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Short communication

Peptide mapping by capillary electrophoresis with Pluronic F127

Ivan Mikšík*, Jana Charvátová, Adam Eckhardt, Zdeněk Deyl

Institute of Physiology, Academy of Sciences of the Czech Republic, Videnska 1084, 14220 Prague 4, Czech Republic

Abstract

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. To minimize the protein/peptide–capillary wall interaction we tried to use 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. The analytes studied were enzymatic digests (obtained by trypsin or pepsin treatment) of insoluble matrix proteins from avian eggshell. The best separations were obtained by a system exploiting 10% Pluronic F127 in 20 mmol/l phosphate buffer, pH 2.5. Electrophoretic peptide profiles obtained were very complex owing to the complicated nature of the samples (the exact composition of the proteinous insoluble part of the eggshell is still unknown). The separation in phosphate buffer only offered complex maps of incompletely resolved peaks. The use of Pluronic F127 distinctly improved the separation with a considerably better resolution regarding both the number of peaks obtained and the quality of the separation.

Keywords: Peptide mapping; Pluronic F127

1. Introduction

In addition to two-dimensional gel electrophoresis the alternative technique for studying proteins, peptides and peptide mapping is capillary electrophoresis (CE).

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 such adverse effects 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 the nature of which is not clearly understood so far. The most easy approach to minimize the protein/peptide–capillary wall interactions is to lower the dissociation of the silanol groups by running the separation at very acid pH. Alternatively it is possible to run the separations at very high pHs (at decreased dissociation of the proteins/peptides separated) which, however, is less efficient in terms of selectivity though this approach leads to rather short running times. While the former 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 endo-osmotic flow [1].

Another reason for the application of acidic conditions for capillary separation may be the need to separate proteins that are soluble in acid media only (typically proteins of connective tissue, namely collagens and their fragments [2]).

Alternatively peptides/proteins can be also separated by micellar electrokinetic chromatography. Since the first application of this technique the preferred surfactant used has been SDS. By using SDS as the pseudophase it was possible to separate a broad spectrum of analytes inclusive peptides and proteins by exploiting the hydrophobic domains of the separated molecules and the negative charge of protein–SDS adducts. Cationic pseudophases as well as neutral surfactants have been used only occasionally [3]. Using SDS implies that only a single type of the hydrophobic domain (C-12) in the micellar pseudophase is available.

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

* Corresponding author. Tel.: +420-2-96442534; fax: +420-2-96442558.
E-mail address: miksik@biomed.cas.cz (I. Mikšík).

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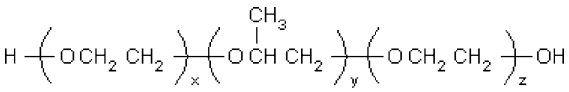
oxide)$_x$(polypropylene oxide)$_y$(polyethylene oxide)$_z$:



where $x = 106$, $y = 70$ and $z = 106$. This polymeric structure 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 of these analytes by using (i) capillary zone electrophoresis, (ii) SDS micellar electrokinetic chromatography [10] and (iii) capillary electrophoresis with the Pluronic F127 modifier (pseudophase) [8,9] at acid pH.

Pluronic F127 offers clear-cut separations of standard proteins up to relative molecular mass 5×10^4 and allows to reveal protein/polypeptide microheterogeneity where applicable [10].

Avian eggshell can be divided in a few basic components: the thin cuticle (outside layer) and thick calcified layer that is formed from elonged calcite structures named palisade layer which is terminated by the mammillary layer that contains anchor points for the inner and outer shell membranes. These membranes envelope the yolk and albumen. Proteins from avian eggshells have been studied mainly during the last decade, however, these studies were limited to the soluble species only. Recently [11] we made the first attempt to analyze the insoluble protein/peptide-based constituents of the eggshell matrix by capillary electrophoresis and HPLC/MS. It was proven that these matrices are really complex mixtures of proteins/peptides, some of which are glycosylated. Lipoproteins appear to represent another category of protein structures involved.

In the case of a complex peptide mixture arising after digestion of the insoluble eggshell proteins it is obvious that we need to use more than a single enzyme digest. For example in the previous paper [11] we concluded that in all three layers of the eggshell matrix none of the currently described soluble proteins from the eggshell were present. When we look at the peptide profiles, particularly at the molecular mass of the peptides arising after trypsin cleavage, we found a few peptides the molecular mass of which was identical with peptide fragments of soluble eggshell's ovocleidin 116 [12]; on the other hand some peptides had a similar molecular mass of the peptides/proteins of completely different origin as is, e.g., bovine serum albumin. For this reason it appeared important to use another type of enzymatic digestion (e.g. pepsin cleavage and consequent treatment by collagenase used in our work [11]) and combine these sets of data. This combination was foreseen to reveal possible similarity of the investigated mixture of proteins/peptides to other, better known species.

In our previous investigation we could see some similarity to ovocleidin 116 in the case of trypsin digest but peptides arising after pepsin (and subsequent collagenase) digest were totally different (collagenase was used in order to reveal whether or not typically collagenous sequences are involved in the eggshell structural proteins). As a number of peaks in the obtained profile need not necessarily represent pure entities a need for having an alternative separation procedure emerged; it is obvious that any proposed alternative procedure must possess a high selectivity (it should be capable of revealing at least 100 peaks from the complex peptide mixture arising from the enzymatic cleavage of the studied eggshell structural proteins).

In this report we tried to apply a Pluronic-based system to peptide mapping of eggshell structural proteins arising after pepsin or trypsin digestion that would be better (or complementary) as compared to the conventional system that exploits standard capillary electrophoresis conditions in acid background electrolyte.

2. Experimental

2.1. Instrumental

Capillary electrophoresis experiments were done with Beckman P/ACE 5000 system (Fullerton, CA, USA) with UV detection set on 214 nm. The instrument was controlled, data collected and manipulated by Beckman P/ACE Station program version 1.21. Fused-silica capillary of 37 cm total length (30 cm to the detector), 75 μm i.d., 375 μm o.d. was used for all experiments.

2.2. Chemicals

Calcium chloride, pepsin (Pepsin A, activity 3460 U/mg protein), trypsin (type N-S from porcine pancreas, 15,450 U/mg) and Pluronic F127 were from Sigma (St. Louis, MO, USA), Tris, sodium dihydrogen phosphate, hydrochloric acid, sodium hydroxide were purchased from Lachema (Brno, Czech Republic) and were of p.a. quality. 2-Mercaptoethanol and ethylenediaminetetraacetic acid disodium salt (EDTA, Titriplex III) were from Merck (Darmstadt, Germany). All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA). Eggs used in experiments were commercially available hen eggs.

2.3. Sample preparation

2.3.1. Preparation of fractions of a eggshell

Preparation of various insoluble layers followed the method published previously [11]. Whole eggs were washed with water and methanol and two types of samples were prepared.

2.3.1.1. Cuticle layer. Eggs were treated by 5% EDTA (pH 7.6) containing 10 mmol/l 2-mercaptoethanol (three times the egg volume) for 60 min at room temperature. The insoluble organic layer resulting on the egg surface after this partial decalcification was scratched and accumulated by washing

with water and centrifuged (1000 × *g*, 15 min). The resulting pellet was resuspended in water and centrifuged at the above conditions (repeated three times) and then lyophilized. This resulted in the removal of the outer eggshell layer (the cuticle).

2.3.1.2. Palisade layer I. In this step, the egg that has passed the previous step was treated with 0.6 mol/l EDTA (pH 7.6) containing 10 mmol/l 2-mercaptoethanol (three times the egg volume) for 90 min at laboratory temperature. The insoluble material (layer) on eggs was scratched and the material was subjected to the same procedure as described in Section 2.3.1.1.

2.3.2. Enzyme digestion

Enzyme digestion was done as follows: 1.25–6.3 mg of the eggshell layer samples was treated by pepsin or trypsin solution (5 mg/ml layer, 50:1 substrate:enzyme ratio), in 0.01 mol/l HCl (pH 2) or in 20 mmol/l ammonium bicarbonate buffer (pH 7.8), respectively. Two categories of samples were obtained: the trypsin digest and pepsin digest. Blank samples of each of these groups were prepared by incubation of the enzyme solutions only under identical conditions. Vials were incubated at 37 °C for 36 h. After finishing the incubation the vials were centrifuged for 5 min at 2000 × *g* and the supernatants removed and washed twice with 1 ml of Milli-Q water, lyophilized and stored at −18 °C. The solid residue was washed with Milli-Q water (4 × 0.5 ml) and frozen.

2.4. Conditions for capillary electrophoresis

2.4.1. Conditioning

Before running the sample, the capillary was washed with 1 mol/l NaOH (20 min), followed by 20 min wash with water and 20 min wash with 1 mol/l HCl. Then it was washed with water again for 20 min and finally with the running buffer (20 min). Between runs the capillary was conditioned with 1 mol/l HCl (3 min), water (1 min) and running buffer (3 min).

2.4.2. Running conditions

Capillary electrophoresis separations were run at 6 kV, the samples were injected hydrodynamically (5 s at 3.45 kPa overpressure). Through all separations 0.02 mol/l phosphate buffer with 10% Pluronic F127 of pH 2.5 (adjusted with 1 M HCl) was used as the background electrolyte.

Comparative separations (without Pluronic) were run at 10 kV, the samples were injected hydrodynamically (1 s at 3.45 kPa overpressure). Through all separations 0.05 mol/l phosphate buffer of pH 2.5 (adjusted with 1 M HCl) was used as the background electrolyte.

It is necessary to emphasize that the run voltage that has shown optimum resolution (the largest number of peaks) under both conditions (i.e. in the presence and absence of Pluronic) was different (6 and 10 kV, respectively) and was carefully optimized.

3. Results and discussion

As was demonstrated previously by our group [11], the composition of the insoluble layers of the eggshell is very complex. Peptide mapping of enzymatic digests (after pepsin or trypsin cleavage) by capillary electrophoresis by the standard procedure, i.e. in pure phosphate buffer at acid pH, revealed a complex profile with a plethora of peaks (Fig. 1A). Not all of the peaks were well resolved, particularly in the case of the tryptic digest.

When Pluronic F127 was added to the background electrolyte (10% Pluronic F127 in 20 mmol/l phosphate buffer, pH 2.5) the separation obtained was better (Fig. 1B). When we simply compare the number of peaks obtained (Table 1), the latter separation resulted in about 10% increase of observed number of peaks and regarding the total peak number was comparable to the results obtained by the HPLC procedure [11]. A distinct improvement is particularly noticeable in the case of the tryptic digest of the proteins present in the palisade layer. It should be stressed that some of the peaks are not visible on the printed total electropherograms, they can be visualized after appropriate enlargement of the electropherograms only, as is possible to see on the enlarged section in Fig. 2.

It is also to be emphasized that, according to our previous results on model mixtures and collagenous peptides [8–10], the separation order of peptides is different for the system exploiting phosphate buffer only as compared to the runs effected in the presence of Pluronic. It was proposed that the separation of proteins/peptides in the presence of Pluronic in the background electrolyte occurs on the charge:mass ratio basis with molecular sieving effects acting as secondary partition mechanism. We found out that separation in Pluronic was really improved in case of CNBr peptides of collagen when separation was comparable to the separation made by HPLC [8]. The separation made by capillary electrophoresis in phosphate buffer only was worse. It was also demonstrated that the Pluronic system is suitable for separation of shorter peptides [9]. For example it is possible to see the

Table 1
Comparison of the number of discernible peaks in various method of peptide mapping analysis of eggshell layers

Enzyme digestion	Eggshell layer	CE–Pluronic	CE–phosphate	HPLC
Pepsin	Cuticle	90	80	95
	Palisade	100	90	110
Trypsin	Cuticle	80	70	100
	Palisade	80	58	90–95

Capillary electrophoresis exploiting background electrolyte with Pluronic F127 (CE–Pluronic), phosphate buffer only (CE–phosphate) and HPLC (separation on Zorbax 300Extend-C18 column using gradient elution between 20 mM NH_4OH, pH 9.7 and acetonitrile).

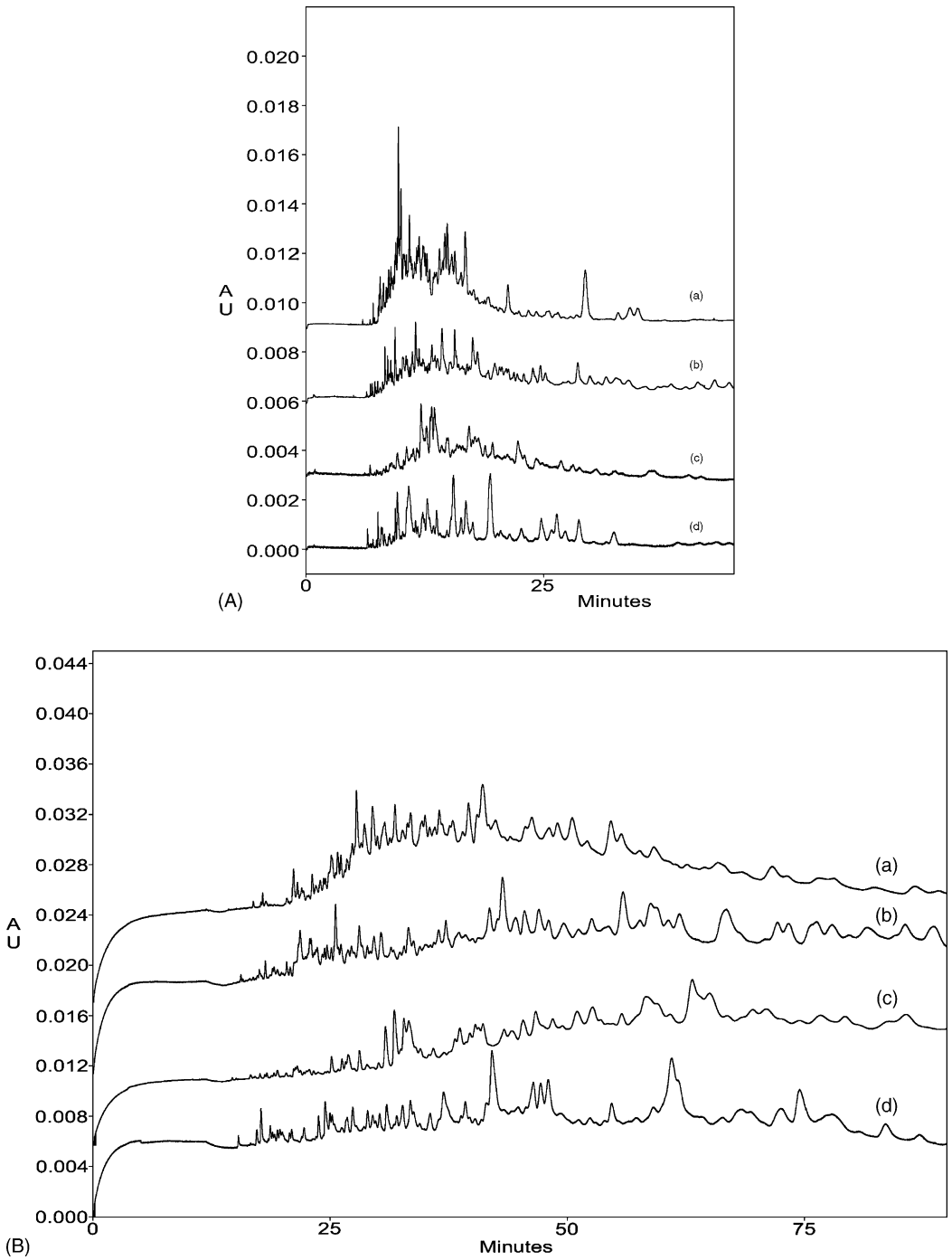


Fig. 1. (A) Capillary electrophoretic profiles of enzymatic digests of individual insoluble fractions from eggshell in system with phosphate buffer only (50 mM phosphate buffer, pH 2.5, 10 kV). Pepsin digest (a and b), trypsin digest (c and d), cuticle layer (a and c), palisade layer (b and d). (B) Capillary electrophoretic profiles of enzymatic digests of individual insoluble fractions from eggshell in system with Pluronic F127 (10% Pluronic, 20 mM phosphate buffer, pH 2.5, 6 kV). Pepsin digest (a and b), trypsin digest (c and d), cuticle layer (a and c), palisade layer (b and d).

heterogeneity of polylysine with average molecular mass 3300; in the case of longer polylysine (average $M_r = 28\,200$) we could see the separation of lower peptides (the front part of the electropherogram), separation of the longer peptides was not revealed [9]. Comparing the separations in the presence of Pluronic F127 in phosphate buffer, phosphate buffer only and with that in which SDS was present in the background electrolyte it was possible to conclude that Pluronic F127 offers clear-cut separations of standard proteins up to relative molecular mass 5×10^4 and allows to reveal protein/polypeptide microheterogeneity where applicable [10].

The fact that the Pluronic system is usable mainly for separation of peptides possessing lower relative molecular mass was noticeable in the current results—peaks with longer

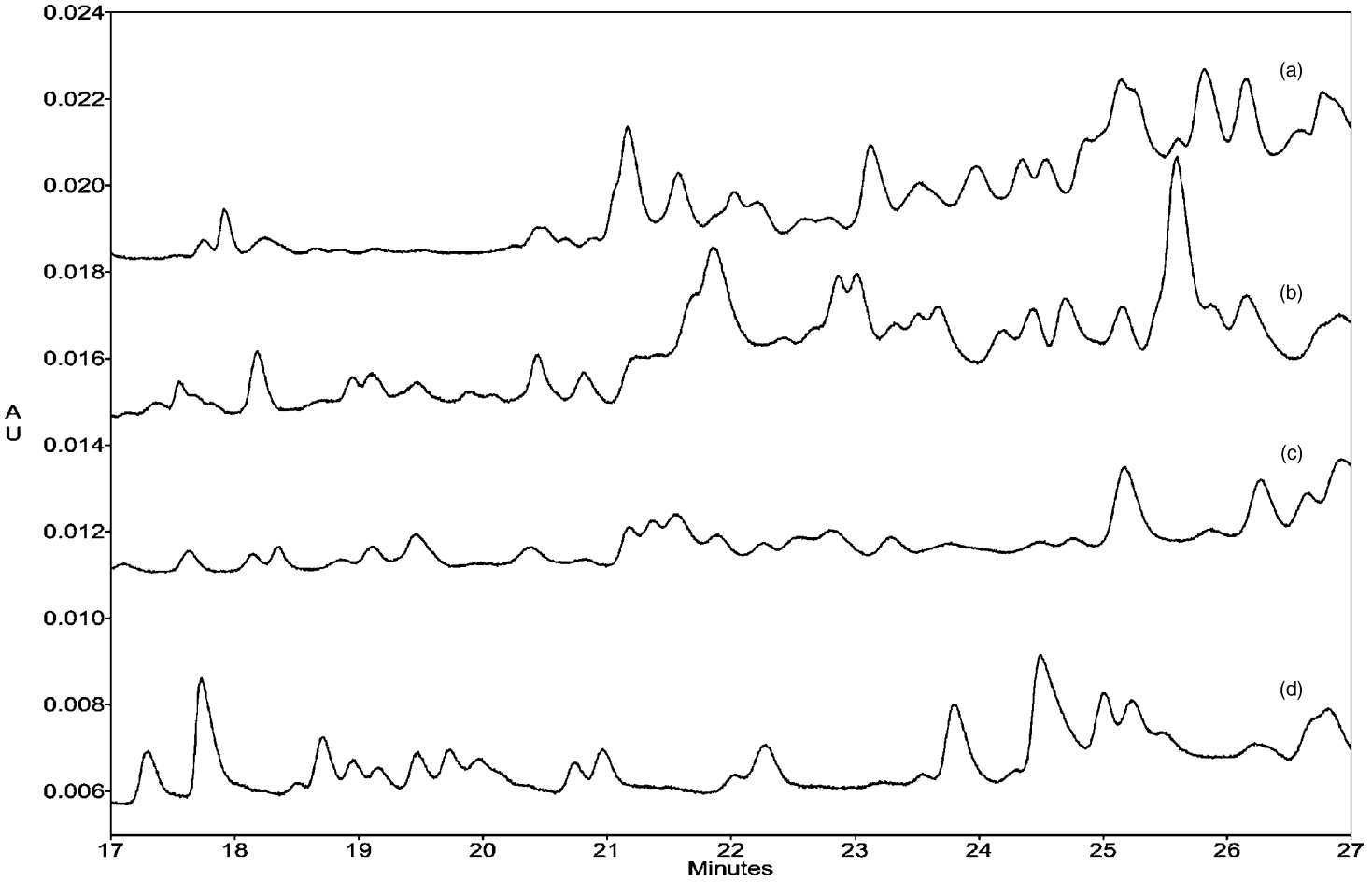


Fig. 2. Enlarged electropherograms from Fig. 1 region of migration time from 17 to 27 min. Conditions and description are the same as in Fig. 1.

migration time are broader and not so well resolved as those running fast. In particular peaks in the first part of the electropherogram are really well resolved and a number of compounds with low abundance can be observed. This confirms our previous findings [8–10].

Based on the results presented we can conclude that the use of Pluronic F127 in peptide mapping by capillary electrophoresis can be a convenient alternative to other separation methods. It has to be remembered that the separation mechanisms involved are apparently different from those governing capillary zone electrophoresis and the results obtained can be complementary to CZE or HPLC peptide mapping.

Acknowledgements

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