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Analysis of major ovine milk proteins by reversed-phase high-performance liquid chromatography and flow injection analysis with electrospray ionization mass spectrometry

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Abstract

Ovine milk proteins were analyzed both by coupling HPLC and electrospray ionization mass spectrometry (ESI-MS) and by flow injection analysis and ESI-MS detection after separation and collection of fractions from gel permeation chromatography. These methods resolved the four ovine caseins and whey proteins and made it possible to study the complexity of these proteins associated with genetic polymorphism, post-translational changes (phosphorylation and glycosylation) and the presence of multiple forms of proteins. The experimental molecular masses of ewe milk proteins were: 19 373 for κ-casein 3P; 25 616 for α_{s2}-casein 10P; 23 411 for α_{s1}-casein C-8P; 23 750 for β-casein 5P; 18 170 and 18 148 for β-lactoglobulins A and B; 14 152 for α-lactalbumin A and 66 322 for serum albumin.

Keywords: Milk; Proteins; Casein; Whey proteins

1. Introduction

Among the hoofed animals, ewe milk is the richest in total solids and casein. The proteins of ewe milk are classified into two groups: caseins (α_{s1}-, α_{s2}-, β- and κ-CN) and whey proteins (α-lactalbumin: α-La, β-lactoglobulin: β-Lg and serum albumin: SA).

The primary structures of ovine κ-, β- and α_{s1}-CN and β-Lg have been determined on the protein and confirmed through the study of their copy DNA (cDNA), and those of α_{s2}-CN and α-La have only been studied on the cDNA precursors.

To analyze ovine milk proteins and genetic polymorphism, various electrophoretic and chromatographic techniques have been used [1–5]. Recently, the main protein fractions and protein polymorphism have been studied in ewe milk by capillary electrophoresis [6,7].

In recent years, electrospray ionization mass spectrometry (ESI-MS) has become a powerful alternative tool for the determination of the relative molecular mass (M_r) of bovine milk proteins [8,9], detection of any changes in proteins caused by, e.g., insertion, deletion or modification of amino acids [10], identification of genetic variants [11,12] and post-translational modifications such as phosphorylation [13], glycosylation [14], etc.

In this investigation, ESI-MS was used to de-

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termine the molecular masses of major ovine milk proteins and to study the great complexity of these proteins by coupling high-performance liquid chromatography (HPLC) and ESI-MS (HPLC–ESI-MS) for on-line separation, detection and mass determination of ovine proteins from whole skim milk. Some whey milk proteins, not resolved by HPLC–ESI-MS, were purified by fast protein liquid chromatography (FPLC) and analyzed by ESI-MS via flow injection analysis (FIA).

2. Experimental

2.1. Preparation of ovine milk proteins

Ewe raw milk (Manchega breed) was obtained from the Experimental Farm of the Universitat Autònoma de Barcelona (Spain). Skim milks were prepared by centrifugation at 2500 *g* and 30°C for 30 min. Whey milks were obtained from skim milks by isoelectric precipitation at pH 4.6 and 20°C by the addition of 1 *M* HCl. Major whey proteins (β-Lg, α-La and SA) were purified from whey milks by gel permeation FPLC according to Felipe and Law [15]. Whey samples were fractionated at 20°C on a column (950×26 mm, bed volume 504 ml) of Superdex 75 prep grade (Amersham Pharmacia Biotech, St. Albais, UK) in 100 m*M* Tris–HCl buffer (pH 7.0) containing 0.5 *M* NaCl at a flow-rate of 2.5 ml/min. Absorbance of the eluate was monitored at 280 nm.

The sample buffer (pH 7.5) consisted of 17.5 m*M* 1,3 - bis[tris(hydroxymethyl) - methylamino]propane containing 7 *M* urea and 0.5% of 2-mercaptoethanol. A 200-μl volume of skim milk was mixed with 800 μl of sample buffer. After 1 h incubation at room temperature, the reduction reaction was finished and the sample was applied to the HPLC column without further preparation.

2.2. Reversed-phase HPLC separation

A modification of the method proposed by Visser et al. [16] was used to separate the major proteins of ewe milk (caseins, β-Lg and α-La) in a single run. Proteins were separated on an Apex WP ODS reversed-phase column (7 μm particle, 300 Å porosity, 250×4.6 mm, Jones Chromatography, Mid-Glamorgan, UK) at 46°C using a gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid. The acetonitrile gradient was 33 to 49% in 30 min. Flow-rate was 1 ml/min and detection was at 214 nm.

2.3. FIA–ESI-MS

In order to study the molecular mass of some whey milk proteins which were not resolved by HPLC–ESI-MS, ovine whey proteins (β-Lg, α-La and SA) were purified from whey milk by gel permeation FPLC. The corresponding peaks from FPLC analysis were collected and freeze-dried.

Each isolated whey milk protein was dissolved with a solution of acetonitrile and water [acidified with 0.1% (v/v) trifluoroacetic acid] at a ratio of 1:1 (v/v) and then introduced in the fluidic way via FIA, using the same solvent as carrier, to a mass spectrometer Micromass, model Platform II (Micromass, Manchester, UK), fitted with an electrospray source. A Phoenix 20 syringe pump (C.E. Instruments, Milan, Italy) set at 50 μl/min was used. A 10-μl volume of each sample solution, containing approximately 0.1 mg of protein per milliliter, was injected into the fluidic way with a Rheodyne 7125 injector (Rheodyne, Cotati, CA, USA).

The main working conditions of the source and mass spectrometer were the following: capillary at 4.5 kV, counter-electrode at 500 V, sample cone at 80 V, source temperature at 80°C, scan range for the mass-to-charge ratio (m/z) from 1000 up to 3000 during 2 s with an inter scan time of 0.25 s, scan mode continuum and photomultiplier at 650 V. The spectrometric data were collected and analyzed by using the MassLynx software (Micromass).

2.4. HPLC–ESI-MS

An Alliance Waters 2690 separation module (Waters Chromatography, USA) fitted with the column was used as chromatograph. The elution was performed with the gradient mode described above at a flow of 1 ml/min. That flow was too strong to reach the desired sensitivity with the ESI source used. A laboratory-made flow divisor of 1:20 ratio was added to the end of the chromatographic column which

allowed us to have greater spectroscopic signals for protein peaks. A T-union of zero dead volume (Waters) was used. A polyether ether ketone (PEEK) (254 μm I.D.) tubing from the column was connected to one end of the T-union, at the other end a fused-silica capillary tubing (1 m × 50 μm I.D.) connected the T-piece with the ESI source, and at the third end a coil of approximately 3 m length of PEEK tubing (254 μm I.D.) was connected to divert the main part of the liquid to waste.

Some of the samples were too rich in urea and 2-mercaptoethanol. This led to the crystallization of the urea at the top of the sample cone, inside the ESI source, with the consequent obstruction of its capillary hole; this made it difficult to obtain any signal from the mass spectrometer. To solve this problem a pneumatically-assisted Rheodyne 7010 valve was introduced between the column and the flow divisor. The valve allowed us to derive the eluate to waste from the injection time to the complete elution of the urea (~5 min).

3. Results and discussion

Table 1 shows the M_r of major ovine proteins obtained by HPLC–ESI-MS, and those from whey proteins by FIA–ESI-MS.

In Fig. 1 we can see the chromatographic patterns obtained from a skimmed milk sample. With the HPLC method used, separation can be achieved between caseins and whey protein components.

Table 1
M_r determination of major ewe milk proteins

Proteins	Experimental M_r [a]	Calculated M_r
κ-CN 3P	19 373	19 373
α_{s2}-CN 10P	25 616	25 622
α_{s1}-CN C-8P	23 411	23 401
β-CN 5P	23 750	23 751
β-Lg A	18 170	18 171
β-Lg B	18 148	18 145
α-La A	14 152	14 158
Serum albumin	66 322	66 327

[a] The average of relative molecular mass (M_r) from five milk samples.

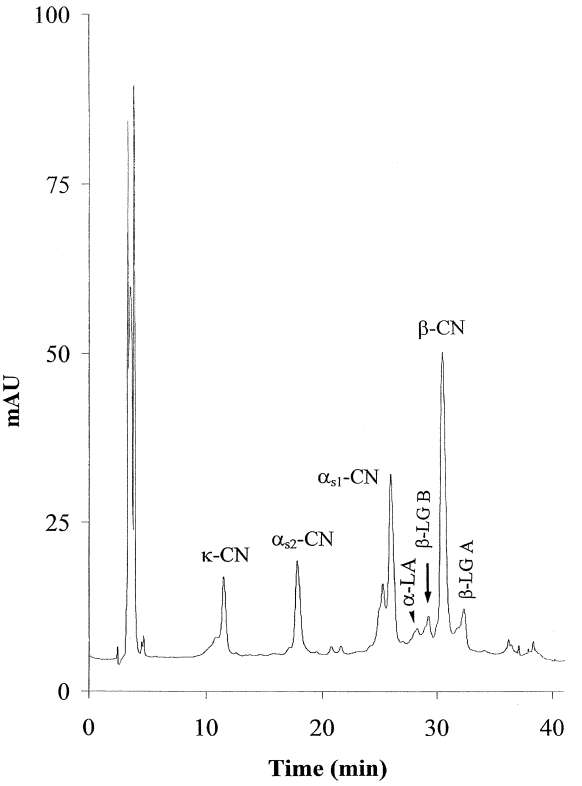

Fig. 1. Chromatographic patterns (RP-HPLC), of skim milk proteins from a ewe milk sample. Milk proteins were separated on an Apex WP ODS reversed-phase column (7 μm, 300 Å, 250×4.6 mm) at 46°C using a gradient (33 to 49% in 30 min) of acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow-rate of 1 ml/min and absorbance at 214 nm was recorded. κ-CN=κ-Casein; α_{s2}-CN=α_{s2}-casein; α_{s1}-CN=α_{s1}-casein; β-CN=β-casein; β-Lg=β-lactoglobulin; α-La=α-lactalbumin.

3.1. κ-Casein

The primary structure of κ-CN A has been determined [17,18] and it has been confirmed by the corresponding sequences of the cDNA [19] except for the residues Gln_2→Glu and Glu_7→Gln. From the amino acid sequencing this protein contains 171 residues and only one residue phosphate.

κ-CN heterogeneity has been described by numerous authors [1,20,21] in different ovine breeds by using electrophoretic and immunochemical techniques. According to these authors the high degree of κ-CN heterogeneity probably derives from different degrees of phosphorylation and glycosylation.

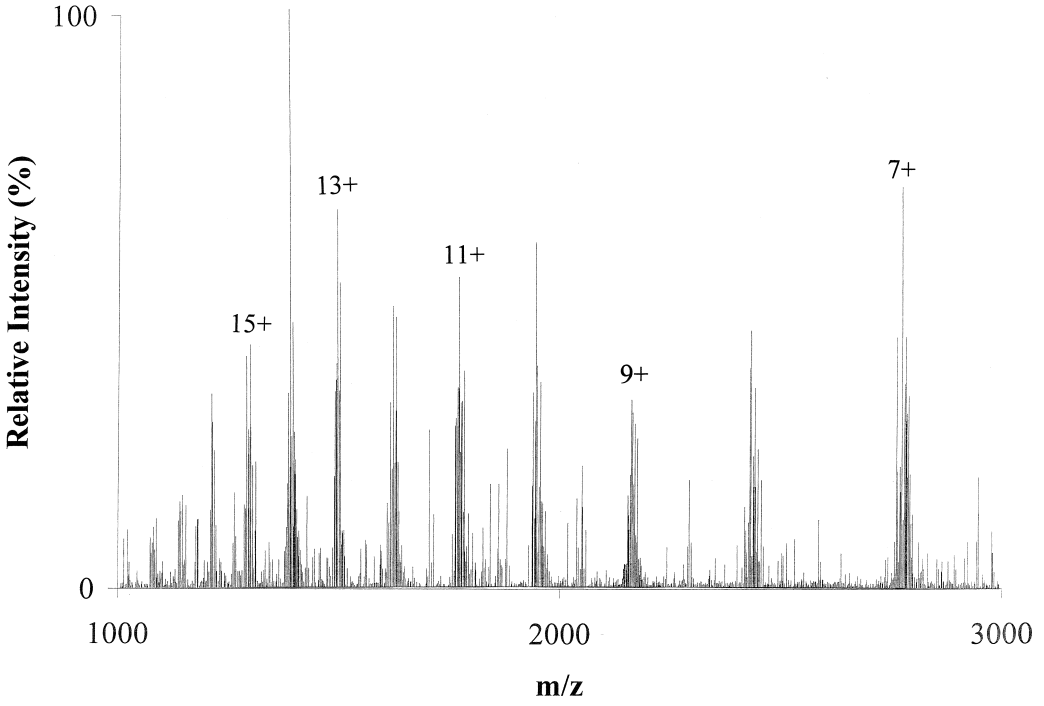

Fig. 2. Electrospray mass spectrum of ovine κ-casein after RP-HPLC separation. m/z=Mass-to-charge ratio.

Fig. 2 shows the ESI mass spectrum of κ-CN which had two main components with M_r 19 374.82±3.17 and 19 448.79±3.32. However, the calculated M_r from the protein sequence of κ-CN 1P is 19 213. The difference in mass between the observed and calculated M_r is consistent, taking into account the following two possibilities: (1) the presence of a different number of phosphate residues (ΔM_r 80) in the same protein. Mercier [22] stated that the configuration Ser/Thr-X-A, where X represents any amino acid and A an acidic residue or Ser/Thr(P), is required for phosphorylation. Sites where A is a dicarboxylic amino acid are considered primary sites and those with a phosphorylated residue in that position secondary sites. Four primary sites of phosphorylation can be found in ovine κ-CN (positions 127, 137, 151 and 168). The mass difference (ΔM_r 80) between the two components found by ESI-MS can be accounted for by the presence of a different number of phosphate residues in the same protein (3 and 4, respectively). (2) In addition to the heterogeneity due to the phosphorylation level, it appears that ovine κ-CN, like cow, buffalo and goat κ-CNs, is composed of several fractions having identical peptide chains and differing in their carbohydrate content [20]. Therefore, the differences between the observed and calculated M_r could be explained in part by the presence of glycosylated components.

3.2. α_{s2}-Casein

The primary structure of α_{s2}-CN has only been determined on the cDNA precursor [23] and it contains 208 amino acids. As for other species, ovine α_{s2}-CN to be appear the most heterogeneous fraction due to the multiphosphorylation level. However, one of the sources of heterogeneity of ovine α_{s2}-CN depends on the presence of multiple forms of protein produced from different forms of messenger RNA (mRNA) [24]. Two co-existing protein forms have been described and they differ for the deleted peptide of nine amino acid residues at positions 34–42 or 35–43 of the α_{s2}-CN chain. The deletion leads to the absence on two cysteinyl residues, one phosphoseryl and three other charged residues.

The occurrence of polymorphism in α_{s2}-CN has been demonstrated by genetic sequencing [24] and by electrophoretic techniques and immunoblotting [2]. cDNA analysis from α_{s2}-CN revealed five single mutations from which only two involved amino acid replacements: Asn_{49}→Asp and Lys_{200}→Asn.

The main forms of α_{s2}-CN detected by ESI-MS (Fig. 3) had M_r 25 615.38±7.88, 24 222.35±10.17 and 24 291.30±7.67. The first M_r is consistent with the calculated mass from the long sequence for α_{s2}-CN 10P (M_r 25 622) and the other M_r are consistent with the deleted forms of α_{s2}-CN 9P and 10P (M_r 24 226 and 24 306, respectively).

3.3. α_{s1}-Casein

The primary structure of ovine α_{s1}-CN was first deduced from the cDNA analysis [25] and later from protein sequencing for the ovine α_{s1}-CN variants A, C and D [11]. Five α_{s1}-CNs (A to E) have now been identified by electrophoretic techniques [26]. The most frequent allele in Spanish and Italian breeds is α_{s1}-CN C [26,27].

The mature α_{s1}-CN is composed of a mixture of different molecular species differing in molecular mass and degree of phosphorylation produced from different forms of mRNA [11,28]. The main component represents more than 50% of total α_{s1}-CN and it corresponds to the full-length (199 residues) protein. The other components are shorter forms of α_{s1}-CN and they differ for the detected peptides 110–117, 140–148 or Gln_{78} [10]. Another source of heterogeneity for α_{s1}-CN is due to the partial phosphorylation of both Ser_{41} and Ser_{115} giving three different phosphorylation levels [11].

Ovine α_{s1}-CN was separated into two peaks in the chromatographic system used (Fig. 1). ESI-MS analysis (Fig. 4) demonstrated that the minor peak and the major peak corresponded to the short and the full-length proteins, respectively. The main M_r found for the first peak had M_r 22 394.82±9.43 which corresponds to the calculated mass from the sequence of the 191-residues form of α_{s1}-CN C 8P (M_r 22 389). The M_r found for the second peak were 23 410.74±9.46 and 23 489.63±9.43 which correspond to the calculated mass from the sequences of

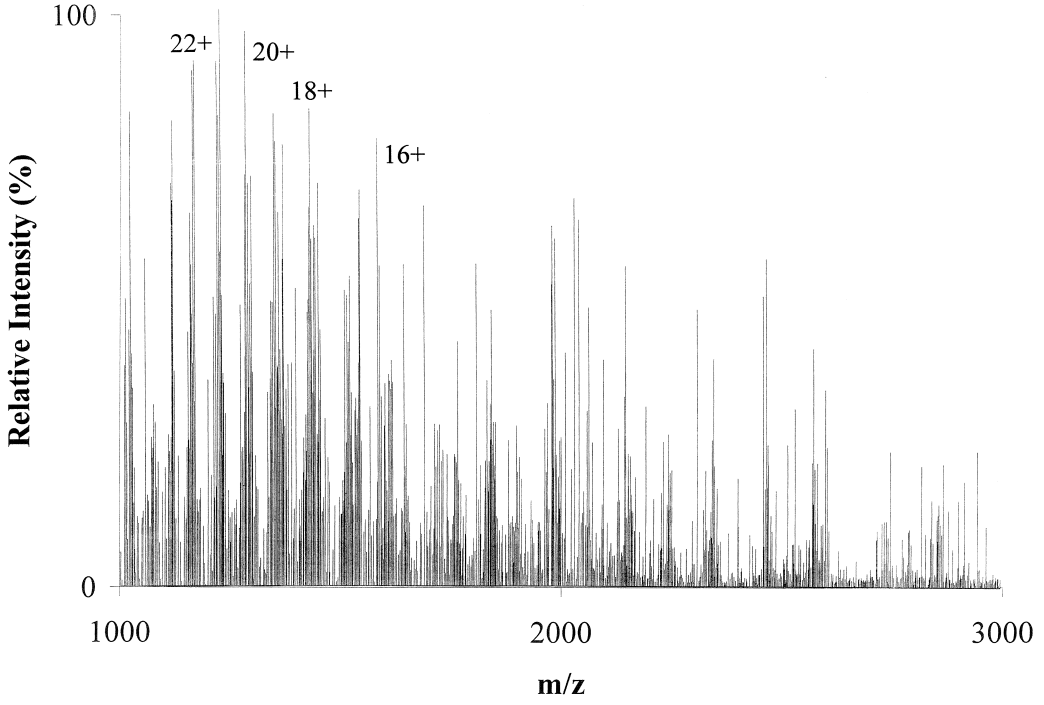

Fig. 3. Electrospray mass spectrum of ovine α_{s2}-casein after RP-HPLC separation. m/z=Mass-to-charge ratio.

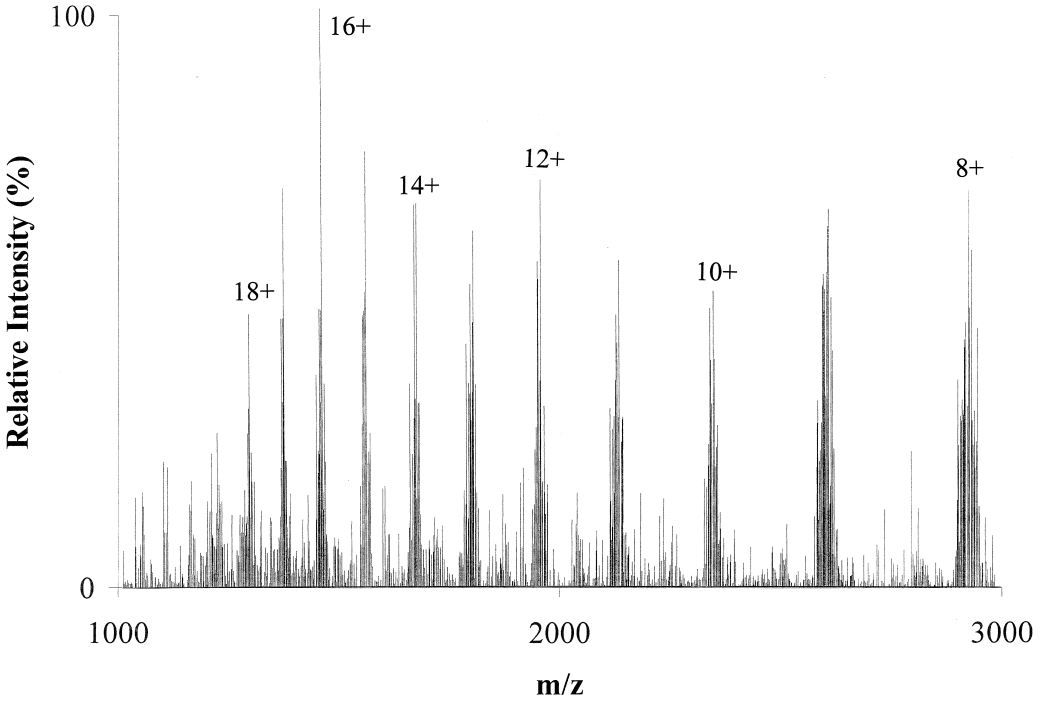

Fig. 4. Electrospray mass spectrum of ovine α_{s1}-casein C after RP-HPLC separation. m/z=Mass-to-charge ratio.

α_{s1}-CN C 8P (M_r 23 401) and α_{s1}-CN C 9P (M_r 23 481), respectively, from the 199-residues chain or main component described by Ferranti et al. [11].

3.4. β-Casein

The electrophoretic patterns of ovine β-CN at alkaline pH is characterized by the presence of two main bands (β_1 and β_2) attributed to the different numbers of phosphate groups, 6 and 5, respectively.

No β-CN variant has been characterized until now, but a great heterogeneity has been observed. This heterogeneity is dependent on the multiple phosphorylation of the protein chain having 1–7 phosphate groups per molecule [13].

Primary sequence was described by Richardson and Mercier [29] and it consists in 207 amino acids. Protein sequence has been confirmed by the study of the cDNA [30] except for the residues Thr_{55}→Ala and Ala_{69}→Pro.

ESI-MS analysis (Fig. 5) gave two main M_r (23 749.44±4.14 and 23 834.50±9.73). The mass difference between the β-CN components can be accounted for by the presence of a different number of phosphate residues (5 and 6). The results agree with those expected from the cDNA deduced protein sequence (M_r 23 751 and 23 831, respectively) and with those obtained by Chianese et al. [13] from isolated β-CN by using ESI-MS.

3.5. β-Lactoglobulin

The major whey proteins in ewe milk are β-Lg and α-La. The complete β-Lg sequence which has been confirmed by its genomic sequence, is well known [31,32]. Ovine β-Lg has 162 amino acids, it binds retinol and is probably involved in the transport of that molecule. The best documented polymorphism in the sheep is that of β-Lg. Three variants (A, B and C) have been described, β-Lg A and B being normally predominant [3]. They differ by the substitution of His_{20} (B)→Tyr (A and C) and Arg_{148} (A and B)→Gln (C).

Genetic variants of β-Lg were well resolved by the chromatographic method used in heterozygote

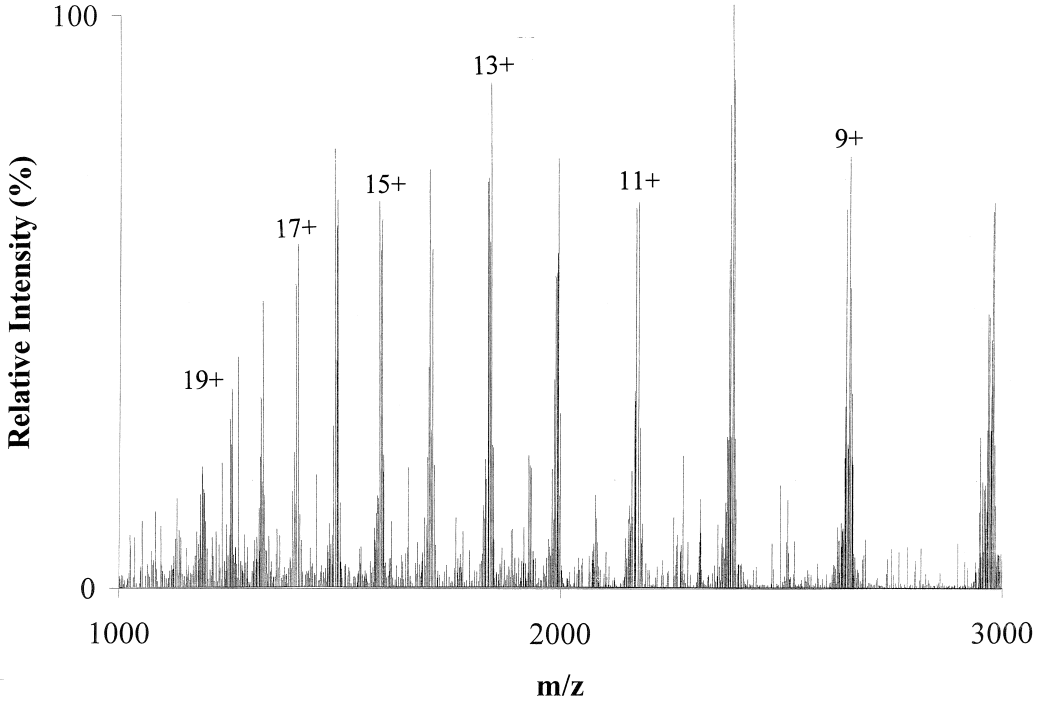

Fig. 5. Electrospray mass spectrum of ovine β-casein after RP-HPLC separation. m/z=Mass-to-charge ratio.

(A/B) milk samples (Fig. 1). However, in the analytical conditions used hyphenating HPLC and ESI-MS techniques only variant A of β-Lg raised a clear mass spectrum, and we were no able to obtain clear mass spectrum corresponding to variant B of β-Lg.

Fig. 6 shows the ESI mass spectrum β-Lg A. From the spectrum it is clear that β-Lg A exhibits heterogeneity with two different components. The main component had a M_r 18 170.24±2.78 which corresponds to that obtained from the protein sequence (M_r 18 171). The minor component had a M_r 18 495.36±4.96. The difference in mass of 325 between components is consistent with the covalent linkage of a lactosyl residue to the protein.

FIA–ESI-MS of a β-Lg B solution gave a ESI mass spectrum close to β-Lg A with two different components. The main and minor components had M_r 18 148.70±2.90 and 18 472.59±5.55, respectively, which correspond to the calculated M_r from the protein sequence (M_r 18 145) and a lactosyl residue linked to the protein.

3.6. α-Lactalbumin

For the α-La, two variants (A and B) have been found, A being the most common allele, while variant B has rarely been detected and confined to very specific breeds. In Manchega breed only variant A has been detected [3].

The primary structure of ovine α-La A has been deduced from the corresponding sequences of the cDNA [33]. This protein contains 123 amino acids and 8 cysteine residues connected by four disulfide bonds.

α-La A was resolved by HPLC but it was not possible to detect from skim milk samples by on-line HPLC–ESI-MS in the conditions used, perhaps due to the low concentration found in milk or to the partial overlapping with β-Lg B. However, direct injections of a purified α-La A solution using FIA–ESI-MS made the detection and mass determination of α-La A possible. Fig. 7 shows the ESI mass spectrum of purified α-La A. Like β-Lg, this protein presented heterogeneity. The main component had a

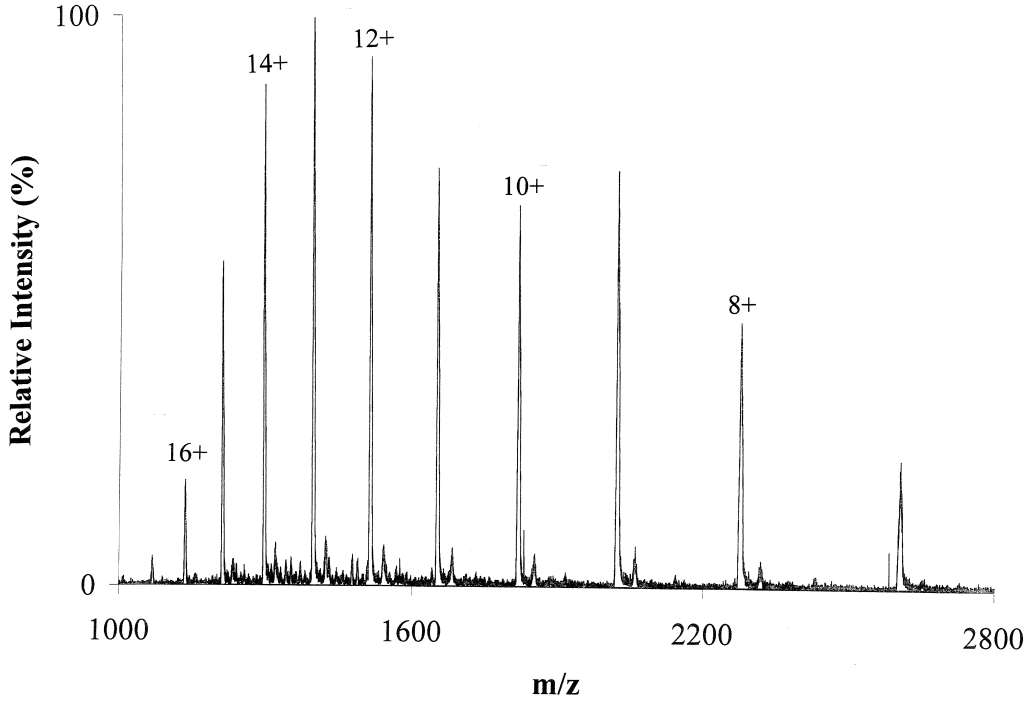

Fig. 6. Electrospray mass spectrum of ovine β-lactoglobulin A after RP-HPLC separation. m/z=Mass-to-charge ratio.

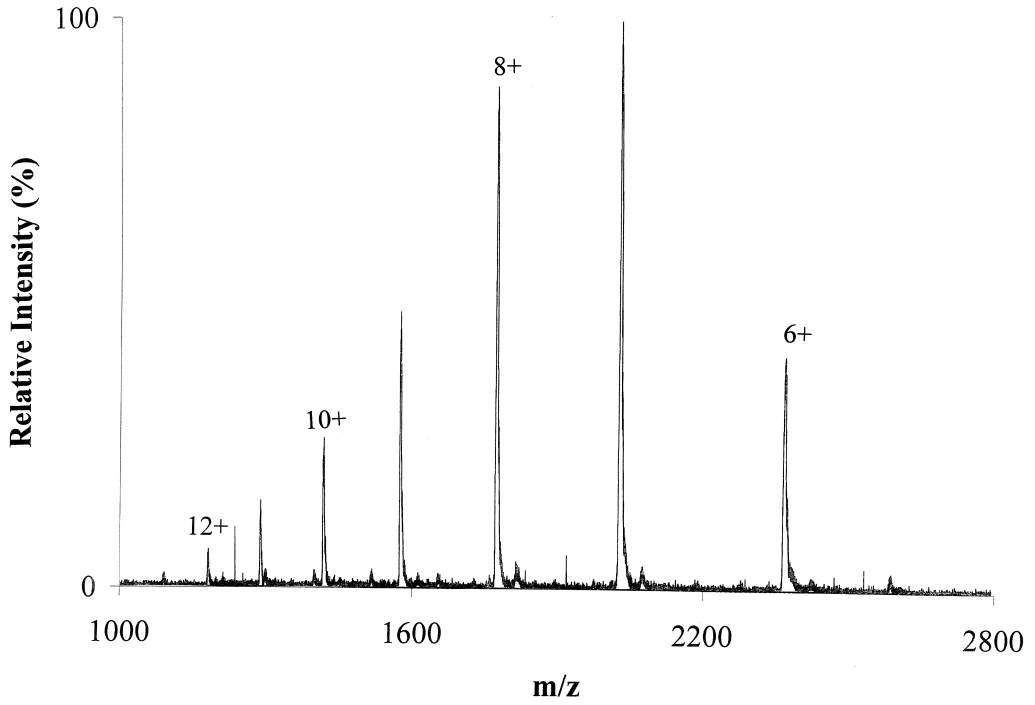

Fig. 7. Electrospray mass spectrum of isolated ovine α-lactalbumin A obtained by flow injection analysis. m/z=Mass-to-charge ratio.

M_r 14 152.38±1.32 which correspond to the calculated mass from the deduced protein component (M_r 14 158) and the minor component had a M_r consistent with the presence of a lactosyl residue linked with α-La (M_r 14 471.75±3.67).

3.7. Serum albumin

Electrophoretic patterns of the whey proteins of milks of all species that have been examined exhibit a band with the mobility of the blood SA of that species. SA has been isolated from the milk of two species, human and cow, and they behave chemically and immunologically like the albumins isolated from blood of those species. This protein is synthesized in the liver and it is transported into milk through the mammary cells by processes probably involving specific receptors.

There is no available information about the protein or gene sequence of ovine milk SA, to our knowledge. On the contrary, the nucleotide and the deduced amino acid sequence of ovine SA from liver tissue is known [34]. From this study ovine SA contains 583 amino acids and 17 disulfide bonds.

This protein was neither detected in milk by the HPLC method used, possibly due to the low concentration present in milk or due to the overlapping with other milk protein. FIA–ESI-MS spectrum of a purified SA solution (Fig. 8) gave a M_r 66 322.23±35.40 which agrees with the M_r calculated from the deduced amino acid sequence (M_r 66 327) reported by Brown et al. [34].

4. Conclusions

The HPLC–ESI-MS method made separation and detection of ovine caseins possible. Although the major whey proteins (β-Lg and α-La) were well separated by the HPLC method used, only β-LG A was detected by coupling HPLC and ESI-MS. β-LG B, α-La and SA were resolved from purified protein solutions by FIA–ESI-MS.

This method has allowed us to study the great heterogeneity of ewe milk proteins mainly due to the

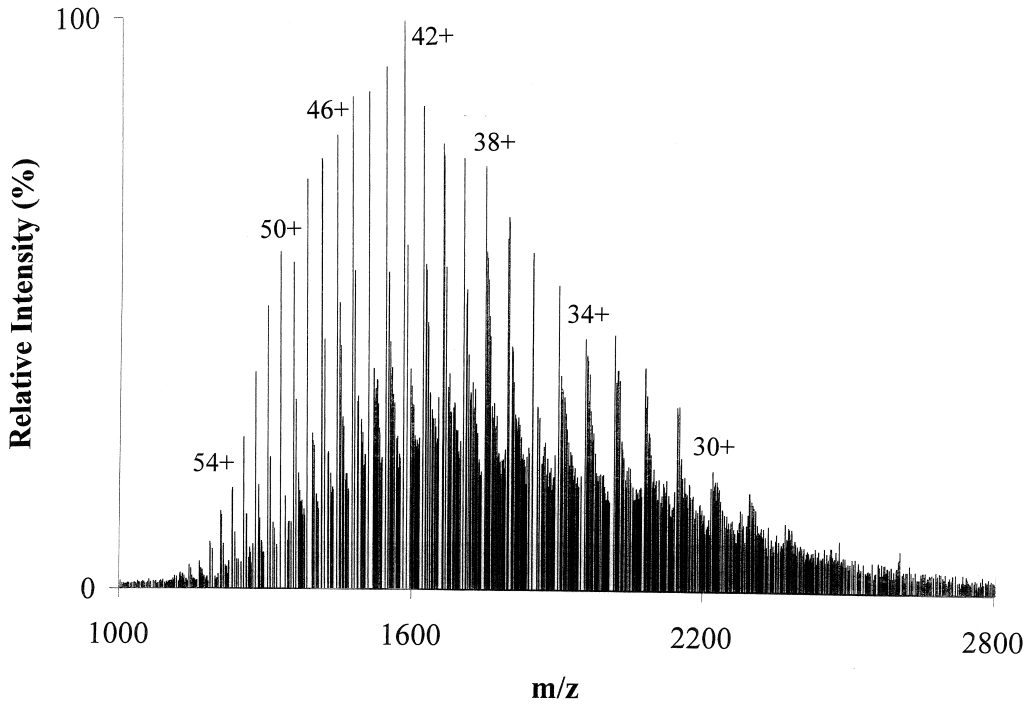

Fig. 8. Electrospray mass spectrum of isolated ovine serum albumin obtained by flow injection analysis. m/z=Mass-to-charge ratio.

genetic polymorphism, post-translational modifications as different levels of phosphorylation and glycosylation, and the presence of multiple (long and short) forms seen with α_{s2}- and α_{s1}-CNs. It may also be possible to use this method to characterize individual or bulk milks, study some proteolytic processes in milk or in model milk systems produced by the action of endogenous or added enzymes.

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