Journal of Chromatography A, 652 (1993) 207–213
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 179

Determination of milk proteins by capillary electrophoresis

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ABSTRACT

The determination of milk proteins by capillary zone electrophoresis (CZE) is hampered by the adsorption of the solute on the capillary wall. The effects of pH, ionic strength of the buffer and polymeric additives were studied in combination with a hydrophilically coated capillary. Optimum separations were obtained at low pH (2.5–3) in aqueous solutions containing 6 *M* urea and methylhydroxyethylcellulose, resulting in a complete separation of the serum proteins and caseins, including some genetic variants. The results were compared with those achieved with reversed-phase HPLC. With CZE, theoretical plate numbers in the range 300 000–700 000 were obtained. The relative standard deviations for migration times were below 0.08% and for peak areas were 2–4%. The separation of cow, goat and sheep milk proteins and of heat-damaged casein is reported.

INTRODUCTION

Bovine milk contains 3–3.5% of protein, of which 80% consists of caseins, insoluble at their isoelectric pH (pH 4.6). The serum proteins, soluble at pH 4.6, make up the remaining 20%. The casein fraction can be subdivided into 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 serum proteins are comprised mainly of β-lactoglobulin (βLg) and α-lactalbumin (αLa) in the ratio *ca.* 3:1.

The determination of milk proteins, including the separation of genetic variants, has been achieved by classical gel electrophoresis [1,2], isoelectric focusing [3–5] and high-performance liquid chromatography (HPLC) in the ion-exchange [6], hydrophobic interaction [7] and reversed-phase [8] modes. Each method has its own merits, but no single method gives an excellent separation between serum proteins and caseins in combination with possibilities for good quantification.

Capillary zone electrophoresis (CZE), a rapidly evolving technique, has the potential to give rapid separations with high resolutions and good quantification. The separation of proteins by CZE, however, might be hampered by adsorption on the negatively charged fused-silica surface of the capillary, leading to broad and/or tailing peaks and sometimes poor quantification [9]. These problems can be (partly) overcome by using a high pH buffer [10], a high ionic strength buffer [11,12], coated capillaries [13,14] or buffer additives [15,16].

In this paper, we describe the development of a CZE method by which the serum proteins and caseins are well separated from each other and some of their genetic variants.

EXPERIMENTAL

Reagents and chemicals

Phosphate buffer (10 m*M*) was prepared by dissolving in a 50-ml volumetric flask 69 mg of NaH_2PO_4 (Merck, Darmstadt, Germany) and 25

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0021-9673/93/$06.00

mg of methylhydroxyethylcellulose (MHEC, 30 000) (Serva, Heidelberg, Germany) in 37.5 ml of 8 *M* urea (Riedel-de Haën, Seelze, Germany); the pH was adjusted to 2.50 with 4 *M* phosphoric acid (AnalaR; BDH, Poole, UK) and the volume was made up with water. For some experiments MHEC was replaced with the same amount of methylhydroxypropylcellulose (MHPC, 15 000) (Serva) or poly(vinyl alcohol) (PVA, 15 000) (Fluka, Buchs, Switzerland).

Citrate buffer (10 m*M*) was prepared by dissolving in a 50-ml volumetric flask 147 mg of trisodium citrate dihydrate (Merck) and 25 mg of MHEC in 37.5 ml of 8 *M* urea. The pH was adjusted to 2.45 with 2.5 *M* citric acid solution and the volume was made up with water. Citrate buffer (20 m*M*) (pH 3.00) was prepared analogously.

Buffers were filtered through a 0.22-μm Millex GV filter (Millipore, Molsheim, France). Urea solution (8 *M*) was passed over a mixed-bed ion exchanger [AG 501-X8 (Bio-Rad Labs., Richmond, CA, USA)] in order to remove isocyanate and other ionic impurities.

Capillary zone electrophoresis

Electromigration was carried out with a Beckman P/ACE system 2050 controlled by a Laser 386/2 computer with Beckman P/ACE v. 2.0 software using a hydrophilically coated fused-silica capillary, either from SGE (Milton Keynes, UK) or CElect P1 from Supelco (Bellefonte, PA, USA), both with dimensions of 57 cm × 50 μm I.D. and fitted in a cartridge with a modified mandrel in order to improve cooling. A new SGE capillary required extensive flushing with methanol–water mixtures and with the buffer in order to obtain good electropherograms. In contrast, the Supelco capillary gave immediately repeatable electropherograms of high quality. Migrations were run at 45°C and the voltage across the capillary was maintained at 25 or 20 kV in the case of phosphate buffers, with ground at the detector side. Injections were carried out by pressure (duration 10–30 s). After each separation the capillary was flushed with the appropriate buffer for 4 min. Prior to storage the capillary was flushed for 10 min with water.

UV detection was performed at 214 nm (data collection rate 5 Hz). Peaks were identified by comparison with authentic samples and by analysing milks with a known genetic protein composition. Peak areas and peak heights were obtained from the same raw data after processing with Caesar NT software (v. 2.0, B*Wise, Geleen, Netherlands).

Liquid chromatography

Sample preparation and separations were carried out as described previously [17].

Sample treatment for CZE analysis

The reduction buffer was prepared by dissolving in a 50-ml volumetric flask 73 mg of trisodium citrate dihydrate (Merck) and 38 mg of DL-dithiothreitol (DTT) (Sigma, St. Louis, MO, USA) in 37.5 ml of 8 *M* urea. The pH was adjusted to 8 with dilute sodium hydroxide solution and the volume was made up with water. Skim milk (0.5 ml) was diluted with 2.5 ml of reduction buffer and incubated for 1 h at room temperature. The resulting clear solution was used for CZE analysis.

RESULTS AND DISCUSSION

The majority of caseins are present in milk in the form of micelles. The micelles can be disrupted by the combined action of a reducing agent (DTT) and 6 *M* urea. The reformation of micelles during electrophoresis can be prevented by using 6 *M* urea in the electrophoresis buffer. We investigated previously published electrophoretic separation methods [10,14,15] with and without the addition of 6 *M* urea to the buffers. The results obtained with serum proteins and caseins were in general disappointing, as broad and tailing peaks were observed or the selectivity was insufficient.

A Beckman application note showed an interesting separation of caseins at a low pH (10 m*M* phosphate, pH 2.5) in combination with a modified cellulose and an uncoated silica capillary. These conditions proved to be a good starting point for a systematic optimization of the analy-

sis. At pH 2.5 silica is almost without negative charge, and therefore the positively charged proteins are not attracted to the silica surface. Modified celluloses tend to adsorb on silica surfaces, shielding the polar nature and residual charges of the surface. Under these conditions adsorption of proteins is obviously prevented to a great extent.

We reasoned that the application of a hydrophilically coated capillary could suppress the adsorption of proteins even more, which indeed appeared to be the case. Elevation of the temperature to 45°C also improved the separation. In both instances the peaks became narrower and the migration times decreased. Electroosmotic flow is virtually zero under these conditions.

Fig. 1 shows the pH dependence of the electrophoretic pattern of bovine milk proteins using the phosphate buffer. It should be noted that the ionic strength was not the same at the different pH values, because the pH was adjusted starting with 10 m*M* sodium phosphate solution. At pH 2 and 2.5 serum proteins and caseins were well separated.

Fig. 2 shows the influence of various polymeric additives to the phosphate buffer of pH 2.5. MHEC gave the best performance, closely followed by MHPC. PVA gave rather broad peaks with long migration times. Recently Belder and Schomburg [18] described the beneficial properties of PVA and hydroxyethylcellulose on separations with bare silica capillaries. We investigated the effect of the average molecular mass of MHPC (viscosity of 2% solution in water of 5, 100 and 15 000 mPa s) on the separation. When the molecular mass of the polymer decreased the peak width increased. This cannot be overcome by using a higher mass percentage of the low molecular mass material.

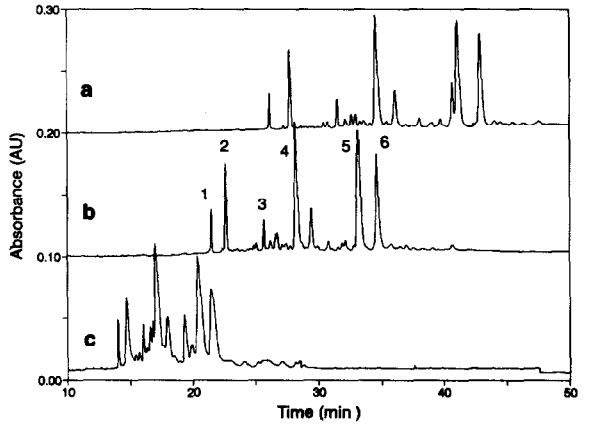

Fig. 1. Electropherograms of bovine milk proteins at (a) pH 2.0, (b) pH 2.5 and (c) pH 3.0. Buffer, 10 m*M* sodium phosphate containing 6 *M* urea and 0.05% MHEC; electromigration in a hydrophilically coated SGE capillary at 20 kV with ground at the detector side. Peaks: 1 = αLa; 2 = βLg; 3 = α_{s2}CN; 4 = α_{s1}CN; 5 = βCN-Al; 6 = βCN-A2.

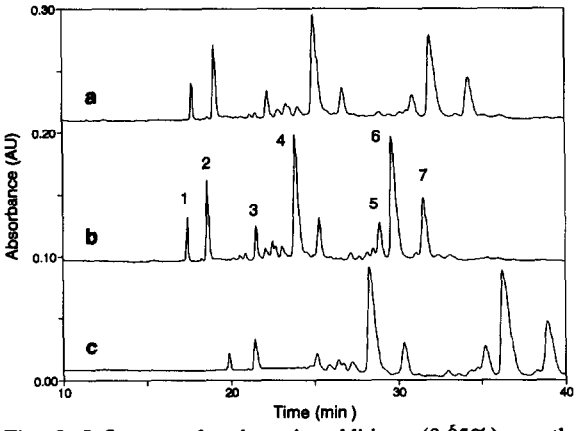

Fig. 2. Influence of polymeric additives (0.05%) on the electromigration of bovine milk proteins at pH 2.5. (a) MHPC; (b) MHEC; (c) PVA. Experimental conditions as in Fig. 1. Peaks: 1 = αLa; 2 = βLg; 3 = α_{s2}CN; 4 = α_{s1}CN; 5 = κCN; 6 = βCN-Al; 7 = βCN-A2.

The replacement of phosphate with citrate increased the theoretical plate number, calculated for the proteins in Fig. 3A, to the range 300 000–700 000. The conductivity of this buffer is lower than that of the phosphate buffer of the same concentration and pH, permitting an increase in the separation voltage to 25 kV, which generates a current of only *ca.* 60 μA. An excellent separation between serum proteins and caseins is observed. The A and B genetic variants of βLg and of κCN are not separated, and only a slight separation is observed for the B and C variants of α_{s1}CN. These genetic variants differ only in an acidic amino acid residue (Table I), which is almost not ionized at this pH, inducing no charge differences. The genetic variants of the β-caseins differ in the amount of

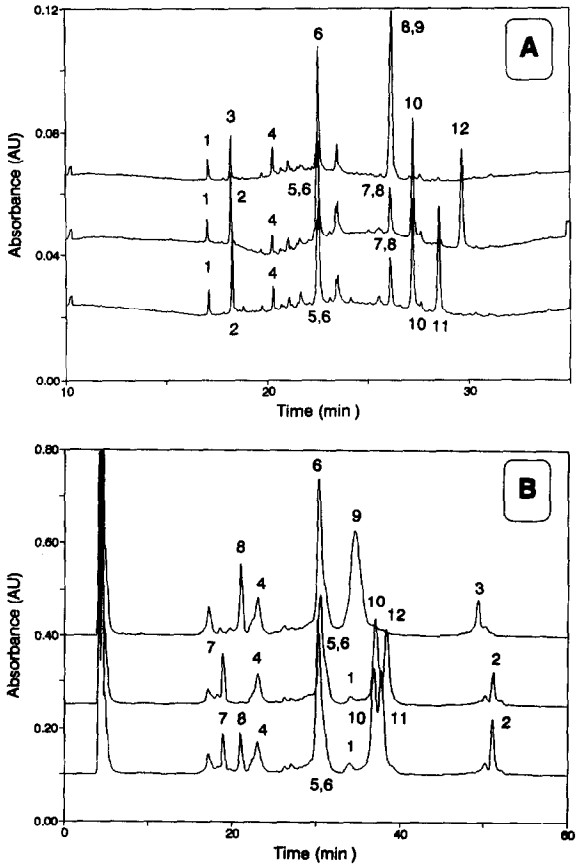

Fig. 3. Separation of genetic variants of proteins of three bovine milks by (A) capillary electrophoresis using a 10 m*M* sodium citrate buffer (pH 2.45) containing 6 *M* urea and 0.05% MHEC in combination with a hydrophilically coated SGE capillary run at 25 kV and (B) reversed-phase HPLC with an acetonitrile gradient on a Hi-Pore RP 318 column (Bio-Rad Labs.). Peaks: 1 = αLa; 2 = βLg-A; 3 = βLg-B; 4 = α_{s2}CN-A; 5 = α_{s1}CN-C; 6 = α_{s1}CN-B; 7 = κCN-A; 8 = κCN-B; 9 = βCN-B; 10 = βCN-Al; 11 = βCN-A2; 12 = βCN-A3.

basic amino acids residues. An excellent separation is observed (Fig. 3A). For comparison the reversed-phase HPLC results are shown in Fig. 3B. A completely different pattern is found, indicating that the two techniques are complementary to each other.

Increasing the pH of the electrophoresis buffer should allow the separation of genetic variants differing in acidic amino acid residues. However, a slight increase in the pH from 2.45 to 2.50 decreased the theoretical plate number by *ca.*

TABLE I

AMINO ACID DIFFERENCES IN GENETIC VARIANTS OF BOVINE MILK PROTEINS

Protein	Variant	Substitution
α_{s1}-Casein (α_{s1}CN)	B	Glu
	C	Gly
β-Casein (βCN)	A1	His His Ser
	A2	Pro His Ser
	A3	Pro Gln Ser
	B	His His Arg
κ-Casein (κCN)	A	Thr Asp
	B	Ile Ala
β-Lactoglobulin (βLg)	A	Asp Val
	B	Gly Ala

20%. At pH 3.00 we could obtain similar plate numbers (Fig. 4) if the concentration of trisodium citrate was increased from 10 to 20 m*M*, the conductivity of this buffer being the same as that of pH 2.45. This suggests that there is a minimum ionic strength needed to suppress adsorption of proteins on the capillary surface, similarly to the situation encountered in gel permeation chromatography. Fig. 4 shows a

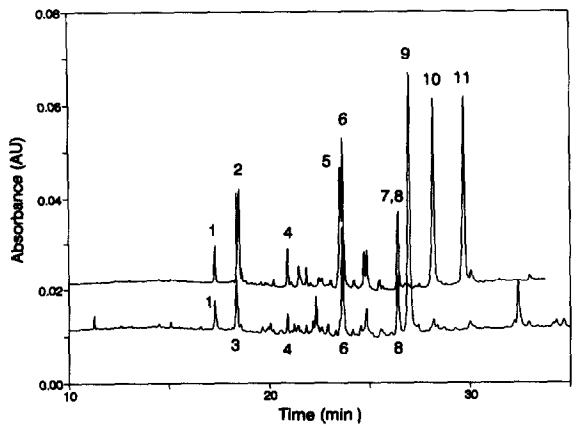

Fig. 4. Electromigration of genetic variants of proteins of two bovine milks at pH 3.00 (20 m*M* sodium citrate, 6 *M* urea and 0.05% MHEC) using a hydrophilically coated Supelco capillary at 25 kV. Peaks: 1 = αLa; 2 = βLg-A; 3 = βLg-B; 4 = α_{s2}CN-A; 5 = α_{s1}CN-C; 6 = α_{s1}CN-B; 7 = κCN-A; 8 = κCN-B; 9 = βCN-B; 10 = βCN-Al; 11 = βCN-A2. The dotted line near peak 2 in the upper trace indicates the position of βLg-B.

slight separation for the A and B forms of βLg and an improved separation for α_{s1}CN-B and -C. The A and B forms of κCN were not separated, but βCN-B was well separated from κCN.

A possible application is the determination of proteins from goat, sheep and, for comparison, cow milk (Fig. 5). For each species a distinctive pattern is found, which potentially could be used to detect the adulteration of each by addition of the other down to 1%.

During our investigations we observed that caseins, believed to be heat stable, were changed considerably on heating in milk. Caseins, isolated by isoelectric precipitation at pH 4.6 from evaporated milks (EVAP), show a dramatic change in their patterns (Fig. 6). All the caseins from EVAP show an additional peak after the main casein peaks. When the EVAP received an additional heat treatment, the peak of α_{s1}CN disappeared almost completely, leaving several partly separated peaks. The β-caseins show three additional peaks in addition to the original peak. This phenomenon could be used to detect heat treatments of milk more intensive than pasteurization, and to detect the addition of milk powder to fresh milk.

The repeatability of the electrophoresis separation system was assessed by analysing eight times a sample composed of standards (Table II). Excellent repeatabilities were found for the migration times (R.S.D. <0.085%). The absence of electroosmotic flow could be important for these results. For peak areas R.S.D.s of 2–4% were found. In real samples the identification of peaks is easy, owing to the insensitivity of migration times to matrix effects. In other electrophoretic systems these matrix components might influence the electroosmotic flow and thus the migration times. The response factors, calculated for peak areas, showed larger variations for the proteins studied, than expected for detection at 214 nm. Therefore, an equal response for every protein cannot be assumed.

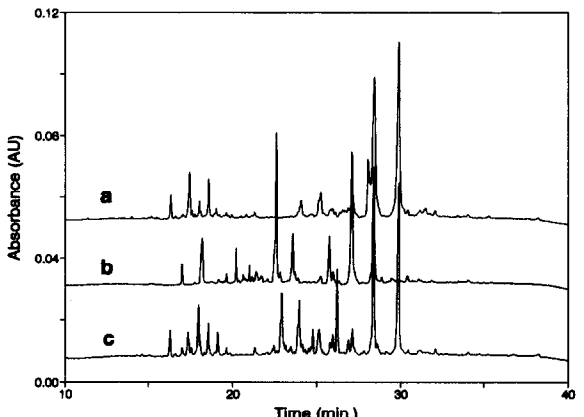

Fig. 5. Electropherograms of proteins from (a) goat, (b) cow and (c) sheep milk using a buffer of pH 2.45. Conditions as in Fig. 3.

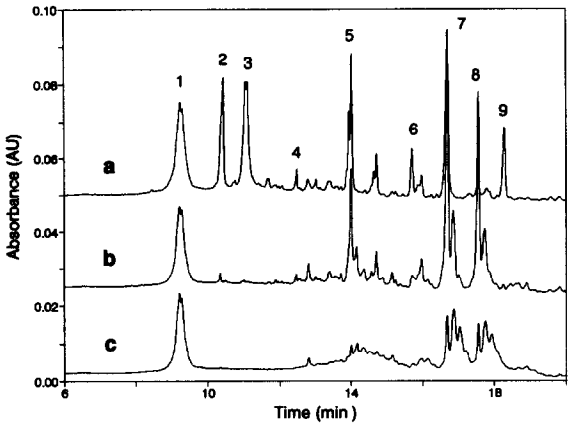

Fig. 6. Electropherograms of heat-damaged caseins. (a) Standard sample composed of serum proteins and caseins; (b) caseins isolated by isoelectric precipitation from EVAP; (c) caseins from EVAP that had received an additional heat treatment. A hydrophilically coated Supelco capillary (47 cm × 50 μm I.D.) was used; other conditions as in Fig. 4. Peaks: 1 = BSA (added as an internal standard); 2 = αLa; 3 = βLg-A and -B; 4 = α_{s2}CN; 5 = α_{s1}CN-B and -C; 6 = κCN; 7 = βCN-A1; 8 = βCN-A2; 9 = βCN-A3.

CONCLUSIONS

The CZE method described here is the first method for determining simultaneously serum proteins and caseins with high resolution and good possibilities for quantification in combination with a simple sample treatment.

Capillary electrophoresis proved to be a reliable method for the determination of milk proteins. The life of the coated capillary was at least 2 months, during which many hundreds of samples were analysed.

TABLE II

REPEATABILITY OF MIGRATION TIMES AND PEAK AREAS OF A STANDARD SAMPLE RUN WITH THE CITRATE BUFFER (pH 3.00) AT 25 kV

Injection No.	Migration time (min)									Peak area (area counts)						
	BSA-1	BSA-2	αLa	βLg-B	βLg-A	α_{s1}CN	κCN	βCN-A1	βCN-A2	BSA[a]	αLa	βLg[a]	α_{s1}CN	κCN	βCN-A1	βCN-A2
1	12.82	12.92	14.51	15.45	15.51	19.48	21.94	23.28	24.48	0.1315	0.0282	0.1204	0.0768	0.0216	0.1200	0.0483
2	12.82	12.93	14.51	15.47	15.53	19.48	21.94	23.28	24.48	0.1377	0.0290	0.1201	0.0782	0.0229	0.1224	0.0499
3	12.82	12.92	14.50	15.43	15.50	19.46	21.94	23.27	24.46	0.1303	0.0282	0.1203	0.0763	0.0210	0.1216	0.0504
4	12.83	12.93	14.51	15.45	15.51	19.48	21.94	23.28	24.48	0.1367	0.0284	0.1200	0.0753	0.0228	0.1203	0.0501
5	12.82	12.92	14.50	15.44	15.51	19.47	21.94	23.27	24.46	0.1294	0.0274	0.1146	0.0733	0.0206	0.1159	0.0473
6	12.83	12.93	14.51	15.45	15.51	19.48	21.94	23.28	24.48	0.1354	0.0277	0.1161	0.0756	0.0218	0.1188	0.0486
7	12.82	12.92	14.50	15.43	15.50	19.46	21.93	23.26	24.46	0.1289	0.0269	0.1155	0.0725	0.0218	0.1150	0.0477
8	12.82	12.92	14.50	15.44	15.50	19.46	21.93	23.26	24.46	0.1303	0.0270	0.1158	0.0736	0.0221	0.1160	0.0477
Mean	12.82	12.92	14.51	15.45	15.51	19.47	21.94	23.27	24.47	0.1325	0.0279	0.1178	0.0752	0.0218	0.1187	0.0488
S.D.	0.005	0.005	0.005	0.013	0.010	0.010	0.005	0.009	0.011	0.0035	0.0007	0.0026	0.0019	0.0008	0.0028	0.0012
R.S.D. (%)	0.036	0.040	0.037	0.085	0.064	0.051	0.021	0.038	0.044	2.6	2.6	2.2	2.6	3.7	2.3	2.5

[a] Sum of two partly separated peaks.

ACKNOWLEDGEMENTS

We are grateful to Dr. H.M. Farrell (Eastern Regional Research Center, Philadelphia, PA, USA) and Dr. H. Bovenhuis (Agricultural University, Wageningen, Netherlands) for providing samples of casein genetic variants and whole milk samples, respectively. We thank J.A.M. van Riel, K.J. Slangen, P. Tienstra and M. Casella for technical assistance.

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