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Characterisation of the heterogeneity of ovine deleted variant α_{S1}-casein E by a proteomic approach

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ABSTRACT

The micro-heterogeneity of the ovine α_{S1}-casein (α_{S1}-CN) E variant, occurring in Leccese ewe milk, was characterised using a bottom-up, shotgun proteomic approach using two-dimensional gel electrophoresis and reversed phase-high performance liquid chromatography as separation techniques and polyclonal antibodies against α_{S1}-CN and nano-liquid chromatography-electrospray ionisation-time-of-flight tandem mass spectrometry as identification methodology. Comparing results with the most common genetic variant α_{S1}-CN C, α_{S1}-CN E variant showed a reduced number of phosphorylated serine residues (α_{S1}-CN E -4P versus α_{S1}-CN C -9P) and a lower measured molecular mass (22241.7 Da versus 23481.5 Da). These differences were due to the α_{S1}-CN (70–77) amino acid sequence deletion, coded by exon 10, thus triggering the phosphate loss on serine residues located at positions 64, 66, 68 and 75. The loss of four phosphate groups, associated with the lower content of α_{S1}-CN E, could be negatively related both to cheese-making aptitude of milk and mineral carrier activity.

1. Introduction

The study of genetic polymorphisms of milk proteins is helpful to define the genetic evolutionary process taking place in species diversification, its impact on milk production traits (Calvo et al., 2013; Giambra, Brandt, & Erhardt, 2014) and on technological properties of milk (Amigo, Recio, & Ramos, 2000; Chianese et al., 1997; Martin, Szymanowska, Zwierzchowski, & Leroux, 2002; Ng-Kwai-Hang, 2006), as well as to find genetic markers of typical dairy products (Chianese et al., 2009).

The available results on the milks of ruminant species, particularly cow, goat, ewe and buffalo milks, showed the occurrence of several alleles at each casein locus. Generally, genetic variants could differ by a single amino acid substitution or a deletion of the entire sequence coded by an exon, as found in goat milk (Grosclaude & Martin, 1997; Leroux, Mazure, & Martin, 1992). The study of the ovine α_{S1}-casein (α_{S1}-CN) genetic polymorphism by electrophoretic techniques and Coomassie Blue or/and specific immunostaining with polyclonal antibodies discriminated nine α_{S1}-CN genetic variants (A→I), of which A, B, C, D, E, and F in ewe milk were from Italian and Spanish breeds (Chianese et al., 1996; Lopez-Galvez, Amigo, & Ramos, 1999; Pirisi et al., 1999) and H and I were from German breeds (Giambra, Chianese, Ferranti, & Erhardt, 2010a, b). Among them, to date, the primary structures of A, C, D, and E (Chianese, Caira, Garro, & Addeo, 2008; Ferranti et al., 1995) and H and I (Giambra et al., 2010a, b) have been determined using mass spectrometry techniques by a classic bottom-up approach. These results allowed for the establishment of a different hierarchy of the α_{S1}-CN genetic variants, i.e., A > C > H > D > I > E or A < C < H < D < I < E, based on the negative net charge (urea-polyacrylamide gel electrophoresis; urea-PAGE at pH 8.6) or isoelectric point (ultra-thin layer isoelectric focusing, UTLIEF), respectively.

Moreover, the proteomic approach enabled us to determine the complex composition of ovine α_{S1}-CN due to two main events taking place at the genic level: (i) the alternative splicing processes leading both to casual exon skipping events (i.e., exons 7, 8 and 10 in α_{S1}-CN; Giambra et al., 2010a,b) and multiple forms of mature α_{S1}-CN (Ferranti et al., 1998, 2001); (ii) the discrete phosphorylation phenomenon after the transcriptional process, depending either on

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the activity of native mammary kinases (Mercier, 1981) or on abnormally high alkaline phosphatase activity (Mauriello et al., 2008; Pinto et al., 2013).

The aim of present study was to define the composition of the α_{S1}-CN E genetic variant, occurring only in heterozygous form (α_{S1}-CN CE), in Leccese ewe milk. The objective was achieved with a proteomic approach based on the synergy of two high-resolution separation techniques [two-dimensional (2-DE) gel electrophoresis and reversed phase-high performance liquid chromatography (RP-HPLC)] and nano-liquid chromatography-electrospray ionisation-time-of-flight tandem mass (nanoLC-ESI-MS/MS) analysis for the identification of the components responsible for the micro-heterogeneity of the α_{S1}-CN variant E.

2. Materials and methods

2.1. Extraction of casein from milk and sample preparation for electrophoresis

Individual whole casein samples from 126 ewes of Leccese breed were obtained by acid precipitation from skimmed milk, followed by centrifugation at 2500×*g* for 15 min (Aschaffenburg & Drewry, 1959); the pellet obtained was rinsed twice with distilled water to eliminate whey, then freeze-dried and stored at −20 °C before use. Casein samples for electrophoretic analysis were dissolved in a 9 M urea solution (20 g L^{-1}) containing 2-mercaptoethanol (10 mL^{-1}).

2.2. Gel electrophoresis

2.2.1. Urea-PAGE at pH 8.6

Urea-PAGE was performed with vertical electrophoretic apparatus Protean II (Bio-Rad, Richmond, CA 94804, USA) at 200 V and 6 °C for 7 h. The gels (0.75 mm thick, 180 × 140 mm) consisted of stacking gel (3% T and 1% C) containing 3.6 M urea, 7.5% (w/v) glycerol in 0.5 M Tris-HCl buffer at pH 6.8 and running gel (7.5% T and 2.5% C) containing 6.1 M urea in 1.5 M Tris-HCl buffer at pH 8.6. N,N,N′,N′-tetramethylethylenediamine (TEMED) and ammonium persulphate (PER), 0.04% (v/v) and 0.07% (w/v) final concentrations, respectively, were added to running and stacking gel, as activator and catalyst agents of polymerisation. The migration buffer consisted of 0.19 M glycine and 0.024 M Tris-HCl. The gel was stained overnight with a methanolic solution of Coomassie Brilliant Blue R-250 (1.5 g L^{-1}) and destained with an aqueous solution containing 25% (v/v) methanol and 10% (v/v) glacial acetic acid.

2.2.2. Densitometric analysis

The relative percentage of α_{S1}-CN genetic variants to total casein was evaluated by scanning Coomassie Blue stained gel patterns with a laser densitometer Ultroscan XL (Amersham Biosciences AB, Uppsala, Sweden).

2.2.3. Ultra-thin layer isoelectric focusing on polyacrylamide gel

UTLIEF was performed on a 2117 Multiphor II Apparatus (LKB, Bromma, Sweeden) at 10 °C using a Multitemp II (LKB, Bromma, Sweeden). Homemade polyacrylamide gel (265 × 125 × 0.25 mm; 4.5% T and 0.3% C) consisted of 7.2 M urea, 1% (w/v) glycerol and 1% (v/v) Ampholine (GE Healthcare Amersham Bioscience, Buckinghamshire, UK). To obtain the pH gradient 2.5–6.5 Ampholine 2.5–5, 4.5–5.4 and 4–6.5 in the ratio 1.6:1.4:1 (by vol) were mixed. TEMED and PER 0.04% (v/v) and 0.07% (w/v) final concentrations, respectively, were added to the gel, as activator and cathalyst agents of polymerization. UTLIEF analysis consisted of three steps: pre-focusing set, 2000 V, 15 mA, 4 W, 30 min; sample focusing set, 2000 V, 15 mA, 4 W, 60 min; final focusing set, 3000 V, 5 mA, 20 W, 130 min. The gel was stained with Coomassie Brilliant Blue G-250 as described by Neuhoff, Arold, Taube, & Ehrhardt, 1988.

2.2.4. Two-dimensional electrophoresis

The two-dimensional electrophoresis (2-DE, urea-PAGE versus UTLIEF) procedure was achieved by combining the first dimension urea-PAGE with the second dimension UTLIEF: a strip of unstained gel, excised along the direction of the first run, was equilibrated in 10 mL 9 M urea containing 100 μL 2-mercaptoethanol for 15 min, rinsed twice with distilled water, then laid in the acid region of the prefocused gel (see section 2.2.2). The proteins transfer from urea-PAGE onto UTLIEF gel was due to applied voltage (see section 2.2.3). The 2-DE map was stained with Coomassie Brilliant Blue G-250 as described by Neuhoff et al., 1988.

2.3. Immunoblotting

The casein fractions separated either by UTLIEF analysis transferred by capillary diffusion from the gel onto a nitrocellulose membrane (0.45 μm, Trans-Blot, Bio-Rad, Richmond, CA, USA) and stained with antisera preparations raised against synthetic peptides (as antigen) reproducing C-terminal sequence of α_{S1}-CN produced by Primm (Milano, Italy).

2.4. In-gel tryptic digestion

The in-gel tryptic digestion was performed following the procedure described by Mamone et al. (2003). Essentially, the 2-DE spots, Coomassie Blue G-250 stained, belonging to α_{S1}-CN E were carefully excised (to avoid gel contamination with foreign proteins), transferred in an Eppendorf tube and twice rinsed with Milli-Q water. The gel pieces, were dipped into a solution NH_4HCO_3 50 mM in 50% (v/v) aqueous acetonitrile up to destaining. After dehydrating by submersion into acetonitrile and drying under vacuum to remove acetonitrile, the dried pieces were covered by 0.4% NH_4HCO_3 (~5 μL mm^{-3}) containing 12 ng μL^{-1} trypsin (Roche, Mannheim Germany) maintained in ice-cold tube. After 45 min, the supernatant containing the solution of extracted proteins was removed and incubated for 16 h at 37 °C. The enzymatic reaction was stopped adding 2 μL 4 N trifluoroacetic acid (TFA), then frozen at −20 °C and lyophilised.

2.5. Alkaline phosphatase hydrolysis

Alkaline phosphatase (Roche) hydrolysis was carried out on the 2-DE spots in situ before flow-injection-analysis-electrospray-ionisation-quadrupole-time-of-flight-mass spectrometry (FIA-ESI-Q-TOF-MS) analysis. The dried pieces were covered by 0.4% NH_4HCO_3 (~5 μL mm^{-3}) containing alkaline phosphatase. The reaction was conducted at pH 8.5, 37 °C, for 4 and 16 h, at a substrate: enzyme ratio of 50:1 (w/w). The enzymatic reaction was stopped adding 2 μL 4 N TFA, then frozen at −20 °C and lyophilised.

2.6. HPLC separation of α_{S1}-casein

Pure α_{S1}-CN fractions were isolated by RP-HPLC using an HP 1100 Agilent Technology modular system (Palo Alto, CA, USA) equipped with a Vydac (Hesperia, CA, USA) C_4 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. Native and dephosphorylated whole caseins were dissolved in 1 mL 10 mM dithiothreitol (DTT); for each injection, 100 μL of the resulting solution was used. 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 flow injection method either directly or after concentration under vacuum. Alternatively, the samples were lyophilised prior to enzymatic digestion.

2.7. Liquid chromatography electrospray ionization mass spectrometry

Liquid chromatography electrospray ionisation mass spectrometry (LC-ESI-MS) analysis of casein tryptic digests were performed by using the same instrument as that used for RP-HPLC separation of intact proteins. A Vydac C18 218TP52 column (5 μm, 250–2.1 mm) was utilised 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 inter-scan 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.

2.8. Analysis of protein gel bands by nano-ESI/MS and of derived tryptic peptides by MS/MS

Nano-ESI-MS/MS of proteins were performed with a Q-TOF ULTIMA mass spectrometer (Waters Corporation) equipped with a nano-electrospray source. Samples were desalted using ZipTip C4 microcolumns (Millipore, Bedford, MA, USA) and sprayed from coated silica PicoTip EMITTER capillaries (New Objective, USA). The capillary voltage used was 800 V. For the peptides, the dephosphorylated tryptic samples were desalted using a Zip-Tip™ C18 microcolumn (Millipore), the doubly charged ion isotopic clusters selected by the quadrupole mass filter (MS1) and fragmentation induced by collision. The collision energy was 30 V. The tryptic peptides collision-induced dissociation spectra were deconvoluted using MaxEnt III and interpreted using Peptide Sequencing both from MassLynx 4.0 software (Waters Corporation).

2.9. 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. 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. 2DE-ESI/MS analysis

The ovine milk sample containing the α_{S1}-CN CE phenotype was previously determined by urea-PAGE pH 8.6 and UTLIEF analysis followed by classic Coomassie Blue staining (BCS) and specific anti-α_{S1}-CN immunoblotting (Chianese et al., 1996). The results indicated a lower net negative charge and a higher pI value of α_{S1}-CN E compared with reference α_{S1}-CN C. To isolate the α_{S1}-CN E components, the α_{S1}-CN CE sample was submitted to 2-DE analysis (urea-PAGE pH 8.6 versus UTLIEF). The casein fractions, first

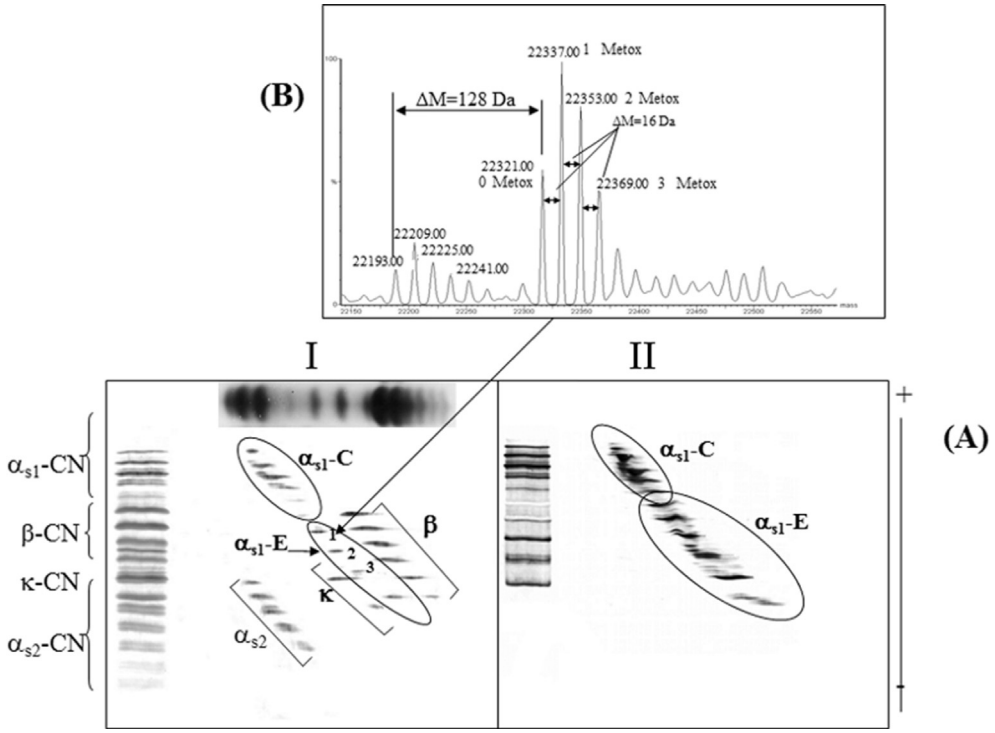

Fig. 1. Two-dimensional electrophoresis (2-DE) analysis of whole ovine casein sample containing the α_{S1}-CN CE variant (A). Coomassie brilliant blue staining (I) and immunoblotting with polyclonal antibodies against α_{S1}-CN (II). Electrospray-ionisation-quadrupole-time-of-flight-mass spectrometry (ESI-Q-TOF-MS) analysis of the intact protein extracted from spot 1 (B).

Table 1
Identification of tryptic peptides from spots 1, 2 and 3 by ESI-qTOF MS-MS analysis.

Spot	Molecular mass (Da)		Peptide sequence	Peptide
	Measured	Expected		
1	2575.6	2576.0	(-)RPKHPIKHQGLSPEVLNENLLR(F)	1–22
	1306	1306.7	(R)FVVAPFPEVFR(K)	23–33
	1434.9	1435.8	(R)FVVAPFPEVFRK(E)	23–34
	2391.9	2392.1	(K)AGSSSSSEQKYIQKEDVPSER(Y)	62–90 -(70–77)1P
	2471.9	2472.1	(K)AGSSSSSEQKYIQKEDVPSER(Y)	62–90 -(70–77)2P
	1266.5	1266.7	(R)YLGYLEQLLR(L)	91–100
	1298.9	1298.7	(K)YNVPQLEIVPK(S)	104–114
	3189.2	3189.6	(K)EGNPAHQKQPMIAVNQELAYFYPQLFR(Q)	125–151
	759.3	759.4	(K)ITMPLW (-)	194–199
2	2575.6	2576.0	(-)RPKHPIKHQGLSPEVLNENLLR(F)	1–22
	1306	1306.7	(R)FVVAPFPEVFR(K)	23–33
	1434.9	1435.8	(R)FVVAPFPEVFRK(E)	23–34
	2362.4	2363.3	(R)FVVAPFPEVFRKENINELSK(D)	23–42
	2391.9	2392.1	(K)AGSSSSSEQKYIQKEDVPSER(Y)	62–90 -(70–77)1P
	1266.5	1266.7	(R)YLGYLEQLLR(L)	91–100
	1298.9	1298.7	(K)YNVPQLEIVPK(S)	104–114
	1668.8	1669.0	(R)LKKYNVPQLEIVPK(S)	101–114
	1158.8	1159.5	(K)SAEEQLHSMK(E)	115–124
	3189.2	3189.6	(K)EGNPAHQKQPMIAVNQELAYFYPQLFR(Q)	125–151
	759.3	759.4	(K)ITMPLW (-)	194–199
3	2311.5	2312.4	AGSSSSSEQKYIQKEDVPSER	62–90 -(70–77)
	2391.9	2392.1	(K)AGSSSSSEQKYIQKEDVPSER(Y)	62–90 -(70–77)1P
	1266.5	1266.7	(R)YLGYLEQLLR(L)	91–100
	1298.9	1298.7	(K)YNVPQLEIVPK(S)	104–114
	2343.6	2344.2	(K)QPMIAVNQELAYFYPQLFR(Q)	133–151

separated by urea-PAGE at alkaline pH analysis were orthogonally focused in four well separated zones of the 2-DE map (Fig. 1A). After BCS, the heterogeneity of each casein family, due to different degrees of phosphorylation (α_{S1}- α_{S2}-, β-CN) or glycosylation (κ-CN), and the presence of non-allelic protein forms, were well visualised in the 2-DE map (Fig. 1A, panel I).

The immunostaining with polyclonal antibodies against α_{S1}-CN (Fig. 1A, panel II) showed almost five components for each α_{S1}-CN

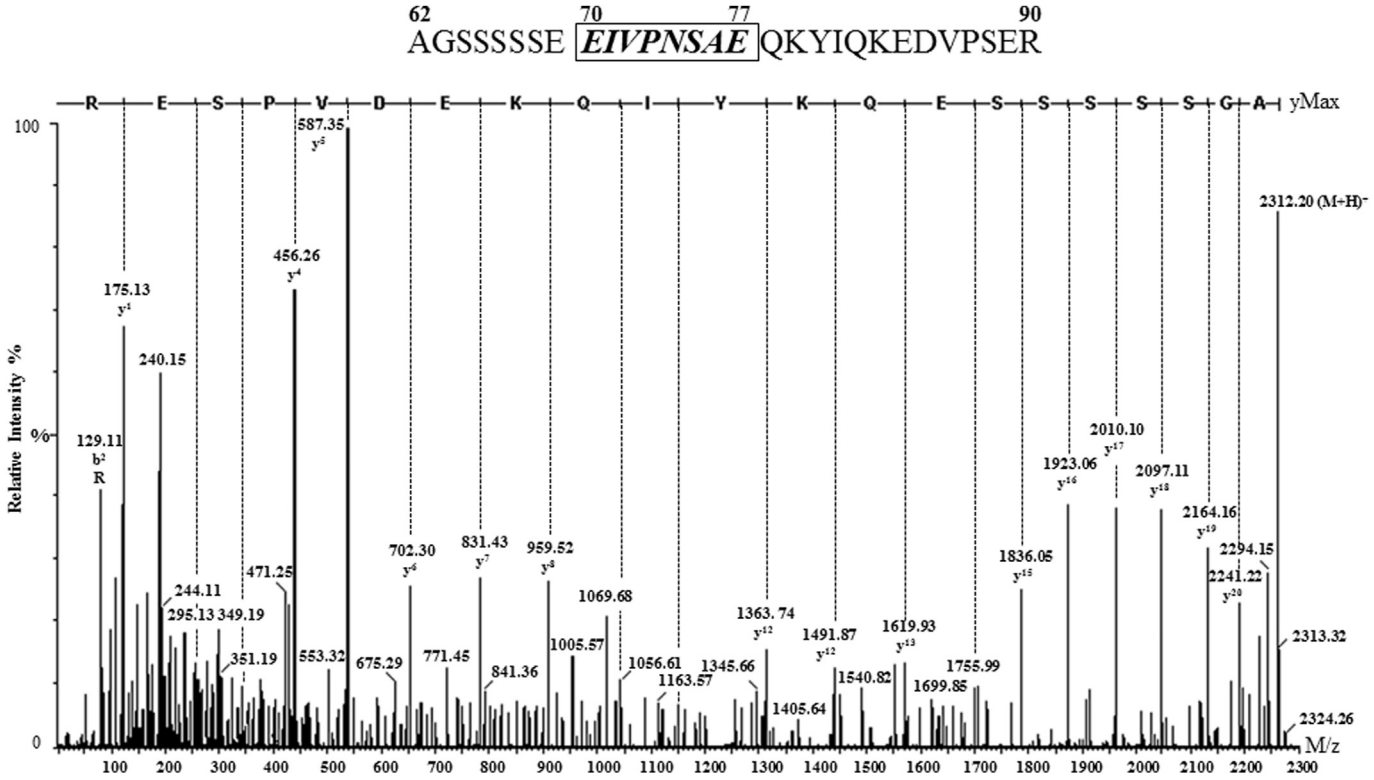

Fig. 2. Deconvoluted mass spectrum of MS/MS analysis of peptide at 2312.4 Da elucidating the amino acid sequence of α_{S1}-CN (f62–90)-(70–77) deleted peptide.

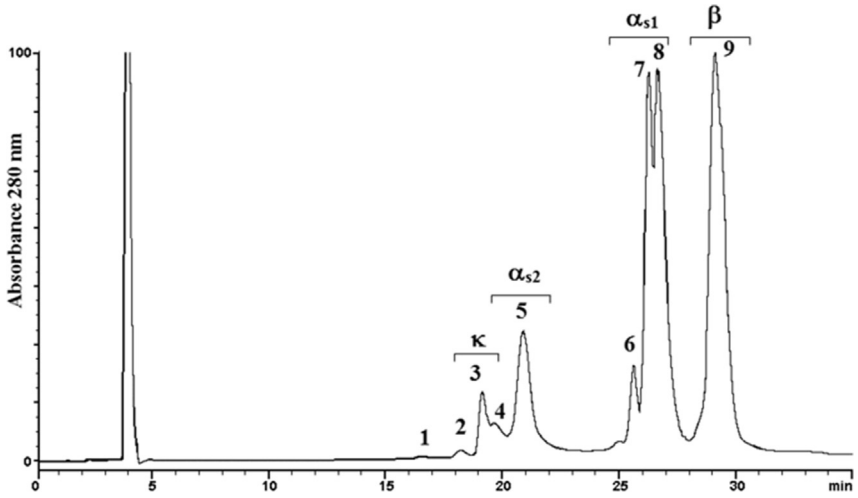

Fig. 3. RP-HPLC of whole individual casein sample containing α_{S1}-CN CE after alkaline dephosphorylation.

genetic variant and was very helpful to accurately cut the α_{S1}-CN E gel spots. The intact proteins extracted from spots 1, 2 and 3 (Fig. 1A, panel I) were analysed by ESI/MS. The deconvoluted mass spectrum of spot 1 (Fig. 1B) showed two components, one measuring 22193.0 and the other measuring 22321.0 Da. The molecular mass difference, accounting for 128 Da, allowed us to identify the smaller component as the non-allelic form of α_{S1}-CN E lacking Gln^{78} and the greater one as the main component (α_{S1}-CN E). Moreover, almost three components having 1, 2 and 3 metox (oxidation methionine) are shown in Fig. 1B. The same analysis on proteins extracted from both spots 2 and 3 gave the same qualitative profile (results not shown) except for the molecular mass values decreasing by 80 Da each. Finally, the three spots contained the same protein with a decreasing number of phosphate groups.

Tryptic digests analysis of native protein, extracted from spot 1, 2 and 3, were performed by nanoLC/ESI-MS-MS and the identified components reported in Table 1. In the tryptic digests of spot 1, two peptides differing by other by 80 Da (i.e., 2472.1 Da and 2392.1 Da, respectively), replaced the theoretical trypsin missed cleavage peptide α_{S1}-CN(f62-90)6P of 3631.3 Da.

The same analysis on peptide digest extracted from spots 2 and 3 confirmed the above results, but with 1 and 0 phosphate groups (Table 1). Finally, tandem MS analysis of the dephosphorylated tryptic peptide at 2312.4 Da, replacing the α_{S1}-CN(f62-90), provided information about the internal deletion of (f70–77) amino acid residues, as shown in Fig. 2, whose mass of 840 Da matched with the mass difference value between α_{S1}-CN E and C, as reported in preliminary results (Chianese et al., 1996, 2008).

The loss of four phosphate groups (located at sites 64, 66, 68 and 75 sites) and the lower content of α_{S1}-CN E, determined by densitometric analysis (19% versus 30% for α_{S1}-CN E and C, respectively) could be negatively related both to cheese-making aptitude of milk and mineral carrier activity (Pirisi et al., 1999).

3.2. HPLC/ESI/MS analysis

The RP-HPLC profile of α_{S1}-CN CE after alkaline dephosphorylation (Fig. 3) showed nine chromatographic peaks, with peaks 6, 7, 8 belonging to the α_{S1}-CN family. The components of each manually collected peak were analysed by ESI-qTOF/MS, and the deconvoluted mass spectra is shown in Fig. 4. The molecular masses of the casein components in each peak were identified by matching the measured mass value with the theoretical value calculated from the ovine α_{S1}-CN C amino acid sequence present in the UniProt Database (accession number P04653); the relative results were reported in Table 2. The ΔM values calculated on the basis of different molecular weight before and after alkaline phosphatase action (Table 2) led us to identify the α_{S1}-CN as having 2–6 phosphorylation degrees for peaks 6 and 7 and, as expected, with 8–10 phosphorylation degrees for peak 8; the same was found for its main non-allelic form 198aa (-Gln^{78}). Moreover, the three calculated mass values were in increasing order of 6 < 7 < 8. As a minor eluting peak, peak 6 contained the α_{S1}-CN E-deleted non-allelic form lacking the α_{S1}-CN(f141–148) sequence (1011 Da). The complete sequence of the two variants of α_{S1}-CN, i.e., the deleted (α_{S1}-CN E, peak 7) and the reference (α_{S1}-CN C, peak 8) variants, was achieved by submitting each collected RP-HPLC peak to alkaline phosphatase processing and trypsin hydrolysis, followed by LC-ESI/MS analysis of the resulting peptide mixture (see Supplementary material Fig. S1 and Table S1). By comparing the UV-HPLC profiles, all assessed molecular masses were similar except for peak 3 (Fig. S1, panel b). Here, the molecular mass of 2312.4 Da in the α_{S1}-CN E profile α_{S1}-CN(f62–90)-(70–77) replaced the corresponding peptides at 1805.8 Da, with α_{S1}-CN(f62–79) and 1363.5 α_{S1}-CN(f80–90) belonging to the α_{S1}-CN C counterpart, thus confirming the above result.

The isolation of single α_{S1}-CN E components at different phosphorylated rates by 2DE analysis reduced problems regarding the

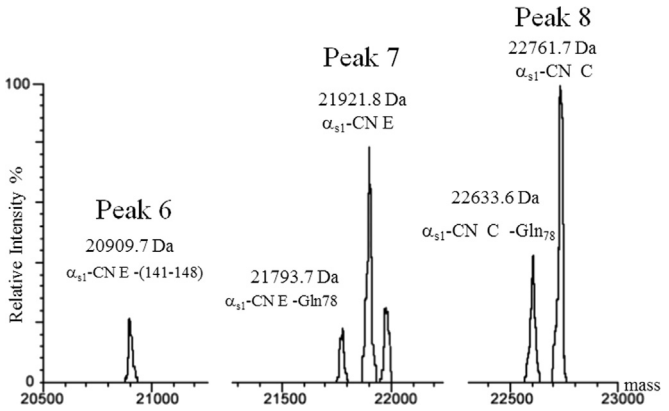

Fig. 4. Deconvoluted spectra of identified dephosphorylated components in HPLC peaks 6, 7, 8 shown in Fig. 3.

Table 2
ESI-qTOF/MS analysis and identification of manually collected α_{S1}-CN CE variant ovine casein HPLC peak, before and after alkaline phosphatase (AP) action.

Peak	Molecular mass (Da)				Protein	ΔM	Number of phosphate group
	Before AP		After AP				
	Measured	Theoretical	Measured	Theoretical			
6	21068.4	21069.6	20908.2	20909.7	α_{S1}-CN E -(141–148)	160	2P
	21148.2	21149.6				240	3P
	21228.4	21229.7				320	4P
	21309.4	21309.6				400	5P
	21392.1	21389.6				480	6P
7	22080.2	22081.8	21921.6	21921.8	α_{S1}-CN E	160	2P
	22160.2	22161.8				240	3P
	22234.0	22241.7				320	4P
	22319.7	22321.7				400	5P
	22395.2	22401.7				480	6P
	22112.0	22113.6	21793.7	21793.7	α_{S1}-CN E -Gln^{78}	320	4P
8	23400.0	23401.6	22761.5	22761.7	α_{S1}-CN C	640	8P
	23480.0	23481.5				720	9P
	23559.8	23561.5				800	10P
	23272.9	23273.4	22633.5	22633.6	α_{S1}-CN C -(Gln^{78})	640	8P
	23351.5	23353.4				720	9P

poor ionisation capability of ESI-MS/MS in positive mode analysis of minor phosphorylated components belonging to the same protein. At the same time, there are many problems concerning either the elution of the intact protein from a gel or the determination of the exact molecular mass due to the prevalence of an oxidative phenomenon occurring during electrophoresis analysis (Mamone et al., 2013).

It must be highlighted that the higher effectiveness and capability of HPLC separation technique coupled with mass spectrometry analysis in molecular characterisation of the amino acid sequence of α_{S1}-CN casein variants also would apply to analysis of the co-elution phenomena. Moreover, the 2-DE proteomic approach could be effective in resolving doubtful variants attributed with the immunostaining tools.

4. Conclusions

In this study the potential of proteomics has been highlighted to describe milk quality on molecular terms, and the quality concept has been illustrated considering the underphosphorylated and fully phosphorylated α_{S1}-CN E and C variants. The internally deleted α_{S1}-CN E variant has both the lowest number of phosphorylated residues and chain length and the shorter reference compared to α_{S1}-CN C. The loss of four phosphate groups, and the lower content of α_{S1}-CN E could produce a detrimental effect on the cheese-making aptitude of milk and mineral carrier activity.

Although the 2-D electrophoresis could be effective in resolving doubtful variants attributed with the immunostaining tools, in comparison with HPLC analysis it is too time consuming and depends on operator skill.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2018.09.007.

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