Study of the polymorphism of ovine α_{s1}- and α_{s2}-caseins by capillary electrophoresis

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Summary. Polymorphism of ovine α_s-caseins was studied by capillary electrophoresis at pH $3{\cdot}0 \pm 0{\cdot}1$. Individual caseins (CN) were selected according to their genetic variants, as determined by PAGE and isoelectric focusing. The ovine caseins, containing different genetic variants of α_{s1}-CN and α_{s2}-CN, were fractionated by cation-exchange FPLC®. The α_{s1}-CN variants A, B and C, and the fast moving α_{s2}-CN variant were identified by a capillary electrophoresis method. The fast moving α_{s2}-CN variant, so-called for its behaviour in PAGE, also had a faster electrophoretic mobility than the common α_{s2}-CN when analysed by the present technique. The capillary electrophoresis method gave excellent, rapid, automated separation of α_{s1}-CN and α_{s2}-CN variants and was suitable for screening studies.

Studies on genetic polymorphism in ovine milk proteins, including methods of analysis and potential technological applications, have attracted considerable attention in recent years (Sánchez-Bonastre, 1993; López-Gálvez *et al*. 1994*b*, 1995). Although most of the available information concerns β-lactoglobulin polymorphism (Erhardt, 1989; López-Gálvez *et al*. 1994*a*; Recio *et al*. 1997*b*), new genetic variants of α_{s1}-casein (CN) and α_{s2}-CN have recently been identified in various ovine breeds by electrophoretic and immunological techniques (Chianese *et al*. 1992, 1993, 1996). There is little information from genetic studies, and variants derived from these differ from those discovered by electrophoretic and immunological techniques. Boisnard *et al*. (1991) have found two non-allelic forms of ovine α_{s2}-CN that differ by an internal deletion of nine amino acid residues in the mature chain. Both long and short forms occurred within the normal genetic variation of α_{s2}-CN owing to amino acid substitution. Ferranti *et al*. (1995) have reported the existence of two forms of ovine α_{s1}-CN, with 191 and 199 amino acid residues respectively, that differ by the deletion of segment 141-148 in the shorter form. The discovery of two forms of ovine α_{s1}-CN in the variants A, C and D indicates that these forms could originate from abnormal mRNA splicing rather than from allelic variations.

Because of the relevance of these genetic variants to the technological properties of milk, it is necessary to characterize them by using effective analytical techniques. Moreover, studies of the genetic variants have always been carried out using conventional electrophoretic techniques. Although these achieve excellent separation, they do not permit good quantitative analysis. As in the case of caprine milk, it would be relevant also to determine whether the genetic variants are related to their content in ovine milk.

Capillary electrophoresis (CE) is an alternative separation technique that has

great potential for the analysis of milk proteins (Chen *et al.* 1992; De Jong *et al.* 1993), and its application to milk protein analysis has been reviewed by Olieman (1993) and Recio *et al.* (1997*a*). Cattaneo *et al.* (1996) have characterized some ovine milk proteins by capillary zone electrophoresis. A CE method has been used to identify the different protein fractions and some genetic variants of caprine (Recio *et al.* 1997*c*), bovine and ovine milk (Recio *et al.* 1997*d*). The genetic variants of β-lactoglobulin and the α_{s1}-CN D variant (also known as the Welsh variant) have been identified by CE in ovine milk.

The present paper describes the application of a rapid and automated CE method to the study of the main genetic variants of α_s-caseins in ovine milk.

materials and methods

Milk samples

Two hundred individual samples of ovine milk were collected from Merino and Manchega breeds of sheep from several local breeders in Spain. Various individual samples that had been analysed by PAGE at pH 8·8 and isoelectric focusing (IEF) using a pH gradient of 2·5–8·0 (López-Gálvez, 1993) were selected according to their genetic pattern. The casein variants were identified by reference to the electrophoretic pattern reported by Chianese *et al.* (1993, 1996).

Caseins were obtained by precipitation from skim milk with acetic acid (100 ml/l) at pH 4·6, followed by centrifugation at 4500 ***g*** and 5 °C for 15 min and freeze drying. FPLC fractions and lyophilized casein ($\sim$ 18 mg) were dissolved in 1 ml diluted CE sample buffer (CE sample buffer : water, 1·5 : 1, v/v) and injected directly on to the CE column.

Isolation of the casein fractions by FPLC

Whole ovine casein fractions were reduced with 2-mercaptoethanol and then fractionated by cation exchange using a Fast Protein Liquid Chromatography apparatus (FPLC®, Pharmacia LKB Biotechnology S-75182, Uppsala, Sweden) on a prepacked Mono S HR 5/5 column (Pharmacia) following the method described by Law *et al.* (1992). Representative material from the peaks obtained was collected after single or multiple runs, depending on the quantity. When necessary, fractions were concentrated by filtration (molecular mass cut-off 10 kDa, Centricon-10; Amicon, Beverly, MA 01915, USA) before their qualitative identification by CE.

Electrophoretic techniques

Whole ovine casein and the fractions collected from the FPLC system were examined by alkaline-PAGE at 0·7 mm thickness, following the method described by Ramos *et al.* (1977). For SDS-PAGE PhastSystem® electrophoresis equipment (Pharmacia Biotech) was used. Homogeneous precast PhastGels 20 were used according to the manufacturer's instructions (Pharmacia, 1986, 1987). IEF of whole ovine casein and the collected fractions from the FPLC separation was carried out using the PhastSystem (Pharmacia) as described by Bovenhuis & Verstege (1989).

Capillary electrophoresis

CE buffers were prepared following the method of Recio & Olieman (1996). Sample buffer (pH 8·6 $\pm$ 0·1) consisted of 167 mM-Tris(hydroxymethyl)-aminomethane (reagent grade, Sigma, St Louis, MO 63178, USA), 42 mM-3-morpholinopropanesulphonic acid (BioChemica MicroSelect; Fluka, CH-9470 Buchs, Switzerland), 67 mM-ethylenedinitrolotetra-acetic acid disodium salt dihydrate (Tritiplex

III; Merck, D-64293 Darmstadt 1, Germany), 17 mM-DL-dithiothreitol (Sigma), 6 M-urea (Sigma) and methylhydroxyethyl cellulose (0·5 g/l, 30000; Serva, D-69042 Heidelberg 1, Germany). The electrophoresis buffer was 0·32 M-citric acid–20 mM-sodium citrate–6 M-urea buffer, pH 3·0 ± 0·1 containing 0·5 g methylhydroxyethyl cellulose/l. Before use, all buffers were filtered (0·22 μm, Sterile Acrodisc® with HT Tuffryn membrane; Gelman Sciences, Ann Arbor, MI 48106, USA).

CE was carried out using a Beckman P/ACE System 2050 controlled by a System Gold Software data system version 810 (Beckman Instruments Inc., San Ramon CA 94583-0701, USA). The sample was injected at the anode using N_2 (3·4 kPa) for 15 s. The separations were performed using a hydrophilically coated fused-silica capillary column (CElect P1, 570 mm × 50 μm i.d., Supelco; Bellefonte, PA 16823, USA) fitted in a cartridge (slit opening 100 × 800 μm). The distance between the detection window and the outlet was 70 mm, resulting in an effective length of 500 mm. Separation conditions were as described by Recio & Olieman (1996), with final applied voltage 25 kV and final current ~ 52 μA. The u.v. detection was at 214 nm. Peaks were identified by injecting the FPLC collected fractions together with lyophilized caseins.

RESULTS AND DISCUSSION

Identification and separation of the casein fractions

Seventy individual samples of ovine caseins were selected according to their genetic variants, as determined by PAGE and IEF. Caseins were fractionated by FPLC and the different casein fractions identified according to the method of Law *et al*. (1992). The elution profiles of a whole casein sample and a sample containing α_{s1}-CN A obtained by cation-exchange FPLC are shown in Fig. 1(*a*) and Fig. 1(*b*) respectively. The FPLC elution profile of samples containing α_{s1}-CN A gave an additional peak (F3*) with a shorter elution time than the usual α_s-CN peak (F3). The F3 peak was always accompanied by a shoulder with a higher elution time (F4), which was collected separately. The fractions collected from FPLC were analysed by IEF. F3 and F3* were identified as α_{s1}-CN and F4 as α_{s2}-CN.

Fig. 2(*a*) shows the capillary electropherograms of an ovine casein and its fractions collected from FPLC. Because of the presence of different ions in the whole casein sample and its collected fractions, the migration time varied slightly. Identification was therefore achieved by injecting the caseins together with the fractions collected from FPLC. In CE, the β-CN fraction (Fig. 2*a*, fraction 1) gave two major peaks and another minor peak with a shorter migration time. The major peak with shorter migration time was assigned to β_2-CN, which contains five phosphate groups (Richardson & Creamer, 1976). The κ-CN fraction (Fig. 2*a*, fraction 2) was resolved by CE as one major peak and a number of minor peaks, which probably corresponded to different glycosylated or phosphorylated forms of κ-CN. Fraction 3, corresponding to α_{s1}-CN, was resolved as three peaks: I, II and III (Fig. 2*a*, *b*). Fraction 3* contained, in addition to peaks II and III, an extra peak with a longer migration time than κ-CN (marked as α_{s1} A in Fig. 2*b*, fraction 3*), which was close to the minor peak in the β-CN fraction. These results agree with those obtained by Chianese *et al*. (1996) and López-Gálvez (1993), who found a fourth band in samples containing the α_{s1}-CN A variant using IEF in ultrathin layer gels. This band, corresponding to α_{s1}-CN A, had the lowest pI of the α_{s1}-CN fraction and, therefore, a longer migration time in CE at acid pH. α_{s2}-CN (Fig. 2*a*, *b*, fraction 4) appeared at the early part of the electropherogram, giving two main peaks and a

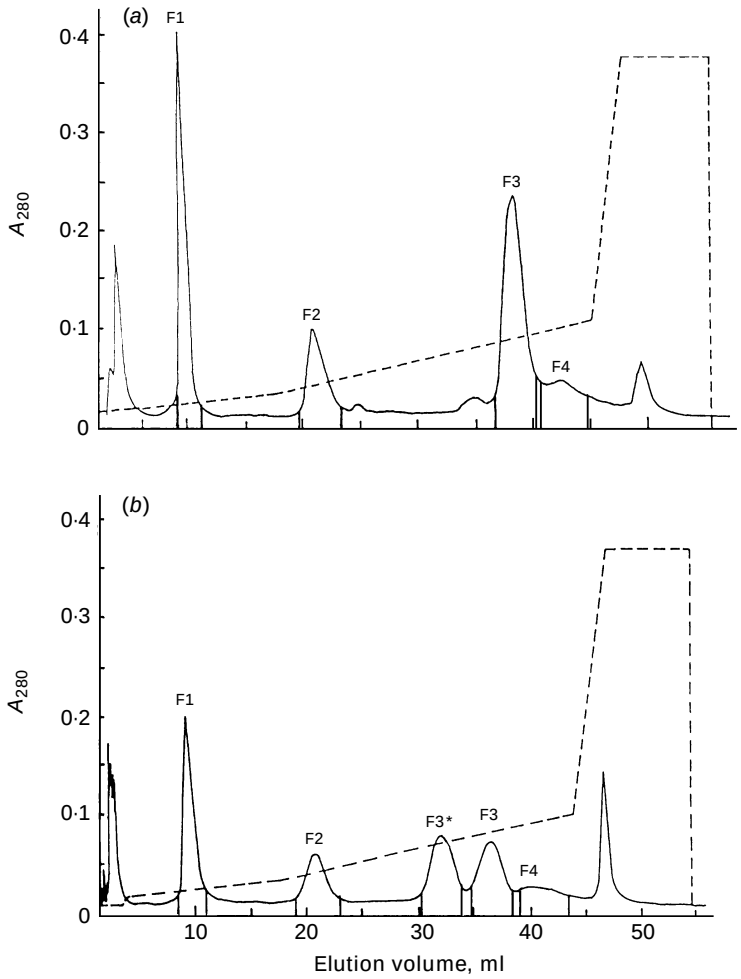

Fig. 1. Elution profiles of (*a*) a reduced whole ovine casein (CN) and (*b*) a reduced whole ovine casein containing α_{s1}-CN A variant obtained by FPLC® on a Mono S HR 5/5 column with 20 mM-sodium acetate–6·0 M-urea buffer, pH 5·0; ––––, NaCl gradient. Fractions F1–F4 and F3* are discussed in the text.

number of minor peaks. Law *et al.* (1992) and Cattaneo *et al.* (1996) did not collect the shoulder of the α_s-CN peak obtained by FPLC (F4) separately, and since this corresponded to the α_{s2}-CN fraction, α_{s2}-CN was not identified in either case. In Fig. 2(*b*), the presence of small amounts of α_{s1}-CN in fraction 4, α_{s2}-CN in fraction 3, and peaks II and III in fraction 3* was probably due to incomplete FPLC separation of the α_s-CN peaks, so that each fraction collected contained some material corresponding to the adjacent peak.

The CE migration order (α_{s2}-CN < α_{s1}-CN < κ-CN < β-CN) agreed with that obtained by PAGE at pH 4·0, described by Dall'Olio *et al.* (1989), Chianese *et al.* (1992) and López-Gálvez (1993), and with that obtained by this CE method in studies of cows' and goats' milk (Recio *et al.* 1997*d*).

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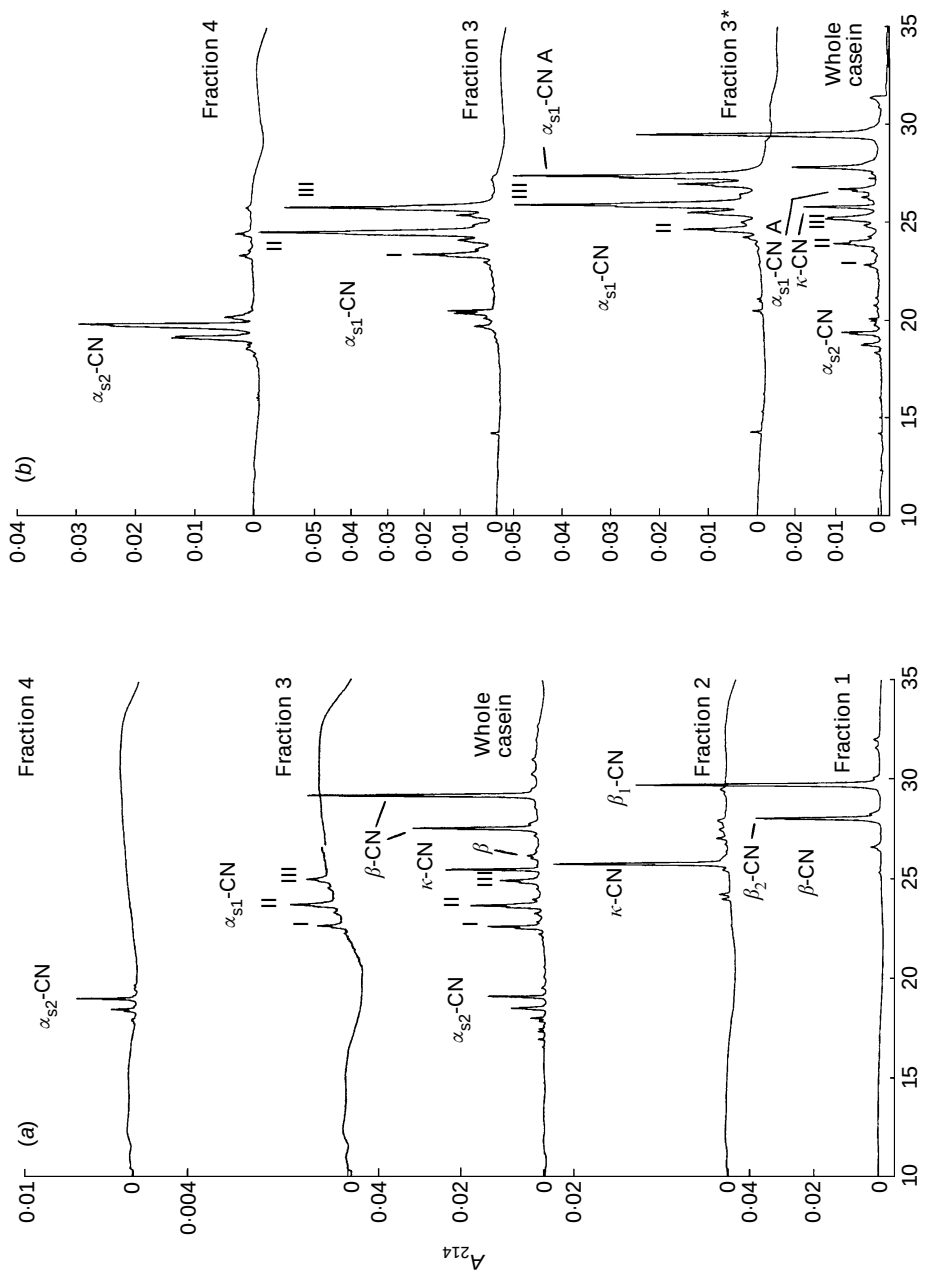

Fig. 2. Capillary electropherograms of (*a*) the whole casein sample and fractions collected from FPLC® and (*b*) the ovine casein (CN) sample containing α_{s1}-CN A variant and fractions collected from FPLC (see Fig. 1). I, II and III indicate the three components of α_{s1}-CN and β the minor component of the β-CN fraction.

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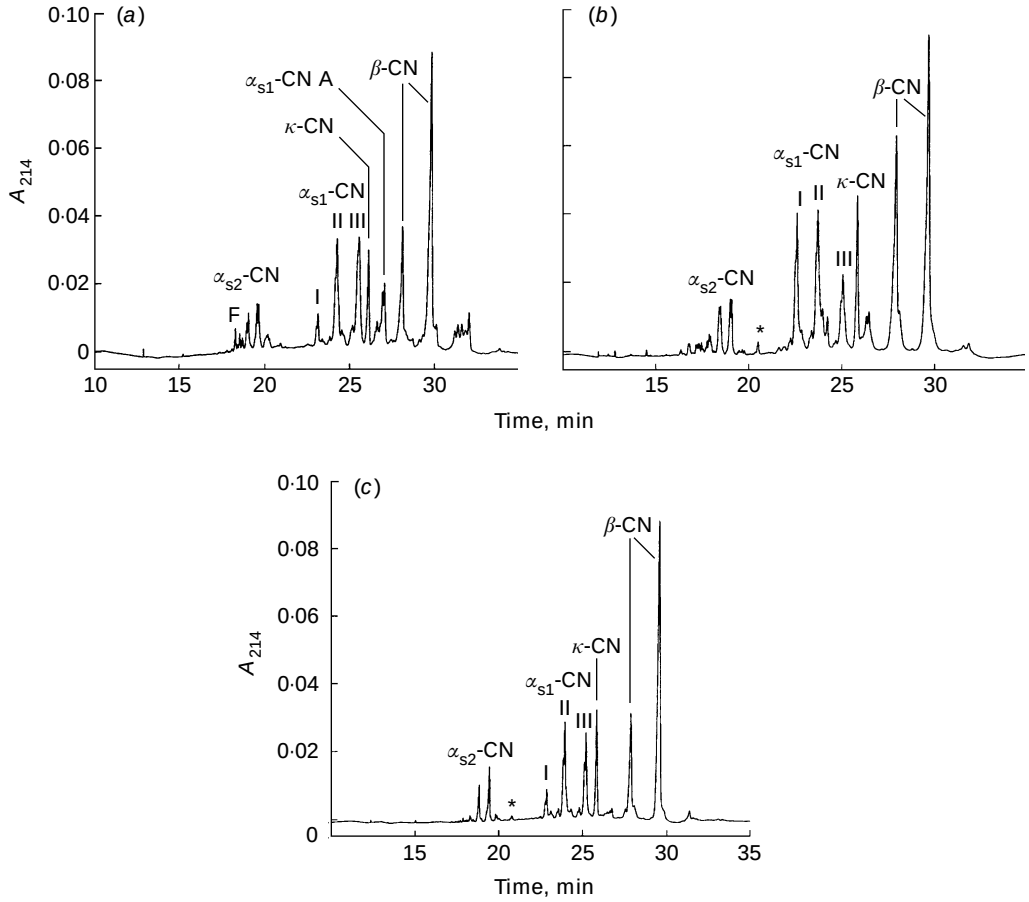

Fig. 3. Capillary electropherograms of three individual ovine casein (CN) samples with (*a*) phenotype α_{s1}-CN AA, (*b*) phenotype α_{s1}-CN CC and (*c*) phenotype α_{s1}-CN BB. F indicates the fast moving α_{s2}-CN variant and * a minor form of the κ-CN fraction. I, II and III indicate the three components of α_{s1}-CN.

Genetic variants of α_{s1}-casein

Fig. 3 shows the electropherograms obtained by CE of three individual samples of casein containing different genetic variants of α_{s1}-CN (as determined by alkaline-PAGE and IEF). It is possible to deduce the phenotype of a sample by CE by considering the intensity of the α_{s1}-CN peaks. Samples corresponding to the homozygous form AA had a CE pattern in which the α_{s1}-CN A peak was very intense, and peak I was small (Fig. 3*a*). A sample phenotyped as α_{s1}-CN CC gave a CE pattern in which peaks I and II had the same intensity, and there was no α_{s1}-CN A peak (Fig. 3*b*). The electropherogram of Fig. 3(*c*) shows a sample containing α_{s1}-CN BB, because intensities of peaks II and III were the same and there was no α_{s1}-CN A peak. Identification of the α_{s1}-CN variants in whole casein samples from heterozygous animals would be expected to be more difficult. In spite of this, in those samples studied we found an excellent agreement between the phenotype determined by IEF and CE. This is shown in Fig. 2(*a*), which corresponds to the heterozygous form of α_{s1}-CN BC. Nevertheless, further studies on the different levels of phosphorylation and expression of the α_{s1}-CN variants are necessary.

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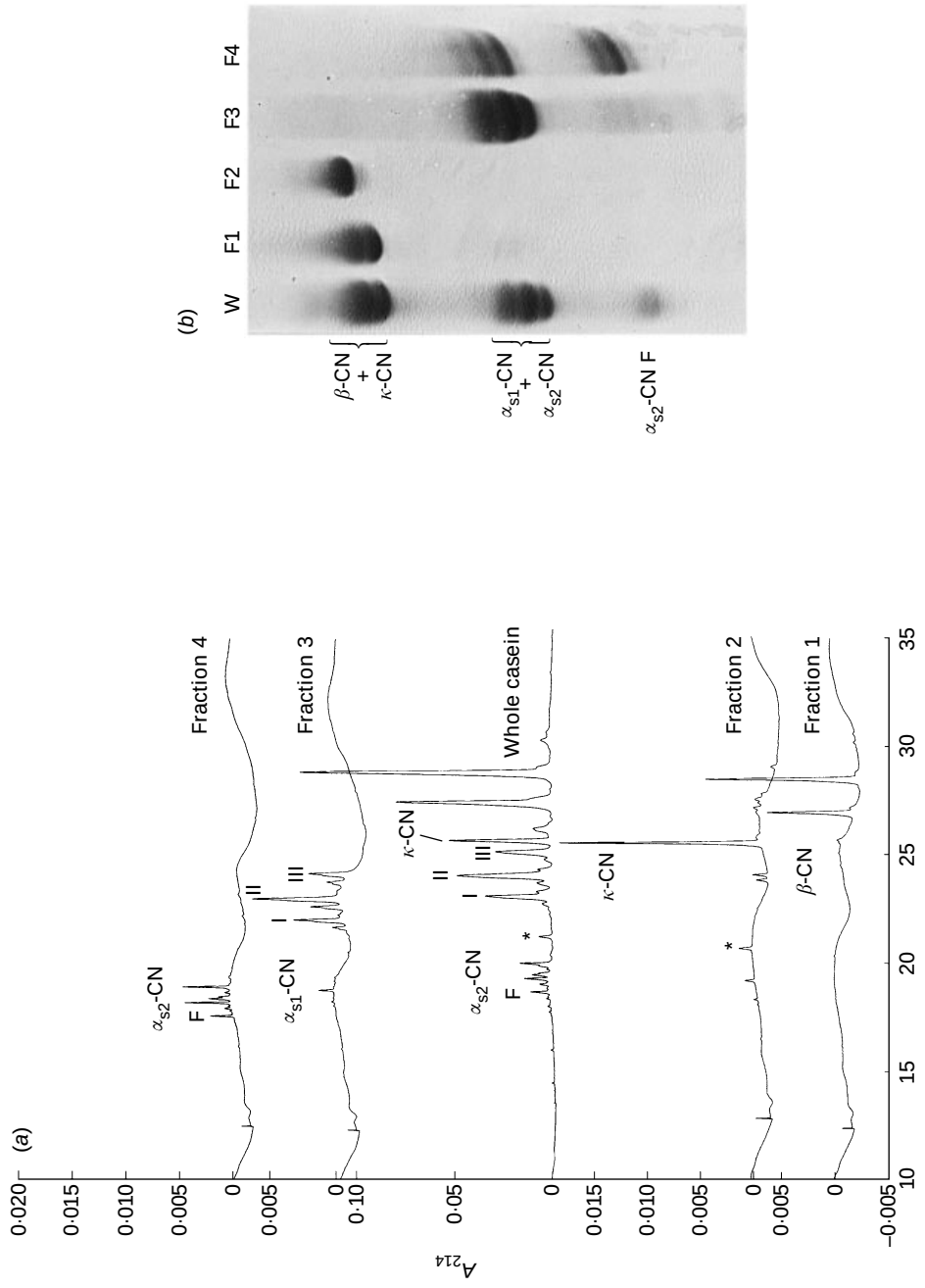

Fig. 4. Electropherograms of an ovine casein (CN) sample containing the fast moving α_{s2}-CN variant and its fractions collected from FPLC®: (*a*) capillary electropherograms and (*b*) PAGE at alkaline pH (8·6) *W*, whole casein; F1, *β*-CN fraction; F2, *κ*-CN fraction; F3, α_{s1}-CN fraction; F4 α_{s2}-CN fraction. F indicates the fast moving α_{s2}-CN and * a minor form of the *κ*-CN fraction. I, II and III indicate the three components of α_{s1}-CN.

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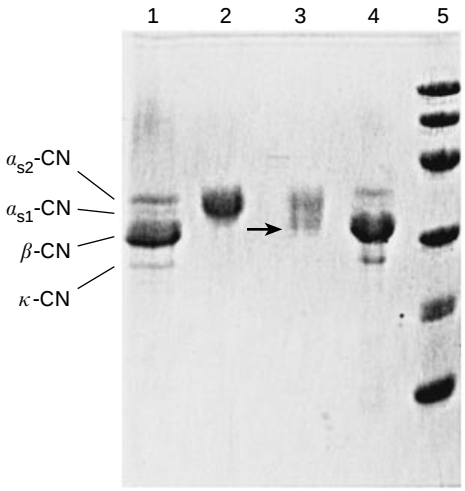

Fig. 5. SDS-PAGE pattern of whole ovine caseins (CN) and the α_{s2}-CN fraction collected from FPLC®. Lane 1, whole casein sample containing the common α_{s2}-CN; lane 2, isolated α_{s2}-CN fraction of a sample containing the common α_{s2}-CN; lane 3, isolated α_{s2}-CN fraction of a sample containing the fast moving α_{s2}-CN variant; lane 4, whole casein sample containing the fast moving α_{s2}-CN variant; lane 5, molecular mass marker (Pharmacia, S-751 28 Uppsala, Sweden), containing (molecular masses, kDa, in parentheses) phosphorylase B (95), bovine serum albumin (64), ovalbumin (43), carbonic anhydrase (30), trypsin inhibitor (20·1) and α-lactalbumin (14·4). →, Band with a lower relative mass than the common α_{s2}-CN.

Genetic variants of α_{s2}-casein

The α_{s2}-CN fraction displayed considerable heterogeneity (Fig. 3). In particular, the two major peaks of the common α_{s2}-CN were resolved either as single (Fig. 3*c*) or split peaks (Fig. 3*a*, *b*). In addition, a number of other minor peaks were observed.

Although there is little information on the gene structure of α_{s2}-CN, Boisnard *et al.* (1991) reported the existence of two ovine genetic variants resulting from substitution of Asn^{49} and Lys^{200} for Asp^{49} and Asn^{200} respectively. Both variants were present as non-allelic long and short forms of α_{s2}-CN. Chianese *et al.* (1993) found a new ovine α_{s2}-CN variant in the Manchega breed that was characterized by higher electrophoretic mobility and lower pI, as determined by alkaline-PAGE and IEF respectively, than the common α_{s2}-CN. CE analysis of nine individual samples containing this fast moving α_{s2}-CN variant, selected on the basis of their alkaline-PAGE pattern, gave an extra peak with a shorter migration time than that of the common α_{s2}-CN, which was tentatively identified as the fast moving variant α_{s2}-CN (marked F in Fig. 3*a*). Using a similar CE method, Cattaneo *et al.* (1996) have recently assigned this variant to a peak present between α_{s2}- and α_{s1}-CN on the grounds that, in a previous FPLC fractionation, the fast moving α_{s2}-CN eluted together with κ-CN from a Mono S column. However, as illustrated in Fig. 4, CE and alkaline-PAGE analyses of the different protein fractions collected from FPLC separation of a sample containing the fast moving α_{s2}-CN provided evidence of its coelution with the common α_{s2}-CN (Fig. 4*a*, *b*, fraction 4), and not with κ-CN (Fig. 4*a*, *b*, fraction 2). Therefore, the peak identified by Cattaneo *et al.* (1996) between α_{s2}- and α_{s1}-CN was likely to have been a minor form of κ-CN (probably with a different glycosylation or phosphorylation pattern), which coeluted with κ-CN by cation-exchange FPLC. Moreover, we found this peak in individual ovine milk samples that did not present the fast moving α_{s2}-CN (marked with an asterisk in Fig. 3*b*, *c*).

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The short migration time of the fast moving α_{s2}-CN variant by CE is consistent with its high electrophoretic mobility in PAGE at acid pH (López-Gálvez, 1993). However, this protein also had high mobility in alkaline-PAGE (Chianese *et al*. 1993), which is indicative of lower molecular mass relative to that of common α_{s2}-CN, probably caused by a deletion in the amino acid sequence. The SDS-PAGE pattern of the isolated α_{s2}-CN fraction of a sample containing the fast variant (Fig. 5, lane 3) gave two bands, one with the same mass as the common α_{s2}-CN (Fig. 5, lane 2) and another (marked with an arrow in Fig. 5) with a lower molecular mass. A distinct band of fast moving α_{s2}-CN could not be detected in the SDS-PAGE analysis of whole casein because it migrated together with α_{s1}-CN (Fig. 5, lanes 1 and 4).

The CE method made possible the simultaneous separation of all the genetic variants described in ovine α_s-CN in a single run. The main genetic variants of α_{s1}-CN A, B and C, and the fast moving α_{s2}-CN variant, were identified. Great heterogeneity was found in α_{s2}-CN, and other protein fractions. The rapid and easy identification of the ovine genetic variants makes this method suitable for screening and complementary to other existing phenotyping methods. It may be particularly useful because of the complexity of the genetic polymorphism of ovine α_s-CN associated with different levels of phosphorylation and non-allelic (long and short) forms. It may also be possible to use this method to determine the level of expression of α_{s1}-CN and α_{s2}-CN.

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