Reverse-phase HPLC analysis of goat caseins. Identification of αs1 and αS2 genetic variants

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Original article

Reverse-phase HPLC analysis of goat caseins. Identification of α_{s1} and α_{s2} genetic variants

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Summary — Separation of caprine α_{s1}-, α_{s2}-, β- and κ-caseins was achieved by a simple and reliable HPLC method, using a reverse-phase C4 column and water/acetonitrile/TFA as solvent. Identification of the fractions separated by this method was performed by both alkaline PAGE and SDS–PAGE, and confirmed by analyses of purified casein fractions, prepared using cation-exchange chromatography. Analysis of individual milk from goats homozygous at the α_{s1}- and α_{s2}-Cn loci revealed that most of the α_{s1}-casein variants (A, B, C, D, F) and the null phenotype, as well as α_{s2}-casein variants A and B are distinguishable. In addition, our method permits the separation of caseinomacropeptide and para κ-casein.

goat milk / casein / chromatography / genetic variant / RP-HPLC

Résumé — Analyse des caséines caprines par HPLC en phase inverse. Identification des variants génétiques des caséines α_{s1} et α_{s2}. La séparation des caséines caprines α_{s1}, α_{s2}, β et κ a été obtenue par une méthode de HPLC simple et fiable, à l'aide d'une colonne de phase inverse C4 et d'un système de solvants eau/acétonitrile/TFA. L'identification des fractions séparées par cette méthode a été effectuée par électrophorèse en gel de polyacrylamide à pH alcalin et par électrophorèse en gel de polyacrylamide en présence de SDS, et confirmée par les analyses de caséines purifiées, préparées par chromatographie d'échange de cations. L'analyse de laits individuels de chèvres homozygotes aux loci α_{s1}- et α_{s2}-Cn a démontré que la plupart des variants (A, B, C, D, F) et le phénotype nul de la caséine α_{s1} peuvent être distingués, ainsi que les variants A et B de la caséine α_{s2}. En outre, notre méthode permet la séparation du caséinomacropeptide et de la para caséine κ.

lait de chèvre / caséine / chromatographie / variant génétique / HPLC en phase inverse

INTRODUCTION

In ruminant milk, whole casein, which accounts for 83% (sheep) to 77% (goat) of milk proteins, consists of 4 proteins (α_{s1}-, α_{s2}-, β- and κ-caseins). Combined with inorganic material, it forms large, stable colloidal particles, referred to as casein micelles. Among ruminants, interspecies comparison of the primary structures of homologous caseins reveals a large similarity, especially between ovine and caprine caseins (Grosclaude, 1991).

Total casein content, as well as the relative proportions of each casein, are characteristic of the species. In ovine, bovine and caprine milks, casein accounts for 45, 25 and 21 g/l, respectively. On the other hand, α_{s1}-, α_{s2}-, β- and κ-caseins are present in bovine milk in the approximate proportions 4:1:4:1, respectively, whereas ovine (2:2:5:1) and caprine (1:2:5:2) milks can be considered as β-casein-rich milks (Assenat, 1985; Remeuf and Lenoir, 1986). The composition of the casein fraction may strongly influence structural characteristics and properties of the micelle and, consequently, lead to variations in technological properties. Physico-chemical parameters of caprine and bovine micelles have been compared by Remeuf *et al* (1989), who reported in particular the effect of the α_s/β-casein ratio on rennetability of goat milk.

Moreover, genetic polymorphism of milk proteins has been shown to be responsible for changes in milk composition and to influence its technological properties (Grosclaude, 1988). Particularly, Losi *et al* (1973) first reported that bovine κ-casein B improves renneting properties of milk. These remarks also apply to goats' milk, for which qualitative as well as quantitative, variability is observed, especially for α_{s1}-casein (Grosclaude *et al*, 1987).

Over the past few years, several studies have been devoted to genetic polymorphism of goat caseins. Following the work of Boulanger *et al* (1984), caprine α_{s1} and α_{s2} fractions were further studied. α_{s2}-Casein exhibits a rather simple genetic polymorphism with two variants (A and B) (Boulanger *et al*, 1984; Grosclaude *et al*, 1987). As far as α_{s1}-casein is concerned, a very unique and complex pattern of genetic polymorphism was demonstrated (Boulanger *et al*, 1984; Grosclaude *et al*, 1987; Mahé and Grosclaude, 1989), for which variants are associated with different protein contents in milk. At present, 6 protein variants, A, B, C, D, E and F, are known, of which the primary structure has been recently established (Brignon *et al*, 1989; 1990). Variants D and F have been shown to have internal deletions of 11 and 37 amino acid residues, respectively. These proteins correspond, at the gene level, to a minimum of 9 different alleles at the locus α_{s1}-Cn, designated A, B, C, D, E, F, F', O and O' (Leroux *et al*, 1990). Alleles O and O' appear to be null alleles; milk from goats O/O, O/O' and O'/O' at the α_{s1}-Cn locus contains very small, if any, amounts of α_{s1}-casein. β-Casein has also been shown to be absent from individual milk of an Italian Garganica breed (Dall'olio *et al*, 1989) and of a Creole breed (Grosclaude and Mahé, personal communication). Finally, a possible genetic polymorphism of κ-casein was inferred from electrophoretical studies carried out on individual goat milks (Di Luccia *et al*, 1986, 1990; Russo *et al*, 1986).

To analyse genetic polymorphism of caprine caseins, various electrophoretic techniques have been used such as SDS–PAGE, alkaline or acid starch gel electrophoresis (Boulanger *et al*, 1984; Russo *et al*, 1986; Grosclaude *et al*, 1987; Dall'olio *et al*, 1989) but none of them appears to be fully satisfactory for both the resolution of α_{s1}- and α_{s2}-caseins and the identification of the relevant variants. Two-dimensional electrophoresis, including or

not isoelectric focusing (IEF) in the second dimension (Addeo *et al*, 1988; Tutta *et al*, 1991), seems to be, to date, potentially the most powerful method.

As an alternative, chromatographic techniques, such as reverse-phase HPLC (RP-HPLC) has been shown to achieve casein fractionation of bovine (Barrefors *et al*, 1985; Visser *et al*, 1986) and caprine milk (Mikkelsen *et al*, 1987). The genetic variants of bovine β-casein have been resolved by RP-HPLC (Carles, 1986). In addition, separation of the genetic variants of bovine κ-casein by anion-exchange chromatography has been reported (Guillou *et al*, 1987).

We are currently studying the physicochemical characteristics of caprine casein micelles, particularly casein dissociation in relation to technological parameters such as pH and temperature. In this connection, a reliable analytical method was needed to determine qualitatively the composition of whole casein and to identify genetic variants in individual goat milks. This paper presents a rapid and reproducible RP-HPLC method by which separation of caprine caseins is reliably achieved with high resolution. It also permits the identification of most of the α_{s1}- and α_{s2}-casein variants, but the quantitative aspect remains to be investigated.

MATERIALS AND METHODS

Deionized water was prepared with a Milli-Q water purification system (Millipore, St-Quentin en Yvelines, France). All common reagents were of analytical grade and purchased from Merck (Darmstadt, Germany). Methanol and ethanol were NORMAPUR analytical reagents from Prolabo (Paris, France).

Preparation of casein samples

Two types of milk, *ie* either raw bulk milk collected in a local herd of mixed breed, near Rennes (Pacé, France), or raw milk from individual goats homozygous at the α_{s1}-Cn locus: O/O, F/F, D/D, E/E and A/A, obtained from the Station de Testage Caprin (48110 Ste Croix Vallée Française, France), were used in this study.

Skim milk was separated by centrifugation at 2500 *g* and 30 °C for 20 min. Whole casein was prepared by acid precipitation (pH 4.2) at 30 °C of skim milk, previously diluted with an equal volume of distilled water, and centrifuged for 10 min at 500 *g*. The precipitate was dispersed in distilled water and dissolved by adjusting the pH to 7.0. Three successive precipitations and washes were performed; the casein was finally freeze-dried.

Other samples, whole caseins containing the A or B variant of α_{s2}-casein and purified α_{s1}-caseins B and C were kindly supplied by Dr MF. Mahé (Laboratoire de Génétique Biochimique, INRA, Jouy-en-Josas, France).

A sample containing para κ-casein and caseinomacropeptide (CMP) was prepared as follows: a solution (20 g/l) of whole casein was treated with a 5% (w/v) rennet solution (rennet powder HALA, Chr Hansen's Laboratory Ltd, St Germain les Arpajon, France) in 50 mmol/l-Na acetate buffer, pH 5.5 to a final concentration of 0.075‰ (w/v). The casein solution was hydrolysed for 30 min at 30 °C and pH 6.5, treated with dithiothreitol (DTT) as described below, and diluted before injection on the HPLC. S-Carboxymethylation of casein was performed essentially as described by Hirs (1967).

Chromatography

The equipment for HPLC consisted of a Waters 600 multisolvent delivery system, a 481 variable wavelength LC spectrophotometer and a 740 data module (Waters, Milford, MA, USA). Separations were carried out on a 15 cm Vydac C4 column 214 TP 54 (Touzart et Matignon, Vitry-sur-Seine, France), maintained at 40 °C with a Prolabo Sup-Rs Stabitherm oven (Paris, France). A 25-cm Vydac C18 column 218 TP 54 was also tested.

Solvents, degassed with helium, were: solvent A, 0.1% (v/v) trifluoroacetic acid (TFA, Pierce) in water; solvent B, 0.096% (v/v) trifluoroacetic acid in 80% (v/v) acetonitrile. For casein separation, elution was achieved using a linear gradient from 37 to 53% solvent B for 30 min

at a flow rate of 1 ml/min, and the absorbance at 214 nm was recorded. For separation of CMP and para κ-casein, the linear gradient was from 27 to 53% solvent B in 50 min.

Before analysis, the casein solution (10 mg/ml) was reduced with 10 mmol/l-DTT for 1 h at 37 °C, then diluted with solvent A (adequate dilution to reach pH 2.0) and filtered through an 0.45 μm Acrodisc filter (Labo Standa, Caen, France). The amount of protein injected was about 50 μg.

Preparative chromatography was performed using Pharmacia equipment: a GP 250 gradient programmer, a single-path 280 nm UV-1 detector, a two-channel REC-482 recorder and a peristaltic pump (Pharmacia LKB Biotechnology, Uppsala, Sweden).

A solution of whole casein (4 g isoelectric freeze-dried casein in 350 ml 0.8 mmol/l-DTT, 25 mmol/l-Na formate, 7.5 mol/l-urea buffer, pH 4.0) was reduced for 2 h at 37 °C and then applied to an XK 50/30 column (5 x 30 cm) containing 230 ml of S-Sepharose Fast-flow cation exchanger (Pharmacia). The column was equilibrated with 0.064 mmol/l-DTT, 75 mmol/l-Na formate, 7.5 mol/l-urea buffer, pH 4.0. Elution was carried out at room temperature at a flow rate of 2 ml/min by a linear NaCl gradient from 0 to 300 mmol/l for 22 h. Casein fractions were collected with a FRAC-300 collector (Pharmacia), ultrafiltered and diafiltered with a CH2A Amicon concentrator (Amicon Corporation, Lexington, MA, USA) fitted with an H1P10-20 hollow fiber cartridge (cut-off 10 000 Da) to remove excess urea. Finally, fractions were dialysed, adjusted to pH 7.0 and freeze-dried.

Electrophoretic techniques

Electrophoresis was performed in a Bio-Rad Mini Protean II apparatus (Bio-Rad Laboratories, Richmond, CA, USA) with miniature gels (70 x 80 x 0.75 mm).

SDS–PAGE was performed according to the method of Laemmli (1970), modified as follows: gels containing 3.6% (stacking gel) or 14% (separation gel) acrylamide were prepared from 29.2% (w/v) acrylamide (Bio-Rad) and 0.8% (w/v) *NN'* methylene bis-acrylamide (Bio-Rad) stock solutions. The final concentrations in the separation gel were 0.3 mol/l–Tris–HCl (Trizma base Sigma), pH 8.9 and 0.1% (w/v) SDS (Sigma). The stacking gels were prepared in 0.06 mol/l-Tris-HCl, pH 6.8, and 0.1% (w/v) SDS. Gels were polymerized by the addition of 0.5‰ (v/v) tetramethylethylenediamine (TEMED) and 0.5‰ (w/v) ammonium persulphate.

The electrode buffer contained 50 mmol/l-Tris, 0.38 mol/l-glycine and 0.2% (w/v) SDS. Samples were diluted with the denaturing solution and kept overnight at 4 °C. Just before analysis, β-mercaptoethanol was added (5%; v/v) to the samples which were heated in a boiling water-bath for 5 min.

Electrophoresis was carried out for 45 min at 200 V. Gels were stained for 0.5 to 1 h at room temperature in a 0.1% (w/v) solution of Coomassie blue R-250 in methanol/water/acetic acid (50/40/10; v/v). The gels were destained by repeated washing for 1 to 3 h in a 30% (v/v) ethanol, 10% (v/v) acetic acid solution.

PAGE at pH 8.9 was performed as described by Andrews (1983). Staining and destaining were performed as described previously.

RESULTS AND DISCUSSION

RP-HPLC fractionation of whole caprine casein

The first separations were performed on whole casein prepared from bulk milk.

The chromatographic profiles from a C4 column, presented in figure 1, show: (a) the separation of casein fractions with and (b) without DTT. As already reported by Mikkelsen *et al* (1987), these patterns clearly demonstrate the influence of a reduction treatment. Indeed, without DTT, total goat casein was separated into 3 main fractions (I, II and III), whereas in the presence of DTT a fourth peak occurred. Peaks A and B probably arise from peak I; peaks II and III (fig 1a), which appear not to be affected, likely correspond to peaks C and D (fig 1b). In order to check the efficiency of this reduction treatment, caseins were alkylated. *S*-Carboxymethylated

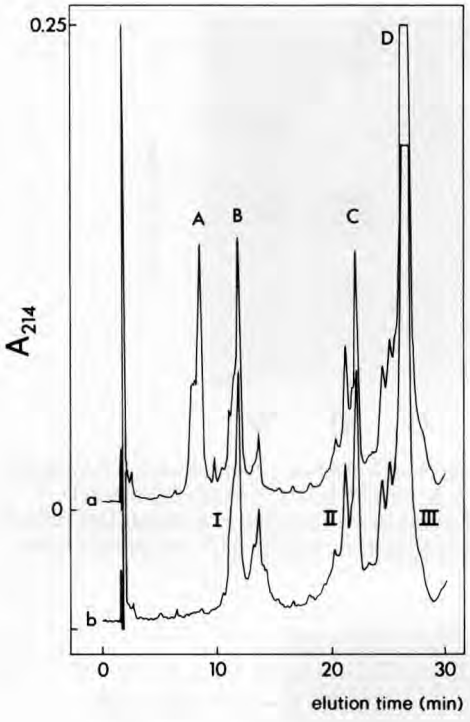

Fig 1. Separation of whole caprine casein by RP-HPLC (Vydac C4 column). Effect of DTT treatment: 10 mmol/l DTT-treated casein **(a)**, unreduced casein **(b)**. Amount of protein injected: 70 µg.
*Séparation par HPLC en phase inverse sur colonne Vydac C4 de caséine entière caprine. Effet du traitement de réduction : **(a)** caséine réduite avec 10 mmol/l-DTT, **(b)** caséine non réduite. La quantité de protéine injectée était de 70 µg environ.*

caseins gave the same profile as figure 1a (results not shown). Different concentrations of DTT (1 mmol/l to 30 mmol/l) were tested in the preparation of samples. Although 1 mmol/l is sufficient to achieve complete reduction, we have chosen 10 mmol/l for safety purposes. The effect of temperature (20 to 60 °C) on peak separation was also examined: appreciable differences in the pattern were observed. At 20 °C, fractions C and D were not satisfactorily resolved, whereas 60 °C did not allow a good separation of the first two fractions. We finally chose to operate at 40 °C, which seems the best compromise to achieve good separation of all peaks, at a 1 ml/min optimal flow rate.

A C18 column, also tested for this application, gave identical profiles but it was decided not to use it because small amounts of β-casein were always retained on the column.

Identification of fractions separated by RP–HPLC

Fractions A, B, C and D, separated by RP-HPLC were collected for analysis by both alkaline PAGE (pH 8.9) and SDS–PAGE.

Patterns obtained by PAGE at pH 8.9 (fig 2) allowed unambiguous identification of fractions A and D as κ- and β-casein, respectively, with reference to Addeo *et al* (1978) and Boulanger *et al* (1984). In alkaline PAGE, β-casein migrates as 2 bands, corresponding to β_1- and β_2-caseins, which differ in their level of phosphorylation (6/5) (Richardson and Creamer, 1974). Fractions B and C migrate ahead of κ-casein, in the region where Boulanger *et al* (1984) have located α_s-caseins. Indeed, these authors have shown that α_{s1}- and α_{s2}-caseins co-migrate at this position under these conditions, confirming that electrophoresis at alkaline pH was unsatisfactory for the separation of this α_s-casein complex.

Patterns obtained by SDS–PAGE of HPLC fractions are shown in figure 3. Fraction D occurred as the more intense band, which corresponds to β-casein. κ-Casein, which migrated just ahead of β-casein in whole casein sample, was separated as fraction A. SDS–PAGE allows

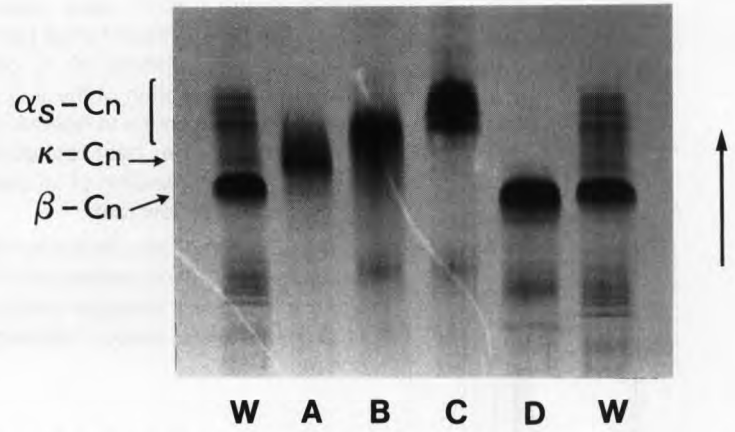

Fig 2. Alkaline PAGE (pH 8.9) in the presence of 4.5 mol/l-urea of whole caprine casein and fractions separated by RP-HPLC. W: whole casein; A: fraction A; B: fraction B; C: fraction C; D: fraction D.
Électrophorèse en gel de polyacrylamide à pH alcalin en présence d'urée 4,5 mol/l de caséine entière caprine et des fractions séparées par HPLC. W: caséine entière; A : fraction A; B : fraction B; C : fraction C; D : fraction D.

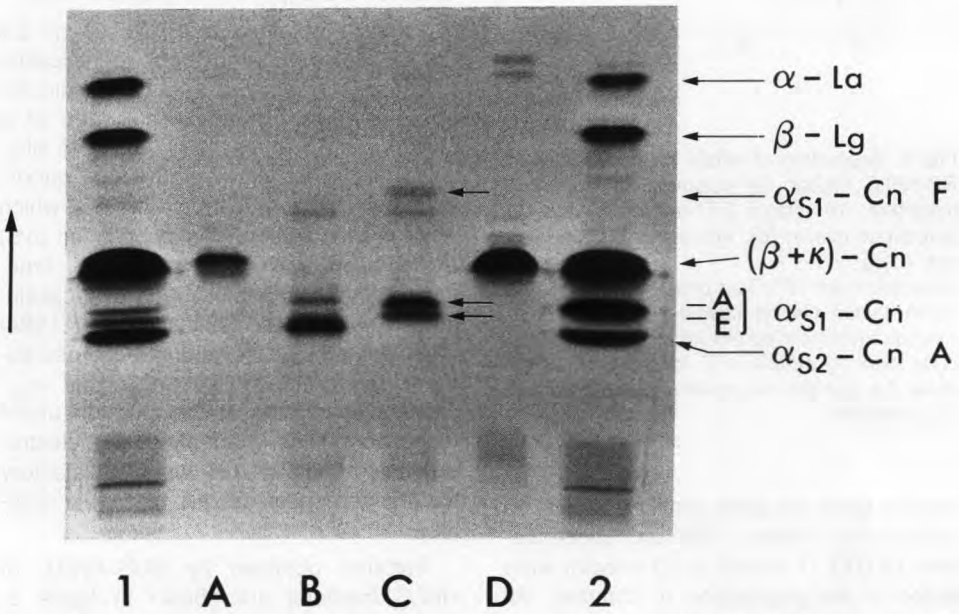

Fig 3. SDS–PAGE of milks from individual goats and fractions separated by RP-HPLC (samples A, B, C and D as in figure 2; samples 1 and 2 are milks from individual goats heterozygous E/F and homozygous A/A at the locus α_{s1}-Cn respectively).
Électrophorèse en gel de polyacrylamide en présence de SDS de laits de chèvre individuels et des fractions séparées par HPLC (Les échantillons A, B, C et D sont les mêmes qu'en figure 2; 1 : lait de chèvre hétérozygote E/F; 2 : lait de chèvre homozygote A/A au locus α_{s1}-Cn).

identification of fraction B, the slowest-migrating band, as α_{s2}-casein, while fraction C which gave a set of four bands, corresponds to α_{s1}-casein. The fastest-migrating doublet was further shown to represent variant F of α_{s1}-casein, whereas the lower bands migrate at the same level as α_{s1}-casein A and E variants (see *Separation of genetic variants).*

In both electrophoretic systems, fraction D (β-casein) appeared to be contaminated by small amounts of other components, which migrated more slowly than β-casein in alkaline PAGE, and behaved as low molecular mass compounds in SDS-PAGE. These components, which correspond to shoulders preceding the β-casein peak in the RP-HPLC pattern, were further identified as γ-caseins (manuscript in preparation).

Preparative fractionation of goat caseins using ion-exchange chromatography

In order to prepare purified casein fractions, whole caprine casein was chromatographed on S-Sepharose cation exchanger. Four fractions, S1, S2, S3 and S4, were separated (fig 4), collected and identified by both PAGE at pH 8.9 and SDS–PAGE. Caseins are eluted in the following order: β, κ, α_{s1}, α_{s2}, which is consistent with previous results (Brignon, personal communication). These fractions (S1 to S4) were analysed in RP-HPLC (fig 5). Although they are not homogeneous and required an additional purification step, each fraction S1, S2, S3 and S4 gave elution profiles characteristic for β-, κ-, α_{s1}- and α_{s2}-casein, respectively, thus confirming our first identification.

Anion-exchange chromatography, under conditions usually used to fractionate bovine caseins (Guillou *et al,* 1987), does not allow separation of the 4 caprine caseins. Conversely, cation-exchange chromatography, using the conditions previously described, proved to be satisfactory. This method could have been applied on an analytical scale, using as exchange support a Mono-S HR 5/5 column, equivalent to S-Sepharose, but it was decided not to use it for the following reasons. Firstly, working at pH 4.0, near caprine casein pI, requires the use of buffers containing a high molarity of dissociating agent (at least 7.5 mol/l-urea), which, consequently, generates a high pressure on the column because of

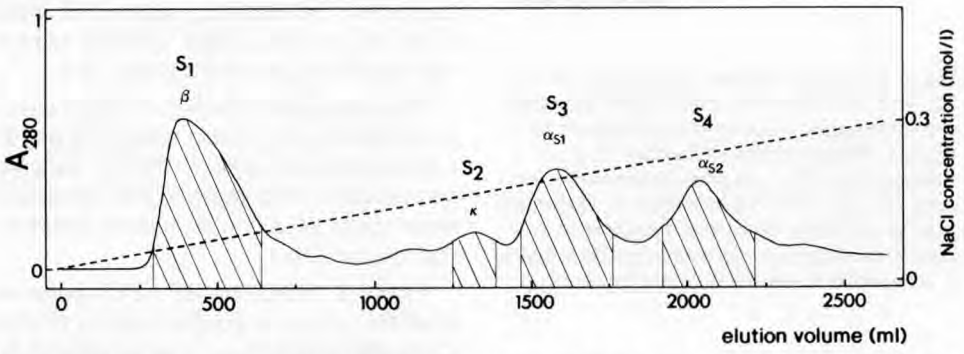

Fig 4. Fractionation of whole caprine casein by cation-exchange chromatography on S-Sepharose Fast flow.
Fractionnement de caséine entière caprine par chromatographie d'échange de cations sur résine S-Sépharose Fast-flow.

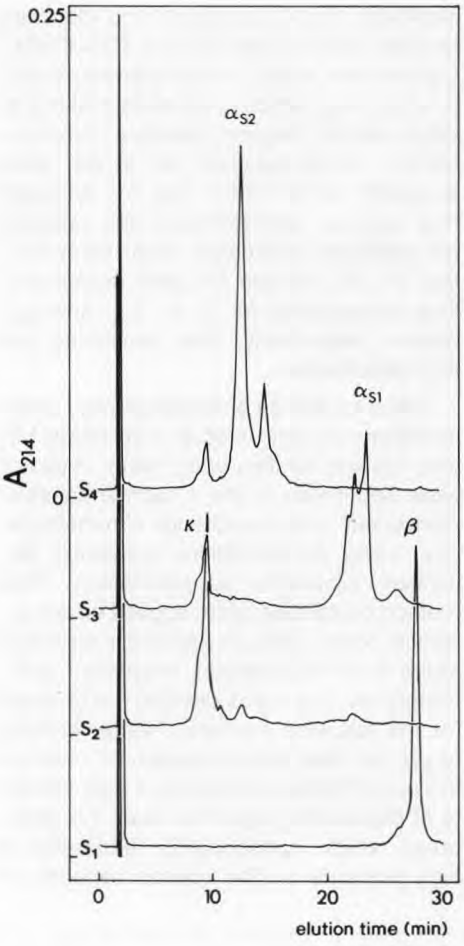

Fig 5. RP-HPLC analyses of fractions S1, S2, S3 and S4 collected from cation-exchange chromatography. Experimental conditions as in figure 1. Amount of protein injected: 15 μg.
Analyses par HPLC en phase inverse des fractions S1, S2, S3 et S4 collectées en chromatographie d'échange de cations. Les conditions expérimentales sont identiques à celles de la figure 1. La quantité de protéine injectée est de 15 μg.

increased buffer viscosity. Secondly, results were shown not to be very reproducible as has been reported recently (Collin *et al,* 1991) for anion-exchange chromatography on a Mono-Q HR 5/5 column.

Fractionation of rennet-treated whole casein

RP-HPLC patterns of whole casein (a) and the same whole casein treated with rennet (b) are compared in figure 6. As for the bovine protein, caprine κ-casein is specifically cleaved by chymosin between the amino acid residues 105 and 106 (Mercier *et al,* 1976), producing a highly hydrophobic peptide 1-105 (para κ-casein) and an acidic, soluble peptide 106-171 (caseinomacropeptide). Recording the absorbance at 280 nm allowed us to identify the CMP peak: CMP eluted ahead of all the casein components, whereas para κ-casein was eluted just after intact κ-casein. Therefore, para κ-casein and CMP are clearly distinguished.

Resolution of genetic variants

Caseins obtained from individual milks of homozygous goats at the α_{s2}- and α_{s1}-Cn loci were analysed, using the optimized RP–HPLC method. The results reported here show that both α_{s2}-casein and most of the α_{s1}-casein genetic variants can be reproducibly resolved in a single step.

Chromatographic profiles of whole caseins containing α_{s2}-casein variants A and B are presented in figure 7. The two variants can be easily distinguished and identified, since there is a 1-min interval between their elution times.

Figure 8 shows the RP-HPLC patterns of whole caseins or purified caseins (B and C) corresponding to α_{s1}-casein variants A, B, C, D and F, the amino acid substitutions which are given in table I. From bottom to top casein from bulk milk and α_{s1}-casein

variants are compared in their order of elution. To simplify this figure, the chromatographic profile of 'type null' whole casein obtained from goats homozygous (O/O) at the locus α_{s1}-Cn is not given. However, such a pattern, which is characterized by the absence of peaks in the region of α_{s1}-casein, is shown in figure 7. Separation of most of the variants was effective, and might be significantly improved by flattening the elution gradient. However, variant E, which was present in whole casein from bulk milk (fig 8W), co-elutes with variant B. This result is consistent with amino-acid sequence data, since these 2 variants only differ by 2 substitutions (Arg100 —> Lys and Thr195 —> Ala). Conversely, α_{s1}-casein variants D and F, which have inter-

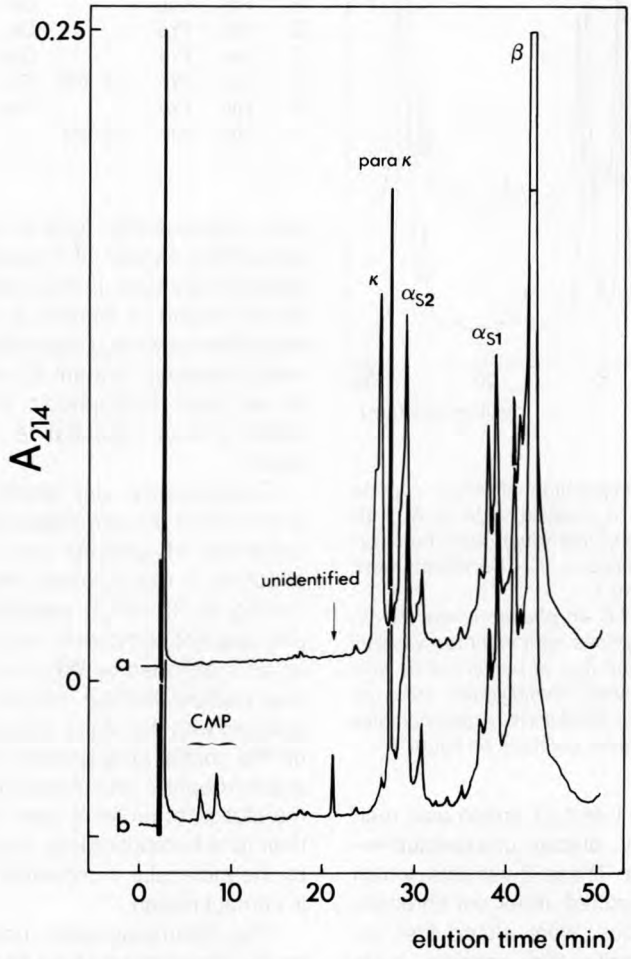

Fig 6. RP-HPLC separation of whole caprine casein. Effect of rennet treatment: untreated whole casein (a), rennet-treated casein (b). Amount of protein injected: 70 µg.
Séparation par HPLC en phase inverse de caséine entière caprine. Effet du traitement par la présure : (a) caséine témoin, (b) caséine additionnée de présure. La quantité de protéine injectée était de 70 µg.

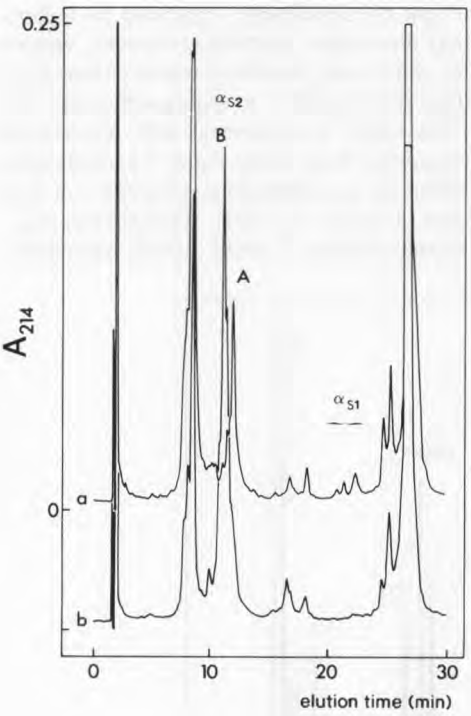

Fig 7. RP-HPLC separation of whole caprine caseins containing α_{s2}-casein variants A or B, isolated from milks of individual goats homozygous (O/O) at the locus α_{s1}-Cn. Conditions were the same as in figure 1.
Séparation par HPLC en phase inverse de caséines entières caprines contenant les variants A et B de la caséine α_{s2}, et provenant de laits individuels de chèvres homozygotes (0/0) au locus α_{s1}-Cn. Les conditions expérimentales sont identiques à celles données en figure 1.

nal deletions of 11 and 37 amino acid residues respectively, display unexpected retention behaviour. These 2 variants, which can easily be identified under our chromatographic conditions, were eluted first, although theoretically they should have been eluted after the other variants since they were expected to be more hydrophobic than the complete α_{s1}-casein. Indeed, both deletions, which start at the same position (residue 59), lead to the loss of the hydrophilic cluster of 5 contiguous phosphoseryl residues. In the same way, the order of elution of variants A and C should have been reversed, according to their primary structure. Variant C, which appears to be more hydrophobic than variant A (table I), was expected to elute after the latter.

Table I. Positions of the amino acid substitutions occurring within the primary structure of the different caprine α_{s1}-casein variants.
Positions des substitutions d'acides aminés rencontrés dans la structure primaire des différents variants de caséine as1 caprine.

	8	*16*	*Deletion*	*77*	*100*	*195*
A	His	Leu		Gln	Arg	Thr
B	His	Pro		Glu	Arg	Thr
C	Ile	Pro		Glu	Lys	Ala
D	His	Pro	(58–69)	Glu	Arg	Thr
E	His	Pro		Glu	Lys	Ala
F	His	Pro	(58-95)		Arg	Thr

Consequently, our results confirm that prediction of the chromatographic retention behaviour of proteins from their primary structure is not possible. Mechanisms occurring in RP-HPLC separation are complex and not completely explained. However, as mentioned by Wilce *et al* (1991), it is now well established that the interaction of proteins with the stationary phase depends on the spatial arrangement of hydrophobic and hydrophilic amino acid residues. Thus, the elution of proteins does not result from their total hydrophobicity, but is determined by the molecular composition of the specific contact region.

The chromatographic profiles obtained by this analytical method showed great repeatability and reproducibility. However, it should be stated that although differences between elution times of different variants remain constant, elution times may vary

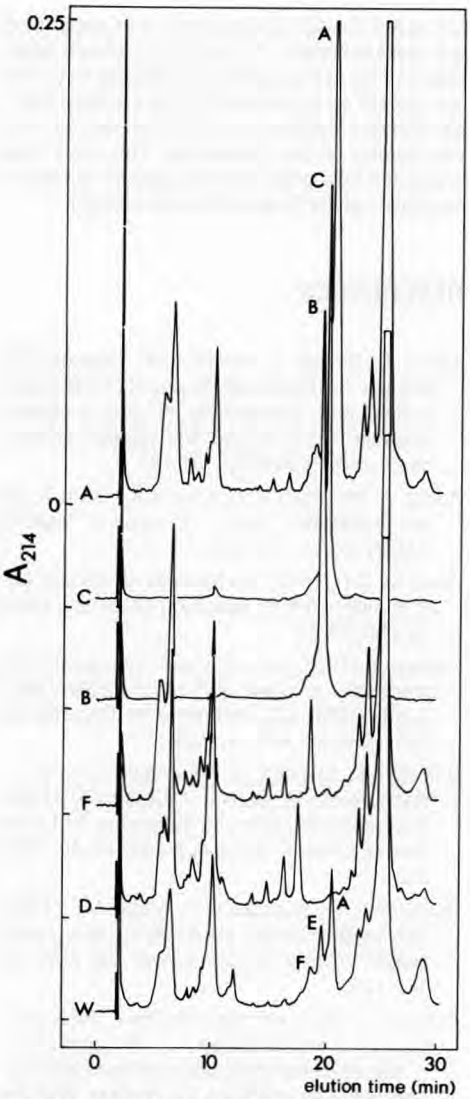

Fig 8. RP-HPLC separation of caseins containing α_{s1}-casein variants A, B, C, D and F. Conditions are as in figure 1. W: whole casein from bulk milk. B and C: purified caseins. Amount of protein injected: 50 μg for variants A, D, F, W; 10 μg for variants B and C.
Séparation par HPLC en phase inverse de caséines contenant les variants A, B, C, D et F de la caséine α_{s1}. Conditions identiques à celles de la figure 1. W: caséine entière de lait de mélange. Les quantités de protéine injectées étaient de 50 μg pour les variants A, D, F et W, et de 10 μg pour les variants B et C.

slightly from day to day. Therefore, the identification of α_{s1}-casein variant with confidence may require analysis, under the same conditions, of a standard sample containing 1 or several variants.

Analysis of a bulk milk (profile W in figure 8) allowed us to determine its casein variant composition. This milk probably contained α_{s2}-caseins A and B (variant A was the major fraction), and at least 3 α_{s1}-casein variants (F, E or B and A). These results are in agreement with the frequencies of the α_{s1}- and α_{s2}-casein alleles found by Boulanger *et al* (1984) and Grosclaude *et al* (1987). Indeed, α_{s2}-casein A is predominant (about 85%), and α_{s1}-casein F, E and A are the most frequent alleles in both Alpine and Saanen breeds (close to 40%, 37% and 10%, respectively).

By analysing an individual milk from a Corsican goat breed, κ-casein occurs as 2 peaks, which after chymosin treatment, gave 2 peaks probably corresponding to 2 para κ-caseins (results not shown). Our results may be connected with those of Di Luccia *et al* (1990), who first detected, by IEF and titration curve, the presence of 2 κ-casein in a local Italian population. They demonstrated that the substitution differentiating variants A and B is located within the para κ-casein moiety. These results strongly suggest that a genetic polymorphism also exists as far as goat κ-casein is concerned. However, further analyses are needed to conclude definitively.

CONCLUSION

The RP-HPLC method described here allows the complete separation of the caprine caseins, α_{s1}, α_{s2}, β, κ, as well as CMP and para κ-casein. In addition, this method permits the resolution of the α_{s2}-casein A and B variants, of most of the α_{s1}-casein variants (A, B, C, D, F) and the identification of the null phenotype.

In comparison with electrophoretic techniques, our method is probably more time-consuming for the analysis of many samples, since it requires the preliminary preparation of isoelectric casein. Moreover, although the RP-HPLC method gave very high resolution, α_{s1}-casein B and E variants remained, as with SDS–PAGE, indistinguishable. On the other hand, it allowed separation of α_{s1}-casein C and B, which is not possible by SDS–PAGE. Thus, it can be considered as a complementary technique for the identification of genetic variants. In addition, this chromatographic method appears to be especially interesting, because it provides, in a single step, unlike electrophoretic methods, fractionation and genetic variant resolution of both α_{s1}- and α_{s2}-casein. Furthermore, separation of α_{s1}- and α_{s2}-caseins is a real advantage for the quantitative aspects that we are currently investigating. It seems that quantification of the 6 main milk proteins might be reasonably expected, since β-lactoglobulin, which is eluted after β-casein, can now be easily quantified. On the other hand, α-lactalbumin, which co-elutes with α_{s1}-casein under our chromatographic conditions, still poses a problem.

Finally, it is worth noting that this method can also be applied to bovine and ovine casein fractions separation (results not shown).

NOTE ADDED DURING THE EDITING

Concurrent to the editing of this manuscript, Visser *et al* (1991; *J Chromatogr* 548, 361-370) published a very similar RP-HPLC method for the separation of bovine casein fractions and the main genetic variants of bovine proteins.

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