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Characterization of ewe's milk by capillary zone electrophoresis

Tiziana Maria Piera Cattaneo*, Franca Nigro, Piero Maria Toppino, Viviana Denti

Istituto Sperimentale Lattiero-Caseario, Via Lombardo 11, 20075 Lodi (MI), Italy

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Abstract

The purpose of the present work was to develop a procedure able to separate and identify the major protein components of ewe's milk by capillary zone electrophoresis (CZE). Thirty-five individual milk samples of Massese breed were analyzed using a coated capillary. The analyses were performed at pH 3.0 at a temperature of 40°C in the presence of 6 *M* urea. The purification of casein fractions was carried out by preparative fast protein liquid chromatography and the CZE results were confirmed by polyacrylamide agarose gel electrophoresis (PAAGE). The identification of whey proteins was also carried out by comparison with high-performance liquid chromatography data. The present study permitted the identification of the major components of ewe's milk by high-resolution electropherograms and characteristic migration times (t_M). It was also possible to detect the presence of genetic variants of β-lactoglobulin. The t_M of k-casein was determined after enzymatic action of chymosin by verifying the simultaneous formation of *p*-k-casein. In most of the samples a fast moving α-$_{S2}$-casein variant was identified by comparison with PAAGE results. Minor genetic differences were found in other casein fractions for this pool of samples.

Keywords: Capillary electrophoresis; Milk; Proteins; Caseins; Whey proteins

1. Introduction

Capillary zone electrophoresis (CZE) with its high resolving power and unique selectivity plays an important role in the separation and characterization of proteins [1].

Recently, optimal separations of milk proteins in aqueous solutions containing 6 *M* urea and methylhydroxyethylcellulose (MHEC) were obtained at low pH using a hydrophilically coated capillary by Olieman and co-workers [2,3]. Moreover, electromigration patterns of milk proteins from goats, ewes and cows showed considerable differences, although the authors could not identify the peaks in other than cow's milks.

The aim of the present work was to optimize the procedure of Olieman and co-workers [2,3] for the separation of the major protein components of ewe's milk. Identification was carried out by analysis of individual milk samples and comparison with the results obtained for the same samples by well-known separation techniques, such as high-performance liquid chroma-

* Corresponding author.

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tography (HPLC), fast protein liquid chromatography (FPLC) and polyacrylamide agarose gel electrophoresis (PAAGE).

2. Experimental

2.1. Samples and sample preparation

The sample buffer consisted of 5 m*M* trisodium citrate dihydrate (PRS, Montplet & Esteban, Barcelona, Spain), 9 *M* urea (Merck, Darmstadt, Germany), purified by Amberlite-MB1 (Pharmacia LKB, Uppsala, Sweden) for 45 min and then filtered by Whatman 540 filter paper (Whatman International, Maidstone, UK), and 30 m*M* D,L-dithiothreitol (DTT) (Merck). Milk samples were diluted 1:4 (v/v) in sample buffer, incubated for at least 1 h at room temperature under shaking and filtered over Minisart RC25 hydrophilic membranes (0.45 μm) (Sartorius, Göttingen, Germany) before use.

Thirty-five individual ewe milk samples of Massese breed were analyzed by CZE. Some samples were chosen according to their genetic pattern to carry out the identification of the major whey protein components from their characteristic migration times (t_M) and were also analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) [4] in order to identify β-lactoglobulin (β-lg) genetic variants.

Acid and rennet caseins were prepared by adding 0.1 *M* HCl to pH 4.6 and by clotting with 0.01% liquid calf rennet (Hansen, Kobenhavn, Denmark) at 37°C, respectively. Casein samples were dissolved in sample buffer at 0.8% (w/v).

The purification of casein fractions was carried out by preparative cation-exchange FPLC analysis [5], carried out on a Mono S HR5/5 column using urea–acetate buffer (pH 5.0; 0.02 *M* acetate; 6 *M* urea), 0–0.26 *M* NaCl gradient and a Pharmacia FPLC System (Pharmacia LKB). UV detection was performed at 280 nm. Each collected fraction was analyzed by PAAGE under alkaline conditions [6], using as separation buffer Tris–glycine pH 8.6 [0.3 *M* glycine; 0.01 *M* tris(hydroxymethyl)aminomethane], and as sample buffer 9 *M* urea with 1% 2-mercaptoethanol. Separations were performed using a 2-step procedure: pre-run, 100 V (hold) for 15 min; run, 200 V (hold) for 2 h. Voltage was applied using negative to positive polarity. The staining solution consisted of 0.25% Coomassie Bleu G250 in destaining solution [acetic acid–methanol–water (100:500:400, v/v)]. The same fractions were analyzed by CZE, after a desalting step [7].

2.2. Capillary zone electrophoresis

Electromigrations were carried out by using a BioFocus 3000 capillary system (Bio-Rad Laboratories, Richmond, CA, USA), controlled by a DELL 425_s/L computer (Dell Computer Corporation, Raheen, Limerich, Ireland) with BioFocus & Spectra v.3.01 Software (Bio-Rad).

Separations were performed using a 55 cm (50 cm to detection point) × 50 μm I.D. CElect P1 hydrophilically coated fused-silica capillary (Supelco, Bellefonte, CA, USA); voltage was applied using positive to negative polarity.

As electrolyte was used a citrate buffer [2], containing 20 m*M* trisodium citrate dihydrate (PRS), 6 *M* urea (Merck) and 0.05% MHEC (Serva, Heidelberg, Germany); the pH value was adjusted to pH 3.00 with concentrated citric acid (Bio-Rad). Sample injection was performed by pressure (34 474 Pa × 8 s). Separation conditions were 25 kV applied voltage and 40°C.

Protein components were detected by UV absorbance (AU) at 214 nm and data collected at a rate of 2 Hz.

3. Results and discussion

Fig. 1 shows the FPLC pattern of the acid casein sample. This method [5] permitted us to collect five fractions (0–4); each fraction was successively analyzed by both PAAGE [6] and CZE.

The FPLC fraction analyses were carried out by PAAGE on the same gel together with whole casein samples (A–H). In comparison with the electrophoretic patterns of whole casein samples and on the basis of literature data [5,8], from Fig. 2 it can be observed that fraction 0 did not

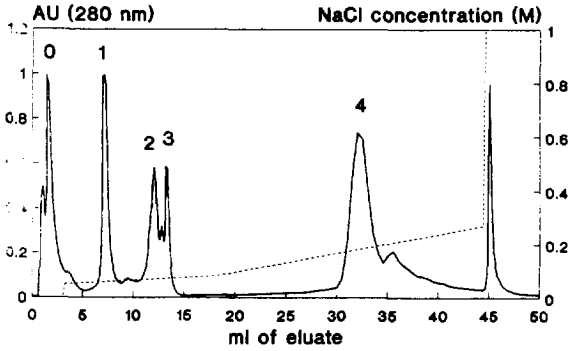


Fig. 1. FPLC analysis of ewe's milk. Preparative FPLC analysis [5] of whole acid casein. Mono S HR5/5 column; urea–acetate buffer (pH 5.0; 0.02 *M* acetate; 6 *M* urea); NaCl gradient, 0–0.26 *M*; UV detector, 280 nm. Abbreviations: 0–4 = FPLC fractions.

contain any protein component, corresponding to the breakthrough peak (mainly consisting of β-mercaptoethanol) [5]. Fraction 1 contained the first major peak and a subsequent minor peak: this fraction was found to be particularly rich in β-casein (β-cn). k-Casein (k-cn) was found in next two major peaks (fractions 2 and 3), eluting together with small amounts of γ-casein (γ-cn) and fast moving α-$_{S2}$-casein (α-$_{S2}$-cn) variant [8]. Fraction 4 was found to correspond with α-$_{S}$-casein fractions. It contained the last major peak

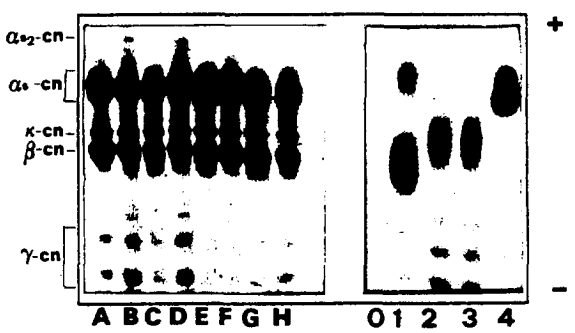


Fig. 2. PAAGE analysis of ewe's milk. PAAGE analysis of whole casein and FPLC fractions under alkaline conditions [6]. Separation buffer, Tris–glycine pH 8.6 [0.3 *M* glycine; 0.01 *M* tris(hydroxymethyl)aminomethane]; sample buffer, 9 *M* urea, 1% 2-mercaptoethanol; separation conditions: pre-run, 100 V (K) for 15 min; run, 200 V (K) for 2 h; staining, 0.25% Coomassie Bleu G250 in destaining solution [acetic acid–methanol–water (100:500:400, v/v)]. Abbreviations: A–H = whole casein samples; 0–4 = FPLC fractions; α_{S2}-cn = α_{S2}-casein fast moving; α_S-cn = α_S-casein complex; k-cn = k-casein; β-cn = β-casein; γ-cn = gamma-casein.

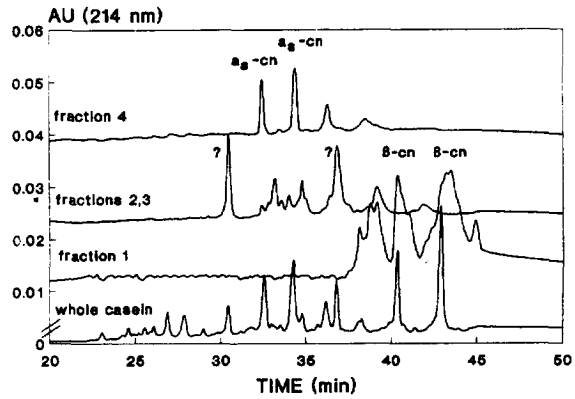


Fig. 3. CZE analysis of ewe's milk. CZE analysis of FPLC fractions and whole casein. Hydrophilically coated fused-silica capillary, 50 cm (to the detection point), 55 cm total length × 50 μm I.D.; electrolyte [2], pH 3.0; injection by pressure, 34 474 Pa × 8 s; applied voltage, 25 kV; T = 40°C; UV detection, 214 nm. (See Experimental section for details.)

as well as a minor peak eluting immediately after it. So, a definite differentiation between α-$_{S1}$-casein and α-$_{S2}$-casein fractions could not be made.

Results from FPLC fractions 1–4, obtained with the same CZE procedure as proposed for whole milk, are reported in Fig. 3. On the basis of PAAGE information, it was possible to identify two specific ranges of t_M, corresponding to α-$_S$-cn (29–36 min) and β-cn (38–45 min) fractions, respectively.

Fig. 4 shows the identification of k-casein and

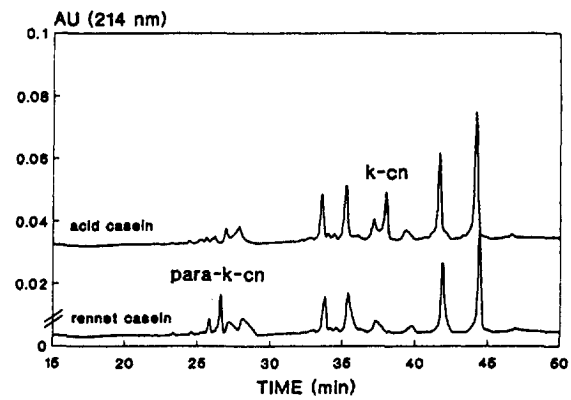


Fig. 4. CZE analysis of ewe's milk. Identification of k-casein (k-cn) and *p*-k-casein (*para*-k-cn) migration time (t_M). CZE analysis of acid and rennet caseins. (Experimental conditions: see Fig. 3.)

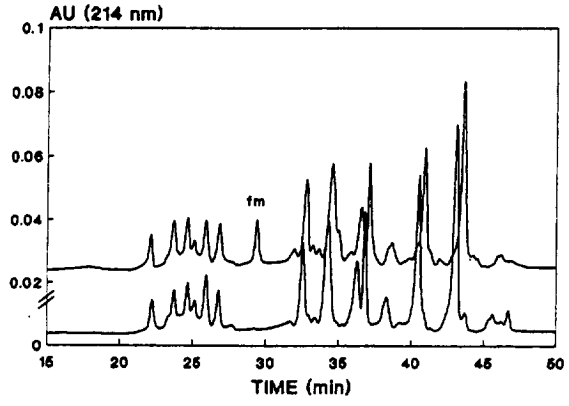
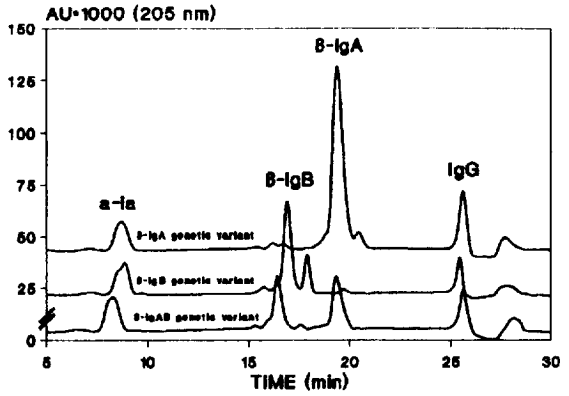


Fig. 5. CZE analysis of ewe's milk. Identification of fast moving α-$_{S2}$-casein variant (fm) migration time (t_M). CZE analysis of two individual milks. (Experimental conditions: see Fig. 3.)



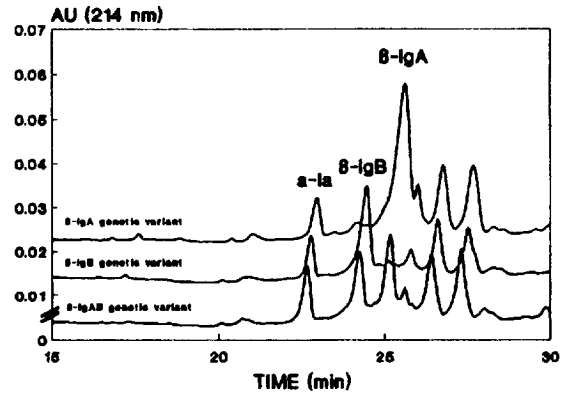


Fig. 6. HPLC and CZE analysis of ewe's milk. HPLC analysis of whey proteins [3] and CZE analysis of the same samples, as whole milk. (Experimental conditions: see Fig. 3.)

p-k-casein at t_M of 37 and 26 min, respectively. The electropherograms obtained for both acid and rennet casein samples showed good separation and high resolution also for samples other than whole milk. These results suggest that a wider application of CZE in the dairy field may be envisaged.

The electropherograms in Fig. 5 show the identification of fast moving α-$_{S2}$-cn, $t_M = 30$ min. The presence of a fast moving α-$_{S2}$-casein variant in ewe's milk, detected in the Manchega breed by Chianese et al. [8], was confirmed by PAAGE data [6] (an example is reported in Fig. 2, samples B and D). In this case, good qualitative correspondence between the two techniques was found. In fact, only for two of the 35 individual milk samples analyzed the results did not correspond.

Fig. 6 shows a comparison of the results obtained by RP-HPLC analysis on whey proteins [9] with those from CZE on the same samples, but analyzed as whole milk. Identification of the major whey proteins was possible: peaks corresponding to α-lactalbumin (α-la) and β-lgB, β-lgA were well resolved. Genetic variants of β-lg were also well separated in heterozygote milk samples, as expected based on the differences in amino acid composition [10]. Good correspondence between CZE and RP-HPLC results for identification of genetic variants of β-lg was found: the CZE method proposed allowed all

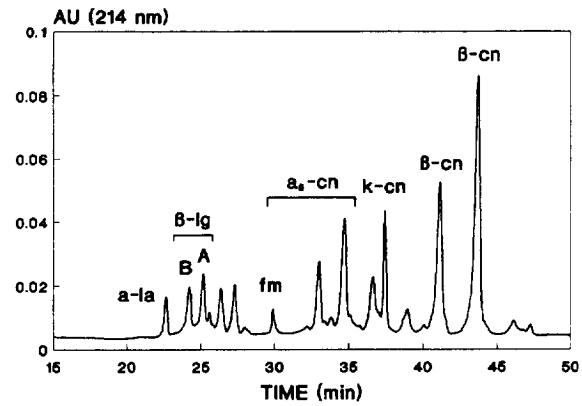


Fig. 7. CZE analysis of ewe's milk. Identification of the major protein components in individual milk. (Experimental conditions: see Fig. 3.)

samples to be correctly recognized. The last two peaks ($t_M > 26$ min) are still unknown.

Fig. 7 shows the high resolving power of this technique. Under the analytical conditions used, the major protein components of ewe milk were well-separated within 45 min, based on differences in their amino acid composition, as reported in literature [10,11].

4. Conclusions

CZE proved to be a suitable separation technique for the analysis of milk proteins. The major protein components of ewe milk were well resolved within 45 min. The urea and DTT concentrations used in this study gave complete sample solubilization during the incubation period, thus minimizing problems related to sample homogeneity in the loading step.

Identification of α-la, β-lg genotypes, α-$_S$-cn complex, fast moving α-$_{S2}$-cn variant, k-cn and β-cn fractions was possible by their characteristic t_M. The CZE results for both β-lg genotypes and fast moving α-$_{S2}$-cn in Massese breed showed good correspondence with those obtained by RP-HPLC and PAAGE. CZE separations permit the simultaneous determination of whey proteins and caseins. Sample preparation for CZE, requiring less manipulation, was easier than for RP-HPLC and PAAGE.

Finally, application of CZE in the dairy field may be broadened to the analysis of dairy products. Preliminary analyses on rennet casein samples showed satisfactory results.

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