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Fundamental and practical aspects of coupled capillaries for the control of electroosmotic flow in capillary zone electrophoresis of proteins

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ABSTRACT

This article represents an extension to a new approach, which was introduced very recently by our laboratory, for the control of the magnitude of electroosmotic flow in capillary zone electrophoresis. In this new approach, short fused-silica capillaries having different ζ potentials were coupled in series, and the amount of the electroosmotic flow was conveniently varied by changing the lengths of the individual capillary segments. The different coupled capillary systems evaluated in this study comprised various combinations of untreated fused-silica capillaries and polyether-coated capillaries having various electroosmotic flow characteristics. A general equation relating the average electroosmotic flow velocity in the coupled capillaries to the intrinsic electroosmotic velocity of the connected segments and their corresponding lengths has been derived and verified experimentally. The rate of the electroosmotic flow in a given system of coupled capillaries could be tuned over a range bordered by the lowest and highest intrinsic flow-rates of the connected capillary segments. In addition, a system of coupled capillaries that permitted a stepwise change in the rate of the electroosmotic flow during analysis was introduced and evaluated. These elution schemes were useful in the rapid separation of oppositely charged proteins in a single electrophoretic run and in the rapid analytical determination of the various components of heterogeneous proteins.

INTRODUCTION

In capillary zone electrophoresis (CZE), the observed migration behavior of charged species is determined by the electroosmotic flow (EOF) of the bulk solution inside the capillary and the electrophoretic mobilities of the analytes. While separation in CZE is based on differences in the electrophoretic mobilities of the analytes, the amount of time the solutes spend in the capillary is affected by the direction and magnitude of the EOF, which contribute to the migration of all analytes to the same extent regardless of their charges. While a relatively low level of electroosmotic flow is advantageous for the separation of closely related positively or negatively charged species, the analysis of basic, neutral and acidic solutes in a single electrophoretic run would require a relatively strong electroosmotic flow velocity.

Recently, Lee and co-workers [1,2] demonstrated that the direction and velocity of the EOF can be changed by the application of an external electric potential across a buffer-filled sheath capillary surrounding the separation capillary. More recently, Hayes and Ewing [3] proposed the use of a radial voltage with capillaries having an external film of conductive polymer for the control of EOF in CZE. However, the control of the electroosmotic flow by virtue of electronic means, *i.e.,* application of an external electric field, is only effective at low pH or with the use of surface modified capillaries whereby the concentration of the ionized surface groups are quite low and thus can be overcome by the electrostatic charges induced by the external potential.

Very recently, we have introduced a new electrophoretic system for the control of EOF in CZE. In this system, two capillaries having different wall ζ

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potential were coupled in series beyond the detection point [4]. When fused-silica capillaries with polyether coatings were connected to untreated fused-silica capillary tubes, the average electroosmotic flow velocity throughout the tandem capillary system was found to be a linear function of the fractional length of the individual connected capillary segments. This enabled the tuning of the rate of EOF independently of the applied voltage within the upper and lower limits of the intrinsic electroosmotic mobilities in the individual connected capillaries. The tandem operation allowed the rapid analysis of acidic proteins that would repulse electrostatically from the negatively charged fused-silica surface.

The aim of the present article is to investigate the potentials of other coupled capillary systems in the control of EOF and to extend the utility of the new approach to the rapid analysis of oppositely charged proteins in a single electrophoretic run with minimum solute-wall interaction. This was accomplished by connecting in series fused-silica capillaries of different intrinsic electroosmotic flow characteristics whose inner surfaces were either naked or modified with polyether chains of various length. Such coupling has reduced the analysis time of model proteins by a factor of 2 on the average. In addition, a configuration of coupled capillaries which allowed a stepwise increase in the electroosmotic flow during analysis was introduced. This elution scheme permitted a 3-fold decrease in the analysis time of model proteins without sacrificing the high separation efficiencies that can be achieved with surface-modified capillaries.

EXPERIMENTAL

Apparatus

The instrument for capillary electrophoresis used in this study was assembled in-house from commercially available components, and resembled that reported earlier [4,5]. It comprised two high-voltage power supplies of positive and negative polarity Models MJ30P400 and MJ30N400, respectively, from Glassman High Voltage (Whitehouse Station, NJ, USA) and a Linear (Reno, NV, USA) Model 200 UV-VIS variable wavelength detector equipped with a cell for on-column capillary detection. The elctropherograms were recorded with a Shimadzu computing integrator Model C-R6A (Columbia, MD, USA) equipped with a floppy disk drive and a cathode-ray tube (CRT) monitor.

TABLE I

PROTEINS USED IN THIS STUDY

Proteins	M_r	p*I*
Lysozyme	14 100	11.0
Ribonuclease A	13 700	9.40
Carbonic anhydrase	31 000	6.20
r-Lactalbumin	14 200	4.80
β-Lactoglobulin A	35 000	5.23
Albumin, egg	45 000	4.63

Reagents and materials

Lysozyme from chicken egg white, ribonuclease A from bovine pancreas, carbonic anhydrase from bovine erythrocytes, Ix-lactalbumin and β-lactoglobulin A from bovine milk and albumin from chicken egg were purchased from Sigma (St. Louis, MO, USA). Table I compiles the molecular weights (M_r) and isoelectric points (p*I*) of these proteins. Reagent grade sodium phosphate monobasic, hydrochloric acid, sodium hydroxide and HPLC grade methanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Phenol which was used as an inert tracer for measuring the electroosmotic flow (EOF) was obtained from J. T. Baker (Phillipsburg NJ, USA).

Capillary columns

Polyimide-coated fused-silica capillary columns of 50 μm I.D. and 365 μm O.D. were obtained from Polymicro Technology (Phoenix, AZ, USA). Both untreated and coated capillaries were used in this study. The coated capillaries were modified in-house with surface-bound hydroxylated polyether chains of various length according to previously described procedures [6]. The capillary coding I-200 and F-2000 is used to denote capillaries with interlocked coatings having polyethylene glycol 200 chains and capillaries having fuzzy coatings with polyethylene glycol 2000 moieties, respectively.

Other procedures

The capillary segments of the tandem capillary systems were connected butt-to-butt using PTFE tubes the inner diameter of which matched the outer

diameter of the connected capillary columns. In all cases the connection was made beyond the detection point. While the length of the individual capillaries having different surface characteristics was varied to span a wide range of fractional length, the total length of the coupled capillaries was kept constant at 80 cm with a detection window at 30 cm from the inlet reservoir. It has to be noted that in all experiments, the background electrolyte was 0.1 *M* sodium phosphate, pH 6.50. Solute introduction was by hydrodynamic flow (i.e., by gravity flow) at a differential height of 5 cm between the electrolyte reservoirs for 10 s. The detection wavelength was set at 210 nm for sensing the various proteins as well as the inert tracer.

To ensure reproducible separations the capillary column was flushed successively with fresh buffer, water, methanol, water, and again running buffer. In addition, the running electrolyte was renewed after each run and the capillary was allowed to equilibrate with the new buffer for 10 min before each injection.

RESULTS AND DISCUSSION

Control of elctroosmotic flow by coupled capillaries

We have previously shown [4] that for a system of n coupled capillaries having the same inner diameter but differing in their ζ potentials, the average electroosmotic mobility, μ_{eo}, across the tandem system is a weighted average of the intrinsic or local electroosmotic mobilities in the individual connected segments as follows [4]:

$$\mu_{eo} = \frac{\sum_{i=1}^{n} \mu_{eo,i} l_i}{l_t} \quad (1)$$

where l_i is the length of the individual capillary segment, i, l_t is the total length of the connected capillaries and $\mu_{eo,i}$ is the intrinsic electroosmotic mobility in each capillary segment, i, measured on a separate length l_t. Eqn. 1 can be rearranged as a linear function of the fractional length of a given capillary segment, i, in the tandem capillaries as in eqn. 2.

where j and k are random variables. It follows then that the average electroosmotic flow or bulk flow across the tandem capillary system can be in principle controlled to any desired value bordered by the lowest and highest intrinsic electroosmotic mobilities in the individual capillaries.

Due to the differences between the intrinsic electroosmotic flows in the connected segments and the uniform bulk flow across the tandem capillary system, a compensating hydrostatic pressure would develop. Consequently, a change from a plug flow profile to a laminar or poiseuille profile would result. As the difference between the intrinsic electroosmotic flow and the bulk flow increases, the laminar flow profile would become more pronounced. Although a purely pressure driven laminar flow is known to introduce band broadening, it has been shown recently that the superposition of poiseuille flow on an electroosmotic flow may not necessarily deteriorate the separation efficiencies [7]. In addition, under conditions whereby the pressure driven flow opposes the bulk flow, the electrokinetic dispersion coefficient may be even reduced [7]. A detailed treatment concerning the effects of superposed laminar and electroosmotic flow profiles on electrokinetic dispersion in CZE can be found in ref. 7.

Eqn. 2 was verified experimentally by measuring the EOF in 3 different tandem capillary systems, *i.e.*, F-2000 → I-200, F-2000 → untreated and I-200 → untreated, using phenol as the inert tracer. The results are shown in Fig. 1 by plots of the electroosmotic mobility *versus* the fractional length of the capillary segment having the highest intrinsic EOF. This will be the untreated capillary segment in the F-2000 → untreated and I-200 → untreated tandem capillaries, and I-200 capillary in the F-2000 → I-200 coupled capillaries (see legend of Fig. 1 for details). As expected, the EOF changed linearly with the fractional length of the coupled capillaries and fitted quite well to eqn. 2 with correlation coefficients of 0.997 on average. The data points in Fig. 1 show clearly that the EOF can be changed in a predictable manner by varying the length of the connected capillary segment(s). The slope of these curves,

$$\mu_{eo} = \left(\mu_{eo,i} - \sum_{j=1;j\neq i} \mu_{eo,j}\right)\frac{l_i}{l_t} + \sum_{j=1;j\neq i} \mu_{eo,j} \left| 1 - \frac{\sum_{k=1;k\neq i,j} l_k}{l_t} \right| \quad (2)$$

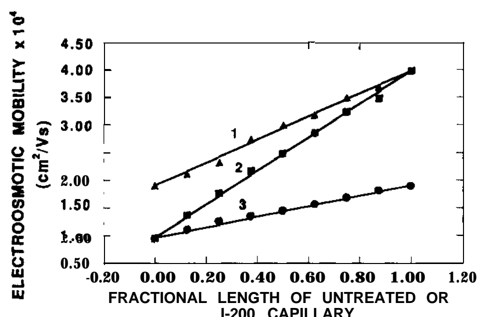

Fig. 1. Plots of the electroosmotic mobility *versus* the fractional length of the untreated capillary segment in (1) and (2) or *versus* the fractional length of the I-200 capillary segment in (3). (1) I-200 → untreated; (2) F-2000 → untreated; (3) F-2000 → I-200 tandem capillaries, 30 cm (to the detection point), 80 cm (total length) x 50 μm I.D.; running voltage. 20 kV; background electrolyte, 0.1 *M* sodium phosphate solution pH 6.50. lnert tracer, phenol.

$\mu_{eo,i} - \sum_{j=1;j\neq i}^{n} \mu_{eo,j}$, is indicative of the range over which the EOF can be varied. The greater the slope, the wider is the range of variation. For instance, and at the voltage of the experiments of Fig. 1, the F-2000 → untreated coupled capillaries with the highest slope allowed the tuning of the EOF to any value between 28 and 115 nl/min, while with the F-2000 → I-200 tandem capillary system the change in the EOF was limited to the interval 28 to 58 nl/min.

Evaluation of the F-2000 → I-200 coupled capillaries with proteins

An important feature of coupled capillaries is their ability to adjust the analysis time to suit a particular separation problem. To realize this attractive advantage, the tandem system should meet three major criteria: (i) solute-wall interactions must be absent, (ii) the coupled capillaries should not introduce band broadening and (iii) the connected capillary segments should allow the tuning of the EOF over a wide range to accommodate the electrophoresis of a broad range of analytes.

Although, our initial studies with the I-200 → untreated capillaries showed promise in the rapid and efficient analysis of acidic proteins [4], this system was not suitable for the simultaneous separation of both acidic and basic proteins. In fact. as the basic proteins entered the untreated segment of the I-200 → untreated tandem system, the EOF started to decrease during the electrophoretic run due to solute adsorption on the inner surface of the capillary, and concomitant decrease in the magnitude of the negative ζ potential of the capillary wall. This phenomenon drastically delayed and in some instances inhibited the elution of the later eluting acidic proteins.

A better approach that would enable the analysis of oppositely charged species in the absence of solute-wall interactions is the use of the F-2000 → I-200 coupled capillaries. The polyether chains of these coated capillaries have been shown effective in minimizing solute-wall interaction [6]. Furthermore, due to the difference in the length and the way in which the polyether chains are attached to the capillary inner surface, the F-2000 and I-200 capillaries possess different levels of electroosmotic flow [6]. Therefore, their coupling would allow the tuning of the EOF so that oppositely charged solutes could be analyzed in a single electrophoretic run with high separation efficiencies.

The feasibility of the F-2000 → I-200 tandem capillaries in the rapid analysis of biopolymers was demonstrated by using a mixture of 5 model proteins the p*I* values of which ranged from 4.8 to 11 .O (see Table I). Fig. 2a depicts a typical electropherogram of this protein mixture performed on an F-2000 capillary at an applied voltage of 20 kV using 0.1 *M* sodium phosphate solution, pH 6.50, as the running electrolyte. Due to the low electroosmotic flow obtained with the F-2000 capillary (*ca.* 28 nl/min), carbonic anhydrase was eluted after cu. 65 min while the elution of cc-lactalbumin would require 235 min as calculated from the EOF and the electrophoretic mobility of this solute. On the other hand, β-lactoglobulin A, having an electrophoretic mobility greater in magnitude and opposite in direction to the electroosmotic flow, would leave the F-2000 capillary and enter the inlet buffer reservoir after introducing the sample by gravity-driven flow. Although high separation efficiencies could be achieved, the F-2000 capillary can be only used for the analysis of basic and moderately acidic solutes. Fig. 2b and c illustrates the electropherograms of the same protein mixture performed on the F-2000 → I-200 coupled capillaries with 0.25 and 0.5 fractional length of the I-200 capillary segment. respectively. under other-

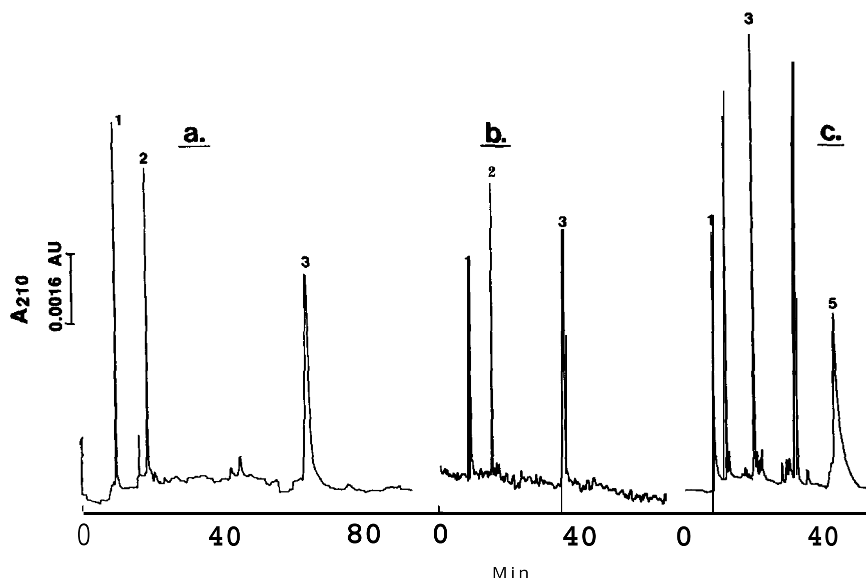

Fig. 2. Typical electropherograms of proteins obtained on F-2000 coated capillary in (a) and on tandem F-2000 → I-200 capillaries with 0.25 and 0.5 fractional length of the I-200 capillary segment in (b) and (c), respectively. Proteins: 1 = lysozyme; 2 = ribonuclease A; 3 = carbonic anhydrase; 4 = a-lactalbumin; 5 = β-lactoglobulin A. All other conditions are as in Fig. 1.

wise identical conditions as in Fig. 2a. It can be seen from these electropherograms that the analysis time of the model proteins decreased as the fractional length of the I-200 capillary increased. At 0.5 fractional length of the I-200 capillary, the EOF generated (43 nl/min) by the tandem capillaries was enough to bring about the analysis of all 5 proteins within 50 min.

Fig. 3 illustrates typical plots of the overall and electrophoretic mobilities of two proteins as well as plots of the electroosmotic mobility *versus* the fractional length of the I-200 capillary segment in F-2000 → I-200 coupled capillaries. In both cases, the electrophoretic mobility of the two test proteins remained practically unchanged while the overall and electroosmotic mobilities increased linearly and paralleled each other. This observation indicates that the variation in the analysis time of the model proteins is solely due to the change in the EOF, and no significant interaction between the proteins and the inner surface proper of the capillaries was present.

The effect of the tandem system on the bandwidth of the separated proteins was also investigated. Table II summarizes the plate height obtained with three model proteins at each fractional length stud-

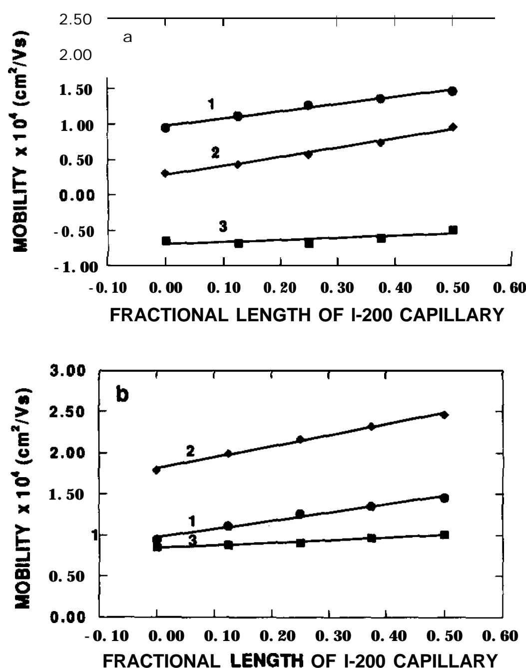

Fig. 3. Plots of overall (2) and electrophoretic (3) mobilities of (a) carbonic anhydrase and (b) lysozyme as well as plots of the electroosmotic (1) mobility of phenol *versus* the fractional length of the I-200 capillary segment in the F-2000 → I-200 tandem system. All other conditions are as in Fig. 1.

TABLE II

VALUES OF PLATE HEIGHT (H) MEASURED FROM SELECTED PROTEIN PEAKS

Protein	H (μm)				
	Fractional length of I-200 capillary in the F-2000 → I-200 system				
	0.00	0.125	0.25	0.375	0.50
Lysozyme	7.05	6.12	6.70	7.59	7.50
Ribonuclease A	3.50	4.10	5.00	5.80	6.67
Carbonic anhydrase	20.4	18.8	15.2	16.5	19.2
Average plate height[a]	10.3	9.67	8.97	9.96	11.1

[a] Average plate height was calculated for all three proteins at each fractional length. Conditions are as in Fig. 2.

ied. It can be seen from Table 11 that while the average plate height of the three model proteins remained almost the same, the analysis time decreased by a factor of cu. 2.0 at 0.5 fractional length of each of the connected capillaries. It is believed that shortening the residence time of the separated analytes has decreased the amount of molecular diffusion, and subsequently may have compensated for band broadening caused by the laminar flow profile.

Analysis of ovalbumin

Ovalbumin, the major protein constituent of egg white, is a heterogeneous protein due to the variation in its phosphate content [8]. About 75% of this protein possesses two phosphate groups per molecule of the protein, and is designated A,. The remaining ovalbumin consists largely of another component, A_2, having only one phosphate group per molecule. In addition, small amounts of phosphate-free component, A_3, are also present. Obviously,whenovalbuminisanalyzedbyelectrophoresis it shows different bands [9].

Therefore, ovalbumin was a good example to evaluate the effect of the magnitude of EOF as far as the quality of the analytical information generated during CZE separation is concerned. In other words, the goal was to determine whether there would be a loss in the analytical information when the speed of separation is increased. On this basis two tandem capillary systems of moderate and relatively high EOF were evaluated. As demonstrated in Fig. 1, F-2000 → I-200 offers a tunable flow over a moderate range whereas F-2000 → untreated allows a higher EOF to be realized. Fig. 4a and b represents typical electropherograms of ovalbumin performed on the F-2000 → I-200 and F-2000 → untreated tandem capillaries, respectively. Similarly to previous observations in free-boundary electrophoresis [9], this protein showed different electrophoretic components due to the variation in its phosphorous content. Comparison of Fig. 4a and b reveals that both electropherograms clearly show all the differently phosphorylated components of ovalbumin; the only difference is that the analysis time on the F-2000 → untreated system was decreased by a factor of *ca.* 4.0. Thus, while drastically decreasing the analysis time, the analytical information remained almost the same when going from low to high EOF by the use of coupled capillaries.

Rapid separation of proteins by stepwise increase in the EOF

The analysis time of oppositely charged proteins was further decreased by increasing the EOF during the electrophoretic run. This was carried out by switching manually from one tandem capillary system of relatively low EOF to another set of coupled capillaries of higher EOF, see Fig. 5. At the beginning of the run, the tandem system consisted of three capillary segments connected in series and containing F-2000, I-200 and untreated capillary at

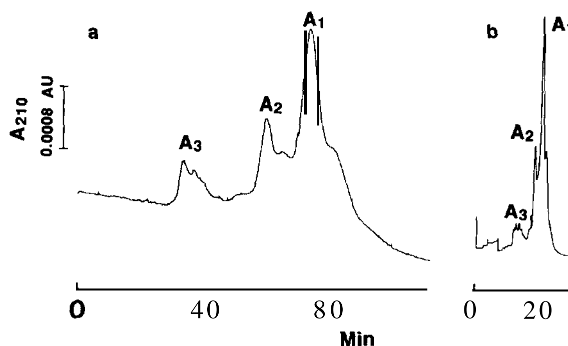

Fig. 4. Typical electropherograms of ovalbumin obtained on (a) F-2000 → I-200 and (b) F-2000 → untreated at 0.5 fractional length of the I-200 and untreated capillary, respectively. All other conditions are as in Fig. 1.

0.5, 0.25 and 0.25 fractional lengths, respectively. Thereafter, a stepwise increase in EOF was carried out by connecting in series F-2000 and untreated capillary at 0.5 fractional length each. In both tandem capillary systems, the F-2000 capillary having the most inert surface toward proteins, was selected as the separation capillary. Under these conditions, the EOF was increased stepwise from 58.2 to 72.5 nl/min. Fig. 5 is a typical electropherogram of the five model proteins using the stepwise increase in the EOF. The arrow in Fig. 5 indicates the time at which the stepwise increase in EOF was performed. It should be noted that the voltage was turned off at the time the first set of coupled capillaries was disconnected and replaced by the untreated capillary segment. This manual operation required less than 20 s.

As can be seen in Fig. 1, a relatively high level of EOF can only be achieved with the incorporation of an untreated capillary segment in the tandem capillaries. But, no basic solute should come into direct contact with the inner surface of the untreated fused-silica capillary to avoid solute-wall interactions, and in turn the unpredictable change in the level and polarity of EOF. For this reason, the length of the I-200 segment in the first set of coupled capillaries was adjusted to trap all the basic proteins as they elute from the fuzzy capillary after being separated and detected. Thus, while the I-200 segment provided the shielding from wall interaction, its presence in conjunction with the untreated segment afforded a moderate EOF. Once all basic solutes entered the I-200 capillary, a stepwise increase in EOF was performed whereby both the I-200 and untreated capillaries were disconnected and replaced by one untreated segment at 0.5 fractional length to speed the net migration of the acidic solutes.

The above elution schemes involving two-step coupled capillaries yielded an increase in the EOF by a factor of 1.35 in I and 1.69 in II (see Fig. 5), when compared to the F-2000 → I-200 tandem capillary system at 0.5 fractional length of the I-200 capillary segment in Fig. 2c. Furthermore, comparison of Figs. 2a and 5 reveals that the average plate height for lysozyme, ribonuclease A and carbonic anhydrase increased slightly from 10.3 to 12.5 μm, a factor of 1.2, while the migration time for these proteins decreased by factors of 1.9, 2.4 and 4.7, respectively. The slight increase in the plate height

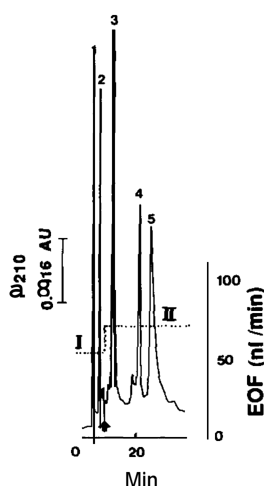

Fig. 5. Rapid capillary zone electrophoresis of proteins with stepwise increase in the electroosmotic flow. I, initial flow: F-2000 → I-200 → untreated coupled capillaries at 0.5, 0.25 and 0.25 fractional length of the F-2000, I-200 and untreated capillaries, respectively. II: F-2000 → untreated capillaries at 0.5 fractional length each. All other conditions are as in Fig. 1. The arrow is to indicate the time at which the stepwise increase in EOF occurred.

may be attributed to the presence of a more pronounced laminar flow as a result of the large difference between the bulk flow and the intrinsic EOF in the F-2000 capillary segment.

To develop this concept further and permit the realization of several stepwise increases in the EOF during analysis, a multiple capillary device which will allow the switching between several coupled capillary systems having different electroosmotic flow characteristics is under development [10].

The two-step tandem capillaries provided the rapid analysis of proteins at relatively moderate applied voltage. To obtain nearly the same EOF (i.e., 65.0 nl/min) with the F-2000 capillary as with the two-step coupled capillaries, the F-2000 capillary had to be operated at an applied voltage of 28 kV whereby the current was relatively high (155 μA). Fig. 6 illustrates a typical electropherogram of the protein mixture obtained at high voltage with F-2000 capillary. As can be seen in Fig. 6, with the exception of ribonuclease A the proteins underwent severe sample degradation. The peak of β-lactoglobulin A disappeared, lysozyme eluted as a tailing peak and only short peaks were observed for

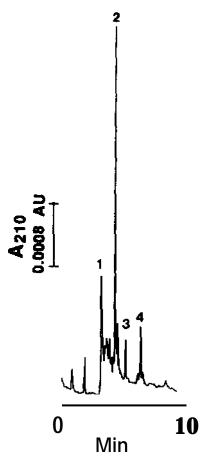

Fig. 6. Electropherogram of model proteins on fuzzy 2000-coated capillary at an applied voltage of 28 kV. All other conditions are as in Fig. 2a.

carbonic anhydrase and a-lactalbumin. These observations may be attributed to system overheating [11,12] and/or to the binding of the proteins to the coating proper by hydrophobic interactions at elevated temperatures due to heat-induced conformational changes as previously shown in liquid chromatography [13,14]. Very recently, it has been shown that capillary temperature can dramatically affect the electrophoretic patterns of proteins in CZE via the reduction of the structural metal in metalloproteins and by inducing conformational changes [15].

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