Ivan Mikšík[1]
Adam Eckhardt[1]
Esther Forgács[2]
Tibor Cserháti[2]
Zdeněk Deyl[1]

[1]Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic
[2]Institute of Chemistry, Chemical Research Centre, Hungarian Academy of Sciences, Budapest, Hungary

The effect of sodium dodecyl sulfate and Pluronic F127 on the electrophoretic separation of protein and polypeptide test mixtures at acid pH

Using a test mixture consisting of standard proteins (cytochrome *c*, chymotrypsinogen A, hen egg albumin, bovine serum albumin, aldolase, catalase and ferritin) and synthetic polypeptides (polylysine, polyaspartic, polyglutamic acid and polyproline) it was revealed that using sodium dodecyl sulfate (SDS) as background electrolyte modifier at acid pH (2.5) allows selective separation of highly positively charged polypeptides (polylysine) provided that their relative molecular mass is sufficiently low (3300 Da). The altered elution sequence of standard proteins as compared to a separation done without SDS may help their identification. Addition of Pluronic F127 offers clear-cut separations of standard proteins up to a relative molecular mass of 5 $\times$ 10^4 Da and allows to reveal protein/polypeptide microheterogeneity where applicable. None of the systems tested is suitable for the separation of acidic polypeptides and polyproline.

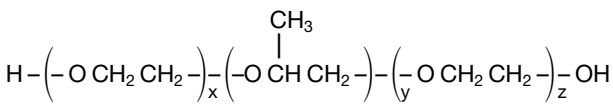
Keywords: Capillary electrophoresis / Low pH / Peptide analysis / Pluronic F127 EL 4913

1 Introduction

Separation of peptides and proteins by capillary zone electrophoresis suffers from the interaction of these solutes with the capillary wall which results in the formation of broad peaks and low resolution. The reason for this adverse effect is the negative charge of the free silanol groups on the inner surface of the capillary and, perhaps, the involvement of other types of interaction as well. There are several methods how to minimize the protein/peptide-capillary wall interaction. The most easy approach is to minimize the dissociation of the silanol groups by running the separation at very acid pH. While this arrangement decreases the interaction of separated solutes with the capillary wall, it concomitantly increases the running time as the result of a considerable decrease of the endoosmotic flow [1].

An alternative approach to the separation of peptides/proteins is micellar electrokinetic chromatography. Since the first application of this technique the preferred surfactant used has been SDS. This operational mode allows successful separation of a broad spectrum of analytes inclusive peptides and proteins by exploiting the hydrophobic domains of the separated molecules. Cationic pseudophases as well as neutral surfactants have been exploited considerably less [2]. The main limitation of using SDS is the availability of only a single type of the hydrophobic domain (C-12) in the micellar pseudophase. Another reason for the application of acidic conditions for capillary separation may be the need to separate proteins that are soluble only in acid media. These regard particularly proteins of connective tissue, namely collagens and their fragments [3].

The existing possibilities of micellar electrokinetic chromatography for the separation of biopolymers (*i.e.*, nucleic acids, proteins and their fragments [4–9]) have been broadened rather recently by introducing Pluronic F127, a triblock copolymer of the general formula (polyethylene oxide)$_x$(polypropylene oxide)$_y$(polyethylene oxide)$_z$:



when $x = 106$, $y = 70$ and $z = 106$ which can be considered a surfactant capable of self-association both into isotropic and anisotropic gels. Because our group is interested in physiology and analysis of structural proteins (particularly collagens and their fragments), we have in the past investigated the separation possibilities of this category of analytes by using (i) capillary zone electrophoresis, (ii) SDS micellar electrokinetic chromatography [10] and (iii) capillary electrophoresis with Pluronic F127 modifier [8, 9] at acid pH. In this report, we summarize (and compare) our experiments obtained with model protein/peptides separated by the above specified operational modes.

Correspondence: Dr. Ivan Mikšík, Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1083, CZ-14220 Prague, Czech Republic
E-mail: miksik@biomed.cas.cz
Fax: +420-2-4106-2534

 0173-0835/02/1206–1882 $17.50+.50/0

2 Materials and methods

2.1 Chemicals

Sodium dihydrogen phosphate and hydrochloric acid were products of Lachema (Brno, Czech Republic) and were of p.a. quality. Pluronic F127 ((polyethylene oxide)$_x$ (polypropylene oxide)$_y$(polyethylene oxide)$_z$ triblock copolymer, x = 106, y = 70 and z = 106) and all peptides were from Sigma (St. Louis, MO, USA). SDS was from Merck (Darmstadt, Germany). All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA).

2.2 Capillary electrophoresis

All runs were performed on a Beckman P/ACE instrument system 5500 (Beckman, Fullerton, CA, USA). A bare fused-silica capillary of 57 cm total length (50 cm to the detector) with an ID of 75 μm was used except for the separation with Pluronic liquid crystals where also a capillary with 27 cm (20 cm to the detector) × 75 m ID was used (Polymicro Technologies, Phoenix, AZ, USA). Detection was done by UV absorbance recording at 214 nm. Before analysis the capillary was washed 3 min with the background electrolyte. The sample was injected hydrodynamically (1 s, 3.45 kPa overpressure). The separations were run at 20°C at an applied voltage of 15 kV (except for the Pluronic F127 experiments in which 20 or 10 kV were used with longer or shorter capillary, respectively). 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). Three background electrolyte systems were used (pH was in all cases adjusted by 1 M HCl after the surfactant was added): (A) phosphate buffer (50 mmol/L), pH 2.5; (B) SDS (50 mmol/L) in 50 mmol/L phosphate buffer, pH 2.5; (C) Pluronic F127 (5% w/v) in 50 mmol/L phosphate buffer, pH 2.5. Before analysis, the background electrolyte was filtered using Millex-HV filter (Millipore), 0.45 μm. Because of the low pH values in the runs EOF could not be estimated (it is usually larger than 1 h). Owing to the strongly acidic pH of the background electrolyte all analytes were positively charged.

2.3 Peptide and polyamino acid samples

The set of test proteins comprised the following proteins: cytochrome *c* (M_r 12 500), chymotrypsinogen A (M_r 25 000), hen egg albumin (M_r 45 000), bovine serum albumin (M_r 68 000), aldolase (M_r 158 000), catalase (M_r 240 000) and ferritin (M_r 450 000). All these proteins were products of Boehringer (Mannheim, Germany). Two poly-L-lysines were obtained from Sigma: the first sample had an average relative molecular mass of 22 700 Da (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); the second polymer had an average relative molecular mass of 4000 Da (by viscosity; DP: 19) or 3300 (by SEC-LALLS; DP: 16). Polyaspartic acid (Sigma) had an average relative molecular mass of 8600 Da (by viscosity; DP: 63) or 7000 (by SEC-LALLS; DP: 51). Two poly-L-glutamic acids were obtained from Sigma: the first had an average relative molecular mass of 13 600 Da (by viscosity; DP: 90) or 13 700 (by SEC-LALLS; DP: 91); the second polymer had an average relative DP: 10 (estimated by capillary electrophoresis). Poly-L-proline (Sigma) had an average relative molecular mass of 5000 Da (by viscosity; DP: 52) or 3700 (by SEC-LALLS; DP: 38). Samples were dissolved in Milli-Q water to the concentration of 1 mg/mL.

3 Results and discussion

Using bare silica capillary and phosphate buffer at pH 2.5 (positive polarity at the injection side) the separation of the model compounds shown in Fig. 1 was obtained. Cytochrome *c* was already separated from the rest of the mixture which was only partly separated though the individual members of the set could be clearly distinguished except for aldolase and catalase the peak of which were hidden in the terminal part of the electropherogram. If the latter two proteins were run separately, they offered broad peaks only in the region between 20–25 min running time. On the other hand synthetic polylysines (over M_r 4000 and 22 700) offered, as expected, two sharp peaks in the front of the electropherogram. Samples of polyaspartic and polyglutamic acids, no matter what their average M_r was, could not be brought in front of the detector's window at positive polarity at the injection side. These amino acids exhibit a strong tendency to adhere to the capillary wall at acid pH and they result in broad peaks obtained under reversed polarity. These "bump" peaks are visible only when polyamino acids are injected at a high amount (*e.g.*, 3 s at 1 mg/mL concentration). Polyproline sticks in acid pH strongly to the capillary no matter which operational mode (positive or negative) was used. At pH 7 this sample gave a single unresolved peak (result not shown).

All these experiments served for comparison only as the pH used was far from being optimal for the separation of the tested mixture (except for polylysines). In the next step of our experiments we have repeated the separations using 50 mmol/L SDS in the background electrolyte with the idea that binding of an anionic surfactant to the

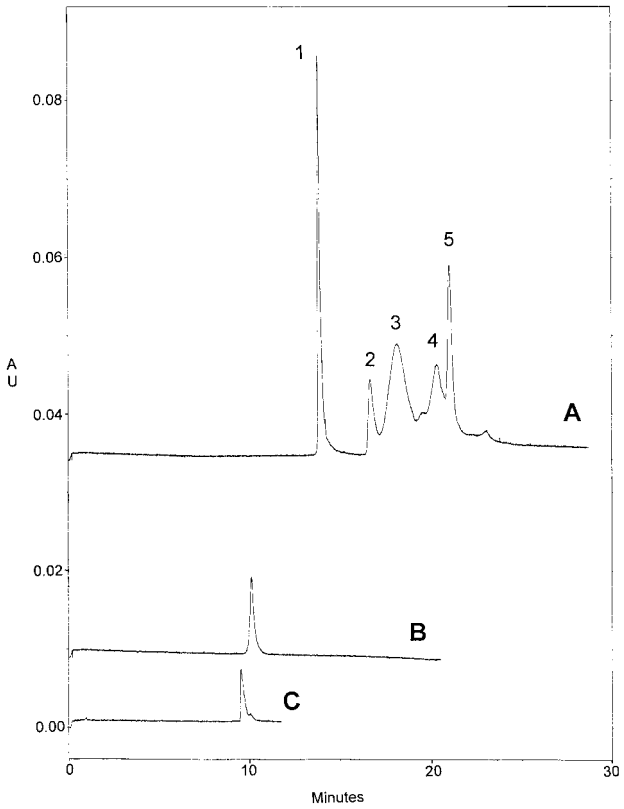


Figure 1. Comparative separations of the tested solutes in phosphate buffer pH 2.5 under conditions specified in Section 2. (A) Set of test proteins; (B) polylysine (M_r 4000), (C) polylysine (M_r 22 700). Peak identification: 1, cytochrome *c*; 2, hen egg albumin; 3, ferritin; 4, bovine serum albumin; 5, chymotrypsinogen A (positive polarity at the injection side).

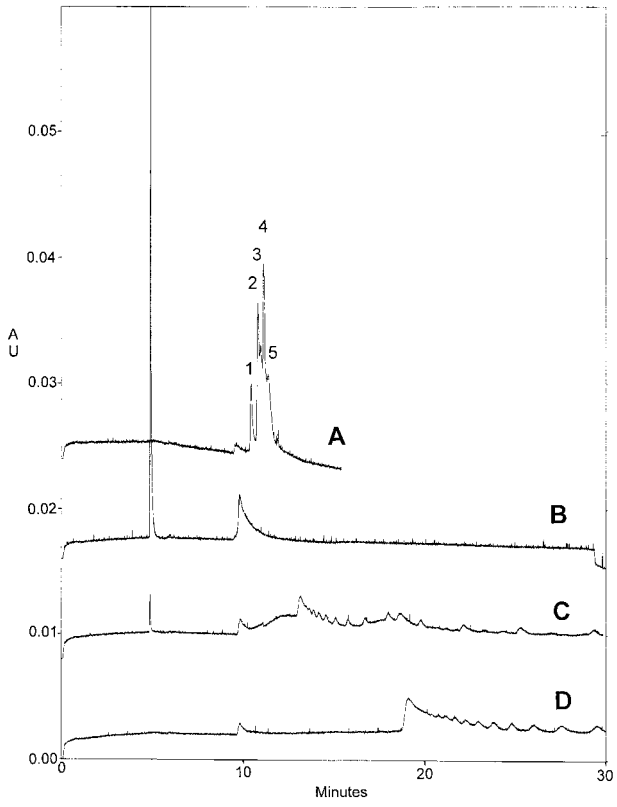


Figure 2. Separations of the test solutes using 50 mmol/L SDS as background electrolyte modifier at reversed polarity (negative polarity at the injection side). All conditions as specified in Section 2, except for polyglutamic acids when sample had concentration 10 mg/mL (in contrast to 1 mg/mL). (A) Set of test proteins; (B) polylysine (M_r 4000); (C) polyglutamic acid (M_r 1000); (D) polyglutamic acid (M_r 13 600). Peak identification: 1, ferritin; 2, chymotrypsinogen A; 3, aldolase; 4, cytochrome *c*, bovine serum albumin and catalase; 5, hen egg albumin. The early emerging peak in (C) and peaks at 10 min are system peaks of unknown nature.

test solutes will bring a negative charge to the molecules to be separated. Consequently these separations had to be run in the reversed polarity mode. As shown in Fig. 2, indeed, the test proteins offered a set of anodically moving peaks, however, their separation remained partial only except for ferritin which was separated best and run at the beginning of the electropherogram. Taking into consideration the well-known fact that the amount of SDS bound per unit mass of protein is practically constant, one would have expected that large proteins bearing the highest negative charge would exhibit the highest migration towards anode. As indicated by Pitt-Rivers and Impiombato [11], the amount of SDS bound to a protein ranges between 90–100% w/w and can increase up to 140% in proteins devoid of disulfide bonds (for more detailed discussion see [12]). If in the first approximation 100% w/w SDS binding is considered, one would expect that generally the proteins would emerge roughly in the order of decreasing molecular mass (in reversed-polarity mode) which, however, is obviously not the case (typically cytochrome *c*, bovine serum albumin and catalase possessing relative molecular masses of 12 500, 68 000 and 240 000 Da comigrate as a single peak, chymotrypsinogen of a relative molecular mass of 25 000 is considerably faster than hen egg albumin of a relative molecular mass of 45 000). It is, perhaps, feasible to ascribe these differences to hydrophobic interactions with the capillary wall, though involvement of other effects cannot be excluded.

Low-molecular-mass polyglutamic acid samples moved in the reversed order than would have been expected which may be caused by the fact that short polyglutamic acid chains were adhering less to the capillary wall. On the other hand, polyaspartic acid of the relative molecular mass 8600 could not be brought in front of the detector's

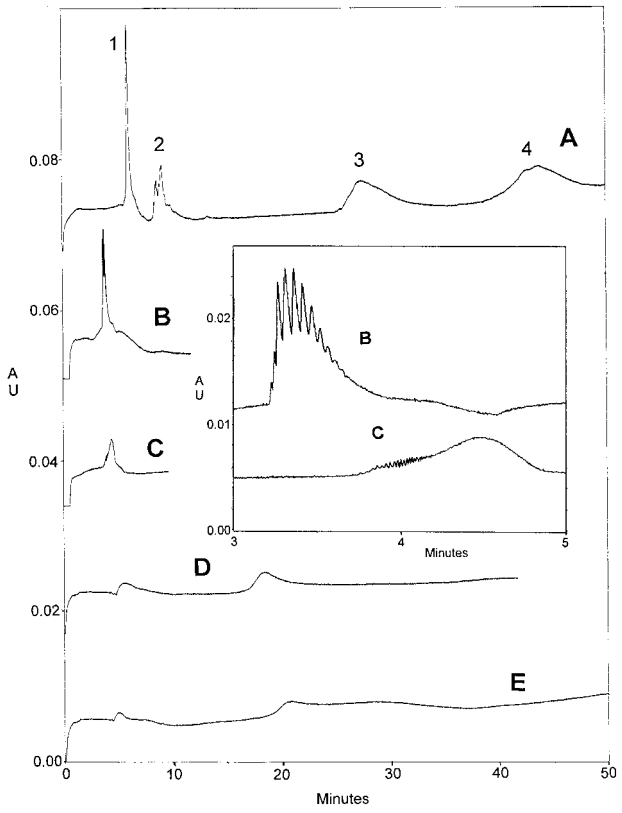


Figure 3. Separations of the test solutes in 5% w/v Pluronic F127 under condition specified in Section 2 (capillary 27/20 cm to the detector; 10 kV) except for aspartic and glutamic acids, when reversed polarity (negative polarity at the injection side) was used. (A) Set of test proteins (1, cytochrome *c*; 2, hen egg albumin; 3, chymotrypsinogen A; 4, bovine serum albumin); (B) polylysine (M_r 4000); (C) polylysine (M_r 22 700); (D) polyaspartic acid; (E) polyglutamic acid (M_r 1000). Inset: enlarged regions of polylysine separation. Note the split peak of hen egg albumin which indicates the microheterogeneity of this protein; compare Fig. 4, line A.

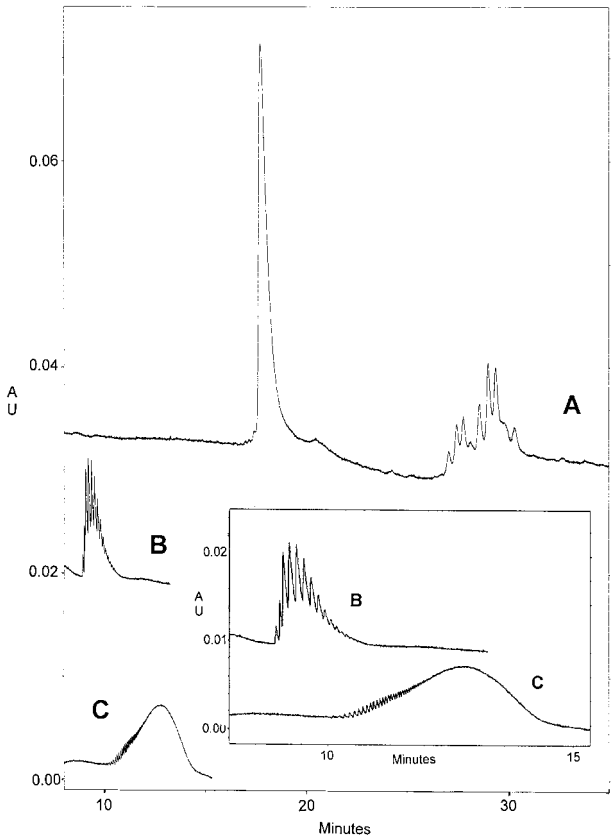


Figure 4. Separation of the microheterogeneity exhibiting solutes under the same conditions as in Fig. 3 at Pluronic F127 (see also Section 2), except that the runs were done in a longer capillary (57/50 cm to the detector) at 20 kV. (A) Cytochrome *c* and hen egg albumin (in order of elution); (B) polylysine (M_r 4000); (C) polylysine (M_r 22 700). Inset: enlarged regions of polylysine separation (B – upper, C – lower recording). Note the polydispersity of hen egg albumin in lane (A).

window at all. Polylysine of the relative molecular mass 4000 exhibited a sharp peak in the front of the electropherogram while polylysine of a relative molecular mass 22 700 could not be eluted at all. Polyproline, as in the case of phosphate buffer, strongly sticks to the capillary wall and cannot be eluted.

Using Pluronic F127 as the background electrolyte modifier does not bring about charge changes, however, exploits the hydrophobic properties of the proteins/peptides separated combined reportedly with some sieving effects [9]. As visualized in Fig. 3 of the set of test proteins, only cytochrome *c*, hen egg albumin, chymotrypsinogen and bovine serum albumin appeared on the electropherogram, while the largest components, *i.e.*, aldolase, catalase and ferritin were not eluted apparently because they have been trapped by the additive. A profile of this kind can be seen only on the short capillary (27/20 cm to the detector), on longer the capillary (57/50 cm to the detector) the last two proteins were spread and no reasonable peaks were observed in 60 min. While hen egg albumin revealed even its microheterogeneity, the peaks of chymotrypsinogen and bovine serum albumin, though clearly separated, resulted in rather broad peaks. Both polylysines, owing to their large positive charge moved in the front of the electropherogram and revealed the expected microheterogeneity. Polyaspartic and polyglutamic acids can be visualized as broad peaks in reversed-polarity mode which mirrors their negative charge. A better image about the microheterogeneity of polylysine samples and the sample of hen egg albumin can be obtained by using a longer capillary (57/50 cm to the detector) as visualized in Fig. 4.

4 Concluding remarks

(i) Using 50 mmol/L SDS in the background electrolyte and operating the system in reversed-polarity mode offers sharper peaks of standard proteins. Changes in the relative position of individual peaks may help identification. (ii) In the above system highly positively charged polypeptides can be brought in front of the detector's window only if they possess an adequately small relative molecular mass (typically polylysine M_r 3300) and can be selectively separated from their high molecular mass possessing counterparts. (iii) Highly negatively charged polypeptides (polyaspartic and polyglutamic acids) are poorly separated no matter whether SDS is present or not. (iv) It was demonstrated that using Pluronic F127 as background electrolyte modifier offers separations superior to CZE or micellar electrokinetic chromatography, however, these results can be obtained only with proteins/peptides of low to medium relative molecular masses (up to 5×10^4 Da). This separation system appears particularly applicable to proteins/peptides requiring an acid electrolyte because of their solubility characteristics. (v) Microheterogeneity of polylysines and hen egg albumin is clearly revealed in Pluronic F127 containing background electrolyte. (vi) At the pH used (2.5) Pluronic containing systems are not suitable for the separation of highly acidic polypeptides. (vii) The systems tested (at pH 2.5) are not suitable for the separation of polyproline.

This work was supported by Grant Agency of the Czech Republic, grants Nos. 203/99/0191 and 203/00/D032.

Received September 13, 2001

5 References

[1] Banks, J. F., in: Deyl, Z., Mikšík, I., Tagliaro, F., Tesařová, E. (Eds.), *Advanced Chromatographic and Electromigration Methods in Biosciences*, Elsevier, Amsterdam 1998, pp. 525–573.

[2] Terabe, S., Deyl, Z. (Eds.), *Micelles as Separation Media in Chromatography and Electrophoresis*, Special Volume J. Chromatogr. Vol. 780, Elsevier, Amsterdam 1997.

[3] Deyl, Z., Mikšík, I., *J. Chromatogr. B* 2000, *739*, 3–31.

[4] Rill, R. L., Liu, Y., Van Winkle, D. H., Locke, B. R., *J. Chromatogr. A* 1998, *817*, 287–295.

[5] Rill, R. L., Liu, Y. J., Ramey, B. A., Vanwinkle, D. H., Locke, B. R., *Chromatographia* 1999, *49*, S65–S71.

[6] Rill, R. L., Locke, B. R., Liu, Y. J., Vanwinkle, D. H., *Proc. Natl. Acad. Sci. USA* 1998, *95*, 1534–1539.

[7] Liu, Y. J., Locke, B. R., Van Winkle, D. H., Rill, R. L., *J. Chromatogr. A* 1998, *817*, 367–375.

[8] Mikšík, I., Deyl, Z., *J. Chromatogr. B* 2000, *739*, 109–116.

[9] Mikšík, I., Deyl, Z., Kašička, V., *J. Chromatogr. B* 2000, *741*, 37–42.

[10] Mikšík, I., Deyl, Z., *J. Chromatogr. A* 1999, *852*, 325–336.

[11] Pitt-Rivers, R., Impiombato, F. S., *Biochem. J.* 1968, *109*, 825–820.

[12] Deyl, Z., in: Deyl, Z., Everaerts, F. M., Prusík, Z., Svendson, P. J. (Eds.), *Electrophoresis, A Survey of Techniques and Applications, Part A: Techniques,* Elsevier, Amsterdam 1979, pp. 45–67.