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Separation of low-molecular mass peptides by capillary electrophoresis with the use of alkylamines as dynamic coating agents at low pH

Adam Eckhardt[a,b,c], Ivan Mikšík[a,c,*], Zdeněk Deyl[a], Jana Charvátová[a]

[a] *Institute of Physiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 14220 Prague 4, Czech Republic*
[b] *Department of Physiology, Second Medical School, Charles University, Prague, Czech Republic*
[c] *Centre for Experimental Cardiovascular Research, Prague, Czech Republic*

Abstract

Modification of the silanophilic activity of the inner surface of the capillary wall was studied in a capillary electrophoretic system using alkylamines containing background electrolytes at acid pH. The effect of the following amine additives was investigated:
(1) alkyl-α,ω-diamines (1,2-diaminoethane, 1,4-diaminobutane, 1,7-diaminoheptane, spermine),
(2) polymeric amines (polyethylenimine, polybrene),
(3) cationic amine surfactants (cetrimide, hexamethonium bromide).

A seven membered test mixture of peptides (Gly–Pro–Ala, Pro–hPro, Gly–Pro–Arg, Gly–Pro–Gln, Lys–Pro–Gly, Asn–Pro–Gly, His–Pro–Gly) possessing one or more amino groups was used for selectivity evaluation. Under optimised concentration of the amine modifiers the selectivity was always improved (except for polybrene), particularly with the fast moving analytes. The best results were obtained with 1,2-diaminoethane and 1,7-diaminoheptane. On the other hand with slowly moving peaks the best separations were obtained with 1,7-diaminoheptane, hexadecyltrimethylammonium bromide and hexamethonium bromide, i.e. with modifiers possessing large aliphatic domains which are likely to be hydrophobically bonded with the separated solutes. The selectivity improvement with fast moving members of the test mixture can be ascribed to the decrease of the electroosmotic flow, while the improved separation with slowly moving peaks appears to reflect the altered interaction with the hydrophobized capillary wall. As expected the endoosmotic flow was in all cases decreased. The practical applicability of using amine based dynamic modifiers of the capillary wall was demonstrated on a natural peptide mixture (bacterial collagenase hydrolysate of collagen types I and III).

Keywords: Amine additives; Selectivity; Collagen fragments; Peptides; Capillary coating

1. Introduction

Fused silica capillary displays approximately 8.31 μmol/m^2 of silanol groups with a pK mean value of 6.3 [1]. These weakly acidic groups generate in electrolyte solutions an electric double layer on the inner surface of the capillary which is responsible for two fundamental phenomena: the electroosmotic flow, and the attraction of positively charged analytes (ions) by the capillary wall which represent the immobilized part of the electric double layer [2].

Modification of the fused-silica surface (both chemical or dynamic) can be used not only to control the electroosmotic flow (EOF) in capillary electrophoresis (CE) but also to alter (generally minimize) the interaction of the analytes with the capillary wall and, consequently to modify the selectivity of the capillary electrophoretic system [3]. In the analysis of proteins and peptides by CE, shielding of the analytes from the active sites of the fused silica capillary wall is often

* Corresponding author. Tel.: +420 296442534; fax: +420 296442558.
E-mail address: miksik@biomed.cas.cz (I. Mikšík).

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necessary. There are several strategies to avoid undesirable interactions of the analytes with the capillary wall (for review see [4]): (i) modification of the ionic conditions in fused silica capillary [5–8], (ii) dynamic coating [9–11] and (iii) permanent coating through chemical modification of the capillary [12]. Replacing standard fused silica capillaries for capillaries made from an organic polymer [13] represents another alternative.

The main reason for protein/peptide sticking to the capillary results from the interaction of the negatively charged silanol groups with countercharged functionalities of the polypeptide chain (amino groups) [14]. A logical way for circumventing these effects is either to oppress the dissociation of the silanol groups by running the separation at very low pH ∼ 2.5 [15,16] or, to depress the dissociation of the protein nitrogen functionalities by using background electrolytes of high pH [17]. However, ionic interactions are not the only ones involved. In proteins possessing large hydrophobic domains also the hydrophobic interactions between the capillary wall and the separated analyte may influence the separation.

Alkylamines are known to interact strongly with the silanol groups [18] and, consequently, they are quite effective at masking the silanolphilic activity in both HPLC [19] and capillary electrophoresis [1,2,9,11,20].

In protein chemistry peptide mapping, both by chromatographic and electromigration methods, is a widely applied approach [21]. Unfortunately, current separation methods frequently do not exhibit sufficient selectivity to offer baseline separations in complex peptide mixtures. Such mixtures typically arise from proteolytic cleavage of the studied proteins, an approach gaining popularity in proteomic studies today [22].

To reveal the possibilities opened by dynamic modification of the capillary wall we focused on the separation of peptides arising from collagen cleavage by *Clostridium histolyticum* collagenase. Application of this very specific enzyme leads to a complex peptide mixture (theoretically about 172 peptides could arise from the naturally occurring collagen types I and III mixture). This set of peptides (typically tripeptides Gly–Pro–X; where X is an arbitrary amino acid) can be partially separated by CE in a bare-silica capillary at acid pH [22].

In this work, the effect of amine additives (alkyl-α,ω-diamines, polymeric amines and amino surfactants) to control both the electroosmotic flow and the peptide migration behaviour in bare fused-silica capillaries was investigated. The investigation was performed in strongly acidic background electrolyte in order to oppress the dissociation of the silanol groups and to have all alkylamines fully protonated, and therefore, behaving as cations differing in their molecular size and hydrophobicity.

A seven-membered set of peptides related to the collagen structure and *Clostridium histolyticum* collagenase digest of a mixture of collagen types I and III was used as test mixture.

2. Experimental

2.1. Apparatus and operating conditions

A Beckman P/ACE 5000 CE system (Fullerton, CA, USA) was used throughout this study.

Separations were run at 10 kV and 20 °C in bare fused standard capillary, 50 μm i.d. (40/47 cm long) (purchased from Composite Metal Services, The Chace, Hallow, Worchester, Great Britain). The injection was done hydrodynamically by overpressure (3.45 kPa, 2 s) and the concentrations of the peptides were 0.1–0.3 mg/ml. The separations were run with positive polarity at the inlet, detection was done at 214 nm. Coating agents were added to the phosphate buffer (50 mmol/l NaH_2PO_4, pH was adjusted by 1 mol/l HCl or NaOH to 2.5).

The running electrolyte was renewed after every run. Before analysis the capillary was conditioned by the run buffer (3 min). After every run the capillary was flushed step-wise with the run buffer (1 min), water (1 min), 1 mol/l NaOH (5 min), water (1 min), 3 mol/l HCl (5 min) and water (1 min). For storage the capillary was rinsed with water (3 min) and then dried by flushing nitrogen for 5 min.

2.2. Chemicals

Standard peptides Gly–Pro–Ala, Pro–hPro were the products of Sigma (St. Louis, MO, USA), Gly–Pro–Arg, Gly–Pro–Gln, Lys–Pro–Gly, Asn–Pro–Gly, His–Pro–Gly were purchased from Polypeptide Laboratories (Prague, Czech Republic). 1,7-Diaminoheptane, ethylenediamine (1,2-diaminoethane), cetrimide (hexadecyltrimethylammonium bromide), hexamethonium bromide (hexane-1,6-bis[trimethylammonium bromide]), polyethylenimine, putrescine (1,4-diaminobutane), spermine (*N*,*N*′-bis[3-aminopropyl]-1,4-butanediamine), and polybrene [poly(*N*,*N*,*N*′,*N*′-tetramethyl-*N*-trimethylenehexamethylenediammonium dibromide)] were the products of Sigma. NaH_2PO_4 was obtained from Lachema (Brno, Czech Republic). Collagenase E.C. 3.4.24.3., 0.8 U/mg, from *Clostridium histolyticum*, was from Fluka (Buchs, Switzerland, c.n.27676).

To obtain peptide profiles, collagen from rat tail tendon (essentially a mixture of collagen types I and III) was digested by bacterial collagenase (collagen/collagenase ratio 100:1, w/w). The samples were incubated at 37 °C for 48 h in the collagenase activating buffer (200 mmol/l NH_4HCO_3, 1 mmol/l $CaCl_2$, pH 7.8).

All chemicals used were of the p.a. or highest purity available. All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA) and before analysis were filtered using Millex-HV filter, 0.45 μm (Millipore).

3. Results and discussion

A model set of seven peptides [His–Pro–Gly (HPG), Lys–Pro–Gly (KPG), Gly–Pro-Arg (GPR), Gly–Pro–Ala

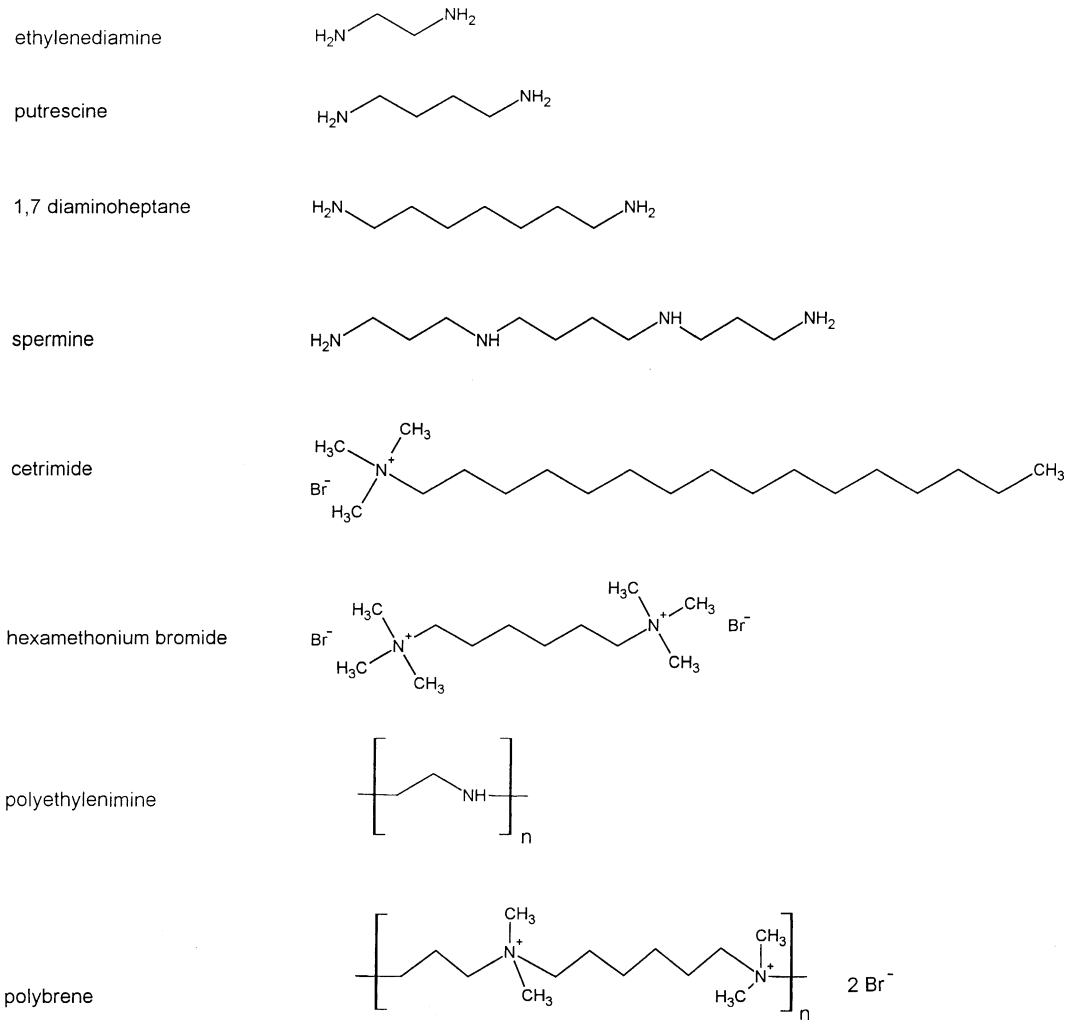

Fig. 1. Structures of amines utilized for dynamic coating: ethylenediamine (1,2-diaminoethane), putrescine (1,4-diaminobutane), 1,7-diaminoheptane, spermine (*N*,*N*′-bis[3-aminopropyl]-1,4-butanediamine), cetrimide (hexadecyltrimethylammonium bromide), hexamethonium bromide (hexane-1,6-bis[trimethylammonium bromide]), polyethylenimine, polybrene [poly(*N*,*N*,*N*′,*N*′-tetramethyl-*N*-trimethylenehexamethylenediammonium dibromide)].

(GPA), Asn–Pro–Gly (NPG), Pro-hydroxyPro (PhP), Gly-Pro-Gln (GPQ)] was used to reveal the influence of the alkylamines on the resulting separation.

Eight amine additives (for structure see Fig. 1) capable of controlling of the electroosmotic flow and peptide migration behaviour in bare fused-silica capillaries were used:

(1) alkyl-α,ω-diamines (ethylenediamine, putrescine, 1,7-diaminoheptane, spermine),
(2) polymeric amines (polyethylenimine, polybrene),
(3) amine surfactants (cetrimide, hexamethonium bromide).

Most of these amino compounds have been already used as coating agents, but under different conditions: putrescine, spermine (pH 8.3, for optimisation of CE [23]), putrescine, spermine and hexamethonium bromide (pH 5, for decreasing protein absorption [9]); hexamethonium bromide (pH 9.2, for reversed polarity separations [24]), polyethylenimine (pH 3–10, for changing the electroosmotic flow [25,26]), polybrene (pH 2.5, for reversed polarity separations [27]).

Under the conditions specified in Section 2 the dissociation of the silanol groups present on the capillary surface was oppressed; on the other hand the alkylamines used as modifiers were fully protonated and behaved as cations. The analytes (peptides) were also fully protonated and behaved as cations as well.

In the absence of the additives in the running electrolyte the separation of the model mixture was incomplete (fused peaks of HPG and KPG, and PhP with GPQ), KPG and GPR were partly separated (Table 1, Fig. 2).

In the presence of all additives (except polybrene), some improvement of separation of the model group of peptides was obtained. The best resolution of the model peptides was observed if ethylenediamine, or 1,7-diaminoheptane were used as modifiers. In this case the separation of HPG/KPG and PhP/GPQ was nearly complete (Table 1, Fig. 2).

Except polyethylenimine, the presence of all other modifiers in the background electrolyte resulted in a slow down of the separation (lower apparent mobility) (Fig. 2).

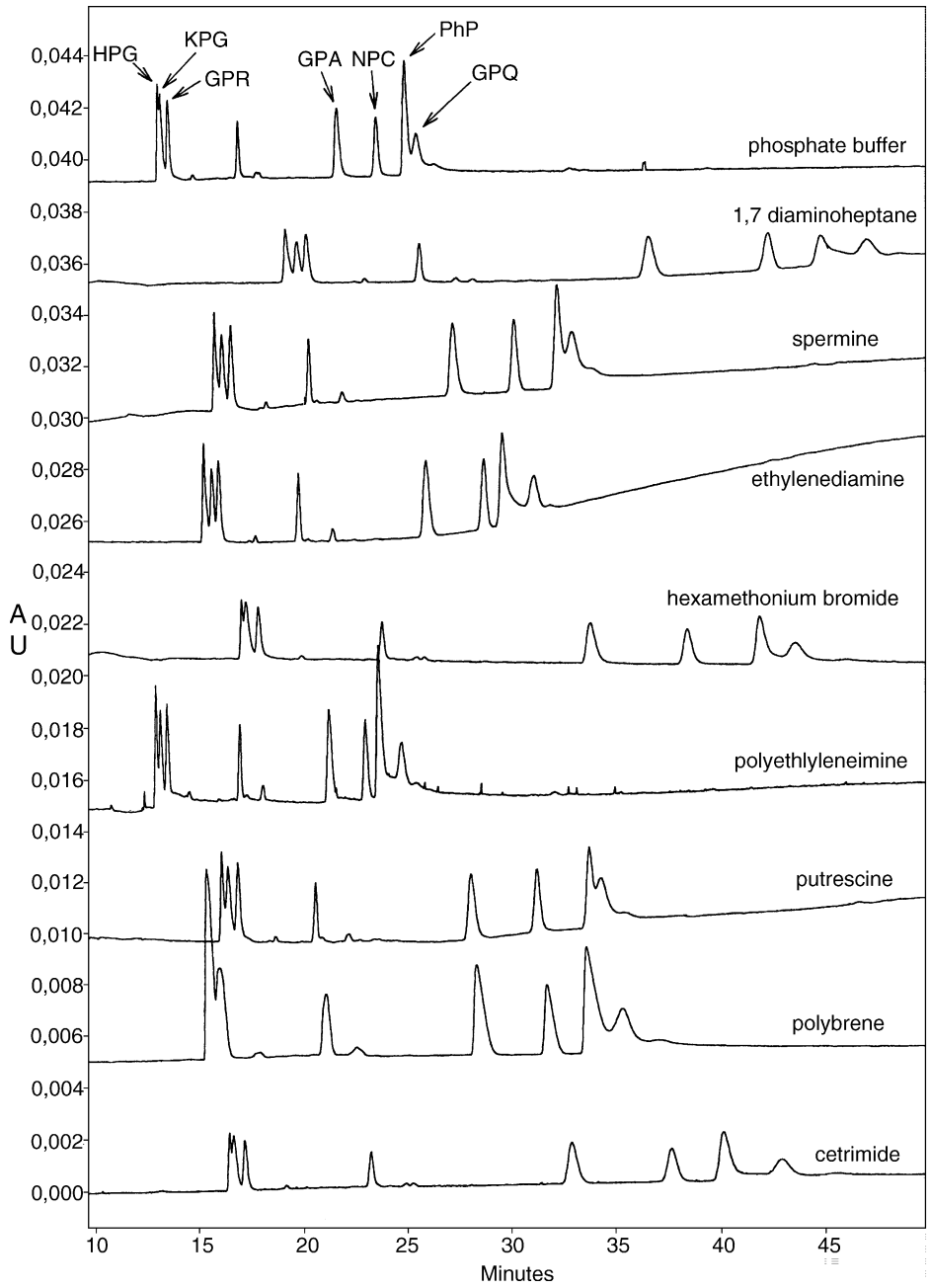

Fig. 2. Capillary electrophoretic separation of a set of model peptides (see Section 2) in the presence of eight types of dynamic coating: 75 mmol/l 1,7-diaminoheptane; 25 mmol/l spermine; 50 mmol/l ethylenediamine; 2.5 mmol/l hexamethonium bromide; 0.1% polyethylenimine; 50 mmol/l putrescine; 0.02‰ polybrene; 0.03 mmol/l cetrimide. The 50 mmol/l phosphate buffer pH 2.5 as background electrolyte. All runs effected at the same voltage, temperature and capillary (for identification of individual peptides see Section 2).

This indicates slowing down of the endoosmotic flow owing to the attachment, perhaps by hydrophobic interactions, of the amine modifiers to the capillary wall. Consequently the separation window for the three fast moving peptides (HPG, KPG, GPR) was spread allowing more time for separation and yielding better results (see the runs effected in the presence of ethylenediamine, putrescine and 1,7-diaminobutane).

On the other hand the separation of the above three fast moving peptides remained poor if hexamethonium bromide, or cetrimide were used to modify the inner surface of the capillary. Concomitantly the separation of the last four peptides, particularly of PhP and GPQ was considerably improved.

From the viewpoint of the quality of separation of the whole test mixture, using of 1,7-diaminoheptane was certainly the best.

The results with different amine modifiers can be divided in two categories. The first category (i) is represented by spermine, ethylenediamine, polyethylenimine and putrescine all of which offer better separation of the fast moving peaks

Table 1
Resolution (R_s) of critical pairs in capillaries coated dynamically with the tested alkylamines

	R_s		
	HPG with KPG	KPG and GPR	PhP with GPQ
Phosphate buffer only	0.31	1.05	0.95
1,7-Diaminoheptane	1.08	0.96	1.71
Spermine	1.03	1.08	0.72
Ethylenediamine	1.21	0.93	2.92
Hexamethonium bromide	0.43	1.22	1.70
Polyethylenimine	0.87	1.07	2.12
Putrescine	0.79	1.07	0.55
Polybrene	0.12	0.53	1.14
Cetrimide	0.47	1.12	2.42

Resolution was calculated according to the formula $R_s = 2\,(t_2 - t_1)/(w_1 + w_2)$ where t_2 and t_1 are migration times of two peptides ($t_2 > t_1$) and w_1 and w_2 are widths of peptide peaks (at 1/10 of peak height) in the electrophoregram.

and (ii) hexamethonium bromide and cetrimide which offer better separation of the more slowly moving members of the set. 1,7-Diaminoheptane appears capable of improving the separation of both (fast and slowly moving) subsets of peptides.

Comparing the results obtained under optimised conditions with putrescine and cetrimide (or hexamethonium bromide), in which apparently the slow down of the endoosmotic flow is about the same, a better separation of the fast moving peaks was observed with putrescine only, while on contrary the separation of the slowly moving peaks is complete with cetrimide and hexamethonium bromide and stays poor with putrescine. This means that there must be another mechanism in addition to the slow down of the electroosmotic flow involved.

This conclusion is further supported by the results with polyethylenimine in which a better separation of the three fast moving peptides is, no doubt, better than in the pure phosphate buffer (devoid of any polyamine modifier) with a small change (if any) in the profile of the four more slowly moving peaks.

Further, with hexamethonium bromide and cetrimide where the fast moving peptides are running about with the same speed as with putrescine, i.e. are slowed down in comparison to the runs effected with phosphate only (no modifier at all), the quality of separation of the fast running peaks remains poor.

It should be stressed that the results shown in Fig. 2 were obtained at optimised concentrations of the background electrolyte modifiers. If the runs were effected at 50 mmol/l of all the used electrolytes, the results shown in Fig. 3 were obtained. With hexamethonium bromide and polybrene no response of the detector was obtained during 1 h running time. This is fully compatible with result reported in refs. [24] and [27] in which both these cationic surfactants were used for reversed polarity runs. With 1,7-diaminoheptane a considerable decrease in running time of the three last moving peptides (HPG, KPG, GPR) was observed.

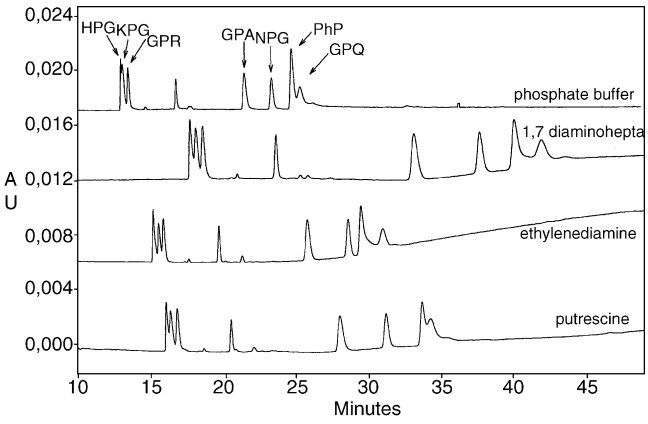

Fig. 3. Separation of the model set of peptides with the same background electrolyte as in Fig. 2 but at an identical concentration (50 mmol/l) of the indicated polyamine modifiers (for identification of individual peptides see Section 2). No peaks could be detected with hexamethonium bromide and polybrene, no further improvement at this higher modification was seen with spermine as compared to the runs effected at 25 mmol/l (see Fig. 2).

It appears feasible to propose that the resulting profiles reflect (in addition to the slow down of the endoosmotic flow) also hydrophobic interactions of the separated solutes with the amine modifiers attached to the capillary wall. If these two mechanisms are harmonized, complete (or nearby complete) separation of all the injected peptides can be obtained as seen with 1,7-diaminoheptane and (to a lesser extent) also with polyethylenimine.

In the next step the role of the amine modifiers upon the separation of collagen peptides was investigated (Fig. 4). Because, as mentioned, the slow down of the separation with some of the modifiers used (typically 1,7-diaminoheptane, hexamethonium bromide and cetrimide) was much to large resulting in considerable spreading of late moving peaks and, consequently, a poor resolution of the slowly moving peptides present in a real sample (collagenase digest of collagen); spermine was used as the dynamic modifier. It was expected that using this modifier improved separation could be obtained for both slowly and fast moving peptide peaks (see enlarged sections of the fast and slowly moving peaks of the collagenase digest in Fig. 5).

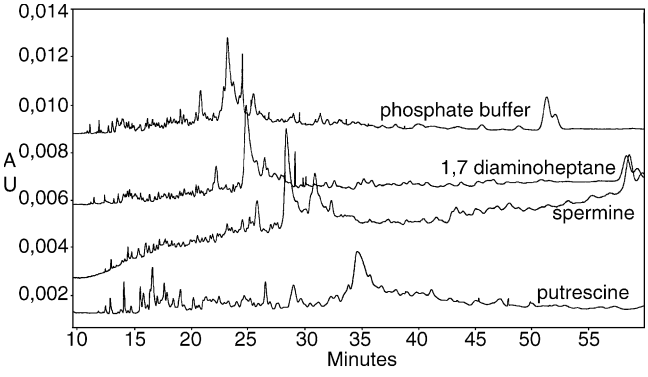

Fig. 4. Comparison of collagen peptide profiles using 1,7-diaminoheptane, spermine and putrescine as dynamic coating agents.

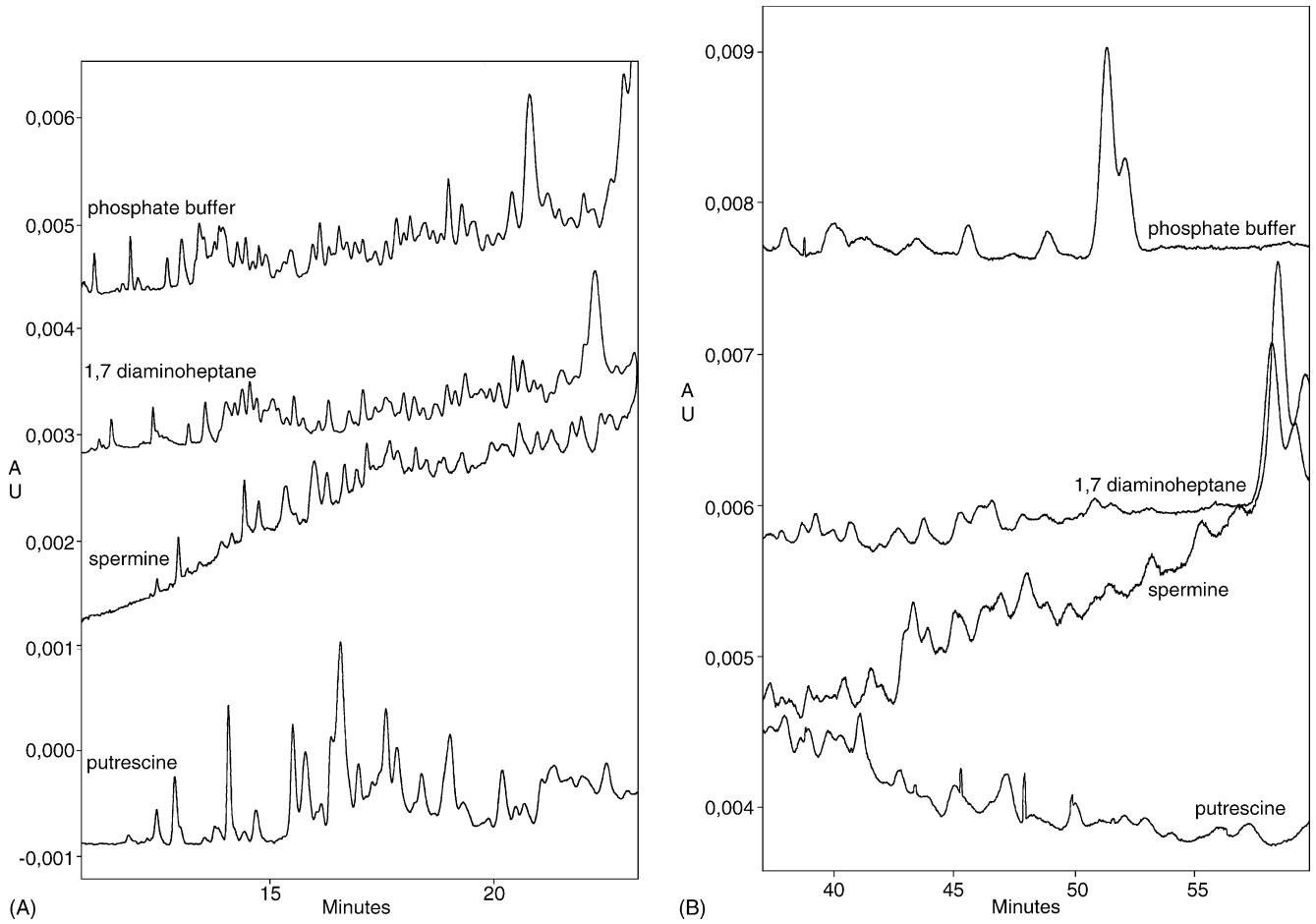

Fig. 5. Detailed view of the fast (A) and slowly (B) moving peaks in Fig. 4 (enlarged sections of the electropherograms).

By using the spiking method we were able to localise some of the peptides of the test mixture in the collagenase digest (see Fig. 6). HPG, KPG and GPR peptides appear as minor peaks in the collagenase digest profile. The GPA peptide being abundant in the collagenase sequence is represented by the first dominant peak. The NPG peptide comprises a part of the second dominant peak of the profile (apparently there are more peptides present in this peak; data not shown). The PhP and GPQ peptides are represented by the shoulder at the front and tail of the third dominant peak. The two peaks occurring at 19.9 and 21.3 min of the spiked run are impurities present in the spiking solutions.

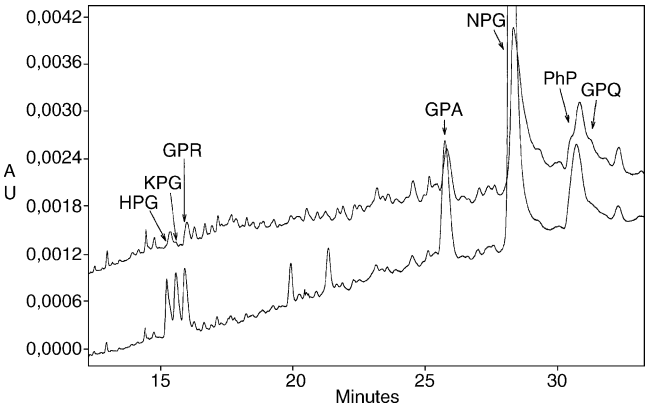

Fig. 6. Electrophoretic profile of the collagenase digest showing the location of six collagenase released peptides. Upper tracing: profile obtained with spermine as dynamic modifier; lower tracing: the same profile spiked with the seven peptides as indicated.

4. Conclusions

Using alkylamines as dynamic coating agents added to the background electrolyte (phosphate buffer, pH 2.5) caused an extension of the electrophoretic migration times and improved the separation of the model mixture of peptides, as well as a complex peptide mixture (collagen peptides obtained after bacterial collagenase cleavage). Ethylenediamine, or 1,7-diaminoheptane as background electrolyte modifiers yielded the best results.

When applying this approach to real samples (typically bacterial collagenase digest of collagen) care must be taken about the modifier used. The most efficient modifiers in slowing down the runs offer improved separations of the fast

moving peptides while they can bring about problems with the separation of slowly moving peptide peaks which are much to broad and overlapping of adjacent peaks would be observed. Spermine used as dynamic modifier appears a reasonable compromise covering the whole separation window. By using the spiking procedure it was possible to localise seven peptides in the profile.

Acknowledgements

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