The application of the high-performance ion-exchange chromatography for the analysis of bovine milk proteins

P. Bican

Swiss Federal Dairy Research Institute, CH-3097 Liebefeld-Berne (Switzerland), 4 June 1984

Summary. A high-performance ion-exchange chromatographic (HPIEC) method for the analysis of milk proteins is described. The technique provides an alternative to other separation methods. The resolution obtained for both the proteins of the casein group and bovine lactoserum is comparable with that of electrophoretic analysis on polyacrylamide gels and low-pressure chromatography.
Key words. Bovine milk proteins; high-performance liquid chromatography; ion-exchange chromatography.

Analyses of milk proteins were introduced during studies on genetic interrelationships, endocrinology, and for other scientific and industrial applications. Much knowledge has accumulated from work using electrophoresis and classical liquid chromatography[1,2]. These methods are, however, laborious and time-consuming. Recently, studies in this laboratory[3,4] and elsewhere[5-7], have shown that high-performance liquid chromatography provides a promising approach to the analysis and preparation of milk proteins.

The early work of Sober and Peterson[8] has shown that ion-exchange chromatography is capable of resolving related biological molecules. The procedure depends on the interactions between the bound phase at the surface of the ion exchanger and the charged solute molecules. HPIEC represents a successful combination of the kinetics of classical ion exchangers with modern column supports and advanced instrumentation. Substantial information on this topic can be found in the studies of Regnier[9].

The primary objective of the present experiments was to assess the potential of HPIEC for the analysis of closely-related milk proteins.

Methods. α-Lactalbumin, grade II, 90% purity, β-lactoglobulin A, and β-lactoglobulin B were all purchased from Sigma Chemical Co. Bovine serum albumin was from Serva Feinbiochemica. The whey proteins were prepared from pooled skim milk by centrifugation (160,000 × g, 3 h). β-Casein 90% and $\varkappa$-casein were prepared according to Zittle and Custer[10]. The whole casein was precipitated at its isoelectric point. Urea was ultra pure grade from Serva. All other chemicals were of Analar grade (BDH).

The liquid chromatograph used was Beckman Instruments 324 chromatograph consisting of a Model 100 A solvent metering

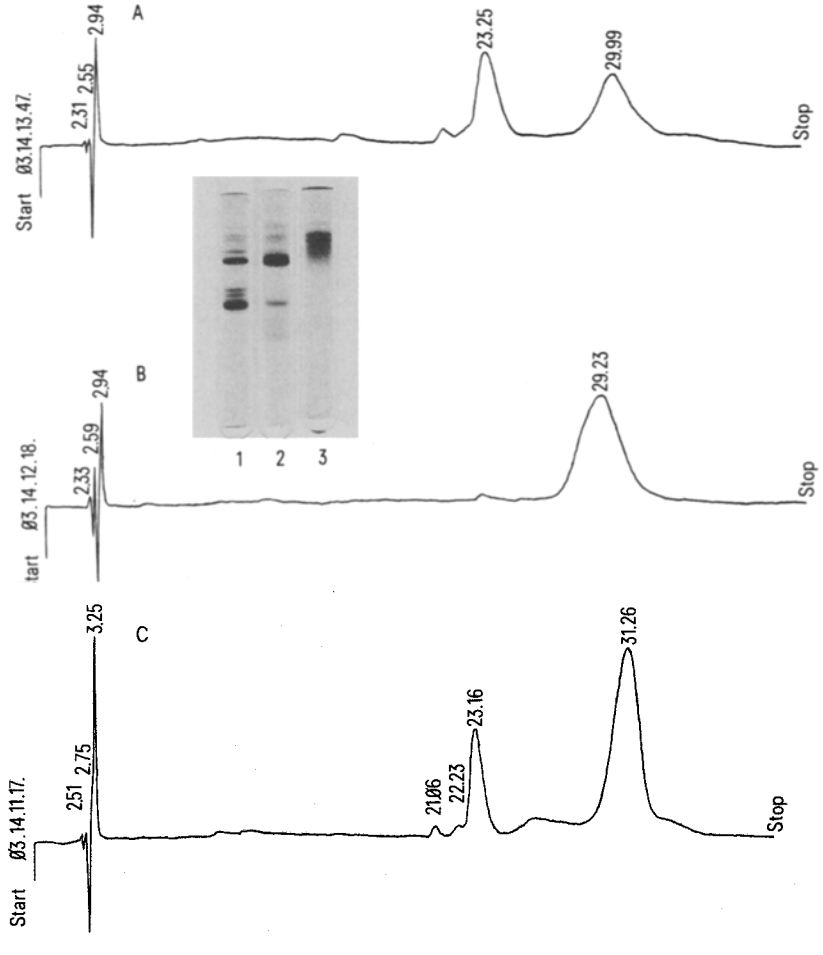

Figure 1. *A* β-Casein; *B* $\varkappa$-casein; *C* whole casein. Column: Protein Pak DEAE-5 PW, ambient temperature. Buffer: solvent A: 0.02 M Tris/Cl, pH 7.2; solvent B: 0.5 M NaCl in solvent A; linear gradient: 0–100% over 40 min. Sample: 50–100 µg of protein were injected onto a column. Detection: 280 nm. The number appearing on each peak is the retention time expressed in min. The insert: polyacrylamide (PAA)-gel electrophoresis of the caseins, 11% PAA-gel, 5 M urea, 25 mM Tris, 190 mM glycine, pH 8.3. 1: whole casein; 2: β-casein-rich fraction; 3: $\varkappa$-casein-rich fraction.

system. An Altex Scientific Model C-R 1 A computing integrator was used to process the data from the chromatography. Protein Pak DEAE-5 PW, 7.5 × 75 mm column was from Waters Associates. Absorbance tracings at 280 nm were measured with a Beckman Model 165 variable wavelength detector. To maintain performance the column was flushed daily with 5 ml of 0.1 N NaOH. The column was equilibrated with new mobile phase conditions for 15 min. The operational details are given in the legends to the figures. The gradient was started 3 min after injection of the sample. The whey protein samples were dissolved in the starting buffer (A), casein was dissolved in the same buffer, containing 7 M urea.

Results and discussion. Figure 1 (traces A–C) shows the separations of different casein components with HPIEC. In general, the chromatographic separation attained can be considered good for such proteins. The retention values are reproducible. Initial attempts to supplement the buffers with urea in concentrations of 3.5–7 M failed. The addition of urea at any concentration resulted in incomplete resolution, severe peak asymmetry and baseline instability. When working with the Protein Pak DEAE column, urea at a concentration of 7 M in 0.02 M Tris/Cl-buffer, pH 7.2 was necessary only for sample preparation. It is assumed that temporary destruction of the casein conformation in the urea containing sample buffer is sufficient for successful resolution. A possible explanation of this phenomenon would be the short length of the column and the rapid binding of the casein monomers with the column support at the begining of the chromatography. The decrease in the urea concentration of the sample preparation due to dilution with the small column volume is probably not sufficient to enable the reassociation of the monomers. No substantial alteration of casein resolution could be observed when agents such as 2-mercaptoethanol or dithiotreitol were added to buffers. It could also be seen that the nonionic surfactant Triton X-100 showed no positive contribution to the resolution. Experiments were carried out in which the gradient elution was accomplished in shorter run times and with a gradient of varying slope. A lower gradient slope and linear elution resulted in better resolution.

The HPIEC-procedure for whey proteins was similar to that for casein analysis. The UV-tracings (depicted in figure 2, A–E) compare with results obtained by the electrophoretic analysis. They whey proteins were distributed on the gradient into as many as nine peaks. The overloading of the column to detect fine peaks brought about tailing at the end of the profiles. Definite differences could be found between elution characteristics for the genetic variants A and B of bovine β-lactoglobulin, reflecting even the minimal difference in the primary structure of the protein. The pattern obtained for α-lactalbumin showed the

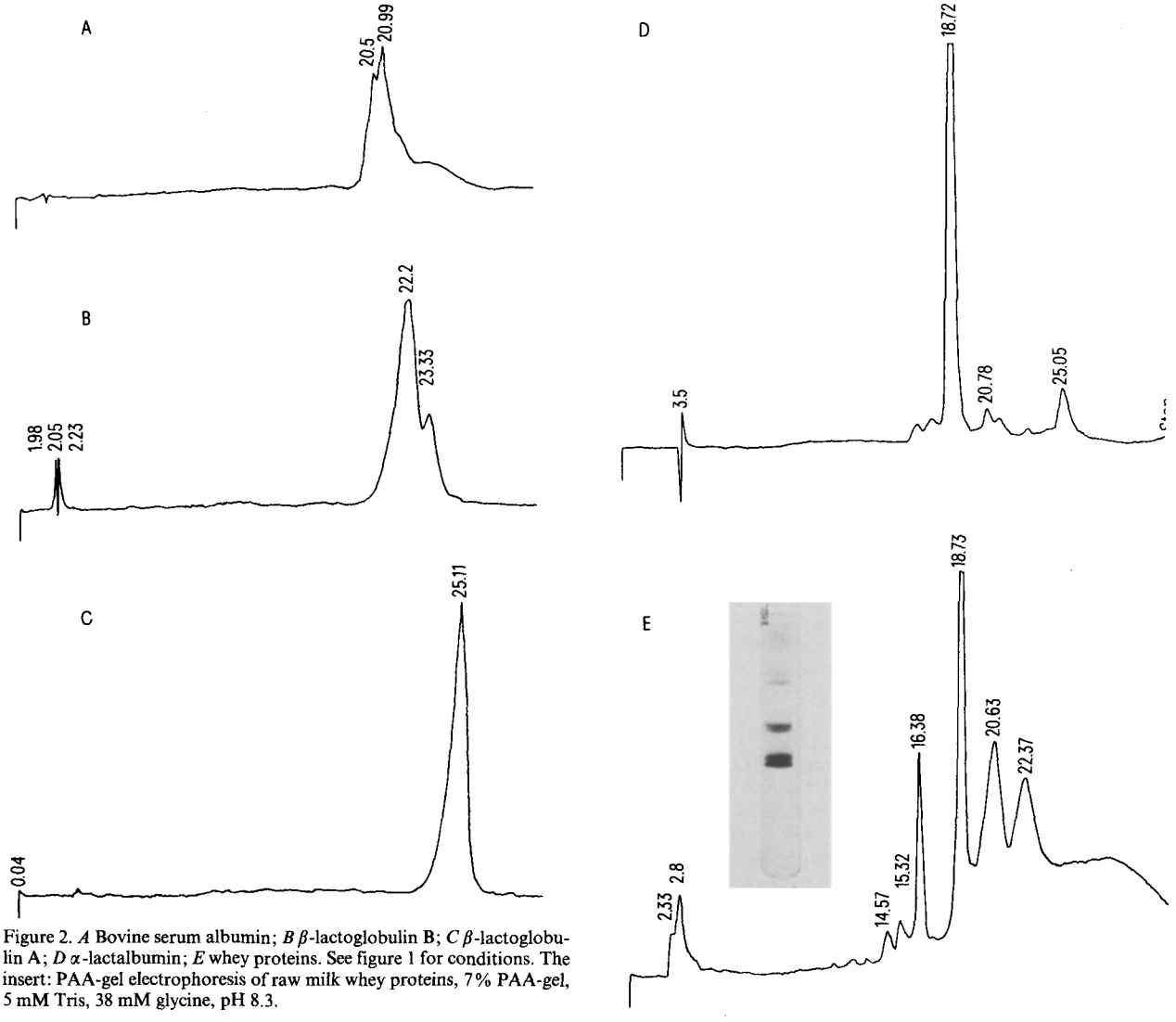

Figure 2. *A* Bovine serum albumin; *B* β-lactoglobulin B; *C* β-lactoglobulin A; *D* α-lactalbumin; *E* whey proteins. See figure 1 for conditions. The insert: PAA-gel electrophoresis of raw milk whey proteins, 7% PAA-gel, 5 mM Tris, 38 mM glycine, pH 8.3.

expected contaminants. The results based on area measurement (values not shown in figure) are consistent with the quality of the standard.

In conclusion, the HPIEC-technique described in this communication exceeds the classical separation procedures in speed of analysis and possibility of automatic operation. Besides its application in biological studies, this can be used for following the proteolytic degradation of milk proteins.

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Tyrosinase-cytalyzed conjugation of dopa with glutathione[1]

S. Ito[2], A. Palumbo and G. Prota

Institute for Comprehensive Medical Science, School of Medicine, Fujita-Gakuen University, Toyoake, Aichi 470-11 (Japan), and Stazione Zoologica di Napoli, Napoli 80121 (Italy), 19 June 1984

Summary. A convenient method is described for the preparation of 5-S- and 2-S-glutathionyldopa, based on tyrosinase oxidation of dopa in the presence of glutathione. The yields of 5-S, 2-S, and 6-S isomers produced were about 76, 12, and 5%, respectively.
Key words. S-glutathionyldopa; tyrosinase; dopa; glutathione.

In recent years, the origin and metabolism of cysteinyldopas[3], i.e. the 2-S, 5-S, and 6-S isomers, have received a good deal of attention following the extensive studies by Rorsman and his associates on the clinical use of these metabolites, as biochemical markers for early diagnosis of metastasizing melanomas[4]. Available evidence suggests that formation of cysteinyldopas in melanocytes may proceed 1) via direct addition of free cysteine to dopaquinone generated by tyrosinase-catalyzed oxidation of dopa[3], or 2) by the analogous reaction of the quinone with glutathione (GSH) followed by enzymic hydrolysis of the resulting glutathionyldopas, or 3) by both mechanisms. Which of these pathways prevails in vivo is not yet clear, but it is noteworthy that both 5-S-cysteinyldopa and 5-S-glutathionyldopa (fig. 1) have been found in melanoma tissues, which also seem to contain the hydrolytic enzymes (γ-glutamyl-transferase and peptidase) capable of converting the latter into the former[5]. This finding, coupled with the fact that GSH is generally the most abundant thiol in cells[6], points to an important role of glutathionyldopas in the biosynthesis of cysteinyldopas. As a part of our continuing studies on the role of sulphydryl compounds in pigment cell metabolism[7,8], we report here a convenient procedure for the preparation on a semi-preparative scale of 5-S- and 2-S-glutathionyldopa by tyrosinase-catalyzed conjugation of dopa with GSH.

Materials and methods. Analytical method. Conditions for high-performance liquid chromatography (HPLC) were as follows: chromatograph, Yanaco Model L-2000; detectors, Yanaco VMD-101 electrochemical detector (750 mV vs Ag/AgCl reference electrode) and Yanaco U-213 UV detector (254 nm); column, Yanaco ODS-A (4.6 × 250 mm); mobile phase, 0.1 M potassium phosphate buffer, pH 2.1–methanol, 96:4 (v/v); column temperature, 45 °C; flow rate, 0.7 ml/min. Peptide samples (ca. 1 mg) were hydrolyzed with 6 M HCl (1 ml) in evacuated, sealed tubes at 110 °C for 24 h. The hydrolysates were evaporated to dryness and analyzed with a JEOL JLC-6AH amino acid analyzer using a 4 lithium citrate buffer system.

Preparation of glutathionyldopas. A solution of L-dopa (197 mg; 1 mmole) and GSH (614 mg; 2 mmoles) in 100 ml of 0.05 M sodium phosphate buffer, pH 6.8, was stirred at room temperature after the addition of mushroom tyrosinase (50 mg; 2000 units/mg from Sigma Chem. Co.). The reaction was monitored by HPLC. After 3 h when most of dopa (> 95%) had disappeared, the reaction was stopped by the addition of 6 M HCl (5 ml). The reaction mixture was passed through a column (2.0 × 8 cm) of Dowex 50W-X2 (200–400 mesh, equilibrated with water). After washing with 0.5 M HCl (100 ml), the column was eluted with 3 M HCl and 20-ml fractions were collected and monitored by HPLC. Fractions 1–7, which contained the glutathionyldopas, were evaporated to dryness in a rotary evaporator at 40 °C; each evaporation was conducted on 40 ml volume or less to minimize hydrolytic cleavage of the peptide bonds. The residue, taken up in 2 M HCl (2 ml), was chromatographed as described in figure 2. Fractions 13 and 14

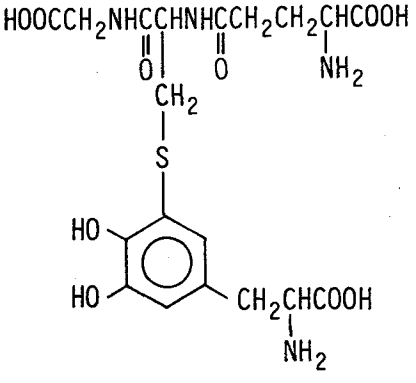

Figure 1. Structure of 5-S-glutathionyldopa.