

Original article

Characterization of mare caseins. Identification of α_{s1} - and α_{s2} - caseins

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Abstract — Whole mare (Mongolian and French breeds) casein was obtained from skim milk by isoelectric precipitation (pH 4.6) at 22 °C. In another series of experiments, equine caseins were fractionated after isoelectric precipitation at 4 °C, according to their sensitivity to temperature, on one hand, the pH of the resulting pellet was adjusted to 6.5 before centrifugation (45 000 *g* for 30 min) at 4 °C; on the resulting casein fraction which precipitated was named the cold precipitated (CP) fraction. On the other hand, the supernatant obtained from the centrifugation of skim milk at pH 4.6 and 4 °C was warmed to 30 °C before being centrifuged at 30 °C, 45 000 *g*, for 30 min. The resulting casein fraction which precipitated was named the thermally precipitated (TP) fraction. Equine caseins were then purified by high resolution gel chromatography on an anion-exchange column followed by reversed phase-high performance liquid chromatography. The casein fractions were analyzed by urea- and SDS-polyacrylamide gel electrophoresis, their amino acid compositions were determined and their first 15 N-terminal amino acids were sequenced. This analysis showed the presence of α_s -like caseins isolated from the CP fraction. α_{s1} -Like-casein showed 4 major double bands by electrofocusing with a pI range of 4.3–4.8. In the same conditions, α_{s2} -like casein showed 2 major bands with a pI range of 4.3–5.1. The TP fraction revealed the existence in equine milk of 6 subfractions of β -like caseins, occurring in the following ratios: 1:5:18:20:15:10, differing at least in their degree of phosphorylation (as shown also by the action of acid phosphatase). Since no evidence of the presence of κ -casein was found in equine milk, it is proposed that part of its functions in the milk of the Equidae could be assumed by the population of less phosphorylated β -caseins.

mare milk / α_{s1} -casein / α_{s2} -casein / β -casein

Résumé — Caractérisation des caséines du lait de jument. Identification des caséines α_{s1} et α_{s2} . La caséine entière de lait écrémé de juments issues de troupeaux de Mongolie et de France a été obtenue par précipitation isoélectrique (pH 4,6) à 22 °C. Par ailleurs, les caséines équines ont été

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fractionnées, après une précipitation isoélectrique à 4 °C, selon leur sensibilité à la température. D'une part, le pH du culot résultant était ajusté à 6,5 avant de réaliser une nouvelle centrifugation (45 000 g pendant 30 min) à 4 °C ; la fraction caséique qui précipitait était nommée « cold precipitated (CP) fraction ». D'autre part, le surnageant résultant de la centrifugation du lait écrémé à pH 4,6 et 4 °C était alors réchauffé jusqu'à 30 °C avant d'être centrifugé à 30 °C, 45 000 g, pendant 30 min. La fraction caséique qui précipitait dans ces conditions était nommée « thermally precipitated (TP) fraction ». Les caséines équine ont été purifiées successivement par chromatographie sur colonne d'échange d'anions et chromatographie liquide haute performance en phase inversée. Les fractions caséiques ont été analysées par électrophorèse sur gel de polyacrylamide en présence de SDS ou d'urée. Leur composition en acides aminés et la séquence des 15 premiers résidus N-terminaux ont été déterminées. Ces analyses et les résultats de l'action de la phosphatase acide révèlent l'existence dans la fraction TP de 6 fractions de caséine β (pI 4,8–5,5), présentes dans les rapports 1:5:18:20:15:10, différant, entre autres, par leur degré de phosphorylation. Les caséines α_{S1} (pI 4,3–4,8) et α_{S2} (pI 4,3–5,1) ont été mises en évidence dans la fraction CP. Aucune fraction caséique assimilable à la caséine κ n'a pu être retrouvée ; il est suggéré qu'une partie des fonctions de la caséine κ dans le lait des équidés soit assumée par les populations de caséines β les moins phosphorylées.

lait de jument / caséine α_{S1} / caséine α_{S2} / caséine β

1. INTRODUCTION

Dairy proteins constitute the main element of the diet in many regions of the world, including those inhabited by the pastoral populations of Central Asia in particular. Knowledge regarding the structure and the functions of the major bovine milk proteins is now fairly complete, though their transformations and possible nutritional and nutraceutical functions are still being intensively studied.

Surprisingly, the situation is quite the opposite in the case of equine caseins, about which even less is known than caseins from different Camelidae [2, 9, 17, 20, 22, 24].

In the past decade numerous studies have examined the effect of milk and fermented milk products on consumers, and the associated health benefits. In this area, mare milk and koumiss (fermented mare milk) appear very promising. Based on its composition in proteins, lactose, macro- and oligoelements [27], mare milk is more similar than bovine milk to human milk. Whey proteins of mare milk, easier to separate than caseins, have been studied extensively [3, 5, 7, 10, 11, 13, 15, 16]. However, equine caseins still remain relatively poorly investigated,

their study being limited to the determination of total casein content [8, 14], amino acid composition or electrophoretic patterns of whole casein fraction [21, 30]. The mare β -casein fraction has been characterized previously by Visser et al. [30] and the analysis of its amino acid composition and the determination of its N- and C-terminal sequence has been carried out, but little is known about the other caseins present in mare milk.

As can be observed from the increase in basic knowledge regarding bovine dairy proteins and its effect on the dairy industry in developed countries, further studies on equine milk proteins could also have a positive effect on transformations, and on the more rational use of this important food product in Mongolia and other Central Asian countries. Furthermore, it is traditionally considered that fresh and fermented equine milk has a number of nutritional and curative properties which should be verified by simple research systems. Consequently, the aim of this study was the isolation and investigation of the physico-chemical characteristics of α_S -casein fractions, and a re-examination of the β -casein fraction of mare milk of Mongolian origin.

2. MATERIALS AND METHODS

2.1. Preparation of samples

Three samples of milk were studied and showed similar results. Milk was either collected from 2 individual mares of Mongolian stock but of different geographical origin, or obtained from 'La Ferme des Minimes', Saint Jean les Deux Jumeaux, France, and the 'Laboratoire Jum'Lac', domaine de la Voie Lactée, Eschviller, Volmunster, France. After defatting and freeze-drying, milk was kept frozen at $-20\text{ }^{\circ}\text{C}$ until further processing. All the experiments described in this paper were carried out using milk from only one Mongolian mare per experiment.

2.2. Preparation and separation of caseins

Whole casein was obtained from skim milk by isoelectric precipitation (pH 4.6) at $22\text{ }^{\circ}\text{C}$, using $1\text{ mol}\cdot\text{L}^{-1}$ HCl. The precipitate was washed twice with distilled water at pH 4.6, solubilized at pH 7 by addition of $1\text{ mol}\cdot\text{L}^{-1}$ NaOH, precipitated again at

pH 4.6 with $1\text{ mol}\cdot\text{L}^{-1}$ HCl and washed 3 times with distilled water. Finally, the whole casein was solubilized at pH 7, freeze-dried and stored at $-20\text{ }^{\circ}\text{C}$. In another series of experiments, mare milk caseins were fractionated after acid precipitation at $4\text{ }^{\circ}\text{C}$ according to their sensitivity to temperature, as described by Visser et al. [30]. The casein fraction which precipitated after centrifugation ($45\text{ }000\text{ g}$ for 30 min) at pH 6.5 and $4\text{ }^{\circ}\text{C}$ of the pellet obtained from the treatment of skim milk at pH 4.6 and $4\text{ }^{\circ}\text{C}$ was named the cold precipitated (CP) fraction. The supernatant resulting from the centrifugation of skim milk at pH 4.6 and $4\text{ }^{\circ}\text{C}$ was then warmed up to $30\text{ }^{\circ}\text{C}$. The casein fraction which precipitated after centrifugation at $30\text{ }^{\circ}\text{C}$ ($45\text{ }000\text{ g}$ for 30 min) was named the thermally precipitated (TP) fraction (Fig. 1).

2.3. High perfusion liquid chromatography (HPLC)

Individual caseins were separated by chromatography on an anion-exchange

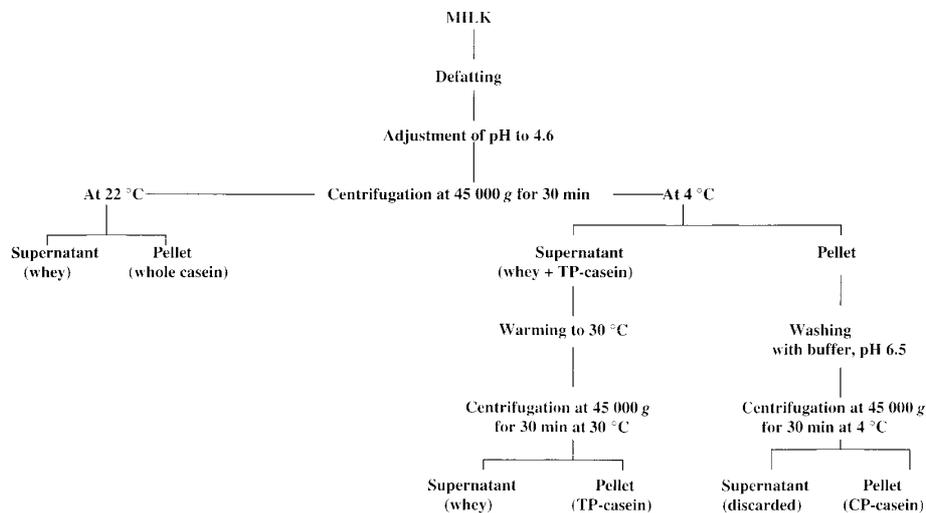


Figure 1. Preparation of mare caseins according to their sensitivity to temperature according to Visser et al. [30].

Figure 1. Préparation des caséines de jument en fonction de leur sensibilité à la température d'après Visser et al. [30].

column. This was carried out on a Biocad Sprint system (PerSeptive Biosystems, Voisins le Bretonneux, France), on an HQ Poros 20 μm column (4.6×100 mm). The column was equilibrated in buffer A (25 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl, pH 7.0, 4.5 $\text{mol}\cdot\text{L}^{-1}$ urea, 0.8 $\text{mmol}\cdot\text{L}^{-1}$ dithiothreitol). Separation was carried out at a flow rate of 5 $\text{mL}\cdot\text{min}^{-1}$, using a linear gradient from 0 to 30% B (25 $\text{mmol}\cdot\text{L}^{-1}$ Tris-HCl, pH 7.0, 4.5 $\text{mol}\cdot\text{L}^{-1}$ urea, 0.8 $\text{mmol}\cdot\text{L}^{-1}$ dithiothreitol, 1 $\text{mol}\cdot\text{L}^{-1}$ NaCl) over 20 column volumes. Absorbency was recorded at 280 and 214 nm.

2.4. Reversed phase-high performance liquid chromatography (RP-HPLC)

Individual caseins were further purified by RP-HPLC. Analytical and preparative RP-HPLC separations were performed on a Waters instrument (Waters Associates, Millford, MA, USA) equipped with an interface module system, assisted by a chromatography workstation Maxima 820. Analytical RP-HPLC of proteins was carried out on a Nucleosil 5 C_{18} column (4.6×250 mm), with a flow rate of 1 $\text{mL}\cdot\text{min}^{-1}$. A LiChro-CART 100 C_{18} column (10×250 mm) was used for preparative runs, with a flow rate of 2 $\text{mL}\cdot\text{min}^{-1}$. Separation was obtained by using a gradient from solvent A (H_2O , 0.1% TFA) to solvent B (20% H_2O , 80% acetonitrile, 0.09% TFA) as described in the corresponding figures. Absorbency was recorded at 214 nm.

2.5. Polyacrylamide gel electrophoresis (PAGE)

SDS- and urea-PAGE were performed in a vertical mini-slab gel apparatus Protean II (Bio-Rad) and in a standard vertical gel electrophoretic apparatus, model SE-400 (Hoeffer Sci. Inst., San Francisco, CA, USA). The SDS-PAGE was carried out according to the method of Laemmli [19] at 20 °C. The running gel ($80 \times 60 \times 0.75$ mm

or $160 \times 140 \times 1$ mm) and the stacking gel contained 150 $\text{g}\cdot\text{L}^{-1}$ and 40 $\text{g}\cdot\text{L}^{-1}$ acrylamide, respectively. The acrylamide gel (100 $\text{g}\cdot\text{L}^{-1}$ in 4 $\text{mol}\cdot\text{L}^{-1}$ urea, Tris-HCl, pH 8.8, buffer solution) for urea-PAGE was prepared as described previously [24]. The silver staining of the gel was carried out according to Nesterenko et al. [23], and staining with Schiff's reagent was according to Grooves et al. [12].

2.6. Isoelectric focusing

Isoelectric focusing was performed on ready-to-use gels (Serva, Gagny, France) in a pH range of 3–10, modified with 8 $\text{mol}\cdot\text{L}^{-1}$ urea and 2.5% Triton X-100 according to Vegarud et al. [29]. The samples were dissolved in 8 $\text{mol}\cdot\text{L}^{-1}$ urea, 1% 2-mercaptoethanol and 10% Triton X-100. Isoelectric focusing was performed at 4 °C on a 2117 Multiphor II apparatus (LKB, Bromma, Sweden), at a constant current of 7 mA.

2.7. Amino acid composition analysis

The purified individual caseins were hydrolyzed with 6 $\text{mol}\cdot\text{L}^{-1}$ HCl (Pierce) for 24 h at 110 °C in a Pico-Tag station (Waters). The amino acids were derivatized with phenylisothiocyanate (PITC) according to the method of Bidlingmeyer et al. [4] and separated by RP-HPLC on a Pico-Tag C_{18} column (3.9×150 mm). The column was equilibrated in solvent A (94% 0.14 $\text{mol}\cdot\text{L}^{-1}$ CH_3COONa , 0.5 $\text{mL}\cdot\text{L}^{-1}$ triethylamine, pH 6.4/6% acetonitrile) and the elution was performed by using a gradient from solvent A to solvent B (40% H_2O /60% acetonitrile) as previously described [24]. Both the column and solvents were maintained at 38 °C. The flow rate was 1.0 $\text{mL}\cdot\text{min}^{-1}$ and absorbency was recorded at 254 nm. Presented results are the means of 3 determinations.

2.8. N-terminal sequencing

The N-terminal amino acid sequence analysis was performed on an Applied

Biosystems model 477A sequencer with on-line identification of the phenyl thiohydantoin derivatives. Reagents used for sequencing were purchased from Perkin-Elmer. In each case, 150 pmol of casein were applied. The identity and originality of the amino acid sequences obtained were checked in the NCBI data base using the Blast network service.

2.9. Chymosin test

This was carried out as previously described [24].

2.10. Determination of protein concentration and phosphorus content

Concentration of proteins in samples was determined by the bicinchoninic acid method according to Smith et al. [28]. Phosphorus content was measured by the method of Bartlett [1].

2.11. Dephosphorylation of mare β -casein

β -Casein fractions were dephosphorylated according to Carles and Ribadeau Dumas [6]; 1 mg of fractions TP-4 to TP-8 (see §3.2) were solubilized in 1 mL 0.1 mol·L⁻¹ acetate buffer, pH 5.8. Acid phosphatase from potato (E.C. 3.1.3.2.; activity: 62 units·mg⁻¹ protein, Sigma) was added to the solution in an enzyme/substrate ratio of 1:2 000. The mixture was incubated at 37 °C for 24 h in the presence of a protease inhibitor (0.1 mmol·L⁻¹ phenylmethylsulfonyl fluoride, PMSF). Samples were then dissolved in electrophoretic sample buffer (1:1, v/v) before running in urea-PAGE.

3. RESULTS AND DISCUSSION

Equine milk caseins were isolated either by precipitation at pH 4.4-4.6 or according

to their sensitivity to temperature, as described above. Mare whole casein, obtained by acid precipitation at room temperature, separated in alkaline urea-PAGE into 3 groups of proteins consisting, according to Bioprofil software, of 40.8% in group Ia and b, 51.4% in group II and 7.8% in group III (Fig. 2, lane 1). The same 3 groups of proteins were also obtained in the CP fraction (Fig. 2, lane 2). Alkaline urea-PAGE patterns showed that TP casein (Fig. 2, lane 3) was completely free from group I. However, all the casein components were globally recovered by adding TP (Fig. 2,

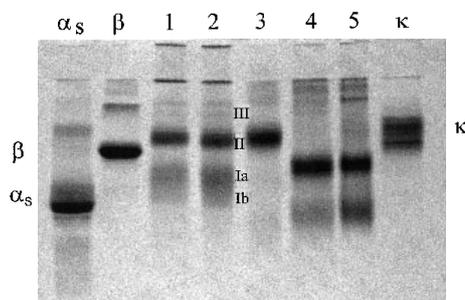


Figure 2. Urea-PAGE of mare milk protein subcomponents obtained by acid precipitation at different temperatures. α_s , β and κ denote cow α_s , β - and κ -casein as references. 1: whole mare casein precipitated at pH 4.6 and 22 °C; 2: cold precipitated (CP) mare casein; 3: thermally precipitated (TP) mare casein; Ia and b, II and III denote mare casein groups as defined in the Results and Discussion; 4: whole whey protein fraction soluble at pH 4.6 and 22 °C; 5: resulting whey protein fraction after separation of CP- and TP-fractions.

Figure 2. Electrophorèse sur gel de polyacrylamide en présence d'urée des composants protéiques du lait de jument obtenus par précipitation acide à différentes températures ; α_s , β et κ représentent les caséines α_s , β et κ de vache, comme références. 1 : caséine entière de jument précipitée à pH 4,6 et 22 °C ; 2 : caséine de jument précipitée à froid (CP) ; 3 : caséine de jument précipitée à température ambiante (TP) ; Ia et b, II et III représentent les groupes de caséines définis en résultats et discussion ; 4 : fraction protéique soluble à pH 4,6 et 22 °C ; 5 : fraction protéique soluble après séparation des fractions CP et TP.

lane 3) and CP (Fig. 2, lane 2) fractions since the resulting whey fractions (proteins soluble at pH 4.6, 22 °C (Fig. 2, lane 4) and proteins soluble after separation of CP- and TP-fractions (Fig. 2, lane 5) were casein-free.

3.1. Cold precipitated casein subfraction

Figure 3 shows the separation of mare CP casein by anion-exchange chromatography on Poros HQ using a linear NaCl gradient. The 7 fractions obtained were combined as shown. Further analyses of these fractions were performed by urea-PAGE

(Fig. 4). Urea-PAGE of aliquot CP-1 showed a pattern of diffuse bands moving in a similar way to the proteins from the first 2 aliquots of TP casein (Fig. 8), being a bit more positively charged. According to the SDS-PAGE, this CP-1 fraction consisted of 3 proteins (or peptides) with an apparent molecular mass of 13.0, 21.0, and 24.8 kg·mol⁻¹ respectively (data not shown). The fractions CP-2 to CP-6 consisted of 2 protein groups, previously termed I and II, free of protein group III present in the crude CP fraction (Figs. 2 and 4). A good separation of these 2 protein groups by anion-exchange chromatography on Poros HQ was particularly difficult mainly because

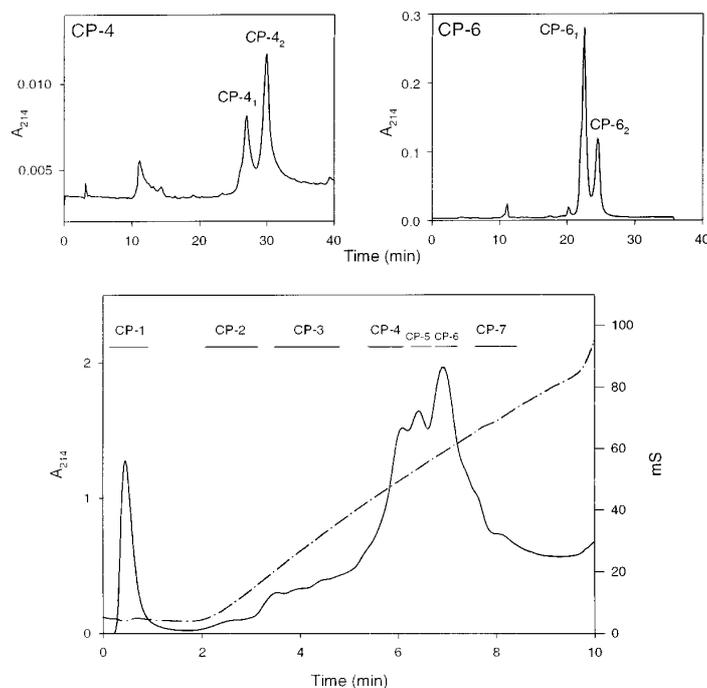


Figure 3. Separation of CP casein subfractions by anion-exchange chromatography on Poros HQ into 7 fractions (CP-1 to CP-7). Further purification of CP-4 and CP-6 was performed by RP-HPLC on a C₁₈ column with linear gradients from 0 to 80% solvent B (20% H₂O, 80% acetonitrile, 0.09% TFA) in 35 min and from 15 to 80% solvent B in 30 min for CP-4 and CP-6, respectively (see Material and Methods for details).

Figure 3. Séparation en 7 fractions, par chromatographie sur colonne Poros HQ, des fractions caséiques précipitées à froid (CP-1 à CP-7). Purification ultérieure de CP-4 et CP-6 par CLHP en phase inversée sur colonne C₁₈, à l'aide de gradients linéaires de 0 à 80 % de solvant B (H₂O 20 %, acétonitrile 80 %, TFA 0.09 %) en 35 min ou de 15 à 80 % de solvant B en 30 min, respectivement pour la purification de CP-4 et de CP-6 (pour plus de détails, voir Matériel et méthodes).

their differences in charge were very close. Nevertheless, chromatography by RP-HPLC on a C_{18} column of fractions CP-4 and

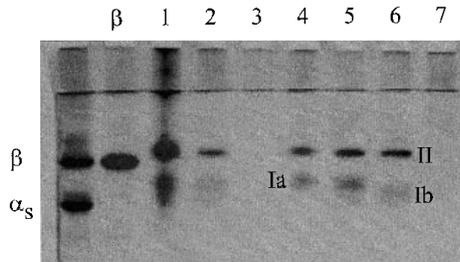


Figure 4. Urea-PAGE at pH 8.8 of CP-1 to CP-7 fractions obtained by anion-exchange chromatography. α_s and β denote cow α_s - and β -casein. 1 to 7 indicate fractions CP-1 to CP-7, respectively; Ia, Ib, and II denote mare casein groups as defined in the Results and Discussion.

Figure 4. Electrophorèse sur gel de polyacrylamide en présence d'urée, à pH 8,8 des fractions CP-1 à CP-7 issues de la chromatographie d'échange d'anions. α_s et β représentent les caséines α_s et β de vache. 1 à 7 représentent respectivement les fractions CP-1 à CP-7. Ia, Ib, et II représentent les groupes de caséines de jument tels que définis en résultats et discussion.

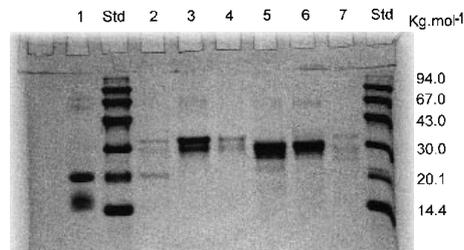


Figure 5. SDS-PAGE. 1: whey proteins obtained after precipitation of caseins at pH 4.6, 22 °C; 2: mare milk; 3: whole casein obtained by precipitation at pH 4.6, 22 °C; 4: CP-fraction; 5: CP-6₁ fraction; 6: CP-4₁ fraction; 7: TP-fraction; Std: molecular mass markers.

Figure 5. SDS-PAGE. 1 : protéines du lactosérum obtenues après précipitation des caséines à pH 4,6, 22 °C ; 2 : lait de jument ; 3 : caséine entière obtenue par précipitation à pH 4,6, 22 °C ; 4 : fraction CP ; 5 : fraction CP-6₁ ; 6 : fraction CP-4₁ ; 7 : fraction TP ; Std : kit de calibration de masses moléculaires.

CP-6 (Fig. 3) first allowed the separation of groups I and II, and secondly the separation of 2 proteins in group I (Fig. 4) named CP-4 Ia (peak CP-4₁ in Fig. 3) and CP-6 Ib (peak CP-6₁ in Fig. 3). With SDS-Tricine-PAGE, these proteins were found to have a molecular mass near 30 000 g·mol⁻¹ (Fig. 5).

The amino acid composition of fractions CP-4 Ia and CP-6 Ib (Tab. I) is very similar, and totally different from the amino acid composition of the TP fractions (Tab. III). Although the main differences between bovine α_{S1} - and α_{S2} -caseins in particular as regards Gly, Thr, Pro, and Lys residues are not observed in their equine counterparts, the results obtained so far favor the hypothesis that CP-4 Ia and CP-6 Ib are mare α_s -like-caseins. Moreover, the homology observed in the N-terminal sequence of the first 15 amino acids of several mammals (Tab. II) indicates that CP-6 Ib and CP-4 Ia are mare α_{S1} - and α_{S2} -like-caseins, respectively.

The results of urea-PAGE show that mare α_{S1} -like-casein has a lower net negative charge than cow α_{S1} -casein, and migrates as a diffuse band. This may result from heterogeneous phosphorylation.

As shown by isoelectrofocusing (Fig. 6), 4 major double bands have been obtained for α_{S1} -casein in a pI range of 4.3-4.8 and 2 major bands for α_{S2} -casein in a pI range of 4.3-5.1.

The N-terminal sequence of protein group II also present in CP fractions CP-4 II (peak CP-4₂ in Fig. 3) and CP-6 II (peak CP-6₂ in Fig. 3) revealed the same sequence as mare β -casein (Tab. II). This protein group II, during electrophoresis in the presence of urea, migrates identically to mare β -casein TP-8.

3.2. Thermally precipitated casein subfractions

As shown in Figure 7, separation of TP casein by anion-exchange chromatography

Table I. Amino acid composition of α_{s1} - and α_{s2} - like-casein fractions.**Tableau I.** Composition en acides aminés des fractions de caséines α_{s1} et α_{s2} .

Fraction	CP-4 Ia*	Cow α_{s2}	CP-6 Ib*	Cow α_{s1}
Asx	8.1	8.7	8.7	7.5
Glx	23.7	19.3	24.0	19.6
Ser	7.1	8.2	6.9	8.0
Gly	0.3	1.0	1.3	4.5
His	3.5	1.4	3.7	2.5
Arg	6.7	2.9	6.1	3.0
Thr	3.7	7.2	4.2	2.5
Ala	3.7	3.9	5.1	4.5
Pro	7.1	4.8	6.9	8.5
Tyr	4.9	5.8	3.9	5.0
Val	3.5	6.8	3.4	5.5
Met	2.0	1.9	1.4	2.5
Cys	nd	1.0	nd	0
Ile	5.1	5.3	4.8	5.5
Leu	9.0	6.3	9.2	8.5
Phe	3.5	2.9	3.2	4.0
Lys	8.0	11.6	7.0	7.0

* Fractions CP-4 Ia and CP-6 Ib correspond to peaks CP-4₁, and CP-6₁, respectively; values are given in number of residues/100 residues.

* Les fractions CP-4 Ia et CP-6 Ib correspondent respectivement aux pics CP-4₁ et CP-6₁; valeurs exprimées en nombre de résidus/100 résidus.

on a Poros HQ-20 μ column gave 8 fractions (TP-1 to TP-8). Alkaline urea-PAGE (pH 8.8) of the separated subfractions (Fig. 8) showed that the first 2 samples contained several high positively charged peptides (or proteins), with an apparent molecular mass between 14 and 25 kg·mol⁻¹, according to SDS-PAGE (results not shown). Visser et al. [30] proposed these peptides to be a mixture of β -casein fragments resulting from post-translational proteolysis by plasmin. According to their amino acid composition (Tab. III) and their N-terminal sequences (Tab. II), the combined fractions TP-3, TP-4, TP-5, TP-6, TP7, plus TP-8 (named β -total in Tabs. II and III) contained mare β -like-caseins. Urea-PAGE of these samples (Fig. 8) indicated clearly that each of them was close to homogeneity and that their mobility increased with the order of their elution from a Poros HQ-20 μ column

(Fig. 7). In an earlier study on equine casein of European origin, Visser et al. [30] found 5 β -like-caseins, all of them showing quite similar amino acid composition and identical N- and C-terminal sequences. Additionally, the phosphate content of at least 4 of these components was found to be fairly constant. In the present work, 6 β -like-casein components were found, differing from each other both in the number of phosphate groups and in their content in some amino acid residues (mainly Asx, Glx, Ala and Pro; Tab. III). Fractions TP-3, TP-4, TP-5, TP-6, TP-7, and TP-8 obtained from Poros HQ chromatography further purified by RP-HPLC on a Nucleosil 5 C₁₈ column (Fig. 7) contain 0, 2, 6, 4, 4, and 5 phosphorus residues, respectively. These results agree well with previously reported data [30]. The detected 6 subfractions of mare β -casein occur in studied equine casein in a

Table II. N-terminal sequence of obtained fractions of α_{s1}^- [CP-6 Ib (peak CP-6₁)], α_{s2}^- [CP-4 Ia, (peak CP-4₁)] and β -caseins [TP-4 to TP-8; CP-4 II (peak CP-4₂) and CP-6 II (peak CP-6₂)] of *Equus caballus*. Comparison with α_{s1}^- , α_{s2}^- and β -caseins of cow*, goat*, mouse*, pig*, *Camelus bactrianus*[†] and *Camelus dromedarius*[§].

Tableau II. Séquence N-terminale des fractions de caséines α_{s1} [CP-6 Ib (pic CP-6₁)], α_{s2} [CP-4 Ia (pic CP-4₁)] et β [TP-4 à TP-8 ; CP-4 II (pic CP-4₂) et CP-6 II (pic CP-6₂)] d'*Equus caballus*. Comparaison entre les caséines α_{s1} , α_{s2} et β de vache*, de souris*, de porc*, de chèvre*, de souris*, de porc*, de *Camelus bactrianus*[†] et de *Camelus dromedarius*[§].

α_{s1} (Protein group Ib)	
cow	R - P - K - H - P - I - K - H - Q - G - L - P - Q - E -
Goat	R - P - K - H - P - I - N - H - R - G - L - S - P - E -
Mouse	R - L - H - S - R - N - A - V - S - S - Q - T - Q - Q -
Pig	R - P - K - P - P - L - R - H - Q - E - H - L - Q - N -
<i>Camelus bactrianus</i>	R/K - P - K - Y - P - L - R - Y - P - E - V - F - Q - N -
<i>Camelus dromedarius</i>	R - P - K - Y - P - L - R - Y - P - E - V - F - Q - N -
Mare CP-6 ₁	R - P - K - L - P - S - R - Q - Q - E - I - I - Q - N -
α_{s2} (Protein group Ia)	
cow	K - N - T - M - E - H - V - S - S - S - E - E - S - I - I -
Goat	K - H - K - M - E - H - V - S - S - S - E - E - P - I - N -
Mouse	K - Q - R - M - E - Q - Y - I - S - S - E - E - S - M - D -
Pig	K - H - E - M - E - H - V - S - S - S - E - E - S - I - N -
<i>Camelus bactrianus</i>	K - H - E - M - D - Q - G - X - X - X - E - E - Q - N - I -
<i>Camelus dromedarius</i>	K - H - E - M - D - Q - G - S - S - S - E - E - S - I - N -
Mare CP-4 ₁	K - H - K - M - E - H - F - A - P - X - Y - X - Q - V - L -
β (Protein group II)	
cow	R - E - L - E - E - L - N - V - P - G - E - I -
Goat	R - E - Q - E - E - L - N - V - V - G - E - T -
Mouse	R - E - T - T - F - T - V - S - S - E - T - D -
Pig	R - A - K - E - E - L - N - A - S - G - E - T -
<i>Camelus bactrianus</i>	R - E - K - E - E - F - K - T - A - G - E - A -
<i>Camelus dromedarius</i>	R - E - K - E - E - F - K - T - A - G - E - A -
Mare β -total	R - E - K - E - E - L - N - V - S - S - E - I -
Mare TP-4 to TP-8	R - E - K - E - E - L - N - V - S - S - E - I -
Mare CP-4 ₂	L - E - K - E - E - L - N - V - P - K - E - Y -
Mare CP-6 ₂	R - E - K - E - E - L - N - V - I - S - Q - I -

* Data on cow, goat, mouse and pig caseins are from the Swissprot database; [†] according to Ochirkhuyag et al. [24]; [§] according to Kappeler et al. [17].

* Les données concernant les caséines de vache, chèvre, souris et porc sont issues de la base de données Swissprot ; [†] selon Ochirkhuyag et al. [24] ; [§] selon Kappeler et al. [17].

ratio of 1:5:18:20:15:10. Fractions TP-4 to TP-8 present the same primary structure in their first 12 amino acid residues. Urea-PAGE of fractions TP-5 to TP-8 after the action of acid phosphatase (Fig. 9) shows that phosphorylation may be one of the factors in the polymorphism of mare β -casein.

Since the fractions TP-3 to TP-8 are not exactly eluted by anion-exchange chromatography according to their degree of phosphorylation, other factors are responsible for the different forms of β -casein observed. These could be a different content in amide amino acid residues of the

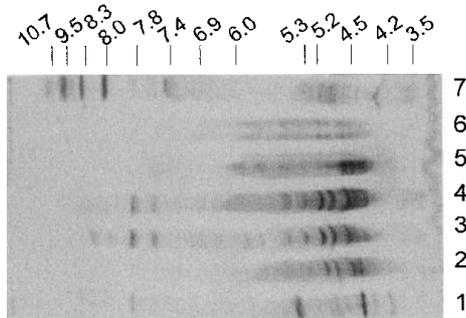


Figure 6. Isoelectrofocusing in the pH range 3-10 of: 1: mare milk; 2: whole casein obtained by acid precipitation at pH 4.6 at 22 °C; 3: TP-fraction (β -casein); 4: CP-fraction (α_S -caseins); 5: peak CP-6₁ (α_{S1} -casein); 6: peak CP-4₁ (α_{S2} -casein) and 7: pI marker.

Figure 6. Isoélectrofocalisation dans la gamme de pH 3-10 de : 1 : lait de jument ; 2 : caséine entière obtenue par précipitation à pH 4, 6, à 22 °C ; 3 : fraction TP (caséine β) ; 4 : fraction CP (caséine α_S) ; 5 : pic CP-6₁ (caséine α_{S1}) ; 6 : pic CP-4₁ (caséine α_{S2}) ; 7 : marqueurs de pI.

caseins, or the result of their deamidation occurring during the purification process (factors which are not detectable by determination of amino acid composition after acid hydrolysis). By electrofocusing of the crude TP-fraction, 6 bands of β -casein could be detected in a pI range of 4.8-5.5 (Fig. 6). Only the determination of the primary structure of the different forms of mare β -casein should permit elucidation of what is responsible for their differences.

3.3. Does a κ -like-casein exist in mare milk?

Evidence for the presence of a κ -like-casein in mare milk is still quite contradictory, despite several studies on the subject [18, 21, 25, 28]. No protein bands that were homologous to bovine κ -casein could be detected in urea electrophoregrams in this study. As is well known, chymosin

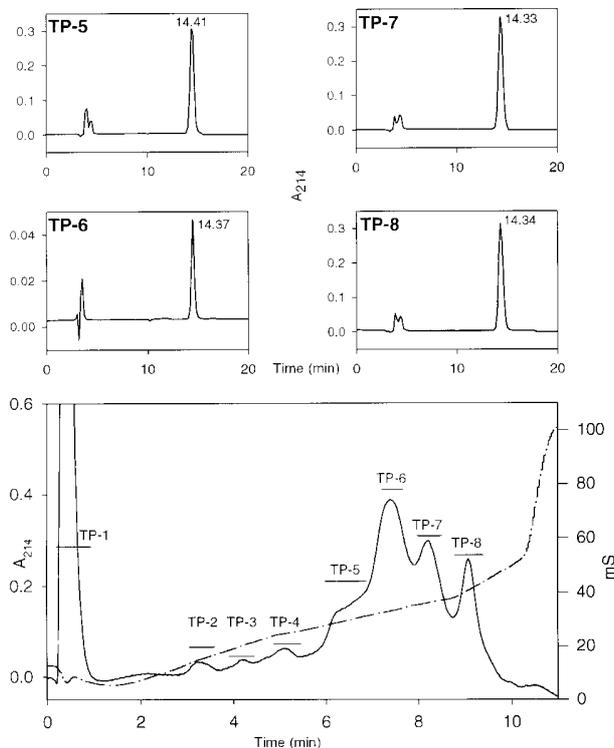


Figure 7. Separation of TP casein subfractions by anion-exchange chromatography on Poros HQ into 8 fractions (TP-1 to TP-8). Further purification of fractions TP-5 to TP-8 by RP-HPLC on a C₁₈ column with a linear gradient from 30 to 70% solvent B (20% H₂O, 80% acetonitrile, 0.09% TFA) in 20 min (see Materials and Methods for details).

Figure 7. Séparation en 8 fractions (TP-1 à TP-8) par chromatographie par échange d'anions (colonne Poros HQ) des caséines précipitées à température ambiante (fractions TP). Purification ultérieure des fractions TP-5 à TP-8 par CLHP en phase inversée sur colonne C₁₈, à l'aide d'un gradient linéaire de 30 à 70 % de solvant B (H₂O 20 %, acétonitrile 80 %, TFA 0,09 %) en 20 min (pour plus de détails, voir Matériel et méthodes).

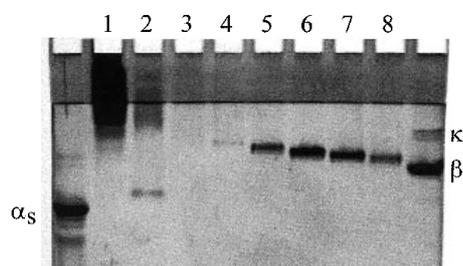


Figure 8. Urea-PAGE at pH 8.8 of TP-1 to TP-8 fractions obtained by chromatography on Poros HQ. 1 to 8 are fractions TP-1 to TP-8, respectively. α_S , β and κ denote cow α_S -, β - and κ -casein.

Figure 8. Electrophorèse sur gel de polyacrylamide en présence d'urée, à pH 8,8 des fractions TP-1 à TP-8 issues de la chromatographie sur colonne Poros HQ. 1 à 8 représentent respectivement les fractions TP-1 à TP-8 ; α_S , β et κ représentent les caséines α_S , β et κ de vache.

(EC-3.4.23.4) splits preferentially bovine κ -casein. Consequently, in this study, treatment with chymosin of TP casein was performed, leading to the appearance of a new band migrating on urea-PAGE in a similar region to cow *para*- κ -casein (data not shown). However, the original band of intact κ -casein could not be observed. Furthermore, the analysis of TP casein by analytical RP-HPLC showed that a minor peak observed on the chromatogram vanished after the action of chymosin (data not shown). Unfortunately, the analysis of the N-terminal sequence of the protein purified from this minor peak demonstrated that it was similar to the minor mare β -like-casein component. Neither the attempt to detect κ -like-casein by affinity chromatography on thiol-activated Affi-gel 501, nor the

Table III. Amino acid composition of β -like-casein fractions.

Tableau III. Composition en acides aminés des fractions de caséines β .

Fractions	TP-3	TP-4	TP-5	TP-6	TP-7	TP-8	β Total	Mare β -CN*	Cow βA^2 -CN§
Asx	3.7	2.4	4.9	5.2	4.7	5.3	4.0	5.4	4.3
Glx	15.2	13.3	21.1	21.3	18.8	22.2	20.3	18.4	18.7
Ser	7.6	8.4	7.9	7.9	7.9	9.5	7.7	7.6	7.7
Gly	3.7	3.0	1.9	1.9	2.0	2.3	1.8	1.9	2.4
His	1.5	1.9	2.0	2.3	2.3	2.5	2.3	2.4	2.4
Arg	5.2	5.1	4.3	4.3	4.0	4.1	4.7	4.0	1.9
Thr	4.6	4.0	3.4	3.5	3.0	3.4	2.9	3.4	4.3
Ala	8.1	7.2	6.5	4.0	8.9	4.0	4.1	3.6	2.4
Pro	12.6	16.4	15.5	15.6	13.7	13.5	15.4	15.1	16.7
Tyr	2.2	1.4	1.6	1.7	1.6	1.4	2.1	2.2	1.9
Val	8.1	9.6	8.9	9.2	8.8	8.6	9.6	10.1	9.1
Met	2.4	1.7	1.7	1.8	1.6	1.4	2.0	1.8	2.9
Cys	nd	nd	nd	nd	nd	nd	0.0	0.0	0.0
Ile	5.4	5.2	4.4	4.8	5.0	4.9	5.0	5.4	4.8
Leu	10.2	10.6	8.9	9.6	9.5	9.3	10.2	9.2	10.5
Phe	4.0	4.1	3.1	3.3	3.5	3.1	3.5	3.6	4.3
Lys	5.8	5.4	3.9	3.5	4.8	4.4	4.4	5.4	5.3
Trp	nd	0	0.5						
MW $\times 10^3$	$\sim 31.0^\dagger$	$\sim 30.0^\dagger$	23.8						
pi $^\times$	0	2	6	4	4	5	0~6	3~6	5

* From amino acid composition of mare β -like-casein [30]; § from sequence data for cow β -casein A² [26]; [†] estimated from SDS-PAGE (results not shown); [×] determined as described in Materials and methods; nd: Not determined. Values are given in number of residues/100 residues.

* À partir de la composition en acides aminés de la caséine β de jument [30]; § à partir de la séquence de la caséine βA^2 bovine [26]; [†] estimé à partir de l'électrophorèse SDS (résultats non montrés); [×] déterminé comme décrit en Matériel et méthodes; nd : non déterminé. Les valeurs sont exprimées en nombre de résidus/100 résidus.

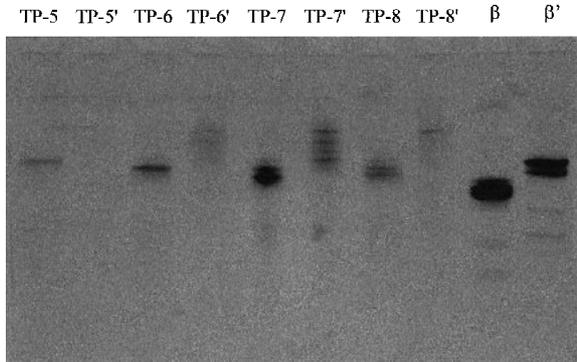


Figure 9. Urea-PAGE of fractions TP-5 to TP-8 before (5, 6, 7, 8) and after the action of acid phosphatase (5', 6', 7', 8'); β and β' denote bovine β-casein before and after the action of acid phosphatase, respectively.

Figure 9. Electrophorèse sur gel de polyacrylamide en présence d'urée des fractions TP-5 à TP-8 avant (5, 6, 7, 8) et après (5', 6', 7', 8') l'action de la phosphatase acide ; β et β' représentent respectivement la caséine β-bovine avant et après action de la phosphatase acide.

separation by capillary electrophoresis and coloration of the gel with periodic acid-Schiff's reagent after native PAGE were successful. Since the Schiff coloration is not universal and is not applicable to all glycan structures, it could be supposed that mare κ-like casein, if it really exists, should be glycosylated with non-reducing carbohydrates which did not stain well with Schiff's reagent. The other possibility which could be considered is that part of the κ-casein functions observed in bovine and other milks may be assumed in equine milk by the fraction of the less phosphorylated β-casein. This assumption is in correlation with the results of Yoshikawa et al. [31] who observed that when β-casein was dephosphorylated and its phosphorus content decreased to 2 mol per mol of protein, then it could behave like κ-casein, being able to form the micelles together with α_{S1} -casein.

4. CONCLUSION

As in bovine milk, mare milk contains the proteins which can be identified as α_{S1} , α_{S2} - and β-caseins. Many of the major physico-chemical properties of the equine caseins, e.g., amino acid composition, sequences and levels of phosphorylation can be compared with their bovine counterparts.

Bovine β-caseins occur in 7 genetic variants differing slightly in their primary struc-

tures and containing from 4 to 5 phosphates. Like the β-caseins issued from mare milk of European origin, β-caseins purified from mare milk of Mongolian origin can be divided into several subfractions varying at least in their degree of phosphorylation. This higher heterogeneity in the degree of β-casein phosphorylation in mare milk than that seen in bovine milk could be due to the total absence in equine milk of κ-casein, or to its presence only in minute and therefore undetectable quantities. In this respect, mare milk appears similar to human milk which also contains 6 β-caseins differing in their phosphorus content, and a low amount of κ-casein. As stated before, a significant amount of less phosphorylated mare β-caseins could compensate for the absence of κ-casein during the formation of superstructures of a suspension and colloid such as milk. If general in the Equidae, the formation of micelles in their milk could also be assumed by less phosphorylated β-caseins.

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