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Effects of electroosmotic flow on zone mobilization in capillary isoelectric focusing

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Abstract

The electroosmotic mobilization of focused protein zones in a fused-silica capillary is investigated using a mixture of model proteins, including α-chymotrypsinogen A (bovine pancreas), myoglobin (horse heart) and carbonic anhydrase II (bovine erythrocytes). The presence of carrier ampholytes in the entire capillary and the adsorption of carrier ampholytes onto the capillary wall almost eliminate the electroosmotic flow in the fused-silica capillary, obviating the need for polymer additives such as methylcellulose and hydroxypropylmethylcellulose. In fact, the electroosmotic displacement of focused protein zones can only be achieved by injecting a mixture of proteins and ampholytes as a plug at the inlet of a capillary that has been pre-filled with the catholyte. Various approaches for protein mobilization in the uncoated capillary completely filled with carrier ampholytes are studied. The addition of methylcellulose to the sample mixture of carrier ampholytes and protein analytes serves as an anticonvective medium during the gravity mobilization step and contributes to the reduction of protein adsorption onto the capillary wall. © 1997 Elsevier Science B.V.

Keywords: Isoelectric focusing; Electroosmotic flow; Proteins

1. Introduction

Isoelectric focusing is one of the most powerful separation modes available for protein characterization. Based on the immobilized pH gradient in gel media, separations of protein analytes differing in isoelectric point (p*I*) by 0.001 pH units have been demonstrated [1]. The transfer of isoelectric focusing from slab gel to the capillary format was first achieved by Hjerten and Zhu [2]. Capillary isoelectric focusing (cIEF) combines the strengths of both conventional gel isoelectric focusing in resolving power and capillary electrophoresis in the ease and speed of separation.

To eliminate the electroosmotic flow and reduce protein adsorption onto the capillary wall, capillaries coated with methylcellulose and linear polyacrylamide were developed by Hjerten for performing cIEF [3]. Experimentally, the capillary not only contains the carrier ampholytes for the formation of a pH gradient but also the protein sample to be analyzed. In the presence of an electric field, the proteins are focused into discrete and narrow zones with local pH values corresponding to their p*I* values. To prevent the carrier ampholytes from migrating into the inlet and the outlet reservoirs by either diffusion or gradient drift [4], solutions of phosphoric acid and sodium hydroxide are generally used as the anolyte and the catholyte, respectively.

Theoretically, there is no movement in the coated capillary when the focusing is completed. Thus, the entire pH gradient, along with the focused protein zones, must be mobilized past the UV detection window. To achieve mobilization of protein zones

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for detection, Hjerten et al. [2–4] described the use of salt mobilization. For example, the anions of added salt in the cathode buffer compete with hydroxide ions for electromigration into the capillary. Since fewer hydroxide ions enter the capillary, the pH gradient drifts downwards. Protein analytes previously focused at their p*I* values become positively charged and migrate towards the cathodic end. Additionally, the contents of the capillary may be pumped or aspirated past the detection window [3]. During hydrodynamic mobilization, an electric field is applied for reducing protein band broadening due to the parabolic velocity distribution of laminar flow.

Mazzeo and Krull [5,6] illustrated the use of electroosmotic mobilization together with protein focusing in uncoated capillaries. Specifically, it has been postulated that methylcellulose, as a polymer additive, acts as a dynamic coating on the capillary wall for the reduction of protein adsorption and electroosmotic flow. The electroosmotic flow is slow enough to ensure that protein focusing is near completion before protein analytes are eluted past the detection window. The main advantage of single-step cIEF is the simplification of the separation procedures. Since fused-silica capillaries are employed for protein separation, the concern for coating stability, especially at alkaline pH, is avoided.

Instead of filling the entire capillary with a mixture of carrier ampholytes, protein sample and polymer additive, Thormann et al. [7] and Molteni and Thormann [8] described the introduction of a mixture containing proteins and ampholytes as a plug at the inlet of a capillary that had been pre-filled with the catholyte. The addition of hydroxypropylmethylcellulose to the catholyte serves to dynamically coat the fused-silica wall, thereby reducing protein adsorption and electroosmotic flow. In the presence of an electric field, the formation of a longitudinal pH gradient and the focusing of protein analytes occur within the injection plug. Simultaneously, cathodic electroosmosis sweeps the sample plug towards the point of detection.

In this study, the impact of carrier ampholytes on electroosmosis in fused-silica capillaries is investigated. The presence of carrier ampholytes alone in the entire capillary is sufficient to eliminate the electroosmotic flow, obviating the need for polymer additives, such as methylcellulose and hydroxypropylmethylcellulose. In fact, the electroosmotic zone displacement of focused proteins can only be achieved by injecting a mixture of proteins and ampholytes as a plug at the inlet of a capillary that has been pre-filled with catholyte [7,8]. Various approaches are studied for mobilizing proteins in an uncoated capillary that has been filled completely with carrier ampholytes. The effects of electroosmotic flow on protein mobilization in cIEF are discussed using a mixture of model proteins, including α-chymotrypsinogen A (bovine pancreas), myoglobin (horse heart) and carbonic anhydrase II (bovine erythrocytes).

2. Experimental

The cIEF apparatus was constructed in-house using a CZE 1000R high-voltage power supply (Spellman High-Voltage Electronics, Plainview, NY, USA). Fused-silica capillaries of various dimensions were obtained from Polymicro Technologies (Phoenix, AZ, USA). Procedures for preparing capillaries coated with linear polyacrylamide have been described elsewhere [9]. A constant electric field of 500 V/cm was employed for both the focusing and the mobilization steps. The protein zones were monitored by an UV detector (Linear Instruments, Reno, NV, USA) at 280 nm. Data collection was performed using a HP 35900D analog-to-digital interface board with HP G1250C General Purpose Chemstation Software (Hewlett-Packard, Fullerton, CA, USA).

Methylcellulose and standard proteins including α-chymotrypsinogen A (bovine pancreas, p*I* 9.1), myoglobin (horse heart, p*I* values of 7.2 and 6.8) and carbonic anhydrase II (bovine erythrocytes, p*I* 5.9) were obtained from Sigma (St. Louis, MO, USA). Carrier ampholytes of pharmalyte 3–10 were purchased from Pharmacia (Uppsala, Sweden). All chemicals, including phosphoric acid, sodium hydroxide and sodium phosphate, were purchased from Fisher (Fair Lawn, NJ, USA). All solutions were filtered through a 1-μm filter (Whatman, Maidstone, UK).

3. Results and discussion

To investigate electroosmotic zone displacement

in the cIEF capillary, experimental conditions identical to those of Mazzeo and Krull [5] were employed for the separation of model proteins, including α-chymotrypsinogen A, myoglobin and carbonic anhydrase II. The first group of protein peaks shown in Fig. 1, similar to those observed by Mazzeo and Krull [5], was caused by the movement of nascent protein zones past the detection point. Nascent protein zones were generated at both ends of the protein–ampholyte mixture, and eventually merged at their p*I* values in the capillary. Additionally, a drop in the absorbance baseline was noticed after the

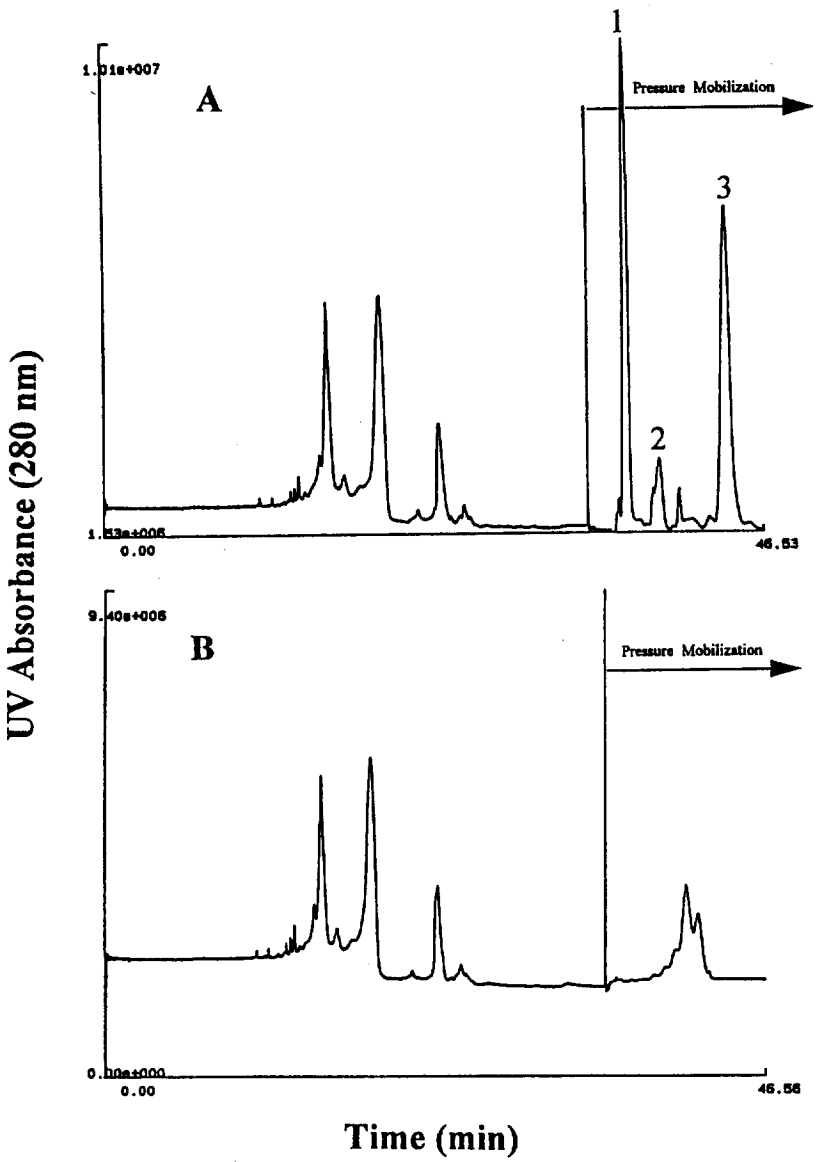

Fig. 1. cIEF separation of model proteins in a fused-silica capillary. Capillary: 60 cm (40 cm from the anodic end to the detection window)×50 μm I.D.×375 μm O.D.. cIEF conditions: Anolyte, 20 m*M* phosphoric acid; catholyte, 20 m*M* sodium hydroxide: voltage, 30 kV; sample, 1 mg/ml for each protein, 5% pharmalyte 3–10, 0.1% methylcellulose; UV detection at 280 nm. (A) Pressure mobilization of (1) myoglobin, p*I*=7.2; (2) myoglobin, p*I*=6.8 and (3) carbonic anhydrase II, p*I*=5.9 at the anodic end. (B) pressure mobilization of α-chymotrypsinogen A, p*I*=9.1 at the cathodic end.

prefocused protein zones passed the detection window. When analysis was initiated, the cIEF apparatus zeroed the detector baseline while proteins and ampholytes were in the light path of the detection point. After the movement of prefocused protein zones, more UV light reached the photomultiplier and the baseline decreased [10].

A constant electric field of 500 V/cm was applied during the first 35 min of the cIEF separation. The electric field was then turned off and a hydrodynamic flow was generated by connecting a nitrogen pressure of 1 p.s.i. at either the anodic or the cathodic ends of the cIEF capillary (1 p.s.i.=6894.76 Pa). Pressure mobilization of focused protein zones (the second group of protein peaks) is shown in Fig. 1A–B for α-chymotrypsinogen A, myoglobin and carbonic anhydrase II. As shown in Fig. 1B, the basic protein of α-chymotrypsinogen A was focused past the detection window and was brought back to the detection point by applying a low pressure in the outlet reservoir, the cathodic end.

The external polyimide coating on a 25-cm section of fused-silica capillary was removed using hot sulfuric acid for monitoring the migration of myoglobin bands during the cIEF protein separation. Two myoglobin bands, at both the anodic and the cathodic sides of the capillary, were formed shortly after the application of a constant electric field of 500 V/cm. These two myoglobin bands migrated towards the same position in the capillary, where the local pH was equal to myoglobin's p*I* value. The emergence of myoglobin bands occurred 10 min after initiation of the analysis and resulted in a 4-mm wide protein zone (see Fig. 2A). As shown in Fig. 2B, residual electroosmosis for the mobilization of the myoglobin zone was either very slow or was close to zero. It took more than 100 min for the electroosmotic displacement of the myoglobin zone over a 1.2-cm distance towards the cathodic end.

The results shown in Figs. 1 and 2 clearly indicated the absence of electroosmotic mobilization of focused protein zones in the fused-silica capillary filled with carrier ampholytes and methylcellulose. In fact, the electroosmotic flow in the fused-silica capillary was already close to zero on filling the capillary with a solution of only 5% carrier ampholytes (data not shown). Thus, the adsorption of carrier ampholytes onto the capillary wall might be sufficient to eliminate the electroosmotic flow in the cIEF capillary.

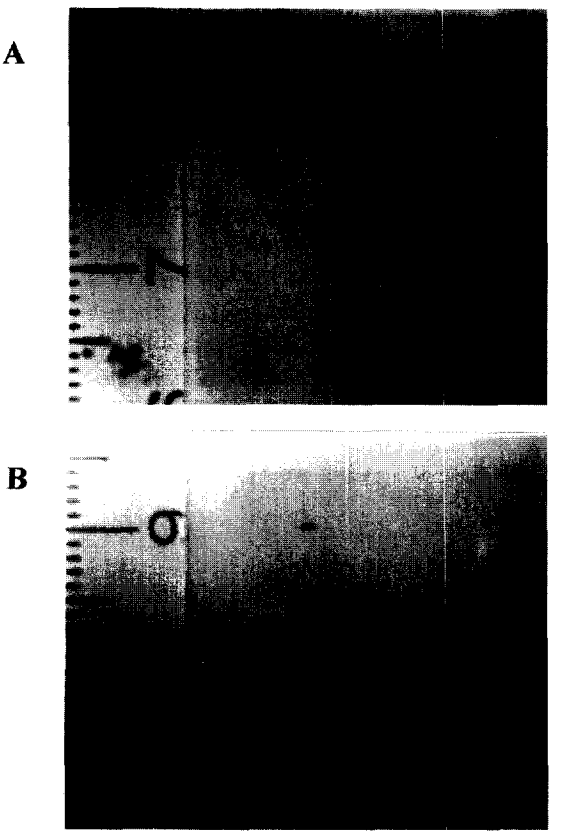

Fig. 2. Myoglobin band at (A) 10 min and (B) 120 min after the initiation of the cIEF separation. Myoglobin, at a concentration of 3 mg/ml, was dissolved in 5% pharmalyte 3–10 and 0.1% methylcellulose. Other conditions were the same as in Fig. 1.

On the other hand, the electroosmotic displacement of focused protein zones in the fused-silica capillary was observed (data not shown) by following the approach described by Thormann et al. [7] and Molteni and Thormann [8]. The sample containing carrier ampholytes and protein analytes only occupied a portion of the capillary, while the rest of the capillary was filled with the catholyte. The electroosmotic flow, originated from the section of the capillary filled with the catholyte, was responsible for the mobilization of the sample plug in the cIEF capillary. Despite the simplicity of this single-step cIEF approach, the reduced detection signal and the generally low separation resolution are attributed

to the limited amount of protein analytes and a steep pH gradient in the sample plug [10].

In the absence of an electric field, the focused protein zones in the fused-silica capillary were mobilized past the detection window by applying a pressure at either the cathodic or the anodic end of the capillary (see Fig. 1). For the coated cIEF capillaries, an electric field was applied during hydrodynamic mobilization for reducing protein band broadening [3]. However, the combination of an electric field with pressure mobilization failed to mobilize the focused protein zones in the fused-silica capillary. As shown in Fig. 3A, the hydrodynamic introduction of the anolyte (20 m*M* phosphoric acid) into the capillary acidified the adsorbed ampholytes near the anodic end. Consequently, the electroosmotic flow generated from the positively charged ampholytes under the influence of an electric field was in the direction of the anodic end and counteracted hydrodynamic mobilization.

The use of cathodic mobilization was also investigated to mobilize the focused protein zones for detection in the fused-silica capillary. The electric field was turned off 15 min after the initiation of the cIEF separation. The sodium hydroxide catholyte was then replaced by a solution of 20 m*M* phosphoric acid. The electric field was turned on again, but failed to mobilize the focused protein zones past the detection point. As shown in Fig. 3B, fewer hydroxide ions entered the capillary and the pH gradient drifted downwards. Protein analytes previously focused at their p*I* values became positively charged and migrated towards the cathodic end. However, the direction of electroosmotic flow induced by the positively charged ampholytes (adsorbed onto the capillary wall) was opposite to that of electrophoretic migration of protein analytes.

Furthermore, the effect of methylcellulose on the cIEF protein separation was studied using both coated and uncoated capillaries. Gravity mobilization of focused protein zones was employed in both the coated and the uncoated capillaries. To induce gravity mobilization, the anodic reservoir was raised 6 cm above the cathodic reservoir. No electric field was applied during the gravity mobilization step. As shown in Fig. 4, the presence of 0.1% methylcellulose increased the migration times of myoglobin and carbonic anhydrase II in the cIEF capillaries, due to the increasing viscosity of the solution. Significant reduction in the peak height of focused proteins was observed in the absence of a polyacrylamide coating and 0.1% methylcellulose (see Fig. 4A).

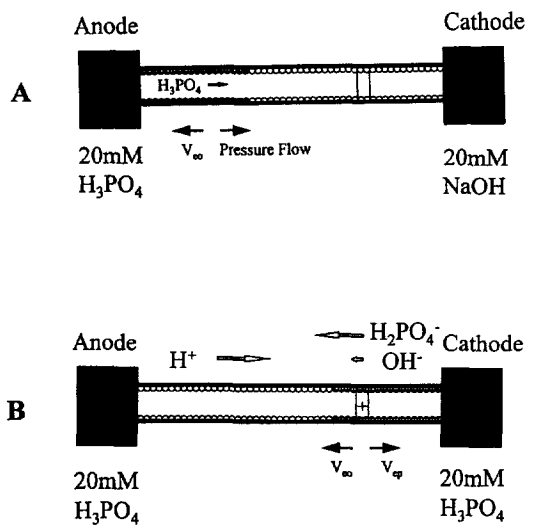

Fig. 3. Schematic diagrams of electroosmotic flow induced by positively charged carrier ampholytes adsorbed onto the capillary wall under the influence of (A) hydrodynamic mobilization and (B) cathodic mobilization.

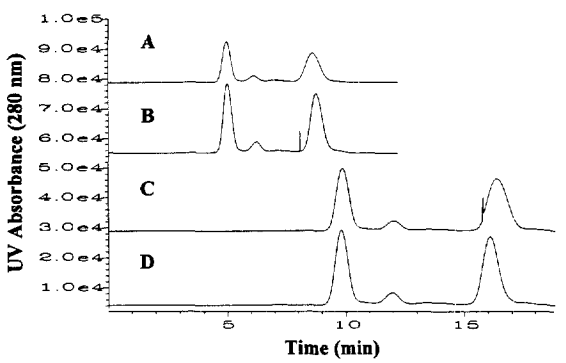

Fig. 4. cIEF separation of model proteins. Capillary: 30 cm (24 cm from the anodic end to the detection window)×50 μm I.D.× 192 μm O.D.; cIEF conditions: Anolyte, 20 m*M* phosphoric acid; catholyte, 20 m*M* sodium hydroxide; voltage, 15 kV. Migration order: Myoglobin (p*I*=7.2), myoglobin (p*I*=6.8) and carbonic anhydrase II (p*I* 5.9). Sample: 1 mg/ml each for myoglobin and carbonic anhydrase II, 2% pharmalyte 3–10; UV detection at 280 nm. (A) uncoated capillary, (B) polyacrylamide-coated capillary; (C) uncoated capillary with 0.1% methylcellulose and (D) polyacrylamide-coated capillary with 0.1% methylcellulose.

Table 1
Effect of methylcellulose on cIEF protein separation[a]

cIEF conditions	Resolution[b] (Myoglobin/carbonic anhydrase)	Peak height (%)[c]	
		Myoglobin	Carbonic anhydrase
Coated capillary, 0.1% methylcellulose	9.0	100	100
Coated capillary	7.7	95.8	86.4
Uncoated capillary, 0.1% methylcellulose	7.6	87.5	77.3
Uncoated capillary	6.7	54.2	41.0

[a]The experimental error in measuring the values of separation resolution and peak height was about 4–8% for various cIEF conditions for over five runs.
[b]Resolution was calculated using $R=2(t_A-t_B)/(W_A+W_B)$.
[c]The peak heights of myoglobin and carbonic anhydrase measured by UV absorbance at 280 nm were set at 100%, using the polyacrylamide-coated capillary in the presence of 0.1% methylcellulose.

The quantitative dependence of methylcellulose on the separation resolution and the peak height of focused proteins in cIEF is summarized in Table 1. The best cIEF protein separation was achieved using a polyacrylamide-coated capillary in the presence of 0.1% methylcellulose. In comparison with the best cIEF protein separation, a 26% loss in the separation resolution and a 46% reduction in the UV absorbance intensity were measured in the fused-silica capillary without the addition of 0.1% methylcellulose. Clearly, the presence of methylcellulose served as an anticonvective medium during the gravity mobilization step and contributed to the reduction of protein adsorption onto the capillary wall.

4. Conclusions

The presence of carrier ampholytes in the entire cIEF capillary and the adsorption of carrier ampholytes onto the capillary wall reduced the electroosmotic flow to a value close to zero. The mobilization of focused protein zones in the fused-silica capillary was established by introducing a gravity- or pressure-induced hydrodynamic flow in the absence of an electric field. The addition of methylcellulose significantly enhanced the separation resolution and the peak height of model proteins, due to the reduction of diffusion band broadening and protein adsorption onto the fused-silica capillary. Finally, the true electroosmotic displacement of focused protein zones in the uncoated capillary could only be achieved by injecting a mixture of proteins and ampholytes as a plug at the inlet of a capillary that had been pre-filled with the catholyte.

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