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Capillary electrophoretic separation of proteins and peptides using Pluronic liquid crystals and surface-modified capillaries

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Abstract

Separation of model mixtures of peptides/proteins carried out in a hydrophilically coated capillary in 10 mmol/l Tris and 75 mmol/l phosphate buffer containing 7.5% (w/w) Pluronic F127 copolymer (apparent pH 2.9) revealed that the separation is predominantly driven by the charge/mass ratio with little or no sieving effect. Using a coated capillary helped to remove current fluctuations that are observed in the fused-silica capillaries in the presence of the Pluronic copolymer. With peptides bearing distinct positive charge (polylysine of M_r around 3300) molecular sieving helps more detailed separation of individual species. Polyamino acids carrying negative charge can be brought to the detector window in the reversed polarity mode, however, no detailed separation of the individual species involved was observed under the conditions used. With a naturally occurring mixture of collagen fragments released by CNBr treatment of the protein the sequence of emerging peptides (positive polarity mode) with no relation to the rel. mol. mass could be revealed. It is concluded that separation of proteins/peptides in the presence of Pluronic in the background electrolyte occur on the charge/mass ratio basis with molecular sieving effects acting as a secondary partition mechanism. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Pluronic modifier; Proteins; Peptides

1. Introduction

Capillary electrophoretic separations of peptides and proteins exploit generally three properties of the solutes involved, namely the effective charge, molecular size (relative molecular mass) and shape (conformation) [1–5]. In capillary electrophoresis dynamic molecular sieving exploiting entangled water-soluble synthetic polymers appears today the approach of choice as permanently gel-packed capillaries suffer a number of drawbacks of which poor packing reproducibility and short life-time of the capillary caused by its clogging after a few runs should be mentioned in the first place.

In dynamic sieving molecular sieving effecting modifiers are added to the background electrolyte at a concentration at which these polymers entangle attaining thereby the properties similar to traditional hydrogel networks. Linear polyacrylamide, polyethylene oxide, polyvinyl alcohol or hydroxyethylcellulose are typical polymers used. Recently, a new category of self-organizing polymers has been introduced in the area of capillary electrophoresis under the name Pluronic (the trade name of BASF Performance Chemicals, Mount Olive, NJ, USA). Chemically it is dealt with (polyethylene oxide)$_x$(polypropylene oxide)$_y$(polyethylene oxide)$_z$ triblock copolymers, which are highly soluble and

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which associate into large micelles. In the case of Pluronic F127 the coefficient numbers are $x=106$ and $y=70$; the relative molecular mass of the polymer is about 13 000. In these micelles the hydrophobic core is formed by the polypropylene oxide part of the molecule while the more polar polyethylene oxide part constitutes the outer layer. From this point of view Pluronic copolymers can be considered as surfactants which self-associate both into isotropic and anisotropic gels. This association is favored by temperature and concentration of the polymer present [6–8].

It is, perhaps, not surprising that owing to their totally different nature (as compared to traditional entangled polymers) also their properties as background electrolyte modifiers would be different. Applications of Pluronic liquid crystals have been so far limited to the separation of DNA and oligonucleotides [9–12]. The first attempt to use this gel for the separation of peptides/proteins is mentioned by Rill et al. [9] but to our best knowledge the only paper dealing with this is our recent report (Mikšík and Deyl [13]). It seemed to us quite challenging to investigate the properties of these gels (liquid crystals) as background electrolyte modifiers in more detail.

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, ammonium hydrogencarbonate was 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). Capillaries 27 cm (20 cm to the detector)×50 μm I.D. were used (CElect H, H1, H2 and P1; Supelco, Bellefonte, PA, USA). Detection was performed by UV absorbance recording at 214 nm. Before analysis the capillary was washed 5 min with the background electrolyte. The sample was injected hydrodynamically (1 s, 3.45 kPa overpressure). The separation was run at 20°C at an applied voltage of 5 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 containing 7.5% (w/w) Pluronic F127, apparent pH 2.9. This electrolyte was extensively mixed at 4°C for 24 h. Before analysis the background electrolyte was filtered using a Millex-HV filter (Millipore), 0.45 μm.

The peptide profile obtained with the small average M_r polylysine sample and the profile obtained with collagen CNBr peptides were evaluated by the PeakFit programme v4 for Windows (Jandel Scientific, San Rafael, CA, USA).

2.3. Protein and peptide samples

The set of test analytes comprised the following proteins: cytochrome *c* (M_r 12 500), chymotrypsinogen A (M_r 25 000), albumin from hen egg (M_r 45 000), albumin from bovine serum (M_r 68 000), aldolase (M_r 158 000) and catalase (M_r 240 000). All these proteins were products of Boehringer (Mannheim, Germany). Two poly-L-lysines were obtained from Sigma, the first had an average relative molecular mass of 22 700 (by viscosity; degree of polymerization, DP: 138) or 28 200 [by size-exclusion chromatography–low-angle laser light scattering (SEC–LALLS); DP: 172] (molecular distribution: $<$10% smaller than 10 800 and $<$10% greater than 46 000) and the second polymer had an average relative molecular mass of 4000 (by viscosity; DP: 19) or 3300 (by SEC–LALLS; DP: 16). Polyaspartic acid (Sigma) had an average relative molecular mass of 8600 (by viscosity; DP: 63) or 7000 (by SEC–LALLS; DP: 51) and polyglycine (Sigma) had an average molecular mass of 4600 (by viscosity; DP: 80). Samples were dissolved in 5% (v/v) formic acid to a concentration of 1 mg/ml.

A set of peptides obtained by CNBr cleavage of

rat tail tendon collagen was also 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 [13]. Briefly, samples (rat tail tendons) were incubated in 0.2 mol/l ammonium hydrogencarbonate, pH 7.0, containing 25% (v/v) β-mercaptoethanol to reduce oxidized methionyl residues and after lyophilization the samples were cleaved by CNBr in 70% (v/v) formic acid under nitrogen. Samples were lyophilised and then reconstituted in 5% formic acid to a concentration of 2 mg/ml.

3. Results and discussion

In the first stage of our experimental work we attempted to separate a set of molecular mass standards specified in Experimental. Under the conditions used [CElect P150 capillary, 10 mmol/l Tris and 75 mmol/l phosphate buffer containing 7.5% (w/w) Pluronic F127] cytochrome c (M_r 12 500) and chymotrypsinogen A (M_r 25 000) moved in the order of increasing molecular mass; however, hen egg albumin (M_r 45 000) appeared before the peak of chymotrypsinogen A in spite of its larger rel. mol. mass (Fig. 1A). In addition three components of the test mixture, namely bovine serum albumin (M_r 68 000), aldolase (M_r 158 000) and catalase (M_r 240 000) did reach the detector window even after 1 h of running time. The charge-to-mass ratio of these analytes in the relatively low pH background electrolyte (2–3 pH units below the isoelectric point, p*I*) should be sufficient for reasonable values of mobilities and migration times. The slow migration of these proteins is probably caused by too dense sieving effect of liquid crystals and/or by their strong interactions with the liquid crystals micellar pseudophase. In order to elucidate which of these two possibilities is more likely we have tried to separate four types of polyamino acids, namely two samples of polylysine differing in their relative average molecular mass, namely 28 200 and 3300, a polyglycine sample (rel. mol. mass in the range 4600) and polyaspartic acid (rel. mol. mass 8600). The polylysine sample with the lower rel. mol. mass moved faster than that possessing the larger M_r. Also the former exhibited a fine structure of the emerging peak while the latter yielded a single broad peak only (Fig. 1B, C). No peaks of the polyglycine and polyaspartic acid samples appeared in this arrangement. Under reversed polarity polyaspartic acid yielded a single broad peak, while polyglycine was not seen. This indicates that polylysine samples are driven to cathode as long as they possess a strong positive charge. Polyaspartic acid moved to anode as even at this low pH used for separation, it still possessed sufficient negative charge to bring it in front of the detector window in the reversed polarity mode (Fig. 2A). Formic acid present in the sample solution had the same direction of mobility (formic acid gave the faster peak) (Fig. 2B). Polyglycine, bearing a considerably lower positive charge compared to polylysine moved very slowly to cathode which precluded it to move in front of the detector in a reasonable time. The fine structure of the low-molecular-mass polylysine peak revealed secondary sieving effect of Pluronate present in the background electrolyte. The profile obtained was the subject to the analysis by the Peak Fit programme which revealed 17 peaks: 15 sharp peaks and two broad ones (Fig. 3A).

The latter two probably reflect high-molecular-mass contaminants of the sample. As no fine structure of the low-molecular-mass polylysine peak was observed in the absence of the Pluronic modifier, it can be concluded that some additional partition mechanism was involved in addition to electromigration with this sample type. It means that, with respect to the micellar-like structure of the Pluronic liquid crystals in the background electrolyte, partition between hydrophobic micellar core and hydrophilic surroundings may be one of the mechanisms participating in the resulting separation effect of this media and contributing to the separation of proteins and peptides.

Using the collagen-released CNBr set of peptides with 50 mmol/l phosphate buffer at pH 2.5 a profile strongly resembling that obtained by reversed-phase chromatography was obtained [13–15]. However in this case the electrokinetic separation was carried out in the positive mode with an untreated capillary. In a hydrophobically-coated capillary the separation was much worse than with bare silica. Using the Pluronic copolymer in the background electrolyte a separation exhibiting several interchanges in the profile in

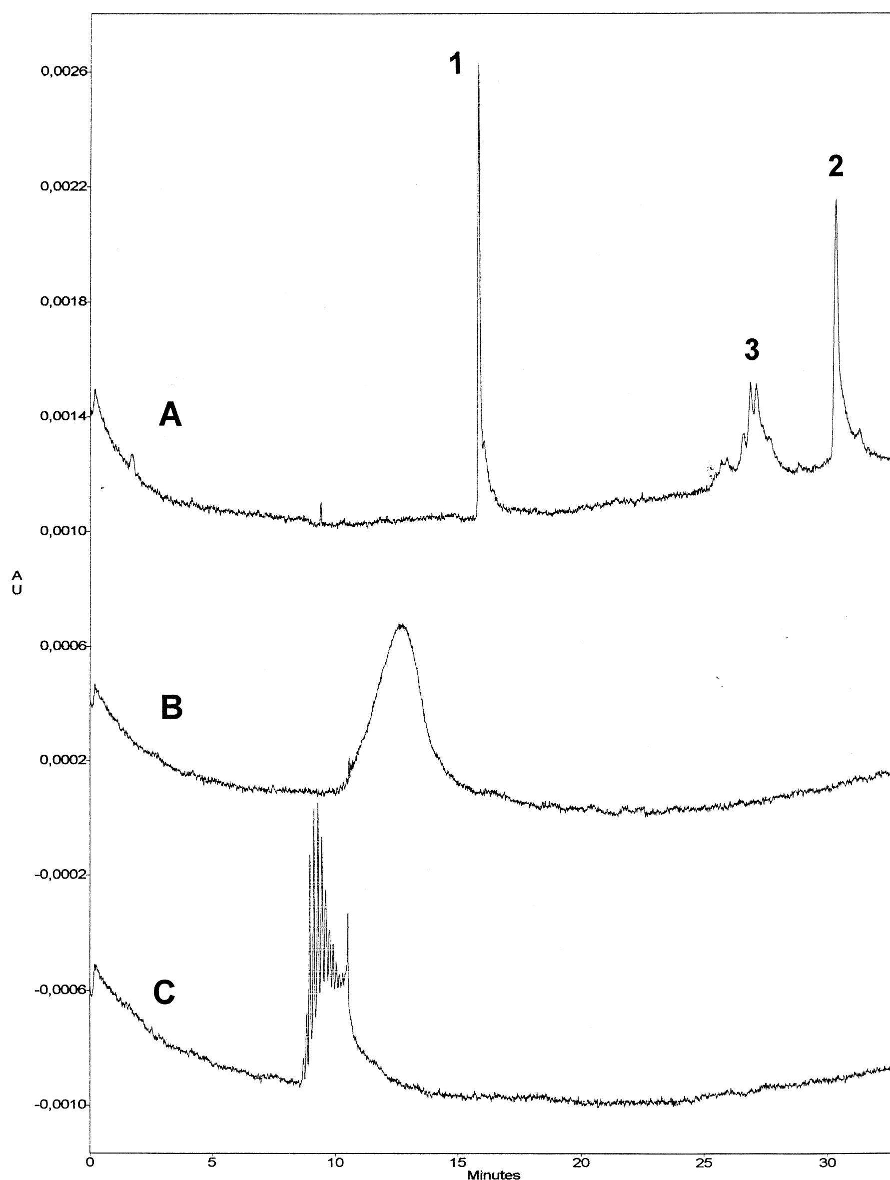

Fig. 1. Separation of protein standards: (A) 1=cytochrome *c* (M_r 12 500), 2=chymotrypsinogen A (M_r 25 000), 3=albumin from hen egg (M_r 45 000), (B) polylysine (average M_r 28 200), (C) polylysine (average M_r 3300). Conditions: CElect P150 capillary 27 cm (20 cm to the detector)×50 μm I.D., 10 mmol/l Tris and 75 mmol/l phosphate buffer containing 7.5% (v/v) Pluronic F127, pH 2.9, 20°C, 5 kV, 22 μA.

comparison with the result of reversed-phase separations was obtained (see our previous communication [13]). However the latter system suffered with considerable current fluctuations that could be in part removed by using either hydrophobic or hydrophilic coating. The results were similar with either type of coating, however, the separation in the case of hydrophilic coating seemed slightly better (data not shown). In the case hydrophilic coating most of the peptides present emerged in a single, poorly resolved bulk peak with the migration time between 40 and 55 min. It is noticeable that this peak comprised pep-

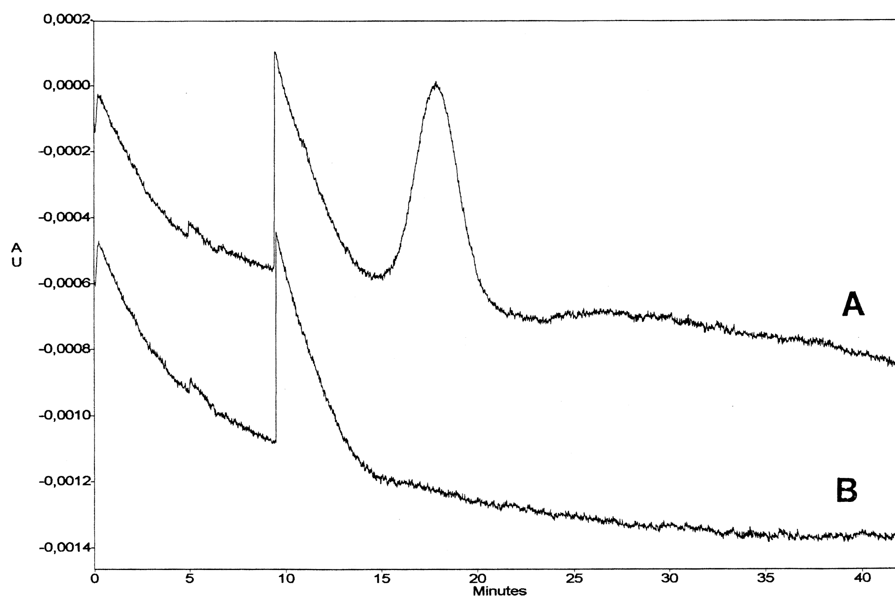

Fig. 2. Separation of polyaspartic acid (average M_r 7000) (A) and blank with 5% (v/v) formic acid (B). Conditions as in Fig. 1, but reversed polarity mode was used.

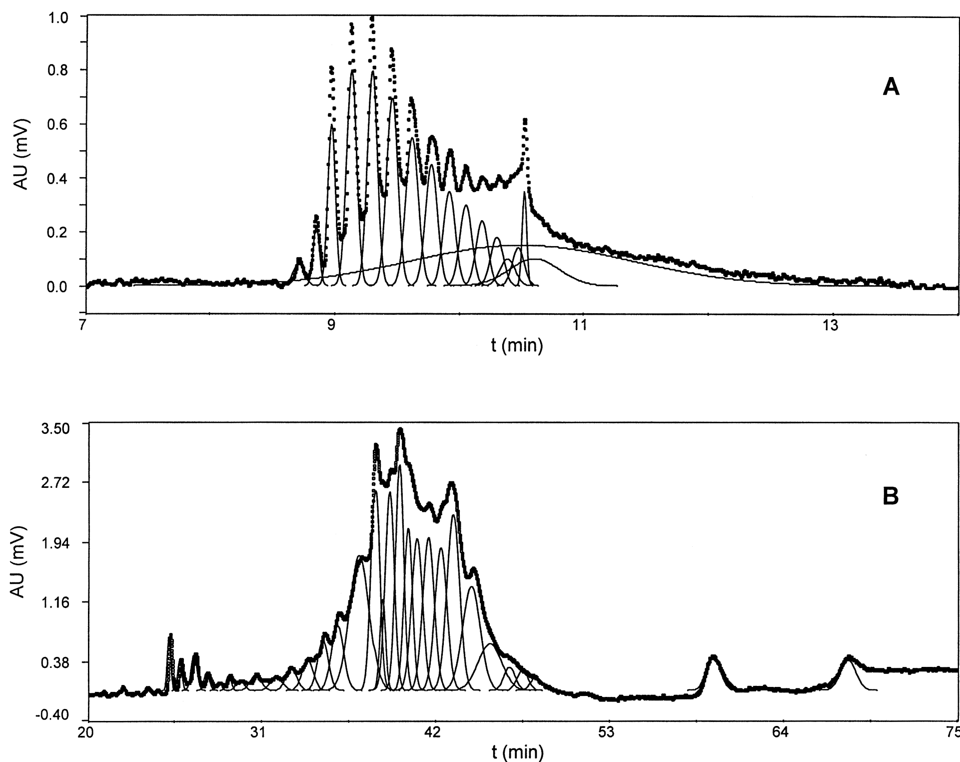

Fig. 3. Analysis of electrophoretic profiles by PeakFit programme. (A) Polylysine (average M_r 3300), (B) rat tail tendon collagen CNBr peptides. Separation conditions as in Fig. 1.

tides covering the relative molecular mass within the range $2–6\cdot10^4$. The largest two peaks, namely that of $\alpha_2(I)CB_{3,5}$ and $[\alpha_1(III)CB_9]_3$ were also fused with the large bulk peak which supports the idea of other partition mechanisms being involved besides molecular sieving as the peak of bovine serum albumin (M_r $6.8\cdot10^4$) did not emerge before 1 h run time. On the other hand at least three distinct peaks appeared in the front of the electropherogram, which by comparison with the reversed-phase chromatography profile should comprise $\alpha_1(I)CB_4$, $\alpha_1(III)CB_3$, $\alpha_1(III)CB_6$, $\alpha_1(I)CB_3$ and $\alpha_1(I)CB_6$ peptides. The resulting separation was evaluated by the PeakFit program with the result shown in Fig. 3B. This evaluation revealed 30 peaks some of which correspond very likely to contaminants and/or uncompletely cleaved fragments as the theoretical number of collagen type I and III (collagens present in the parent tissue) CNBr peptides is 24.

4. Conclusions

The following conclusions can be drawn from the above results:

(1) Separation of peptides/proteins in the Pluronic-containing system using a coated capillary is primarily ruled by the effective charge-to-mass ratio of the solutes with little or no effect of molecular sieving or hydrophobic interactions.

(2) Using a coated capillary helps to remove current fluctuations during the separation.

(3) With highly positively charged peptides of rel. mol. mass around 3000 sieving helps more detailed separation of peptides involved.

(4) Polyamino acids possessing negative charge can be brought in front of the detector window under reversed polarity, while those bearing a small charge only move so slowly that they can be never seen on the electropherogram.

Acknowledgements

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