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Phenotyping of bovine milk proteins by reversed-phase high-performance liquid chromatography

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ABSTRACT

A reversed-phase high-performance liquid chromatographic method for the separation of the most common and some less common genetic variants of the bovine caseins is described. When the method is used for analysing clarified skim milk, simultaneous identification of casein variants and major whey protein variants can be effected in a single run. The potential of the method for quantitative application is discussed.

INTRODUCTION

About 80% of the bovine milk proteins consists of caseins, a heterogeneous fraction which is insoluble at its isoelectric pH (pH 4.6). The casein fraction can be subdivided into the α_{s1}-, α_{s2}-, β- and κ-casein components (α_{s1}CN, α_{s2}CN, βCN and κCN), which in milk occur as a micellar complex in the approximate proportions 4:1:4:1, respectively. The remaining 20% of the milk protein fraction is formed by the whey proteins (soluble at pH 4.6), of which β-lactoglobulin (βLg) and α-lactalbumin (αLa) are the main components (ratio *ca.* 3:1). During the classical cheesemaking process it is the casein fraction which constitutes the cheese curd after the enzyme-triggered milk coagulation step.

The caseins and the major whey proteins have been found to show genetic polymorphism [1]. In western breeds certain genetic variants occur exclusively or are strongly predominant.

During the last decade studies on milk protein polymorphism have gained renewed interest, because the occurrence of certain milk protein genetic variants is correlated with the composition of milk and also with some milk processing parameters. For instance, the genotype κCN-BB has been reported to be associated with enhanced rennetability of the milk [2–6], casein content [4], cheese curd firmness [2,5], curd syneresis [7–9] and overall cheese yield [5,7,9]. It has also been found that the κCN-BB genotype is accompanied by a relatively high Ca^{2+} activity [10] and low citrate concentration [3] in the milk, but the difference between the effects of the κCN A and B variants on the renneting properties can be eliminated by addition of calcium

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chloride [2,10]. The latter facts may provide a more direct explanation for the observed relationship between κCN genotype and rennetability of milk. The B variant of βLg is also associated with some of the above-mentioned favourable technological properties [3,6,10,11]. Consequently, the combined occurrence of κCN-BB and βLg-BB genotypes could be of particular importance for obtaining improved cheese technological parameters [12,13]. Other properties reported to be influenced by milk protein genetic polymorphism are total milk production [12,14,15], heat stability of milk [16–19], water sorption of the casein fraction [18] and fat content of cheese [9,15,20]. Conclusions in reports concerning the relationship between protein genotype and various technological properties are not always consistent [5,20]; the influence of the season should also be considered [18].

From the above it follows that for the optimum selection of milk for further processing, a reliable method for the qualitative and quantitative determination of milk protein genetic variants should be available. Reported procedures for the identification of genetic milk protein variants are classical gel electrophoresis [16,17,21–23], isoelectric focusing [24–28] and, in the case of βLg, high-performance liquid chromatography (HPLC) [29–33]. Recently, for κCN genotypes, identification at the DNA level has been reported [34–36].

In this paper we describe a reversed-phase (RP) HPLC method by which the most common and also some less common casein variants can be separated. The method permits the simultaneous identification of various casein variants and the whey proteins αLa-B, βLg-A and βLg-B in a single chromatographic run. Furthermore, attention is paid to the quantitative aspects of the method.

EXPERIMENTAL

Materials

Whole casein was isolated from skimmed bulk milk or from skim milk from individual cows by isoelectric precipitation at pH 4.6; the soluble fraction contained the whey proteins. Purified casein components were gifts from Dr. D. G. Schmidt. *para*-κCN (the 1–105 fragment of κCN) and the κCN glycomacropeptides (GMP-A and GMP-B, both representing non- and differently glycosylated 106–169 fragments) were obtained by splitting κCN-A and κCN-B with chymosin at pH 6.5, which results in a precipitate (*para*-κCN) and a soluble fraction (GMP-A and GMP-B, respectively).

Analysis

The HPLC equipment consisted of two M 6000A pumps (Waters Assoc.), an ISS-100 automatic sample injector (Perkin-Elmer), a Kratos Model 783G UV detector and a Waters Type 680 automated gradient controller. The equipment was linked to a data acquisition and processing system (Waters Maxima 820). A 250 mm × 4.6 mm I.D. HiPore RP-318 column (Bio-Rad Labs.) was used with a C_{18} cartridge (Bio-Rad Labs.) as a guard column. Solvent A was acetonitrile–water–trifluoroacetic acid (100:900:1 v/v/v) and solvent B was the same mixture with the proportions 900:100:0.7 (v/v/v). The solvent gradient reported for casein separation [37] was adapted for genetic casein variants and whey proteins: starting from 26% of solvent B (equilibration buffer) a gradient was generated immediately after injection by in-

creasing this proportion at 0.60% min^{-1} (15 min), 0% min^{-1} (7 min), 0.67% min^{-1} (3 min), 0% min^{-1} (12 min), 0.44% min^{-1} (18 min), 12.5% min^{-1} (2 min) and 0% min^{-1} (5 min) subsequently, before returning to starting conditions in 5 min. After re-equilibration, the next analysis was carried out under the same conditions. If only casein variants were to be separated, the last three steps of the gradient were replaced by 6.6% min^{-1} (5 min) and 0% min^{-1} (5 min), subsequently, before returning to the starting conditions in 5 min. The column temperature was 30°C and peak detection was at 220 nm. The flow-rate was 0.8 ml min^{-1}, except in the determination of response curves for the caseins, when it was 1.0 ml min^{-1}. The system pressure was 1500 p.s.i.

Freeze-dried samples were dissolved in 0.02 *M* 1,3-bis[tris(hydroxymethyl)-methylamino]propane (Bis–Tris) buffer (pH 7) containing 4 *M* urea and 0.3% of 2-mercaptoethanol (ME). After standing at room temperature for 1 h, the samples were diluted (at least four-fold) with solvent A containing 6 *M* urea. The final sample concentration was about 2 mg ml^{-1} for whole casein and 0.5 mg ml^{-1} for casein components and whey proteins; 50-μl amounts were applied to the column.

Clarified milk samples were obtained by mixing skim milk with a buffer (1:1, v/v) containing 0.1 *M* Bis–Tris, 8 *M* urea, 0.3% ME and 1.3% trisodium citrate dihydrate (pH 7); in some experiments ME was omitted from the buffer. After standing at room temperature for 1 h, the mixture was diluted with solvent A containing 6 *M* urea as above. Whole casein and whey protein fractions were isolated from the same skim milk and prepared for HPLC analysis, the whey protein solution (pH 4.6) being directly diluted with solvent A containing 6 *M* urea.

For the determination of response curves for the various milk proteins, use was made of freeze-dried, purified components of known protein content.

RESULTS AND DISCUSSION

For the main κCN variants, *i.e.*, types A and B, we examined the influence of various glycosylation states on the RP-HPLC pattern. In Fig. 1 patterns for carbohydrate-free fractions and fractions with different levels of glycosylation [38] are depicted. It appears that the first-eluting protein peak of whole casein from bulk milk (reference) represents a mixture of carbohydrate-rich κCN A and B. The carbohydrate-free κCN A and B subcomponents are eluted at different positions and can therefore be distinguished from each other. Minor peaks at intermediate positions originate from partly glycosylated κCN A or B. The small peak(s) immediately following those of carbohydrate-free κCN A and B (also seen in the pattern of whole κCN-B; Fig. 1B, trace *d*) should probably be attributed to remaining non-reduced κCN or to a complex between κCN and α_{s2}CN, formed by intermolecular S–S linkages.

We also established the retention times of the fragments *para*-κCN and GMP formed after specific cleavage of κCN A and B by chymosin during the first step of the milk-clotting process. The two genetically determined amino acid substitutions in these variants are both located in the GMP part [39], so that in the cheese whey from bulk milk essentially two GMP fractions (GMP-A and GMP-B) can be expected; the *para*-κCN fraction is entrapped in the cheese curd. As seen in Fig. 2, *para*-κCN elutes just prior to the carbohydrate-free κCN-A variant, whereas the GMP A and B variants elute at quite different positions ahead of all the casein components.

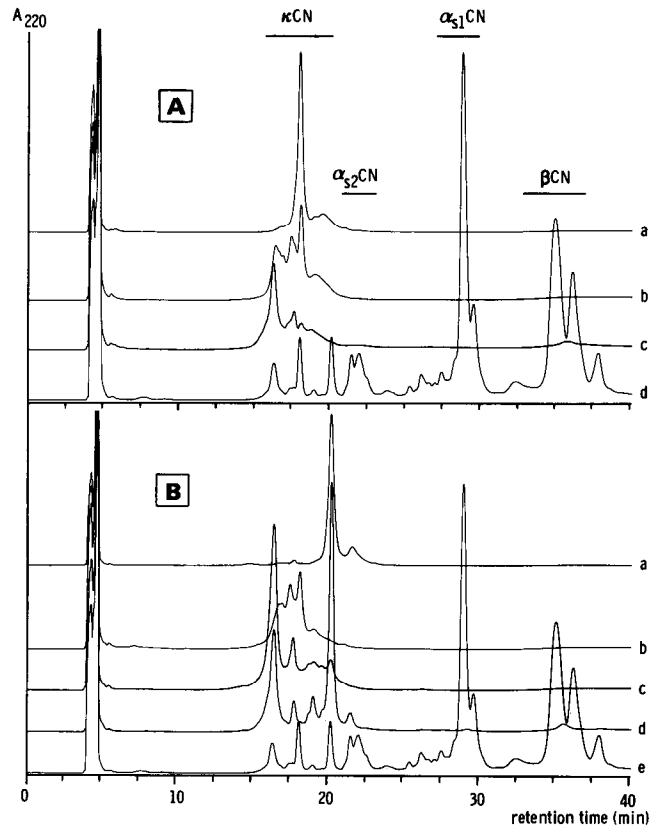

Fig. 1. Effect of degree of glycosylation on the retention of κCN fractions during RP-HPLC. (A) (a) Carbohydrate-free κCN-A; (b) partly glycosylated κCN-A; (c) carbohydrate-rich κCN-A; (d) whole casein from bulk milk (reference). (B) (a) Carbohydrate-free κCN-B; (b) partly glycosylated κCN-B; (c) carbohydrate-rich κCN-B; (d) whole κCN-B (reference); (e) whole casein from bulk milk (reference).

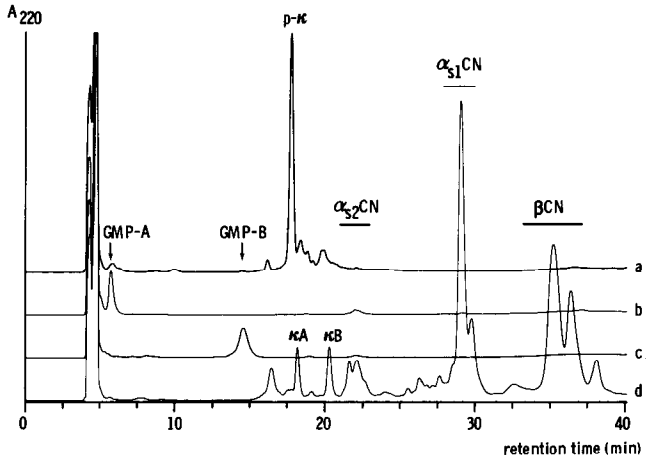

Fig. 2. RP-HPLC patterns of chymosin-generated breakdown products of κCN A and B; carbohydrate-free κCN indicated as κA and κB. (a) *para*-κCN (p-κ); (b) GMP-A; (c) GMP-B; (d) whole casein from bulk milk (reference).

TABLE I

LOCATION OF DIFFERENCES IN THE PRIMARY STRUCTURE OF SOME GENETIC VARIANTS OF THE MAJOR BOVINE MILK PROTEINS [40]

Protein	Variant	Position in amino acid sequence				
α_{s1}CN		14–26	53	192		
(199 residues)	A	Deleted	Ala	Glu		
	B	Present	Ala	Glu		
	C	Present	Ala	Gly		
	D	Present	ThrP	Glu		
βCN		35	37	67	106	122
(209 residues)	A^1	SerP	Glu	His	His	Ser
	A^2	SerP	Glu	Pro	His	Ser
	A^3	SerP	Glu	Pro	Gln	Ser
	B	SerP	Glu	His	His	Arg
	C	Ser	Lys	His	His	Ser
κCN		136	148			
(169 residues)	A	Thr	Asp			
	B	Ile	Ala			
βLg		64	118			
(162 residues)	A	Asp	Val			
	B	Gly	Ala			

The two relatively polar amino acid substitutions in κCN-A as compared with κCN-B (Table I) are in agreement with the earlier elution of the A-variant (including GMP-A compared with GMP-B) from the reversed-phase column.

In Fig. 3 RP-HPLC patterns of whole caseins obtained from milks of single cows are compared. Also, the patterns of two purified (less common) casein variants have been added. The genotypes of the casein components (including that of α_{s2}CN, which occurs predominantly as the A variant in European cattle) had been established by gel electrophoresis [16] or by isoelectric focusing [28]. As was already shown in Fig. 1, the κCN A and B variants can be identified by the positions of their carbohydrate-free subcomponents. As far as α_{s1}CN is concerned, a distinction could be made between the A variant (missing a 13-residue fragment in its primary structure, see Table I), the B/C and the D variant. No separation could be achieved between the B and C variants (Glu ↔ Gly at position 192, Table I), both of which give rise to a double peak, due to a difference in the degree of post-translational phosphorylation (an extra phosphate group at position 41 [40]). The α_{s1}CN-B variant and to a much lesser extent the C variant are predominant in western cattle. βCN mainly occurs as its A^1, A^2 and/or, at a much lower frequency, as its B variant (for structural differences see Table I). These forms could be completely separated using the present RP-HPLC programme. In addition, the less common βCN-A^3 is also separately seen in the elution pattern; it elutes just after βCN-A^2 from the column, owing to a His → Gln replacement at position 106 (Table I). On the other hand, the rare βCN-C coelutes with the βCN-A^1 variant. The minor component βX, which in the reference whole casein appears as the last-eluting peak (just after the position of βCN-A^3), was isolated and shown to be also a βCN, as judged by sodium dodecyl sulphate–po-

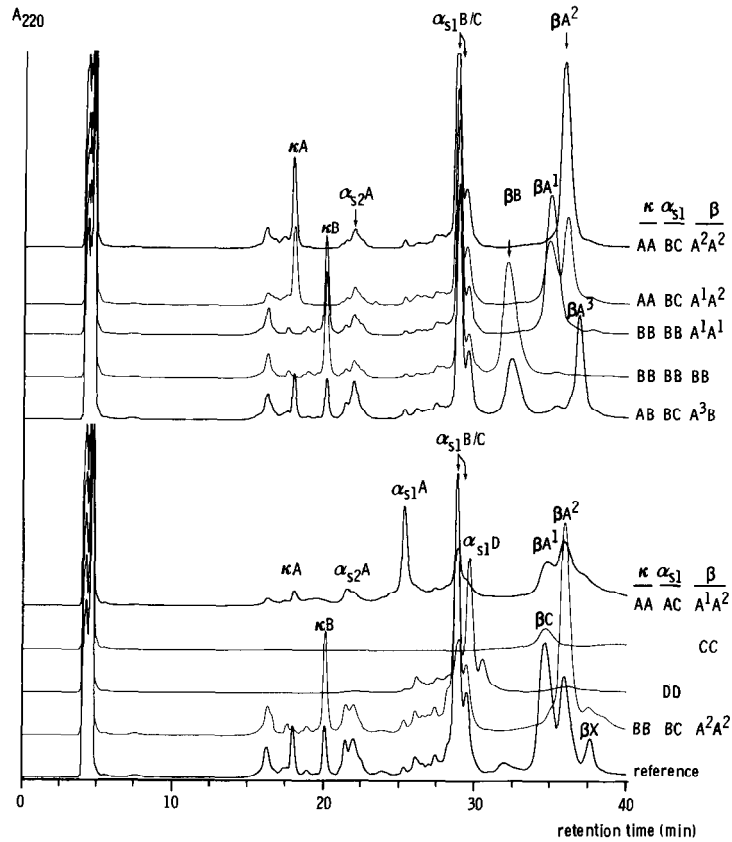

Fig. 3. RP-HPLC patterns of whole caseins and some casein components from milks from single cows. The α_{s_2}CN is present as the A type throughout. The other genotypes are indicated. Whole casein from bulk milk is added as a reference; the unknown component βX herein is referred to in the text.

lyacrylamide gel electrophoresis and amino acid analysis (results not shown). We found this component in several whole caseins from bulk milk, although in different amounts. Re-chromatography of the isolated component under the same conditions (see Experimental) showed a single peak at its original position, so that its designation as some aggregated form of βCN can be excluded. It could be a still unidentified βCN variant [41].

The RP-HPLC separation of αLa-B, βLg-A and βLg-B is well documented [29,32,33]. It generally concerns purified whey proteins or total whey protein preparations. However, identification and phenotyping of these proteins in combination with the caseins directly in milk could be desirable. We have achieved this by analysing samples of skim milk clarified in a buffer containing urea, ME and sodium citrate (for details see Experimental). The RP-HPLC pattern of a total milk protein fraction is shown in Fig. 4. It also shows the patterns of the casein and whey protein fractions isolated from the same milk, together with a pattern of cheese whey, in which the main whey proteins and also GMP-A and GMP-B can be observed. In the total milk

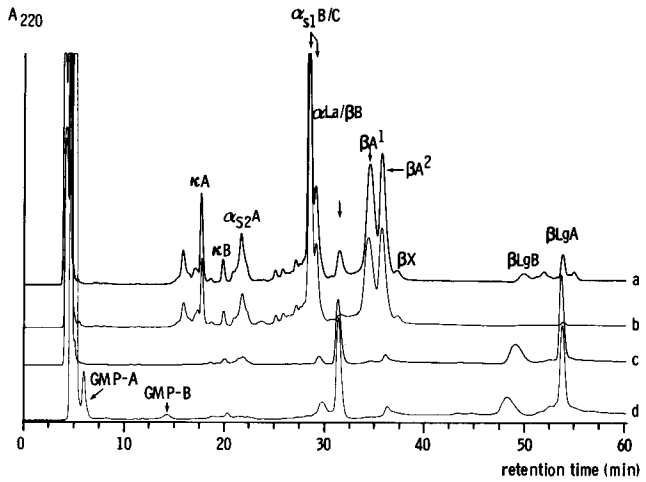

Fig. 4. RP-HPLC patterns of (a) a clarified skim milk sample and of (b) whole casein and (c) acid whey prepared from the same milk. A pattern of a cheese whey has also been added (d).

protein pattern the casein variants can be clearly distinguished from the whey proteins except βCN-B, which is largely obscured by αLa-B, the latter being the only αLa variant found in milk from western cattle [40]. The buffer used to clarify the milk sample contained ME to obtain an optimum separation in the κCN and α_{s_2}CN region of the RP-HPLC pattern. However, this ME-containing clarification buffer tends to deform the βLg peaks in the RP-HPLC pattern, giving rise to small peaks between those of the A and B variants (see Fig. 4, trace *a*). The minor component immediately following the βLg-A peak appears exclusively in the presence of urea in the reaction mixture at pH 7 and could be avoided by using deionized urea (not done in this study). In the whey protein fractions not treated with ME/urea at pH 7 (Fig. 4, traces *c* and *d*) such additional peaks are absent. The αLa-B component seems to be much less subject to deformation by mild ME treatment, probably because of its more compact globular character. Altogether, this means that proper quantification of separate βLg A and B variants from patterns as shown in Fig. 4, trace *a*, is difficult as it is unknown to which of the variants, if both are present, the intermediate peaks should be assigned. The peak of βLg-B can be sharpened by shortening the "hold" period of 12 min in the gradient programme (see Experimental). In that case, however, ME-generated peaks between the A and B variants remain largely hidden.

In principle, the RP-HPLC method lends itself better to quantification of milk protein genetic variants than do electrophoretic methods, because when using the latter procedures problems with quantitative staining may arise. On the other hand, with electrophoretic methods many samples can be handled simultaneously. Therefore, RP-HPLC and electrophoresis or isoelectric focusing may be used as complementary techniques. In Fig. 5 the relationships between RP-HPLC peak areas and concentrations of various isolated casein components (A) and whey proteins (B) are shown. For caseins reasonable linearity was obtained in the concentration range investigated (Fig. 5A). However, the linear α_{s_2}CN curve did not pass through the origin (result not shown), owing to a "memory effect" observed with α_{s_2}CN on the

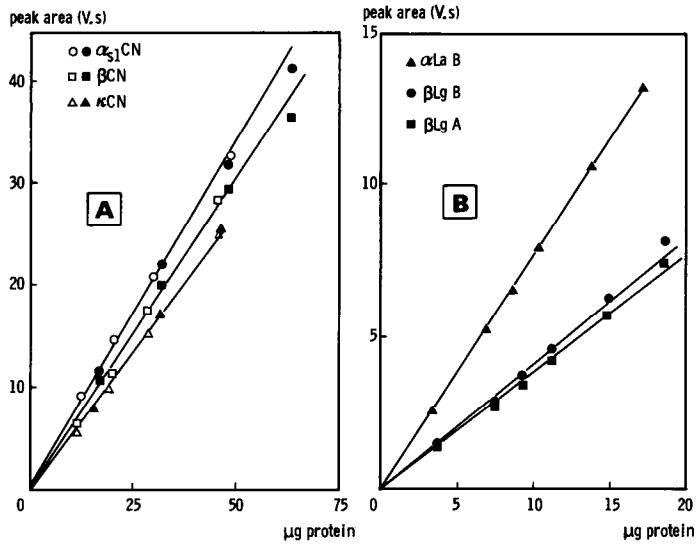

Fig. 5. Relationship between RP-HPLC peak area(s) and amount of milk protein samples injected. (A) Casein components (open and closed symbols represent two independent experiments); (B) whey proteins.

column used. Of the whey proteins, both βLg and αLa show satisfactory linearity with a significantly higher slope observed for αLa (Fig. 5B). To minimize experimental variations, proper standards should be included in each series of analyses. Quantification of RP-HPLC patterns of skim milks after clarification gave problems with βLg, owing to the effect of ME described above; in that case an additional run with a clarified, non-reduced milk sample should provide the analytical data for βLg. The quantitative aspects of the method are being investigated further.

CONCLUSIONS

With the RP-HPLC method described, separation can be achieved between (in order of increasing retention time) carbohydrate-rich κCN-A + -B, carbohydrate-free κCN-A, carbohydrate-free κCN-B, α_{s2}CN-A, α_{s1}CN-A, α_{s1}CN-B/C, α_{s1}CN-D, βCN-B, βCN-C/A^1, βCN-A^2 and βCN-A^3. In addition, the products of κCN cleavage by the milk-clotting enzyme chymosin (*i.e.*, GMP-A, GMP-B and *para*-κCN) can be distinguished from each other and from the above-mentioned casein components. When the whey proteins αLa and βLg are also included in the mixture (for instance, by injecting diluted, clarified skim milk), the βLg A and B variants can be observed separately from the caseins, whereas αLa-B co-elutes with the less common B variant of βCN.

Except for α_{s2}CN, quantification should be possible provided that precautions are taken, such as inclusion of standards in each series of analyses and standardization of the experimental conditions.

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