Isolation and characterization of β - and γ -caseins from horse milk

Servaas VISSER,* Robert JENNESS and Robert J. MULLIN

Department of Biochemistry, College of Biological Sciences, University of Minnesota, St. Paul, MN 55108, U.S.A.

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Three groups of casein components were isolated from horse milk. Group I is almost insoluble at acid and neutral pH, and is rather heterogeneous on alkaline gels with or without sodium dodecyl sulphate. Group II shows strong similarity to β -casein from other species, as concluded from its amino acid composition and its *N*- and *C*-terminal sequences. This group consists of five electrophoretically distinguishable forms, all containing ester phosphate groups but no carbohydrate. Group III is composed of *C*-terminal fragments of the β -like (group II) fraction and probably arises from the action of a plasmin-like enzyme present in horse milk. It does not contain phosphate or carbohydrate. Homology of this group with bovine γ -caseins is demonstrated. Both β - and γ -like caseins are more soluble at 4°C than at room temperature.

During the past few years growing attention has been given to the comparative study of the structure of caseins isolated from milks of different species. Bovine caseins, of which the complete primary structures have been determined (Ribadeau Dumas *et al.*, 1975; Brignon *et al.*, 1977), usually serve as reference proteins in identifying newly discovered caseins from other mammals. Several of those non-bovine proteins have been provisionally named ' α_s ', ' β '- or ' κ '-casein on the basis of resemblance to cow casein components in molecular weight, electrophoretic behaviour, amino acid composition, cleavability by chymosin or Ca^{2+} -sensitivity (cf. Woodward, 1976; Kotts & Jenness, 1976; Green & Pastewka, 1976; Bezkorovainy, 1977; Mulvihill & Fox, 1979; Jenness, 1979). However, definite homology can best be established if at least parts of the amino acid sequence are available for comparison. The latter is the case with casein components isolated from milk of several artiodactyls (Jollès *et al.*, 1974; Chobert *et al.*, 1976; Mercier *et al.*, 1976a,b; Addeo *et al.*, 1977; Datta *et al.*, 1977; Richardson & Mercier, 1979), one primate (Chobert *et al.*, 1976; Greenberg *et al.*, 1976; Groves & Greenberg, 1978) and one rodent (Pélissier *et al.*, 1980; Visser *et al.*, 1981) species.

Until now, studies on caseins of the perissodactyls have been restricted to determination of total casein content (Jenness & Sloan, 1970) and amino acid composition or electrophoretic patterns of

whole casein (Woodward, 1976). The present paper describes the isolation and purification of casein fractions from horse (*Equus caballus*) milk, and their characterization by gel electrophoresis, amino acid analysis, *N*- and *C*-terminal sequence determination and behaviour towards attack by some enzymes. For two of the fractions a relationship with cow caseins is demonstrated.

Experimental

Isolation and fractionation

Milk was collected from individual mares and kept refrigerated for a few hours. Some of the samples were kept frozen until further processing. After a short warming to 40°C the milk was defatted by centrifugation at 1000g for 30 min and then adjusted to pH 4.6 by slow addition of 1 M-HCl. During subsequent dialysis against water at 4°C, a precipitate appeared. This cold-precipitated (CP-) casein was collected by centrifugation at 45 000g for 30 min at 4°C. By warming up the supernatant to 30°C a second precipitate could be obtained, which was collected by centrifugation at 45 000g for 30 min at room temperature. This fraction is designated as temperature-sensitive (TS-) casein, as it is much more soluble at 4°C than at room temperature. The combined CP- and TS-caseins, obtained by centrifuging directly at room temperature, are referred to below as whole casein.

The CP-casein was repeatedly washed, at room temperature as well as at 4°C, with 20 mM-imidazole/HCl buffer, pH 6, containing 4 M-urea.

Abbreviation used: SDS, sodium dodecyl sulphate.

* Present address: Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands.

The remaining insoluble material is denoted group I casein.

The TS-casein was fractionated in 220mg portions on a 20cm × 1.5cm column of DEAE-cellulose (Whatman DE-52) equilibrated with 20mM-imidazole/HCl buffer, pH 7.3, containing 4M-urea and 20mM-2-mercaptoethanol. Sample solutions (approx. 4ml) were adjusted to pH 7.3 before application to the column. A linear salt gradient (0–0.25 M-NaCl; total volume 600ml) was used for development of the column at a flow rate of 30ml/h. This resulted in the isolation of fractions referred to as group II casein and group III casein.

Further separation of group II casein was achieved by rechromatography on DEAE-cellulose

with a shallower salt gradient (0.05–0.17M; total volume 600ml). Group III casein was further purified on a 17cm × 1.0cm column of CM-cellulose (Whatman CM-52) with a phosphate buffer, pH 7.1 (0.28mM-KH₂PO₄/0.24mM-K₂HPO₄), containing 4M-urea; in this case a linear gradient of 0–15mM-NaCl was generated in the above buffer in 8h by using the LKB Gradient Mixer, followed by two steps (each about 40ml) of 25mM- and 2M-NaCl subsequently. In all fractionations the effluent fractions were monitored continuously at 280nm with an LKB Uvicord III detector equipped with a 3mm cell.

The isolation and the designation of fractions are summarized in Scheme 1.

Scheme 1. *Pathway of casein isolation and fractionation*

Electrophoresis

Starch-gel electrophoresis at pH 8.6 in the presence of urea and 2-mercaptoethanol was performed as described by Schmidt (1964); staining was with Amido Black. Polyacrylamide-gel electrophoresis was performed on slab gels in the presence of SDS (Laemmli, 1970) or on cylindrical gels (pH 9.5) in the presence of 4M-urea (Groves, 1974) with 2-mercaptoethanol included in the sample solutions; gels were stained with Aniline Blue Black (urea/polyacrylamide gels) or Coomassie Brilliant Blue G-250 (SDS/polyacrylamide gels). Occasionally, the periodic acid/Schiff reagent for carbohydrate staining (Kapitany & Zebrowski, 1973) was applied on SDS/polyacrylamide gels. For molecular-weight estimation by SDS/polyacrylamide-gel electrophoresis the following reference proteins were used: bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 29000), chymotrypsinogen (M_r 26000), trypsinogen (M_r 24000), soya-bean trypsin inhibitor (M_r 21000), bovine β -lactoglobulin (M_r 18000) and bovine α -lactalbumin (M_r 14000).

Amino acid analysis

Proteins were analysed with a JEOL JLC-5AH automatic amino acid analyzer by the procedures of Moore & Stein (1963). Before analysis, samples were hydrolysed with 6M-HCl at 110°C for 24–96 h in sealed evacuated tubes. L-Norleucine and L- α -amino- β -guanidinopropionic acid were used as internal standards for the long column and the short column respectively. Tryptophan was determined via separate analysis on the short column after sample hydrolysis in 4M-methanesulphonic acid at 115°C for 22 h (Simpson *et al.*, 1976).

Homology with cow casein components was established from the amino acid compositions by using the parameter $S\Delta Q$ of Marchalonis & Weltman (1971). $S\Delta Q = \sum_j (X_{ij} - X_{kj})^2$, where X_{ij} and X_{kj} are the contents (residues/100 residues) of the amino acid of type j . For homologous protein pairs $S\Delta Q$ is generally less than 100.

Amino acid sequence determination

C-Terminal sequences were identified by hydrolysis with di-isopropyl phosphorofluoridate-treated carboxypeptidase A (Ambler, 1972), followed by automatic amino acid analysis.

N-Terminal sequences were obtained with a Beckman 890 C Automatic Sequencer by using the dimethylallylamine/trifluoroacetic acid program (Beckman Instruments, 1975). Phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography with a Waters Series 200 Chromatograph (Downing & Mann, 1976). A gradient of acetonitrile/1.5% (v/v) acetic acid from 15:85 to 75:25 (v/v) was achieved in 33 min at room

temperature by following curve 5 of the Waters solvent programmer. This program did not resolve the pairs valine/methionine, isoleucine/phenylalanine or leucine/lysine.

Occasionally, end-group analysis was performed by the dansyl (5-dimethylaminonaphthalene-1-sulphonyl) procedure (Gray, 1972).

Phosphate and sialic acid contents

These were determined as described by Griswold *et al.* (1951) and Warren (1959) respectively.

Enzyme assays

The assay for chymosin (EC 3.4.23.4) activity has been described elsewhere (Visser *et al.*, 1981).

Plasmin (EC 3.4.21.7) isolated from pig blood was obtained from Sigma Chemical Co. (0.45 unit/mg; lot no. 77c-0053). For studying the sensitivity towards this enzyme, casein fractions (approx. 3 mg) were dissolved or suspended in 250 μ l of 10 mM-Tris/HCl buffer, pH 7.8, containing 1 mM- CaCl_2 ; 10 μ l of enzyme solution (0.25 mg/ml of buffer) was added at 37°C. After 2 and 5 h of incubation, 110 μ l samples were taken, to each of which 135 μ l of 7M-urea in 75 mM-Tris/citrate buffer, pH 8.6, was added. These samples were then immediately subjected to starch-gel electrophoresis.

Acid phosphatase (EC 3.1.3.2) from potato was purchased from Boehringer Mannheim (approx. 60 units/ml of suspension; grade I, lot no. 1267214). For dephosphorylation with this enzyme we modified the procedure of Bingham *et al.* (1976), because the rather long incubation period (2 h at 38°C, followed by dialysis for 65 h at 4°C) used by those authors caused our preparations to be completely degraded, probably as a consequence of proteolytic activity present in our enzyme or substrate preparation. Dephosphorylation was performed in a small dialysis bag (approx. 1 ml volume) while dialysing against water (pH 7) at 38°C. In this way the inhibiting phosphate ions are removed from the beginning of the reaction. The incubation time generally was 2 h, after which the sample was immediately freeze-dried.

Results

Separation

In the cylindrical polyacrylamide gels (Fig. 1) whole casein clearly shows three zones of bands, represented by groups I, II and III. In addition, a band is often observed near the front of the gel; this probably represents a component occurring predominantly in the whey fraction (cf. also Bell *et al.*, 1981). Whole caseins from individual mares exhibited differences in the number and/or intensity of bands within the various groups (Fig. 1, cf. gels *a* and *b*). Group I is nearly insoluble unless the pH is

Fig. 1. Electrophoresis on cylindrical alkaline polyacrylamide gels of whole horse casein and its subfractions denoted in Scheme 1

Gels: (a) and (b), whole caseins from milks of different individual mares; (c), CP-casein; (d), TS-casein; (e) group III casein; (f), group II casein; (g), group I casein. Caseins shown in gels (c)–(g) were prepared from the whole casein preparation shown in gel (b). For full details see the text.

raised to above 10 or high concentrations (6M) of urea are added. This group consists of seven to ten discrete bands in the alkaline urea gel. In addition, a weak component migrating about halfway between the groups II and III is isolated with group I. In Fig. 1 (gel f) group II mainly shows one diffuse band, but actually it contains at least five electrophoretic components (see Fig. 1, gels a–d). Group III exhibits two main bands and one or two minor ones (Fig. 1, gel e). On SDS/polyacrylamide-gel (results not shown) group I shows several bands, and groups II and III are nearly homogeneous; we have done nothing further with group I.

The isolation of groups II and III was achieved starting from TS-casein (Fig. 1, gel d), which is essentially free of group I. The solubility in water of TS-casein is highly temperature-dependent. This effect, which is more pronounced at higher concentrations, is eliminated by addition of urea or by raising the pH to 10–12. Therefore TS-casein was fractionated on DEAE-cellulose in a urea-containing buffer (Fig. 2). Complete separation of group II from group III was achieved (Fig. 1, cf. gels e and f). Group II comprised 86% and group III 8% of the overall yield.

Further purification was monitored by alkaline starch-gel electrophoresis, which separates the in-

dividual bands somewhat better than does polyacrylamide gel. Rechromatography of group II fractions on DEAE-cellulose resulted in the isolation of five components (Fig. 3). The components are denoted II-1 to II-5 in order of decreasing electrophoretic mobility.

Further separation of group III on CM-cellulose gave more or less distinct peaks (Fig. 4), but starch-gel-electrophoretic analysis (results not shown) showed a strong overlap of components, particularly in the middle peaks, C and D, of the elution pattern. Nevertheless, fractions enriched in one of the electrophoretic components of group III were obtained from each of peaks A, B and E. These fractions are designated III-1, III-2 and III-3 respectively (Fig. 5, slots a–d). It should be noted that group III components, which in the cylindrical polyacrylamide gels (run at pH 9.5) migrate slowly towards the positive electrode (Fig. 1), behave on starch gels at pH 8.6 like neutral or even slightly positively charged fractions.

Molecular-weight estimation by SDS/polyacrylamide-gel electrophoresis

On SDS/polyacrylamide-gel electrophoresis group I casein shows several bands and diffuse zones in the region M_r 23 000–33 000. Whole group II as well as its isolated components had M_r approx. 30 000; in each preparation faint bands at M_r approx. 27 000 and approx. 24 000 were also present. For group III an M_r of approx. 23 000 was found, with a minor band at M_r approx. 24 000 and a very faint one at M_r approx. 42 000. Under identical conditions the cow α_{s1} -, β - and γ_2/γ_3 - (R-, S- and TS-)caseins showed approximate M_r values of 32 000, 25 000 and 12 000–13 000 respectively (for nomenclature of bovine caseins, see Whitney *et al.*, 1976).

We attempted to influence the relative mobilities by changing the conditions of sample preparation (addition of urea; increasing the percentages of SDS and mercaptoethanol; varying the degree of heat treatment) or gel preparation (9% and 12% acrylamide), but M_r values were essentially the same in all cases.

Composition and sequence

The amino acid compositions of the isolated (sub-)fractions of groups II and III are shown in Table 1.

Since group II appears, on the basis of both its amino acid composition and its partial sequence (see below), to be a homologue of the β -caseins, we calculated its amino acid composition on the basis of 209–210 residues/molecule (Ribadeau Dumas *et al.*, 1975; Woodward, 1976), corresponding to M_r 23 600. A high degree of similarity was found between the compositions of the individual sub-

Fig. 2. Fractionation of TS-casein on a DEAE-cellulose column

The sample (220mg) was dissolved in approx. 4 ml of equilibration buffer (20 mM-imidazole/HCl buffer, pH 7.3, containing 4 M-urea and 20 mM-2-mercaptoethanol). Column dimensions were 20 cm $\times$ 1.5 cm; the flow rate was approx. 30 ml/h, and the fraction size approx. 5 ml. The broken line indicates the NaCl gradient applied. For full details see the text. Group III and the various pools of group II were further fractionated (see the text and Figs. 3–5).

Fig. 3. Electrophoresis on alkaline starch gel of purified fractions obtained by rechromatography of group II on DEAE-cellulose

Slots: (a), whole group II; (b) II-1; (c) II-2; (d), II-3; (e), whole group II; (f), II-4; (g), II-5; (h), whole group II. For full details see the text.

components of group II. The same holds true for the components of group III, the composition of which has been based on about 105 residues/molecule (M_r 11 500), because this fraction appears to be homologous to cow γ_2/γ_3 -casein. Both groups II and III lack tryptophan and cystine/cysteine. Group III is devoid of phosphate, whereas the number of

phosphate groups attached to group II components is close to six, except that of component II-5, for which three or four residues/molecule were found. The latter component was present in low amount, and after isolation its electrophoretic purity was somewhat less satisfactory (Fig. 3, slot g). No component of either group II or group III casein was stained with the periodic acid/Schiff reagent or was found to contain sialic acid; both groups appear to be carbohydrate-free.

The *N*-terminal sequence of group II casein resembles that of cow β -casein (Table 2). The *N*-terminus of group III casein was not accessible to Edman degradation, whereas separate end-group determination by the dansyl procedure revealed trace amounts of valine; the latter might, however, be attributable to contamination. All components isolated from group II or group III casein have C-terminal -Ile-Val, which also forms the C-terminus of cow β - and γ -caseins.

Sensitivity towards enzymic attack

Several fractions obtained were investigated for their susceptibility towards the action of acid phosphatase, plasmin and chymosin. In most cases cow casein fractions were used as reference substrates.

With a modification of the procedure of Bingham *et al.* (1976), cow α_{s1} -casein (2.5 mg/ml) was completely dephosphorylated by acid phosphatase (7 μ l of suspension/ml) within 2 h (result not shown). Under identical conditions cow β -casein was nearly completely dephosphorylated (Fig. 5, slots *i* and *j*). Horse group II casein as well as its individual components were converted by acid phosphatase

Fig. 4. Separation of whole group III casein on a CM-cellulose column

The equilibration buffer was 0.28 mM-KH₂PO₄/0.24 mM-K₂HPO₄ buffer, pH 7.1, containing 4 M-urea. The sample (120 mg), dissolved in 2–3 ml of equilibration buffer, was adjusted to pH 7 and dialysed for 2 h against the buffer before being applied to the column. Column dimensions were 17 cm × 1.0 cm; the flow rate was approx. 20 ml/h, and the fraction size approx. 3 ml. A linear NaCl gradient (----) from 0 to 15 mM was generated in 8 h by the LKB Gradient Mixer, followed by two stepwise additions of 25 mM- and 2 M-NaCl subsequently. For full details see the text.

Fig. 5. Electrophoresis on alkaline starch gel showing the purified fractions obtained from group III as well as the action of plasmin and acid phosphatase on horse group II and bovine β -casein

Slots: (a), whole group III; (b), III-1; (c), III-2; (d), III-3; (e), whole group II; (f), whole group II + plasmin (2 h); (g), whole group II + plasmin (5 h); (h), bovine β -casein + plasmin (5 h); (i), bovine β -casein; (j), bovine β -casein + acid phosphatase; (k), whole group II + acid phosphatase; (l), whole group II. The patterns of individual group II components + acid phosphatase were identical with the pattern of slot (k). For full details see the text.

into two components, both of lower electrophoretic mobility (Fig. 5, cf. slots *k* and *l*); the starch-gel pattern shows no difference between the dephosphorylated protein after 2 h and after 8 h of incubation. Horse group III casein was not modified by acid phosphatase.

Plasmin converts cow β -casein into a mixture of fragments (Fig. 5, cf. slots *h* and *i*), of which some proteose/peptone components (Andrews, 1978*a,b*, 1979; Jenness, 1979; Eigel & Keenan, 1979) and the complementary γ_1 - (γ -), γ_2 - (S-, TS-A²) and γ_3 - (R-, TS-B) caseins (Kaminogawa & Yamauchi, 1972; Gordon *et al.*, 1972; Groves *et al.*, 1972, 1973; Eigel, 1977) form the early degradation products. Whole horse group II casein is completely converted by plasmin into group III casein, as judged by the gel-electrophoretic pattern; at the same time faint bands with a mobility identical with that of the bovine proteose/peptone fraction become apparent in the gel (Fig. 5, slots *e*–*g*). Isolated group II subcomponents are affected by plasmin in exactly the same manner (results not shown), each being converted into the combined components of group III casein. In its turn group III was, on longer incubation, further degraded by the enzyme.

The milk-clotting enzyme chymosin, which cleaves cow κ -casein into positively charged para- κ -casein and a negative macropeptide part (MacKinlay & Wake, 1971), slowly attacked horse group III casein, giving more positively charged ('para- κ -casein-like') components on the starch gel (results not shown). Under identical conditions groups I and II remained essentially unaffected.

Table 1. *Composition of whole group II and III caseins and their individual components*
Abbreviation: N.D., not determined.

	Content (mol/23 600 g of protein)							Content (mol/11 500 g of protein)				
	II	II-1	II-2	II-3	II-4	II-5	Cow βA^2	III	III-1	III-2	III-3	Cow $\gamma_2 A^2/\gamma_3 A$
Asx	11.3	11.2	11.3	11.1	11.8	13.0	9	5.7	5.3	4.8	4.4	3
Thr*	7.0	7.0	7.3	7.4	7.6	8.0	9	3.9	4.4	4.0	3.7	4
Ser*	16.0	15.9	16.4	16.1	16.5	16.2	16	5.1	5.2	6.1	5.8	7
Glx	40.5	38.3	39.2	38.3	37.8	39.0	39	16.2	15.1	15.4	15.4	15
Pro	32.3	31.4	31.6	31.5	30.1	30.0	35	18.4	19.6	19.8	20.1	21
Gly	3.8	4.0	4.3	4.2	5.6	5.5	5	2.6	3.1	2.9	2.2	2
Ala	7.4	7.4	7.5	7.4	8.0	8.4	5	5.2	5.4	5.2	4.8	2
Cys	0	0	0	0	0	0	0	0	0	0	0	0
Val†	21.1	21.0	21.0	20.9	21.2	19.3	19	11.6	11.2	11.3	11.8	10
Met	3.4	3.7	3.4	3.8	3.5	3.6	6	2.2	1.9	2.0	2.1	4
Ile†	10.8	11.3	10.8	11.5	10.7	10.9	10	5.5	5.3	5.5	5.7	3
Leu	20.8	20.7	20.6	20.5	20.8	20.5	22	12.1	12.2	11.8	11.5	14
Tyr*	4.2	4.6	4.4	4.5	3.7	4.7	4	2.4	2.8	2.5	2.7	3
Phe	7.3	7.6	7.6	7.4	7.7	7.4	9	4.2	4.0	4.0	4.2	5
Trp‡	0	0	0	0	0	0	1	0	N.D.	N.D.	N.D.	1
Lys	10.4	11.2	10.8	11.0	11.7	11.0	11	3.0	3.0	3.6	3.3	4/3
His	4.6	5.1	5.0	5.0	4.9	4.3	5	1.7	1.8	2.0	1.8	4/3
Arg	8.1	8.3	8.1	8.3	8.5	7.9	4	3.4	3.6	3.3	3.9	2
Phosphorus	5.5	6.2	5.9	5.8	6.3	3.5	5	0	N.D.	N.D.	N.D.	0
Sialic acid	0	0	0	0	N.D.	N.D.	0	0	0	0	0	0
$\Delta Q\delta$	14	15	14	14	19	19		49	42	38	38	

* Derived from extrapolation to zero hydrolysis time.

† Obtained from 96 h hydrolysates.

‡ Obtained from separate analyses after hydrolysis in 4 M-methanesulphonic acid (22 h, 115°C). For bovine β -casein we found 0.9 residue/molecule with this method (theoretically 1 residue/molecule).

§ Group II and III were compared with cow βA^2 - and $\gamma_3 A$ -casein respectively. Group III subcomponents were assumed to have no tryptophan.

Table 2. *N- and C-Terminal sequence of horse and cow β - and γ -caseins compared*

Fraction	N-Terminus				C-Terminus
	Lys*	Lys*	Met*	Phe* Met*	
Horse β -casein	Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val- ?			Arg-Glu-Ile-Val-	Ile-Val
Cow β -casein†	Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-				Ile-Val
Horse γ -casein	N-Terminus not accessible for Edman degradation				Ile-Val
Cow $\gamma_1/\gamma_2/\gamma_3$ -casein†	Lys-Ile-/His-Lys-/Glu-Met-				Ile-Val

* Peaks of phenylthiohydantoin derivatives not resolved with the high-pressure liquid-chromatography program used.

† See Ribadeau Dumas *et al.* (1975).

Discussion

Earlier attempts to fractionate horse casein were not successful (O'Connor & Fox, 1973), probably because of its low solubility. In the present study a first separation between the various fractions was brought about by making use of the reversibly temperature-dependent solubility of part of the casein. Further fractionation, by selective extraction and ion-exchange chromatography, led to three

groups of casein components (I, II and III) in the roughly estimated proportions 1:1:0.1.

Group I, which is largely insoluble in water at acid and neutral pH, consists of at least seven discrete bands in the alkaline urea/polyacrylamide gel, corresponding to electrophoretic patterns of horse casein published previously (Antila *et al.*, 1971; Lauer & Baker, 1972; Jenness, 1973; O'Connor & Fox, 1973).

Group II is water-soluble at pH 7 and low temperature (4°C), but is precipitated when the solution is brought to room temperature. At higher concentrations, solutions tend to become turbid even at low temperature. Group II consists of five components, all having similar amino acid composition and identical *N*- and *C*-terminal sequences. Since the phosphate content of at least four of these components is fairly constant and no sialic acid was determined in them, their different electrophoretic mobilities should probably be explained in terms of amino acid substitution or different amide content. Homology with β -casein from cow is concluded for group II because: (a) its *S* Δ *Q* value, as calculated (Marchalonis & Weltman, 1971) from the respective amino acid compositions, is only about 15 (Table 1); (b) its *C*-terminal sequence (-Ile-Val) is identical with that of cow β -casein; (c) its *N*-terminal sequence shows, as compared with the cow β -casein sequence, only one or two substitutions in the 13 positions determined (if one assumes that the three overlap peaks in the high-pressure liquid chromatogram stand for the phenylthiohydantoin derivatives of valine, isoleucine and leucine respectively). The substitution glycine (cow)→arginine (horse) in position 10 of the sequence is also found in β -casein from human (Greenberg *et al.*, 1976) and rat (Visser *et al.*, 1981). Horse group II (β)-caseins differ from cow β -casein principally in lack of tryptophan, higher arginine and alanine contents and lower methionine contents. It should be noted, however, that most of the β -like caseins known at present (Ribadeau Dumas *et al.*, 1975; Woodward, 1976; Mulvihill & Fox, 1979; Pélissier *et al.*, 1980; Visser *et al.*, 1981) are devoid of tryptophan.

Group III is composed of several parts of the group II (β -casein) sequence. The composition and *C*-terminal sequence of these fragments are similar to those of cow γ -caseins. Apart from the compositional differences mentioned above for the β -homologues, the horse group III proteins have lower histidine and somewhat higher isoleucine contents than cow γ -caseins. The *S* Δ *Q* value obtained by comparison with cow γ -caseins is approx. 40 (Table 1). Further support for homology with γ -caseins is that the group III components are formed as breakdown products from group II (β)-casein on incubation of the latter with plasmin, as is the case with the cow β -casein homologues (Eigel, 1977). Plasmin has been found to be very similar to or probably even identical with a proteinase naturally occurring in bovine milk (Kaminogawa *et al.*, 1972; Halpaap *et al.*, 1977; Eigel *et al.*, 1979). The present results indicate that such a plasmin-like enzyme is also present in horse milk. Lack of phosphate in group III suggests homology with cow γ_2/γ_3 -casein rather than with γ_1 -casein, although the presence of

the latter cannot be excluded. The solubility of the group III fraction is highly temperature-dependent, which corresponds to that of the cow γ_2/γ_3 (TS) species. Reversibly heat-precipitable γ -like fractions were also reported in earlier studies on human (Schade & Reinhart, 1970) and dog (Swanson & Sanders, 1974) milk.

The approximate molecular weights of 30 000 and 23 000 for horse β - and γ -caseins respectively, as determined by comparison with a series of standard proteins on SDS/polyacrylamide gels, cannot be explained easily in the light of the actual molecular weights of the cow casein homologues, which are approx. 24 000 and approx. 11 500 respectively. Possibly the conditions of SDS/polyacrylamide-gel electrophoresis as applied in the present study are less appropriate for the M_r determination of highly unordered proteins such as caseins. Although in our study the bovine β - and γ_2/γ_3 -caseins showed M_r values (approx. 25 000 and 12 000–13 000 respectively) that are close to the actual molecular weights, we found the M_r of bovine α_{s1} -casein to be much too high (approx. 32 000). The latter observation is in agreement with SDS/polyacrylamide-gel electrophoretic results obtained by Creamer (1978), El-Negoumy (1980) and Kaminogawa *et al.* (1980). Swanson & Sanders (1974) found by SDS/polyacrylamide-gel electrophoresis the M_r of the temperature-sensitive casein fraction from dog milk to be 25 000, which is about twice the value of 13 400 they determined by sedimentation-equilibrium studies. A dimeric form, maintained under the conditions of SDS/polyacrylamide-gel electrophoresis, could, as with our horse γ -caseins, be an explanation for the discrepancy.

In the present study we were not able to locate a κ -like casein in horse milk. Accepting the idea that horse casein is present in the milk in the micellar form, one should expect the occurrence of such a potentially micelle-stabilizing component. We might have overlooked it because of its possible inability to stain with Amido Black (Kotts & Jenness, 1976). Since the cleavability by calf chymosin may be one of the criteria for the characterization of a κ -like casein (cf. also Kotts & Jenness, 1976), we should note here that group III casein is our only fraction susceptible to the action of chymosin under conditions specified for the rapid splitting of cow κ -casein. However, group III was clearly found by other criteria to be a γ - rather than a κ -like casein. The group I fraction, which has not been further investigated, may include a κ -casein-like component; the insensitivity of this fraction towards attack by chymosin could then be ascribed to its very low solubility at pH 6–7.

Identification of the group I components and location of a possible κ -like casein would be of interest for further study.

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