Application of Pluronic copolymer liquid crystals for the capillary electrophoretic separation of collagen type I cyanogen bromide fragments

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Abstract

A capillary electrophoretic method exploiting the properties of Pluronic copolymer liquid crystals (F127) was developed for the separation of collagen cyanogen bromide (CNBr) fragments. The separations obtained were at least comparable (if not better) to those obtained by other methods applicable to this category of compounds. In the optimized version a bare silica capillary [47 cm (40 cm to the detector)×75 μm I.D.] was used with 10 mM Tris and 75 mM phosphate buffer (pH 2.5) containing 7.5% Pluronic F127 copolymer. The separation mechanism which involves both the molecular sieving and surfactant properties of the Pluronic F127 gel phase is discussed.   © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Though capillary electrophoretic separations of proteins have been highlighted by analytical chemists for a long time and considerable advances in this direction can be traced in the literature [1], no generally applicable approaches are yet available at the moment. Most of the methods applied suffer from protein analytes sticking to the capillary wall and low selectivity, particularly if closely related proteins/fragments are subjected to electromigration separations. Collagens represent in this context an interesting subject to study. Not only do they constitute about one-third of all body proteins but they also represent a set of closely related proteins (about 19 isotypes have been recognized so far) that are difficult to separate. The most common collagen molecule, type I, is constituted of three polypeptide chains called α_1 (two chains) and α_2 which form chain polymers (dimers, β_{12}, β_{11} and trimers, γ). Characterization and identification of the constituting α chains is possible by using cyanogen bromide (CNBr) fragmentation; owing to the limited amount of methionine residues a relatively simple peptide mixture arises. The nomenclature of these peptides refers to the parent polypeptide chain; α_1 constituting polypeptide chain yields a set of α_1CNBr peptides (α_2 chain yields in analogy a set of α_2CNBr peptides). The index, e.g., α_1CB$_1$, identifies a particular peptide within the set. The number in parentheses refers to the collagen type, e.g., α_1(I)CB$_1$ means CNBr peptide of collagen type I. The complete sequence of collagen type I is known; the protein exhibits a high internal homogeneity with highly abundant sequence Gly–X–Y, where X is most frequently proline (for a review see Ref. [2]).

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Cyanogen bromide collagen fragments are traditionally separated by gel electrophoresis and high-performance liquid chromatography (HPLC), however the possibilities of capillary electrophoresis (CE) are now investigated with the aim of a more easy quantitation of the arising peaks (for a review see Ref. [3]). These protein fragments were separated at acidic conditions (pH 2.5) either in pure buffer or in buffers containing a polymer modifier in combination with both sub- and supramicellar concentrations of sodium dodecyl sulfate (SDS) [4–7].

Pluronic polymers are triblock uncharged copolymers with the general formula [poly(ethylene oxide)]$_x$[poly(propylene oxide)]$_y$[poly(ethylene oxide)]$_x$ (Pluronic is a registered trade name of BASF Performance Chemicals, Mount Olive, NJ, USA). In the case of Pluronic F127 the coefficient numbers are $x=106$ and $y=70$ and molecular mass is about 13 000. These copolymers have the typical features of surfactants and self associate into large micelles [8–12]. Self-association is favored by increasing concentration and temperature. The less polar poly(propylene oxide) chain segments are desolvated and segregate into a hydrophobic micelle core surrounded by a soft "brush" of highly hydrated, flexible poly(ethylene oxide) chains. Pluronic copolymers form both isotropic and anisotropic liquid crystalline "gels". The type of the phase (isotropic, cubic, hexagonal and lamellar) depends, not only on the structural features of the polymer but also on its concentration and temperature. It means, in practice, that Pluronic which is soluble at low temperature gellifies with a temperature increase, i.e., Pluronic F127 at a concentration of 20% is a freely flowing liquid at refrigerator temperature (5°C). At this stage the polymer can be easily introduced into the capillaries. At room temperature (20°C) this liquid forms gels [13–16]. It was proposed that these copolymers, owing to their unique features, might represent useful media for electrophoretic separations of biological macromolecules (reviewed by Rill et al. [16]). So far double-stranded DNA [13–15] and oligonucleotides [14,17] were investigated but the possibility of separation of proteins in 20–25% Pluronic F127 [13,16] has also been mentioned. A common problem in these separations is time consuming operations: it is necessary to introduce the Pluronic gel into the capillary in a cold room,

followed by temperature equilibration to ensure gel formation.

In this study we attempted to simplify the preliminary operations and exploit the unique properties of the Pluronic copolymer for the separation of a model set of proteins/peptides (collagen fragments). We also attempted to compare proteins/peptides profiles obtained by the Pluronic-containing electrophoretic system and reversed-phase HPLC.

2. Experimental

2.1. Chemicals used

Sodium dihydrogenphosphate, Tris and hydrochloric acid were products of Lachema (Brno, Czech Republic) and were of analytical-reagent grade quality. Pluronic F127, heptafluorobutyric acid (HFBA) and ammonium hydrogencarbonate were from Sigma (St. Louis, MO, USA), bromcyan (cyanogen bromide) and 2-mercaptoethanol were purchased from Merck (Darmstadt, Germany), formic acid was a product of Fluka (Buchs, Switzerland). All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA).

2.2. Capillary electrophoresis

All runs were done with Beckman P/ACE instrument system 5500 (Beckman, Fullerton, CA, USA). A bare fused-silica capillary 47 cm (40 cm to the detector)×75 μm I.D. was used. Detection was done by UV absorbance recording at 214 nm. Before analysis the capillary was washed for 5 min with the background electrolyte. The sample was diluted by buffer (2:1, v/v) and injected either hydrodynamically (3 s, 3.45 kPa overpressure) or electrokinetically (8 s, 10 kV). The separation was run at 20°C at an applied voltage of 15 kV (except for the analyses at 50°C where because of the current limit the voltage applied was 11 kV). After the separation came to its end, the capillary was washed step-wise with the background electrolyte (1 min), water (1 min), 3 mol/l HCl (3 min), and water (1 min). The background electrolyte consisted of 10 mmol/l Tris and 75 mmol/l phosphate buffer, pH 2.5 containing 7.5% Pluronic F127. This electrolyte was extensively

mixed at 4°C for 24 h. Before analysis the background electrolyte was filtered using a 45-μm Millex-HV filter (Millipore).

2.3. Model mixtures

A set of peptides obtained by CNBr cleavage of rat tail tendon collagen was used as model mixture. This sample contained fragments of both type I and III collagen and was prepared by the procedure described in our previous communication [4], based on the classical paper of Scott and Veis [18]. Briefly, samples (rat tail tendons) were incubated in 0.2 mol/l ammonium hydrogencarbonate, pH 7.0 containing 25% β-mercaptoethanol to reduce oxidized methionyl residues and after lyophilization the samples were cleaved by CNBr in 70% (v/v) formic acid under N_2. Samples were lyophilized and than reconstituted in 5% formic acid to a concentration of 10 mg/ml.

2.4. HPLC

Separation of collagen CNBr peptides was done by the HP 1100 system (Hewlett-Packard, Palo Alto, CA, USA) consisting of a degasser, binary pump, autosampler, thermostatted column compartment and diode array detector. The column used was Zorbax 300SB-C_4, narrow-bore, 150×2.1 mm, 5 μm [Rockland Technologies, USA (Hewlett-Packard)] with a precolumn (12.5×2.1 mm) packed with the same material. Elution was done by a linear gradient using mobile phase A (water with 0.1% HFBA) and B (water–acetonitrile, 50:50, v/v, with 0.1% HFBA). Elution was made by a gradient from 0 to 100% B at 40 min followed by a linear elution by B (10 min). Next the column was equilibrated 10 min with solution A before the next run. The flow-rate used was 0.25 ml/min, the column temperature was held at 60°C. UV detection was done at 214 nm and injection volume was 3 μl.

3. Results and discussion

Separation of CNBr fragments of collagen using Pluronic copolymer-containing background electrolyte at acidic pH gave the separation seen in Fig.

1. Differences between the separation order in CE and HPLC are indicative of a different mechanism of separation (compare Fig. 1A and B). The most noticeable difference is the changed elution order of fractions 3 and 4 and splitting of fraction 5. The resolution obtained for the collagenous fragments exhibited mainly different selectivity when compared to results published so far in the field of capillary electrophoresis with other types of background electrolytes, namely it was possible to distinguish between fragments possessing a rel. mol. mass in the range around M_r 20×10³.

To obtain the result shown it was necessary to optimize the buffer composition. If pure phosphate buffer containing Pluronic was used, the voltage was fluctuating and irreproducible values were obtained. On the contrary, if only Tris was present as buffer the current stability was good, however the separation was totally unsuccessful (data not shown). The optimized composition of buffer finally found was 75 mM phosphate and 10 mM Tris [different concentrations of both phosphate (10–100 mmol/l) and Tris (5–50 mmol/l) were tested]. Addition of organic modifiers (acetonitrile, isopropanol or methanol) did not improve the separation. Also concentration of the Pluronic copolymer was optimized. Its high concentration (18–30%) as recommended by Rill et al. [16] for the separation of nucleotides and DNA, proved unsuitable for the separation of collagen fragments because the elution time exceeded 2 h and resulted in broad ''bumps''. Optimized concentration of Pluronic F127 (7.5%) gave adequate separation. Temperature effect was also examined (Fig. 2). At 50°C, the Pluronic solution formed a gel phase–cubic liquid crystalline phase. In this experiment capillary was filled with buffer at 20°C and then the capillary was heated to 50°C followed by conditioning at this temperature for 30 min. Separation at this elevated temperature was faster (it must be mentioned that separation voltage was 11 kV instead 15 kV owing to the imposed current limit of 250 μA). However better separations were obtained at 20°C – compare Fig. 2A and B. The fact that lower Pluronic concentration satisfies the separation needs is a favorable result because 7.5% Pluronic F127 is at 20°C freely flowing viscous liquid which can be introduced into the capillary and used for flushing as well. In this case the tedious replacement
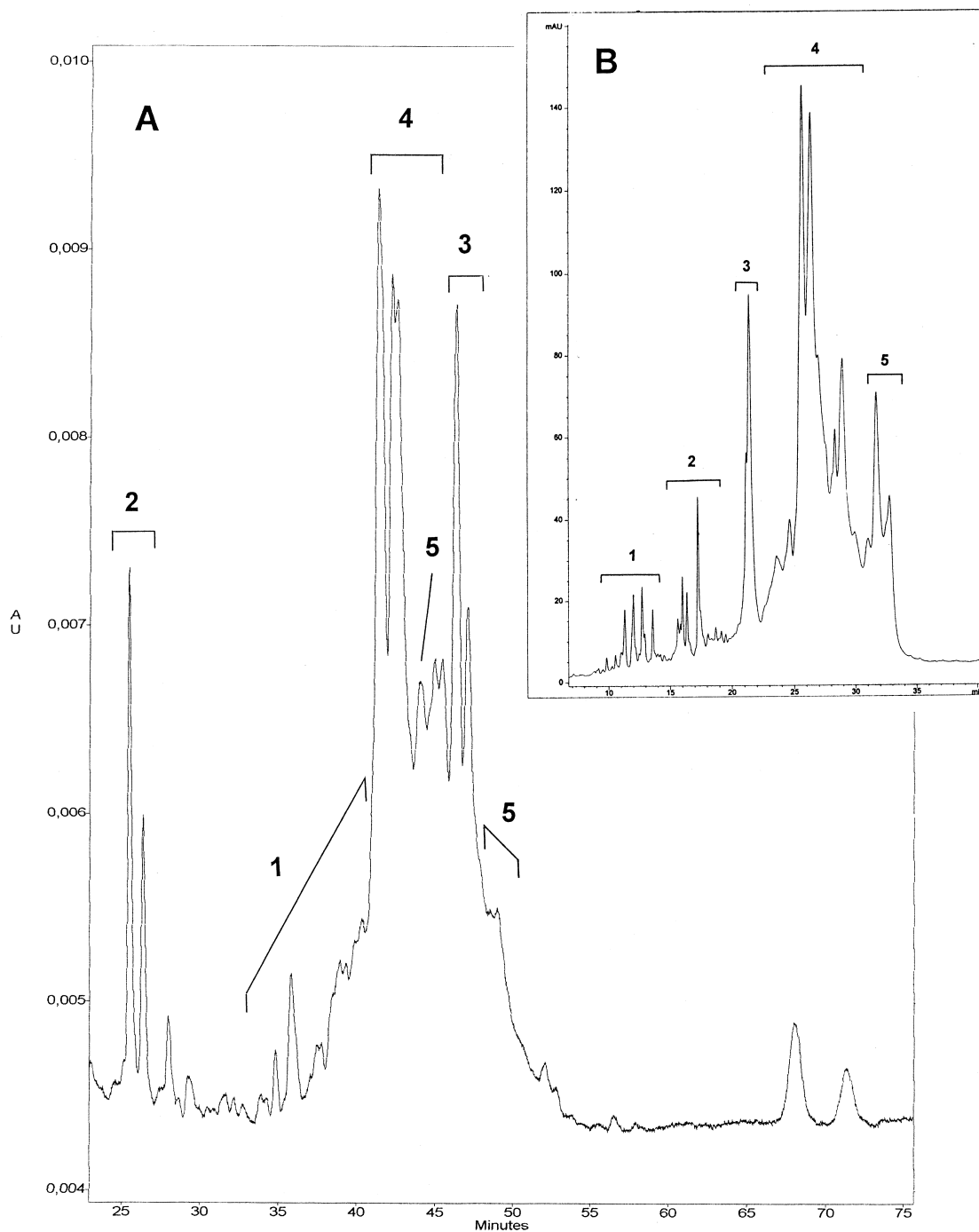
Fig. 1. Separation of rat tail tendon collagen CNBr peptides by (A) capillary electrophoresis in the Pluronic copolymer media and by (B) high-performance liquid chromatography (for details see Experimental). Identification of individual fractions: 1, $\alpha_2(I)CB_2$, $\alpha_1(I)CB_2$, $\alpha_1(I)CB_5$; 2, $\alpha_1(I)CB_4$, $\alpha_1(III)CB_3$, $\alpha_1(III)CB_6$, $\alpha_1(I)CB_3$, $\alpha_1(III)CB_4$; 3, $\alpha_1(I)CB_6$; 4, $\alpha_1(III)CB_5$, $\alpha_1(I)CB_7$, $\alpha_1(I)CB_8$, $\alpha_2(I)CB_4$ and incomplete cleavage products; 5, $\alpha_2(I)CB_{3,5}$, $[\alpha_1(III)CB_9]_3$. Peak identification was done in the same way as described in Ref. [19].
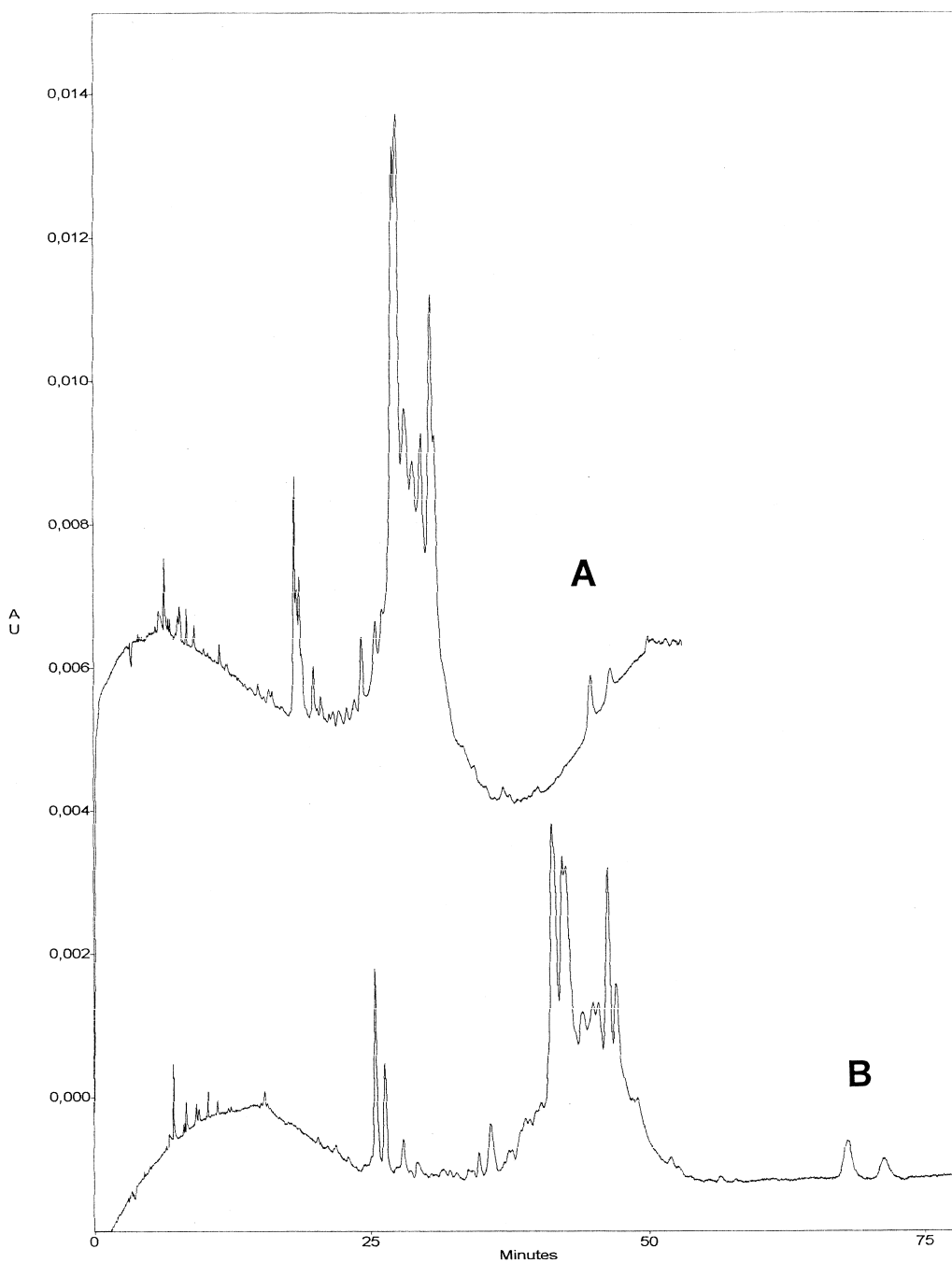
Fig. 2. Temperature effect on the separation of rat tail tendon collagen CNBr peptides in 7.5% Pluronic F127 at (A) 50°C, (B) 20°C. Separation voltage was 15 kV for 20°C and 11 kV for 50°C (for details see Experimental).
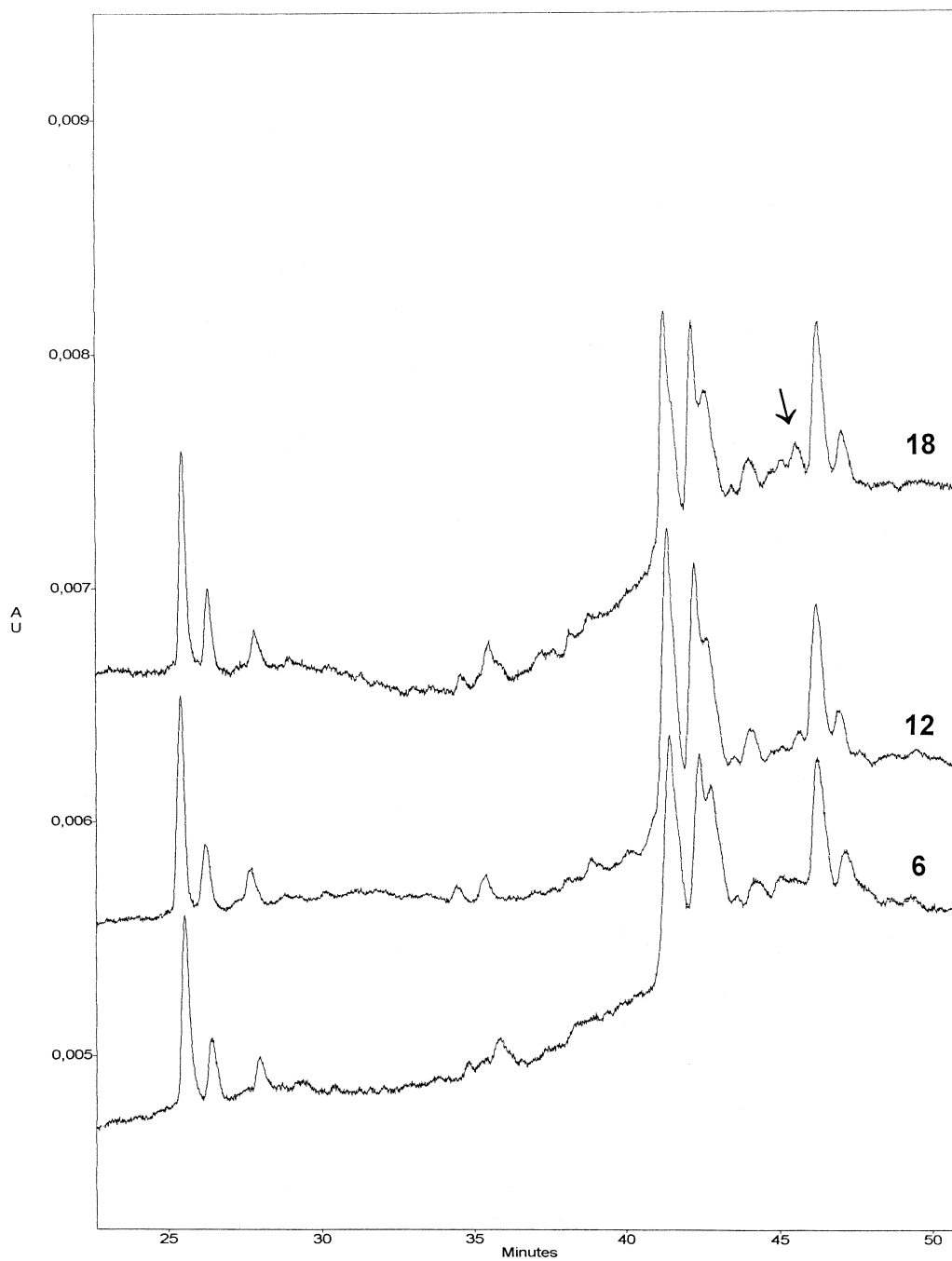
Fig. 3. Electrophoretic profiles of tail tendon collagen CNBr peptides from rats 18, 12 and 6 months old. Arrow indicates the peak that is age-dependent. For details see Experimental.

of the gel involving removal of the capillary cartridge and cooling it off in a cold room can be omitted.

The influence of the injection mode was also investigated. In capillary gel electrophoresis electrokinetic sample introduction of the sample appeared better for sample application and therefore it was used in all experiments. It was observed that the result was comparable when hydrodynamic injection for 3 s (3.45 kPa) or electrokinetic injection for 8 s (10 kV) were used.

The electrophoretic separations obtained in the Pluronic copolymer-containing background electrolyte is superior to simple electrophoresis under acidic conditions (pH 2.5), even if the latter separation is done in the presence of a polymer additive or a surfactant [4–7]. It seems that this separation combines favorably the features of all previously mentioned methods. The electrophoretic profile resembles the result of capillary zone electrophoresis in pure buffer (pH 2.5) or in a buffer containing sieving polymer as well as the result of micellar electrokinetic chromatography (in which an altered order of peaks, i.e., 1, 2, 5, 4 and 3 was observed, Ref. [7]). It supports the idea about the synergistic effects of capillary gel electrophoresis (molecular sieving) and micellar electrokinetic chromatography (association with the micelles). The differences in selectivity of electrophoretic profiles of collagen type I CNBr peptides obtained with capillary electrophoresis in plain phosphate buffer or with an SDS-containing background electrolyte can be gained from the results published in Refs. [4–7].

Rill et al. [14,16] described the process of separation in Pluronic as being based on a partition equilibria between the aqueous phase and the hydrophobic domains during the electrophoretic transport (''hydrophobic interaction electrophoresis''). These authors have described Pluronic liquid crystals as a tripartite structure defined by the hydrophobic micelle cores, hydrated brushes and interbrush domains. It is noticeable, however, that in our model peptide mixture, owing to the large internal homogeneity, larger peptides possess more hydrophobic domains compared to the smaller ones.

The separation of CNBr peptides with Pluronic offered a separation with different selectivity when compared to the HPLC profile. Consequently, combination of the Pluronic based profiles with HPLC profile can bring about new information about modifications of collagenous molecules.

To investigate the above idea we tried to apply this electrophoretic method for the determination of the presence of age-associated changes in CNBr peptides. Fig. 3 shows a change in the amount of the small peak at 45 min (marked by an arrow) that is only hardly seen in collagen fragments preparation obtained from 6-month-old rats but is increasing with advancing age. This increasing peak is not visible in the HPLC profile. This observation is in agreement with the idea of a different separation mechanism in capillary electrophoresis with Pluronic and reversed-phase chromatography.

4. Conclusions

In conclusion it is possible to say that the unique properties of Pluronic copolymers (a special combination of capillary gel electrophoresis and micellar electrokinetic chromatography) appears useful in peptide analysis. Separation of collagenous CNBr peptides/fragments can be achieved with this copolymer used in the form of a freely flowing liquid, i.e., 7.5% gel at 20°C.

Acknowledgements

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