Rapid separation of peptides and proteins by isocratic capillary electrochromatography at elevated temperature

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Abstract

The use of capillary electrochromatography (CEC) for the separation by isocratic elution of synthetic peptides, proteins as well as the tryptic digest of cytochrome *c* has been demonstrated. The monolithic porous stationary phase was prepared from silanized fused-silica capillaries of 75 μm I.D. by in situ copolymerization of vinylbenzyl chloride and ethylene glycol dimethacrylate in the presence of propanol and formamide as the porogens. The chloromethyl groups at the surface of the porous monolith were reacted with *N,N*-dimethylbutylamine to form a positively charged chromatographic surface with fixed *n*-butyl chains. Results of studies on the influence of temperature and mobile phase composition on the retention and selectivity of separation by CEC demonstrated the feasibility of rapid polypeptide analysis and tryptic mapping at elevated temperature with high resolution and efficiency. Typically the chromatography of a tryptic digest of cytochrome *c* took about 5 min at 55°C and 75 kV/m with hydro–organic mobile phases containing acetonitrile in 50 mM phosphate buffer, pH 2.5. For peptides and proteins plots of logarithmic k'_{cec} against acetonitrile concentration were nonlinear, whereas Arrhenius plots for the mobilities were nearly linear. Comparison of the separation of such samples under conditions of CEC and capillary zone electrophoresis (CZE) indicates that the mechanism of separation in CEC is unique and leads to a chromatographic profile different from that obtained by CZE.   © 2001 Elsevier Science B.V. All rights reserved.

1. Introduction

Despite the recent interest in capillary electrochromatography (CEC) at large, the separation of proteins and peptides by CEC has received scant attention. Only a few reports discussed the separation of proteins by using open tubular column [1,2] or by gradient elution with monolithic column [3]. The work presented here is the continuation of our earlier work on the CEC of peptides and proteins employing various types of granular or monolithic packed capillary columns described below [4–9].

CEC separation of proteins was first carried out in our laboratory with a novel porous layer open tubular (PLOT) column prepared by in situ polymerization of vinylbenzyl chloride (VBC) and divinylbenzene (DVB) and followed by functionalization of the chromatographic surface with *N,N*-di-methyldodecylamine [4]. Subsequently two kinds of packed monolithic columns were prepared with styrenic [5] and acrylic [6] supports and functionalized with alkyl amines for separating proteins and

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synthetic peptides. Furthermore novel columns packed with silica beads, which had strong anion (SAX) or weak cation (WCX) exchanger groups at the surface, were prepared by reacting the siliceous support first with a bifunctional reagent and then with a vinyl monomer containing quaternary ammonium [7] or carboxylic functions [8], respectively. The separations of proteins by CEC was carried out with high efficiency on the SAX column while the WCX column was used for the separation of angiotensin type peptides with favorable results. A novel method for the coating of the tube innerwall was also introduced to facilitate CEC of peptides and proteins [9].

In the monolithic columns described above the chromatographic surface had both fixed positive charges for generating electrosmotic flow (EOF) and alkyl moieties serving as hydrophobic binding sites. In this report we describe the preparation of a new column with porous styrenic monolith. The heteropolymeric porous support was prepared in situ by copolymerization of VBC and ethylene glycol dimethacrylate (EGDMA) with a suitable porogen. Finally the chromatographic surface was formed in a reaction with N,N-dimethylbutylamine. This styrenic column was compared to the acrylic column prepared in similar way and described earlier [6] and the chromatographic properties of the two columns were also rather similar. For this reason the styrenic column was used in the present study to explore the possibility of rapid separation of peptides and proteins by CEC at elevated column temperatures. Although the significance of temperature as a major operational variable in liquid chromatography was recognized already in the 1960s [10] and reaffirmed in the reversed-phase chromatography of proteins [11,12], the use of elevated temperature in CEC elicited only little attention [13,14]. In order to gain further insight into the effect of the temperature in capillary electrochromatography, experiments were carried out at elevated temperature and the results will be presented in this work.

2. Experimental

2.1. Materials

Fused-silica capillary tubing of 75 μm I.D. × 375

μm O.D. with a polyimide outer coating was purchased from Quadrex Scientific (New Haven, CT, USA). 3-(Trimethoxysilyl) propyl methacrylate and EGDMA were from Polysciences (Warrington, PA, USA). 2,2-Diphenyl-1-picryhydrazyl hydrate (DPPH), glycidyl methacrylate (GMA), methyl methacrylate (MMA), N,N-dimethylbutylamine (99%), formamide, tris(hydroxymethyl)aminomethane (Tris), calcium chloride and acetic acid were from Aldrich (Milwaukee, WI, USA). VBC was from Dow (Midland, MI, USA). Analytical reagent-grade monobasic, dibasic and tribasic sodium phosphates, dimethylformamide (DMF) (99%) and hydrochloric acid were from J.T. Baker (Phillipsburg, NJ, USA) and azobisisobutyronitrile (AIBN) (98%) from Pfaltz & Bauer (Waterbury, CT, USA). Insulin and ribonuclease A (bovine pancreas), α-lactalbumin (bovine milk), myoglobin (horse skeletal muscle), bovine serum albumin (BSA), cytochrome c (horse heart), morphiceptin, substance P (free acid), bradykinin (acetate salt), angiotensin I (acetate salt), angiotensin II (acetate salt), [Sar[1]Ala[8]] angiotensin II were purchased from Sigma (St. Louis, MO, USA). Trypsin [bovine, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated] was from Worthington (Freehold, NJ, USA). Dimethyl sulfoxide (DMSO) was purchased from Burdick & Jackson (Muskegon, MI, USA). Phosphoric acid (85%) and sodium hydroxide (98.8%) were of analytical reagent grade from Mallinckrodt (Paris, KY, USA). HPLC-grade methanol, acetone, acetonitrile (ACN), propanol and methylene chloride were purchased from Fisher (Fair Lawn, NJ, USA). The materials were used without further purification. Water was purified and deionized with a NANOpure system (Barnstead, Boston, MA, USA).

The protein digest was prepared as follows: A 2.0 mg/ml solution of cytochrome c in 50 mM Tris–HCl, 1 mM CaCl$_2$ (pH 8.1) was incubated with trypsin at a substrate-to-enzyme ratio of 50:1 at 37°C for 4 h. Digestion was halted by acidifying the solution to pH 4 by adding acetic acid and then storing the digest at −20°C.

2.2. Column preparation

2.2.1. In situ polymerization
The pretreatment and the silanization procedure

for the fused-silica capillary were the same as described previously [9]. A solution containing 20% (v/v) each of vinylbenzyl chloride and ethylene glycol dimethacrylate, 40% (v/v) of *n*-propanol and 20% (v/v) of formamide as well as 0.3% (w/v) of the initiator (AIBN) was prepared and degassed with nitrogen for 10 min. The solution was filled into the silanized capillary. After both ends were sealed by butane flame of a Veriflo Air-gas Torch (Macalaster Bicknell, New Haven, CT, USA), the capillary tube was heated at 75°C for 16 h in the oven of a Model Sigma 2000 gas chromatograph (Perkin-Elmer, Norwalk, CT, USA). Subsequently, the column was purged with nitrogen to remove *n*-propanol and formamide. The capillary was washed with methanol and then heated at 120°C for 2 h with a nitrogen stream.

2.2.2. Functionalization of the chromatographic surface

The capillary containing the porous monolithic support was filled with *N,N*-dimethylbutylamine and heated at 70°C for 30 min. Subsequently it was washed with methanol and dried with a nitrogen stream.

2.2.3. Preparation of the detection window

A 1–2 mm wide segment of the polyimide outer coating was scraped off using a small blade. The polymeric packing inside of the segment was removed by burning it off with a butane fueled Archer Torch Model B microtorch (Radio Shack, New Haven CT, USA), while the tube was purged with oxygen at 160 p.s.i. (1 p.s.i.=6.895 kPa) inlet pressure. Subsequently, the column was washed with acetonitrile.

2.3. Capillary electrochromatography: apparatus and procedure

The experiments were conducted using a Model MDQ P/ACE system capillary electrophoresis unit equipped with a P/ACE system MDQ photodiode array detector (Beckman, Fullerton, CA, USA). A Model 6588-12U IBM personal computer with Beckman MDQ capillary electrophoresis software version 2.2. and Windows 95 (Microsoft, Redmond, WA, USA) installed was used to control the instrument and to acquire and process the data.

In all experiments 20–70 m*M* aqueous sodium phosphate buffers, pH 2.5, containing acetonitrile of various concentrations were used. The proteins were dissolved in 20 m*M* sodium phosphate buffer, pH 2.5, to obtain a solution containing 1 mg/ml of each protein. Concentration of each peptide in 20 m*M* sodium phosphate buffer, pH 2.5, was 0.2 mg/ml. The samples were injected at −2 kV for 2 s. Between runs the column was rinsed with acetonitrile for 10 min followed with the running mobile phase for 20 min at 100 p.s.i. inlet pressure. Then with both ends pressurized at 80 p.s.i. the column was equilibrated electrokinetically at the operating voltage for 5 min. The EOF marker, DMSO, at a concentration of 2 μl/ml in water, was injected at −2 kV for 1 s. All samples were detected at 214 nm. All experiments were carried out at 25°C unless otherwise specified.

2.4. Comparison of columns with styrenic and acrylic monoliths

Two columns were prepared by the same procedure described in Sections 2.1–2.2 but using monomer mixtures of different composition as shown in Table 1. The main difference is that the monofunctional monomer component of the mixture is VBC in the styrenic column (Column A) and GMA in the acrylic column (Column B). Fig. 1 shows chromatograms of proteins obtained in CEC and it can be seen that despite some modest selectivity differences both columns yield good separation of the proteins under identical operating conditions. Since the properties of the acrylic column have been described [6], in the following sections of the present study the styrenic column will be used exclusively.

Table 1
Comparison of the monomer mixtures employed in the preparation of styrenic Column A and acrylic Column B[a]

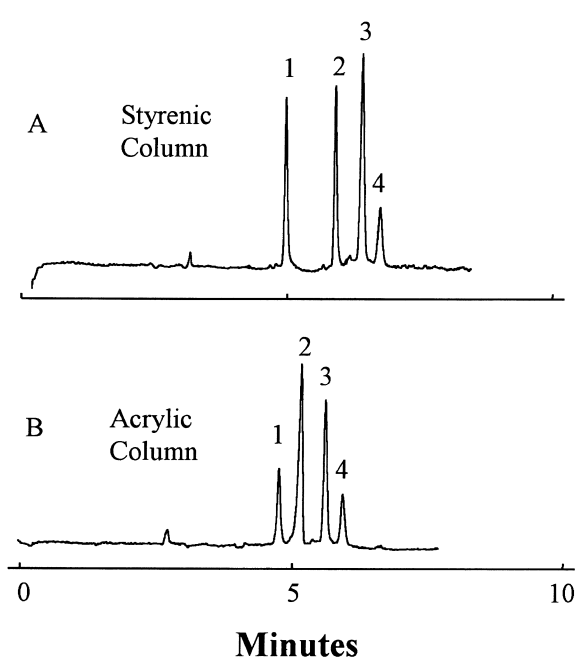| Components of the monomer mixture (%, v/v)	Styrenic column A	Acrylic column B
VBC	20	0
GMA	0	10
MMA	0	10
EGDMA	20	20
Propanol	40	15
Formamide	20	45

[a] The acronyms are defined in Section 2.1.

Fig. 1. Comparison of CEC of proteins with two columns which differ mainly in the chemical nature of the polymeric monolithic support. (A) Styrenic column, 40 cm (effective length 30 cm)×75 μm, fused-silica with VBC–EGDMA monolith having quaternary ammonium functions; (B) acrylic column, 40 cm (effective length 30 cm)×75 μm, fused-silica with GMA–MMA–EGDMA monolith having tertiary amino functions. Mobile phase, 30% (v/v) acetonitrile in 60 mM phosphate buffer, pH 2.5; applied voltage, −30 kV; detection, 214 nm; temperature, 25°C. Peaks: (1) insulin, (2) ribonuclease A, (3) α-lactalbumin, (4) myoglobin.

3. Results and discussion

3.1. Theory

In CEC the overall mobility of a charged sample component, μ_{cec}, is expressed [15] as:

$$\mu_{cec} = (\mu_{eof} \pm \mu_{ep})/(1 + k'_{lc}) \tag{1}$$

where μ_{eof} is the EOF mobility and μ_{ep} is the electrophoretic mobility of the migrant whereas k'_{lc} is the chromatographic retention factor. The sign in the numerator of the right hand side is positive in codirectional and negative in counterdirectional CEC. In the following we shall use the notation for counterdirectional CEC.

Another way to express μ_{cec} is:

$$\mu_{cec} = \mu_{eof}/(1 + k'_{cec}) \tag{2}$$

where k'_{cec} is the CEC migration factor [6] of the migrant under consideration.

By combining Eqs. (1) and (2) we can express the CEC migration factor, which is formally analogous to the chromatographic retention factor:

$$k'_{cec} = (1 + \mu_r) k'_{lc} + \mu_r \tag{3}$$

where μ_r is called the reduced mobility [16,17] so that:

$$\mu_r = \mu_{ep}/(\mu_{eof} - \mu_{ep}) \tag{4}$$

Inspection of Eqs. (3) and (4) shows that μ_r measures the relative contributions of chromatographic and electrophoretic migration to the overall migration factor. In the limiting case where the solute does not have electrophoretic mobility, μ_r is zero, then the migration is solely chromatographic, i.e., $k'_{cec} = k'_{lc}$. Alternatively, when the migrant does not have chromatographic retention, i.e., $k'_{lc} = 0$, then k'_{cec} equals the reduced mobility μ_r.

In the following the dependence of the electrokinetic mobilities, which are expected to reflect mainly changes in the viscosity of the medium, on the temperature is examined. The EOF mobility in the interstices of a packed column [18,19] has been expressed as:

$$\mu_{eof} = \epsilon\epsilon_0 \zeta_s/\eta \tag{5}$$

where ϵ is the dielectric constant of the medium, ϵ_0 is the permitivity of the vacuum, η is the viscosity of the bulk solution and ζ_s is the zeta potential at the surface of the packing. Despite its simplicity Eq. (5) has been found useful to evaluate the EOF mobility in packed beds having sufficiently wide pores.

Under the experimental conditions of this study, the temperature dependence of the viscosity of the mobile phase can be described by the Andrade equation [20] as:

$$\eta = A \exp(B/T) \tag{6}$$

where A and B are empirical constants for a given liquid and T is the absolute temperature.

Neglecting changes in the pH, the dielectric constant of the medium and the zeta potential at the

surface with the temperature, the dependence of μ_{eof} and μ_{ep} can be expressed as [21]:

$$\mu_{eof} = A_{eof} \exp(-B_{eof}/T) \tag{7}$$

and

$$\mu_{ep} = A_m \exp(-B_m/T) \tag{8}$$

In Eq. (7) the pre-exponential factor, A_{eof}, depends on the dielectric properties of the buffer and the zeta potential at the surface of the packing. On the other hand, in Eq. (8) A_m reflects the dielectric properties of the buffer and the molecular properties of the migrant of interest. Parameters B_{eof} and B_m represent the activation energies associated with the migration process. In most cases A_{eof} and A_m can be regarded as constants at a given temperature.

3.2. Effect of organic strength on CEC separation of peptides and proteins

The effect of the organic strength of the mobile phase on the CEC separation of peptides and proteins was investigated by varying the acetonitrile concentration from 0 to 80% in aqueous phosphate buffer, pH 2.5. The separation was performed at -30 kV (reversed polarity) with the monolithic column that was prepared from silanized fused-silica capillaries of 75 μm I.D. by in situ copolymerization of a mixture of vinylbenzyl chloride and ethylene glycol dimethacrylate in the presence of propanol and formamide as the porogens. The chloromethyl groups at the surface of the porous monolith were reacted with N,N-dimethylbutylamine to form a positively charged chromatographic surface for generating anodic electrosmotic flow upon applying the electric field. The surface bound butyl chains served as the retaining sites for the chromatographic separation of peptides and proteins. The peptide mixture contained insulin, angiotensin II, angiotensin I and [Sar[1],Ala[8]]angiotensin II while the protein mixture consisted of insulin, α-lactalbumin and myoglobin. The dependence of EOF mobility on the acetonitrile concentration from 0 to 80% in 60 and 70 mM phosphate buffer, pH 2.5, is illustrated in Fig. 2. It is seen that in this range of acetonitrile concentration the EOF mobility was practically invariant.
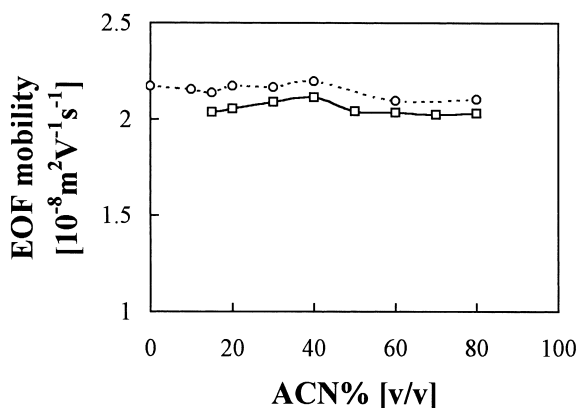

Fig. 2. Plots of the EOF mobility measured with DMSO as the unretained neutral marker against the acetonitrile concentration in the eluent. Column, 40 cm (effective length 30 cm)×75 μm, fused-silica with styrenic monolith having quaternary ammonium functions; Mobile phase, acetonitrile (%, v/v) in (○) 60 (□) 70 mM sodium phosphate buffer, pH 2.5; temperature, 25°C; applied voltage, -30 kV; detection, 214 nm.

Electrochromatograms showing the isocratic separation of peptides with mobile phase having ACN concentrations ranging from 0 to 30% are illustrated in Fig. 3. On the other hand, Fig. 4 shows the electrochromatograms of proteins obtained with eluents having ACN concentration in the range from 15 to 40%. As expected, the organic strength of the mobile phase affect not only the migration time but also the selectivity of the separation. This is illustrated in Fig. 3 by the selectivity reversal of insulin and angiotensin II when the acetonitrile concentration was increased from 10 to 20%. As far as the separation of proteins is concerned, it can be seen from Fig. 4 that with increasing acetonitrile concentration the proteins elute in the order of increasing hydrophobicity.

In order to gain insight of the separation mechanism under these conditions, the migration of peptides and proteins in CEC was measured with different acetonitrile concentrations in the eluent at -30 kV applied voltage and 25°C. The results are illustrated in Fig. 5 that reveals that plots of the logarithmic migration factors against the acetonitrile concentration are non-linear. The non-linear dependence of the migration factor as well as that of the logarithmic migration factor on acetonitrile concentration could be explained in the light of Eq. (3). In
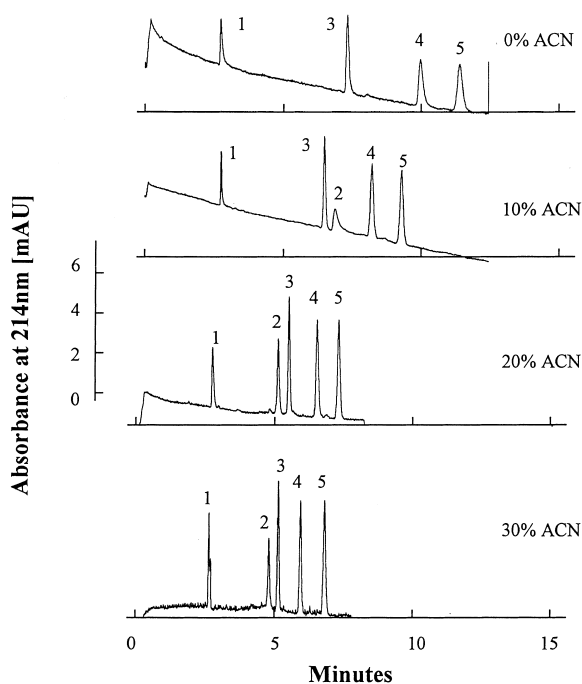
Fig. 3. Capillary electrochromatograms of four peptides obtained under isocratic elution conditions. Mobile phase, 60 mM phosphate buffer, pH 2.5, containing different concentrations of ACN; other conditions as in Fig. 2. Sample: (1) DMSO, (2) insulin, (3) angiotensin II, (4) angiotensin I, (5) [Sar[1],Ala[8]]angiontensin II.
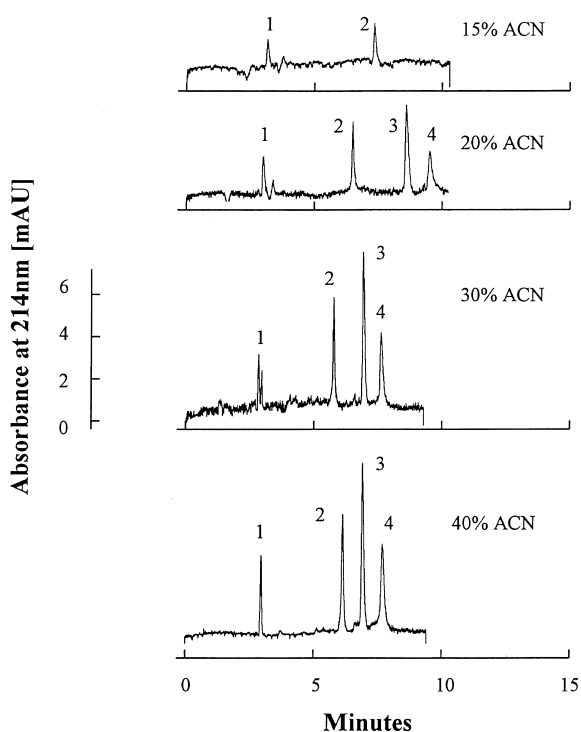

Fig. 4. Capillary electrochromatograms of three proteins obtained under isocratic elution conditions. Mobile phase, 70 mM phosphate buffer, pH 2.5, containing different concentrations of ACN; other conditions as in Fig. 2. Sample: (1) DMSO, (2) insulin, (3) α-lactalbumin, (4) myoglobin.

our CEC system the separation is brought about by a dual mechanism involving both chromatographic retention and electrophoretic migration. When the organic strength of the mobile phase is low, the migrants are strongly retained by the stationary phase so that the magnitude of k'_{lc} plays an important role in determining the rate of migration. At high organic strength of the mobile phase, chromatographic retention is low and k'_{lc} assumes very small values. In this case only the second term on the right hand side of Eq. (3) affects the value of k'_{cec}. The above argument is supported by the results shown in Fig. 5a where the k'_{cec} of peptides decreased almost linearly with increasing ACN concentration from 0 to 20% and then became plateaus in the range of 20 to 60% acetonitrile concentration in the mobile phase. For the separation of proteins Fig. 5b shows similar trend for the dependence of k'_{cec} on the acetonitrile concentration.

However, as shown in Fig. 5, the chromatographic retention factor is a weaker function of the acetonitrile concentration than in reversed-phase HPLC [22]. The results apparently contradict the tenet first enunciated by Tiselius [23] that the retention factors of large molecules decrease faster than those of small molecules with increasing eluent strength. The apparent contradiction arises from the relatively much greater complexity of the CEC separation process than the simple chromatographic processes such as ion-exchange or reversed-phase chromatography. As it was mentioned before, the meaning of the overall migration factor, k'_{cec}, is not comparable to the retention factor in HPLC except that both can be used as a dimensionless peak locator in the ways shown here to explore the dependence of the migration behavior on the operating conditions.
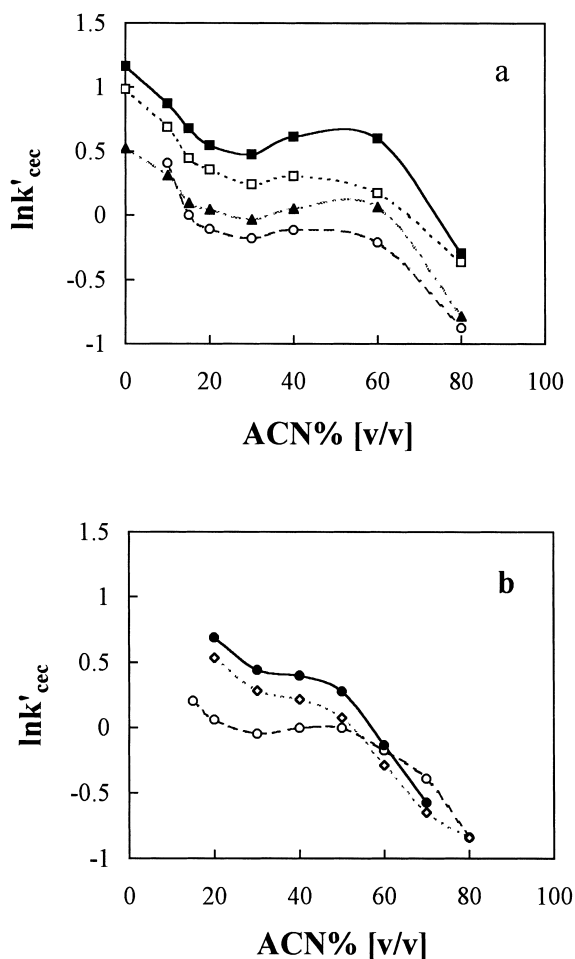
Fig. 5. Plots of the logarithmic CEC migration factors against the acetonitrile concentration in the eluent. (a) Peptides, mobile phase, ACN (%) in 60 mM phosphate buffer, pH 2.5; sample: (○) insulin, (▲) angiotensin II, (□) angiontensin I, (■) [Sar[1],Ala[8]]angiotensin II. (b) Proteins, mobile phase, ACN% in 70 mM phosphate buffer, pH 2.5; sample: (○) insulin, (◇) α-lactalbumin, (●) myoglobin; other conditions as in Fig. 2.

3.3. Selectivity of protein separation by CEC and capillary zone electrophoresis (CZE)

For CEC to become a widely used separation technique it is essential to exhibit selectivity different from that shown by CZE. In order to make such a comparison, a mixture of three proteins was separated by using (a) open fused-silica capillaries in raw and in poly(vinyl alcohol) (PVA)-coated forms in CZE, and (b) a styrenic monolithic column with quaternary ammonium functions on the chromatographic surface, which was used throughout this study, in CEC. The settings of the operational conditions were the same in all three cases. The resulting electrochromatogram and two electropherograms of the mixture of the three proteins are shown in Fig. 6 whereas the column efficiency is summarized in Table 2. The selectivity, α_{21}, of the chromatographic system for migrants 1 and 2 is given by the ratio of the corresponding migration factors as:

$$\alpha_{21} = k_2'/k_1'$$

where subscripts 1 and 2 refer to the faster and the slower migrating component, respectively. Thus it conforms to the convention that the selectivity in a separation process is never less than unity.

In CZE, the selectivity or separation factor can be expressed as [15]:

$$\alpha_{21} = \mu_{ep,1}/\mu_{ep,2}$$

where $\mu_{ep,1}$ and $\mu_{ep,2}$ are the mobilities of the two components and the selectivity, α_{21}, is by convention greater than unity. The results in Table 2 and Fig. 6 show that the counterdirectional CEC system with the monolithic column having positively charged chromatographic surface exhibits high separation efficiencies both in terms of band spreading and selectivity.

3.4. Effect of temperature on the CEC separation of peptides and proteins

More than often the speed of chromatographic separations can be increased by increasing the temperature [12] and thus enhancing the transport properties, such as diffusivity and fluidity. Here we make an attempt to explore the effect of elevated temperature on the separation of peptides and proteins by CEC using the monolithic capillary column in the range from 25 to 55°C. The results are shown in Fig. 7 by plots of the electrosmotic mobility against the reciprocal absolute temperature upon varying the acetonitrile concentration from 10 to 30% in 60 mM phosphate buffer, pH 2.5. The EOF velocity was
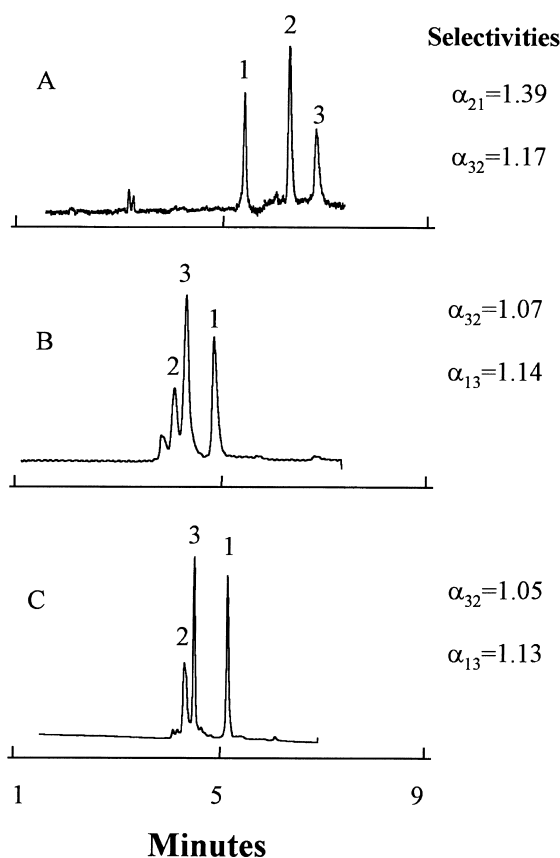
Fig. 6. Chromatograms illustrating the separation of three proteins by CEC and CZE. The selectivities of the three methods for the proteins are also shown. (A) CEC, 40 cm (effective length 30 cm)×75 μm, fused-silica with monolith having quaternary ammonium functions; (B) CZE, 40 cm (effective length 30 cm)×75 μm, raw fused-silica capillary; (C) CZE, 40 cm (effective length 30 cm)×75 μm, PVA coated fused-silica capillary. Mobile phase, 30% (v/v) acetonitrile in 70 mM phosphate buffer, pH 2.5; injection, −2 kV for 1 s for CEC and 0.2 p.s.i. for 2 s for CZE; applied voltage, −30 kV for CEC and +30 kV for CZE; detection, 214 nm. Peaks: (1) insulin, (2) α-lactalbumin, (3) myoglobin.
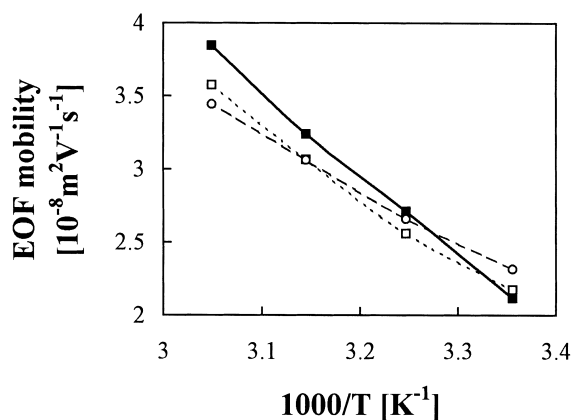

Fig. 7. Plots of the EOF mobility, measured with DMSO as the unretained neutral marker, against the reciprocal absolute temperature. Mobile phase, (■) 10% ACN, (□) 20% ACN, (○) 30% ACN in 60 mM sodium phosphate buffer, pH 2.5; other conditions as in Fig. 2.

measured with DMSO as the marker at −30 kV of applied voltage. It is seen that the EOF velocity increased almost linearly from 1.95 to 2.90 mm/s when the temperature was raised from 25 to 55°C. This increase is attributed mainly to a decrease in the viscosity of the mobile phase with increasing temperature.

The effect of temperature on the CEC separation of peptides and proteins in the range from 25 to 55°C is illustrated in Figs. 8 and 9, respectively. The peptide mixture contained insulin, angiotensin II, angiotensin I and [Sar[1],Ala[8]]angiotensin II while the protein mixture consisted of insulin, α-lactalbumin, myoglobin and bovine serum albumin. The chromatograms were obtained by isocratic elution at pH 2.5 with 30% acetonitrile in 60 mM phosphate buffer for peptides and 70 mM for proteins. As shown by the chromatograms, an increase of the temperature

Table 2
Plate efficiency of the columns used for protein separation by CEC and CZE[a]

Proteins	Peak number	Number of theoretical plates per column		
		CEC	CZE–raw	CZE–PVA
Insulin	1	49 614	11 022	61 862
α-Lactalbumin	2	38 276	5 237	14 676
Myoglobin	3	35 923	8 163	50 229

[a] The mobile phase was the same in both cases, 30% (v/v) acetonitrile in 70 mM phosphate, pH 2.5. The other conditions were the same as in Fig. 6.

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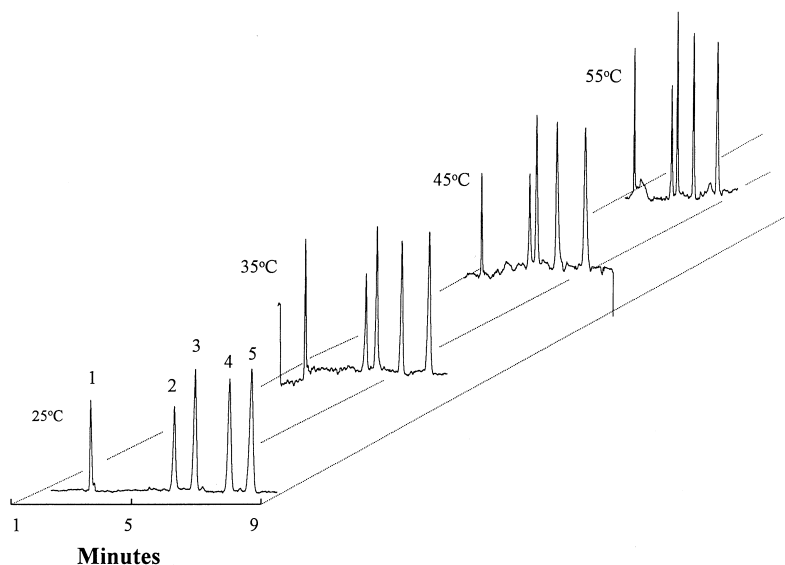
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Fig. 8. Capillary electrochromatograms illustrating the effect of temperature on the separation of peptides. Mobile phase, 30% ACN in 60 mM phosphate buffer, pH 2.5; other conditions as in Fig. 2. Sample: (1) DMSO, (2) insulin, (3) angiotensin II, (4) angiotensin I, (5) [Sar[1],Ala[8]]angiontensin II.

from 25 to 55°C resulted in an almost 2-fold increase in the speed of analysis. This effect is similar to that observed in reversed-phase chromatography upon raising the column temperature [24]. Arrhenius plots of logarithmic μ_{cec} against the reciprocal absolute temperature for CEC of peptides and proteins are shown in Fig. 10. As expected the plots were nearly straight lines. The slopes, and the activation energies
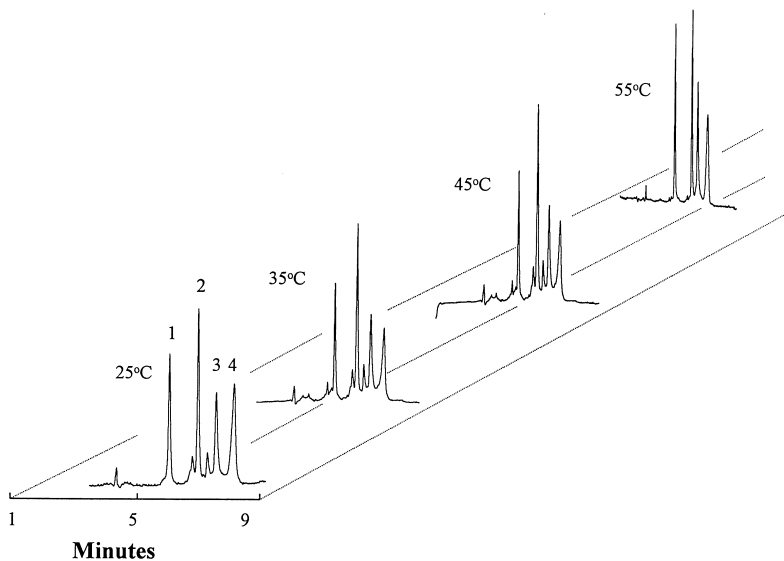
Fig. 9. Capillary electrochromatograms illustrating the effect of temperature on the separation of proteins. Mobile phase, 30% ACN in 70 mM phosphate buffer, pH 2.5; the other conditions were the same as in Fig. 2. Sample: (1) insulin, (2) α-lactalbumin, (3) myoglobin, (4) BSA.
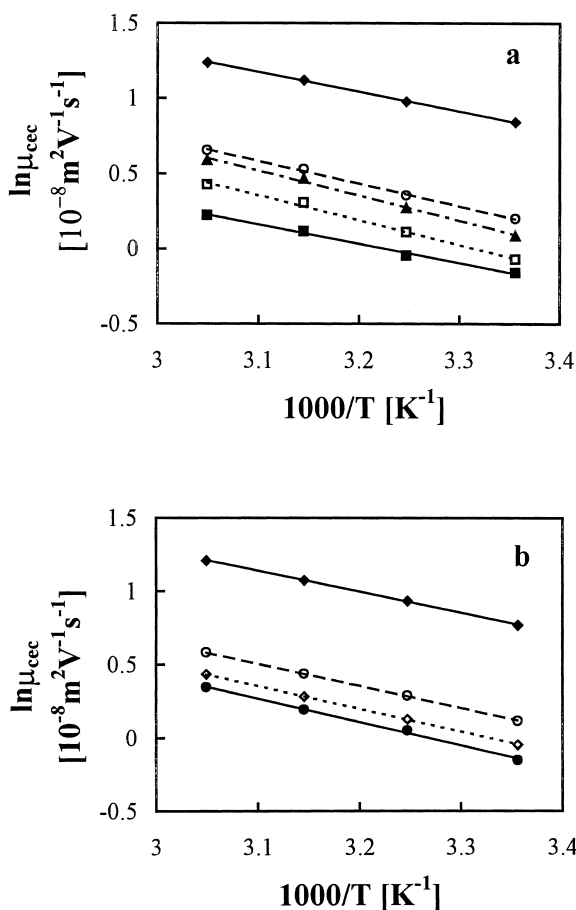
Table 3
Slopes and activation energies derived from the plots in Fig. 10

	Slope	Activation energy (kJ/mol)
Insulin	−1.507	12.53
Angiotensin II	−1.671	13.89
Angiotensin I	−1.655	13.76
[Sar[1],Ala[8]]Angiotensin II	−1.283	10.67
Insulin	−1.515	12.60
α-Lactalbumin	−1.560	12.97
Myoglobin	−1.596	13.27

Fig. 10. Arrhenius plots of logarithmic μ_{cec} against reciprocal temperature for CEC of peptides and proteins. (a) Peptides, mobile phase, 30% ACN in 60 mM phosphate buffer, pH 2.5; sample: (♦) DMSO, (○) insulin, (▲) angiotensin II, (□) angiotensin I, (■)[Sar[1],Ala[8]]angiontensin II. (b) Proteins, mobile phase, 30% ACN in 70 mM phosphate buffer, pH 2.5; sample: (♦) DMSO, (○) insulin, (◇) α-lactalbumin, (●) myoglobin. Other conditions as in Fig. 2.

derived from the slopes, for various migrants are listed in Table 3.

3.5. Effect of temperature on the migration factor for separation of peptides and proteins by CEC

It is believed that in CEC systems such as that described here, chromatographic retention is governed predominantly by solvophobic interactions [6]

or by electrostatic interactions that can be conveniently modulated by appropriately adjusting the organic or ionic strength of the mobile phase or both. The column under investigation in this study falls into the first category and its retentive behavior is modulated by the concentration of acetonitrile in the hydro–organic mobile phase. For this reason, it is of interest to plot the logarithm of the overall migration factor, k'_{cec}, against the reciprocal absolute temperature. In order to do so, data were obtained with peptides and proteins by using mobile phases lean and rich in acetonitrile, the mobile phase modulator. The peptide results in Fig. 11a were obtained with insulin and angiotensin II and the mobile phase contained 15 or 45% (v/v) ACN in 60 mM phosphate buffer, pH 2.5. The proteins (Fig. 11b) in these experiments were α-lactalbumin and myoglobin and the mobile phase had 20 or 50% (v/v) ACN in 70 mM phosphate buffer, pH 2.5.

Examination of the data reveals that the van't Hoff/Arrhenius plots of the overall CEC migration factors are quasi linear. We shall attempt to explain this behavior in the light of Eq. (3) as follows. With eluents rich in organic modulator, the value of the chromatographic retention factor, k'_{lc} in Eq. (3), will have a small value and be often negligible. In such cases the magnitude of k'_{cec} approaches that of μ_r in Eq. (3) and this may explain that k'_{cec} is practically constant as illustrated by the dotted lines in Fig. 11. On the other hand when the hydro–organic mobile phase is lean in the organic modifier, the chromatographic retention will be predominant in determining the value of k'_{cec}. Therefore, in view of Eq. (3) the quasi linear solid lines in Fig. 11 manifest a behavior that resembles reversed-phase chromatography.
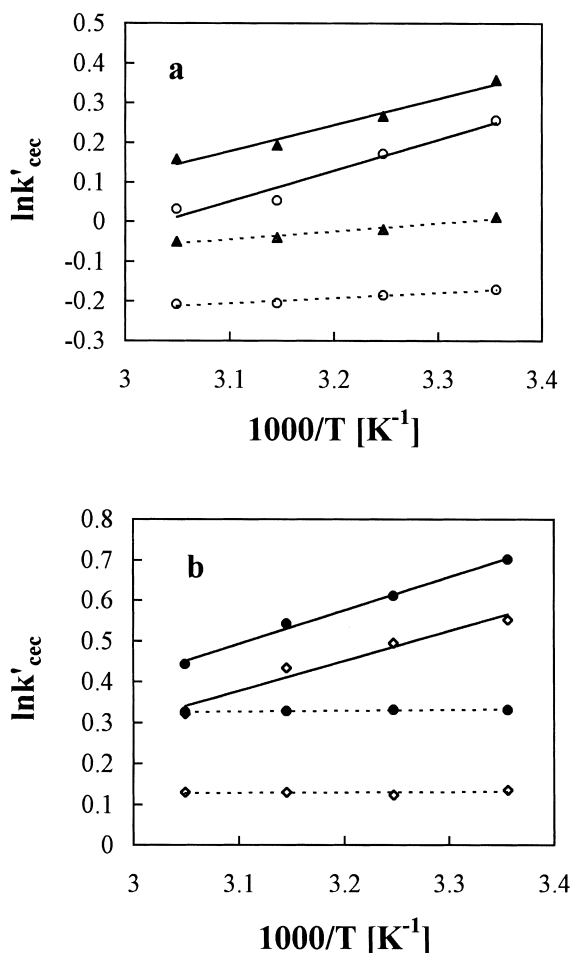
Fig. 11. Plots of the logarithmic CEC migration factors against reciprocal absolute temperature at different ACN concentrations. (a) Peptides, mobile phase, 45% ACN (dotted line) and 15% ACN (solid line) in 60 mM phosphate buffer, pH 2.5; sample: (○) insulin, (▲) angiotensin II. (b) Proteins, mobile phase, 50% ACN (dotted line) and 20% ACN (solid line) in 70 mM phosphate buffer, pH 2.5; sample: (◇) α-lactalbumin, (●) myoglobin; other conditions as in Fig. 2.

3.6. Fast analysis of tryptic digest by CEC at elevated temperature

Improving the speed of separation, increasing column efficiency and enhancing selectivity are the main driving forces in the development of new analytical techniques. In HPLC various approaches have been taken to reduce the separation time of
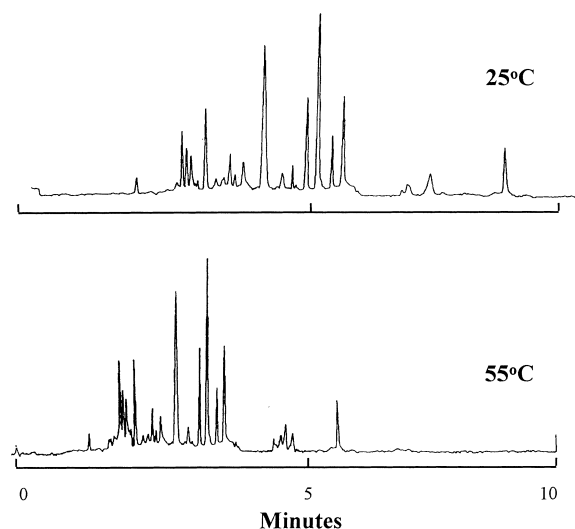

Fig. 12. Capillary electrochromatograms illustrating the separation of tryptic digest of cytochrome c obtained by isocratic elution at 25°C and 55°C, respectively. Mobile phase, 40% ACN in 50 mM phosphate buffer, pH 2.5; other conditions as in Fig. 2.

biopolymers in a major way [11,12]. These include the use of small particles with favorable mass transfer properties, pellicular sorbents and short columns operated at high flow-rates, steep gradients and high column temperatures. Despite the promise of CEC, no attempts have been made before to exploit the potential of this technique for high speed separation of proteins and peptides at elevated column temperature. In order to gain further insight of this matter, we separated the components of the tryptic digest of horse heart cytochrome c. The results are shown in Figs. 12 that depict the isocratic separation at 25 and 55°C, respectively. The mobile phase was 40% acetonitrile in 50 mM phosphate buffer, pH 2.5 and the applied voltage was −30 kV. The EOF velocity was 3.00 mm/s. It can be seen from these two chromatograms that the analysis time was reduced from 9 to less than 6 min upon increasing the temperature from 25 to 55°C without compromising the resolution.

4. Conclusions

This study was carried out with a novel column

packed with a porous styrenic monolith prepared by the polymerization of vinylbenzyl chloride with ethylene glycol dimethacrylate as the crosslinker. The positively charged chromatographic surface was formed by reacting the chloromethyl groups with *N,N*-dimethylbutylamine at the support surface. The column was found suitable to separate peptides and proteins. The observed effect of temperature and mobile phase composition on the separation by CEC supports our earlier postulate [6] that the migration of the sample components in this type of column is brought about by a dual mechanism involving both chromatographic retention and electrophoretic migration. Comparison of this column to a similar one packed with porous acrylic monolith, which was described earlier [6], showed very similar behavior in the CEC of proteins. This suggests that such column architecture and chemistry offers a chromatographic system of broad utility in the CEC of peptides and proteins. The performance of the column in protein separation was also compared to that of open capillaries in CZE, and both the plate efficiency and selectivity of CEC was found to be superior with this new column.

The final goal of our investigation will be to develop a rapid method for protein analysis on a CEC platform. The results presented here suggest that elevated temperature offers a means to accelerate the separation of slowly diffusing species as it does in HPLC [12,22]. This is demonstrated by rapid tryptic mapping of cytochrome *c* at elevated temperatures.

A particularly significant feature of this study with the new type of column is that it confirmed that the CEC platform offers a relative simple way to protein separations as we can forgo gradient elution and carry out the separation isocratically with a concomitant simplification of the instrumental requirements. Therefore, it is expected that isocratic protein separation will become an important application of CEC in the future.

Acknowledgements

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