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Capillary electrophoresis of peptides and proteins with plug of Pluronic gel☆

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Abstract

Electromigration capillary methods are promising techniques in proteomics and they are still under research. We used a partial filling approach, i.e. a combination of gel and non-gel separation mechanisms in a single dimension. We tried using an interesting gel, Pluronic F 127, which can be considered as a surfactant capable of self-association both with isotropic and anisotropic gels. The Pluronic was inserted inside the capillary as a plug at the start of the capillary, and it provided separation at the first time. Separation by this gel was achieved according to molecular weight and/or hydrophobicity. The applicability of this method was demonstrated in the separation of real samples—peptides arising from collagen after CNBr or collagenase cleavage and albumin after trypsin cleavage (peptide mapping). Some peptides and proteins were selectively retained by the Pluronic gel. These interactions with the gel did not depended on their molecular weight alone, but they probably depend on a combination of both principles. It was confirmed that capillary electrophoresis with Pluronic plug can give us another new separation option, complementary to free solution capillary electrophoresis. The CE method presented here, consisting of a partial filling approach with combine gel and non-gel separation mechanisms seemed to be a promising method for the separation of complex mixtures of peptides.

Keywords: Pluronic; Partial filling; Proteins; Peptide

1. Introduction

The analysis of proteins and peptides are now, in the era of proteomic research, the most important area of analytical chemistry. One promising category of techniques is that of electromigration capillary methods. There are many forms of these techniques, but in protein/peptide analysis the most common modes are capillary zone electrophoresis and capillary gel electrophoresis.

Protein/peptide samples (even samples of animal tissue) are complex mixtures of compounds of low and high molecular mass. The resolution of these complex samples into their components requires the use of sophisticated technologies. We have to realize that most separation techniques are capable of resolving several dozen components at most. The combination of two separation techniques is important in the resolution of complex mixtures [1]. The most popular and very frequently used method is two-dimensional (2D) gel electrophoresis (for details, see e.g. [2–4]).

In our experiments, we do not use 2D electrophoresis, but we tried to use a partial filling technique. Partial filling techniques are frequently used for affinity capillary electrophoresis and chiral (enantiomer) separations. Affinity capillary electrophoresis (ACE) can be designed in a number of ways depending on the properties of the affinity system and the problem under investigation. In the classical affinity capillary electrophoresis approach the affinity ligand is added to the background electrolyte (BGE) and the sample is injected as a narrow zone. Components in the sample that interact with the affinity ligand will exhibit a mobility shift during the passage through the capillary filled with the affinity ligand, provided that the mobility of the affinity complex is different from the mobility of the sample. There is one disadvantage in ACE compare to affinity chromatography—affinity ligand is not immobilized. It is for instance impossible to couple a mass detector to CE with this approach. To overcome these problems the "partial filling technique" was introduced [5]; with this technique the capillary is only partly filled with the affinity ligand in BGE prior to injecting the sample. The pH of the BGE

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is selected to have the affinity ligand and the sample to move in opposite directions in a neutral coated capillary with a low electroosmotic flow (EOF) [6]. The sample migrates through the affinity ligand zone when voltage is applied, leaving the affinity ligand behind, and reaches the detector window without any affinity ligand that can disturb the detection. The partial filling technique has been also used in enantiomer separations of small drug molecules with proteins as chiral affinity ligands (selectors). Affinity constants are possible to estimate from the change in migration time when the absolute amount of the affinity ligand is varied [7].

In our experiments we used a partial filling technique, concrete a partial filling approach with combine gel and non-gel separation mechanism in a single dimension. We used Pluronic F 127 as the gel matrix. Pluronic polymers are triblock uncharged copolymers with the general formula $[\text{poly(ethylene oxide)}]_x[\text{poly(propylene oxide)}]_y[\text{poly(ethylene oxide)}]_x$ (Pluronic is a registered trade name of BASF Performance Chemicals, Mount Olive, NJ, USA). In the case of Pluronic F 127, the coefficient numbers are $x = 106$ and $y = 70$, and its 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 segregated 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 phase (isotropic, cubic, hexagonal or lamellar) depends not only on the structural features of the polymer, but also on its concentration and temperature. This means in practice that a Pluronic which is soluble at a low temperature gellifies with a temperature increase, e.g., Pluronic F 127 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 laboratory 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]). Pluronics can also be used for the slab gel electrophoresis of peptides [17].

In previous reports, we developed capillary electrophoretic methods usable for the analysis of proteins and peptides using Pluronic F-127 (5–10%, w/v) in the background electrolyte to gellify the media at a lower, laboratory temperature (20 °C) [18–21].

In the paper presented here, we tried to develop a combination of gel and non-gel separation mechanisms in a single capillary by using a plug of gel (a higher concentration of Pluronic at high temperature) inside the capillary.

2. Materials and methods

2.1. Chemicals

Sodium dihydrogen phosphate and hydrochloric acid were produced by Lachema (Brno, Czech Republic) and were of p.a. quality. Pluronic F 127 and all peptides (except cytochrome *c* and chymotrypsinogen A) were from Sigma (St. Louis, MO, USA). Cytochrome *c* and chymotrypsinogen A were produced by Boehringer (Mannheim, Germany). Bacterial collagenase (from Clostridium histolyticum) and trypsin were from Sigma. All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA) and filtered through a 45 μm Millex-HV filter (Millipore).

2.2. Capillary electrophoresis

All runs were performed on a Beckman P/ACE system 5500 (Beckman, Fullerton, CA, USA). A bare fused-silica capillary of 37 cm total length (30 cm to the detector) with ID of 75 μm was used. Detection was done by UV absorbance recording at 214 nm. Before analysis the capillary was washed for 2 min with the background electrolyte. The sample was injected electrokinetically (2 s, 10 kV). The separations were run at 40 °C at an applied voltage of 10 kV. At the start of analysis (every day) the capillary was washed step-wise with water (5 min), 1 mol/l NaOH (5 min), water (5 min), 1 mol/l HCl (5 min), water (5 min) and the background electrolyte (5 min). A phosphate buffer was used (0.1 mol/l) pH 2.5 (pH was adjusted with 3 M HCl). Before analysis, the background electrolyte was filtered using a 45 μm Millex-HV filter (Millipore).

The samples were analyzed with or without Pluronic. The Pluronic F 127 was dissolved in 0.1 mol/l phosphate buffer (pH 2.5) at a concentration of 15 or 20% (w/v) at 5 °C and intensively stirred overnight. The Pluronic was inserted into the capillary as a plug at the start of capillary under pressure (3.45 kPa) for 2, 5 or 10 s. The capillary was subsequently warmed for 30 min at 40 °C, and then used for the separation of peptides and proteins. This plug of Pluronic served as a gel separation mechanism.

The gel was removed by cooling the cartridge in a refrigerator, followed by flushing with cold water.

2.3. Protein and peptide samples

The set of test analytes was comprised of the following proteins: cytochrome *c* (M_r 12, 500) and chymotrypsinogen A (M_r 25, 000) (produced by 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 poly-L-lysine had an average relative molecular mass of 4000 (by viscosity; DP: 19) or 3300 (by SEC-LALLS; DP: 16).

The following sets of peptides were tested:

(a) Tripeptides: (APG = Ala-Pro-Gly; FW = 243.3), (TYS = Thr-Tyr-Ser; FW = 369.4), (GPP = Gly-Phe-Phe; FW = 369,4)
(b) Tetrapeptides: (GGTA = Gly-Gly-Tyr-Arg; FW = 451.5), (4G = Gly-Gly-Gly-Gly; FW = 246.2), (PGPG = Phe-Gly-Phe-Gly; FW = 426.5)

(c) Pentapeptide: (the bradykinin fragment 1-5 = Arg-Pro-Pro-Gly-Phe; FW = 572.7)
(d) Hexapeptides: (AGPHPI = Arg-Gly-Pro-Phe-Pro-Ile; FW = 685.8), (6TYR = hexa-L-tyrosin; FW = 997.1), (6A = Ala-Ala-Ala-Ala-Ala-Ala; FW = 444.5)

Samples were dissolved in Milli-Q water to a concentration of 2.5 mg/ml.

The applicability of this method was demonstrated in the separation of real samples—peptides arising from collagen after CNBr or collagenase cleavage and peptides arising from bovine serum albumin (BSA) after trypsin cleavage.

2.3.1. Collagenase digest

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 a collagenase-activating buffer (200 mmol/l NH_4HCO_3, 1 mmol/l $CaCl_2$, pH 7.8).

2.3.2. CNBr digest

A set of peptides obtained by the CNBr cleavage of rat tail tendon collagen was also used as a test mixture. This sample contained fragments of both types I and III collagen and was prepared by the procedure described in our previous report [22]. Briefly, samples (rat tail tendons) were incubated in 0.2 mol/l ammonium bicarbonate, pH 7.0, containing 25% (v/v) β-mercaptoethanol to reduce the oxidised methionyl residues and after lyophilisation the samples were cleaved with CNBr in 70% (v/v) formic acid under nitrogen. Samples were lyophilised and then reconstituted in water to a concentration of 2 mg/ml.

2.3.3. Ultrafiltration

The low molecular fraction (below 10,000 relative molecular mass) of CNBr collagen fragments was prepared by ultrafiltration through Microcon centrifugal filter devices (Microcon YM-10, regenerated cellulose with a nominal molecular mass limit of 10,000 relative molecular mass) made by Amicon (Millipore) at 2000 × *g* for 10 min.

2.3.4. Enzymatic digestion of BSA by trypsin

BSA samples were diluted to the concentration 3 mg/ml with 20 mmol/l ammonium bicarbonate buffer (pH 7.8) and treated with trypsin (1:50 enzyme:substrate ratio). Blank samples were prepared by incubation of the enzyme solution only under identical conditions. Incubation was done at 37 °C for 36 h. Next the samples were centrifuged for 5 min at 2000 × *g* and the supernatants removed and stored at −18 °C.

3. Results and discussion

The routine procedure in capillary electrophoresis is the optimization of all parameters. With a Pluronic copolymer, it is necessary to optimize its concentration and temperature of separation. Liu et al. [23] used 25% Pluronic F127 at 30 °C for the separation of short oligonucleotides in liquid crystalline Pluronic. In our previous experiments [18–21] we developed a method for the separation of peptides/proteins with 5–10% Pluronic F-127 in the background electrolyte producing fluid gel media at 20 °C. It was observed that a higher concentration of Pluronic is not relevant for the analysis of proteins (collagens and albumin) due to the long separation time (exceeded 2 h) and resulted in broad "bumps". The baseline was also unstable in this case. It was also concluded that the separation of proteins/peptides in the presence of Pluronic in the background electrolyte occurs on a charge/mass ratio basis, with molecular sieving effects acting as a secondary partition mechanism, and the hydrophobic properties of the peptides also having some influence.

In combining gel plug and free solution capillary electrophoresis (separation in the phosphate buffer), we optimized the duration of gel introduction (i.e. length of plug), concentration of Pluronic (this factor influences the viscosity of gel, and so the character and again length of plug) and the temperature of separation (this factor affects the viscosity and structure of the gel plug). The optimum conditions were found to be 15% Pluronic F-127 injected for 10 s and a separation temperature of 40 °C.

The separation of the test mixture of proteins and peptides (see Section 2) is shown in Fig. 1. It is interesting to compare the separation achieved by free solution capillary electrophoresis (without gel plug – A) and by CE with a complex separation mechanism (with gel plug – B). In the case of free solution capillary electrophoresis (in the phosphate buffer only), the two sharp peaks at the beginning of the electropherogram are synthetic polylysines (M_r 4000 and 22,700). Then we can see the sharp peak of tetrapeptide GGTA and cytochrome *c*. At the end of the electropherogram we can see a broad peak of 6TYR. The set of test proteins and peptides was comprised of the following peaks: two polylysines, GGTA, cytochrome *c*, the bradykinin frag-

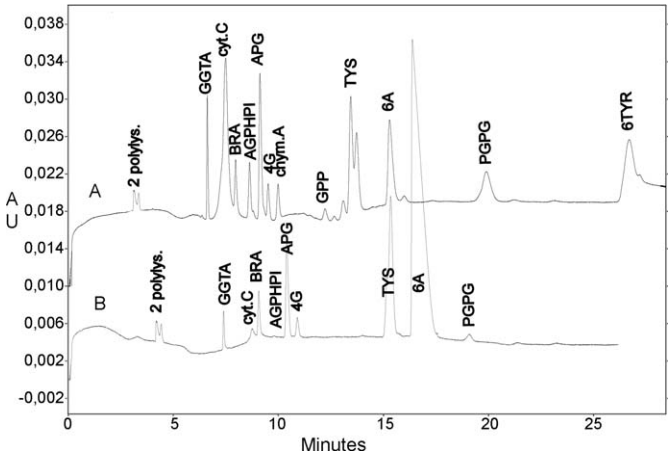

Fig. 1. Separation of test mixture of proteins and peptides. (A) Without gel plug, (B) with gel plug (15%, 10s). Peak identification: two poly-L-lysines (molecular mass 4000 and 22,700), GGTA (Gly-Gly-Tyr-Arg), cytochrome *c* (M_r 12,500), BRA (bradykinin fragment 1-5, Arg-Pro-Pro-Gly-Phe), AGPHPI (Arg-Gly-Pro-Phe-Pro-Ile), APG (Ala-Pro-Gly), 4G (Gly-Gly-Gly-Gly), chym.A (chymotrypsinogen A, M_r 25,000), GPP (Gly-Phe-Phe), TYS (Thr-Tyr-Ser), 6A (Ala-Ala-Ala-Ala-Ala-Ala), PGPG (Phe-Gly-Phe-Gly) and 6TYR (hexa-L-tyrosin).

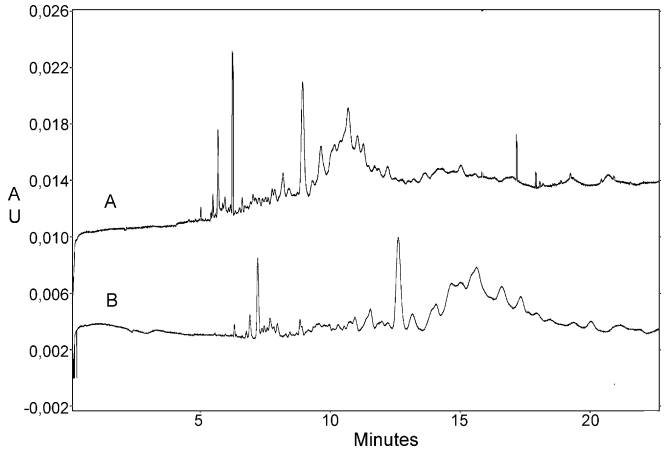

Fig. 2. Analysis of mixture of peptides arising from collagenase digest of collagen. (A) Before application of Pluronic plug and (B) after application of Pluronic plug (15%, 10 s).

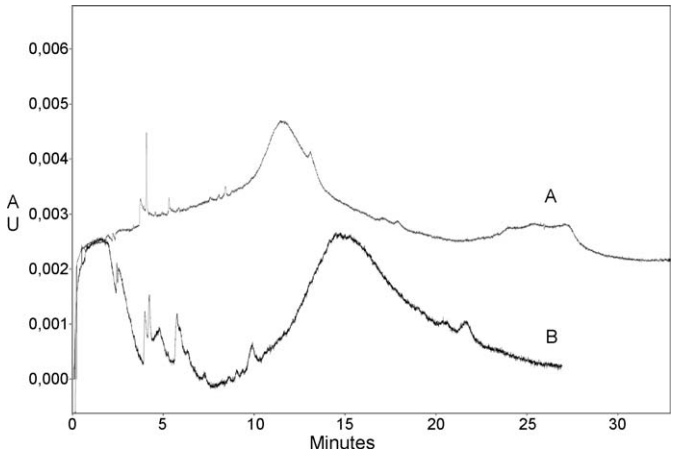

Fig. 3. Analysis of peptides arising from CNBr cleavage of collagen. (A) Before application of Pluronic plug and (B) after application of Pluronic plug (15%, 10 s).

ment, AGPHPI, APG, 4G, chymotrypsinogen A, GPP, TYS, 6A, PGPG and 6TYR. Using a plug of Pluronic F 127 gel produced significant changes. Some peptides and proteins were selectively retained by the Pluronic gel. These interactions with the gel did not depend on their molecular weight or hydrophobicity alone, but they probably depended on the combination of both methods. After applying the Pluronic plug, the set of test proteins and peptides was comprised of the following peaks: two polylysines, GGTA, cytochrome *c*, bradykinin peptide, AGPHPI, APG, 4G, TYS, 6A and PGPG. Chymotrypsinogen A, tripeptide GPP and hexapeptide 6TYR were totally reduced and their peaks did not appear.

The method developed was also tested on the analysis of low-molecular mass peptides (peptide mapping). The mixture of peptides analysed was that arising from the collagenase digest of collagen. The main peptides in the arising mixture are tripeptides (and it was calculated that more then 170 peptides arise from a tissue containing collagens types I and III). Collagens are interesting and highly abundant proteins with a relatively rigid structure where every third amino acid is glycine. For this reason, the separation of these peptides is not a simple matter. The separation is demonstrated in Fig. 2. It is obvious that separation after applying the Pluronic plug is improved and is more complex—more peaks can be determined. The main improvement is located in the first section of the electropherogram—many new peptide peaks can be seen due to the higher resolution of this system. The improvement is also detectable in the final section of the electropherogram, but in this case the peaks that probably contain peptides with a higher molecular mass (and/or with a larger hydrophobic section that sticks to the wall of the capillary) are wider. Overall we can conclude that the use of a plug in collagen analysis improves the separation of collagenous peptides.

The applicability of this method was also tested on the peptides arising from CNBr cleavage (Fig. 3). The range of molecular masses of this set of peptides is really broad: from a relative molecular mass of a few thousand to one of 30×10^3 or 60×10^3 if the splitting of the collagen molecule is incomplete. After applying the Pluronic plug we can see a better distribution (in the area of low-molecular mass peptides) and less migration by longer peptides. This approach enables a better separation of low and high molecular mass peptides, since high molecular items are retained in the plug to a greater extent. We have to mention that better separation is not caused by merely retaining the higher molecular mass peptides, but also by other interactions (probably hydrophobic). It is the same situation as in the test peptide/protein separation (Fig. 1) where retention in the plug does not depend on molecular mass alone.

This assumption was verified by the analysis of CNBr peptides after ultrafiltration (i.e. the separation of peptides with molecular masses below 10,000) (Fig. 4). From this figure it is obvious that many peptides with higher molecular mass are not retained in the plug, but others (e.g. the peak with a migration time of around 10 min) are at least noticeably trapped by the plug.

The applicability of this method was demonstrated in the separation of peptides arising from bovine serum albumin (BSA)

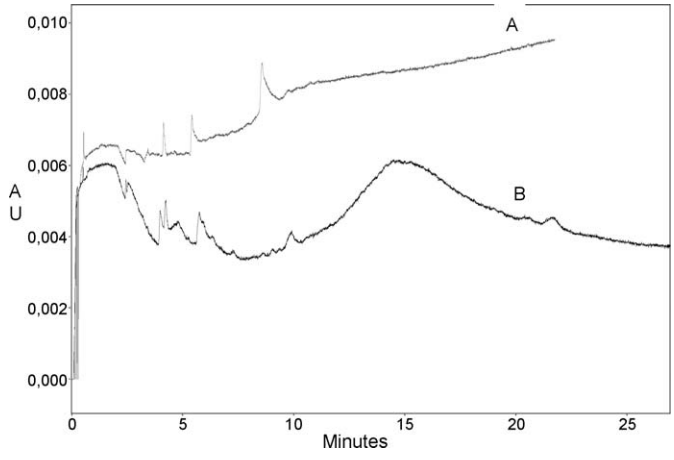

Fig. 4. Analysis of CNBr peptides before and after ultrafiltration (i.e. separation of peptides with molecular mass below 10,000) with gel plug (15%, 10 s). (A) After ultrafiltration and (B) without ultrafiltration.

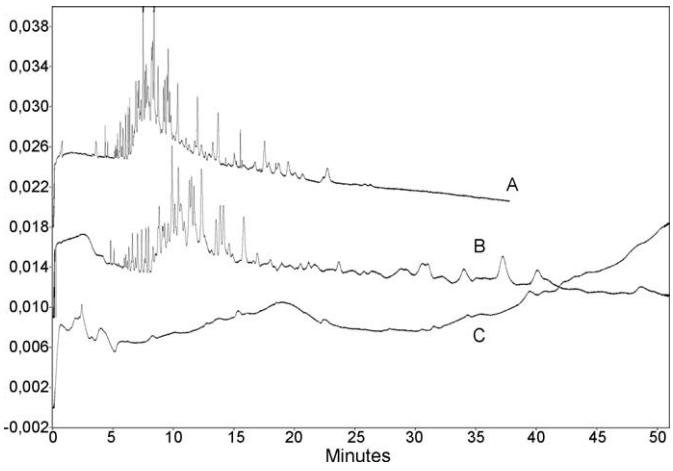

Fig. 5. Analysis of peptides arising from trypsin cleavage of BSA. (A) Free solution capillary electrophoresis, (B) capillary with Pluronic plug (15%, 10 s), (C) capillary fully filled by Pluronic gel (15%, 40 °C).

after trypsin cleavage (Fig. 5). It is interesting to compare the separation achieved by free solution capillary electrophoresis (without gel plug – A) and CE with a complex separation mechanism (with gel plug – B). There are some significant changes in the separation using a plug of Pluronic F 127 gel. The two main improvements are: (1) the better resolution of peaks at the first half of the electropherogram (to the ca. 12 min) and (2) new peptide peaks at the final section of the electropherogram. We have to mention that these peptides were probably selectively retained by the Pluronic gel. These interactions did not depend on their molecular weight or hydrophobic properties alone but they depend on a combination of both principles. This approach enables another separation of peptides in comparison to free solution capillary electrophoresis. Overall, we can conclude that the use of a plug appears to be useful in the albumin analysis. We also compared results obtained on the plug with the separation in the capillary fully filled with the Pluronic gel (Fig. 5C). At the separations conditions used (15% gel, 40 °C), we did not obtain any reasonable peaks and unstable baseline. The possible reason of this behavior is the interaction of peptides with Pluronic gel when they are retained on this matrix. This finding is supported by our previous work [20] when we observed broadening and retaining of protein peaks at 5% Pluronic F127 (at 20 °C). We can suppose that this broadening and retaining is more obvious in the more viscous gel (at higher concentration and temperature). For this reason it was impossible to see peptide peaks at tryptic digest of albumin. The same situation is in the case of artificial mixture of peptides.

We can conclude (on the basis of the presented results as well as on the basis of previously published papers [18–21]) that interactions with the Pluronic gel do not depend on the molecule's molecular mass or hydrophobicity alone, but probably depended on a combination of both methods. It was confirmed that our electrophoretic method with gel plug can provide us with another new possible separation method that can complement one-dimensional CE. It is obvious that this partially filled method is suitable for the separation of the complex mixture of low and high molecular mass peptides (or proteins). Because in this separation there are probably involved two types of the separation (according molecular mass and hydrophobicity) decision about suitability of this method depends on the properties of peptides' mixture of interest. Of course, for all these samples optimization of plug conditions could be made.

4. Conclusions

In conclusion, it can be said that capillary electrophoresis with a complex separation mechanism in one capillary (a partial filling approach with gel and non-gel separation mechanisms in a single dimension) appears to be useful in peptide analysis. The applicability of this method was demonstrated in the separation of a test peptide/protein mixture as well as on real samples containing peptides arising from collagen after CNBr or collagenase cleavage and albumin after trypsin cleavage (peptide mapping). Some peptides and proteins were selectively retained by the Pluronic gel. These interactions with the gel did not depend strictly on their molecular mass or hydrophobicity only, but they probably depend on a combination of both methods. It was confirmed that this method can give us another new separation option that is complementary to the free solution capillary electrophoresis method. This CE method with a complex separation mechanism presented here, based on a combination of Pluronic plug and free solution capillary electrophoresis, seemed to be a promising method for the separation of complex mixtures of peptides.

Acknowledgements

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