International Dairy Journal 19 (2009) 181–189

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Occurrence of genetic polymorphism at the α_{s1}-casein locus in Mediterranean water buffalo milk

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ARTICLE INFO

Article history:
Received 19 May 2008
Received in revised form
8 September 2008
Accepted 25 September 2008

ABSTRACT

A study on casein genetic polymorphism in water buffalo milk of Mediterranean breed, reared in Southern Italy, was carried out by means of immunoelectrophoretic and chromatographic techniques coupled with mass spectrometry analysis. When compared with reference α_{s1}-CN variant A, the results showed the occurrence of a new α_{s1}-casein (α_{s1}-CN) B genetic variant having a single silent amino acid substitution Leu^{178}(A) → Ser^{178}(B). In Mediterranean buffalo milks the allelic frequency of new α_{s1}-CN B was very similar to that determined in Mozzarella di bufala campana (MBC), a Protected Denomination of Origin (PDO) cheese, made exclusively from whole raw buffalo milk of this breed. The relative percentage of the two α_{s1}-CN variants, either in bulk milk or MBC cheese, can be used in defining the authenticity of PDO MBC cheese.

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1. Introduction

The Mediterranean buffalo "caseinome" has been found, to date, to be very monomorphic, as the heterogeneity of the main four casein (CN) fractions is only due to their discrete phosphorylation (α_{s1}-, α_{s2}- and β-CN) or degree of glycosylation (κ-CN), rather than the occurrence of genetic polymorphism. As determined by Ferranti et al. (1998), water buffalo α_{s1}-CN consists of three main components containing eight, seven and six phosphate groups per molecule, as obtained by PAGE (polyacrylamide gel electrophoresis) at pH 8.6 and ultra-thin-layer isoelectric focusing (UTLIEF) analysis. In this regard, water buffalo α_{s1}-casein (W) exhibits a lower anodic mobility than its bovine counterpart (C), due to the substitution PSer^{115}(C) → Leu^{115}(W).

With UTLIEF analysis, by comparison, cow and buffalo α_{s1}-CN exhibited a very similar pI (ΔpI = 0.15 pH unit with respect to theoretical value), PSer^{115}(C) being counterbalanced by Hys^{4}(W), yielding no effect on the negative charge of the protein by PAGE analysis at pH 8.6. However, the primary structure of water buffalo α_{s1}-CN (Ferranti et al., 1998) was different from that resulting from nucleotide sequencing of Indian river buffalo α_{s1}-CN cDNA (GenBank Acc. No. O62823), for the amino acid substitution Ser^{178}(Indian buffalo) → Leu^{178} (Mediterranean buffalo).

The primary structure of buffalo β-CN consists of 209 amino acids, with five phosphoserine residues located at the same positions of N-terminus of β-CN as in the most common (A_1, A_2 and B) variants of bovine β-CN counterpart. Two genetic variants of buffalo β-CN occur, β-CN A being found only in Venezuelan buffalo milk and β-CN B found in Mediterranean breed. These two variants move towards the anode during PAGE at alkaline pH in decreasing order A > B, due to both PThr^{41}(A) → Met^{41}(B) and Asn^{68}(A) → Lys^{68}(B) substitutions, which also allow their discrimination by UTLIEF analysis (Ferranti et al., 1998). Furthermore, with PAGE analysis at alkaline pH, β-CN B variant showed very similar anodic mobility to that of bovine β-CN A_2, as well as very similar pI value. Recently, β-CN B, determined by means of DNA sequencing (GenBank Acc. No. Q9TSI0), was shown to occur in both Indian river buffalo and in German water buffalo (Klotz et al., 2000).

In terms of the genetic polymorphism of buffalo α_{s2}-CN, three electrophoretic phenotypes A, B and C occurred in Mediterranean buffalo (Chianese et al., 1996a); the genetic sequence of Indian river buffalo α_{s2}-CN has also been deposited (GenBank Acc. No. O62825).

Just one κ-CN phenotype has been found in the Mediterranean breed, having an electrophoretic mobility at alkaline pH very similar to that of the bovine κ-CN B counterpart (Addeo et al., 1977); the resulting amino acid sequence has been recently determined by means of DNA sequencing (GenBank Acc. No. P11840). On the other hand, genotyping of κ-CN in Indian river buffalo milk by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) showed the existence of genetic polymorphism at this locus for the occurrence of two alleles A and B in Murrah buffalo (Mitra et al., 1998). Taking into account the silent amino acid substitution Thr^{135}(A) → Ile^{135}(B) affecting the two expressed

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0958-6946/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.idairyj.2008.09.008

proteins, they cannot be discriminated using electrophoretic techniques such as PAGE at alkaline pH or UTLIEF. From a homology point of view, to determine a common phylogenetic origin of casein genetic variants in buffalo specie, it was noted that the substitution occurring in buffalo κ-CN A/B variants was also characteristic of cow κ-CN A/B variants.

Regarding whey protein, genetic polymorphism was recently found by our group at the α-lactalbumin (α-La) locus, for the occurrence of a novel variant, named α-La A (Chianese et al., 2004), which can be related to authenticity of Mediterranean breed.

Buffalo breeding in Campania (southern Italy), has been mainly aimed at producing Mozzarella di bufala campana (MBC) cheese, known worldwide for its distinctive flavour, which is quite different from that of Mozzarella made with bovine milk (Moio et al., 1993). MBC achieved the label of Protected Denomination of Origin (PDO) (E. C. Regulation No. 1107/96) and numerous analytical methodologies have been set up to determine the fraudulent addition of other milks, such as bovine or ewe, to buffalo milk. These methods involve gel and capillary electrophoresis (PAGE, UTLIEF, capillary Isoelectric Focusing (cIEF)) or chromatographic reverse phase-high performance liquid chromatography (RP-HPLC) techniques coupled to mass spectrometry (MS) analysis, to discriminate species-specific molecular markers (Addeo et al., 1989; DPR, 1994; E. C. Regulation No. 213/2001; Pellegrino et al., 1991; Somma et al., 2008).

Recently, "foreign" buffalo milk or derived frozen curd (imported from East European countries) has been fraudulently added to buffalo bulk milk in the making of MBC. This fraud may be performed more frequently than the well-known addition of cow or sheep milk to bulk buffalo milk. Very few studies have examined geographical origins of Mozzarella. Recently, Brescia et al. (2005) proposed discriminating the geographical origin of MBC cheese samples by evaluating, by chemometric methods, the isotopic parameters ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) with nuclear magnetic resonance (NMR) data, determined for aqueous Mozzarella extracts.

To adopt a standard as a "sufficient" basis for genetic traceability of PDO Mozzarella cheese, a phenotyping screening of casein fractions extracted from individual buffalo milks of Mediterranean breed and PDO MBC samples was carried out by means of immunoelectrophoretic and chromatographic techniques, coupled with mass spectrometry analysis, aimed at defining the actual casein genotype of this breed.

2. Materials and methods

2.1. Chemical analysis

The content of total nitrogen and casein-nitrogen was determined according to the method of Rowland (1938).

2.2. Extraction of casein from milk and mozzarella di bufala campana cheese

In typical production areas of Salerno and Caserta, individual milk samples from 180 water buffaloes, taken at random from different herds, twenty bulk buffalo milks from twenty plants (representative of total production of MBC) and twenty MBC cheeses taken at random from markets in Caserta (ten samples) and Salerno (ten samples), were analysed. Each casein sample was prepared by acid precipitation from skimmed milk, followed by centrifugation at 2500 × g for 15 min, as described by Aschaffenburg and Drewry (1959).

After dispersing cheese using an Ultra-Turrax (mod. T25 B Ika-Werke, Germany), the casein fraction was extracted from MBC by centrifugation at 2500 × g for 15 min to eliminate fat and rinsed twice with distilled water in order to eliminate whey. Each casein sample was freeze-dried and then stored at −20 °C before use.

2.3. Electrophoretic and immunoblotting analysis

Casein samples for electrophoretic analysis were dissolved in a 9 M urea solution (0.2 g L^{-1}), containing 2-mercaptoethanol (1 mL L^{-1}). Urea-polyacrylamide gel electrophoresis (urea-PAGE) at pH 8.6 was carried out with a vertical electrophoretic apparatus (Protean II, Bio-Rad, Richmond, CA 94804, USA) at 200 V and 6 °C for 7 h. The gels used (0.75 mm thick, 180 × 140 mm) consisted of stacking gel containing 3.6 M urea, 7.5% (w/v) glycerol in 0.5 M Tris–HCl buffer at pH 6.8, layered on a running gel containing 6.1 M urea in 1.5 M Tris–HCl buffer at pH 8.6; tetra-methyl-ethylene-diamine and ammonium persulphate, at 0.04% (v/v) and 0.07% (w/v) final concentrations, respectively, were added to gels as polymerization agents. The electrode and migration buffers consisted of 0.19 M glycine and 0.024 M Tris–HCl. Staining was performed with Coomassie Brilliant Blue R-250.

UTLIEF on polyacrylamide gels (0.25 mm) was carried out according to the procedure of Chianese et al. (1996b). The pH gradient in the range 2.5–6.5 was obtained by mixing Ampholine buffers (GE Healthcare Amersham Bioscience, Buckinghamshire, UK), 2.5–5, 4.5–5.4 and 4–6.5 (1.6:1.4:1 by volume). The gel was stained with Coomassie Brilliant Blue G-250 as described by Krause et al. (1988).

For immunoblotting analysis, the casein fractions separated either by PAGE or UTLIEF analysis were transferred by capillary diffusion from the gel onto a nitrocellulose membrane (0.45 μm, Trans-Blot, Bio-Rad, Richmond, CA 94804, USA) and immunostained using home-made polyclonal antibodies against bovine α_{s1}-CN, α_{s2}-CN, and κ-CN as primary antibodies (Chianese et al., 1992). Two polyclonal antibodies against β-CN, specifically recognising in addition to native β-CN its fragments containing the N-terminus or C-terminus of the protein, were produced using, as antigen, the synthetic peptides β(1–28) and β(195–209), respectively (Pizzano et al., 2000). These β-CN peptides were linked to ovalbumin through the sulphydryl group of C-terminal or N-terminal cysteine residue, according to the coupling procedure described by Mattson et al. (1993); the ovalbumin conjugates were used to immunize two rabbits (Primm, Milano, Italy). Finally, the antisera were filtered using 0.45-μm filters (Millipore, Bedford, MA), divided into aliquots of 1 mL, and stored at −20 °C.

2.4. Enzymatic hydrolyses

Hydrolysis by trypsin (Roche, Mannheim, Germany) and alkaline phosphatase (Roche) was carried out in 0.4% ammonium bicarbonate, pH 8.5, at 37 °C, for 4 and 16 h, respectively, using a 1:50 (w/w) enzyme/substrate (E:S) ratio. Enzymatic reactions were stopped by adding 2 μL of 4 M trifluoroacetic acid (TFA). Samples were frozen at −20 °C and lyophilised. Hydrolysis by liquid calf rennet (Chr. Hansen S.p.A., Milan, Italy) was carried out as reported by Addeo et al. (1984). Whole buffalo casein was dissolved in 0.1 M sodium citrate, pH 6, and added of liquid calf rennet at 37 °C was added using a 1:10,000 E/S ratio. The enzyme reaction was stopped after 1 h by acidification with 12% trichloroacetic acid (TCA). After centrifugation at 2500 × g for 5 min, the pellet was stored at −20 °C.

2.5. Reversed-phase high performance liquid chromatography analysis

Casein samples were fractionated by reversed-phase (RP) HPLC on a 214TP54, 5 μm Vydac C4, 250 × 4.6 mm internal diameter column (Vydac, Hesperia, CA, USA) at a detection

wavelength of 220 nm. Solvent A was 0.1% trifluoroacetic acid (TFA) in ultra-pure water (v/v) and solvent B 0.1% (v/v) TFA in acetonitrile. Two hundred microliters of a solution containing 1 mg (casein sample) mL^{-1} (solvent A) were loaded onto a C4 column, equilibrated with solvent A. The elution gradient program involved a gradient from 30 to 50% solvent B in 40 min, then from 50 to 100% B in 2 min, at a flow rate of 1 mL min^{-1}. Each eluted casein fraction was manually collected, freeze-dried, and stored at $-20\,^{\circ}C$.

2.6. Electrospray-time-of-flight tandem mass spectrometry

Native and dephosphorylated whole casein as well as derived tryptic digests, dissolved at a concentration of 1 μg mL^{-1} in acetonitrile–water (v/v) containing 0.1% trifluoroacetic acid, were analysed by mass spectrometry (MS). The analyses were performed with electrospray (ESI)-tandem mass spectrometry using a Q-TOF™ hybrid quadrupole/time-of-flight (QTOF) mass spectrometer (Micromass Ltd., Manchester, UK) equipped with a Z-spray ion source in the positive ion mode (ESI-QTOF and MS/MS). The nano flow was executed with a syringe pump at 0.5 μL min^{-1} and the TOF mass analyser was used to acquire data in both MS and MS/MS modes.

The source temperature was 100 °C and the desolvation temperature was 200 °C. The TOF operated at an acceleration voltage of 9.1 kV, a cone voltage of 100 V, a cone gas (N_2) of 13 L h^{-1} and a collision energy in MS mode of 10 eV. Collision-induced dissociation (CID) spectra were acquired in a data-dependent method on the most abundant ions having mass to charge ratios (m/z) from 600 to 1800. Collision energy was dependent on the m/z ratio and the charge state of the parent ion, generally between 25 and 40 V. The collision cell was pressurized with 10.34 Pa ultra-pure Ar (99.999%). Raw MS/MS data were combined and processed using the MaxEnt 3 algorithm prior to de novo sequence analysis using Mass Seq software (Waters, Manchester, UK).

2.7. Data processing and data analysis

All nanoscale MS/MS data were processed automatically with the ProteinLynx Global Server 1.0 (Micromass) module of the data acquisition software of the mass spectrometer. The Mascot database searching software (Matrix Science Ltd., London, UK; http://www.matrixscience.com/) was employed for peptide and protein identification.

3. Results and discussion

3.1. Urea-PAGE at pH 8.6 and immunoblotting analysis

The Coomassie Blue-stained urea-PAGE profiles of the most representative casein phenotypes from milk samples of Mediterranean water buffalo, are shown in Fig. 1A. All casein samples showed a similar composition for each casein family; in particular α_{s1}- and α_{s2}-CN, partially overlapping due to very similar net negative charges, consisted of more components than β- and κ-CN because of their high degree of phosphorylation.

Since the separation between α_{s1}-CN and α_{s2}-CN appeared to be unsatisfactory on urea-PAGE at pH 8.6, the composition of buffalo whole casein was determined by immunostaining PAGE profiles with specific polyclonal antibodies against α_{s1}-CN, α_{s2}-CN, β-CN C-

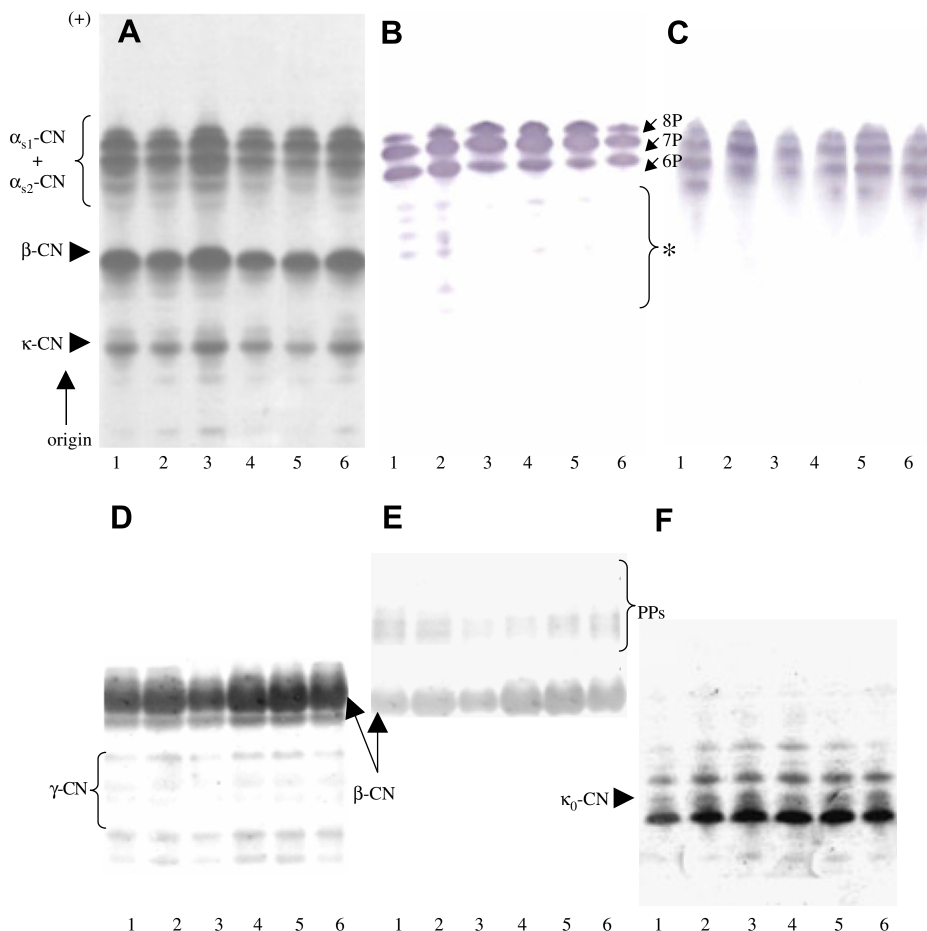

Fig. 1. Urea-PAGE analysis at pH 8.6 (A) and immunoblotting with polyclonal antibodies against individual whole casein samples from water buffalo milk of Mediterranean breed: (B) α_{s1}-CN, (C) α_{s2}-CN, (D) β-CN C-terminus, (E) β-CN N-terminus, (F) κ-CN.

terminus, β-CN N-terminus and κ-CN (Fig. 1B–F, respectively). In each sample (Fig. 1B), buffalo α_{s1}-CN consisted of three main components whose increasing mobility towards the anode depended on number of phosphate groups per molecule (6, 7 and 8P) (Ferranti et al., 1998). However, slower additional migrating components (marked with an asterisk in the figure) were also displayed.

At least three or four phosphorylated components were recognised by use of anti-α_{s2}-CN serum (Fig. 1C). Comparing the anodic mobility of both α_{s2}-CN and α_{s1}-CN (Fig. 1B and C), it can be observed that at least two main components of α_{s2}-CN overlapped with α_{s1} 6P and 7P.

Different β-CN components were observed after immunostaining with antibodies against the C-terminus or N-terminus of protein (Fig. 1D and E). In both cases, the antibodies recognised, in addition to native protein, new products moving faster towards the anode according to their specific antigenicity: buffalo γ_1-, γ_2- and γ_3-CNs by anti-β C-terminus (Fig. 1D) and their complementary hydrophilic proteose-peptones containing PSer residues, by anti-β N-terminus serum (Fig. 1E). Both β-CN-derived peptides, γ-CNs and proteose-peptones (PPs) originated from specific action of plasmin on bonds β-CN 27–28, 105–106, 107–108; the enzymatic activity of plasmin, the main indigenous protease in milk, increases during mastitis and in late lactation (Fox and Kelly, 2006).

The κ-CN fraction consisted of almost five components having an increasing mobility towards the anode (Fig. 1F). This heterogeneity was due to different numbers of glycosylated oligosaccharides attached to the Thr residue of caseinomacropeptide (CMP); the main component (κ_0) with the lowest anodic mobility was lacking sugar chains (Addeo et al., 1977).

Using this technique, the water buffalo population examined seemed to be monomorphic at each casein locus, since no polymorphism was observed, apart from post-translational modification, such as phosphorylation and/or glycosylation, resulting in a high heterogeneity of casein composition.

3.2. Ultra-thin-layer isoelectric focusing and immunoblotting analysis

To determine buffalo casein composition on the basis of relative pI, the selected water buffalo casein phenotypes were analysed by UTLIEF in a pH gradient 2.5–6.5, as shown in Fig. 2A after Coomassie Blue staining.

Each casein family, e.g., α_{s1}-, α_{s2}-, β- and κ-CN, focused at different pI values along the pH gradient and was identified according to the method of Chianese et al. (1996a). As shown in Fig. 2A, the components of α_{s1}- and α_{s2}-CN, having the same negative net charge at pH 8.6, can be differentiated after UTLIEF according to relative different pI values. The three main phosphorylated components in the α_{s1}-CN area seemed to be affected by the degree of phosphorylation degree, in the order $\alpha_{s1}8P < \alpha_{s1}7P > \alpha_{s1}6P$. This result can likely be related to an insufficient activity of kinases responsible for the phosphorylation of large amounts of casein (Mercier, 1981), or to a higher post-secretory activity of milk alkaline phosphatase, depending on somatic cell count (SCC) increase in milk (Kelly et al., 2006). To identify the

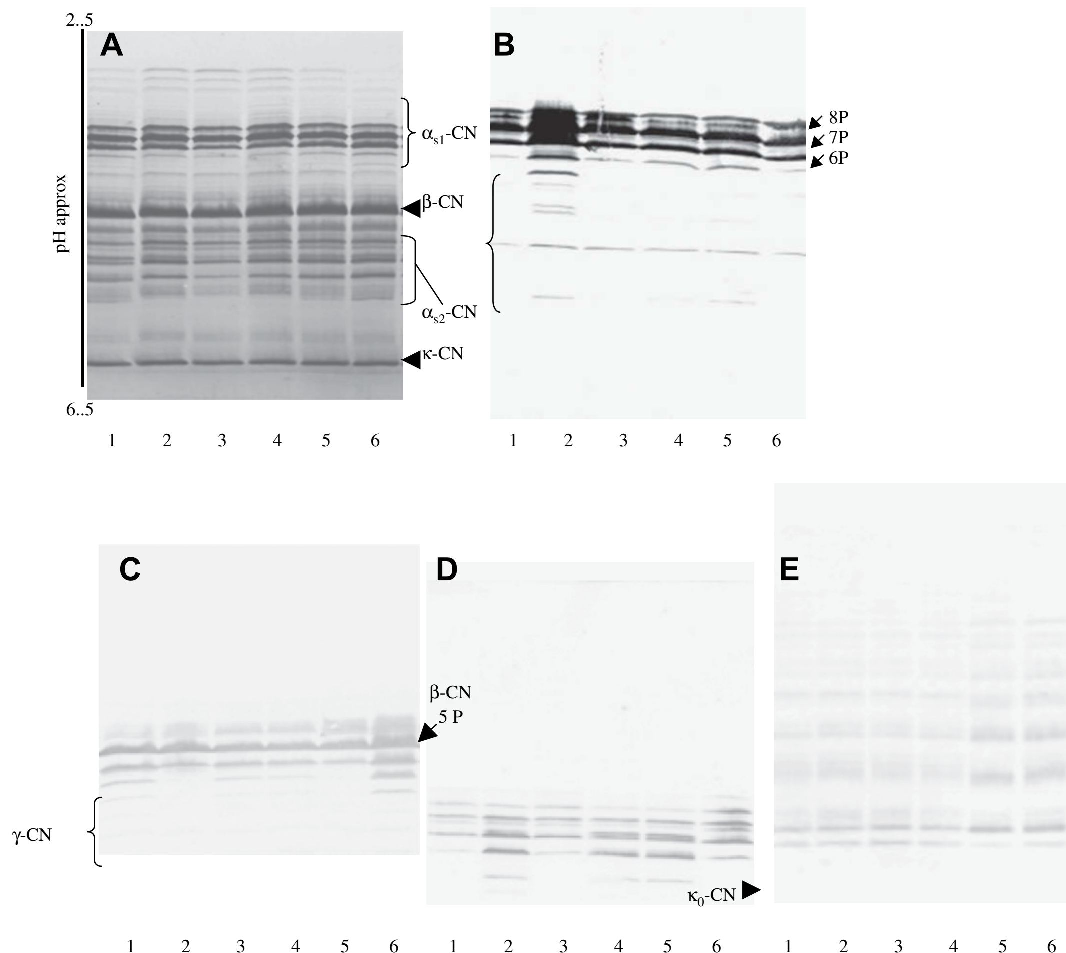

Fig. 2. Isoelectric focusing in pH gradient 2.5–6.5 of individual milk samples. Staining was with (A) Coomassie Brilliant Blue or polyclonal antibodies against (B) α_{s1}-CN, β-CN C-terminus (C), α_{s2}-CN (D) and κ-CN (E).

components belonging to each casein family, the electrophoretic profiles were also immunostained with antisera raised against α_{s1}-, β-, α_{s2}- and κ-CN (Fig. 2B–E, respectively). Consistent with the results of PAGE analysis, an expected higher heterogeneity of α_{s1}-CN composition was displayed (Fig. 1B), due to the presence of minor components (bracket in Fig. 2B) focusing at higher pH values. Taking into account the higher heterogeneity of ewe and goat α_{s1}-CN composition than their bovine counterpart, due to additional presence of non-allelic deleted proteins (Ferranti et al., 1997; Martin et al., 2002), it can be proposed that a similar phenomenon occurs in this species; on the other hand, a non-allelic form lacking in Gln[78] has already been found in buffalo milk (Ferranti et al., 1999).

The immunoblotting of β-CN composition showed more heterogeneity than Coomassie Blue staining, probably due to the discrete phosphorylation degree (higher in samples 1 and 6) and the concomitant presence of γ-CNs at the bottom of the figure (Fig. 2C). These results can be correlated with a higher activity of alkaline phosphatase and plasmin, both depending on SCC in milk, as reported above (Kelly et al., 2006). The same enzymatic activity can also explain the higher number of α_{s2}-CN components in lanes 2, 4 and 6 than α_{s2}-CN samples in lanes 1 and 3. The higher resolving power of UTLIEF analysis, coupled with staining with specific antibodies, allowed us to detect at least eight components of the κ-CN fraction (Fig. 2E) in all samples analysed.

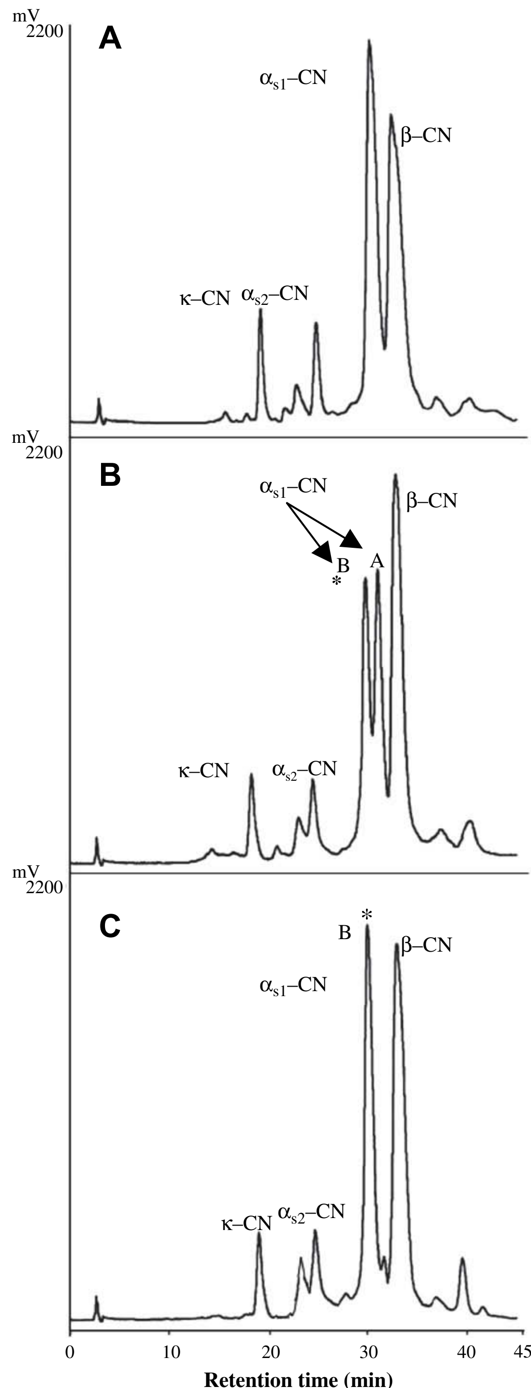

Fig. 3. RP-HPLC profiles of water buffalo casein samples containing the reference genotypes A/A (panel A), A/B (panel B) and B/B (panel C) at the α_{s1}-CN locus.

3.3. *Reversed-phase high performance liquid chromatography analysis*

From a statistical point of view, the number of "electrophoretic" genetic variants (due to different protein positive or negative net charge) should be lower than those produced by silent amino acid substitutions, as suggested by Grosclaude et al. (1988). Therefore, the phenotypic screening of water buffalo individual whole caseins was carried out by RP-HPLC on C4 column. Three representative chromatographic profiles were obtained (Fig. 3), where the main casein fractions eluted in the order: κ-, α_{s2}-, α_{s1}- and β-CN. The first RP-HPLC profile contains the known buffalo α_{s1}-CN A, the second its heterozygous form with a more hydrophilic new α_{s1}-CN B variant, which displayed in homozygous state in the third profile; these three phenotypes showed the same PAGE or UTLIEF patterns (samples No. 1, 3, 6 in Figs. 1 and 2). These results suggest at least an amino acid substitution in α_{s1}-CN B involving a neutral polar residue. On the other hand, the determined average percentage of both α_{s1} A and B variants by HPLC analysis, was very similar, accounting for a mean value of 45% of total casein; since the mean value of casein content in buffalo milk was 3.5%, the absolute amount of each α_{s1} variant corresponds to 15.7 g L^{-1} milk. These results confirmed the occurrence of a qualitative genetic polymorphism at the α_{s1}-CN locus in Mediterranean water buffalo not affecting the total casein content.

3.4. *Mass spectrometry and sequence analysis*

The molecular mass (*M*) values obtained by use of ESI/MS of eluted α_{s1}-CN from samples 1 and 3 (Fig. 3), before and after hydrolysis with alkaline phosphatase are shown in Table 1. The different mass values accounted for three phosphorylated α_{s1}-CN components (8P, 7P and 6P), while a Δ*M* of 26 Da indicated that at

Table 1
Determination of molecular mass of components of α_{s1}-CN peaks of RP-HPLC profiles presented in Fig. 3. Peaks A and B (asterisk in Fig. 3) corresponded to the reference and the new α_{s1}-CN variant, respectively.

Casein	Molecular mass (Da)				Identified casein
	Native		Dephosphorylated		
	Measured	Expected	Measured	Expected	
α_{s1}-CN variant	23,279	23,279			α_{s1}-CN 6P
(Peak B)	23,359	23,359			α_{s1}-CN 7P
	23,439	23,439			α_{s1}-CN 8P
			22,798	22,799	α_{s1}-CN
α_{s1}-CN reference	23,253	23,253			α_{s1}-CN 6P
(Peak A)	23,334	23,334			α_{s1}-CN 7P
	23,413	23,413			α_{s1}-CN 8P
			22,772	22,773	α_{s1}-CN

Table 2
Peptide mapping of water buffalo α_{s1}-CN components[a] resulted from hydrolysis with trypsin. D* occurred in a new α_{s1}-CN B variant.

Fragment[a]	α_{s1}-CN A measured mass (Da)	α_{s1}-CN B measured mass (Da)	Theoretical mass (Da)	Peptide sequence
T17 (194–199)	747.35	747.35	747.36	(K)TTMPLW(–)
A (145–150)	813.42	813.42	813.41	(Y)FYPQLF(R)
T9 (84–90)	830.48	830.48	830.38	(K)EDVPSER(Y)
T1–2 (1–7)	865.51	865.51	865.55	(–)RPKQPIK(H)
T14 (125–132)	909.57	909.57	909.47	(K)EGIHAQQK(E)
T10 (91–100)	1266.67	1266.67	1266.57	(R)YLGYLEQLLR(L)
B (155–165)	1269.57	1269.57	1269.37	(Y)QLDAYPSGAWY(Y)
T8–9 (80–90)	1336.70	1336.70	1336.67	(K)HIQKEDVPSER(Y)
T4 (23–34)	1383.73	1383.73	1383.72	(R)FFVAPFPEVFGK(E)
C (133–145)	1509.45	1509.45	1509.72	(K)EPMIGVNQELAYF(Y)
T3 (8–22)	1686.88	1686.88	1686.92	(K)HQGLPQGVLNENLLR(F)
T2–3 (4–22)	2153.21	2153.21	2153.21	(K)QPIKHQGLPQGVLNENLLR(F)
T9–11 (84–102)	2319.20	2319.20	2320.24	(K)EDVPSERYLGYLEQLLRLK(K)
T13 (104–124)	2451.29	2451.29	2451.25	(K)YNVPQLEIVPNLAEEQLHSMK(E)
T12–13 (103–124)	2579.45	2579.45	2580.35	(K)KYNVPQLEIVPNLAEEQLHSMK(E)
T5–6 (35–58)	2667.28	2667.28	2667.21	(K)EKVNELSTDIGSESTEDQAMEDIK(Q)
D (166–193)	2975.59		2975.46	(Y)YVPLGTQYPDAPLFSDIPNPIGSENSGK(T)
D*		2950.39	2950.40	(Y)YVPLGTQYPDAPSFSDIPNPIGSENSGK(T)

[a] T, tryptic peptides; A, C and D peptides arising from non-specific cleaveages.

least a single amino acid substitution occurred. To this objective, the two dephosphorylated α_{s1}-CN fractions were hydrolysed by trypsin and the resulting peptide mixture was analysed by ESI-QTOF/MS. Both the molecular mass and the amino acid sequence of tryptic digests shown in Table 2. The tryptic peptides determined for the novel variant were identical with that yielded by the reference one, except for the peptide (166–193) with a molecular mass of 2950.39 Da (D* in Table 2) instead of 2975.59 Da as in reference α_{s1}-CN A. MS/MS analysis of the double-charged positive ion at *m*/*z* 1476.21 of this peptide, displayed in Fig. 4, showed a Ser[178] replacing a Leu residue in the reference peptide, as reported in preliminary results (Chianese et al., 2006); such an event has been reported to occur at gene level in Indian river buffalo (GenBank Acc. No. O62823). The same genetic polymorphism at α_{s1}-CN locus could be explained taking into account the fact that Mediterranean water buffalo and Indian river buffalo were closely related (Mason, 1974).

3.5. Phenotypic frequencies of α_{s1}-CN A and B genetic variants by HPLC analysis

Since the allelic frequencies of each α_{s1}-CN genotype could be related to the authenticity of Mediterranean breed as genetic marker, they have been calculated on the basis of RP-HPLC data obtained by chromatographic screening. The allelic frequencies of buffalo α_{s1}-CN B and A were 0.69 and 0.31, respectively, indicating that the most common genetic variant in Mediterranean breed reared in Campania region was the new α_{s1}-CN B variant. The same analytical screening was carried out on twenty whole caseins from bulk milks and twenty MBC cheese samples, taken at random from

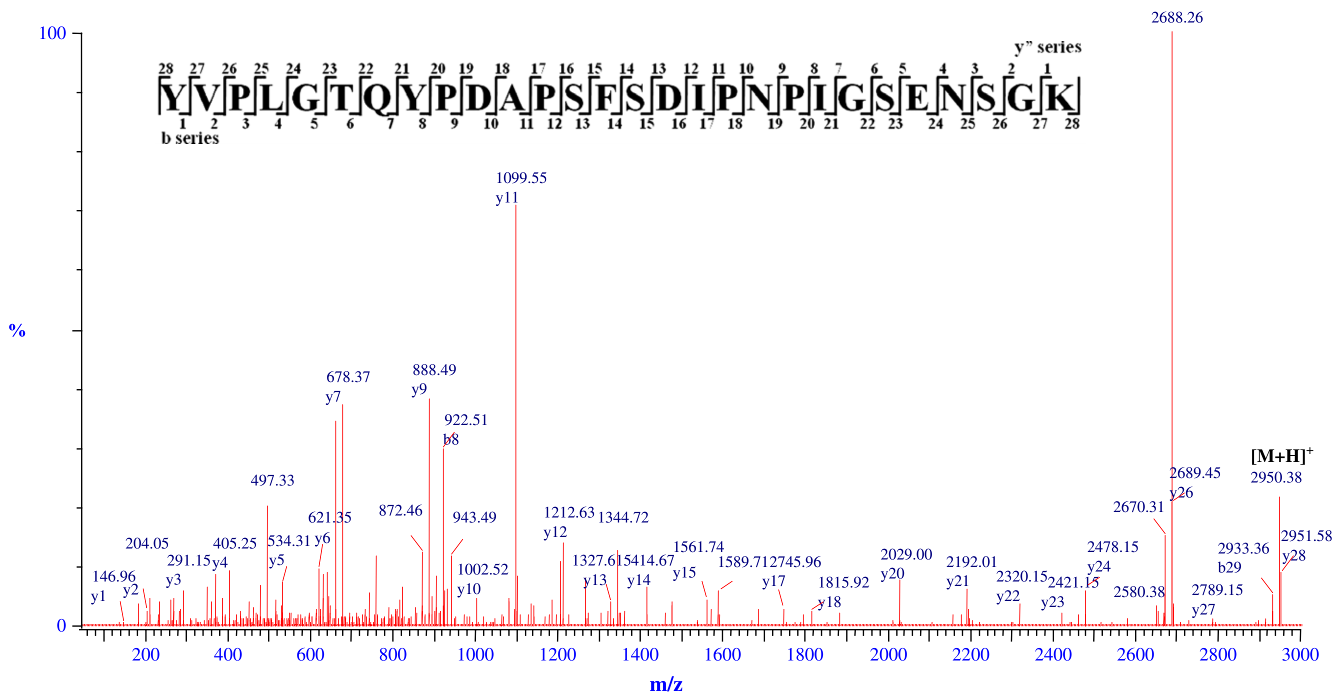

Fig. 4. ESI-MS/MS spectrum of double-charged ion at 1476.21 *m*/*z* of the 28-residue-long tryptic peptide (166–193) (D* in Table 2): the reported peptide sequence is displayed with the ion fragments observed in the spectrum: only *b* and *y* ions are labelled.

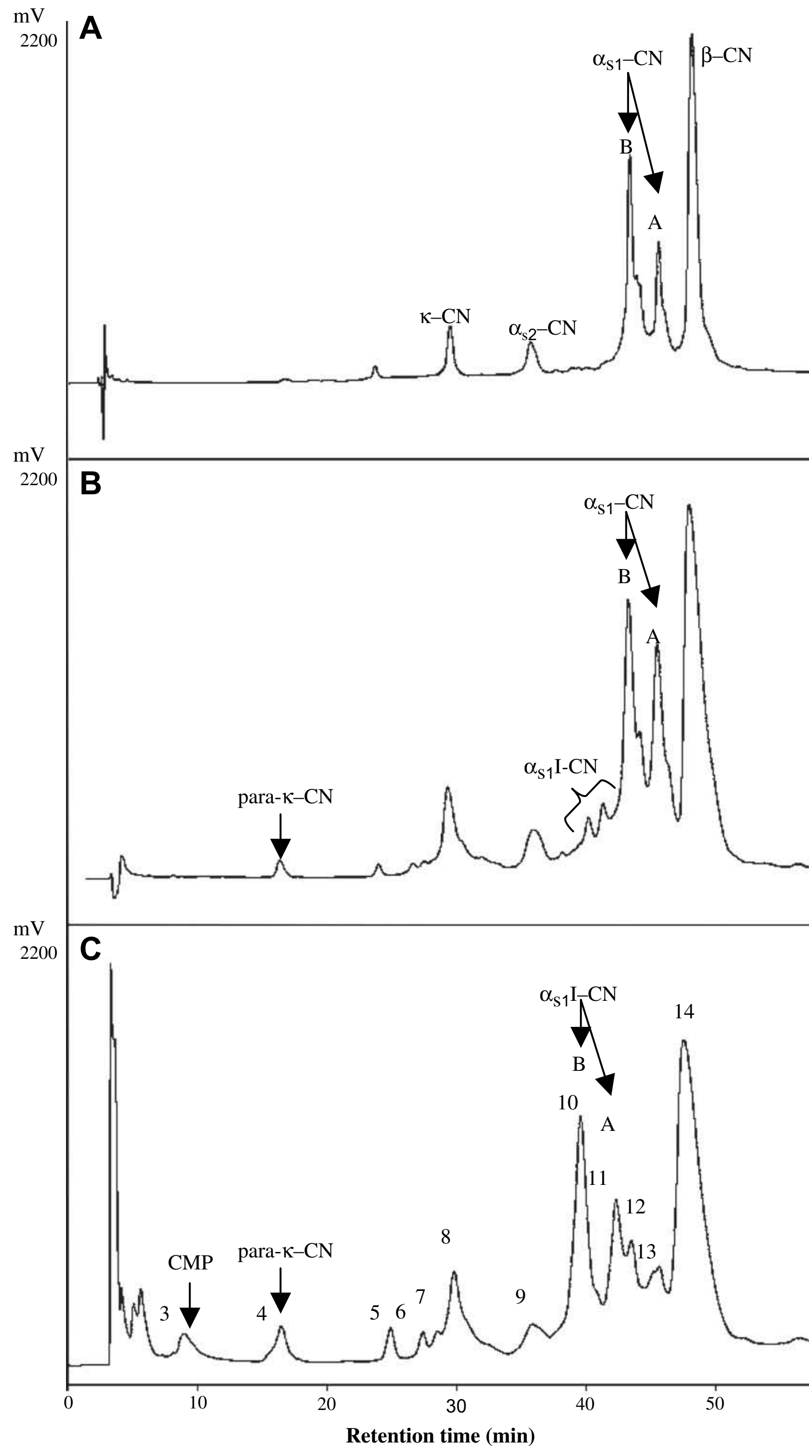

Fig. 5. RP-HPLC analysis of (A) whole casein from bulk buffalo milk, (B) Mozzarella di bufala Campana cheese and (C) whole casein containing α_{s1}-CN A/B after in vitro hydrolysis with chymosin.

markets in both the typical production areas, Caserta (ten samples) and Salerno (ten samples). The most representative profile of whole casein from bulk buffalo milk is shown in Fig. 5A, where the variants α_{s1}-CN A and B are well distinguishable among the other casein fractions, on the basis of their elution time values. In particular, the ratio α_{s1}-CN B/A determined in bulk milk sample by HPLC analysis was 70:30, confirming the higher frequency of allele B in Mediterranean buffalo, as discussed above. The same analysis has been carried out on MBC cheese samples and the representative RP-HPLC profile is shown in Fig. 5B. Taking into account the technological procedure and the very short storage time of cheese before sale (1–2 days), the native α_{s1}- and β-CN fractions are the main components of RP-HPLC profile. The determined α_{s1}-CN B/A ratio accounted for 70:30, indicating a constant frequency of each α_{s1} variant in this population. Among the new formed products eluted in the first part of gradient (0–42 min), para-κ-CN has been only identified with ESI/MS. Moreover, the proteases acting on casein fraction (i.e., chymosin, plasmin or microbial proteases)

Table 3
Peptides identified in the hydrolysate with chymosin of buffalo casein by RP-HPLC/ESI-MS analysis.

HPLC peak	Molecular mass		Protein
	Measured (Da)	Expected (Da)	
1			NI
2			NI
3	6836.5	6834.6	CMP (106–169)P
4	12,363.5	12,366.2	para κ-CN (1–105)
5			NI
6			NI
7			NI
8			NI
9			NI
10	20,667.2	20,667.6	α_{s1}-CN -I 7P Ser^{178}
	20,587.2	20,587.6	α_{s1}-CN-I 6P Ser^{178}
11	20,692.0	20,693.6	α_{s1}-CN-I 7P Leu^{178}
	20,612.2	20,613.7	α_{s1}-CN-I 6P Leu^{178}
12	23,331.2	23,332.7	α_{s1}-CN 7P Ser^{178}
	23,251.9	23,252.7	α_{s1}-CN 6P Ser^{178}
13	23,356.5	23,358.8	α_{s1}-CN 7P Leu^{178}
14	23,980.4	23,982.5	β-CN 5P

NI, not identified.

during cheese-making process, can generate new products which could coelute with the two α_{s1}-CN variants affecting their estimated percentage. To exclude this interference, an in vitro hydrolysis with calf rennet of whole individual casein containing heterozygous α_{s1}-CN AB was carried out; the obtained 12% TCA insoluble fraction was analysed by HPLC (Fig. 5C). In the chromatographic profile 14 peaks were observed, of which seven were identified by ESI/MS analysis (Table 3). Among the new products formed, the caseinomacropeptide (CMP) and para-κ-CN were respectively identified in peaks 4 and 5 and the two α_{s1}-I-CN f(24–199) A and B were identified in the peaks 10 and 11, respectively. A larger amount of these α_{s1} fragments than native proteins (eluted in peaks 12 and 13) has been detected due to the high susceptibility to chymosin action of peptide bond Phe_{23}–Phe_{24} located in amino acid sequence of buffalo α_{s1}-CN, similarly to bovine counterpart. Furthermore, the amounts of α_{s1}-CN fragments and native caseins were in the order α_{s1}-I-CN B > α_{s1}-I-CN A and α_{s1}-CN B > α_{s1}-CN A, respectively. These results indicated that no derived fragments, including α_{s1}-I-CN B and α_{s1}-I-CN A, coeluted in α_{s1}-CN B and A peaks.

4. Conclusion

A proteomic strategy, combining the use of electrophoretic, chromatographic and MS techniques, represents a powerful tool for the detection and characterization of components present in complex protein mixtures. Such as approach was used to characterize and locate the amino acid involved in the mutation resulting in the novel α_{s1}-CN B variant occurring in water buffalo of Mediterranean breed. On the other hand, the studies involving genetic polymorphism of milk proteins are important either for defining the evolutionary mammal pathway or optimising the cheese-making process, since the presence and/or the amount (depending on different expression of the gene) of some genetic variants is positively or negatively related to cheese-making properties. In this case, the results presented can be a satisfactory scientific basis for defining genetic markers of Mediterranean water buffalo, consisting of relative percentage of α_{s1}-CN A and B variants in bulk milk and MBC cheese evaluated by RP-HPLC.

Acknowledgements

We acknowledge Prof. Ferranti for the useful scientific discussion.

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