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Selectivity change in the separation of proteins and peptides by capillary electrophoresis using high-molecular-mass polyethyleneimine

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Abstract

A study of the capillary electrophoretic separations of proteins and peptides using high-molecular-mass polyethyleneimine (PEI) is presented. Experiments were performed in the PEI-coated capillaries together with the use of this polymer as a buffer additive under different separation conditions. The effects of pH and the concentration of PEI in the buffer on the electroosmotic flow and the migration orders of biopolymers were investigated. The use of the cationic polymer offers an alternative for the modification of the separation selectivity and resolution of biopolymers.

Keywords: Proteins; Peptides; Polyethyleneimine

1. Introduction

Polymers have found several applications in CE. The use of polymeric coatings has led to high efficiency and reproducible protein separations preventing the surface adsorptions. Polymers ranging from highly hydrophobic to highly hydrophilic were used for this purpose to obtain efficient separations of proteins in CE [1–16].

It is also well known that polymer additives to the buffer solutions play an important role in CE separation of molecules according to their molecular mass. In this case the entangled polymers form a

sieving matrix for the size-dependent separation of biopolymers such as DNA or proteins. Recently, a comprehensive review of the use of entangled polymer solutions in CZE was reported by Kenndler and Poppe [17].

A different approach to the applications of polymers in CE has been introduced by Terabe and Isemura [18], i.e., ion-exchange electrochromatography using charged polymers to enhance separation selectivity of organic isomer ions. Following this idea, Stathakis and Cassidy proposed cationic polymers for selectivity control in the CE separations of inorganic anions [19]. This enables different selectivity control from that normally employed, i.e., variation of the buffer pH, and the use of complexing or ion-pairing agents for the separation of small ions.

Polyethyleneimine (PEI), a cationic polyelectrolyte, has been used as a coating agent by several authors [5–8]. Recently, we reported a fast and

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simple method for coating the capillaries using high-molecular-mass PEI, i.e., 600–1000 kDa [9]. High-molecular-mass PEI has a positive net charge over a wide pH range, as can be deduced from the anodal electroosmotic flow obtained with coated capillaries. This property of PEI can be useful not only for reducing the adsorption problem, but also for modifying the selectivity of small and large ions in CE.

To the best of our knowledge, the use of polymers for the selectivity control in CE of the large molecules, such as peptides and proteins, has not been reported. The goal of this work was to carry out a preliminary study on the selectivity control of the CE separation of proteins and peptides by using a high-molecular-mass PEI polymer as a buffer additive.

2. Experimental

2.1. Instrumentation

Separations were carried out using a PRINCE (Lauer Labs, Emmen, Netherlands) injection system with temperature controller, connected to a LINEAR M-200 variable-wavelength UV–VIS detector (Linear Instruments, Reno, NV, USA) operated at 214 nm. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) with 75 μm I.D. and 360 μm O.D. were coated with PEI according to the procedure given in Ref. [9]. The injection was carried out at the cathodic side using controlled pressure for a determined time.

2.2. Samples and chemicals

Lysozyme (chicken egg white), cytochrome *c* (horse heart), ribonuclease A (bovine pancreas), trypsinogen and α -chymotrypsinogen (bovine pancreas), myoglobin (horse heart), β -lactoglobulin A (bovine milk) and trypsin inhibitor (soybean) were purchased from Sigma (St. Louis, MO, USA) and used as received. All the short peptides were from Nutritional Biochemicals (Cleveland, OH, USA) and the long ones from Bachem (Bubendorf, Switzerland) and used as received. Proteins and peptides were dissolved at the concentrations indicated in each case (ranging from 0.2 to 2 mg/ml) in water,

previously purified by passage through a PSC filter assembly (Barnstead, Boston, MA, USA). The samples were stored at -20°C and heated to room temperature before use. Polyethylenimine (PEI, molecular mass range from 600 to 1000 kDa) was from Fluka (Fluka, Buchs, Switzerland). Acetic acid (Merck, Darmstadt, Germany), chloroacetic acid, Tricine, MES and CAPS (all from Aldrich, Axel, Netherlands), were used in the different running buffers. The concentration of the buffers was 50 mM and the pH values were the following: 3 (chloroacetic acid), 5.5 (acetic acid), 7 and 7.5 (MES), 8 and 8.5 (Tricine), and 9–11 (CAPS). The buffers were stored at 4°C and heated to room temperature before use.

3. Results and discussion

Prior to the study of the use of PEI as selectivity modifier for proteins and peptides, we investigated the reproducibility of PEI-coated capillaries and the changes of electroosmotic mobility vs. pH using different buffer solutions with and without PEI.

3.1. Reproducibility

Reproducibility experiments were carried out at pH 5.5 using acetone as a neutral marker. Three columns with the same length and coating, i.e., 10% PEI according to the procedure described in Ref. [9], were used for testing column-to-column reproducibility. Within-day reproducibility, column-to-column reproducibility and day-to-day reproducibility were studied. In the absence of PEI, the within-day relative standard deviations (%R.S.D.) for retention time was 0.5% ($n=6$). The %R.S.D. value for column-to-column reproducibility was 1.9% ($n=18$). Day-to-day reproducibility was studied using a 10% PEI capillary for 5 days. The %R.S.D. for the retention time of acetone, used as a neutral marker, was 2% over that period ($n=20$).

Similar experiments were performed by using a buffer at pH 5.5 and 0.1% PEI. Within-day, day-to-day and column-to-column reproducibility experiments were carried out. No noticeable differences in terms of %R.S.D. values were observed compared to those obtained using a buffer without PEI.

3.2. The effect of pH on the electroosmotic flow

The pH dependence of electroosmotic flow was examined for two capillaries coated using different PEI concentration, i.e., 5 and 10% (w/v). Eight different buffers were used covering a pH range from 3 to 10.4. As seen in Fig. 1A, for all the pH range employed the electroosmotic flow was always in the anodal direction. This is due to the protonation of the amine groups of the PEI adsorbed on the capillary wall which changed the sign of the surface charge. The electroosmotic flow was constant between pH 3 and 6. At higher pH values a gradual decrease was observed due both to deprotonation of amine groups and higher ionization of silanol groups on the capillary wall. Capillaries coated with 5 and 10% of PEI showed similar behaviour (Fig. 1A).

Next, we tested the effect of PEI as an additive to the eight running buffers used above. As seen in Fig. 1B, by systematic use of 0.1% PEI in the buffer, slightly higher electroosmotic flow values were obtained than when no PEI was used in the buffer (Fig. 1A). However, these differences were never larger than 10% for all pH values and columns tested.

3.3. Selectivity control of protein and peptide separations depending on pH and PEI concentration in the running buffer

Four different types of biopolymers in a broad range of molecular masses and acidic–basic properties were used to carry out our study: short peptides (i.e., GE, GGG, LGF, AA and SGG); long peptides (i.e., WAGGDASGE, ELAGAPPEPA, SYSMEHPRWG and ELQAAPALDKL); basic proteins (i.e., lysozyme, cytochrome *c*, ribonuclease A, trypsinogen and α -chymotrypsinogen) and myoglobin and acidic proteins (β -lactoglobulin A and trypsin inhibitor).

An optimal separation of basic proteins was achieved in a 50 mM acetic buffer at pH 5.5 using PEI-coated capillary. A typical electropherogram obtained under these conditions is shown in Fig. 2A. The peak shapes and efficiencies for this separation were good, showing values near to 500 000 plates/m for cytochrome *c* and lysozyme.

Employing different buffers from pH 3 to 10.4 for the separation of basic proteins, it was observed that the selectivity between the first three proteins, namely trypsinogen, α -chymotrypsinogen and ribonu-

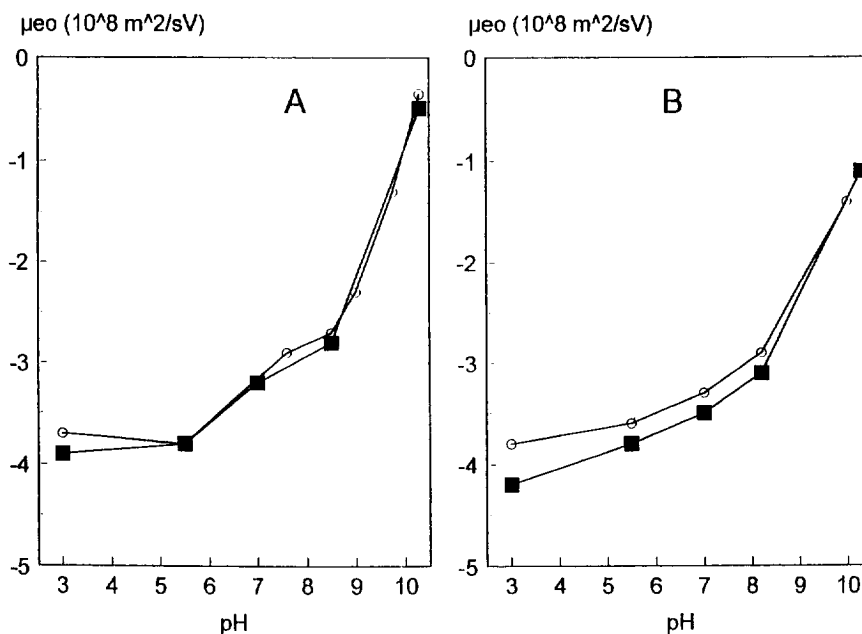


Fig. 1. Plots of electroosmotic flow against pH. Buffers without PEI (A) and with 0.1% PEI as additive (B). Coated capillaries with 5 (■) and 10% (○) PEI were used. Acetone was used as a neutral marker.

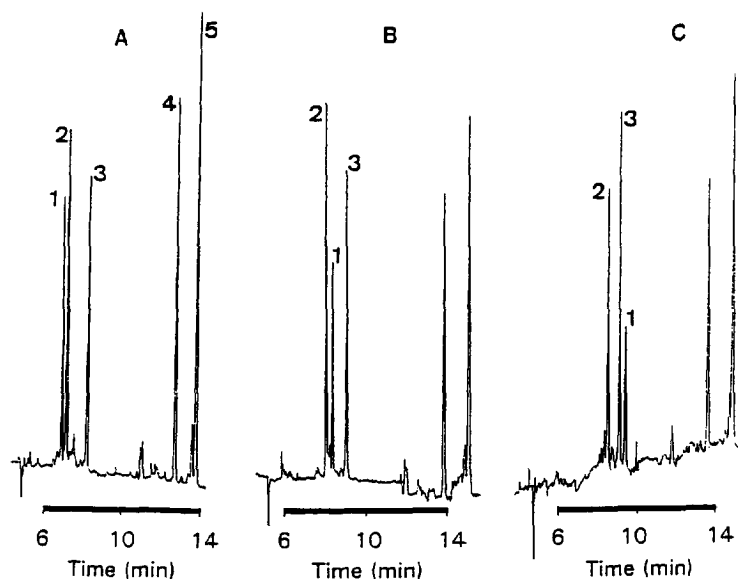


Fig. 2. Separation of five basic proteins. 10% PEI-coated capillary, total length 65 cm, effective length 50 cm, I.D. 75 μm . Buffer: 50 mM acetate at pH 5.5. Voltage -28.8 kV . Injection $1 \times 10^{-3}\text{ MPa}$ for 6 s. Sample: (1) trypsinogen, 0.16 mg/ml; (2) α -chymotrypsinogen, 0.32 mg/ml; (3) ribonuclease A, 0.88 mg/ml; (4) cytochrome *c*, 0.32 mg/ml; (5) lysozyme, 0.32 mg/ml. UV detection 214 nm. (A) 0, (B) 0.1 and (C) 0.25% PEI added to the buffer.

lease A changed as shown in Fig. 3. This study was carried out using buffers without PEI and buffers containing 0.1% PEI as additive. In Fig. 3 the

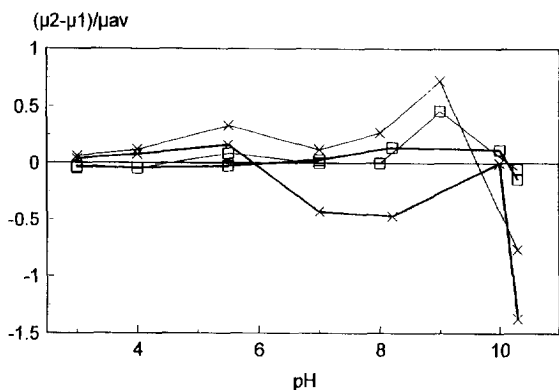


Fig. 3. Relative mobility difference vs. pH for some basic proteins. Narrower lines represent the change in selectivity observed using buffers without PEI. Thicker lines represent the change in selectivity using buffers containing 0.1% PEI. The changes in selectivity represented are between α -chymotrypsinogen and trypsinogen (\square) and between ribonuclease A and trypsinogen (\times).

relative mobility differences (α) were calculated using the equation

$$\alpha = \frac{\mu_2 - \mu_1}{\mu_{av}} \quad (1)$$

where μ_1 and μ_2 are the effective electrophoretic mobilities (electroosmotic mobility minus apparent mobility) for compounds 1 and 2 respectively and μ_{av} is the average effective electrophoretic mobility calculated as $(\mu_1 + \mu_2)/2$. Thus, a change in the sign of the value of a positive to negative or vice versa, indicates a change in migration order between the compounds 1 and 2. As can be seen, the peak order between ribonuclease A and trypsinogen did not change in the pH range 3–9 when buffers without PEI were used. However, several small changes in selectivity are observed for α -chymotrypsinogen and trypsinogen. This change of selectivity is determined by the amphoteric behaviour of these biopolymers whose global charge depends strongly on the pH of the running buffer. The peak order between cytochrome *c* and lysozyme did not change, lysozyme being the last at all the pH values tested (data not shown).

When buffers containing PEI were employed different changes in selectivity were observed. As seen in Fig. 3, the selectivity can be manipulated by using a buffer containing 0.1% PEI at different pH values. Moreover, the differences in selectivity between buffers containing PEI and buffers without PEI were more pronounced in the pH range 5.5–9.

A more systematic study of the use of PEI as a buffer additive and its effect on the selectivity of protein separation was carried out using buffers containing a different percentage of PEI. Buffers were prepared by adding small concentrations of PEI (range 0.01–0.5%, w/v) to a solution containing 50 mM acetic acid and the pH adjusted to 5.5 with 1 M NaOH. The electric current ranged from 46 μ A with 0% of PEI in buffer, to 21 μ A with 0.5% of PEI. Since PEI is a very basic compound, the quantity of NaOH needed to adjust the pH is smaller than for a buffer without PEI. Thus, lower PEI electrical conductivity compared to that from NaOH is advantageous to reduce the heating effects, as can be deduced from the smaller electrical currents obtained with the buffers containing PEI.

Fig. 4 shows the relative mobility differences measured as before, vs. the percentage of PEI in the buffer. A change in the migration order was observed for α -chymotrypsinogen and trypsinogen around 0.05% PEI. The same change was seen for ribonuclease A and trypsinogen between 0.20 and

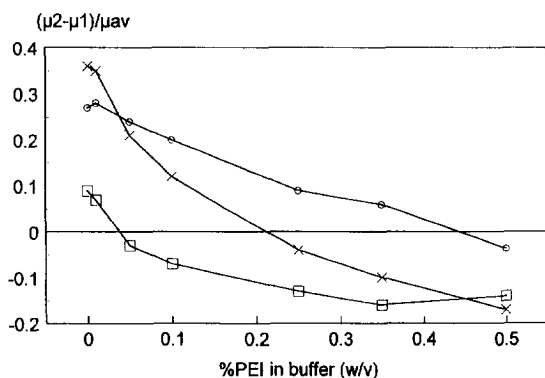


Fig. 4. Relative mobility difference against %PEI in buffer for basic proteins using a 10% PEI-coated capillary. The changes in selectivity represented are between α -chymotrypsinogen and trypsinogen (\square), between ribonuclease A and trypsinogen (\times) and between ribonuclease A and α -chymotrypsinogen (\circ). All the conditions as in Fig. 3.

0.25% PEI in the buffer. A change in the order for ribonuclease A and α -chymotrypsinogen was observed at 0.45% of PEI in the buffer.

The effect of the addition of PEI to the running buffer on the selectivity and efficiency is illustrated in Fig. 2 which shows the separation of the test basic proteins with 0%, 0.1 and 0.25% PEI in the running buffer. The efficiencies of the test proteins dropped till 20% compared to the efficiencies obtained when no PEI was present in the buffer.

The same experiments were performed with the long peptides. The optimum separation of the four peptides obtained at pH 9.75 is shown in Fig. 5. The presence of very small concentrations of PEI in the running buffer also induces selectivity changes for long peptides, as can be seen in Fig. 6. The addition of only 0.02% PEI to the running buffer causes the reversal of the migration order of the peptides WAGGDASGE and ELAGAPPEPA, and with the increasing concentration of PEI, the difference in mobilities between these two peptides increases gradually. Under the same conditions, the relative

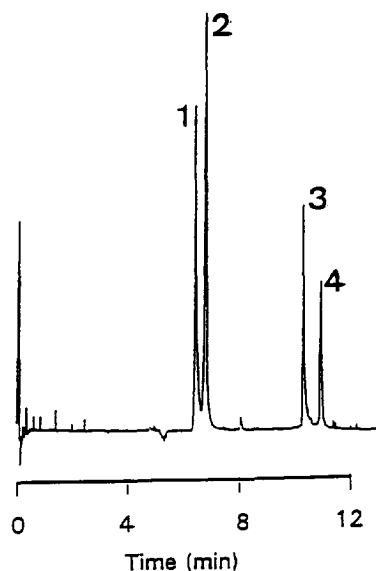


Fig. 5. Separation of long peptides in a 5% PEI-coated capillary, total length 85 cm, effective length 50 cm, I.D. 75 μ m. Buffer: 50 mM CAPS pH 9.75. Run voltage -24 kV. Injection 1×10^{-3} MPa for 6 s. Sample concentration: (1) WAGGDASGE, 0.25 mg/ml; (2) ELAGAPPEPA, 0.3 mg/ml; (3) SYSMEHPRWG, 0.25 mg/ml; (4) LQAAPALDKL, 0.4 mg/ml. UV detection at 214 nm. Modified from Ref. [9].

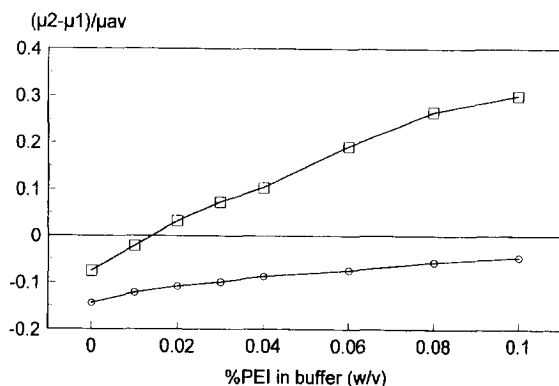


Fig. 6. Relative mobility difference against %PEI in buffer for long peptides using a 5% PEI-coated capillary. The changes in selectivity represented are between peptides WAGGDASGE and ELAGAPPEPA (□), and between SYSMHEPRWG and LQAAPALDKL (○). All the conditions as in Fig. 5.

mobility differences between the peptides SYSMHEPRWG and LQAAPALDKL decreased slightly but no reversal of the migration order occurs. The efficiency of the peptides decreases gradually with increasing PEI concentration and becomes more or less constant at about 0.1% PEI. Under these conditions, the loss in plate number is about 25% compared to the efficiency obtained with the buffer.

The approximate *pI* values of the peptides (calculated as in Ref. [20]) are: WAGGDASGE, 3.30; ELAGAPPEPA, 3.67; SYSMHEPRWG, 6.76 and LQAAPALDKL, 6.71. As can be deduced from these values, the most negative peptides (WAGGDASGE and ELAGAPPEPA) interact more strongly with PEI at the separation pH bringing about a variation of their migration order with a similar trend as observed for basic proteins.

The effect of the addition of PEI to the running buffer on the migration behaviour was also studied with tripeptides at pH 8.2 and with acidic proteins at pH 10.7. At these pH values the optimum separation of the tripeptides was obtained in the absence of PEI in the running buffer. It appears that, under these conditions, the addition of PEI to the running buffer has no influence on the selectivity of these substances.

Since the addition of PEI to the running buffer hardly changes the electroosmotic flow, it is likely that the nature of the dynamically generated PEI

layer has not changed much. Therefore, the changes in migration behaviour can be largely attributed to interactions between the solutes and PEI molecules in the solution.

The effects of the addition of PEI to the running buffer on the migration of proteins and peptides could be explained through a possible electrostatic interaction occurring between the negative groups of these biopolymers and the positive groups from PEI. This interaction could give rise to the formation of ion pairs or complexes [21] which should be responsible for the change in selectivity observed for the proteins and peptides with a higher negative charge. At very low or very high pH values the interaction would be smaller, since at low pH the number of negative groups in the proteins decreases, while the number of positive groups in PEI decreases at high pH values. Moreover, some other effects, such as hydrophobic interactions [22] and hydrogen binding, may play a role as well.

This approach can also explain the different behaviour observed for small peptides and acidic proteins, i.e., no changes in selectivity. Firstly, the short peptides at the pH used (8.2) present a very low negative character, which might be insufficient for interacting with the positive charges of PEI. Secondly, the acidic proteins did not show any change in selectivity at pH 10.7, apparently because at that pH the number of positive groups on PEI would be very small, thus diminishing the interaction between both compounds. Moreover, the possible contribution of the other effects which have been commended on before, i.e., hydrophobic interactions and/or hydrogen bonds, seems not to have an effect on the selectivity for the different peptides and acidic proteins. We are carrying out several experiments in our laboratory in order to gain more insight into this point.

4. Conclusions

We reported our preliminary study on the selectivity changes between the basic proteins and peptides by using PEI-coated capillaries and the buffers with and without PEI as an additive. Relative changes in electrophoretic mobilities of proteins and peptides vs. selected parameters, i.e., pH and the

concentration of PEI in the buffer were investigated. It appears that the concentration of PEI and the pH of the carrier electrolyte influence the migration order of basic proteins and long peptides. Thus, the use of a cationic polymer, PEI offers an alternative way to control the separation selectivity and also the resolution of proteins and peptides. However, more experiments are needed to elucidate the possible interaction mechanism.

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