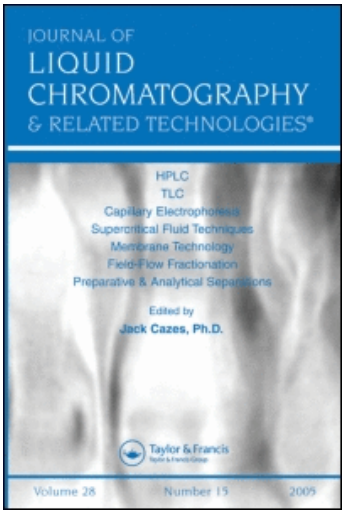

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SEPARATION OF PROTEINS BY CAPILLARY ELECTROPHORESIS USING AN EPOXY BASED HYDROPHILIC COATING

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ABSTRACT

Protein adsorption onto the inner walls of capillaries is a widely recognized problem in the separation of proteins by capillary electrophoresis. Chemical modification of the wall has been an effective method for suppressing this effect. The performance of an epoxy based hydrophilic coating has been evaluated under different experimental conditions. High efficiency separation of proteins (up to 200,000 theoretical plates per meter) was achieved using this coating. The recoveries of both cationic and anionic proteins were very high (over 90% for several positively charged proteins) on this capillary coating. The influence of both pH and potential on efficiency, resolution and peak capacity were examined.

Separation of model proteins at various pH values indicated that this epoxy coating was well suited for protein separations over the range of pH 4-10. Applications using this coating for the separation of specific industrial proteins are also presented.

INTRODUCTION

Capillary electrophoresis (CE) has proven to be one of the best techniques available for the separation of proteins.[1-2] Millions of theoretical plates can be achieved when extremely high voltage is applied.[3] However, CE separation of protein is often complicated by the interaction between the analytes and the silanol groups on the inner capillary surface. This interaction often results in broad asymmetrical peaks, poor efficiency, altered electro-osmotic flow, reduced protein recovery, and poor reproducibility. Various attempts have been reported to solve this problem. Some of those approaches focused on modifying the buffer by using extreme pH,[4] high ionic strength,[5] zwitterions,[6-7] additives,[8-9] or surfactants.[10-12] Other attempts focused on modifying the surface of the capillary with coatings.[13-25] Capillary coating has the advantage of allowing the analysts to freely modify the composition of the buffer to optimize the separation.

Different methods have been developed to make various coatings. The simplest one is to coat the surface dynamically by adsorption of polymeric materials directly onto the inner surface of the capillaries.[10,13,17] However. these coatings suffered from poor stability. An alternative is to add cross-linker along with the surface derivatization reagent to stabilize the surface.[21] Another approach is to attach simple organosilanes to cap the silanol groups on the surface.[4] However, this approach also experienced poor stability and the capillaries deteriorated rapidly due to the reversible reaction. A third approach is to use a Grignard reaction for coupling organic moieties to the silica surface through a C-Si bond.[17] A stable surface is created, but the reaction process is difficult to carry out. Some other new approaches, such as self-assembled bilayer coating[26] and living polymerization for capillary coating[27] have also been reported.

However, the most common approach is still to derivatize the surface first with a derivatization reagent, which has a secondary functional group to participate in the next step reaction.[13-16] A polymerization reaction creates a cross-linked surface. Using a similar approach, we previously reported the synthesis of a covalently bounded epoxy based hydrophilic coating.[19] The performance of this coating is further evaluated for the separation of both model proteins and industrial research proteins at various conditions. This study presents the results of these evaluations.

EXPERIMENTAL

Apparatus

CE experiments were carried out using two different instruments. One was built in-house without a temperature control system. Most of the testing for

model proteins were performed on this system. The other one was a fully automated Beckman 2000 system (Beckman, Pola Alto, CA). Components of the in-house CE system were contained in a Lucite cabinet fitted with a safety interlock that would interrupt electrical power to the instrument when the cabinet door was opened. A Spellman Model FHR 30P 60/EI (Spellman High Voltage Electronics Corp., Plainview, New York) power supply was used with the 22 gauge platinum electrodes immersed in 3 mL buffer reservoirs.

The fused silica capillaries (Polymicro Technologies, Phoenix, AZ) of 50-75 mm ID and 360 mm OD were used for coating. Detection was achieved with a variable wavelength UV detector (Model V4 Isco Inc., Lincoln, NE), that was linked to a Linear 2000 (Linear, Reno, Nevada) strip chart recorders.

Reagents

Model protein samples were purchased from Sigma Chemical Co. (St. Louis, MO). Prepro-rhBMP-2 (BMP) and IL-2 were proprietary recombinant proteins of Genetics Institute (Cambridge, MA). Ethyleneglycol diglycidyl ether (EGDE), g-glycidoxypropyltrimethoxysilane (GOX), 1,4-diazabicyclo-[2.2.2]-octane (DABCO), mesityl oxide (MO), and other solvents and buffer reagents were obtained from Aldrich (Milwaukee, WI). Sodium phosphate (analytical reagent) was purchased from Mallinckrodt, Inc. (Paris, KY) and sodium acetate was from MCB Manufacturing Chemists, Inc. (Cincinnati, OH). Buffers were prepared with double distilled and deionized water.

Capillary Coating

The procedures for preparing this coating were modified based on the procedures reported earlier.[19] Basically, the fused silica capillaries were activated with 1.0 M NaOH solution for 10-20 min and then washed with dilute HCl and water for another 20 min each. The washed capillaries were then heated in an oven for 3 hours at 120°C with N_2 slowly passing through. A GOX solution in CH_2Cl_2 was pushed into the pretreated capillary and heated for 3 hours. Next, a solution of EGDE and DABCO was forced through the column and allowed to react at 120°C for 3 hours. The capillary was washed with methanol after the reaction.

Electrophoresis

Samples were injected by either siphoning 2 - 4 s (on in-house system) or by pressure (on Beckman CE). Most of the separations were run using a low ionic strength buffer, such as 10 mM phosphate, at neutral pH to evaluate the effec-

tiveness of the coating. Acetate (pH 3 to 5), phosphate (pH 6 to 8), and diaminopropane (pH 9 to 11) were also used as buffers over the range indicated. The ionic strength of these buffers was adjusted to approximately the same level as that of the 10 mM phosphate buffer at pH 7. Operating current was generally controlled within 15 to 40 mA.

RESULTS AND DISCUSSION

The separation of proteins in CE proves to be particularly challenging. Proteins, especially those with large hydrophobic regions and positively charged, tend to adsorb onto the capillary surface and create numerous separation problems. Wall coating has been an effective way of suppressing this effect.[13-27] It is hypothesized that a neutral and hydrophilic capillary surface will eliminate protein adsorption.[13-16,19] For example, epoxy coating is a hydrophilic coating due to the high content of ether and hydroxyl groups and is expected to reduce protein adsorption. It is also expected that significant amount of electro-osmotic flow (EOF) will remain with this coating. This EOF can sweep both cations and anions to the detector and allow their analysis in a single run. In addition, this coating should be reasonably stable. Separations of protein samples at various experimental conditions have confirmed the above expectations.

Separation of Model Proteins at Neutral pH

Separation of Positively Charged Proteins

Several model proteins were evaluated in a 10 - 50 mM phosphate buffer near pH 7 using an epoxy coating. The model proteins were lysozyme (pI 11), cytochrome C (pI 10.2), ribonuclease-A (pI 9.3), a-chymotrypsinogen (pI 8.8), trypsinogen (pI 8.7), a-chymotrypsin (pI 8.4, 8.8) and myoglobin (horse heart, pI 7.3). They are all positively charged at pH 7 and have high tendency to adsorb onto the negatively charged walls of uncoated capillaries. But, a good separation of lysozyme, cytochrome C, ribonuclease A, a-chymotrypsinogen A and myoglobin (horse heart) was achieved on the epoxy treated surface (Figure 1a).

All five proteins were baseline resolved with the theoretical plate number as high as 200,000 per meter (for myoglobin). On the other hand, very poor separation was seen with the same sample on an uncoated capillary (Figure 1b).

Separation of Both Positively and Negatively Charged Proteins

The epoxy coating retains about 1/3 of the EOF of an uncoated capillary at pH 7. This EOF is critical for the separation of samples containing negatively

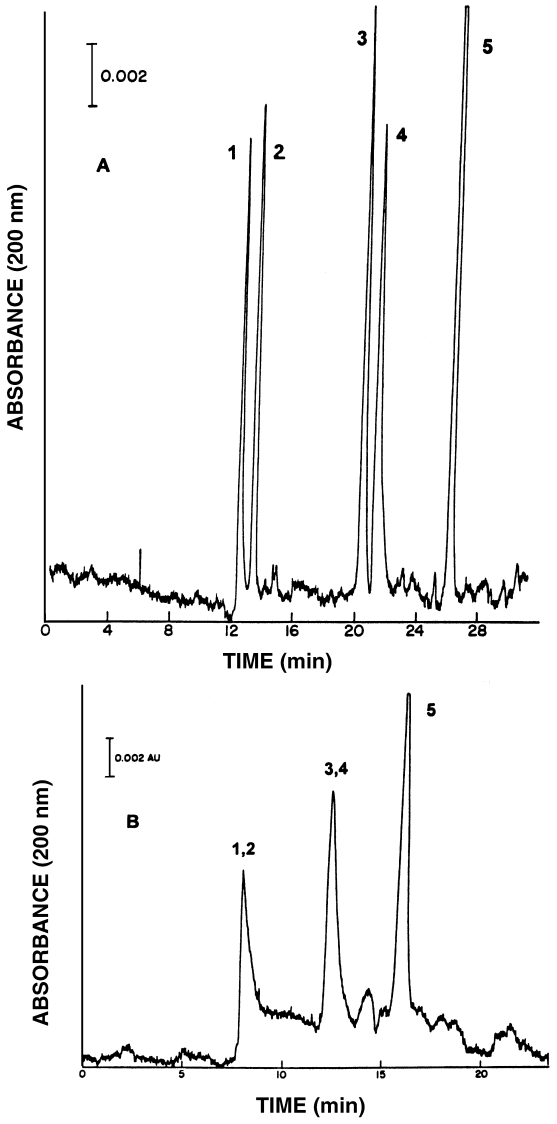

Figure 1. Capillary electrophoretic separation of five basic model proteins in (a) an epoxy coated capillary and (b) an uncoated capillary. Experimental conditions: 65 cm separation length, ID/OD 50/363 μm, 0.01 M phosphate buffer, pH 7, 300 V/cm, 17 μA. Peaks: 1) lysozyme, 2) cytochrome C, 3) ribonuclease A, 4) a-chymotrypsonagen, and 5) myoglobin (horse heart).

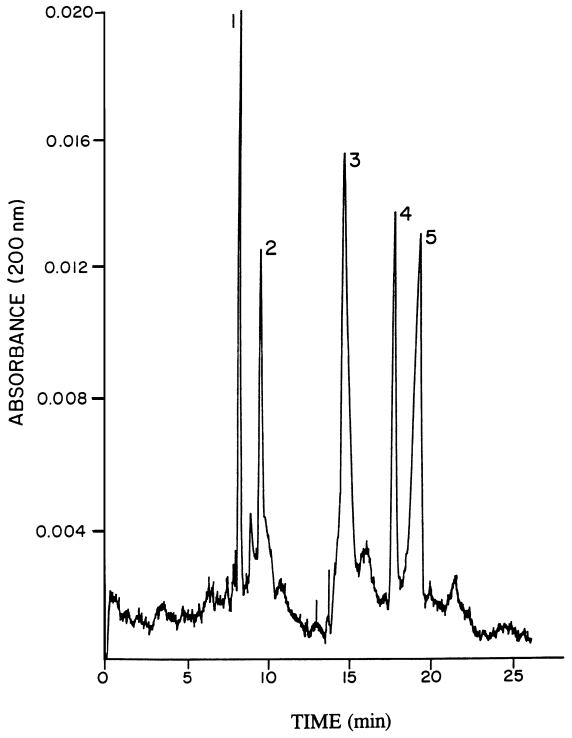

Figure 2. Electrophoregram showing the separation of five proteins with mid pI values. Experiment conditions: 60 cm epoxy coated capillary (ID/OD 50/363) with a separation length of 42 cm; 0.01 M phosphate buffer, pH 7; 300 V/cm. Peaks: 1) trypsinogen, 2) myoglobin (whale), 3) conalbumin, 4) carbonic anhydrase, 5) a-amylase.

Table 1

Run to Run Reproducibility of Migration Times (min) for Proteins at pH 7*

Proteins:	Lysozyme	Cytochrome C	Ribonuclease A	α-Chymotrypsinogen A	Myoglobon
Average	12.44	13.28	20.36	21.26	26.34
SD	0.167	0.164	0.167	0.152	0.134
R.S.D. (%)	1.35	1.24	0.82	0.71	0.51

* n = 5.

charged proteins. Figure 2 shows the separation of a-chymotrypsin, myoglobin (whale, pI 6.9), conalbumin (pI 6.3), carbonic anhydrase (pI 6.1) and a-amylase (pI 5.9) at pH 7. This electrophoregram indicates that both anionic and cationic proteins can be separated and detected in a single run at pH 7 due to the residual EOF.

Reproducibility of the Coating

The reproducibility of protein separation from run to run, day to day, segment to segment, column to column, and chemist to chemist is very high. From run to run, the %RSD (n=5) of migration times for neutral marker (MO) and lysozyme are 0.94% and 0.71%, respectively (Table I). The % RSD in EOF for day to day (n =5) and segment to segment (n=6) are 2% and 3.5%, respectively. From column to column, EOF at pH 7 varied from $0.5x10^{-4}$ cm^2/VS to 2 $x10^{-4}$ cm^2/VS with an average of 1.14 $x10^{-4}$ cm^2/VS for 9 columns coated over a 3 month period. Several other researchers also prepared this coating and obtained similar results.

Separation of Model Proteins at Other pH

One of the major advantages of coating is to allow one to freely adjust the pH to achieve the best separation. Coating reduces the effect of pH on EOF, electrophoretic mobility and protein separation. From pH 3 to 11, the EOF in the coated capillary had less than half (from 0 to 3 x 10^{-4} cm^2/VS) the variation as seen in the uncoated capillary (from 1 to 8 $x10^{-4}$ cm^2/VS).

The five positively charged model proteins shown in Figure 1 were also separated at several other pHs (pH 4-10). However, the migration times of these individual proteins did vary with pH (Figure 3a). Because the electrophoretic mobility of these proteins changes with pH (Figure 3b), the net effect of pH on both EOF and electrophoretic mobility provides a basis for optimizing pH for the separation of these proteins.

Table 2 lists the theoretical plate number, average resolution, and peak capacity for ribonuclease A and myoglobin in the separation of these proteins at different pHs. The maximal theoretical plate number was found at pH 7 for both proteins. Since the separation window between lysozyme and myoglobin increased with pH but the peak widths did not change significantly, peak capacity also increased with pH. From the electropherograms (data not shown), the resolutions between two pairs of proteins, ribonuclease A and a-chymotrypsinogen, and a-chymotrypsinogen and myoglobin, increased with pH. However, the resolutions between lysozyme and cytochrome C increased slightly from pH 5 to 7, dropped to zero at pH 8 (no separation at all), and then increased at higher pH.

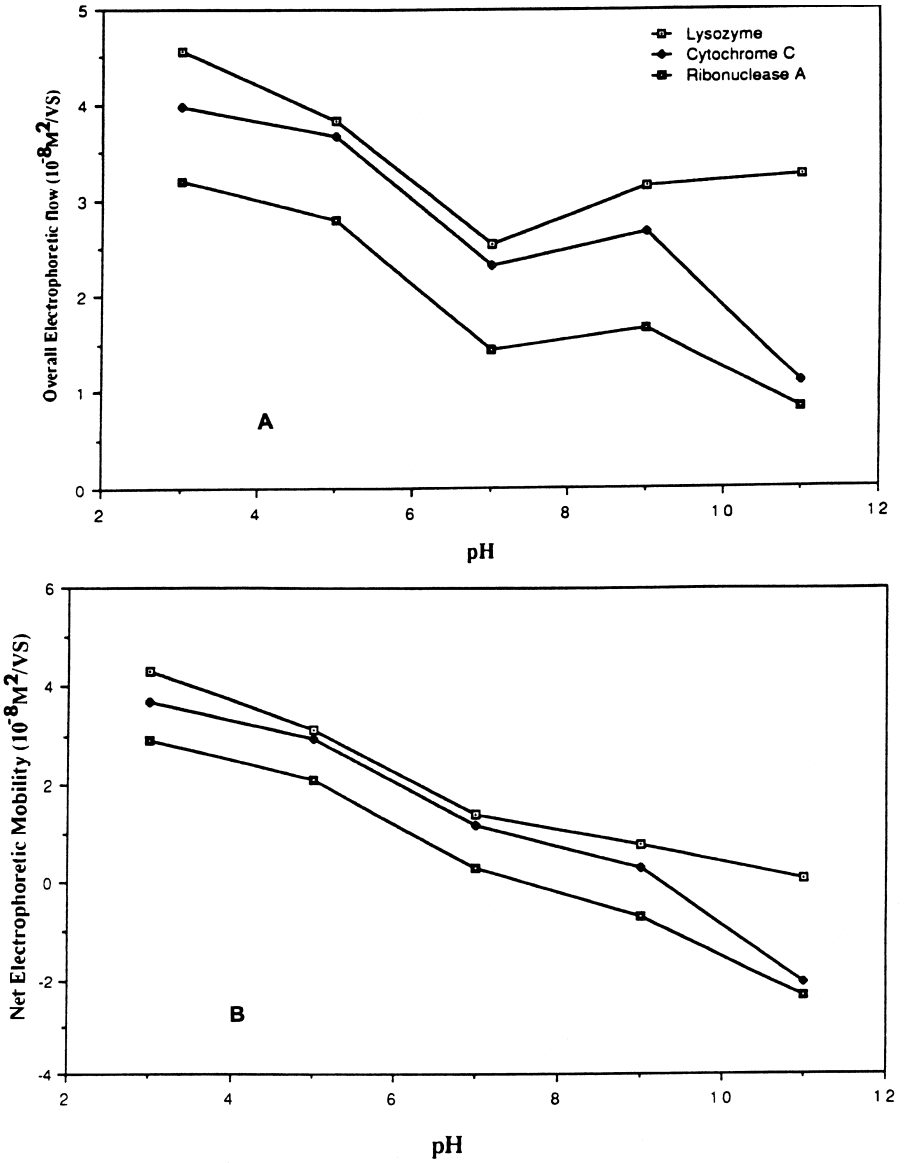

Figure 3. The overall (a) and the net (b) mobilities of proteins in the epoxy coated capillaries at different pHs. The net mobility is the mobility after subtracting EOF from the overall mobility.

Table 2

Effect of pH on Separation Parameters

pH	Theoretical Plate Number[a] Ribonuclease A	Myoglobon	Average Resolution R_{ave}	Peak Capacity Number[b]
5	9,200	2,200	2.31	7.4
6	15,000	18,800	3.81	15.1
7	18,400	36,500	4.87	19.1
8	11,500	28,600	5.69	23.3
9	7,700	18,400	6.73	28.9
10	3,200	13,000	6.06	24.5

[a] $N = 5.54 \times (t/w_{1/2})^2$. [b] Peak capacity number is calculated from the window between lysozyme and myoglobin.

The average resolution listed in Table 2 is the average of the resolutions between the four pairs of consecutive peaks. The average resolution is used to represent the overall effect of pH on resolution. It seems that the best resolution is obtained at pH 9. For ribonuclease A and myoglobin, the highest theoretical plates are obtained near pH 7-8.

The major challenge to capillary coatings, especially hydrophilic coatings, is stability. Adding a hydrophobic moiety into the coating may increase coating stability but interferes with protein separations. Epoxy coating provides a balance between hydrophilicity and stability. Epoxy is well known for its chemical and mechanical stability. The epoxy was covalently bound to the silica surface and was cross-linked to generate a stable surface.

This coating is suitable for protein separations at pH 4 - 10. This column can also be stored at room temperature for several months without a stability problem.

Potential Effects on Protein Separation

A linear relationship is expected between the reciprocal of the elution time and the applied potential,[31] i.e. $1/t = (m/lL)*V$, where t is the migration time, m is the mobility of the analyte, l is the separation length, L is the total length, and V is the applied potential. The existence of such a relationship is a good indication that the proteins are freely moving inside the coated capillary (Figure 4a). In general, peak capacity (Figure 4b) increases while resolution decreases with potential increase.

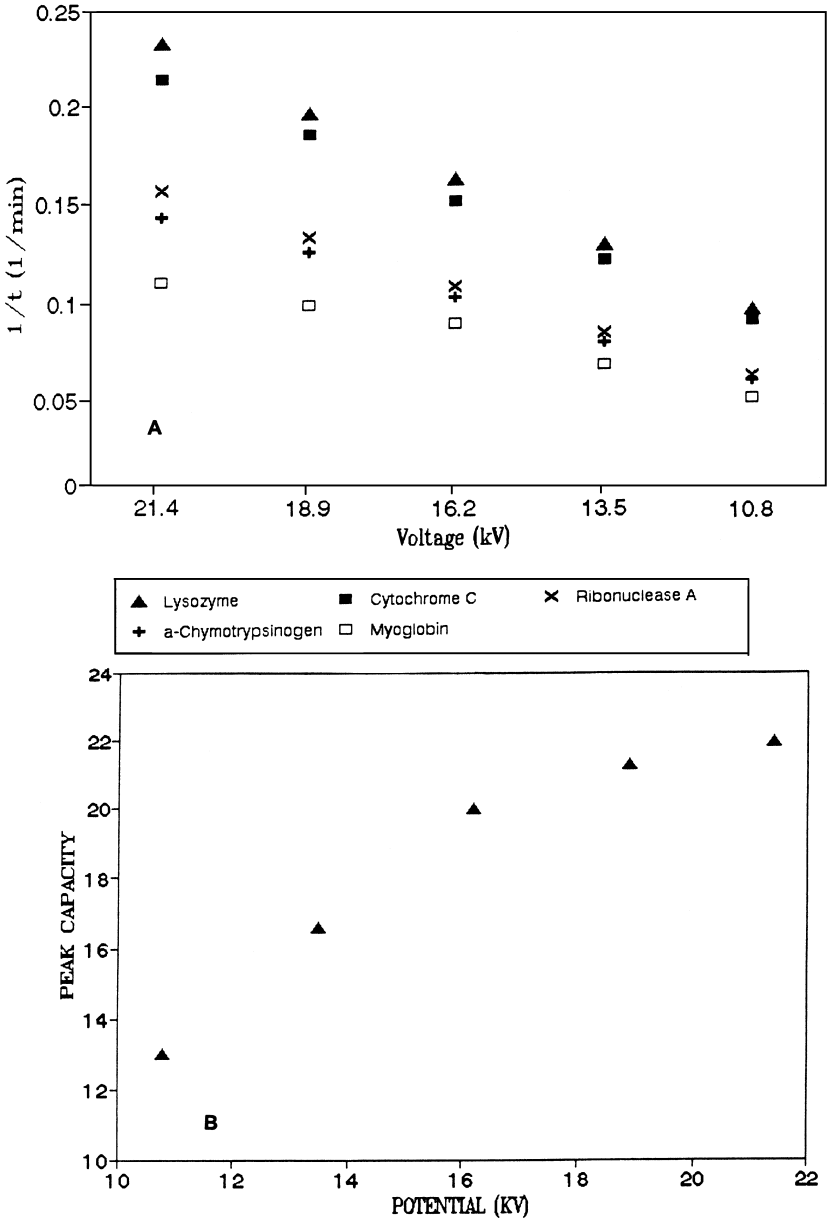

Figure 4. Effect of potential on (a) the retention time and (b) peak capacity.

Table 3

Effect of Potential on Theoretical Plate Number of Proteins[a]

Potential (kV)	Lysozyme	Cytochrome C	Ribonuclease A	α-Chymotrypsinogen A	Myoglobin (Whale)
20.4	24,000	31,000	46,000	31,000	105,000
18.9	36,000	34,000	53,000	32,000	112,000
16.2	55,000	39,000	59,000	36,000	129,000
13.5	57,000	44,000	70,000	45,000	137,000

[a] Data were obtained when protein was injected individually. Experimental conditions: pH 7, 0.01 M phosphate buffer, injection to detection 35 cm.

Table 4

Comparison of the Separation Length in Protein Separation

Proteins	Theoretical Plate Number 20 cm	65 cm		Resolution[a] 20 cm	65 cm
Lysozyme	23,400	61,500	R1	1.75	3.85
Cytochrome C	30,300	70,800	R2	12.73	26.97
Ribonuclease A	59,500	77,100	R3	2.08	4.29
α-Chymotrypsinogen	68,500	155,100	R4	17.59	41.03
Myoblobin (horse)	97,300	162,500			

[a] R1, R2, R3, and R4 are the resolutions between lysozyme, cytochrome C, ribonuclease A, α-chymotrypsinogen A and myoglobin, respectively.

The potential effect on the theoretical plate number is much more complicated and difficult to predict. For example, theory predicts more theoretical plates at high potential. In practice, Joule heating at high potential affects the viscosity and causes bubble formation in the buffer solution. Thus, the experimental theoretical plate number was always much lower than the theoretically predicted values.14,15 The values in Table 3 indicate that theoretical plate number actually dropped at higher potentials. This result is in agreement with the literature.[16]

The efficiency of separation in CE should be directly proportional to the separation length. Results obtained by locating two detectors at 20 and 65 cm from the injection end show that longer separation length gave much better separation (Figure 5a-b). When compared with the results from 20-cm separation length, much higher than expected separation efficiency was obtained from the 65-cm separation length (Table 4).

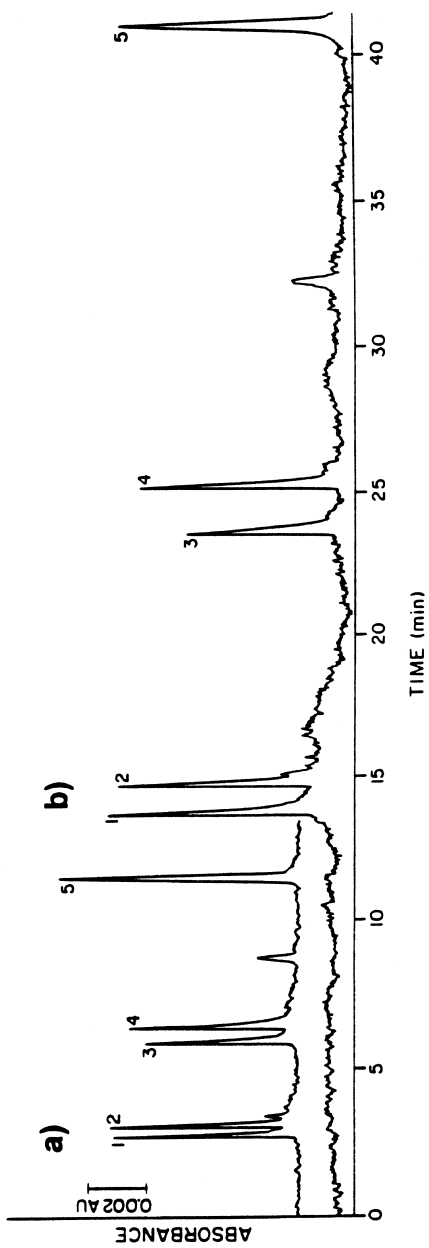

Figure 5. Electrophoregrams showing the separation of five basic proteins on a double detector system. Experiment conditions: 85-cm epoxy coated capillary with separation lenghts at a) 20 and b) 65 cm, respectively; 10 mM phosphate buffer, 300V/cm, 17 μA.

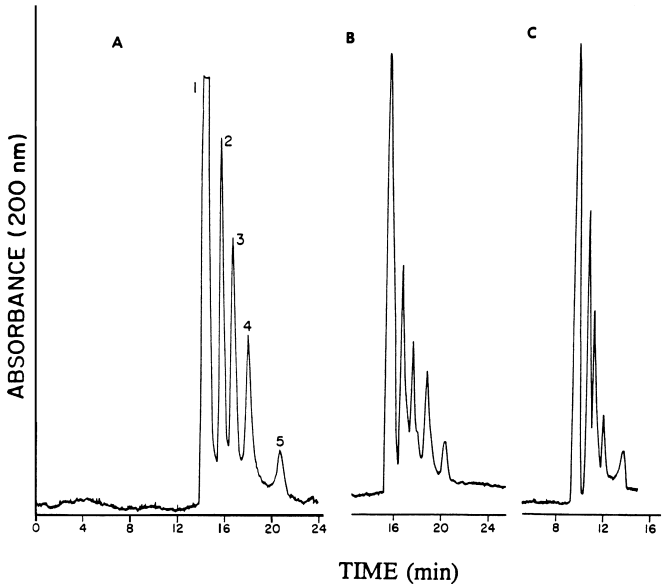

Figure 6. The electrophoregrams of cytochrome C variants at different pHs. 1. horse+dog, 2. chicken, 3. pig, 4. tuna, 5. impurity. A. pH 8, B. pH 7, C. pH 6. Experimental conditions: 50 cm 75 mm ID epoxy coated capillary with 35.5 cm separation length. 250 V/cm, 25 mA, 0.01 M phosphate buffer.

Table 5

Percentage Recovery of Protein from an Epoxy Coated Capillary

Proteins:	Lysozyme	Cytochrome C	Ribonuclease A	α-Chymotrypsinogen A	Myoglobin
% Recovery	55.5	84.4	95.0	88.7	93.7

Therefore, a longer capillary should be used when better resolution and higher efficiency are desired. However, the highest potential available on most instruments is limited to 30 KV. Increasing capillary length may lead to reduced electrical field strength across the capillary and reduce the separation efficiency. In addition, experimental data has shown that no significant improvement in separation efficiency was achieved when the capillary was increased to over 1 meter.[17]

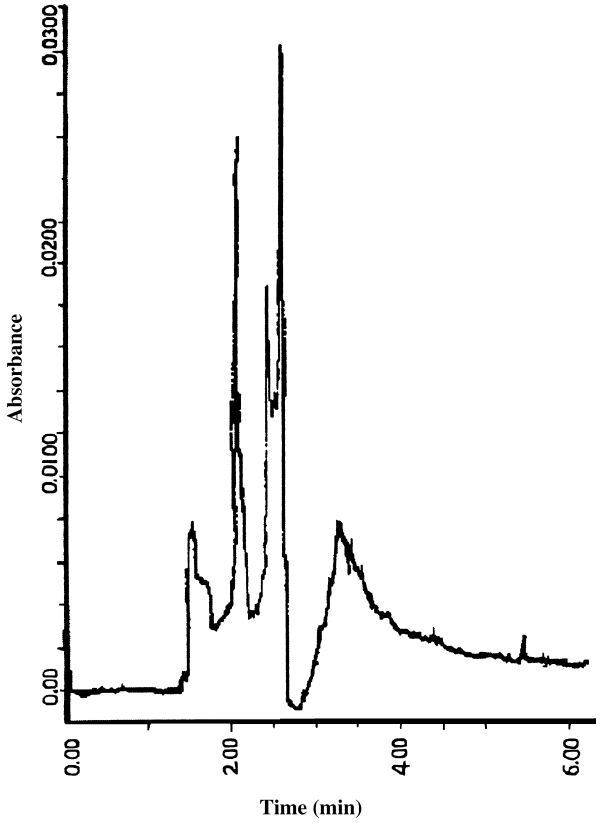

Figure 7. Separation of recombinant protein BMP-2. Experimental conditions: 47 cm 50 mm ID epoxy coated capillary with 39.5 cm separation length, 10 mM acetate buffer with 5 mM SDS, pH 4.5 at 18.6 kV, Beckman CE instrument.

On the other hand, a shorter capillary is preferred when separation time is a major concern. For example, a reasonable separation of the five model proteins was achieved within 12 minutes using a 20 cm effective length capillary (Figure 5a).

Protein Recoveries

The adsorption of positive proteins on to the epoxy capillary surface was quantitatively evaluated using two on-line detectors. Zero percent recoveries were reported on an uncoated capillary at pH 7 for lysozyme, cytochrome C,

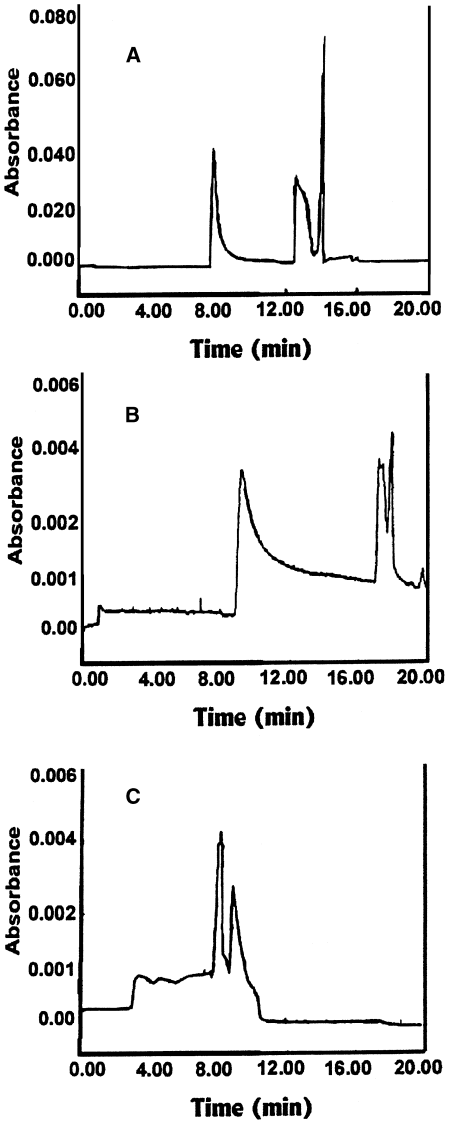

Figure 8. Monitoring the stability of IL-11 under different storage conditions. a) IL-11 was stored at -80°C and was 100% active, b) IL-11 was stored at 50°C for one week, and c) IL-11 was stored at 50°C for 4 weeks. Experimental conditions: 10 mM phosphate, pH 7.0, 37 cm 50 mm ID epoxy coated capillary with 30 cm separation length. Note: 0.01% Brij 35 was added in the running buffer for the separation of sample b).

ribonuclease A and -chymotrypsinogen.17 However, most of these proteins had recoveries between 84.4% and 95%, except lysozyme (55.5%) when using the coated capillary (Table 5). This result indicated that protein adsorption was significantly reduced on this coating.

Separation of Various Cytochrome C

The separation of cytochrome C variants was challenging because of their similarity in structure. Each of them differs by only a few amino acids in their sequences. When five cytochrome C variants were injected, only four peaks were obtained, with the horse and dog variants migrating together (Figure 6). Varying the experimental conditions still did not help the separation of the horse and dog variants. Very reproducible separations were obtained at pH 6-8 (Figure 6).

Separation of Recombinant Proteins

BMP-2 is a recombinant protein which has the potential to develop into a biotech drug. Previous experiments with an uncoated capillary and a commercially available hydrophilic coated column (Celect P150) failed to separate the BMP-2 components with reproducible results. By using the epoxy coating, the various BMP-2 components were separated (Figure 7). IL-11 is another recombinant protein under investigation. Using this epoxy coating, a CE method was developed to monitor the stability of IL-11 at different storage conditions. Figure 8 shows that there are some significant differences in the CE profile of the three IL-11 samples stored at different temperatures for different periods of time.

CONCLUSIONS

The separation of protein samples under different conditions has demonstrated that the epoxy coating is well suited for the purpose of separating proteins. This coating is easy to prepare and suited for a broad range of pH values. High recoveries of proteins prove that this epoxy modified surface has reduced the protein adsorption significantly.

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