Mass spectrometry-based procedure for the identification of ovine casein heterogeneity

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Summary. The efficiency of reversed-phase HPLC, capillary electrophoresis (CE), PAGE and isoelectric focusing with immunoblotting in separating ovine caseins has been evaluated. The assessment was carried out by employing electrospray ionization–mass spectrometry (ESI–MS) and matrix-assisted laser desorption ionization–time of flight as reference tools for identifying protein components. Ovine casein was fractionated by HPLC into four major peaks. With ESI–MS, each peak contained components belonging to only one of the four casein families. On-line liquid chromatography–ESI–MS allowed us to determine each fraction's composition by detecting thirteen α_{s1}-, eleven α_{s2}-, seven β-, and three κ-casein (CN) components. The α_{s1}-CN and α_{s2}-CN consisted of eight and two protein chains respectively of lengths differing through the deletion of one or more peptide sequences; they were also discretely phosphorylated as κ-CN and β-CN. By CE at pH 2·5, each casein fraction was as heterogeneous as that resulting from ESI–MS for the single HPLC-derived fractions. The separation of α_{s1}-CN and α_{s2}-CN proved to be excellent, with the exception of a co-migration of κ_0-CN with a minor α_{s1}-CN component and of a glycosylated κ-CN form with low-phosphorylated α_{s1}-CN and β-CN components. Dephosphorylation of whole casein was used to reduce the heterogeneity of the native fractions and by applying currently used analytical techniques it was possible to visualize the protein moiety difference along the CE profile. CE, HPLC, and immunoblotting were all equally capable of effecting an accurate separation of the four dephosphorylated casein families. The spectra obtained by ESI–MS directly on dephosphorylated whole ovine casein samples contained the signals of the four casein families and the relative α_{s1}-CN variants, the non-allelic α_{s1}-CN and α_{s2}-CN forms, dimeric κ-CN and other newly formed peptides. We suggest using this procedure for rapid characterization of whole casein.

Keywords: Ovine casein, casein heterogeneity, electrospray ionization–mass spectrometry, capillary electrophoresis.

Dairy technologists are frequently faced with variations in milk properties such as clotting time and curd consistency. Both factors are strongly linked to the casein

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genotype, which in turn determines the relative casein level and composition. The allele form of caprine α_{s1}-casein (CN) is directly matched to the casein level: the 'strong' alleles are associated with milk with higher casein levels while the 'low' and 'null' alleles result in decreased levels (Grosclaude *et al.* 1987, 1994). From a technological perspective, casein level is a clear indicator of consistency: lower levels result in softer curd. With regard to casein composition, caprine milks that lack α_{s1}-CN yield cheese with a spreadable consistency and a creamy texture, whereas firmer cheeses are obtained as the level of this casein component increases (Addeo *et al.* 1989; Remeuf, 1993). So far, caprine α_{s1}-CN variants have been shown to differ by one or more amino acid residue substitutions or deletions along the chain (Brignon *et al.* 1989, 1990). The existence of deleted protein species has also been demonstrated for bovine, water buffalo, ovine, and caprine α_{s1}-CN, and in the last two species up to seven forms forms of α_{s1}-CN with deletions have been identified (Ferranti *et al.* 1999). Morover, in ovine α_{s2}-CN the mature protein coexists with a deleted form that lacks sequence 34–42 or 35–43 (Boisnard *et al.* 1991). Further, the complexity of the protein mixture is increased by the co-presence of caseins with a wide range of phosphorylations (Chianese *et al.* 1993; Ferranti *et al.* 1997*a*). The availability of routine methods is essential for rationalizing this heterogeneity in casein from different species. One traditional analytical approach was based on the application of HPLC, capillary electrophoresis (CE) and gel electrophoresis in various forms (Jaubert & Martin, 1992; Cattaneo *et al.* 1996; Chianese *et al.* 1996, 1997*a*, *b*; Recio *et al.* 1997*a*, *b*). Although casein heterogeneity was clearly demonstrated (Chianese *et al.* 1993, 1995), neither the variously sized nor the discretely phosphorylated casein forms were identified by using one dimensional techniques alone. The advantages of two dimensional techniques for describing casein heterogeneity (Addeo *et al.* 1988, 1992; Chianese *et al.* 1992, 1996) by labelling the components using two coordinates are offset by their inability to identify the separated spots, HPLC or CE peaks. The aim of the present study was to evaluate the possibility of using HPLC and CE methods to identify casein heterogeneity by using mass spectrometry (MS) in a second dimension. Since ovine casein has so far received the least attention and therefore awaits both structural and quantitative characterization, we have chosen this casein for a detailed study.

Materials and methods

Materials

Sequence grade bovine alkaline phosphatase (EC 3.1.3.1) from calf intestine was purchased from Boehringer (D-68298 Mannheim 31, Germany). HPLC-grade solvents and reagents were provided by Carlo Erba (I-20195 Milan, Italy). A Vydac C_4 column (214TP52, 5 μm, 250 × 2·1 mm; Hesperia, CA 92345, USA) was used for liquid chromatography (LC)–MS of caseins. Whole ovine casein was prepared by isoelectric precipitation of individual skimmed milks and reduced with 2-mercaptoethanol (10 ml/l). Single casein fractions were prepared from whole casein by reversed-phase HPLC using the procedure of Ferranti *et al.* (1997*a*).

Alkaline phosphatase hydrolysis

Before the dephosphorylation step, casein samples were suspended in water and held at 100 °C for 5 min to inactivate plasmin. Dephosphorylation was carried out at 37 °C and pH 8·5 for 18 h with calf intestine alkaline phosphatase in ammonium bicarbonate (4 g/l) at 1 mU enzyme/mg casein. The reaction was stopped by freeze drying and complete dephosphorylation verified by MS.

Gel electrophoresis and immunoblotting

PAGE at pH 8·6 and immunoblotting with polyclonal antibodies against single casein fractions were carried out according to Chianese *et al.* (1992). The preparation of ultra-thin polyacrylamide gel layers (0·25 mm) and isoelectric focusing (UTLIEF) were carried out using the EC method (Commission Regulation, 1996). The pH gradient (2·5–6·5) was obtained by adding Ampholine 2·5–5·0, 4·5–5·4 and 4·0–6·5 (Pharmacia Biotech AB, S-751 84 Uppsala, Sweden) in the volume ratio 1·6 : 1·4 : 1.

Analysis of casein by liquid chromatography–mass spectrometry

Liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) was performed using an HP1100 modular system on line connected with a Platform single quadrupole mass spectrometer (Micromass, Altrincham, WA14 5RZ, UK). The procedure for separating the caseins was essentially that of Ferranti *et al.* (1995): reversed-phase HPLC on a Vydac C_4 column. Solvent A was 0·3 ml trifluoroacetic acid/l water and solvent B 0·2 ml trifluoroacetic acid/l acetonitrile. Dried sample (100 μg) was dissolved in 20 μl water and injected on to the HPLC column previously equilibrated with solvent A. A linear gradient from 300 to 600 ml B/l was applied over 60 min with a flow rate of 0·5 ml/min. The column effluent was split 1 : 25 to give a flow rate of $\sim$ 4 μl/min into the electrospray nebulizer. The bulk of the flow was run through the detector for peak collection monitored by following the absorbance at 220 nm. The electrospray mass spectra were scanned from 1800 to 400 m/z at a scan cycle of 5 s/scan. The source temperature was 120 °C and the orifice voltage 40 V. Mass values are reported as average masses. Signals recorded in the mass spectra were associated with the corresponding casein fraction on the basis of the molecular mass, taking into account the reported amino acid sequence of the ovine casein fractions. Quantitative analysis of components was made by measuring and averaging the intensity of the multiply charged ions for each component, and normalizing the values to 100 % (Ferranti *et al.* 1997*a*, 1998).

Selected ion monitoring by electrospray ionization–mass spectrometry

For acquisition in selected ion monitoring mode, the multi-charged protein ions ranging from 18 to 25 positive charges were monitored with a residence time of 0·1 s for each ion.

Matrix-assisted laser desorption ionization–time of flight

Using sinapinic acid (Fluka, CH-9471 Buchs, Switzerland) as matrix; the casein sample (1 μl of a 1 g/l solution in water) was loaded on the target and dried. Then 1 μl trifluoroacetic acid (1 ml/l water)–ethyl alcohol–matrix (10 mg/ml water) (1 : 1 : 1 by vol.) was added. The sample was analysed with a Voyager mass spectrometer (PerSeptive Biosystem, Framingham, MA 01701, USA) operating in linear mode.

Capillary zone electrophoresis

CE analysis was performed by using a BioFocus 3000 System (BioRad Laboratories, Hercules, CA 94547, USA), equipped with a pre-assembled BioFocus Capillary Cartridge (360 mm $\times$ 50 μm i.d.; BioRad Laboratories). The experimental procedure, including buffer and sample preparation, capillary conditioning and storage, was essentially that of De Jong *et al.* (1993). Briefly, samples were dissolved in a reducing buffer (whole casein at 10 g/l and HPLC-purified fractions at 2 g/l in

5 mM-trisodium citrate buffer–5 mM-dithiothreitol–6 M-urea, pH 8·0), incubated at 37 °C for 1 h, filtered (0·22 μm filter, Millipore, Bedford, MA 01730, USA) and injected at the anode using nitrogen at 69 kPa for 1 s. Electromigration was carried out at 40 °C in 0·5 M-citric acid–10 mM-trisodium citrate–6 M-urea, pH 2·5 containing 0·5 g hydroxypropylmethyl-cellulose/l by applying a constant voltage of 18 kV and a final current of $\sim$ 40 μA. Detection was at both 220 and 280 nm.

RESULTS

Preparation of reference ovine casein fractions by HPLC separation

HPLC fractionation of an individual ovine casein sample containing α_{s1}-CN A gave four main peaks (Fig. 1), each of which was collected manually and subjected to ESI–MS analysis. The molecular masses of the casein components in each peak were similar to those reported by Ferranti *et al.* (1997*b*). As indicated here, the casein components were identified by matching the measured mass value with the theoretical value calculated from the amino acid sequence (Jollès *et al.* 1974; Richardson & Mercier, 1979) or deduced from the cDNA sequence (Mercier *et al.* 1985; Boisnard *et al.* 1991). Identification obtained by ESI–MS allowed us to establish that elution of the ovine casein fractions on a C_4 column was in the decreasing order β-CN > α_{s1}-CN > α_{s2}-CN > κ-CN, similar to that of the corresponding caprine caseins (Jaubert & Martin, 1992). To monitor the relative elution of the short-chain and long-chain forms of α_{s1}-CN and α_{s2}-CN, the casein sample was subjected to LC–ESI–MS, and the total ion current profile (results not shown) was similar to that in Fig. 1. Table 1 gives the measured and expected values of the HPLC casein components, tentatively identified by comparison with their molecular masses. This procedure allowed us to deduce the presence of thirteen α_{s1}-CN, eleven α_{s2}-CN, seven β-CN and three κ-CN components. The casein fractions' high heterogeneity was consistent with that detected by ESI–MS analysis on the single HPLC peaks and that apparent from the two dimensional gel map staining with polyclonal antibodies specific to the single casein fractions. Only κ-CN appeared more heterogeneous in the two dimensional profile (Chianese *et al.* 1992).

The β-CN comprised seven forms, ranging from zero to six phosphate groups/molecule in proportions calculated from the relative intensity of the multicharged ions (Fig. 2). β-CN 6P was the most abundant form, followed by β-CN 5P and then by less phosphorylated species (Fig. 2, inset). The non-allelic forms of α_{s2}-CN (Boisnard *et al.* 1991) and α_{s1}-CN (Ferranti *et al.* 1995) were localized in the first part of the respective HPLC peaks, and both fractions contained a wide range of discretely phosphorylated forms. This heterogeneity, correlated with the presence of differently phosphorylated forms, was eliminated by dephosphorylation. Individually dried HPLC peaks or whole casein were treated in the appropriate buffer with alkaline phosphatase preparation and then evaluated by comparing native and dephosphorylated casein mobility by PAGE, UTLIEF, ESI–MS and CE.

Polyacrylamide gel electrophoresis, isoelectric focusing and immunoblotting of native and dephosphorylated ovine caseins

The PAGE and UTLIEF patterns of native and fully dephosphorylated caseins stained with Coomassie R-250 brilliant blue are shown in Fig. 3(*a*) and (*d*) respectively. Since mobilities were assumed to be quite different for a casein fraction and its dephosphorylated counterpart, further tests were performed to localize the dephosphorylated caseins by polyclonal antibodies raised against native casein

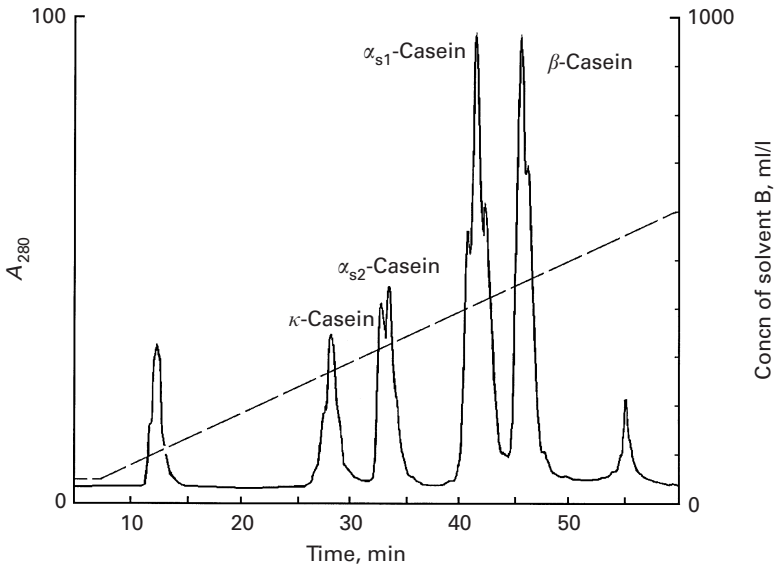

Fig. 1. Reversed-phase HPLC separation (Vydac C_4 column, 100 μg protein; for experimental details, see text) of a sample of whole ovine casein containing α_{s1}-casein A. — — —, Acetonitrile gradient.

fractions. Dephosphorylated α_{s1}-CN A, C and D variants were actually stained as well, revealing lower mobilities than their native counterparts owing to reduced negative charges resulting from the loss of phosphate groups (Fig. 3*b*). Dephosphorylated α_{s1}-CN still appeared heterogeneous, irrespective of the variant. The multiple bands, constituting each dephosphorylated α_{s1}-CN variant, gave the same pattern of migration, since the three variants A, C and D differed only by uncharged amino acid residues (Ferranti *et al.* 1995) and this did not affect electrophoretic mobility. However, if we consider the deleted forms occurring in each sample (Ferranti *et al.* 1998), the full-length native α_{s1}-CN should have a greater negative charge than the corresponding non-allelic form lacking peptide 141–148 (Ferranti *et al.* 1995). As shown by PAGE at alkaline pH, the long form led and the short form followed, owing to the difference in negative charges (Chianese *et al.* 1996). After dephosphorylation, given the persistent charge difference between these two forms, the pair migrated alternately towards the anode irrespective of the variant considered (Fig. 3*b*, lanes 2, 4, and 6). Following immunoblotting (Fig. 3*c*), there were two β-CN with similar phosphorylation patterns (lanes 1 and 5) and a third distinguished by a higher number of protein species (lane 3). The three β-CN had similar mobilities after dephosphorylation (lanes 2, 4 and 6), suggesting the presence of a common chain with varying numbers of phosphorylated groups. The native α_{s2}-CN fraction (Fig. 3*e*) gave a profile with a different heterogeneity (lane 3), implying the presence of a novel variant (lane 3). The dephosphorylated sample had one main component and some secondary components in a narrower pH range together with a pattern similar to those of the other casein fractions, most probably indicating a common protein structure, as discussed below. Dephosphorylated κ-CN samples (Fig. 3*f*) also gave complex patterns like that of the native protein even though three novel components (arrowed in the Figure) were formed at higher pI. Since sugar moieties covalently bound to κ-CN were unaffected by dephosphorylation, heterogeneity due to charged oligosaccharide components persisted along the profiles. Thus dephosphorylation simplified the patterns of the casein fractions, with

Table 1. *Identification of the casein components occurring along an on-line liquid chromatography–electrospray ionization mass spectrometry profile from a native whole ovine casein sample containing α_{s1}-casein A*

	Molecular mass, Da		
HPLC peak	Measured	Predicted	Identification
1	19438	19442	κ-CN(1–171)3P
	19362	19362	κ-CN(1–171)2P
	19285	19282	κ-CN(1–171)1P
2	25702	25702	α_{s2}-CN(1–207)13P
	25622	25622	α_{s2}-CN(1–207)12P
	25542	25542	α_{s2}-CN(1–207)11P
	25462	25462	α_{s2}-CN(1–207)10P
	25382	25382	α_{s2}-CN(1–207)9P
	24706	24706	α_{s2}-CN(1–198)13P
	24626	24626	α_{s2}-CN(1–198)12P
	24546	24546	α_{s2}-CN(1–198)11P
	24467	24466	α_{s2}-CN(1–198)10P
	24387	24386	α_{s2}-CN(1–198)9P
	24307	24306	α_{s2}-CN(1–198)8P
3	23631	23631	α_{s1}-CN(1–199)11P
	23551	23551	α_{s1}-CN(1–199)10P
	23471	23471	α_{s1}-CN(1–199)9P
	23381	23381	α_{s1}-CN(1–199)8P
	23501	23503	α_{s1}-CN(1–198)11P
	23421	23423	α_{s1}-CN(1–198)10P
	23340	23343	α_{s1}-CN(1–198)9P
	22613	22614	α_{s1}-CN(1–191)11P
	22533	22534	α_{s1}-CN(1–191)10P
	22454	22454	α_{s1}-CN(1–191)9P
	22481	22481	α_{s1}-CN(1–190)8P
	22404	22401	α_{s1}-CN(1–190)7P
	22320	22321	α_{s1}-CN(1–190)6P
4	23831	23831	β-CN(1–207)6P
	23751	23751	β-CN(1–207)5P
	23670	23671	β-CN(1–207)4P
	23590	23591	β-CN(1–207)3P
	23511	23511	β-CN(1–207)2P
	23431	23431	β-CN(1–207)1P
	23351	23351	β-CN(1–207)0P

the exception of κ-CN, which gave a higher number of components in the dephosphorylated than in the native form. Thus, dephosphorylation increased the pI of homologous native fractions and made it possible to detect casein variants, which differed by charged amino acid residues, in a simplified electrophoretic pattern.

Analysis of the dephosphorylated single HPLC fractions by mass spectrometry

The molecular masses of the dephosphorylated casein fractions measured by ESI–MS had lower values than the corresponding native caseins. There were several dephosphorylated forms: four α_{s1}-CN (α_{s1}-CN(1–199), 22 751 Da, α_{s1}-CN(1–198), 22643 Da; α_{s1}-CN(1–191), 21734 Da and α_{s1}-CN(1–190), 21607 Da), two α_{s2}-CN (α_{s2}-CN(1–207), 24662 Da and α_{s2}-CN(1–198), 23 666 Da) and one each of β-CN (23351 Da) and κ-CN (19207 Da), the multiplicity being attributable to the varying primary protein structures. The molecular masses of the dephosphorylated caseins shifted by a multiple of -80 Da, an m/z of 80 indicating a phosphate group. The molecular mass difference between a native and the corresponding dephosphorylated fraction was then calculated and when divided by 80 gave the number of phosphate

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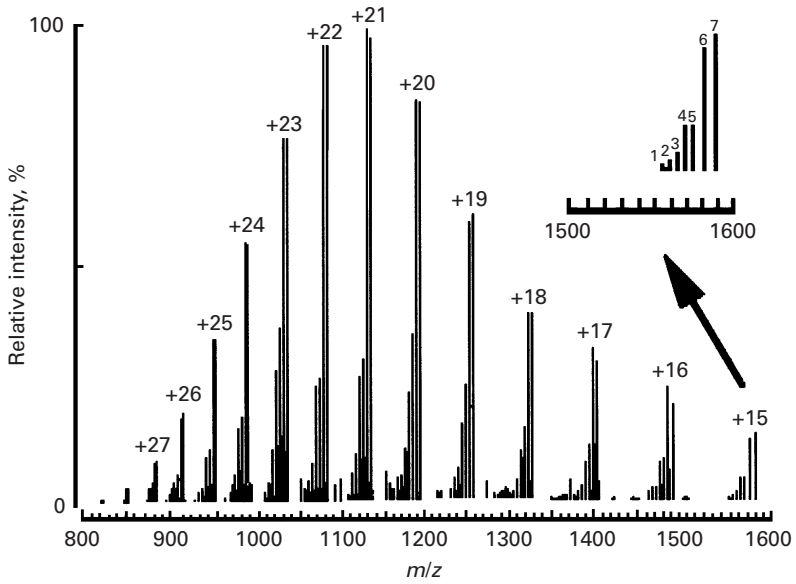

Fig. 2. Electrospray mass spectrum of ovine β-casein showing an ion range of 15–27 positive charges per molecule. Each bar within a cluster corresponds to a protein species. In the inset, the cluster at a charge of +15 is shown and the seven components detected are marked with consecutive numbers. Components 1–7 were β-casein containing zero to six phosphate groups per molecule. The measured molecular masses in Da of each component are given in Table 1.

groups/molecule. These results confirmed the identity of the components reported in Table 1. However, only three of the six deleted α_{s1}-CN forms identified by Ferranti *et al.* (1998) were detected. Like ESI–MS, matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) was effective in identifying HPLC-purified dephosphorylated casein fractions, though it was unable to detect the short α_{s1}-CN forms (results not shown). In order to assign an identity accurately to the protein species, including non-allelic forms, our approach based on the analytical method of on-line LC–ESI–MS encompassed the following three procedures. Firstly, dephosphorylated components in a whole casein sample are separated, then the molecular masses of the single components are determined, and finally protein components are distinguished through different retention times.

On-line liquid chromatography–electrospray ionization–mass spectrometry procedure for separation and identification of the dephosphorylated whole casein components

Analysis of dephosphorylated whole casein by LC–ESI–MS revealed four groups of peaks, irrespective of the casein sample. Fig. 4 shows the total ion current profile of an α_{s1}-CN A sample containing whole dephosphorylated casein. The molecular masses of κ-CN, α_{s2}-CN, α_{s1}-CN and β-CN are given in Table 2 and the components were identified on the basis of their known sequences. In particular, two and eight components were labelled as α_{s2}-CN and α_{s1}-CN respectively and were readily identified from earlier results (Ferranti *et al.* 1997*b*) as the full-length caseins and the corresponding deleted form(s). In contrast to dephosphorylated single HPLC fractions characterized by ESI–MS as a whole, on-line analysis of the column effluent by LC–ES–MS resulted in the detection of four additional deleted α_{s1}-CN forms, giving a total of eight known α_{s1}-CN species. The retention times of short and long α_{s1}-CN species were also compared. The long forms of the dephosphorylated caseins

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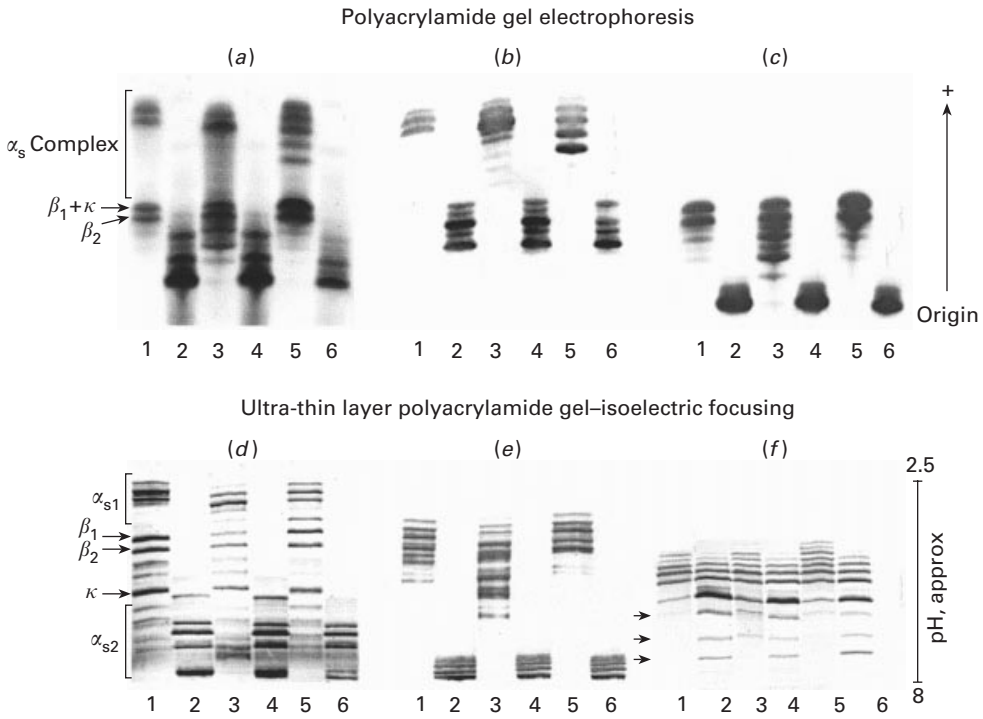

Fig. 3. Polyacrylamide gel electrophoresis PAGE at pH 8·6 and ultra-thin layer polyacrylamide gel–isoelectric focusing in the pH range 2·5–8·0 of native whole ovine casein samples containing α_{s1}-casein variants A, C and D (lanes 1, 3 and 5 respectively) and the corresponding dephosphorylated forms (lanes 2, 4 and 6 respectively). Staining was carried out with Coomassie R-250 brilliant blue (*a*, *d*), and polyclonal antibodies against (*b*) α_{s1}-casein, (*c*) β-casein, (*e*) α_{s2}-casein and (*f*) κ-casein. α_s Complex, α_{s1}-casein and α_{s2}-casein; α_s, α_{s1}-casein; α_{s2}, α_{s2}-casein; β_1, β_1-casein; β_2, β_2-casein; κ, κ-casein. In (*f*), ▶ indicates newly formed κ-casein bands in dephosphorylated samples.

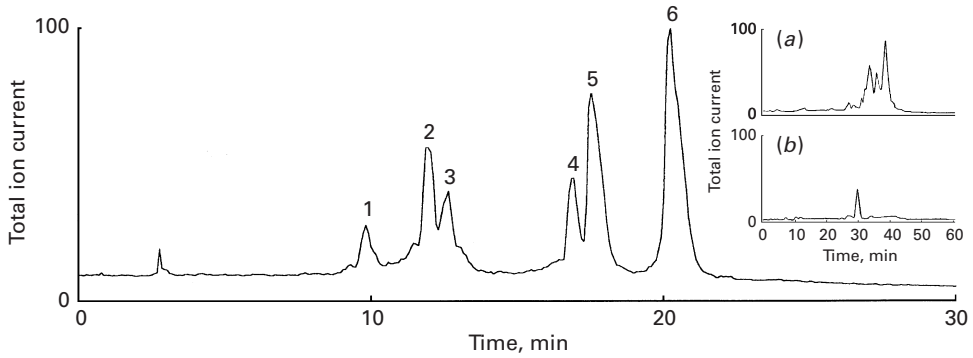

Fig. 4. Liquid chromatography–electrospray ionization mass spectrometry chromatogram of dephosphorylated whole ovine casein containing α_{s1}-casein A. The casein components are identified in Table 2. Inset, total ion current profile of (*a*) dephosphorylated α_{s1}-casein A and (*b*) single ion monitoring of the multicharged ions of the component at 20 864·5 ± 1·2 Da, a non-allelic form of α_{s1}-casein with peptides 110–117 and 141–148 simultaneously deleted.

eluted later than the shorter forms or co-eluted on the basis of their difference in molecular mass and hydrophobicity. Although present in only small proportions, some non-allelic α_{s1}-CN forms (Ferranti *et al.* 1998) were readily detected along the total ion trace by single ion monitoring. Whole dephosphorylated α_{s1}-CN fraction

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Table 2. *Liquid chromatography–electrospray mass spectrometry of the dephosphorylated whole ovine casein containing α_{s1}-casein A and identification of the casein components through molecular mass determination*

		Molecular mass, Da		
HPLC	Retention time	Measured	Predicted	Protein identity
1	9·8	38251	38251	κ-CN dimer
2	11·9	23666	23666	α_{s2}-CN short
3	12·6	24662	24662	α_{s2}-CN
4	16·4	20735	20738	α_{s1}-CN *a*
5	16·9	20864	20866	α_{s1}-CN *b*
5	16·9	21609	21611	α_{s1}-CN *c*
5	16·9	21735	21739	α_{s1}-CN *d*
5	17·0	21748	21750	α_{s1}-CN *e*
5	17·0	21878	21878	α_{s1}-CN *f*
5	17·3	22620	22623	α_{s1}-CN *g*
5	17·5	22751	22751	α_{s1}-CN
6	20·2	23351	23351	β-CN

κ-CN dimer, a dimer form of κ-casein; α_{s1}-CN, α_{s2}-CN, full length α_{s1}-casein and α_{s2}-casein; α_{s2}-CN short, α_{s2}-casein with 198 residues; α_{s1}-CN *a*, *b*, *c*, *d*, *e*, *f* and *g*, α_{s1}-casein forms of different lengths with *a*, 182; *b*, 183; *c*, 190; *d*, 191; *e*, 190; *f*, 191; and *g*, 198 residues. Forms *b*, *c*, *d*, *e*, *f* and *g* were previously identified by Ferranti *et al.* (1998); form *a* has residues 141–148, 110–117 and Gln^{78} simultaneously deleted.

was analysed by LC–ESI–MS and the total ion current profile shows the main components (Fig. 4, inset a). Single ion monitoring was used to detect limited amounts of non-allelic forms, such as the α_{s1}-CN component with peptides 110–117 and 141–148 simultaneously deleted. It was easily measured by specifically monitoring the diagnostic multicharged ions (Fig. 4, inset *b*), and represented 3·6 % of the total α_{s1}-CN (Ferranti *et al.* 1998). Consequently, a significant improvement of signal-to-noise ratio was obtained and this led to an improved detection threshold.

The measured molecular masses (cf. Tables 1 and 2) enabled us to confirm the identities of the casein species in native samples and then to determine their degree of phosphorylation. For example, the 199 residue α_{s1}-CN in Table 2 (dephosphorylated molecular mass, 22 751 Da) was formed from four components with 8, 9, 10 and 11 phosphates/molecule. The other seven non-allelic α_{s1}-CN forms each had components with 9, 10 and 11 phosphates/molecule. The α_{s1}-CN forms with 198 and 191 residues (molecular masses 22 634 and 21 878 Da respectively) differed from the mature form by deletion of the Gln^{78} residue (Ferranti *et al.* 1998) and peptide 141–148 (Ferranti *et al.* 1995) respectively. A novel non-allelic form consisting of 182 amino acid residues with Gln^{78} and simultaneous deletion of peptides 110–117 and 141–148 also was identified in the sample.

Dephosphorylated α_{s2}-CN contained two components of masses 24 662 and 23 666 Da, both consistent with the values calculated from the cDNA precursor sequenced by Boisnard *et al.* (1991). These two coexisting α_{s2}-CN forms differed through the deletion of a peptide of nine amino acids, 34–42 or 35–43 (Boisnard *et al.* 1991). Thus, in view of the results in Tables 1 and 2, one could consider the native long and short α_{s2}-CN forms as containing 9–13 and 8–13 phosphorylated species respectively, making a total of eleven α_{s2}-CN components. The short and the long forms of α_{s2}-CN were in fact detected by UTLIEF because of the difference of three charged amino acid residues: two Glu and one Arg (Fig. 3*e*).

Dephosphorylated β-CN and κ-CN were each composed of a single protein chain as against the seven and three native components, with phosphate contents varying

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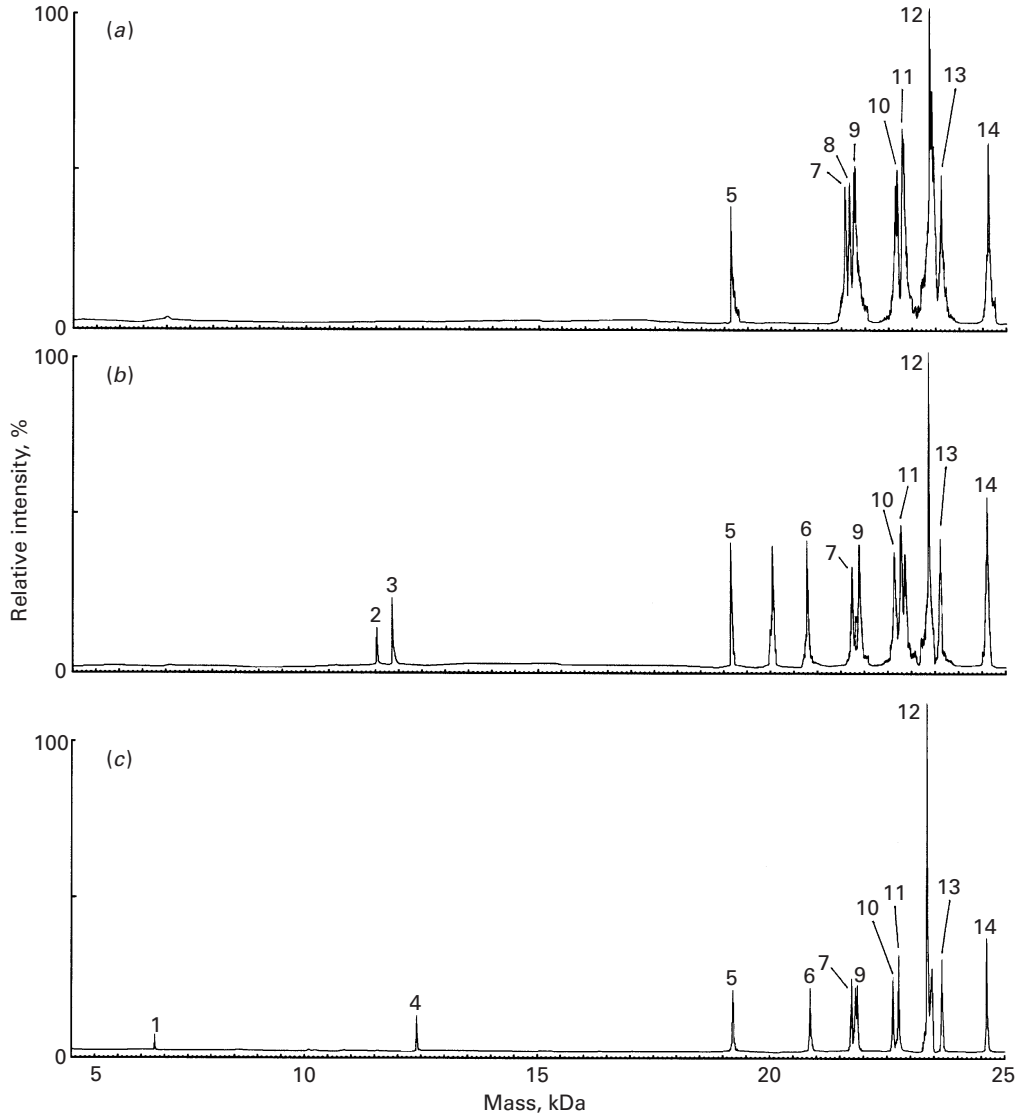

Fig. 5. Deconvoluted electrospray mass spectra of dephosphorylated individual whole ovine casein samples containing (*a*) α_{s1}-casein D, (*b*) α_{s1}-casein C and (*c*) α_{s1}-casein A. The casein components are identified in Table 3. The part of the spectrum relating to dimeric κ-casein is not shown. Unnumbered peaks are unknown.

from zero to six and one to three respectively. Both the monomer and dimer forms of κ-CN were detected in casein samples. In the native samples, the monomer predominated. When a reducing agent (2-mercaptoethanol) was added, the dimer was completely converted into the monomer as a result of the reduction of the disulphide bridge. The dephosphorylation step led to an increase in dimeric species, arising from the possible partial oxidation of the thiol groups during incubation at alkaline pH. In order to detect the presence of native dimer κ-casein, bulk milk was brought to acid pH (different from 4·6) to recover whey containing soluble casein(s). Analysis by LC-ESI-MS of a whey sample at pH 4·9 allowed us to detect only κ-casein together with the whey proteins. κ-Casein consisted of monomer (19447 ± 6;

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Table 3. *Experimental molecular masses compared with predicted values (in parentheses) of the casein fractions in individual samples of dephosphorylated whole ovine casein containing α_{s1}-casein A, C and D obtained by direct electrospray mass spectrometry analysis*

(Values are in Da)

	α_{s1}-Casein variant			
Peak	A	C	D	Identity
1	6796 (6804)	—	—	CMP
2	—	11532 (11534)	—	γ_2-CN
3	—	11832 (11835)	—	β-CN f(1–105)
4	12419 (12419)	—	—	para-κ-CN
5	19203 (19203)	19204 (19203)	19208 (19203)	κ-CN
6	20872 (20866)	20878 (20876)	21914 (21914)	α_{s1}-CN *b*
7	21740 (21739)	21730 (21749)	21774 (21777)	α_{s1}-CN *d*
8	—	—	21792 (21792)	α_{s1}-CN *e*
9	21876 (21878)	21888 (21888)	21914 (21914)	α_{s1}-CN *f*
10	22621 (22623)	22623 (22633)	22666 (22660)	α_{s1}-CN *g*
11	22750 (22751)	22762 (22761)	22788 (22788)	α_{s1}-CN
12	23354 (23351)	23354 (23351)	23351 (23351)	β-CN
13	23605 (23605)	23603 (23605)	23608 (23605)	α_{s2}-CN short
14	24638 (24599)	24595 (24599)	24595 (24599)	α_{s2}-CN
15	38251 (38252)	38249 (38252)	37377 (38252)	κ-CN dimer

CMP, caseinomacropeptide; κ-CN dimer, a dimer form of κ-casein; α_{s1}-CN, α_{s2}-CN, full length α_{s1}-casein and α_{s2}-casein; α_{s2}-CN short, α_{s2}-casein with 198 residues; α_{s1}-CN *b*, *d*, *e*, *f* and *g*, α_{s1}-casein forms of different lengths with *b*, 183; *d*, 191; *e*, 190; *f*, 191; and *g*, 198 residues. Forms *b*, *d*, *e*, *f* and *g* (and form *a* and *c* in Table 2) were previously identified by Ferranti *et al.* (1998).

expected 19442 Da) and a dimer (38890 ± 6; expected 38882 Da) forms in the relative proportion of *ca.* 13 and 87 % respectively. The glycosylated forms of κ-CN detected by immunoblotting were not investigated further. This characterization requires a detailed structural study, which is currently in progress in our laboratory.

Since separation of casein fractions by HPLC and spectral data processing are laborious and time-consuming, a more rapid approach based on direct ESI–MS analysis of the dephosphorylated casein samples was also evaluated.

Direct analysis of dephosphorylated whole ovine casein by ESI–MS

The molecular mass values of the casein components differed sufficiently (Tables 1 and 2) that the signals should in theory be simultaneously detected along an ESI spectrum of whole casein in positive mode. However, assays for a complete ESI spectrum of casein were unsuccessful since only the β-CN signals were significant, suggesting its discrete property of ionizing in a positive mode. By contrast, fully dephosphorylated whole casein gave an ESI spectrum that included the signals of all casein components, regardless of the α_{s1}-CN variant in question. Fig. 5 shows the ESI spectrum of individual dephosphorylated whole casein samples containing α_{s1}-CN A, C, and D. Table 3 gives the mass values of the dephosphorylated protein species, providing a complete set of casein fractions, including α_{s1}-CN variants, non-allelic α_{s1}-CN and α_{s2}-CN forms, and degradation products of κ-CN and β-CN.

Whole dephosphorylated casein was also analysed by MALDI–TOF and this allowed us to recognize α_{s1}-CN, while its deleted forms were under a single unresolved peak with β-CN and γ-CN but not α_{s2}-CN or κ-CN (results not shown). In practice, whole native and dephosphorylated casein samples and the HPLC-derived fractions were characterized by selecting the most suitable approach (MALDI–TOF or

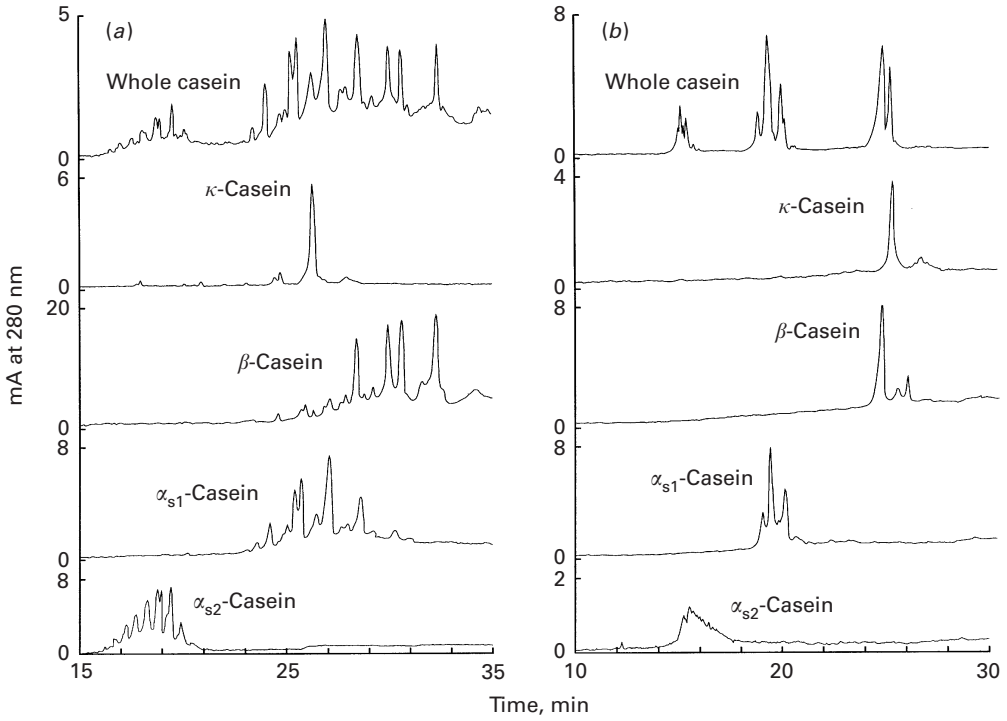

Fig. 6. Capillary electropherograms of (*a*) native and (*b*) dephosphorylated whole casein and of reversed-phase HPLC-purified casein fractions from an individual ovine casein sample containing α_{s1}-casein A. For experimental details, see text.

ESI–MS or both) and this established a starting point for rationalization of the complex CE and UTLIEF profiles.

Fractionation of whole ovine casein by capillary electrophoresis at pH 2·5

The electropherogram at pH 2·5 of a whole ovine casein sample containing α_{s1}-CN A and those of the four native HPLC-derived fractions are compared in Fig. 6(*a*). The separation of the protein species, similar to that obtained by Cattaneo *et al.* (1996) and Recio *et al.* (1997*b*), indicated a high heterogeneity. Four distinct ranges of migration time towards the anode were identified for each casein family, in the order α_{s2}-CN > α_{s1}-CN > κ-CN > β-CN and these were partly consistent with those obtained by PAGE at pH 2·9 (Chianese *et al.* 1997*b*) and 4·0 (Chianese *et al.* 1992). At these values, κ-CN and α_{s1}-CN partly co-migrated by gel electrophoresis. The protein migrating fastest towards the cathode, α_{s2}-CN, was composed of eleven relevant peaks, a value identical to that given by ESI–MS. This suggested that short-chain and long-chain components migrated separately as a result of the different net charges at pH 2·5. The α_{s1}-CN fraction consisted of ~ 13 components, similar to the number detected by ESI–MS. The CE profile of κ-CN, less complex than that from the immunoblotted UTLIEF (Fig. 3*f*), gave a main peak that was probably the κ_0-CN 1P component, which partly co-migrated with one of the secondary α_{s1}-CN components. The other secondary κ-CN components co-migrated with both β-CN and α_{s1}-CN components with lower numbers of phosphate groups. The partial co-migration of some casein fractions prompted us to separate ovine caseins by CE of dephosphorylated samples.

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Fractionation of dephosphorylated whole ovine casein by capillary electrophoresis at pH 2·5

The CE profiles of the dephosphorylated whole ovine casein sample analysed in Fig. 6(a) and the four dephosphorylated HPLC-derived fractions are compared in Fig. 6(*b*). CE analysis revealed a number of protein species consistent with that expected from ESI analysis, confirming that alkaline phosphatase treatment effectively released phosphate groups from caseins. Each casein fraction with its own counterpart in the whole sample provided clear identification of the four fractions migrating separately in the order α_{s2}-CN > α_{s1}-CN > β-CN > κ-CN. The profile of the dephosphorylated sample was greatly simplified compared with that of native whole casein because the heterogeneity resulting from the multiple phosphorylation of caseins was eliminated. Separation of single casein fractions by CE in dephosphorylated samples could provide quantitative results on casein composition.

DISCUSSION

These results show that the advantages and limitations of using HPLC, CE, and gel electrophoresis to study casein heterogeneity are essentially equivalent in that they all need to be combined with one of the available mass spectrometric techniques to identify separated components. Accepting this, we have evaluated the analytical power of each technique. HPLC proved to be effective in fractionating whole casein into the four main casein families, but was unable to separate the closely related species of each casein family. However, separation with a shallower HPLC gradient made it possible to fractionate α_{s1}-CN into single sub-components (Chianese *et al.* 1996). CE was more capable than HPLC of efficiently resolving the sub-components of each casein family, but also failed to provide structural information on the components. It must be pointed out that the very subtle differences in negatively charged density among the casein species of the same family allowed us to obtain reproducible CE profiles from low-nanogram quantities of protein (see Fig. 6). The co-migration of secondary casein components – κ-CN with α_{s1}-CN and β-CN with α_{s1}-CN – meant that CE separation failed to provide single casein components from whole casein fractionation. PAGE also proved to have major drawbacks since α_{s1}-CN and α_{s2}-CN partly co-migrated, making it impossible to separate these major casein fractions. After complete casein dephosphorylation with alkaline phosphatase, both PAGE and CE patterns were simplified, as the protein heterogeneity due to differently phosphorylated proteins was eliminated. However, the dephosphorylated casein fractions had more electrophoretic bands than expected from mass spectrometry. At present, we can give no explanation of this discrepancy. Despite this limitation, dephosphorylation made it possible to distinguish the long and some of the short forms of α_{s1}-CN and between the long and short forms of α_{s2}-CN by UTLIEF. In contrast, by dephosphorylation of the long and short forms of α_{s1}-CN, migration by CE at low pH made it possible for us to obtain a main α_{s1}-CN peak surrounded by to two minor peaks, which probably corresponded to dephosphorylated non-allelic forms. This did not occur with the two dephosphorylated forms of α_{s2}-CN with different numbers of basic residues, which had been partly resolved by relying on the different positive charges. As a result, the dephosphorylation of whole casein made it possible to separate α_{s1}-CN, α_{s2}-CN, κ-CN and β-CN by CE at acid pH ready for evaluation by peak integration. HPLC gave equivalent results when applied to the analysis of native or dephosphorylated casein and gave good

resolution of phosphatase-treated caseins. CE provided accurate information on single families, though it required preliminary treatment to dephosphorylate casein samples. Further, CE gave clear information on polymorphism and different degrees of phosphorylation. The latter is particularly useful for detecting differences in individual and bulk milks since it requires neither preliminary treatment nor separation of casein and whey proteins, as demonstrated for bovine milk by De Jong *et al.* (1993). Thus, CE appeared to be more appropriate than immunoblotting for analysing whole casein, since unlike the latter, it does not require preliminary separation or staining with polyclonal antibodies.

Potential of mass spectrometry in defining casein composition

ESI–MS allowed us to characterize the protein forms of different molecular masses and degrees of phosphorylation occurring in whole casein. Thus, more than other analytical techniques, ESI–MS facilitates access to information on structural elements linked to the origins of protein microheterogeneity. This heterogeneity results from the presence of firstly, casein variants and non-allelic casein forms with amino acid or peptide deletions; secondly, forms with post-translational modifications, such as the different degrees of phosphorylation; thirdly, peptides derived from any proteolysis and, finally, dimer or polymer caseins formed by disulphide bonding.

Casein variants and non-allelic casein forms

With MS information available, comparing measured and calculated molecular masses should ensure immediate identification of the casein variant. Indeed, the full-length α_{s1}-CN A, C and D were easily identified since the experimental mass was consistent with the expected value (Table 3). The additional peaks that often occurred in the spectrum were readily identified since their molecular masses were consistently greater by single or multiple values of the experimental masses of protein-bound sodium, potassium or calcium ions (22, 38 or 40 Da; De Caterina *et al.* 1992). Careful desalting of the protein solution before analysis by ESI–MS could eliminate this inconvenience. In other cases, the mass difference was unrelated to phosphorylation since it persisted even after dephosphorylation. Such differences may have been caused by the presence of genetic variants, which could be confirmed by further structural investigations, or by other unknown factors. With this procedure, some of the 'silent' variants could be revealed, as already demonstrated for water buffalo haemoglobin variants by Ferranti *et al.* (1992). MALDI–TOF was useful for analysing single dephosphorylated casein fractions but failed to detect the complexity of non-allelic α_{s1}-CN forms and was useless for analysing whole dephosphorylated casein owing to the huge differences in ionization among the four casein families.

Thus, an accurate molecular mass determination provides compelling confirmation of the identity of the casein variant as well as information on the subtle protein modification(s) of non-allelic species. On-line LC–ESI–MS examination fulfils two objectives: firstly, fractionation of whole casein by HPLC and accurate molecular mass measurement of the components and, secondly, simultaneous preparative recovery of a protein sample for subsequent structural characterization. The value of ESI–MS for assessing the heterogeneity of α_{s1}-CN and α_{s2}-CN, both containing some non-allelic forms, is illustrated by the results for the three samples of dephosphorylated whole casein reported in Table 3. There were at least seven α_{s1}-CN and one α_{s2}-CN deleted forms. However, there were two components needing further

identification in the sample containing α_{s1}-CN C together with the putative dimer κ-CN with an anomalous molecular mass (37377 Da) lower than that found in the other two samples and predicted by the amino acid sequence. Clearly, ESI–MS offers one of the most rapid tools for identifying protein heterogeneity arising from allelic and non-allelic variations. These latter can be clarified by simple molecular mass determination followed, if necessary, by more detailed peptide mapping.

Post-translationally modified caseins

Subjecting casein to dephosphorylation before analysis could be useful not only for detecting genetic variants by PAGE, UTLIEF or CE but also for determining phosphorylation levels. Using this procedure, it was possible to demonstrate that, depending on the genetic variant, native α_{s1}-CN occurs in three or four discretely phosphorylated forms, α_{s2}-CN in five or six and β-casein in six.

From an analytical point of view, removing the phosphate groups by alkaline phosphatase treatment produced proteins with a decreased negative charge and an increased pI. These changes resulted in a lower electrophoretic mobility of the casein fractions in PAGE at alkaline pH and a higher mobility towards the cathode in UTLIEF, thereby providing a simple means for detecting novel casein variants. Furthermore, as the dephosphorylated forms were less complex, they were better suited for densitometric scanning of the gel.

Enzyme-mediated casein degradation products

Measuring molecular masses by ESI–MS can be used to confirm the integrity of the casein fractions and detect any protein degradation products present. In the sample with α_{s1}-CN A (see Table 3), the two degradation products of κ-casein, para-κ-casein and caseinomacropeptide were found. In the α_{s1}-CN C sample, γ_2-CN and its complementary peptide β-CN f(1–105), formed by plasmin action, were detected. The presence of γ_2-CN indicated that in this case the β-CN was partly hydrolysed by plasmin, unlike the other two samples.

Disulphide bridge-mediated association

In addition to monomer κ-CN, whole ovine samples containing α_{s1}-CN A and C also contained dimer κ-CN, which was absent in the other sample. Dimer formation may have depended on the three cysteine residues of the κ-CN molecule (Jollès *et al.* 1974), which could have given rise to one intermolecular disulphide bond between two protein molecules during incubation with alkaline phosphatase. However, evidence that the dimer form of κ-casein pre-existed as a native component of raw milk was demonstrated by its occurrence in the freshly prepared acid whey.

In conclusion, a comprehensive two dimensional method of coupling ESI–MS with HPLC and with CE to rationalize first dimensional profiles was evaluated. In terms of rapidity and resolving power, we consider CE the most promising candidate for evaluating the casein composition of native and dephosphorylated samples. However, CE profiling requires rationalization in order to search for novel casein variants, and this could be achieved by coupling ESI–MS with CE as shown for LC. At the present, analysis by ESI–MS of a previously dephosphorylated sample indeed seems better suited to casein characterization.

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REFERENCES

Addeo, F., Masi, P. & Rubino, R. 1989 The aptitude of caprine milk to cheesemaking. Relationship between α_{s1}-casein content and rheological properties of curd and cheese. *Proceedings, Philostios Symposium, Fonte-Boa*, 1987 *Agriculture* (Eurostat) pp. 291–311

Addeo, F., Mauriello, R. & Di Luccia, A. 1988 A gel electrophoretic study of caprine casein. *Journal of Dairy Research* **55** 413–421

Addeo, F., Mauriello, R., Moio, L., Laezza, P. & Di Luccia, A. 1992 Ovine casein variant identification using electrophoretic, immunochemical and chromatographic techniques. *Milchwissenschaft* **47** 283–287

Boisnard, M., Hue, D., Bouniol, C., Mercier, J-C. & Gaye, P. 1991 Multiple mRNA species code for two non-allelic forms of ovine α_{s2}-casein. *European Journal of Biochemistry* **201** 633–641

Brignon, G., Mahé, M-F., Grosclaude, F. & Ribadeau-Dumas, B. 1989 Sequence of caprine α_{s1}-casein and characterization of those of its genetic variants which are synthesised at a high level, α_{s1}-CN A, B and C. *Protein Sequence and Data Analysis* **2** 181–188

Brignon, G., Mahé, M-F., Ribadeau-Dumas, B., Mercier, J-C. & Grosclaude, F. 1990 Two of the three genetic variants of goat α_{s1}-casein which are synthesised at reduced level have an internal deletion possibly due to altered RNA splicing. *European Journal of Biochemistry* **193** 237–241

Cattaneo, TMP., Nigro, F., Toppino, P. M. & Denti, V. 1996 Characterization of ewe's milk by capillary zone electrophoresis. *Journal of Chromatography A* **721** 345–349

Chianese, L., Ferranti, P., Garro, G., Mauriello, R. & Addeo, F. 1997*a* Occurrence of three novel α_{s1}-casein variants in goat milk. *International Dairy Federation Bulletin* no. 9702 259–267 (*Proceedings, International Dairy Federation Seminar 'Milk Protein Polymorphism II'*, Palmerston North)

Chianese, L., Garro, G., Ferranti, P., Malorni, A., Addeo, F., Rabasco, A. & Molina Pons, P. 1995 Discrete phosphorylation generates the electrophoretic heterogeneity of ovine β-casein. *Journal of Dairy Research* **62** 89–100

Chianese, L., Garro, G., Mauriello, R., Laezza, P., Ferranti, P. & Addeo, F. 1996 Occurrence of five α_{s1}-casein variants in ovine milk. *Journal of Dairy Research* **63** 49–59

Chianese, L., Garro, G., Nicolai, M. A., Mauriello, R., Ferranti, P., Pizzano, R., Cappuccio, U., Laezza, P., Addeo, F., Ramunno, L., Rando, A. & Rubino, R. 1993 The nature of β-casein heterogeneity in caprine milk. *Lait* **73** 533–547

Chianese, L., Mauriello, R., Ferranti, P., Tripaldi, C., Taibi, L. & Dell'Aquila, S. 1997*b* Relationship between α_{s1}-casein variants and clotting capability of ovine milk. *International Dairy Federation Bulletin* no. 9702 316–323 (*Proceedings, International Dairy Federation Seminar 'Milk Protein Polymorphism II'*, Palmerston North)

Chianese, L., Mauriello, R., Moio, L., Intorcia, N. & Addeo, F. 1992 Determination of ovine casein heterogeneity using gel electrophoresis and immunochemical techniques. *Journal of Dairy Research* **59** 39–47

Commission Regulation 1996 Reference method for the detection of cows' milk and caseinate in cheeses from ewes' milk, goats' milk and buffalo milk or mixtures of ewes', goats' and buffalo milk. EC no. 1081/96 of 14 June 1996. *Official Journal of the European Communities* **L142** 15–25

de Caterina, M., Esposito, P., Grimaldi, E., Di Maro, G., Scopacasa, F., Ferranti, F., Parlapiano, A., Malorni, A., Pucci, P. & Marino, G. 1992 Characterization of hemoglobin lepore variants by advanced mass-spectrometry procedures. *Clinical Chemistry* **38** 1444–1448

de Jong, N., Visser, S. & Olieman, C. 1993 Determination of milk proteins by capillary electrophoresis. *Journal of Chromatography A* **652** 207–213

Ferranti, P., Addeo, F., Malorni, A., Chianese, L., Leroux, C. & Martin, P. 1997*a* Differential splicing of pre-messenger RNA produces multiple forms of mature caprine α_{s1}-casein. *European Journal of Biochemistry* **249** 1–7

Ferranti, P., Chianese, L., Malorni, A., Migliaccio, F., Stingo, V. & Addeo, F. 1998 Copresence of deleted protein species generates structural heterogeneity of ovine α_{s1}-casein. *Journal of Agricultural and Food Chemistry* **46** 411–416

Ferranti, P., Di Luccia, A., Malorni, A., Pucci, P., Ruoppolo, M., Marino, G. & Ferrara, L. 1992 River buffalo (*Bubalus bubalis* L.) AA phenotype haemoglobins: characterization by immobiline polyacrylamide gel electrophoresis and high performance liquid chromatography and determination of the primary structure of the constitutive chains by mass spectrometry. *Comparative Biochemistry and Physiology* **101** 91–98

Ferranti, P., Garro, G., Laezza, P., Chianese, L. & Addeo, F. 1997*b* Identification of ovine casein heterogeneity by HPLC and electrospray mass spectrometry. *International Dairy Federation Bulletin* no. 9702 297–302 (*Proceedings, International Dairy Federation Seminar 'Milk Protein Polymorphism II'*, Palmerston North)

Ferranti, P., Lilla, S., Chianese, L. & Addeo, F. 1999 Alternative nonallelic deletion is constitutive of ruminant α_{s1}-casein. *Journal of Protein Chemistry* **18** 595–602

Ferranti, P., Malorni, A., Nitti, G., Laezza, P., Pizzano, R., Chianese, L. & Addeo, F. 1995 Primary structure of ovine α_{s1}-caseins: localization of phosphorylation and characterization of genetic variants A, C and D. *Journal of Dairy Research* **62** 281–296

Grosclaude, F., Mahé, M-F., Brignon, G., Di Stasio, L. & Jeunet, R. 1987 A Mendelian polymorphism underlying quantitative variation of goat α_{s1}-casein. *Génétique, Séléction, Evolution* **19** 399–412

Grosclaude, F., Ricordeau, G., Martin, P., Remeuf, F., Vassal, L. & Bouillons, J. 1994 [From gene to

cheese: polymorphism of α_{s1}-caseins in goat milk, its effects and development.] *INRA Productions Animales* **7** 3–19

Jaubert, A. & Martin, P. 1992 Reversed-phase HPLC analysis of goat caseins. Identification of α_{s1} and α_{s2} genetic variants. *Lait* **72** 235–247

Jollès, J., Fiat, A-M., Schoentgen, F., Alais, C. & Jollès, P. 1974 The amino acid sequence of sheep κ_A-casein. II. Sequence studies concerning the κ_A-caseinoglycopeptide and establishment of the complete primary structure of the protein. *Biochimica et Biophysica Acta* **365** 335–343

Mercier, J-C. Gaye, P., Soulier, S., Hue-Delhaie, D. & Vilotte, J-L. 1985. Construction and identification of recombinant plasmids carrying cDNAs coding for ovine α_{s1}-, α_{s2}-, β- and κ-casein and β-lactoglobulin. Nucleotide sequence of α_{s1}-casein cDNA. *Biochimie* **67** 959–971

Recio, I., Pérez-Rodríigues, M. L., Amigo, L. & Ramos, M. 1997*a* Study of the polymorphism of caprine milk caseins by capillary electrophoresis. *Journal of Dairy Research* **64** 515–523

Recio, I., Ramos, M. & Amigo, L. 1997*b* Study of the polymorphism of ovine α_{s1}- and α_{s2}-caseins by capillary electrophoresis. *Journal of Dairy Research* **64** 525–534

Remeuf, F. 1993 [Influence of the genetic polymorphism of caprine α_{s1}-caseins on the physicochemical and technological characteristics of goats' milk.] *Lait* **73** 549–557

Richardson, B. G. & Mercier, J-C. 1979 The primary structure of the ovine β-casein. *European Journal of Biochemistry* **99** 285–297