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Capillary electrophoretic analysis of genetic variants of milk proteins from different species

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Abstract

Polymorphism of bovine, ovine and caprine milk proteins was studied by CE. Identification of some rare bovine variants was carried out by isoelectric focussing (IEF) using PhastSystem. Genetic variants A and D of bovine α_{s2}-casein, β-casein variants A^1, A^2, A^3, B and C and α_{s1}-casein variants B and C were determined by CE. In addition, the different casein fractions including some genetic variants of ovine and caprine milk were identified by CE. In order to carry out this identification, collected fractions from a cation-exchange FPLC separation were injected by CE.

Keywords: Milk; Proteins; Caseins

1. Introduction

Milk composition and the main characteristics of its various constituents are now well known. In particular the amino acid sequences of its seven main protein components have been elucidated. At the moment, there is no other food product whose proteins are so well characterized. Moreover, for six of these seven protein types a qualitative polymorphism has been described [1].

Genetic polymorphism in the milk proteins is due to either substitutions of amino acids, or deletion of a certain amino acid sequence along the peptide chain, consequently these mutations are caused by changes in the sequence of base pairs of the DNA molecule which constitute the protein gene. It has been described that polymorphism is also due to post-transcriptional modifications, such as different degrees of phosphorylation and glycosylation of the protein. Besides the structural polymorphism, a quantitative allelic variability has been found which renders differences in protein expression. This phenomenon has been described in cow [2] and goat milk [3].

Polymorphism of milk proteins is related to production traits, milk composition and milk quality. Rapid screening of milk protein variants is important for the genetic improvement of milk with regard to the potential technological application and breeding selection programs [4].

Until now, the detection of genetic polymorphism of milk proteins has been achieved through various electrophoretic and chromatographic techniques [1]. However, the conventional electrophoretic methods are restricted to the resolution of proteins with differing net charges caused by the substitution of one amino acid for another and it is only possible to achieve semiquantitative results. Peptide mapping by HPLC and DNA mapping have also been used for the detection of silent variants (mutations in proteins

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due to amino acid substitutions that do not lead to a change in the net charge of proteins). These methods are more time consuming than electrophoresis and hence would not be practical for large scale routine screening of genetic polymorphism of milk proteins in animal populations. The search for rapid and high resolution methods for the identification of genetic variants of the major milk proteins would be necessary.

The potential of the CE in its different modes for the analysis of proteins has been demonstrated. Recently, some of the most common genetic variants of cow's milk have been identified in the whey fraction allowing quantitative results [5–7]. De Jong et al. [8] established a CE method using a hydrophilically coated capillary which allowed the simultaneous separation of whey proteins and caseins, including some genetic variants. This method was recently optimized by Recio and Olieman [9] in order to provide a quantitative determination of the proteins separated in the electropherograms, and to detect the ratio of serum protein to casein in different dairy products. A preliminary characterisation of the protein fractions of ewe's milk has been performed by Cattaneo et al. [10].

The objective of this paper is to identify the CE pattern corresponding to the main protein variants in milk from different species. We have performed this study with individual cow's, ewe's and goat's milk samples selected on the basis of their genetic variants determined by conventional electrophoretic techniques. To carry out the identification of the different protein fractions in ewe and goat milk, the casein fraction was fractionated by fast protein liquid chromatography (FPLC) and further analysed by CE.

2. Experimental

2.1. Milk samples

671 individual bovine milk samples, 238 individual ovine milk samples and 16 individual caprine milk samples were collected from different breeds from several local breeders in Spain. Various individual bovine, ovine, and caprine samples were selected according to their genetic pattern determined by polyacrylamide gel isoelectric focussing (IEF) and polyacrylamide gel electrophoresis (PAGE) at alkaline pH.

2.2. PAGE and IEF

The genetic variants of α_{s1}-casein (α_{s1}-CN), α_{s2}-casein (α_{s2}-CN), β-casein (β-CN), κ- casein (κ-CN) and β-lactoglobulin (β-LG) were determined by IEF using PhastSystem (Pharmacia, Uppsala, Sweden) following the method described by Bovenhuis and Verstege [11]. This method allows the simultaneous phenotyping of the different milk protein variants of casein and whey proteins in whole milk. PAGE at alkaline pH was carried out according to the method described by Ramos et al. [12].

2.3. Plasmin and rennin assays

Individual milk samples were digested with plasmin (EC 3.4.21.7), chymosin (EC 3.4.23.4), both from Sigma (St. Louis, MO, USA). Plasmin was added at an enzyme to substrate ratio of $5 \cdot 10^{-2}$ units/ml. Hydrolysis with rennin was performed at an enzyme concentration of $5 \cdot 10^{-5}$ mg/ml. Model systems were incubated at pH 6.5 and 37°C for 30 min. Reactions were stopped with trichloroacetic acid (TCA).

2.4. Isolation of the casein fractions by FPLC

Whole ovine and caprine fractions were isolated by cation-exchange using a FPLC apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) on a prepacked Mono S SR 5/5 column (Pharmacia LKB) following the method described by Law et al. [13]. In order to identify the different casein fractions, they were collected, concentrated with a Centricon-10 (cut-off M_r 10 000, Amicon, Beverly, MA, USA) and analysed by CE.

2.5. CE separation

CE was carried out using a Beckman P/ACE System 2050 controlled by a System Gold Software data system version 810. The separations were

performed using a hydrophilic coated fused-silica capillary column, Supelco CElect P1 (Bellefonte, PA, USA), 57 cm×50 μm I.D., with a slit opening of 100×800 μm. Separations were performed as described by Recio and Olieman [9] with a final applied voltage of 25 kV and a final current of around 52 μA. The injection was carried out at the anode N_2 pressure (0.5 p.s.i; 1 p.s.i.=6894.76 Pa) for 15 s. The electrophoresis buffer (pH 3.0±0.1) was 0.32 *M* citric acid, 20 m*M* sodium citrate, 6 *M* urea, 0.05% MHEC. Before use, the buffer was filtered through a 0.22 μm filter (Sterile Acrodisc with HT Tuffryn membrane, Gelman Sciences, Ann Arbor, MI, USA). Detection was at 214 nm. Identification of the peaks corresponding to the genetic variants of milk proteins, for which standards were available or could be prepared at our laboratory, was done by spiking. Spiking was performed by co-injection of the whole milk with the corresponding isolated fractions or standards using appropriate ratios. The Beckman P/ACE System 1050 allows the co-injection of three different samples in the same run.

2.6. Sample preparation for CE

300 μl of whole or skim milk were mixed with 750 μl of sample buffer. After 1 h incubation at room temperature, the reduction reaction was finished and the sample was injected without further preparation. The sample buffer (pH 8.6±0.1) consisted of 167 m*M* Tris (reagent grade from Sigma), 42 m*M* 3-(N-morpholino)-2-hydroxypropane (BioChemika MicroSelect, Fluka, Buchs, Switzerland), 67 m*M* EDTA (Merck, Darmstadt, Germany), 17 m*M* DL-dithiothreitol (DTT) (Sigma), 6 *M* urea (Sigma) and 0.05% methylhydroxyethyl cellulose (MHEC) (30000, Serva, Heidelberg, Germany).

Milk fat in whole milk samples can deteriorate the electrophoretic separation after the analysis of few (ten) samples. To avoid these problems the flushing time between injections was increased from 3 to 10 min. Moreover, when the capillary is damaged due to an excess of fat, it can be restored by washing it with acetonitrile–water mixtures (50:50 and 100:0), followed by flushing the capillary with the electrophoresis buffer for 10 min.

3. Results and discussion

3.1.1. Genetic variants of cow's milk

For the 30 genetic variants identified in six types of bovine milk protein, mutations leading to amino acid deletions occur in only two cases (α_{s1}-CN A, α_{s2}-CN D) whereas the most frequent mutations leading to amino acid substitutions occur for the other genetic variants [1].

The primary structure of the bovine milk proteins and the exact location of the mutations has been determined. Table 1 shows the positions and amino acid differences in the genetic variants found in the phenotyped bovine milk proteins [1].

Fig. 1A shows the separation patterns of different phenotypes in bovine milk obtained by IEF using PhastSystem. Most of the genetic variants were identified by comparing results with Bovenhuis and Verstege [11]. However, other rare variants were identified by comparison the results obtained by other authors using conventional IEF equipments. Thus, β-CN C identification was done according to Seibert et al. [14] and its presence was confirmed by treatment with plasmin (results not shown), while κ-CN C and α_{s2}-CN D were identified according to Erhardt [15] and they were confirmed by a chymosin treatment.

κ-CN C was focalised between κ-CN B (p*I* 5.83) and κ-CN A (p*I* 5.62), but also the rare α_{s2}-Cn D was in the same zone of the gel [15] (Fig. 1A, lanes 5, 6, and 7). A chymosin assay was performed to further study this result. As shown in Fig. 1B this band could clearly be recognized in some cases as κ-CN C, (Fig. 1B, lanes 2 and 4) because it was proteolyzed by the chymosin treatment (Fig. 1B, lanes 1 and 3, respectively) and as α_{s2}-Cn D (Fig. 1B, lanes 5 and 8) when it was not proteolysed by a light treatment with chymosin (Fig. 1B, lanes 6 and 7).

Fig. 2 shows the capillary electropherograms of four individual whole milk samples containing different genetic variants of β-LG, α_{s1}-CN, α_{s2}-CN, β-CN, and κ-CN (previously determined by IEF). The rare α_{s2}-CN variant D was identified by CE. It migrated close to β-LG peak, although well resolved from it (Fig. 2a). This shorter migration time at acidic pH of variant D compared to variant A is due to a deletion of nine amino acids in comparison to

Table 1
Positions and amino acid differences in the genetic variants found in the phenotyped bovine milk samples [1]

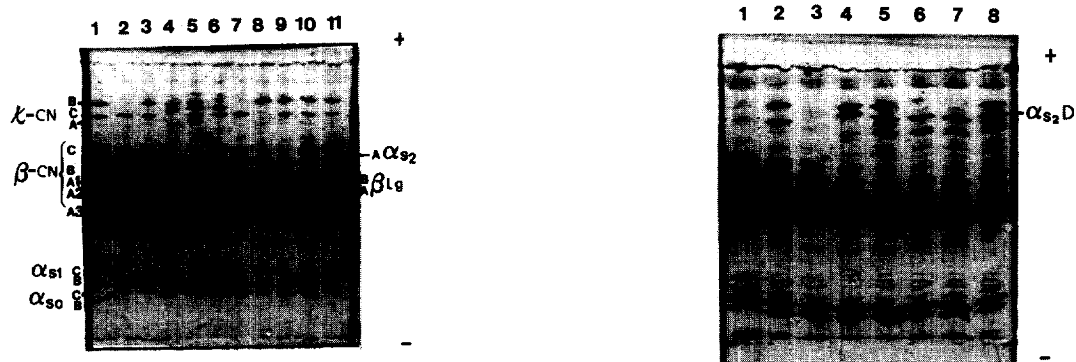
Protein and variants described	Variants found	Position and amino acid in the protein			
α_{s2}-CN		33	47		50–58
(207 aa)	A	Glu	Ala		
A,B,C,D	D	Gly	Thr		Deleted
		35	37	67	106
	A^1			His	
β-CN	A^2	SerP	Glu	Pro	His
(209 aa)	A^3				Gln
A^1,A^2,A^3,B,C,D,E	B			His	
	C	Ser	Lys	His	
α_{s1}-CN		192			
(199 aa)	B	Glu			
A,B,C,D,E	C	Gly			
		97	136	148	
κ-CN	A		Thr	Asp	
(169 aa)	B	Arg	Ile	Ala	
A,B,C,E	C	His			
β-Lg		64	118		
(162 aa)	A	Asp	Val		
A,B,C,D,E,F,G	B	Gly	Ala		

variant A (50–58, 51–59 or 52–60) [16,17], as well as the higher p*I* that the variant D presents with respect to variant α_{s2}-CN A [15] (Table 1). This genetic variant had not been previously identified using CE.

Five different genetic variants of β-CN were identified (Fig. 2). De Jong et al. [8] had identified four of them (A^1, A^2, A^3, B) using conditions similar to our own. The β-CN variant C (Fig. 2b) presented a shorter migration time than the variant B (Fig. 2c). As observed in Table 1, the difference between the primary structures of variants B and C of β-CN is due to the substitutions of Glu and Arg, at positions 37 and 122, for Lys and Ser respectively. The substitution at position 37 affects the phosphorylation of the Ser residue at position 35. These changes cause the shorter migration time observed in CE at acid pH.

Determination of the primary sequence of α_{s1}-CN and α_{s0}-CN has revealed identical amino acid sequences but with different degrees of phosphorylation due to post-translational modifications and they could not be considered as genetic variant. The α_{s0}-CN has an additional phosphate group attached to the Ser residue in position 41, and it must be called α_{s1}-CN-9P [18]. Two genetic variants of α_{s1}-CN were found in our samples: B and C. The difference in migration times between both variants is too small, making identification difficult in the homozygote phenotypes (Fig. 2a and Fig. 2c). However, they could be easily detected in the heterozygote phenotype (Fig. 2b and Fig. 2d). Replacement of an acid amino acid by a neutral one (Table 1), results in a more electropositively charged variant C of α_{s1}-CN, causing a shorter migration time by CE at acid pH. Obviously, α_{s1}-CN-9P (α_{s0}-CN) showed the same genetic variant as α_{s1}-CN, presenting two peaks that are not fully resolved in the heterozygote form (Fig. 2b and Fig. 2d). The excellent separation between the α_{s1}-CN-8P (α_{s1}-CN) and α_{s1}-CN-9P (α_{s0}-CN) demonstrates the potential of this CE method for the separation of different phosphorylated proteins.

The different genetic variants A, B and C of κ-CN previously identified by IEF (Fig. 1) were not resolved by CE. The changes of the amino acid sequence in κ-CN B, result in a κ-CN being more electropositive than κ-CN A due to Asp at position 148 in the variant A (Table 1). However, the Asp residue is not charged at the separation pH (pH 3)



Protein	1	2	3	4	5	6	7	8	9	10	11
κ-CN	AB	AA	AB	BC	AB	AC	AA	BB	AB	AB	AB
β-CN	A^2A^2	A^1A^2	A^1A^3	A^1A^2	A^2A^2	A^1A^2	BB	A^2B	A^1B	A^2C	A^1C
α_{s2}-CN	AA	AA	AA	AA	AD	AA	AA	AA	AA	AA	AA
α_{s1}-CN	CC	BB	BC	BB	BB	BB	BB	BB	BB	BC	BB
β-Lg	AA	AB	BB	BB	AA	AB	AB	AB	AA	BB	AB

A

Protein	1 and 2	3 and 4	5 and 6	7 and 8
κ-CN	AB	BC	AB	AB
β-CN	A^2B	A^1A^1	A^2A^2	A^2A^2
α_{s2}-CN	AA	AA	AD	AD
α_{s1}-CN	BB	BB	BB	BB
β-Lg	AB	AA	AA	AA

B

Fig. 1. (A) Isoelectric focussing (PhastSystem) patterns of whole bovine milk samples containing different phenotypes. (B) Isoelectric focussing patterns of whole bovine milk containing κ-casein variant C and α_{s2}-casein variant D with and without rennin treatment after isoelectric focusing using PhastSystem; lanes 2, 4, 5 and 8 correspond to whole milk samples; lanes 1, 3, 6 and 7 correspond to samples treated with rennin. Bands: κ-CN=κ-casein; β-CN=β-casein; $\alpha_{s1}=\alpha_{s1}$-casein; $\alpha_{s0}=\alpha_{s0}$-casein; $\alpha_{s2}=\alpha_{s2}$-casein; β-Lg=β-lactoglobulin.

(pK_a of the lateral chain =3.96) which prevents the separation of both genetic variants. Similarly, the separation of the κ-CN variant C was not achieved because the substitution of Arg by His at position 97, both basic amino acids, did not cause a change in the net charge at acid pH. Electrophoresis at a basic pH will be more appropiate for the separation of these genetic variants. As β-LG variants, A and B, were only slightly separated, the resolution of both variants was not achieved when, as in our case, the β-LG concentration was high.

3.1.2. Genetic variants of ewe's milk

Fig. 3A shows the IEF patterns of three ovine milks corresponding to the phenotypes BB, BC, AA of β-LG and Fig. 3B shows the capillary electropherograms of three ovine milks containing these same phenotypes of β-LG. As expected, the migration order of β-LG variants was the same by both techniques: β-LG B<A<C. The difference in mobility of β-LG A and B is due to the substitution of Tyr by His at position 20. Cattaneo et al. [10] have described by CE at acid pH the same migration order for the β-LG A and β-LG B. β-LG C is a subtype of ovine β-LG A with a single exchange of Arg by Gln at position 148 [19]. The exchange of a basic amino acid in variant A by a neutral one in variant C, causes a less positively charged variant in variant C, and therefore a lower mobility in the cathodal direction.

The ovine whole milk sample of electropherogram c of Fig. 3B contains the α_{s1}-CN Welsh variant. Recently, Chianese et al. [20] have called this variant α_{s1}-CN D. In order to identify the different casein

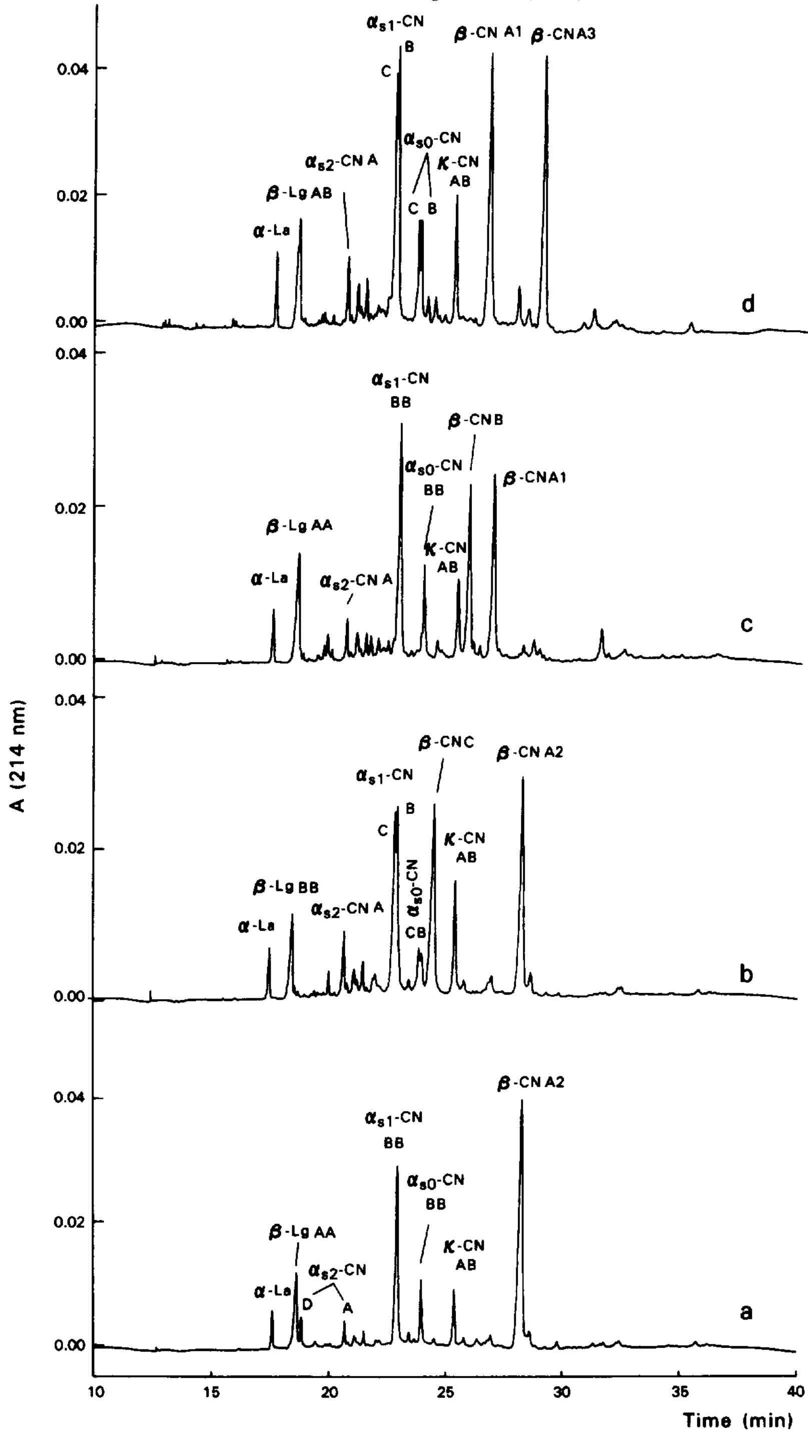


Fig. 2. Capillary electropherograms of four whole individual bovine milk samples containing (a) β-LG AA, α_{s2}-CN AD, κ-CN AB, α_{s1}-CN BB, α_{s0}-CN BB and β-CN A^2A^2; (b) β-LG BB, α_{s2}-CN AA, κ-CN AB, α_{s1}-CN BC, α_{s0}-CN BC and β-CN A^2C; (c) β-LG AA, α_{s2}-CN AA, κ-CN AB, α_{s1}-CN BB, α_{s0}-CN BB and β-CN A^1B; (d) β-LG AB, α_{s2}-CN AA, κ-CN AB, α_{s1}- CN BC, α_{s0}-CN BC and β-CN A^1A^3. Peaks: κ-CN=κ-casein; β-CN=β-casein; α_{s1}-CN=α_{s1}-casein; α_{s0}-CN=α_{s0}-casein; α_{s2}-CN=α_{s2}-casein; β-Lg=β-lactoglobulin; α-La=α-lactalbumin.

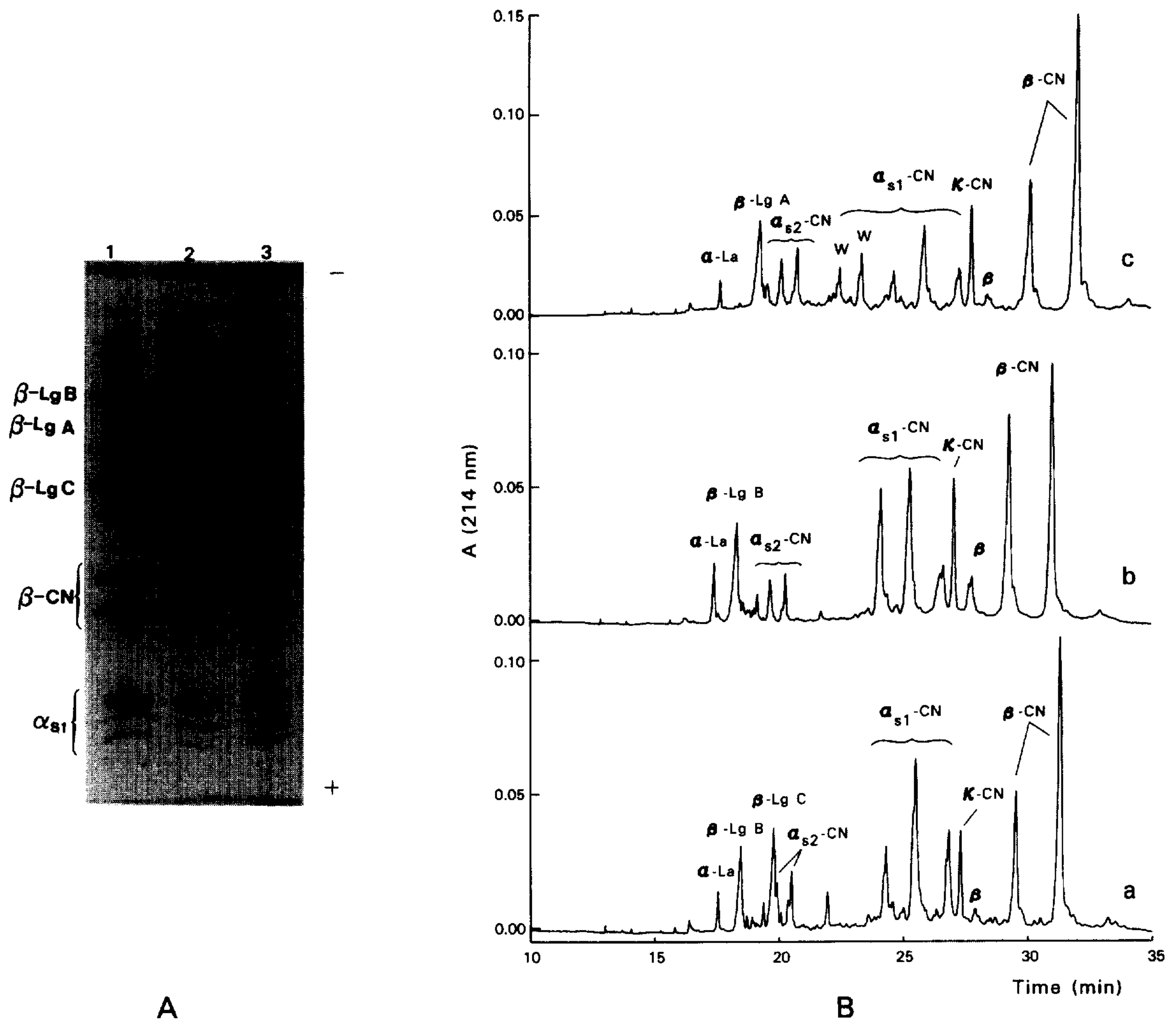


Fig. 3. (A) Separation patterns of whole ovine milk samples after isoelectric focussing using PhastSystem, containing different phenotypes of β-LG: lane 1= β-LG BC, lane 2= β-LG BB and lane 3= β-LG AA. (B) Capillary electrophoregrams of three whole individual ovine milk samples containing different phenotypes of β-LG: (a) β-LG BC, (b) β-LG BB and (c) β-LG AA. W denotes the peaks corresponding to the α_{s1}-CN D variant (also known as Welsh variant). κ-CN=κ-casein; β-CN=β-casein; α_{s1}-CN=α_{s1}-casein; α_{s2}-CN=α_{s2}-casein; β-Lg=β-lactoglobulin; α-La=α-lactalbumin.

fractions by CE, a sample containing this genetic variant was fractionated by cation-exchange FPLC [13]. The whole casein and its fractions collected from FPLC were analysed by alkaline PAGE (Fig. 4A) and by CE (Fig. 4B). By alkaline PAGE, these variants showed two additional bands (marked with 'W') in the α_{s1}-CN fraction (Fig. 4A, lane 4). By CE, the α_{s1}-CN fraction from the sample containing the Welsh variant also showed two additional peaks of α_{s1}-CN (marked with 'W' in Fig. 3B and 4B) with respect to the other milk samples, which did not contain this genetic variant. α_{s1}-CN D is the least phosphorylated α_{s1}-CN variant [21]. The absence of phosphate groups explains the rapid migration of this genetic variant by CE at acidic pH and the slow migration speed by alkaline PAGE. By CE, the β-CN fraction showed two peaks and another minor peak with a shorter migration time. Amino acid sequence studies demonstrated that β_1- and β_2-CN contained six and five phosphate groups respectively [22].

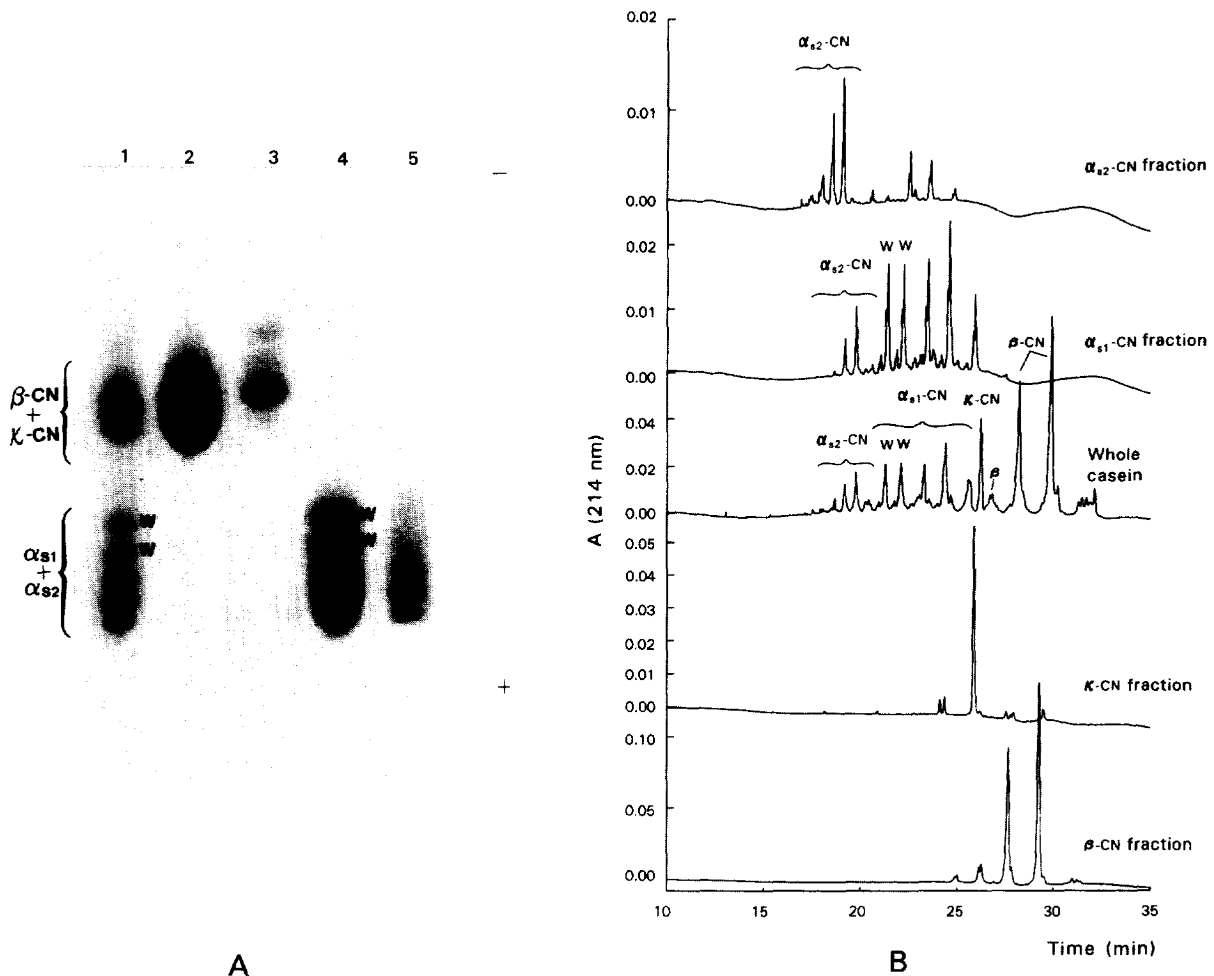


Fig. 4. (A) PAGE pattern of an ovine casein sample containing the α_{s1}-CN D variant (also known as Welsh variant) and its fractions collected from FPLC; lane 1= whole casein sample, lane 2= β-CN fraction, lane 3= κ-CN fraction, lane 4= α_{s1}-CN fraction and lane 5= α_{s2}-CN fraction. (B) Capillary electropherograms of the same ovine casein sample containing the α_{s1}-CN D variant (also known as Welsh variant) and its fractions collected from FPLC. W denotes the bands or the peaks corresponding to the α_{s1}-CN D variant. β denotes the minor component of the β-casein fraction. Bands and peaks: κ-CN=κ-casein; β-CN=β-casein; α_{s1}-CN=α_{s1}-casein; α_{s2}-CN=α_{s2}-casein; β-Lg=β-lactoglobulin; α-La=α-lactalbumin.

Therefore, the peak with shorter migration time would correspond to β_2-CN. The κ-CN fraction was resolved as one major peak and a number of minor peaks, which probably corresponded to different glycosylated or phosphorylated forms.

The presence of small amounts of α_{s2}-CN in the fraction of α_{s1}-CN and vice versa, could be due to the fact that the different components of the α_s-CN were not completely separated by FPLC, and each collected fraction contained some peaks corresponding to the adjacent peak. Ovine α_{s1}- and α_{s2}-CN fractions were well resolved by this CE method, although the variant C of the β-LG interfered with the α_{s2}-CN fraction (Fig. 3B, electrophoregram b): Due to the presence of different ions in the sample matrix between the whole casein and its collected fractions, a slight variation in the migration time could be observed by CE (Fig. 4B). Therefore, the identification was done by spiking, as was mentioned in Section 2.

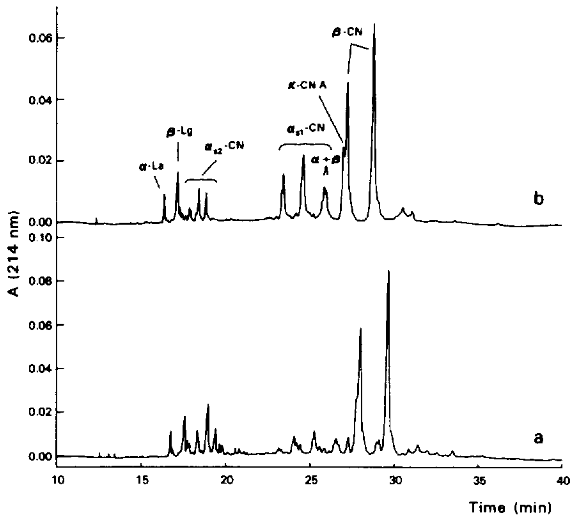


Fig. 5. Capillary electropherograms of two individual caprine milk samples (a) milk sample containing a low expression α_{s1}-CN variant, (b) milk sample containing a high expression α_{s1}-CN variant. Peaks: κ-CN=κ-casein; β-CN=β-casein; α_{s1}-CN=α_{s1}-casein; α_{s2}-CN=α_{s2}-casein; β-Lg=β-lactoglobulin; α-La=α-lactalbumin.

3.1.3. Genetic variants of goat's milk.

The different casein fractions were identified by analysing the collected fractions obtained from the FPLC fractionation by CE [23]. The whey proteins were identified using goat whey protein standards obtained in our laboratory. Fig. 5 shows the identification of the whey and casein fractions in two individual caprine milk samples which presented a quantitative polymorphism of α_{s1}-CN. The sample with a higher amount of α_{s1}-CN corresponded to a sample containing a high expression α_{s1}-CN type (Fig. 5a), while the other sample had a low expression α_{s1}-CN variant (Fig. 5b). α_{s2}-CN fraction was resolved as two major peaks and two minor partially resolved peaks just after the peak corresponding to β- LG. β-CN fraction showed two major peaks and another three minor peaks which comigrate with two peaks corresponding to α_{s1}-CN fraction. κ-CN A migrated close to a peak which belongs to the β-CN fraction.

4. Conclusions

CE using a hydrophilically coated capillary and a low pH buffer containing urea has allowed the identification of different genetic variants in whole cow's, ewe's and goat's milk. An excellent separation of protein variants with different degrees of phosphorylation was achieved (i.e., bovine α_{s1}-CN and α_{s0}-CN and ovine α_{s1}-CN D). Moreover, variants which differ only in a single amino acid (i.e., ovine β-LG variants) could be separated. However, when this substitution did not involve a change in the net charge of the protein at pH 3.0, these genetic variants could not be separated (i.e., bovine κ-CN A, B, and C). The method may be used for routine analysis of whole milk for phenotyping studies allowing the automatic analysis of a great number of samples.

Acknowledgments

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