Occurrence of quantitative genetic polymorphism at the caprine β-CN *locus*, as determined by a proteomic approach

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ABSTRACT

Genetic screening of caseins in caprine milk samples enabled the identification of two novel β-casein (β-CN) phenotypes, C2 and F1, expressed at lower levels (3.3 and 2.7 g L^{-1} per allele, respectively) than reference β-CN C (4.7 g L^{-1} per allele), and another lacking in α_S-complex. The β-CN C2 and F1 primary structures, determined by MS analysis, corresponded to β-CN C and F, respectively. The reasonable hypothesis supporting the low expression of both variants considers the C2 and F1 alleles as deriving from β-CN C1 and β-CN A1 precursors, respectively, and characterised by a genic transition C→T negatively affecting the mRNA stability. Further, phosphorylation of Thr_{41} responsible for the 7P β-CN component was determined by a proteomic approach for the first time. Caprine milk containing low levels of antigenic α_{S1}-, α_{S2}- and β-CN can be used for infant formula production and drinking milk for people with cows' milk protein allergy.

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1. Introduction

Early studies on the biosynthesis of milk caseins in the mammary gland concluded that each was encoded by autosomal codominant alleles expressing the same quantity of different proteins, differing each for one or more amino acidic substitutions. This conclusion was modified when alleles expressing different amounts of related proteins at the same caprine casein locus were observed. In this regard, the most polymorphic casein locus was that of α_{S1}-CN, where 18 alleles have been found to date.

The expressed proteins were categorised into four quantitative classes: 'strong' (A, B_1, B_2, B_3, B_4, C, H, L, M: 3.2 g L^{-1} per allele), 'intermediate' (E, I, D_1: 1.8 g L^{-1} per allele), 'weak' (D, F, G: 0.6 g L^{-1} per allele) and 'null' alleles (0_1, 0_2, N: 0.0 g L^{-1} per allele or trace) (Bevilacqua et al., 2002; Chianese, Ferranti, Garro, Mauriello, & Addeo, 1997; Cunsolo et al., 2005; Garro et al., 2012; Grosclaude, Mahé, Brignon, Di Stasio, & Jeunet, 1987; Martin, 1993). From a structural perspective, the 'intermediate' α_{S1}-CN E and α_{S1}-CN I genetic variants have the same amino acid chain as 'strong' α_{S1}-CN B_4 and A, respectively (Bevilacqua et al., 2002), while the α_{S1}-CN D_1

variant has that of 'weak' variant D (Garro et al., 2012). The genetic event responsible for low expression of the E allele was the insertion of a 457 bp truncated LINE sequence in position 124 within exon 19 (JansàPérez, Leroux, Sanchez Bonastre, & Martin, 1994). The 'weak' α_{S1} genetic variants, instead, have a chain length that is always shorter [α_{S1}D (188 amino acids) > α_{S1}G (186 amino acids) > α_{S1}F (162 amino acids)] than the 'strong' ones (199 aa) due to exon skipping (exon 4 in α_{S1}G, exon 9 in α_{S1}D and 9, 10, 11 concurrently in α_{S1} F) during the translation process (Leroux, Mazure, & Martin, 1992; Martin & Leroux, 1994).

Moreover, in the same way, the alternative exon skipping of both exons 13 and 16, as well as the presence of a cryptic splice site within exon 11 determining the loss of Gln_{78}, gives rise to 'constitutive' non-allelic forms, as in the ovine counterparts (Ferranti et al., 1995, 1997). In this regard, the presence of an additional non-allelic F-like form was recently determined in caprine α_{S1}-CN composition (Garro et al., 2012). The discrete phosphorylation process, involving either genetic α_{S1}-CN variants or the related non-allelic forms, explains the high heterogeneity of this casein, which has been well assessed by proteomic analysis (Ferranti et al., 1997).

The studies carried out on genetic polymorphism of α_{S2}-CN allowed us to find seven alleles, A, B, C (Boulanger, Grosclaude, & Mahé, 1984; Bouniol, Brignon, Mahé, & Printz, 1994), D (Chianese, Mauriello, Intorcia, Moio, & Addeo, 1992; Ramunno, Cosenza,

et al., 2001), E (Chianese et al., 2000, 1998; Lagonigro, Pietrosa, D'Andrea, Veltri, & Pilla, 2001), F and the null allele (Ferranti, Lilla, Chianese, & Addeo, 2000; Ramunno, Longobardi, et al., 2001). The expressed proteins consist of a main chain measuring 208 amino acids in length and a short deleted one lacking the sequence (34−42 or 35−43) otherwise coded by exon 6 (Ferranti et al., 2000), as already found in the ovine counterpart (Boisnard, Hué, Bouniol, Mercier, & Gaye, 1991; Picariello et al., 2009).

At present, ten alleles have been identified at the β-CN locus named A, A1, B, C, C1, D, E, F, 0′, 0. The primary structures of coded proteins as A (Mahè & Grosclaude, 1993; Roberts, Di Tullio, Vitale, Hehir, & Gordon, 1992), A1 (Cosenza Pauciullo, Gallo, Di Berardino, & Ramunno, 2005), C1 (Chessa et al., 2008), E (Caroli et al., 2006), 0 (Persuy, Printz, Medrano, & Mercier, 1999) and 0' (Cunsolo et al., 2005; Ramunno et al., 1995; Rando, Pappalardo, Capuano, Di Gregorio, & Ramunno, 1996), have been elucidated from their cDNA precursor, D (Galliano et al., 2004) and F (Moatsou, Mollé, Moschopoulou, & Gagnaire, 2007) variants from expressed protein using MS analysis and C variant (Chessa et al., 2005; Neveu, Mollé, Moreno, Martin, & Léonil, 2002) using both procedures. Finally, the β-CN B variant (Mahé & Grosclaude, 1993) was only identified on the basis of the electrophoretic pattern. The absence of β-CN, observed early in the milk of some Italian breeds (Chianese et al., 1993; Dall'Olio, Davoli, & Russo, 1989), was explained at the genetic level by the occurrence of stop codons in exon 7 (Persuy et al., 1999; Ramunno et al., 1995).

To date, no phenotypes can be referred to as A1 (Cosenza, Pauciullo, Gallo, Di Berardino, & Ramunno, 2005) and/or C1 (Chessa et al., 2008) variants found in caprine milk, since each of them was derived from A and C alleles originating from the C→T transition at the 180th nucleotide of the untranslated gene sequence of the 9th exon. As the C→T transition has also been found in other ruminant species (Cosenza et al., 2005), the A allele has been considered to be the ancestral allele.

The primary structures of caprine β-CN were shorter than their bovine counterparts, due to the deletion of dipeptide $Pro_{179}-Tyr_{180}$ (Richardson & Creamer, 1974). Specific immunoblotting with anti-β polyclonal antibodies carried out on caprine casein UTLIEF profiles enabled the recognition of almost three phosphorylated β-CN components (6P, 5P, 4P) with a decreased pI value (Chianese et al., 1993). Moreover, phenotypes having one half or trace of β-CN in the milk, relative to the common phenotype, were detected in the milk of caprine breeds reared in Italy (Chianese et al., 1993; Dall'Olio et al., 1989; Ramunno et al., 1995), Guadalupe (Mahé & Grosclaude, 1993) and the Pyrenean zone (Grosclaude & Martin, 1997). In conclusion, the results achieved on genetic polymorphism of α_{S1}- α_{S2} and β-CN have shown the negative impact of their coding 'intermediate', 'weak' and 'null' alleles on the technological (aptitude to cheesemaking) and nutritional quality of milk (Chianese et al., 1993; Remeuf, 1993; Remeuf, Ricordeau, Brignon, & Grosclaude, 2001; Vassal, Delacroix-Buchet, & Bouillon, 1994).

However, taking into account, (i) the antigenicity of phosphorylated caseins, (ii) the notably low level of α_S-complex in human milk and (iii) the superior digestibility of goat milk containing 'weak' and 'null' α_{S1}-CN alleles (Bevilacqua et al., 2000), it is reasonable to consider the use of related milks to be suitable for infant formula production and for patients allergic to bovine milk proteins. Furthermore, an additional health benefit of caprine milk is the presence of Pro_{67} in all known caprine β-CN variants, instead of His_{67} which, in bovine β-CN, has been related to the development of insulin-dependent type I diabetes mellitus (IDDM) in predisposed subjects (Elliott, Wasmuth, Bibby, & Hill, 1997), as well as delayed gastrointestinal transit (Barnett, McNabb, Roy, Woodford, & Clarke, 2014; Ul Haq, Kapila, & Kapila, 2015) and lactose

fermentation in the intestine (Pal, Woodford, Kukuljan, & Ho, 2015). These considerations determined the focus of the present work, i.e., the phenotypic screening of individual caprine milk samples of the Cilentana breed, to select the animals producing suitable and healthy drinking milk as a good substitute for bovine milk. The molecular phenotypic characterisation was performed with a proteomic approach.

2. Materials and methods

2.1. Casein preparation

Individual whole caprine casein (167 samples) was prepared by acid precipitation of skim milk from Cilentana breed reared in the Campania region, followed by centrifugation at 2500× g for 15 min (Aschaffenburg & Drewry, 1959). The casein obtained was rinsed twice with distilled water to eliminate whey, then freeze-dried and stored at −20 °C before use.

2.2. Chemical analysis

The total proteins and casein content in goat milk samples was determined by Kjeldahl method according to IDF (1993).

2.3. Gel electrophoresis

2.3.1. Ultra-thin layer isoelectric focusing

Casein samples from individual milks (20 g L^{-1}), were dissolved in a 9 M urea solution containing 2-mercaptoethanol (10 μL^{-1}). Ultra-thin layer isoelectric focusing (UTLIEF) on polyacrylamide gels (0.25 mm) was carried out in a pH gradient ranging from 2.5 to 6.5 pH units by mixing Ampholine buffers (GE Healthcare, UK) 2.5−5.0, 4.5−5.4 and 4.0−6.5 (1.6:1.4:1, by vol). The gel was stained with Coomassie Brilliant Blue (CBB) G-250 as described by Neuhoff, Arold, Taube, & Ehrhardt, 1988.

2.3.2. Densitometric analysis

The relative percentage of each casein fraction in UTLIEF profiles was determined by scanning Coomassie Blue stained gel patterns with Image J software (http://rsb.info.nih.gov/ij), to perform band aligning/matching between gels.

2.4. Immunoblotting

The UTLIEF casein profiles were transferred by capillary diffusion from the gel onto a nitrocellulose membrane (0.45 μm, Trans-Blot, Bio-Rad, Richmond, CA, USA). Immunodetection was carried out according to the procedure already described by Chianese et al. (1996) using rabbit polyclonal antibodies against bovine synthetic peptide β-CN (195−209) purchased from Primm (Milano, Italy). The casein peptide, linked to ovalbumin, was used as antigen to immunise two rabbits (Primm, Milano, Italy). The obtained antisera were filtered, using 0.45 μm filters (Millipore, Bedford, MA, USA), divided into aliquots of 1 mL, and stored at −20 °C.

2.5. Chemical and enzymatic hydrolysis

Modified trypsin, sequencing grade, was purchased by Promega (Madison, WI, USA), alkaline phosphatase (ALP) by Roche (Mannheim, Germany). Both trypsin and alkaline phosphatase hydrolysis were carried out in 0.4% ammonium bicarbonate, pH 8.0, at 37 °C for 4 and 16 h, using a ~50:1 substrate-to-enzyme ratio (w/w).

2.6. Liquid chromatography electrospray ionisation mass spectrometry

2.6.1. HPLC separation of β-casein

Native and dephosphorylated whole caseins for each genotype, were dissolved in 1 mL 10 mM dithiothreitol (DTT); for injection, 100 µL^{-1} of the resulting solution was used. Pure β-CN fractions were isolated by RP-HPLC using an HP1100 Agilent Technology modular system (Palo Alto, CA, USA) equipped with a Vydac (Hesperia, CA, USA) C4 column (214TP54, 5 µm, 250 × 4.6 mm i. d.). After 5 min of isocratic elution, a linear gradient from 30% to 50% of 0.1% (v/v) TFA in acetonitrile (solvent B), over 60 min at a flow rate of 1 mL min^{-1}, was applied; solvent A was 0.1% (v/v) TFA in water. Column effluents were monitored by UV detection at 220 and 280 nm. Protein fractions were manually collected and used directly for mass spectrometry analysis by a flow injection method either directly or after concentration under vacuum. Alternatively, the samples were lyophilised prior to enzymatic digestion.

For liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS) analysis, tryptic digests of caseins were fractionated using the above HP1100 Agilent Technology modular system equipped with a Vydac C18 218TP52 column (5 µm, 250 × 2.1 mm) at a constant flow rate of 0.2 mL min^{-1}. Elution was carried out with a linear gradient from 5% to 60% of solvent B applied over 60 min (solvent A and B as above) after 5 min of isocratic elution at 5% B. Approximately 100 µg of the peptide mixture, dissolved in 0.1% TFA, was injected for each analysis. The liquid effluent from the column was directly injected into the source of an Agilent 1100LC/MSD single quadrupole instrument via a 0.25 mm PEEK tube connection. The ESI mass spectra scans were acquired from m/z 1600−400 at a scan cycle of 1 s per scan and 0.1 s interscan delay. The source temperature was 350 °C. Spectra were acquired in positive ion mode, with a capillary voltage of 3.5 kV N$_2$ was used as both the drying and nebulising gas. The LC-ESI/MS pattern was elaborated using the LC/MSD ChemStation Data Analysis Software A.08.03, (Agilent Technology) that was supplied with the instrument.

2.6.2. Analysis of derived tryptic peptides by flow injection analysis nano electrospray-ionisation-quadrupole-time-of-flight-mass spectrometry

The amino acid sequences of the peptides of interest, detected during LC-ESI-MS, were determined by tandem mass spectrometry analysis (MS/MS). The mixture of β-CN tryptic digests was dissolved at a concentration of 1 µg µL^{-1} in acetonitrile-water (v/v) containing 0.1% TFA, and analysed by MS. Analysis was carried out using ESI Q-TOF™ hybrid quadrupole/time-of-flight (TOF) mass spectrometer (Micromass Ltd., Manchester, UK) and a Z-spray ion source in the positive ion mode. The nano flow was accomplished with a syringe pump at 0.5 µL min^{-1}, and the TOF mass analyser was used to acquire data in both the 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) ranging from 600 to 1800. The collision energy was dependent on the m/z ratio and the charge state of the parent ion and was generally between 25 and 40 V. The collision cell was pressurised with 10.34 Pa ultra-pure Ar (99.99%). The raw MS/MS data were combined and processed using the MaxEnt 3 algorithm prior to de novo sequence analysis using the Mass Seq software (Waters, Manchester, UK).

3. Results and discussion

3.1. UTLIEF and immunoelectrophoretic analysis

The most representative UTLIEF profiles of casein phenotypes, discriminating the individual caprine milks in the Cilentana breed, are shown in Fig. 1A. These results, on the basis of pI value and intensity of focalising bands, showed the α$_{S1}$-CN genetic variants to be expressed by 'strong' A and B (samples 1, 3, 4, 5), 'intermediate' E (sample 6) and 'null' alleles (samples 2, 7). Moreover, each α$_{S1}$-CN variant consisted of almost three main components, having 9, 8 and 7 phosphate groups in increasing order of pI value (Chianese et al., 1993).

The same post translation event on β-CN gave rise to two main phosphorylated components, β$_1$ (6P) and β$_2$ (5P), exhibiting a similar quantitative level and the same pI value in all the samples, except in sample 5. Moreover, when the UTLIEF profiles were immunostained with polyclonal antibodies against β-CN (Fig. 1B), additional phosphorylated components appeared, exhibiting a lower (asterisk in Fig. 1) and a higher pI value (arrows in Fig. 1) than the main β$_1$ (6P) and β$_2$ (5P) components. These results allowed the identification of the asterisked component as the β-CN 7P occurred at a lower amount than the others ones. However, due to the silent amino acid substitutions characterising the known β-CN variants, it is impossible to discriminate the β-CN genotypes in the samples (Fig. 1), except in sample 5, which exhibited the highest pI value among the others. Comparing the UTLIEF profiles (Fig. 1A), the quantitative level and number of α$_{S2}$-CN components range from 0 (lane 2) to 6 (lane 5).

In particular, the most interesting sample, from an antigenic point of view, can be considered the number 2, since, after staining with CBB (Fig. 1A), it seems to be missing both α$_{S1}$- and α$_{S2}$-CN, as in breast milk, where a very low amount of α$_{S1}$-CN was found (Altendorfer et al., 2015) up to undeterminable traces (our unpublished results). The compositional heterogeneity of α$_{S2}$-CN observed in the other samples was due either to a discrete phosphorylation rate or the presence of a non-allelic form, as reported above.

3.1.1. Densitometric analysis

To determine the quantitative class of the alleles coding for α$_{S1}$-, β- and α$_{S2}$-CN, the amount of each expressed casein in the milk was assessed using densitometric analysis performed on UTLIEF profiles (Fig. 1A). The results obtained are reported in Table 1 as the relative area percentage of each casein UTLIEF profile (first column) and the related expressed protein (g L^{-1}). Due to the overlapping phenomenon taking place in UTLIEF containing heterozygous phenotypes for the phosphorylation process, the expressed g L^{-1} per allele have been deduced only in homozygous phenotypes.

The achieved results showed the higher content of whole casein in milk containing the 'strong' α$_{S1}$-CN variant (samples 1, 3, 4, 5) than in milk containing the lower ones, namely, in quantitative decreasing order, 'intermediate' E (sample 6) and 'null' (samples 2 and 7) alleles according to the literature data (Bevilacqua et al., 2002; Chianese et al., 1997; Grosclaude & Martin, 1997).

The quantitative level of expressed β-CN, instead, was highest in α$_{S1}$ 'null' samples (samples 2 and 7) and lowest in samples 1 and 5 (3.3 and 2.7 g L^{-1} per allele, respectively). These results allowed us to define the occurrence of two intermediate β-CN genetic variants named C2 and F1, respectively, as a consequence of the findings of Section 3.2.

As a result, the quantitative level of α$_{S1}$-CN in milk is positively related to the casein content and is inversely related to that of β-CN. Similarly, three quantitative levels were assessed for α$_{S2}$-CN which, in increasing order, should be expressed by 'null' (sample 2),
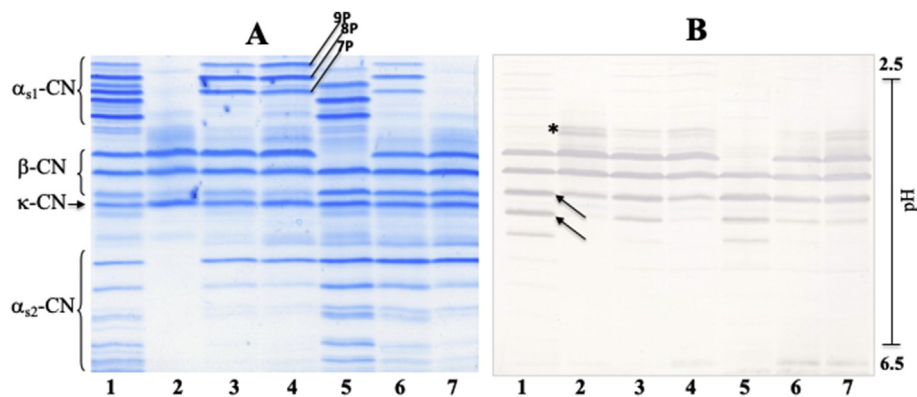
Fig. 1. UTLIEF analysis of whole caprine casein samples (1–7), using (A) Coomassie Brilliant Blue staining and (B) immunoblotting with polyclonal antibodies against β-CN. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Single casein fractions content in comparison with whole casein in Cilentana caprine milk samples.

Sample	α_{S1}-CN		β-CN		α_{S2}-CN		κ-CN		Total casein
	Peak area (%)	g L^{-1}	Peak area (%)	g L^{-1}	Peak area (%)	g L^{-1}	Peak area (%)	g L^{-1}	g L^{-1}
1	43.50	10.79	26.70	6.62	17.40	4.31	4.60	1.14	24.80
2	0.00	0.00	70.30	14.41	0.00	0.0	23.70	4.86	20.50
3	30.50	7.41	40.10	9.74	16.50	4.01	9.20	2.23	24.30
4	30.60	7.59	37.20	9.22	15.50	3.84	12.30	3.05	24.80
5	29.10	7.27	21.90	5.48	35.90	8.98	7.90	1.98	25.00
6	14.30	3.36	36.10	8.48	36.90	8.67	9.80	2.31	23.50
7	0.00	0.00	49.30	11.53	33.40	7.82	12.00	2.81	23.40

Table 2
β-CN genetic variants identified by FIA nano-ESI-Q-TOF analysis of isolated β-CN from individual caprine samples in Fig. 1.[a]

Samples	Genotype	Molecular mass (Da)							
		β-CN native		After alkaline phosphatase action					
		Theoretical	Measured	$\Delta M^{(dp)}$	Phosphate group	Theoretical	Measured	$\Delta M^{(vc)}$
1	C2C2	23,929.00	23,929.01	559.89	7P	23,369.14	23,369.20	
		23,849.02	23,849.07	479.88	6P			
		23,769.02	23,769.08	399.88	5P			
2	CC	23,929.00	23,929.01	559.89	7P	23,369.14	23,369.20	
		23,849.02	23,849.07	479.88	6P			
		23,769.02	23,769.08	399.88	5P			
3, 4	AC	23,900.94	23,901.00	559.92	7P	23,341.08	23,341.08	28
		23,820.96	23,821.00	479.88	6P			
		23,740.98	23,741.00	399.88	5P			
		23,929.00	23,929.01	559.89	7P	23,369.14	23,369.15	
		23,849.02	23,849.07	479.88	6P			
		23,769.02	23,769.08	399.88	5P			
5	F1F1	23,949.03	23,949.2	559.83	7P	23,389.17	23,389.20	20
		23,869.05	23,869.05	479.88	6P			
		23,789.05	23,769.03	399.88	5P			
6	AF1	23,900.94	23,901.00	559.92	7P	23,341.08	23,341.08	28
		23,820.96	23,821.00	479.88	6P			
		23,740.98	23,741.00	399.88	5P			
		23,949.03	23,949.2	559.83	7P	23,389.17	23,389.2	20
		23,869.05	23,869.05	479.88	6P			
		23,789.05	23,769.03	399.88	5P			
7	CF1	23,929.00	23,929.01	559.89	7P	23,369.14	23,369.20	
		23,849.02	23,849.07	479.88	6P			
		23,769.02	23,769.08	399.88	5P			
		23,949.03	23,949.2	559.83	7P	23,389.17	23,389.2	20
		23,789.05	23,769.03	399.88	5P			

[a] $\Delta M^{(dp)}$, difference between the molecular mass of β-CN and the dephosphorylated counterparts; $\Delta M^{(vc)}$, difference between the molecular mass of β-CN genetic variants respect the most common variant C.

Table 3

β-CN genetic variants identification by FIA nano-ESI-Q-TOF analysis of tryptic peptides isolated from individual caprine samples shown in Fig. 1, before and after alkaline phosphatase action.[a]

Peptide	Genotype	Samples	Molecular mass (Da)		AA substitutions	Phosphatase alkaline action			Sequence
						Molecular mass (Da)		Number of phosphate groups	
			Theoretical	Measured		Theoretical	Measured		
1−28	A/C/F	All	3543.41	3543.37		3143.53	3143.53	5	REQEELNVVGETVESLSSSEESITHINK
1−29	A/C/F	All	3671.46	3671.61		3271.65	3271.65	5	REQEELNVVGETVESLSSSEESITHINKK
33−48	A/C	1,2,3,4	2061.83	2061.83	Asp[47]	1981.86	1981.86	1	FQSEEQQQTEDELQDK
33−48	A/C	1,2,3,4	2141.79	2141.79	Asp[47]		1981.86	2	FQSEEQQQTEDELQDK
33−48	F	5,6,7	2109.86	2109.86	Tyr[47]	2029.92	2029.92	1	FQSEEQQQTEDELQYK
33−48	F	5,6,7	2189.83	2189.83	Tyr[47]	3143.53	3143.53	2	FQSEEQQQTEDELQYK
49−97	A/C/F	All	5330.91	5330.91					IHPFAQAQSLVYPFTGPIPNSLPQNILPLTQTPVVVPPFLQPEIMGVPK
98−105	A/C/F	All	931.53	931.53					VKETMVPK
100−105	A/C/F	All	704.36	704.36					ETMVPK
106−107	A/C/F	All	284.17	284.17					HK
108−113	A/C/F	All	748.37	748.37					EMPFPK
114−132	A/C/F	All	2183.08	2183.08					YPVEPFTESQSLTLTDVEK
133−169	A/C/F	All	4155.23	4155.23					LHLPLPLVQSWMHQPPQPLSPTVMFPPQSVLSLSQPK
170−176	A/C/F	All	780.50	780.50					VLPVPQK
177−181	A/F	6,7	570.34	570.34					AVPQR
177−181	C	1,2	598.72	598.72	Val[177]				VVPQR
170−181	A/F	6,7	1331.82	1331.82	Ala[177]				VLPVPQKAVPQR
170−181	C	1,2	1359.85	1359.85	Val[177]				VLPVPQKVVPQR
182−200	A/C/F	All	2186.17	2186.17					DMPIQAFLLYQEPVLGPVR
201−207	A/C/F	All	742.45	742.45					GPFPILV

[a] In the sequence, residues determined as being phosphorylated are underlined.

'intermediate' (samples 1, 3, 4) and 'strong' (the others), as reported in the literature (Boulanger et al., 1984; Bouniol et al., 1994; Lagonigro et al., 2001; Ramunno, Cosenza, et al., 2001,b).

3.2. Molecular characterisation of β-CN genetic variants by MS analysis

3.2.1. Flow injection analysis nano electrospray-ionisation-quadrupole-time-of-flight-mass spectrometry analysis

β-CN genotypes were characterised in all samples (Fig. 1) using flow injection analysis nano electrospray-ionisation-quadrupole-time-of-flight-mass spectrometry analysis of HPLC-isolated β-CN fractions (Table 2). The ΔM values observed, before and after protein dephosphorylation, enabled determination number of β-CN phosphate groups and to prove, according to the electrophoretic results, the presence of the 7P phosphorylated component in each β-CN variant (Table 2). Moreover, using the amino acid sequence of goat β-casein reported in the UniProt database of protein sequence (UniProtKB - P33048 (CASB_CAPHI), the β-CN C variant in homozygous form was recognised in sample 1 and 2 on the basis of ΔM = 28 Da derived from the $Val_{177}(C) \rightarrow Ala_{177}(A)$ substitution. The allele coding for the same aa sequence of β-CN C variant in sample 1, but at lower level, could be characterised by a molecular event taking place in a non-coding region of allele C, as reported for β-CN C1 allele (Chessa et al., 2008) and for this reason named C2. The allele C1, as determined using PCR-SSPCP methodology, differs from C allele in the C→T transition located in the 3' untranslated region of the non-coding ninth exon, previously found in A1 allele deriving from ancestral A allele (Cosenza et al., 2005). In this regard, the lower amount of expressed protein was expected as a consequence of a decreasing mRNA stability (Cosenza et al., 2005; Xu, Chen, & Shyu, 1997).

In a study on quantification of caseins genotype, previously identified with the same PCR protocol (Chessa et al., 2008), in caprine blood, Montalbano, Segreto, Di Gerlando, Mastrangelo, and Sardina (2016) selected and quantified β-CN C and β-CN C1 variants by HPLC analysis. The amounts reported of β-CN per allele were: β-CN C = 3.0 ± 0.8 g L^{-1} and β-CN C1 = 2.0 ± 0.7 g L^{-1}, both lower than reference β-CN C (5.0 g L^{-1}) (Montalbano et al., 2016) and of reference β-CN C = 4.7 g L^{-1} and β-CN C2 = 3.3 g L^{-1} per allele (our findings in present paper). Moreover, there have been no reports about the low content of β-CN C (3.0 ± 0.8 g L^{-1} per allele).

The mass value of the β-CN variant in sample 5 was 20 Da higher than that of the reference variant β-CN C for the two substitutions $Asp_{47}(C) \rightarrow Tyr_{47}$ and $Val_{177}(C) \rightarrow Ala_{177}$ and 48 Da higher than that of variant A for the substitution $Asp_{47}(A) \rightarrow Tyr_{47}$ (Table 2). These results demonstrate the higher pI value of this β-CN variant compared with the others (sample 5, Fig. 1). The same aa substitution characterising the β-CN variant discriminated by PAGE analysis, at the time called E (Chianese et al., 2007), was reported in the same year by Moatsou et al. (2007), and subsequently renamed F by Martin, Bianchi, Cebo, and Miranda (2013). From a genetic point of view, the presence of an Ala residue at position 177 in the amino acid chain of β-CN F indicates, in any case, its derivation from ancestral variant A. However, the low content of β-CN in the milk of sample 5, accounting for 2.7 g L^{-1} per allele (Table 1), reasonably suggests its expression from a derived allele F, named F1, having the same C → T transition of C1 and A1 alleles (Cosenza et al., 2005) with the same implications for mRNA stability.

3.2.2. Determination of the primary structure of the β-CN F1 variant by tandem MS analysis

To confirm the above results, the HPLC-isolated β-CN fractions extracted from samples of Fig. 1 were subjected to trypsin hydrolysis. Each hydrolysate, before and after alkaline phosphatase action, was analysed by LC-ESI-MS analysis and the identified tryptic peptides reported in Table 3. Among the tryptic peptides β-CN (f177−181) (m/z 598.72) and β-CN (f33−48) (m/z 2029.92) were found to exhibit a different molecular mass with respect to the most common reference variant. Moreover, peptide β-CN (f33−48) (m/z 2029.92) was present at two phosphorylation degrees (1P and 2P) (Table 3). The consecutive tandem MS analysis of the tryptic dephosphorylated β-CN (f33−48) peptide of 2029.92 Da demonstrated the amino acid substitution $Asp_{47}(C) \rightarrow Tyr_{47}(F1)$ (Fig. 2).

Using the same analysis, the tryptic peptides β-CN (f170−181) exhibited two different molecular masses, of 1359.85 Da (Fig. 3A)
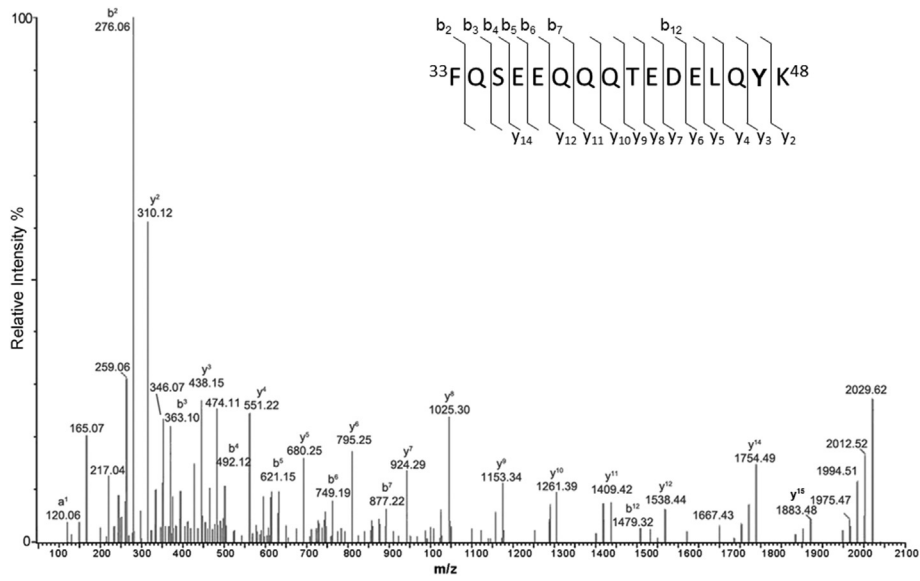
Fig. 2. Tandem MS analysis of β-CN F1 tryptic peptides (f33−48) after alkaline phosphatase action of 2029.92 Da containing amino acid substitution $Asp_{47} \rightarrow Tyr_{47}$.
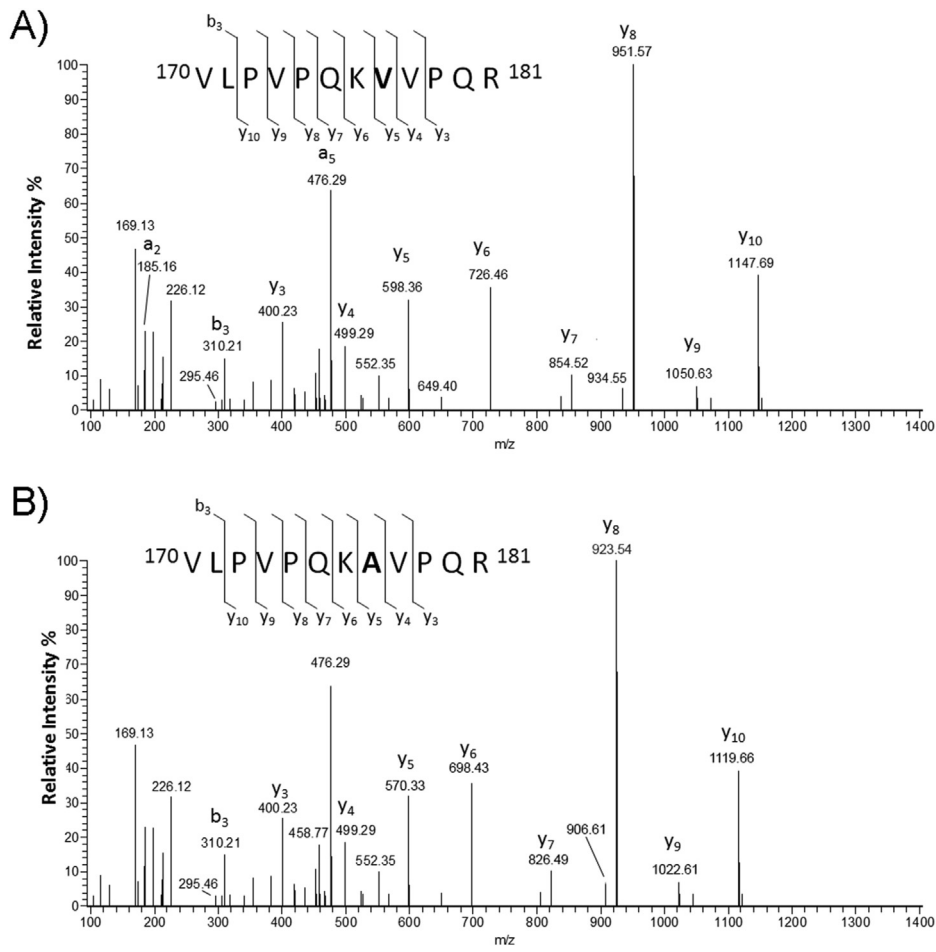

Fig. 3. Tandem MS analysis of β-CN F1 tryptic peptides (f170−181) of (A) 1359.85 Da and (B) 1331.82 Da, confirming the amino acid substitution $Val_{177}(C) \rightarrow Ala_{177}(F1)$.
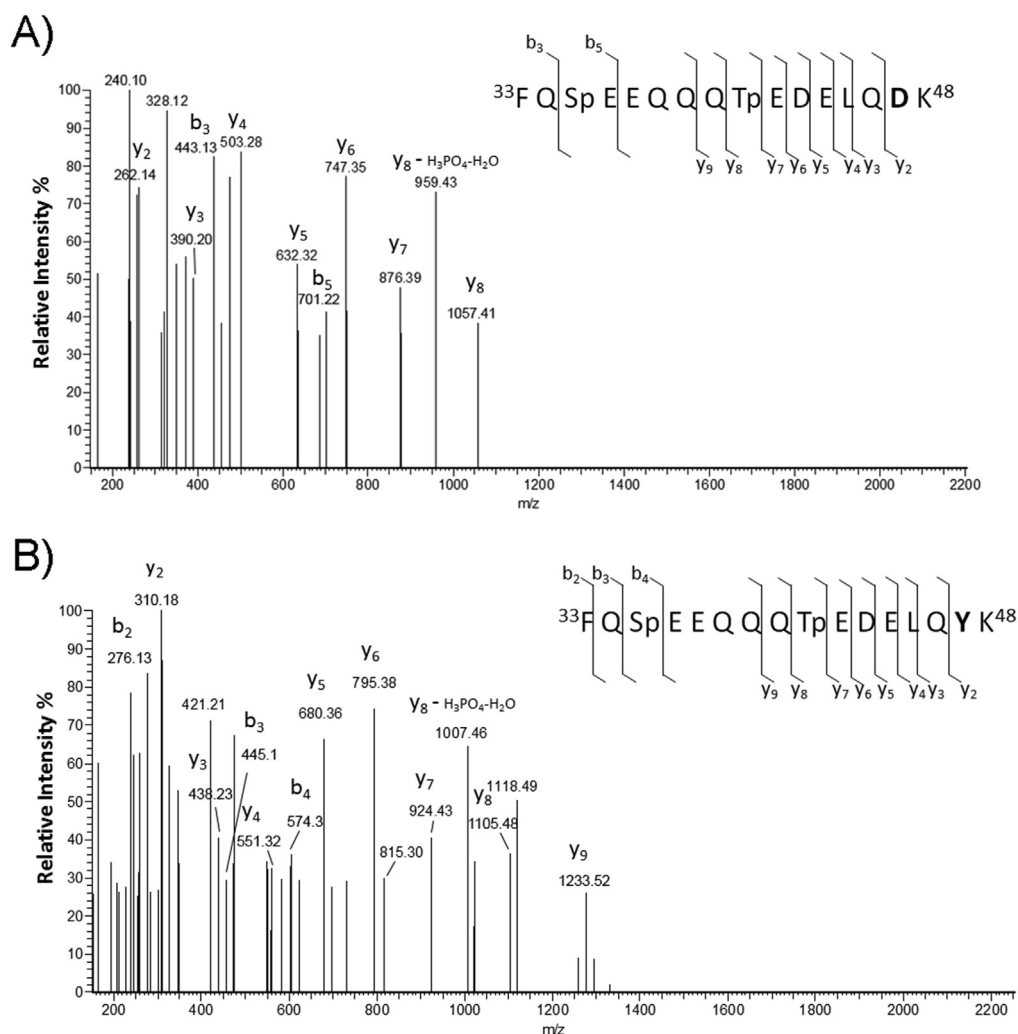
Fig. 4. Tandem MS analysis of the native β-CN F1 tryptic peptides (f33–48) of (A) 2189.83 Da and (B) 2141.79 Da identifying the peptide with two phosphate groups at positions 35 and 41, respectively, and with the amino acid substitution Asp$_{47}$(A) →Tyr$_{47}$(F1).

and 1331.82 Da (Fig. 3B), demonstrating the amino acid substitution Val$_{177}$(C) → Ala$_{177}$(F1).

Furthermore, peptides of 3543.37 Da (Table 3) and 3671.61 Da assigned to β-CN (f1−28)5P and β-CN (f1−29)5P, respectively, confirmed simultaneous phosphorylation at the Ser$_{15}$, Ser$_{17}$, Ser$_{18}$, Ser$_{19}$ and Thr$_{12}$ residues, as previously reported (Neveu et al., 2002). The amino acid sequence of single and double phosphorylated peptides β-CN (f33–48) (Table 3) from the tryptic digest of F1 variant was deduced from the y and b series of the precursor ions of 2109.86 Da (Fig. 4A) and 2189.83 Da (Fig. 4B), thus enabling the location of the sixth phosphate group on Ser$_{35}$ and, for the first time, the seventh phosphate group on Thr$_{41}$, as previously hypothesised by Trujillo, Guamis, and Carretero (1997).

The lower amount of β-CN 7P agrees with the lower efficiency of kinase, probably for steric reasons, in phosphorylating hydroxyl amino acids within the consensus sequences Thr-X-Asp compared with the most effective motif Ser−X−Glu. The results obtained in the field to date have shown a different susceptibility of serine or threonine residues placed within the triplet consensus to be phosphorylated in the following order: Ser−X−Glu > Thr−X−Glu > Ser−X−Asp > Thr−X−Asp (Mercier, 1981). While the phosphorylated threonine in the Thr−X−Glu triplet code has been identified in β-CN of caprine, human and

equine milk (Greenberg, Groves, & Dower, 1984; Matéos et al., 2010; Neveu et al., 2002), the threonine in the Thr−X−Asp counterpart has never been found to be phosphorylated (Lasa-Benito, Marin, Meggio, and Pinna (1996); Meggio et al., 1988; Mercier, 1981), probably due to the limited sensitivity of the instruments, as reported in previous studies. Two different regulatory systems for the phosphorylation of α$_S$-CN in bovine species have been suggested (Fang et al., 2016); one is responsible for isoforms with a lower degree of phosphorylation, and the other is responsible for isoforms with a higher degree of phosphorylation, and responsible for phosphorylating serine or threonine residues in less favourable motifs, such as those within Ser/Thr−X−Asp or Thr−X−Glu motifs.

4. Conclusions

Caprine milk is frequently used for cheesemaking. In contrast to the bovine, ovine and buffalo counterparts, each phosphorylated caprine α$_{S1}$-, α$_{S2}$- and β-CN can be expressed in different amounts in milk up to traces of the casein fraction in milk which, in turn, determines a low cheese yield. In this study, the use of a proteomic methodology based on immunoelectrophoresis coupled to MS analysis allowed us to define the casein phenotyping of intermediate or weak/null alleles at the β-CN, α$_{S1}$-CN and α$_{S2}$ -CN loci. In

particular, a sample simultaneously lacking the α_S complex and two new β-CN variants, named C2 and F1, expressed at lower levels than the most common ones, were found. The lower content of β-CN C2 and β-CN F1 variants in the milk suggested C2 and F1 coding alleles, likely derived from C1 and A1 alleles, respectively, might be due to the presence of C→T transition within the common non-coding ninth exon. Taking into account (i) the positive effects of α_{S1}-CN free caprine milk in infant intolerance to cows' milk (Bevilacqua et al., 2000) and (ii) the high impact of β-CN on the structural and nutritional differences between caprine and bovine milk and its higher degree of tolerance for the presence of Pro_{67} (Haenlein, 2004), the selected 'weak' caprine milk could be used as drinking milk for patients with cows' milk protein allergy.

Acknowledgements

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