Mechanistic Description and Experimental Studies of Electrochromatography of Proteins

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Electrochromatography is a form of gradient liquid chromatography in which an axial electric potential is applied to columns packed with gel-filtration media. Experimental methodology and a mechanistic model are further developed for a system that minimizes Joule heating at electric field strengths of 100 V/cm by dissipating heat through a cooling jacket and use of a cooled, low ionic strength eluting buffer. Focusing of proteins can be achieved in a 15-mm-dia. column by the interplay of eluent velocity, electrophoretic migration rate, and electrically induced concentration polarization when the stationary phase is more conductive than the mobile phase. Voltage gradients of up to 125 V/cm for eluent velocities at 18–25 cm/h separate binary protein mixtures of Bhb-α-lactalbumin, BSA-myoglobin, and α-lactalbumin-myoglobin over Sephadex G-100 and G-50. Retention times are consistent with values obtained from a mechanistic nonlinear model.

Introduction

Electrochromatography (EC) is a form of gradient liquid chromatography in which an electric potential is applied to columns packed with gel-filtration media (Hybarger et al., 1963; Vermeulen et al., 1971; Salak and Roch, 1972; Luzzio, 1975; O'Farrell, 1985; Scott, 1986; Tsuda, 1987; Rudge et al., 1993). The method of Rudge et al. (1993) and Ivory (1988) combines gel electrophoresis and liquid chromatography to resolve biomacromolecules on the basis of molecular weights and electrophoretic mobilities. Separation mechanisms mentioned in the literature include continuous-flow annular-bed electrophoresis, counteracting chromatographic electrophoresis, counteracting flow electrophoresis (Shea et al., 1994) and cocurrent electrochromatography (Ivory, 1988; Rudge and Ladisch, 1988).

The focusing technique of O'Farrell spurred interest in developing new experimental methods and in studying separation mechanisms of preparative electrokinetic systems. Yoshisato et al. (1986) used continuous rotating annular electrophoresis to demonstrate a partial separation of glycine and glutamic acid. Nath et al. (1993) reported the use of continuous zone electrophoresis for the preparative separation of proteins. A continuous free flow electrophoresis apparatus was developed by Richman and Walker (1983) to purify samples containing proteins or cells. Cole and Cabezas, Jr. (1994) have confirmed concentration polarization phenomena, and identified conditions at which this is likely to occur due to electrically driven retention in electrochromatography. They also demonstrated the utility of this technique by separating two genetic variants of β-lactoglobulin.

The use of high-voltage gradients in electrochromatography and other electrophoretic processes can be limited by heat-transfer considerations (Rudge and Todd, 1990). Heat causes loss of zone resolution because of heat-induced convection, and possible denaturation of heat sensitive, biologically active compounds (Raj and Hunter, 1991). In order to obtain good separation of protein mixtures, it is also necessary to consider separation characteristics such as pH, ionic strength, type of stationary phase, buffer composition, and electrode design (Basak et al., 1994).

Mathematical models for electrokinetic systems in literature describe heat-transfer effects (Vermeulen et al., 1971; Lynch and Saville, 1981; Datta et al., 1986; Yoshisato et al., 1986; Ivory and Gobie, 1990; Raj and Hunter, 1991). This article presents a modified electrochromatography system that minimizes Joule heating at electric field strengths up to 125 V/cm by heat dissipation through a cooling jacket (6°C) and use of a cooled, low ionic strength eluting buffer. Consequently, electric field strengths of 125 V/cm can be applied for more than 4 h. The currents generated are less than 15 mA, corresponding to a power dissipation of approximately 1

Table 1. Molecular Properties of Bhb and α-lac Used in a Sephadex G-100 Packed Column

Property	Bhb	α-Lactalbumin	Reference
MW kDa	64	14.2	Gordon and Semmett (1953); Pauling et al. (1949)
Electrophoretic Mobility (10^5) $cm^2/V \cdot s$	−2.3 at pH 8.0	−4.2 at pH 8.5	Same as above
pI	7.0	4.6	Hames and Rickwood (1981)
No. of residues	574	123	Brookhaven Library (1994); Stryer (1981)
Tertiary structure	Globular	Globular	Tanford (1961)
K_{av}	0.34	0.56	This work

W/cm^3 in the column. Thus, while the voltage gradient and total amperage of EC are consistent with those used in capillary electrophoresis (CZE), the power density is about 1,000× lower and is inversely proportional to the much larger cross-sectional area of an electrochromatography column relative to a CZE capillary tube. The system presented in this article facilitates measurement and further interpretation of the mechanism of protein separations in electrochromatography. The polarization adsorption function has been quantitated in terms of velocity, and enables prediction of conditions at which electrically induced concentration polarization occurs.

Experimental

Materials

Reagent grade Trizma base and glycine were obtained from Sigma Chemical Company (St. Louis, MO) and Fisher Scientific (Fair Lawn, NJ), respectively. Distilled deionized water was used to prepare the tris-glycine buffer (3.9 mM tris and 47 mM glycine, pH 8.1). Bovine hemoglobin (Bhb), α-lactalbumin (α-lac), bovine serum albumin (BSA), and myoglobin were obtained from Sigma Chemical Company. Standard protein solutions were made in 3.9 mM tris-47 mM glycine-0.03 M NaCl at concentrations of 2 mg/mL. The Bhb-α-lac and α-lac-myoglobin mixtures were prepared by adding equal quantities of individual standard protein solutions to form 0.25 mL of sample. The BSA-myoglobin mixture was prepared by adding 0.2 mL of BSA standard solution to 0.05 mL of myoglobin standard solution. Blue dextran was obtained from Sigma Chemical Company for measurement of the column void volumes. In this work, three protein mixtures, namely, Bhb-α-lac (over Sephadex G-100, 15 × 450 mm), and BSA-myoglobin, and α-lac-myoglobin (over Sephadex G-50, 15 × 300 mm) were used for conducting electrochromatographic separations. Tables 1 and 2 show their molecular properties. Electrophoretic mobilities have been obtained from the literature that may reflect divergent pH and ionic strength values. However, our assumptions are consistent with the literature (Rudge et al., 1993).

Apparatus and methods

Ace Glass jacketed columns (15 × 450 mm or 15 × 300 mm) were used with the Teflon end caps (Ace Glass Inc., Vineland, NJ) modified to fit 2.5-cm-long platinum electrode loops (Rudge et al., 1993). Columns were packed with Sephadex G-100 or Sephadex G-50 (Sigma Chemical Co.) size-exclusion gel that was swollen in 3.9 mM tris and 47 mM glycine buffer, pH 8.1 for 72 h.

The gravity slurry-sedimentation method typically used for soft gels such as Sephadex was also used here to pack the columns. The swollen gel suspension that is in the form of a thick slurry was degassed under vacuum to remove any air bubbles. The gel was then poured into a vertically mounted column through a packing bulb (Ace Glass Inc.) using a glass rod to direct the flow. Approximately ten column volumes of eluent (3.9 mM tris and 47 mM glycine, pH 8.1) were then passed by gravity flow through the column to pack the bed. After this, the packing bulb was disconnected, and the column top end piece (Rudge et al., 1993) replaced. The last traces of air were removed through the air vent in the top piece.

More than five column volumes of eluent were then pumped at a flow rate of 1 mL/min through the column to stabilize and equilibrate the packed bed. This causes a slight compression of the bed and the position of the electrode coil attached to the top column piece is adjusted such that it rests on top of the packed bed, about 1 cm below the top frit. The other electrode at the bottom of the column is separated from the column packing by the semipermeable frit. An EC 650 power supply (EC Apparatus Corp., St. Petersburg, FL) provided a DC electric field across a column. A constant temperature of 6°C was maintained in the column jacket using a refrigerated water bath (Neslab Instruments Inc., Portsmouth, NH). Eluent passes through tubing submerged in an ice bucket and is delivered to the column head via a Rabbit-Plus peristaltic pump (Rainin Instrument Co. Inc, Emeryville, CA). A four-way Teflon rotary valve (Rheodyne, Cotati, CA) serves as an injector connected to the column head. Effluent from the column travels to a UA-5 spectrophotometric absorbance

Table 2. Molecular Properties of BSA and Myoglobin Used in a Sephadex G-50 Packed Column

Property	BSA	Myoglobin (Horse)	Reference
MW kDa	66	16.9	Hames and Rickwood (1981)
Electrophoretic Mobility (10^5) $cm^2/V \cdot s$	−6.64 at pH 8.6	−2.9 at pH 8.2	Longsworth and Jacobsen (1949); Walbroehl and Jorgenson (1989)
pI	5.74	7.1	Hames and Rickwood (1981); Walbroehl and Jorgenson (1989)
No. of residues	607	153	Brookhaven Library (1994); Stryer (1981)
Tertiary Structure	Globular	Globular	Tanford (1961)
K_{av}	0.0	0.24	This work

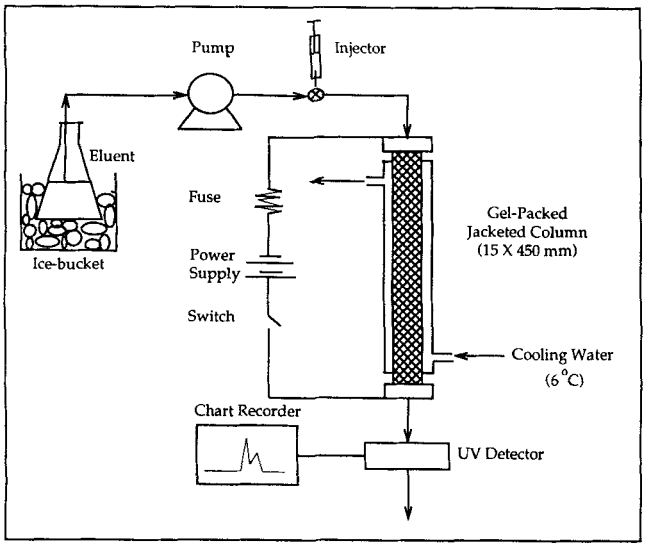

Figure 1. Electrochromatography apparatus with electrodes at column outlets.

fluorescence detector (ISCO, Inc., Lincoln, NE) with a Type 6 optical unit. A diagram of our electrochromatography apparatus is depicted in Figure 1.

The setup of the apparatus considered the placement of the pump, electrode, and flow direction of the eluent. In our attempts to minimize dispersion, the pump was connected to the column outlet. However, this caused the packed bed to crack during application of an electric current. Since the packed gel matrix is not rigid, the passage of buffer through the column became nonuniform in the vicinity of the outlet electrode due to suction caused by the pump. This resulted in removal of the buffer in a localized region, causing bed cracking. When the pump was connected to the column inlet, uniform flow was achieved with an applied pressure of 4,500 Pa, which promoted dissolution of gas within the column and minimized gas bubble formation during periods when an electric potential was applied. The plate count for the 45-cm column was 450, based on BSA in tris-glycine buffer at an eluent flow rate of 0.44 mL/min. This indicates that axial dispersion in the column is typical of a Sephadex stationary phase of this type.

Temperature rise in the column due to Joule heating can cause mixing through natural convection and disruption flow in the packed bed. External cooling by circulating water at 6°C in the jacket and internal cooling by buffer maintained constant temperature, and minimized bubble formation in the column. The downward hydrodynamic flow allowed the buffer to dissolve some of the electrolysis gases.

Column temperatures were measured by inserting a thermocouple (OMEGA Engineering Inc., Stamford, CT) into the packed bed (5 cm from the top) with eluent flowing through it, during application of an electric field. The temperature histories of different cooling strategies for electrochromatography runs with continuously applied electric field presented in Figures 2a and 2b demonstrate their heat dissipation capabilities. A stagnant buffer and water jacket at ambient temperature results in Joule heating, which causes gas to form within the bed. This results in bed cracking (Figure 2a). An autothermal effect described by Ivory and Gobie (1990) is the likely cause of the rapid increase in temperature. The effectiveness of cooling at 6°C with a flowing buffer can be seen by the flat temperature profile (Figure 2b). Once the efficacy of cooled buffer and circulating jacket water was confirmed, the thermocouple was removed and the column repacked as described previously. Table 3 summarizes parameters used in our experiments.

Determination of exclusion coefficients

Experiments were performed with the eluent flowing in the downward direction. Protein retention time in the absence of an electric field was determined by injecting 250 μL of sample into the column head, and measuring the time taken by the peak to emerge from the column. The extraparticle void fraction α (0.39 for the Sephadex G-100 column, 0.41 for the Sephadex G-50 column) was calculated using the definition $(V_e - V_0)/(V_t - V_0)$, where V_t represents the total column void volume; V_0, the extraparticle void volume; and V_e, the elution volume of blue dextran in the absence of an electric field. Using the same definition, the protein exclusion coeffi-

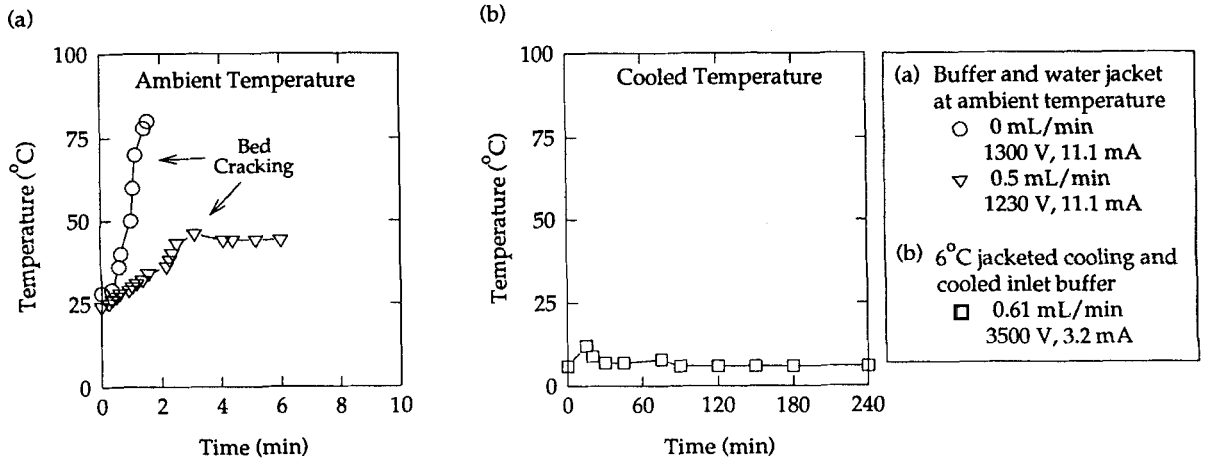

Figure 2. Sephadex G-100(15×450 mm) column temperature histories for EC runs with continuous applied electric field using (a) jacketed cooling and inlet buffer, both at ambient temperature, and (b) 6°C jacketed cooling and cooled inlet buffer.

Table 3. Summary of Experimental Separation Parameters, Associated Protein Properties, and Electrochromatography Selections

Separation Parameter	Protein Property	Selection
Stationary phase porosity	Molecular weight, solute retention	Sephadex G-50 Sephadex G-100
Buffer effectiveness	pK, charge, ionic strength, buffer capacity	3.9 mM tris and 47 mM glycine at pH 8.1
Electrical potential	Charge, zeta potential, electrophoretic mobility, solute retention	0–4,500 V
Flow rate	Solute velocity, solute retention	0.4–0.7 mL/min

cient (K_{av}) was calculated where V_e represents the elution volume of the protein. Immediately after sample injection, buffer flow rate was adjusted visually for several minutes to wash the protein band into the column bed.

The solute velocity in electrochromatography was calculated by

$$u = L/t_r, \tag{1}$$

where L is the length of the packed bed, and t_r is the time taken by the solute to traverse it. This retention time, t_r, is defined to begin after sample injection. The measurement of protein retention time for the case of applied electric fields is similar to that of size exclusion. However, the field is turned on when the protein band has been washed away from the vicinity of the top electrode and turned off before the first eluting protein band reaches the outlet frit. The time of field switch-off can be determined either by visually monitoring colored proteins (colored to the human eye) such as myoglobin and Bhb through the glass column or by use of preliminary runs made to determine specific protein retention times. All binary protein mixtures contain one colored protein (Bhb or myoglobin) to identify protein peaks.

Electrochromatography Theory

Characterization of solute velocity

Pressurized flow, electrophoretic mobility, and electroosmotic flow have been viewed as the main factors contributing to the solute velocity in electrochromatography (Ivory and Gobie, 1990; Rudge and Ladisch, 1988; Hunter, 1988; Tsuda, 1988). However, we hypothesize the velocity of solutes in an electrochromatographic column to be the sum of convective, electrophoretic, electroosmotic, and concentration polarization components similar to the definition of Rudge et al. (1993). The fraction of cross-sectional area, over which electroosmosis occurs, to that of total cross-sectional area, decreases for increasing column diameter. Moreover, flow rates increase by less than 3% when an electric field is applied (Rudge, 1989). Therefore, the electroosmotic velocity component can be assumed to be neglible for our electrochromatographic system. The electrically induced retardation of solute movement in electrochromatography can be attributed to a velocity component due to concentration polarization, v_{cp}. It can be seen that the hydrodynamic flow velocity of the solute represented by the size-exclusion chromatographic velocity (v_c) is opposed by either one or two counteracting forces represented by velocity components v_e and v_{cp}, depending on polarity (Figure 3). For both polarities, the direction of v_{cp} is against that of flow (upward), while, the direction of v_e is dependent on the polarity (upward or downward). Each represents different physical phenomena. Therefore, the decoupling of the concentration polarization velocity term results in a simpler physical interpretation of the separation mechanism. The solute velocity in electrochromatography can be represented by the addition of solute velocities given by

$$\mathbf{u} = \sum_{i=1}^{n} \mathbf{v}_i = v_c + v_e + v_{cp}, \tag{2}$$

where v_i represents a velocity component.

Models have incorporated electrical terms into conventional chromatography theory (Rudge and Ladisch, 1988; Ivory and Gobie, 1990; Rudge et al., 1993), one of which calculates solute velocities in electrochromatography by using a simple expression given by (Rudge et al., 1993):

$$u = \frac{v + \alpha\mu_1 E + (1-\alpha)K_{av}\mu_2 E}{\alpha + (1-\alpha)K_{av} + (1-\alpha)(1-K_{av})k_p}, \tag{3}$$

where v is the superficial velocity. The polarization adsorption function, k_p, was expressed in terms of E/v and given by

$$k_p = \exp\left(a\left|\frac{E}{v}\right|\right) - 1. \tag{4}$$

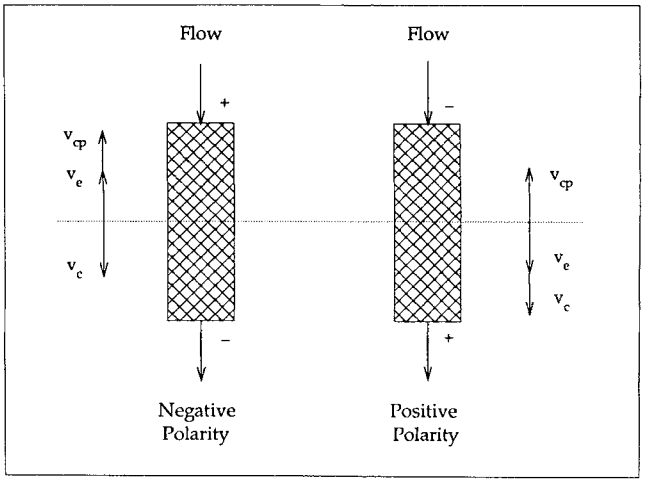

Figure 3. EC column with a mechanistic hypothesis of solute component velocities.

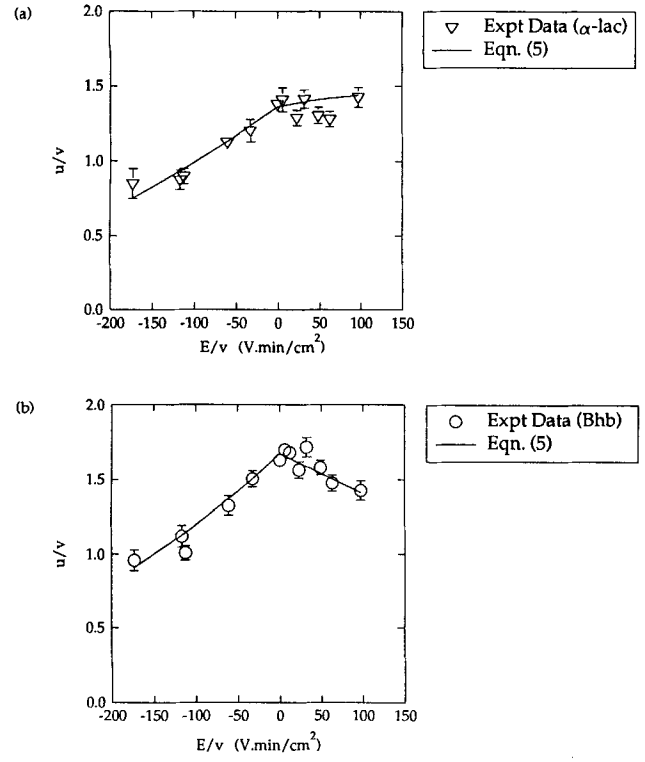

Figure 4. Reduced electrochromatographic solute velocity of (a) α-lac and (b) Bhb as a function of E/v.

Experimental conditions described in text. Sephadex G-100 packed column (15 × 450 mm) was used.

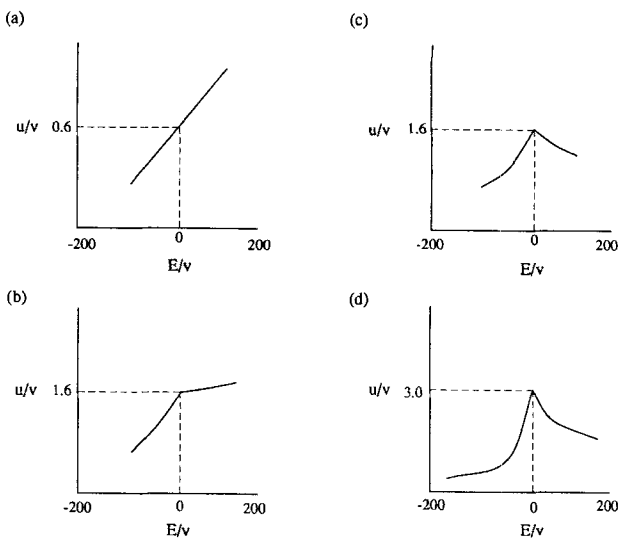

Figure 5. u/v vs. E/v curves for (a) bromphenol blue (670 Da; Rudge et al.); (b) α-lactalbumin (14200 Da); (c) bovine hemoglobin (64,000 Da); (d) blue dextran (2,000 kDa; Rudge et al.).

The values of k_p (or a) and μ's represent unknowns in Eqs. 3 and 4. The exponential form of this equation in general captures the rapid increase of the concentration polarization effect with increasing electric field strength. As a first approximation of electrochromatographic velocities, we assume uniform electrophoretic mobilities in the column and stationary phase, since the Sephadex holds about 50× its weight in water, and in essence, represents an immobilized form of the mobile phase. The $|E/v|$ term is limited by the stationary phase properties, and has been shown to be experimentally limited to 300. The polarization parameter a was physically related to charge-to-mass ratio in our previous publication (Rudge et al., 1993). This parameter increases with higher charge-to-mass ratio. When $a = 0$, there is no concentration polarization ($k_p = 0$). As a increases, the concentration polarization effect increases. The exponential form of k_p captures the rapid increase in concentration polarization, and thereby the decrease in electrochromatographic solute velocity with increasing electric field strengths.

Rudge et al. (1993) incorporated electrophoretic mobilities of solutes from the literature into these equations by assuming μ_1 and μ_2 to be equal. The transport of solute molecules through liquid-filled pores of similar dimensions is restricted and has been theoretically analyzed by Anderson and Quinn (1974) and reviewed by Deen (1987). Pujar and Zydney (1994) studied the effects of electrostatic interactions of a charged solute on the convective and diffusive transport through an asymmetric membrane. At low ionic field strengths (1.5 mM), they found the electrophoretic contribution to the solute flux to be comparable to the convective contribution through these membranes.

Results and Discussion

An electric field ranging from 0 to 4,500 V was applied across a Sephadex G-100 (15 × 450 mm) column for 45 min to obtain experimental data for characterizing solute velocities of α-lac and Bhb. Figures 4a and 4b show consistency between measured and calculated reduced electrochromatographic solute velocity (u/v), where calculated values of u/v are given by

$$\frac{u}{v} = \frac{[\alpha\mu + (1-\alpha)K_{av}\mu]E/v + 1}{\alpha + (1-\alpha)K_{av} + (1-\alpha)(1-K_{av})k_p} \tag{5}$$

and u is given by Eq. 2, while v is the superficial velocity. The proteins α-lac and Bhb have values of k_p determined from Eq. 4 where $a = 0.0029$ and 0.0036, respectively. The polarization adsorption functions are valid for E/v in the range of $+100$ V·min/cm^2 to -170 V·min/cm^2.

The two proteins (α-lac and Bhb) have migration rates that decrease with increasing electric field for negative polarities, as seen in Figures 4a and 4b. Also, the migration rate of α-lac increases gradually while that of Bhb decreases, for increasing positive electric fields. Although the convective and electrophoretic velocity components act in the downward direction (positive polarity), Bhb demonstrates an electrochromatographic velocity that is lower than the convective velocity, and decreases with increasing positive electric field strengths. This provides evidence to the existence of electrically induced concentration polarization phenomenon which is responsible for holding Bhb against the surface of the stationary phase in the presence of an electric field. The difference in behavior of α-lac and Bhb is due to their sizes, caus-

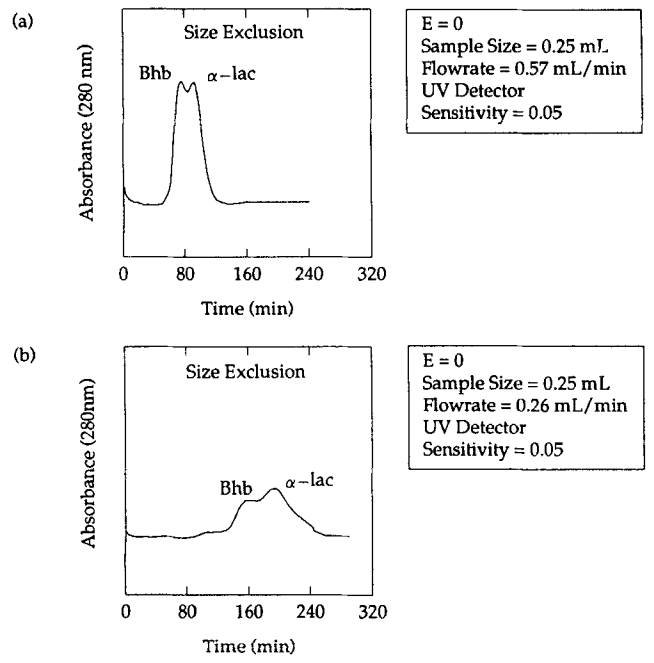

Figure 6. (a) Partial resolution of α-lactalbumin from bovine hemoglobin by size-exclusion chromatography; (b) partial resolution of α-lactalbumin from bovine hemoglobin by size-exclusion chromatography at reduced flow rate.

Sephadex G-100 packed column (15 × 450 mm) was used.

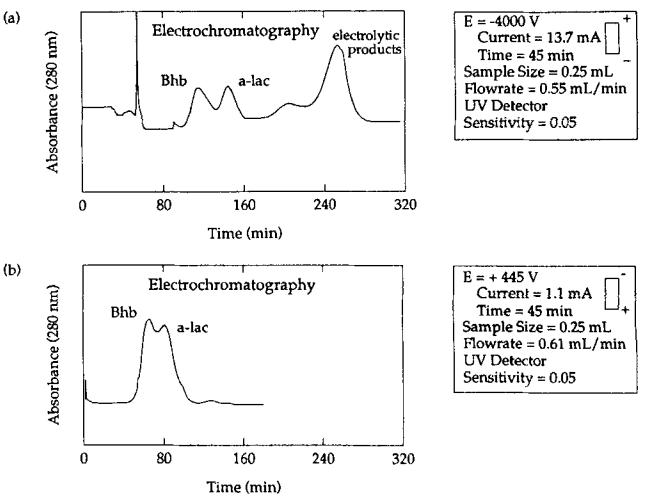

Figure 7. (a) Separation of α-lactalbumin from bovine hemoglobin by electrochromatography with negative polarity; spikes to the left of the peaks are due to gas bubbles in the detector; (b) partial resolution of α-lactalbumin from bovine hemoglobin by electrochromatography with positive polarity.

Sephadex G-100 packed column (15 × 450 mm) was used.

ing them to be subjected to different degrees of concentration polarization. The size of α-lactalbumin is small compared to the nominal exclusion limit of Sephadex G-100 (150 kDa). Thus, it can be thought of as passing into and through the pores under the influence of electric field. The extent of nonlinearity of the reduced electrochromatographic velocity is limited in this case. On the other hand, the size of BSA is comparable to the exclusion limit of Sephadex G-100. Therefore, electrically induced concentration polarization at the surface of the gel could cause restricted movement of BSA through the pores, and result in the nonlinear behavior shown in Figure 4b. The error bars (Figures 4a and 4b) indicate that the solute velocity measurements carried out in duplicate or triplicate are reproducible.

Figure 5 summarizes the patterns of u/v vs. E/v curves observed for molecules having a molecular weight ranging from 670 Da (bromphenol blue) to 2,000 kDa (blue dextran). Bromphenol blue passes unhindered through the gel and is not affected by the electrically induced concentration polarization (Figure 5a). The increase in nonlinearity of the curve begins to occur as the molecular weight of the solute increases from α-lac to Bhb (Figures 5b and 5c). This can be physically interpreted to be due to an increase in electric-field-induced solute partitioning, resulting from increased resistance to passage through the pores. The increasing size of the charged solute causes increased adsorption to the gel surface. Blue dextran has been visually observed to virtually adhere to the surface of the stationary phase, resulting in severely restricted movement through the column.

Cole and Cabezas (1994) show concentration polarization for Sephadex G-75 (regular gel, 40–120-μm dry bead diameter) with respect to the proteins myoglobin (17,800 Da) and BSA (MW 68,000 Da). However, Sephadex G-25 (exclusion limit = 5,000 Da), a coarse gel with 100–300-μm dry bead diameter and lower porosity, did not show concentration polarization. The data for Sephadex G-75 support the hypothesis that concentration polarization occurs for proteins whose molecular weights approximately match the exclusion limit of the stationary phase. The data of Cole and Cabezas (1994) for Sephadex G-25 indicate that dispersion, usually associated with a larger particle size, can mask such an effect.

Electrochromatographic separations

α-Lactalbumin and bovine hemoglobin were partly sepa-

Table 4. Prediction of Retention Time of Bhb and α-lac in Electrochromatography

Component	a $cm^2/V \cdot s$	E V/cm	Field time min	Eluent Flow Rate mL/min	t_r Calc.[1] min	t_r Meas. min
Bhb	0.0036	−100.00	45	0.55[2]	123	116
α-lac	0.0029	−100.00	45	0.55	142	141
Bhb	0.0036	+11.1	45	0.61[3]	69	67
α-lac	0.0029	+11.1	45	0.61	81	82

Assumes [1] $\mu_1 = \mu_2$; [2] $v = 0.31$ cm/min; [3] $v = 0.35$ cm/min.

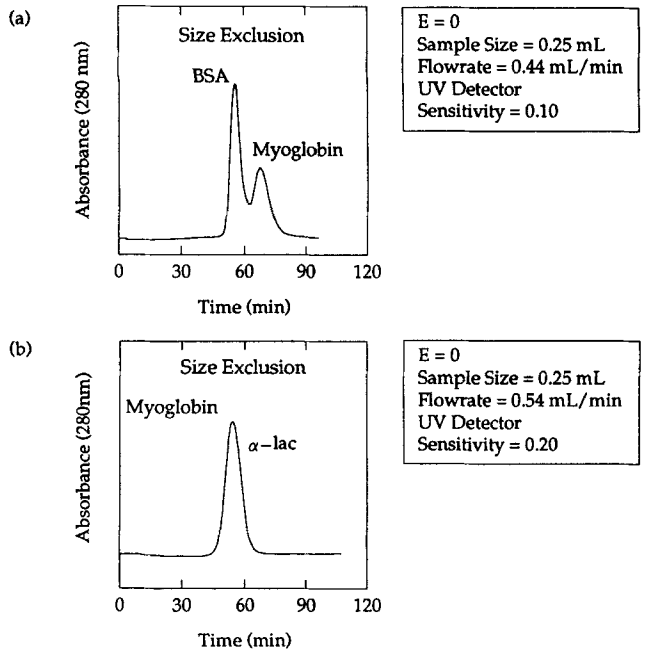

Figure 8. (a) Partial resolution of bovine serum albumin from myoglobin by size-exclusion chromatography; (b) single peak of α-lactalbumin and myoglobin by size-exclusion chromatography.

Sephadex G-50 packed column (15 × 300 mm) was used.

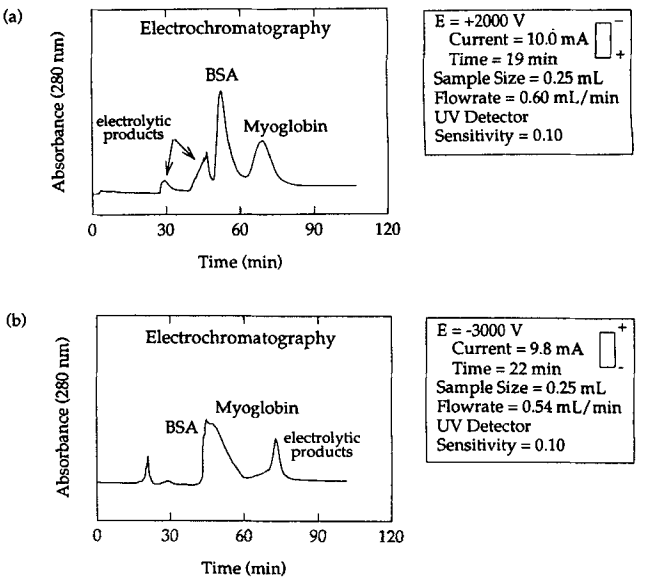

Figure 9. (a) Separation of bovine serum albumin from myoglobin by electrochromatography with positive polarity; (b) single peak of bovine serum albumin and myoglobin by electrochromatography with negative polarity.

Sephadex G-50 packed column (15 × 300 mm) was used. Spikes to the left of the peaks are due to electrolytic products or gas bubbles in the detector.

rated in the Sephadex G-100 size-exclusion column (Figures 6a and 6b). The smaller molecule (α-lac) has a higher electrophoretic mobility (Table 1). Therefore, a negative polarity would cause α-lac to elute more slowly due to its electrophoretic mobility, which would complement its slower chromatographic movement (higher k_{av}) compared to Bhb. On the other hand, a positive polarity would tend to bring the electrophoretically faster α-lac closer to Bhb.

A separation of α-lac and Bhb at an electric field of −4,000 V/cm (negative polarity) applied for 45 min follows anticipated trends (Figure 7a). Thus, for this separation, the v_e aligns itself with v_c making the use of negative polarity preferable (Figure 3).

Partial resolution of α-lac from Bhb occurs at +445 V/cm (positive polarity) applied for 45 min (Figure 7b). Resolution is similar to size-exclusion chromatography, alone, since the electrophoretic mobilities (2.3×10^5 and 4.2×10^5 cm^2/V·s, respectively) cause the proteins to move together (corresponds to positive polarity shown in Figure 3). Thus the experimental results obtained are expected based on our hypothesis. It is evident that the elution time of the proteins in Figure 7a is longer than that in Figures 6a or 7b. Comparison of Figures 6b and 7a show that increase in the elution time of proteins by size-exclusion chromatography does not improve their separation.

Table 4 compares the measured retention time of proteins to that calculated by Eqs. 1 and 3. Since Figure 7a has a $|E/v|$ of −272 V·min/cm^2, which is outside the limits of polarization adsorption functions defined for Bhb and α-lac, we extrapolated the results in Figure 4 to determine the electrochromatographic solute velocity. Good agreement can be seen between the measured and calculated retention time.

Size-exclusion elution profiles of BSA-myoglobin and α-lac-myoglobin mixtures in Sephadex G-50 columns are shown in Figures 8a and 8b. BSA is larger and electrophoretically faster than myoglobin (Table 2). Therefore, a positive polarity should enhance separation of this protein mixture. BSA separated from myoglobin (Figure 9a) for an electrical gradient of +66.7 V/cm. As expected, BSA and myoglobin could not be separated using an electrical gradient with negative polarity (Figure 9b).

Myoglobin is a bigger molecule (16,900 Da), but electrophoretically slower than α-lactalbumin. A negative polarity should enhance the separating capability of size exclusion. As expected, a partial resolution of myoglobin from α-lactalbumin was obtained (Figure 10a) when an electrical gradient of −100 V/cm was applied to the column. On application of +90 V/cm across the column, the two proteins were inseparable, as shown in Figure 10b.

The separation of different protein pairs described earlier illustrates an experimental methodology to align the size, charge, and concentration polarization of the solutes in a manner that increases differences in retention between various components. Poor separations may occur if two or more of these effects counteract the influence of the other. The utility of this technique applied to other separations would depend on the apt alignment of these properties. Further work to quantitate the concentration polarization velocity (v_{cp}) of different solutes would seem appropriate to compare the relative magnitudes of convective, electrophoretic and concentration polarization effects. This would facilitate the calculation of Δv_{cp} (difference in v_{cp} between solutes in a binary protein mixture) to determine the extent to which concentration polarization assists the feasibility of a separation.

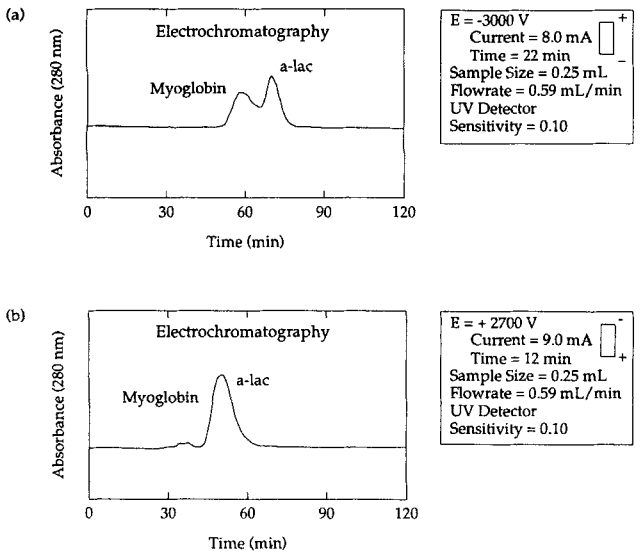

Figure 10. (a) Partial resolution of α-lactalbumin from myoglobin by electrochromatography with negative polarity; (b) single peak of α-lactalbumin and myoglobin by electrochromatography with positive polarity.

Sephadex G-50 packed column (15 × 300 mm) was used.

Conclusions

The combination of convective (v_c), electrophoretic (v_e), and concentration polarization dependent (v_{cp}) solute velocities results in a mechanistic nonlinear equilibrium model. When k_p is combined with the equilibrium model, protein retention consistent with our experiments is obtained. This led to specification of conditions for demonstrating separation of binary protein mixtures of Bhb-α-lactalbumin, BSA-myoglobin, and myoglobin-α-lactalbumin over Sephadex G-100 and G-50 packed columns. These data show that size exclusion, electrophoresis, and concentration polarization are important driving forces in electrochromatography.

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Notation

E = electric field strength, V/cm
t = time, min
$\mathbf{u}$ = vector velocity of solute, cm/min
$\mathbf{v}_i$ = vector velocity component of solute, cm/min
x = axial distance, cm

Greek letters

α = extraparticle void fraction
μ = electrophoretic mobility, $cm^2/V \cdot s$

Literature Cited

Anderson, J. L., and J. A. Quinn, "A Restricted Transport in Small Pores: A Model for Steric Exclusion and Hindered Particle Motion," *Biophys. J.*, **14**, 130 (1974).

Basak, S. K., A. Velayudhan, and M. R. Ladisch, "Characterization of Buffers for Electrokinetic Separations," *Appl. Biochem. Biotechnol.*, **44**, 243 (1994).

Brookhaven Protein Data Bank, National Center of Biotechnology Information (NCBI), National Library of Medicine, NIH, Bethesda, MD (1994).

Cole, K. D., and H. Cabezas, Jr., "Recent Progress in the Electrochromatography of Proteins," *Appl. Biochem. Biotechnol.*, **54**, 159 (1995).

Datta, R., R. A. Yoshisato, and G. R. Carmichael, "Development of a Theoretical Model for Continuous Rotating Annular-Bed Electrophoresis Column for Biochemical Separations," *ACS Symp. Ser.*, 250, Washington, DC, **82**, 179 (1986).

Deen, W. M., "Hindered Transport of Large Molecules in Liquid-Filled Pores," *AIChE. J.*, **27**, 952 (1987).

Gordon, W. G., and W. F. Semmett, "Isolation of Crystalline α-Lactalbumin from Milk," *J. Amer. Chem. Soc.*, **75**, 328 (1953).

Hames, B. E., and D. Rickwood, *Gel Electrophoresis of Proteins. A Practical Approach*, IRL Press, Washington, DC (1981).

Hunter, J. B., "An Isotachophoretic Model of Counteracting Chromatographic Electrophoresis (CACE)," *Sep. Sci. Tech.*, **23**(8, 9), 913 (1988).

Hybarger, R. M., C. W. Tobias, and T. Vermeulen, "Design Principles for Annular-Bed Electrochromatography," *Ind. Eng. Chem. Process Des. Dev.*, **2**(1), 65 (1963).

Ivory, C. F., "The Prospects of Large-Scale Electrophoresis," *Sep. Sci. Tech.*, **23**(8,9), 875 (1988).

Ivory, C. F., and W. A. Gobie, "Continuous Counteracting Chromatographic Electrophoresis," *Biotech. Prog.*, **6**, 21 (1990).

Longsworth, L. G., and C. F. Jacobsen, "An Electrophoretic Study of the Binding of Salt Ions by β-Lactoglobulin and Bovine Serum Albumin," *J. Phys. Colloid. Chem.*, **53**, 126 (1949).

Luzzio, A. J., "Isolation and Purification of Hepatitis B Antigen by Electrochromatography," *J. Inf. Dis.*, **131**(4), 359 (1975).

Lynch, E. D., and D. A. Saville, "Heat Transfer in the Thermal Entrance Region of an Internally Heated Flow," *Chem. Eng. Commun.*, **9**, 201 (1981).

Nath, S., H. Schutte, H. Hustedt, and W.-D. Deckwer, "Application of Continuous Zone Electrophoresis to Preparative Separation of Proteins," *Biotech. Bioeng.*, **42**, 829 (1993).

O'Farrell, P. H., "Separation Techniques Based on the Opposition of Two Counteracting Forces to Produce a Dynamic Equilibrium," *Science*, **227**, 1586 (1985).

Pauling, L., H. A. Itano, S. J. Singer, and I. C. Wells, "Sickle Cell Anemia, A Molecular Disease," *Science*, **110**, 543 (1949).

Pujar, N. S., and A. L. Zydney, "Electrostatic and Electrokinetic Interactions during Protein Transport through Narrow Pore Membranes," *Ind. Eng. Chem. Res.*, **33**, 2473 (1994).

Raj, C. C. B., and J. B. Hunter, "Analysis of Joule Heating in Electrophoretic Processes," *Int. Comm. Heat Mass Transf.*, **18**, 843 (1991).

Richman, D. W., and C. D. Walker, "Electrophoresis Apparatus with Flow Control," U.S. US 4,394,246 (Cl. 204-301; G01N27/26) (July 1983).

Rudge, S. R., and M. R. Ladisch, "Electrochromatography," *Biotech. Prog.*, **4**(3), 123 (1988).

Rudge, S. R., "Applied Electric Fields in the Size Exclusion Chromatography of Proteins," PhD Thesis, Purdue Univ., West Lafayette, IN (1989).

Rudge, S. R., and P. Todd, "Applied Electric Fields for Downstream Processing," in *Protein Purification from Molecular Mechanisms to Large-Scale Processes*, M. R. Ladisch, R. C. Wilson, C. C. Painton, and S. E. Builder, eds., ACS Symp. Series, **427**, Washington, DC, p. 244 (1990).

Rudge, S. R., S. K. Basak, and M. R. Ladisch, "Solute Retention in Electrochromatography by Electrically Induced Adsorption," *AIChE J.*, **39**(5), 797 (1993).

Salak, J., and P. J. Roch, "Isolation of Immunoglobulin M from Human Serum by gel Chromatography and Electrochromatography on a Column of Sephadex G-200," *J. Chromatog.*, **71**, 459 (1972).

Scott, C. D., "Continuous Electrochromatography Using a Rotating Annular System," *Sep. Sci. Tech.*, **21**(9), 905 (1986).
Shea, L. D., D. L. Feke, and U. Landau, "Counteracting Flow Electrophoresis: A Technique for Separating Biochemicals or Charged Macromolecules," *Biotechnol. Prog.*, **10**, 246 (1994).
Stryer, L., *Biochemistry*, 2nd ed., Freeman, New York (1981).
Tanford, C., *Physical Chemistry of Macromolecules*, Wiley, New York (1961).
Tsuda, T., "Electrochromatography Using High Applied Voltage," *Anal. Chem.*, **59**, 521 (1987).
Tsuda, T., "Chromatographic Behavior in Electrochromatography," *Anal. Chem.*, **60**, 1677 (1988).
Vermeulen, T., L. Nady, J. Krochta, E. Ravoo, and D. Howery, "Design Theory and Separations in Preparative-Scale Continuous Flow Annular-Bed Electrophoresis," *Ind. Eng. Chem. Process Des. Dev.*, **10**(1), 91 (1971).
Walbroehl, Y., and J. W. Jorgenson, "Capillary Zone Electrophoresis for the Determination of Electrophoretic Mobilities and Diffusion Coefficients of Proteins," *J. Microcolumn Sep.*, **1**(1), 41 (1989).
Yoshisato, R. A., L. M. Korndorf, G. R. Carmichael, and R. Datta, "Performance Analysis of a Continuous Rotating Annular Electrophoresis Column," *Sep. Sci. Tech.*, **21**(8), 727 (1986).

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