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Capillary zone electrophoresis of proteins with hydrophilic fused-silica capillaries

WASSIM NASHABEH and ZIAD EL RASSI*
Department of Chemistry, Oklahoma State University, Stillwater, OK 74078-0447 (USA)

ABSTRACT

Fused-silica capillaries having surface-bound hydroxylated polyether functions were developed for the separation of proteins by capillary zone electrophoresis. In one approach, the hydrophilic coatings consisted of two layers: a glyceropropylpolysiloxane sublayer covalently attached to the inner surface and a polyether top layer. In a second approach, the capillary wall was coated with polysiloxane polyether chains the monomeric units of which at both ends were covalently attached to the capillary inner surface with possible interconnection. These coatings yielded capillaries with different electroosmotic flow characteristics. The relatively long polyether chains of the various coatings were effective in shielding the unreacted surface silanols, thus minimizing solute–wall adsorption. As a consequence, high separation efficiencies were obtained in the pH range 4.0–7.5, which allowed the separation of widely differing proteins, the characterization of heterogeneous proteins and the fingerprinting of crude protein mixtures. The various coatings were stable and exhibited reproducible separations from run-to-run, day-to-day, and column-to-column. Furthermore, a procedure was developed for restoring collapsed capillaries after prolonged use.

INTRODUCTION

Capillary zone electrophoresis (CZE) with its high resolving power and unique selectivity is well placed to play an important role in the separation and characterization of proteins. However, since its debut [1–3], researchers have soon realized that CZE of proteins with fused-silica capillaries is hampered by solute adsorption onto the capillary inner walls, which often gives rise to band broadening and low recovery of the separated analytes.

Several attempts have been made to alleviate the problem of solute–wall interactions. The major approaches have been: (i) capillary surface treatments [4–11], (ii) the use of running electrolytes at high [12,13] or low pH [6], *i.e.* above or below the isoelectric points of proteins, (iii) the addition of high salt concentrations to the running electrolytes [14] using high pH, and (iv) the use of zwitterionic buffers with high salt concentrations [15]. Among all these approaches, surface modification is the method that affords the highest flexibility as far as selectivity modulation with pH changes is concerned. Indeed, approach (ii) limits the selectivity of the system to a narrow pH range and may result in protein denaturation at low pH or capillary degradation at high pH; approach (iii) may not be effective at medium pH range (5 to

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7) and in addition may produce system overheating due to the high ionic strength of the running electrolyte. On the other hand, method (iv), although effective in reducing solute adsorption, does not allow high sensitivity detection due to increased background in UV of most zwitterions when used at elevated concentrations.

Thus far, several coatings have been developed for CZE of proteins. These coatings are polyacrylamide [4], methylcellulose [4], glycol [5], glycerol-glycidoxypropyl [6], poly(vinylpyrrolidinone) [6], polyethylene glycol (PEG) [7], maltose [8], aryl pentafluoro (AFP) groups [9], polyethyleneimine (PEI) [10], and vinyl-bound polyacrylamide [11]. The PEI coating provided positively charged surface and was useful for the separation of basic proteins. This mimic the idea of operating untreated fused-silica capillaries with high pH, whereby the proteins and the surface have the same charge, which then prevent adsorption by coulombic repulsion. All other coatings were of the neutral type and produced acceptable results in terms of protein separations.

This report is concerned with the investigation of surface modification procedures that yield stable and inert capillaries having moderate electroosmotic flow. The capillary surface modification reported here entails the covalent attachment of polyether chains of various length to the inner surface of fused-silica capillaries via siloxane bonds, either directly using polyether chains with trimethoxysilane groups at both ends or by attaching a top polyether layer to a polysiloxane sublayer already anchored to the capillary inner surface. The first type of coatings is referred to as interlocked coatings, the second as fuzzy coatings. The coated capillaries were evaluated in protein CZE and characterized in terms of efficiency and electroosmotic flow over a wide range of pH. In addition, a procedure was developed for restoration of collapsed capillaries after prolonged use.

EXPERIMENTAL

Instrument

The capillary electrophoresis instrument used in this study resembles that reported earlier [16,17]. It was constructed from a Glassman High Voltage (Whitehouse Station, NJ, USA) Model HP30P3 high-voltage power supply of positive polarity and a Linear (Reno, NV, USA) Model 200 UV–VIS variable-wavelength detector equipped with a cell for on column detection. In all experiments, the wavelength was set at 210 nm. The electropherograms were recorded with a computing integrator interfaced with a floppy disk drive and a CRT monitor from Shimadzu (Columbia, MD, USA).

Reagents and materials

Cytochrome *c* from horse heart, lysozyme from chicken egg white, ribonuclease A and α-chymotrypsinogen A, both from bovine pancreas, myoglobin from horse skeletal muscle, carbonic anhydrase from bovine erythrocytes, iron saturated transferrin from human serum, β-lactalbumin from bovine milk, and crude trypsin inhibitor and lipoxidase, both from soybean were purchased from Sigma (St. Louis, MO, USA). Table I compiles the relative molecular mass (M_r)and p*I* values of these proteins. γ-Glycidoxypropyltrimethoxylsilane (Z-6040) was a gift from Dow Corning (Midland, MI, USA). Polyethylene glycol diglycidyl ether (M_r 600) was purchased

TABLE I

PROTEINS USED IN THIS STUDY

Protein	M_r	p*I*
Lysozyme	14 100	11.0
Cytochrome *c*	12 400	10.7
Ribonuclease A	13 700	9.4
α-Chymotrypsinogen A	25 500	9.5
Myoglobin	17 500	6.8–7.3
Carbonic anhydrase	31 000	5.3
Transferrin	79 550	5.2–6.1
α-Lactalbumin	14 200	4.8
Trypsin inhibitor	20 100	4.5
Lipoxidase	102 400	5.7

from Polysciences (Warrington, PA, USA). Polyethylene glycol (PEG) of M_r 200, 600 and 2000, boron trifluoride etherate and phenol were obtained from Aldrich (Milwaukee, WI, USA). Reagent-grade sodium phosphate monobasic, sodium hydroxide, hydrochloric acid, dioxane, and N,N-dimethylformamide (DMF) were from Fisher Scientific (Pittsburgh, PA, USA). Fused-silica capillaries of 50 μm I.D. and 375 μm O.D. were obtained from Polymicro Technology (Phoenix, AZ, USA). Deionized water was used to prepare the running electrolyte. All solutions were filtered with 0.2-μm Uniperp Syringeless filters from Genex (Gaithersburg, MD, USA) to avoid capillary plugging.

Capillary modification

Fused-silica capillaries of 80 cm long were treated using the following procedures.

Polyether fuzzy coatings. Fused-silica capillaries were first filled with an aqueous solution at 10% (v/v) γ-glycidoxypropyltrimethoxysilane and allowed to react at 96°C for 40 min. This treatment was repeated four times. Subsequently, the capillary was filled with 0.01 *M* HCl and then heated at 95°C for 40 min. After washing successively with water and dioxane, the capillary was flushed with a solution of dioxane at 1% (v/v) boron trifluoride. Following, the capillary was filled with an equimolar solution of polyethylene glycol diglycidyl ether (M_r 600) and PEG 2000 or PEG 600 in dioxane. The modified capillaries were stored in HPLC-grade methanol.

Polyether interlocked coatings. DMF solutions containing PEG 200 with trimethoxysilane at both ends were introduced into the capillary and allowed to react at 100°C for 1 h. This treatment was repeated twice. All capillaries were stored in HPLC-grade methanol.

Restoration of deteriorated capillaries. After prolonged use, the performance of the various hydrophilic capillaries declined due to hydrolytic degradation of some of their coatings. The feasibility of recoating a deteriorated capillary to restore its protective hydrophilic layer was assessed with polyether interlocked coatings. In this regard, the collapsed capillary was flushed with 1.0 *M* NaOH and then heated at 95°C for 30 min; a step that removed the remaining of the coating via nucleophilic cleavage of the siloxane bonds (Si–O–Si–C) [18]. Following, the capillary was successively washed with water, filled with 0.1 *M* HCl and then heated at 95°C for 30 min. After

washing with water and DMF, the capillary was filled with the coating reagent and allowed to react as described above.

Reproducibility. The coated capillaries were evaluated for the reproducibility of protein migration times from run-to-tun, day-to-day and column-to-column. From run-to-run (on the same day), the relative standard deviation (R.S.D.) was calculated based on four successive measurements separated by 20 min equilibration with the running electrolyte. From day-to-day the R.S.D. was calculated based on a total of 9 measurements made on three different days (3 sets of 3 measurements each) and from column-to-column, the R.S.D. was estimated using data collected (2 sets of 6 measurements each) from two columns prepared on different days.

RESULTS AND DISCUSSION

Surface modification

Fig. 1A and B depict the idealized structure of the fuzzy and interlocked hydrophilic coatings, respectively. Both types of coatings are essentially hydroxylated polyether chains covalently attached to the capillary inner surface. The fuzzy coatings (see Fig. 1A) consist of two layers: a cross-linked glyceropropylpolysiloxane sublayer

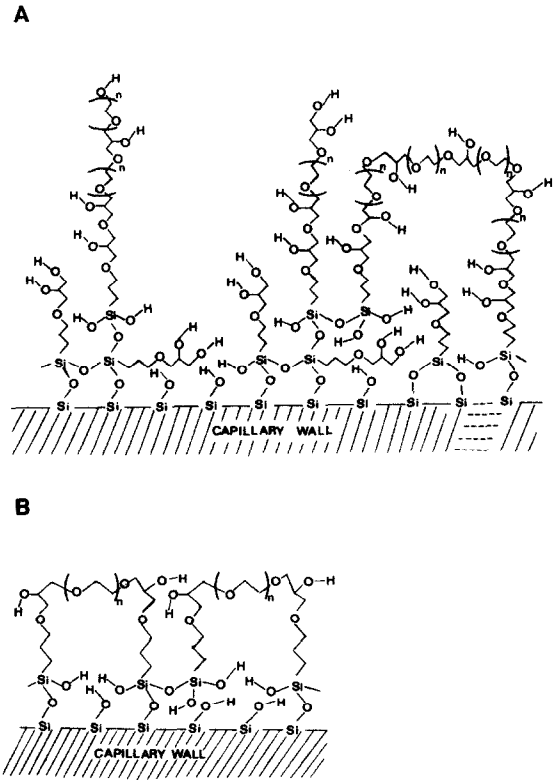


Fig. 1. Schematic illustration of the idealized structures of fuzzy (A) and interlocked (B) polyether coatings of fused-silica capillaries.

covalently attached to the capillary inner-surface, and a hydrophilic polyether top layer. The fuzzy top layer is the result of the different ways in which the PEG 600 diglycidyl ether can react with the PEG 600 or 2000 leading to polyether chains of different lengths with either one end or two ends covalently attached to the sublayer. On the other hand, the interlocked coatings consist of polyether polysiloxane chains whose monomeric units at both ends are covalently attached to the surface with possible interconnection. The following capillary codings will be utilized throughout the text to designate each type of coatings. F-2000 and F-600 denote capillaries with fuzzy coatings having PEG 2000 or PEG 600 moieties, respectively, whereas I-200 refers to capillaries with interlocked coatings having PEG 200 polyether chains.

Electroosmotic flow

The above two schemes of surface modification yielded capillaries with different electroosmotic flow characteristics. Fig. 2A and B illustrate the results obtained at 17 and 20 kV over the pH range 4.0–7.5 using phenol as the inert tracer. At both running voltages, the fuzzy coatings exhibited lower electroosmotic flow than the interlocked ones indicating that the concentration of unreacted surface silanols is relatively low for the capillaries with fuzzy polyether coatings. This is because the fuzzy coatings have an additional cross-linked polysiloxane sublayer that the interlocked coatings do not have. On the other hand, for the two fuzzy coatings, *i.e.* F-600 and F-2000, which should have the same surface concentration of unreacted silanols, F-600 exhibited a higher flow-rate than F-2000 under otherwise identical conditions. The difference in the flow-rate between the two fuzzy coatings may be related to the size of the polar top layer. The polyether chains of the polar top layer in F-600 are shorter than those in F-2000, and as a result they were less effective in masking the ionized surface silanols (*i.e.* unreacted silanols) toward the atmospheric binding of electrolyte cations. In view of Manning's counterion condensation theory [19], salt counterions are

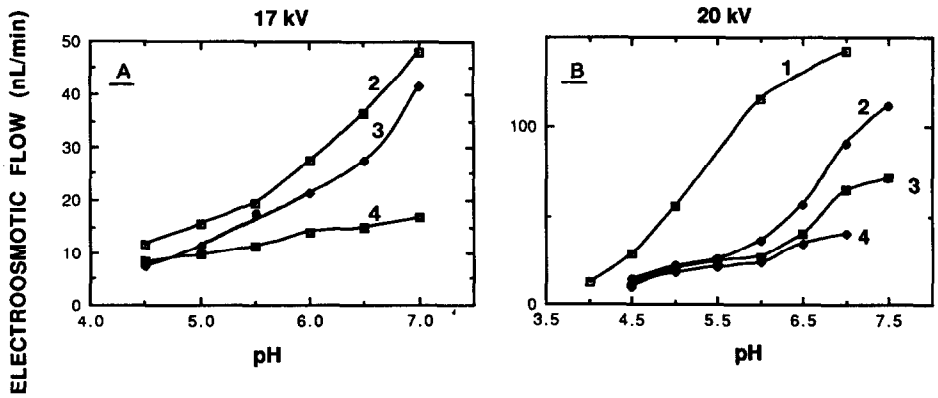


Fig. 2. Plots of electroosmotic flow-rates obtained on coated and uncoated capillaries *versus* pH of the electrolyte at 17 kV in A and 20 kV in B. Fused-silica capillaries: 1 = untreated; 2 = I-200; 3 = F-600; 4 = F-2000. All tubes were 50 cm (to the detection point), 80 cm total length × 50 μm I.D.; electrolyte, 0.1 *M* phosphate solutions at different pH. At 17 kV, currents were 21, 22, 23, 24, 34 and 38 μA at pH 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0, respectively. At 20 kV, currents were 26, 28, 29, 30, 33, 45, 50 and 70 μA at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5, respectively. Inert tracer: phenol.

territorially or atmospherically bound, *i.e.* they are retained by the electrostatic field at the capillary surface, but remain free to move within a certain layer above it. The atmospheric binding of electrolyte cations to the negatively charged silanols generates an electric double layer adjacent to the capillary walls, which migrates toward the cathode when an electric potential is applied to the capillary. Since the cations are solvated, they pull solvent with them, thus producing the electroosmotic or bulk flow.

On the other hand, when compared to untreated fused-silica capillaries at an applied voltage of 20 kV (see Fig. 2B), the electroosmotic flow was reduced on the average by a factor of 3.6, 2.9 and 2.3 for F-2000, F-600 and I-200, respectively. It should be noted that the difference in flow-rate among the various coatings was greater in magnitude at pH 5.0 and above due to increasing ionization of the unreacted surface silanols (see Fig. 2A and B).

In principle, changing the applied voltage should produce proportional changes in the rate of the electroosmotic flow. However, the comparison of Fig. 2A and B shows that increasing the applied voltage by a factor of 1.17, *i.e.* from 17 to 20 kV yielded a larger increase in the electroosmotic flow. On average, the flow increased by a factor of *ca.* 1.5 for the coated capillaries over the pH range studied when going from 17 to 20 kV. Capillary internal temperature was found to increase in non-linear fashion with the applied voltage [20]. On the other hand, solvent viscosity decreases exponentially with increasing temperature and since the electroosmotic flow is inversely proportional to viscosity, this decrease in viscosity may have caused the jump in flow at 20 kV when compared to 17 kV.

Evaluation of coated capillaries with basic proteins

Figs. 3, 4 and 5 depict typical electropherograms of four model basic proteins

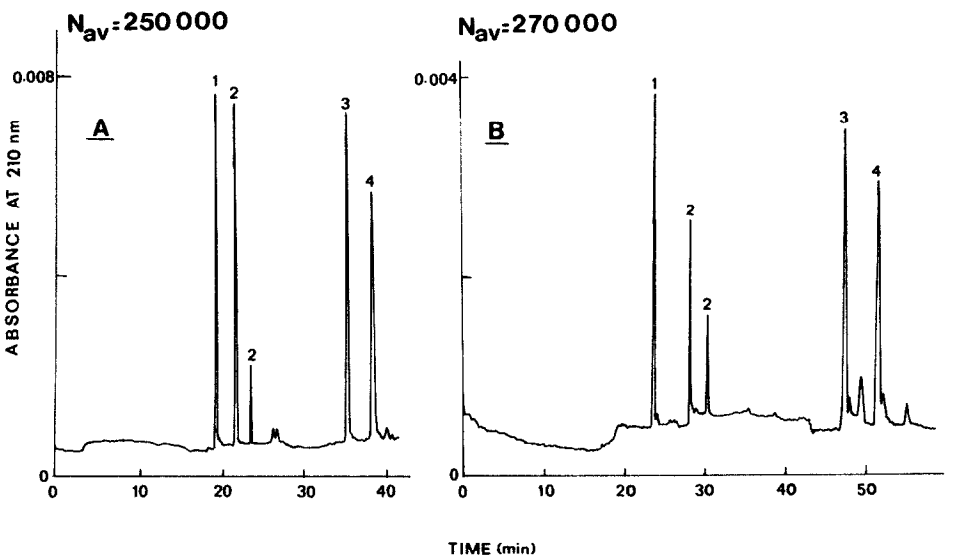


Fig. 3. Electropherograms of basic proteins obtained on the fuzzy 2000 coated capillary. Capillary, 50 cm (to detection point), 80 cm total length × 50 μm I.D.; electrolyte, 0.1 *M* phosphate solutions at pH 6.0 in A and 7.0 in B; running voltage, 17 kV; currents were 24 and 38 μA at pH 6.0 and 7.0, respectively. Proteins: 1 = lysozyme; 2 = cytochrome *c*; 3 = ribonuclease A; 4 = α-chymotrypsinogen A.

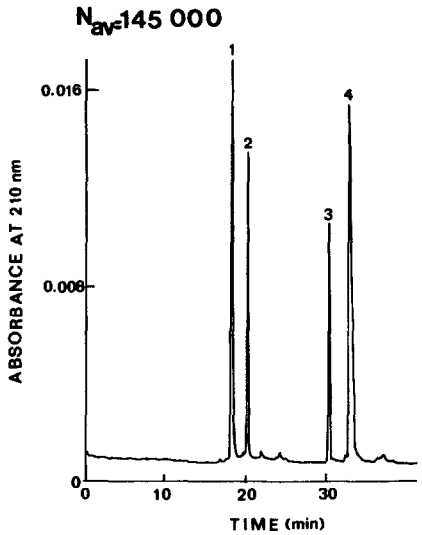


Fig. 4. Electropherogram of basic proteins obtained on fuzzy 600 coated capillary at pH 6.0. All other conditions as in Figs. 2 and 3.

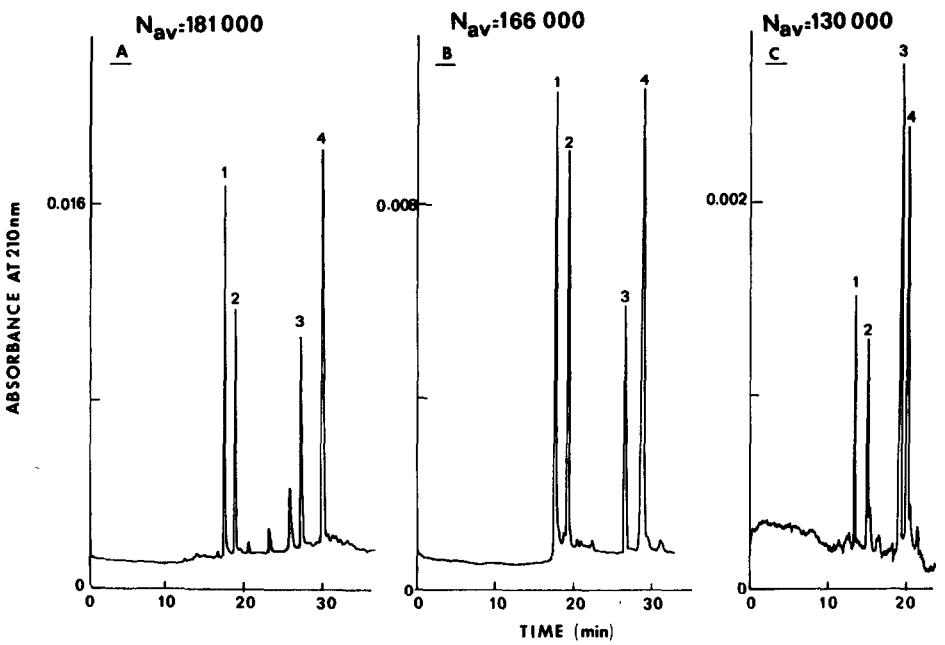


Fig. 5. Electropherograms of basic proteins obtained on interlocked 200 coated capillary at pH 5.0 (A), pH 6.0 (B) and pH 7.0 (C). All other conditions as in Figs. 2 and 3.

performed on F-2000, F-600, and I-200 coated capillaries, respectively, at an applied voltage of 17 kV using 0.1 *M* phosphate solutions as the running electrolyte at different pH. The average plate count per meter, N_{av}, is indicated on each electropherogram. These efficiency values may indicate that interaction between the proteins and the inner surface proper of the capillaries has been greatly reduced. Based on the results of electroosmotic flow with the various capillaries (see Fig. 2), some unreacted silanols remained on the capillary inner surface. These silanols which were accessible for the atmospheric binding of small counterions, may be less exposed for the territorial binding of large polyionic solutes such as proteins. The relatively long polyether chains of the various coatings are believed to hinder the access of macromolecules to the unreacted silanols. Indeed, according to Manning's counterion condensation theory [19], charges must be within the Bjerrum length in order to undergo electrostatic interactions. This length is defined as the distance at which two unit charges interact with an energy of k_BT (k_B is Boltzman's constant and T is absolute temperature) in the relevant dielectric medium and its value is 7.14 Å in water at 25°C.

Referring to Fig. 5, the N_{av} obtained on I-200 was slightly lower at pH 7.0 than at pH 5.0; a trend that was also observed on F-600 and F-2000. This may be due to increasing ionization of unreacted surface silanols and/or Joule heating with increasing pH. Raising the electrolyte pH from 5.0 to 7.0 would lead to increasing protein interactions with the residual ionized silanols, which in turn would cause lower separation efficiencies. On the other hand, due to increasing the ionic strength of the running electrolyte with increasing pH, the current almost doubled when the pH of phosphate solution was raised from 5.0 to 7.0. Higher currents lead to increasing center-to-wall temperature difference, thereby producing a more pronounced radial viscosity gradient inside the capillary. As a result, radial variations in the overall velocity profiles (*i.e.*, the sum of electrophoretic and electroosmotic velocity) of the separated proteins would also increase in magnitude at high currents. These perturbations in the velocity profiles may produce mass transfer resistances that broaden the migrating solute zones and cause lower separation efficiencies. The effect of temperature gradients on CZE separation efficiency is well documented [21–23].

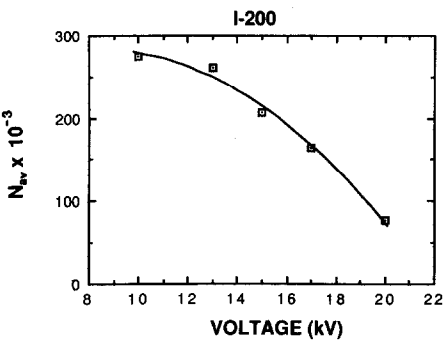
The values of N_{av} obtained on F-600 and I-200 capillaries were quite comparable, but lower than those obtained on F-2000 under otherwise identical conditions (see Figs. 3, 4 and 5). This may be due to the higher electroosmotic flow with F-600 and I-200 when compared to F-2000. Under these conditions, mass transfer resistances due to heat-induced perturbations in the overall velocity profiles are amplified. Another possible explanation is that the F-600 and I-200 coatings are not as effective as F-2000 coatings in shielding the unreacted surface silanols toward the protein solutes. It has been shown that a slight solute–wall adsorption can cause separation efficiency to decline [24].

In CZE of proteins, capillary internal Joule heating due to passage of current through the electrolyte inside the tube could also denature the biomacromolecules. Increasing the voltage from 17 to 20 kV using 0.1 *M* phosphate solutions at pH 6.0, 6.5 and 7.0 resulted in increasing the current from 24 to 33 μA, from 34 to 45 μA and from 38 to 50 μA, respectively. At this higher voltage, and independently of the nature of the coating, α-chymotrypsinogen A did not elute from the capillary when pH 6.5 or 7.0 were used, while the peaks of cytochrome *c* and lysozyme, and to a lesser extent that of ribonuclease A, exhibited a significant drop in efficiency. At pH

6.0, increasing the voltage from 17 to 20 kV did only result in lowering the separation efficiency. This may be due to the binding of proteins to the coating proper by hydrophobic interactions. It has been shown in liquid chromatography of proteins that hydrophobic interactions are induced and/or increased with increasing temperature due to heat-induced conformational changes [25,26]. These interactions which are the result of temperature effects arising from Joule heating can be viewed as the major contributors to band broadening at elevated voltage.

To examine the influence of the running voltage on separation efficiencies, the four basic proteins (*i.e.* lysozyme, cytochrome *c*, ribonuclease A, and α-chymotrypsinogen A) were analyzed on an I-200 capillary at different voltages using 0.1 *M* phosphate, pH 7.0. The results are illustrated in Fig. 6 by a plot of average plate count per meter *versus* applied voltage. As in the preceding experiment, α-chymotrypsinogen A did not elute from the capillary at an applied voltage of 20 kV. It is seen that an applied voltage in the range 10–13 kV yields higher efficiency than at elevated voltage. However, the tradeoff is longer analysis time. A running voltage of 16–17 kV seems to be a compromise, whereby protein separation can be carried out relatively fast without sacrificing separation efficiencies. The relatively high separation efficiency obtained at lower running voltage may result from diminished Joule heating of the system. Similar observations were recently reported by Cobb *et al.* [11] with capillaries having polyacrylamide coatings.

To evaluate the effect of ionic strength on separation efficiencies, the voltage was kept low (*i.e.* 10 kV) so that a wide range of phosphate concentrations in the running electrolyte can be investigated without overheating the system. This set of experiments was performed on an I-200 capillary at pH 7.0. The results are depicted in Fig. 7 by a plot of N_{av} *versus* the phosphate concentrations in the running electrolyte. As can be seen in Fig. 7, N_{av} first increased, passed through a maximum and then decreased with increasing phosphate concentrations. Again, the sharp decrease in separation efficiency at high ionic strength (*i.e.*, at 0.25 *M*; see Fig. 7) can be related to



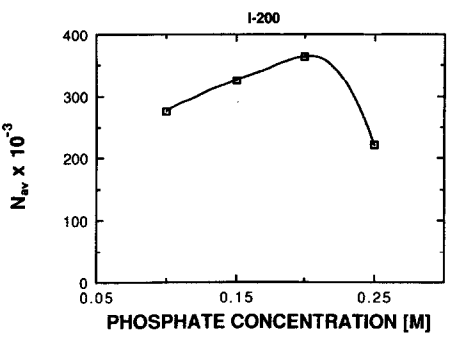


Fig. 6. Plot of average plate count per meter *versus* applied voltage obtained on an I-200 capillary. Electrolyte, 0.1 *M* phosphate solution, pH 7.0; currents were, 7, 20, 28, 38, and 50 μA at 10, 13, 15, 17 and 20 kV, respectively. All other conditions as in Figs. 2 and 3.

Fig. 7. Plot of average plate count per meter *versus* the ionic strength of the running electrolyte obtained on an I-200 capillary. Electrolytes, phosphate solutions at different ionic strength, pH 7.0; running voltage 10 kV; currents were, 7, 18, 35 and 50 μA at 0.1, 0.15, 0.2 and 0.25 *M* phosphate, respectively. All other conditions as in Figs. 2 and 3.

effects associated with system heating. At this lower voltage, *i.e.* 10 kV, and using 0.25 *M* phosphate, α-chymotrypsinogen A eluted from the capillary despite that the current was as high as with 0.1 *M* phosphate and 20 kV (*i.e.* 50 μA); conditions under which the same protein was tightly bound to the capillary (see above). It seems that for the same current passing through the running electrolyte, a higher ionic strength would stabilize protein structure, and consequently protein hydrophobic interaction with the coating due to heat-induced conformational changes would be reduced.

To compare the three types of coatings, peak capacity, n, was calculated for each capillary based on the following assumptions: (i) all capillaries can generate 100 000 theoretical plates per column on the average over the pH range 4.5–7.0, (ii) the hypothetical proteins differ among each others by their pI values, which span 4–11, (iii) lysozyme is the first eluting peak, and (iv) the analysis time is set arbitrarily to 150 min, time at which the center of the last eluting peak appears in the detector. The peak capacity, n, was calculated using the equation $n = 1 + (N_{av}/16)^{1/2} \ln (t_f/t_i)$, and the results are depicted in Fig. 8 for the pH range 4.5 to 7.0. In this equation, t_i is the migration time of lysozyme and t_f is that of phenol (*i.e.* the inert tracer); a migration time interval within which the elution of basic and slightly neutral proteins may take place. On the other hand, the migration times of acidic proteins are assumed to

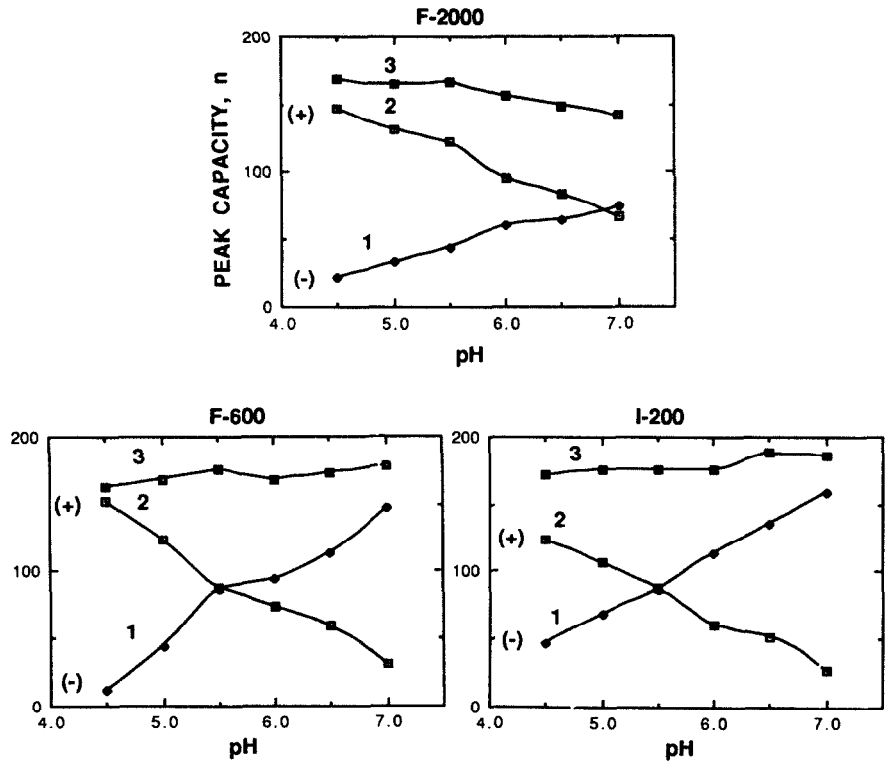


Fig. 8. Comparison of peak capacity, n, of the different coatings over the pH range 4.5 to 7.0 using lysozyme and phenol migration times as 17 kV (see text for details). 1 = n for negatively charged proteins; 2 = n for positively charged proteins; 3 = total n.

lie between the migration times of phenol, t_i, and that of the last eluting peak, t_f, set arbitrarily to 150 min. The migration times of lysozyme and phenol used in calculating peak capacity were those obtained from experiments performed on the various capillaries with a migration distance of 50 cm (*i.e.* distance from injection end to detection point) using 0.1 *M* phosphate solutions at pH ranging from 4.5–7.0, and a running voltage of 17 kV. Referring to Fig. 8, it can be seen that F-2000 capillaries require an electrolyte of pH 7.0 to elute an equal number of positively and negatively charged proteins, whereas this situation is reached at pH 5.5 for I-200 and F-600 capillaries. Due to the oversimplifications in the calculations, the peak capacity may not be realistic. Nevertheless, to obtain similar behavior in terms of peak capacity, it seems wise to state that a tube with relatively low electroosmotic flow requires the use of an electrolyte pH that is two or more units higher in value than with a capillary having 2.5 to 3 times higher electroosmotic flow. Another difference is that at any pH the peak capacity for positively charged proteins on the F-2000 capillary is always greater than that with F-600 and I-200. On the other hand, the peak capacity for the negatively charged proteins is higher with F-600 and I-200 than with F-2000.

Fig. 9 illustrates the overall and the electrophoretic mobilities of two model proteins (*i.e.* lysozyme and ribonuclease A) as well as the electroosmotic mobility of neutral solutes over the pH range studied with F-2000, F-600 and I-200 coated capillaries. In all cases (see Fig. 9), the electrophoretic mobility of the two test proteins decreased sharply between pH 5.5 and 6.0 indicating the deprotonation of an imidazole and/or the dissociation of a carboxylic side chain group. Furthermore, the electrophoretic mobility of proteins was virtually independent of the nature of the coatings under otherwise identical conditions. This is an indication of quasi-homosystems; meaning that the various coatings are quite effective in shielding the surface proper of the capillary toward the protein solutes. On the other hand, the overall mobility, $\mu_{overall}$, which is the sum of electroosmotic and electrophoretic mobilities, varied from one coating to another. The electroosmotic flow was relatively low with F-2000 capillary, and since this flow increased slightly with the pH at 17 kV, the overall mobility of the proteins almost paralleled their electrophoretic mobility (see Fig. 9). This may represent an advantage when dealing with unknown substances in the sense that their basicity or acidity can be assessed from simple $\mu_{overall}$–pH plot. On the other hand, the overall mobility of the same proteins followed different patterns on F-600 and I-200 mainly due to their higher electroosmotic flow. For lysozyme, the overall mobility increased between pH 4.5 and 5.5, decreased at pH 6.0 and then increased between pH 6.0 and 7.0. For ribonuclease A, a sharp increase in the overall mobility occurred in the pH range 6.0–7.0.

Reproducibility and stability of the coatings

These studies were carried out on F-600 and I-200 capillaries that are representative of fuzzy and interlocked coatings, respectively. Table II summarizes the run-to-run, day-to-day and column-to-column migration reproducibility expressed in terms of percent relative standard deviation (R.S.D.) with four basic proteins at pH 6.5. It should be noted that the column-to-column reproducibility reflects columns made on two different days. It can be seen that the interlocked 200 is slightly more reproducible than the fuzzy coatings simply because the surface modification is accomplished in a single step in the former, whereas it takes three steps to make the latter.

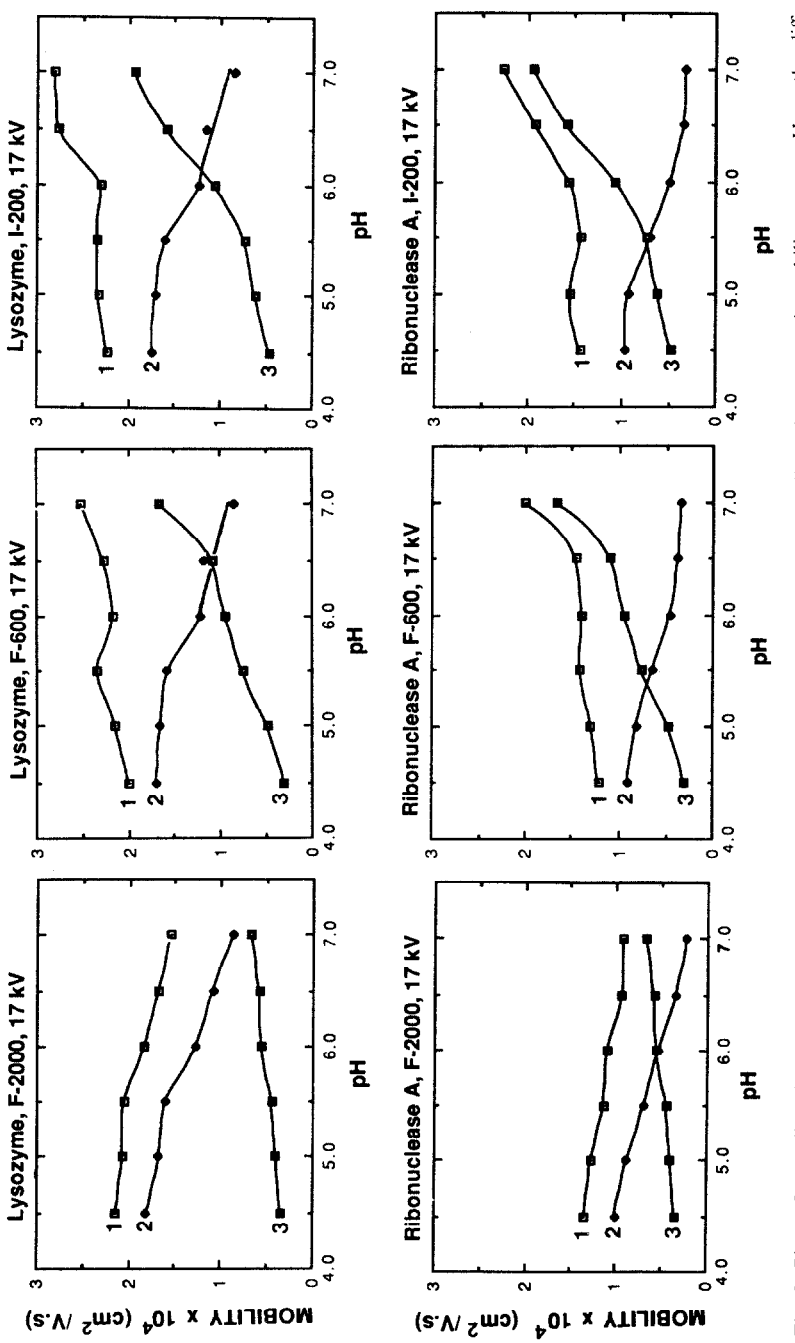


Fig. 9. Plots of overall and electrophoretic mobilities of lysozyme and ribonuclease A as well as electroosmotic mobility *versus* pH on the different coatings. Electrolytes, 0.1 *M* phosphate solutions at different pH. 1 = overall mobility; 2 = electrophoretic mobility; 3 = electroosmotic mobility. All other conditions are the same as in Figs. 2 and 3.

TABLE II

REPRODUCIBILITY OF MIGRATION TIMES OF PROTEINS ON F-600 AND I-200 CAPILLARIES

Capillary, 50 cm (to the detection point), 80 cm total length × 50 μm I.D.; electrolyte, 0.1 *M* phosphate solution, pH 6.5; applied voltage, 17 kV; current, 38 μA (see Experimental for details).

Protein	R.S.D. (%)					
	F-600			I-200		
	Run to run	Day to day	Column to column	Run to run	Day to day	Column to column
Lysozyme	1.6	1.4	4.2	0.9	1.6	1.5
Cytochrome *c*	0.2	0.6	6.7	0.9	1.8	2.2
Ribonuclease A	1.5	1.2	5.4	1.0	1.9	2.8
α-Chymotrypsinogen A	1.8	2.3	4.5	1.0	2.0	2.3

The different coatings were quite stable. In fact, the various columns exhibited constant performance for several weeks when operated at pH 6.0 or below, and for more than 80 h when used in the pH range 6.5–7.0.

Restoration of capillary coatings

After prolonged use, the various capillaries lost some of their coatings by hydrolytic degradation, and consequently they exhibited strong interactions with the proteins and high electroosmotic flow nearing that obtained with uncoated capillaries. Fig. 10A illustrates the separation of 8 acidic and basic standard proteins spanning a wide range of molecular weights. This electropherogram is typical of what can be obtained on an original I-200 coating that is still exhibiting constant performance in terms of efficiency, resolution and electroosmotic flow characteristics. We have chosen several proteins for better assessment of the feasibility of restoring a collapsed capillary. Fig. 10B represents a typical electropherogram obtained on the same capillary after it has lost some of its coating, whereby a significant drop in efficiency and resolution as well as a pronounced increase in electroosmotic flow can be noticed.

In an attempt to restore the performance of the capillary, the coating that remained was stripped off the inner wall of collapsed capillary column by treatment with aqueous solutions of sodium hydroxide. The naked capillary thus obtained was then recoated as described in the experimental section. It can be seen from Fig. 10C that the column can be restored to its orginal performance in every regard. To assess the reproducibility of this procedure, we have performed the restoration several times on collapsed I-200 capillaries and each time obtained similar results to those reported in Fig. 10C. The procedure developed here for restoring capillary coatings is expected to be applicable to other type of coatings provided that they are attached to the capillary inner surface via siloxane bonds.

Referring to Fig. 10A and C, it should be noted that human serum transferrin is a heterogeneous glycoprotein consisting of at least five isoforms [27,28] that differ among each other in sialic acid content. It can be seen that the present electrophoretic

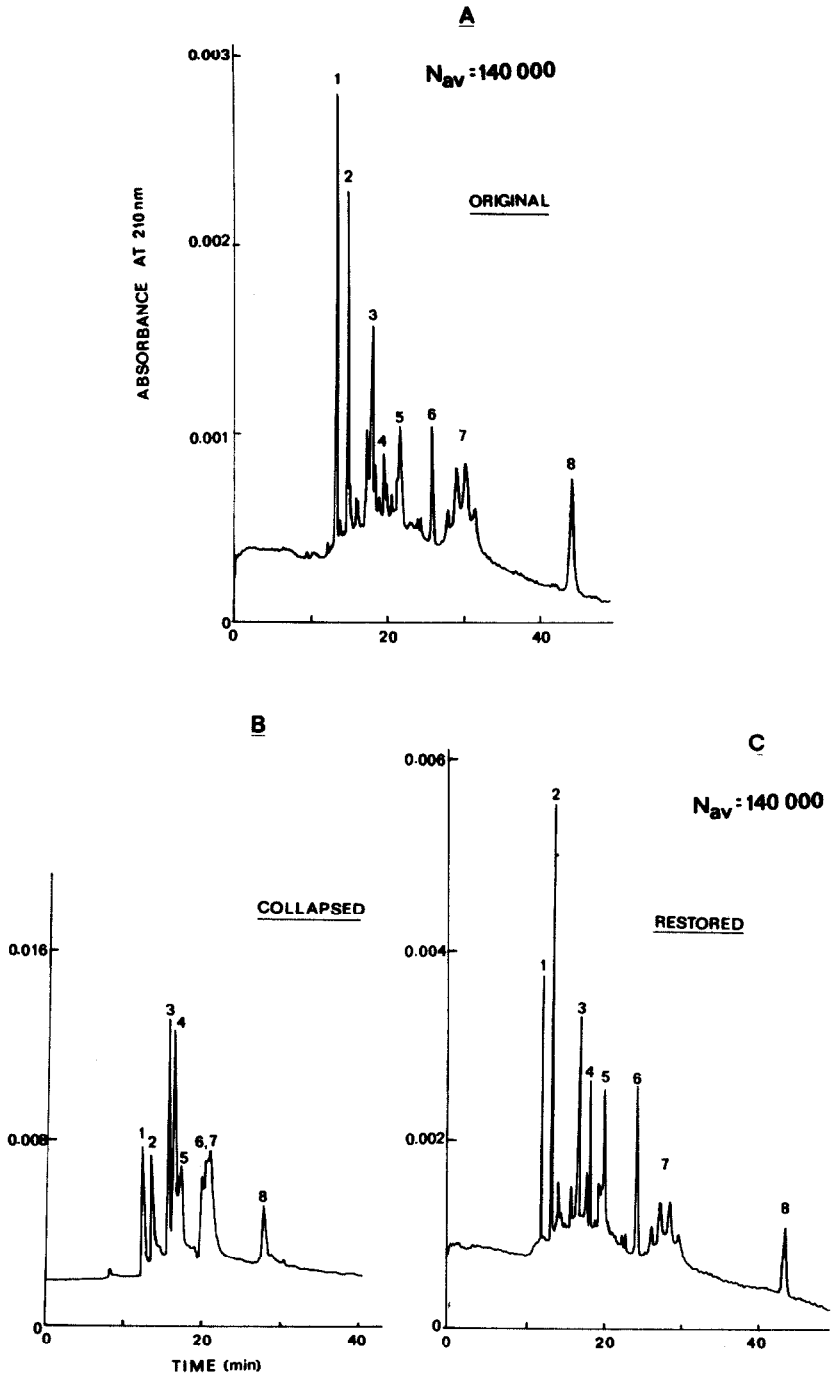


Fig. 10. Electropherograms of basic and acidic proteins obtained on original (A), collapsed (B) and restored (C) I-200 capillaries. Electrolyte, 0.1 *M* phosphate solution, pH 7.0; running voltage, 17 kV. All other conditions as in Fig. 3. Proteins: 1 = lysozyme; 2 = cytochrome *c*; 3 = ribonuclease A; 4 = α-chymotrypsinogen A; 5 = myoglobin; 6 = carbonic anhydrase; 7 = transferrin; 8 = α-lactalbumin. In both cases N_{av} was calculated on the well defined peaks, *i.e.* peaks 1, 2, 4, 6 and 8.

system is capable of separating these isoforms. Furthermore, most of the commercial standard proteins are somewhat microheterogeneous or not highly pure as manifested by the presence of small peaks that elute before or after the main peaks when the selectivity of the electrophoretic system is adequate (*e.g.* myoglobin, cytochrome *c*, etc.; *cf* Figs. 4–6 and 10).

Applications

As discussed above, the I-200 capillaries exhibited relatively higher electroosmotic flow than F-600 and F-2000 capillaries, and similarly to the fuzzy coatings they showed no significant solute–wall interactions over a wide range of pH. In CZE, the primary goal of surface modification of fused-silica capillaries is to minimize protein–wall interactions without completely inhibiting the electroosmotic flow. A reduction in the flow by a factor of 2.0 to 2.5 with respect to the untreated capillary is ideal in order to analyze both positively and negatively charged species in the positive polarity mode. This characteristic added to the "neutrality" of the inner wall is advantageous in capillary electrophoresis with on-line detection. As in slab gel electrophoresis the electropherogram, which is the written record of the experiment, should contain the maximum information about a given mixture. The presence of a moderate electroosmotic flow in capillary zone electrophoresis with coated capillaries permits the achievement of this goal. For a crude mixture whereby the p*I* values of all the components are not known, the presence of electroosmotic flow is essential to analyze and

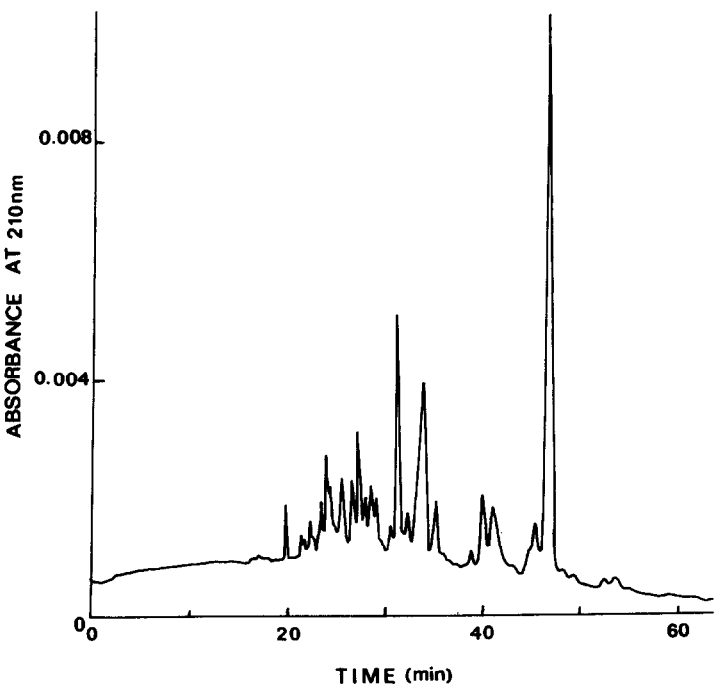


Fig. 11. Electropherogram of crude soybean trypsin inhibitor. Capillary, I-200; electrolyte, 0.1 *M* phosphate solution, pH 6.5; running voltage, 17 kV. All other conditions as in Figs. 2 and 3.

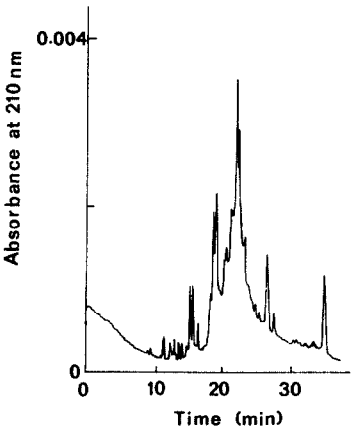


Fig. 12. Electropherogram of commercial soybean lipoxidase. Capillary, I-200, 0.1 *M* phosphate solution, pH 7.0; running voltage 17 kV. All other conditions as in Figs. 2 and 3.

characterize (fingerprinting) the mixture. This is in part the advantage of I-200 capillaries over the fuzzy capillaries.

Fig. 11 represents a typical electropherogram of crude soybean trypsin inhibitor (Sigma T-9128) performed on I-200 capillaries. It can be seen that the mixture contains at least 23 minor components and one major component. Another example of the usefulness of I-200 in analyzing complex protein mixture is shown in Fig. 12, which illustrates the electropherogram of commercial lipoxidase (Sigma L-8383), the unsaturated-fat oxidase. The microheterogeneity of lipoxidase was recently assessed by others [29] using several high-performance liquid chromatography techniques. The commercial protein gave also multiple peaks when chromatographed by reversed-phase, anion-exchange or metal chelate interaction chromatography [29].

The F-600 capillaries would also be useful in the above applications; however, longer analysis time would be expected under otherwise identical conditions. In a recent publication from our laboratory [17], F-2000 capillaries were used successfully at high voltage for the tryptic mapping of glycoproteins and the separation of oligosaccharide chains cleaved from glycoproteins. On the other hand, F-2000 capillaries may prove useful in isoelectric focusing or isotachophoresis whereby the presence of low electroosmotic flow may not be deleterious and good resolution of the solute bands could still be achieved.

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