. Region 21–112 is characterized by positively charged residues in the bovine protein. This positively charged region is much shorter in the equine protein, where it only encompasses residues 45–104. In both proteins, the C-terminal region is negatively charged at neutral pH. However, the density of negatively charged residues is much higher in the bovine than in the equine protein. This latter contains 11 negatively charged residues and 14 positively charged residues compared with 16 and 14, respectively, in the bovine protein. This distribution is reflected in the calculated pI values (equine: 9·02; bovine: 5·93). The particular composition of equine κ-casein is similar to that found in κ-casein from monogastric species, such as human κ-casein (11 negatively charged residues; 13 positively charged residues; pI, 8·68), or pig κ-casein (15 negatively charged residues; 17 positively charged residues; pI, 8·68).

Some other features of equine κ-casein that may be relevant for micelle stabilization can be deduced from Fig. 5, where the hydropathy profiles for equine and bovine proteins are compared. The grand average of hydropathicity (GRAVY) is negative for both proteins, indicating a generally hydrophobic structure (equine: −0·316; bovine: −0·557). Both proteins are highly hydrophilic in their N-terminal region, and are hydrophobic in their C-terminal regions. The most evident differences between the two proteins can be appreciated in the sequence region encompassing residues 61–103 in the equine protein (61–111 in the bovine protein). The average hydrophobicity of bovine κ-casein in this region is higher than that of equine κ-casein. Conversely, the average hydrophobicity of the equine protein is higher in the C-terminal region. These calculations do not take into consideration the possible occurrence of post-translational modifications (glycosylation and phosphorylation) in the C-terminal region.

It may be noticed that both proteins display a hydrophobicity maximum that corresponds to residue pairs that are, or could represent, sites for chymosin action.

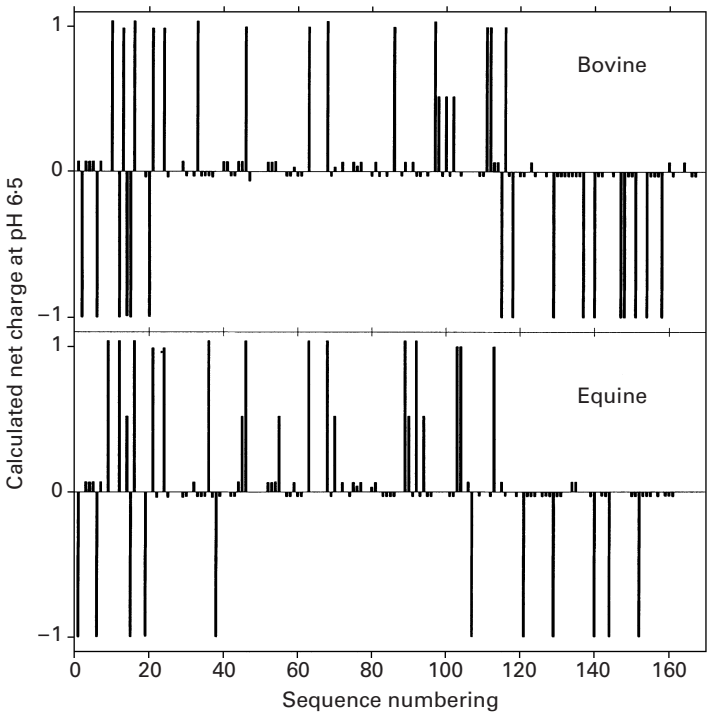

Fig. 4. Charge profiles for bovine and equine κ-casein at pH 6·5. Charges were calculated by using values provided by the PROTSCALE program, and corrected for the tabulated pK_a values of the side-chain groups in the free amino acid.

Indeed, in the equine protein, residues Phe97 – Ile98 are comparable to residues Phe105 – Met106 in bovine κ-casein.

Lectin-binding data indicated that κ-casein is likely to be the only glycosylated casein in mares' milk. It was recognized by a lectin specific for the αGal-(β-1,3)GalNAc motif. This disaccharide is present in the glycosylation patterns reported for bovine κ-casein: the branched AcNeu-(α-2,3)Gal-(β-1,3)GalNAc[β-1,6AcNeu]-(β-1)Thr, or the linear AcNeu-(α-2,3)Gal-(β-1,3)GalNAc-(β-1)Thr (Swaisgood, 1992). However, the glycosylation patterns found in bovine κ-casein are different from those found in κ-caseins from monogastric animals, including the human protein (Saito *et al.* 1988).

No direct information is available on the phosphorylation state of the protein. However, three putative phosphorylation sites, namely Thr 127, Thr 150 and Ser 160, may be suggested based on the sequence similarity studies with other κ-caseins and on prediction analyses. Circumstantially, the possible occurrence of phosphorylation sites at these residues was supported by a noticeable drop in the yield of derivatization for these amino acids in the course of our sequencing experiments, whereas the yield for other hydroxyamino acids in the sequence was normal.

In conclusion, in spite of some peculiarities, some of which also occur in other monogastric species, mares' milk κ-casein appeared to have all the properties found in other κ-caseins and associated with the role of this protein family in the formation and stabilization of the micellar structure of casein in milk.

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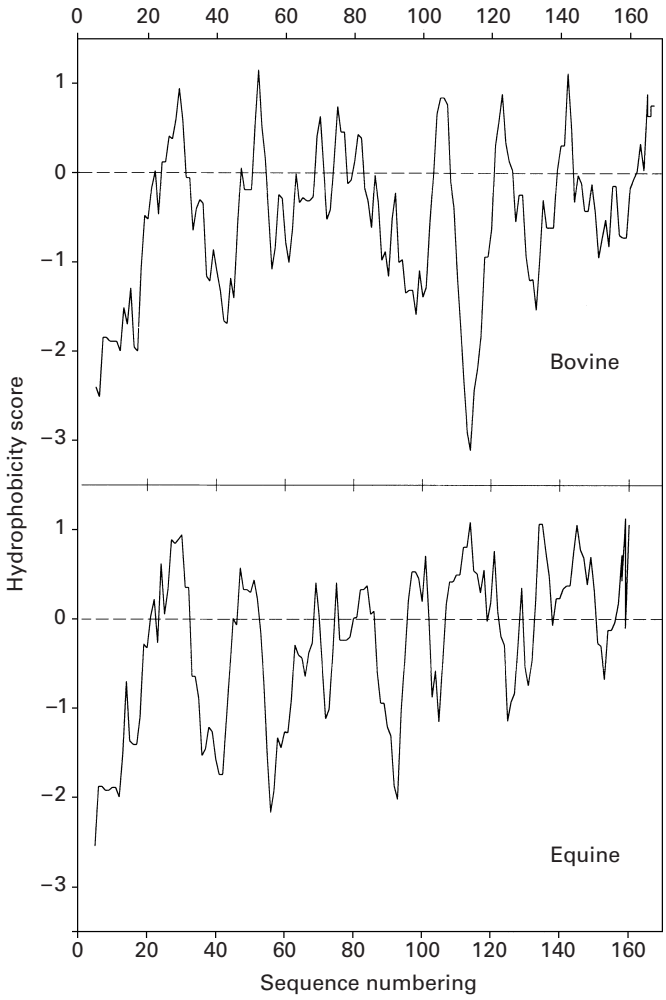

Fig. 5. Hydrophobicity profiles for bovine and equine κ-casein. Hydrophobicity scores were calculated by using the PROTSCALE program with standard settings.

Mass spectroscopy studies were performed at CISESMA-CNR (International Mass Spectrometry Facility Centre), Naples, Italy.

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