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 Bbb-a-lactalbumin, BSA-myoglobin, and a-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 (Hyberger et al., 1962; Vermuelen 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 anular-bed electrophoresis, countercurrent chromatographic electrophoresis, countercurrent flow electrophoresis (Shea et al., 1994) and countercurrent electromicrochromatography (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 anular 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 electrophoretic systems in literature describe heat-transfer effects (Vermuelen 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 Bbb and α-lac Used in a Sephadex G-100 Packed Column

<table>
<thead>
<tr>
<th>Property</th>
<th>Bbb</th>
<th>α-Lactalbumin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW kDa</td>
<td>64</td>
<td>14.2</td>
<td>Gordon and Semmert (1953);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pauling et al. (1949);</td>
</tr>
<tr>
<td>Electrophioretic</td>
<td>2.3 at</td>
<td>4.2 at</td>
<td>Same as above</td>
</tr>
<tr>
<td>Mobility (10^3 cm^2/V·s)</td>
<td>pH 8.0</td>
<td>pH 8.5</td>
<td>Hames and Rickwood (1981)</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
<td>4.6</td>
<td>Brookhaven Library (1994); Stryer (1981)</td>
</tr>
<tr>
<td>No. of residues</td>
<td>574</td>
<td>123</td>
<td>Tanford (1961)</td>
</tr>
<tr>
<td>Tertiary structure</td>
<td>Globular</td>
<td>Globular</td>
<td>This work</td>
</tr>
<tr>
<td>Kav</td>
<td>0.34</td>
<td>0.56</td>
<td></td>
</tr>
</tbody>
</table>

W/cm² 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 x 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 quantified 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 0.9 mM (tris and 47 mM glycine, pH 8.1). Bovine hemoglobin (Bbb), 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 Bbb-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 BBA-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, Bbb-α-lac (over Sephadex G-100, 15 x 450 mm), and BSA-α-lac, and α-lac-myoglobin (over Sephadex G-50, 15 x 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 x 450 mm or 15 x 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 0°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 Rabin Plus peristaltic pump (Rainin Instrument Co. Inc., Emeryville, CA). A four-way Teflon rotary valve (Iko Weldyne, 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

<table>
<thead>
<tr>
<th>Property</th>
<th>BSA</th>
<th>Myoglobin (Heme)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW kDa</td>
<td>56</td>
<td>16.9</td>
<td>Hames and Rickwood (1981)</td>
</tr>
<tr>
<td>Electrophioretic</td>
<td>6.64 at</td>
<td>2.9 at</td>
<td>Longworth and Jacobson (1949);</td>
</tr>
<tr>
<td>Mobility (10^3 cm^2/V·s)</td>
<td>pH 8.6</td>
<td>pH 8.2</td>
<td>Walnboehl and Jorgenson (1989)</td>
</tr>
<tr>
<td></td>
<td>5.74</td>
<td>7.1</td>
<td>Hames and Rickwood (1981);</td>
</tr>
<tr>
<td></td>
<td>155</td>
<td></td>
<td>Wallboehl and Jorgenson (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brookhaven Library (1994); Stryer (1981)</td>
</tr>
<tr>
<td>No. of residues</td>
<td>807</td>
<td>155</td>
<td>Tanford (1961)</td>
</tr>
<tr>
<td>Tertiary Structure</td>
<td>Globular</td>
<td>Globular</td>
<td>This work</td>
</tr>
<tr>
<td>Kav</td>
<td>0.01</td>
<td>0.24</td>
<td></td>
</tr>
</tbody>
</table>
fluorescence detector (ISCO, Inc., Lincoln, NE) with a Type 6 optical unit. A diagram of our electrophorography 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 thermoden (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 electrophorography 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 Coombes (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 thermoden 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 \( q \) (0.39 for the Sephadex G-100 column, 0.64 for the Sephadex G-50 column) was calculated using the definition \( V_p - V_v / V_p \), where \( V_p \) represents the total column void volume, \( V_v \), the extraparticle void volume; and \( V_p \), the elution volume of blue dextran in the absence of an electric field. Using the same definition, the protein exclusion coeffi-
Table 3. Summary of Experimental Separation Parameters, Associated Protein Properties, and Electrochromatography Selections

<table>
<thead>
<tr>
<th>Separation Parameter</th>
<th>Protein Property</th>
<th>Selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stationary phase porosity</td>
<td>Molecular weight, solute retention</td>
<td>Sephadex G-50</td>
</tr>
<tr>
<td>Buffer effectiveness</td>
<td>pH, charge, ionic strength, buffer capacity</td>
<td>Sephadex G-100</td>
</tr>
<tr>
<td>Electrical potential</td>
<td>Charge, zeta potential, electrophoretic mobility</td>
<td>3.9 mM tris and 47 mM glycine at pH 8.1</td>
</tr>
<tr>
<td>Flow rate</td>
<td>Solute retention</td>
<td>0-4,200 V</td>
</tr>
<tr>
<td></td>
<td>Solute velocity</td>
<td>0.4-0.7 mL/min</td>
</tr>
</tbody>
</table>

The solute velocity in electrochromatography was calculated by

\[ u = L/L_t, \]  

where \( L \) is the length of the packed bed, and \( L_t \) is the time taken by the solute to traverse it. This retention time, \( L_t \), 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 Btb 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 (Btb or myoglobin) to identify protein peaks.

**Electrochromatography Theory**

**Characterization of solute velocity**

Presaturated flow, electrophoretic mobility, and electrosomotic flow have been viewed as the main factors contributing to the solute velocity in electrochromatography (Ivory and Grothie, 1990; Rudge and Ladisch, 1988; Hunter, 1988; Tsuda, 1988). However, the velocity of solutes in an electrochromatographic column is the sum of convective, electrophoretic, electrosomotic, 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 negligible for our electrochromatographic system. The electrically induced retardation of solute movement in electrochromatography can be attributed to a velocity component due to concentration polarization, \( u_{cp} \). It can be seen that the hydrodynamic flow velocity of the solute represented by the size-exclusion chromatographic velocity \( u_s \), is opposed by one or two countercarrier forces represented by velocity components \( u_e \) and \( u_{cp} \), depending on polarity (Figure 3). For both polarities, the direction of \( u_{cp} \) is against that of flow (upward), while the direction of \( u_e \) 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

\[ u = \sum \xi \eta = \eta + \eta_e + \eta_{cp}, \]  

where \( \eta \) represents a velocity component.

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

\[ u = \frac{\eta + \alpha \mu_E E + (1 - \alpha) K_{np} \eta_{cp} E}{\alpha + (1 - \alpha) K_{np} + (1 - \alpha)(1 - K_{np}) E}, \]  

where \( \eta \) is the superficial velocity. The polarization adsorption function, \( K_{np} \), was expressed in terms of \( E/\eta \) and given by

\[ K_{np} = \exp\left[\eta \left(\frac{E}{\eta}\right)ight] - 1. \]
The values of $k_p$, for $a$, and $\mu$'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 electrolymographic velocities, we assume uniform electrophoretic mobilities in the column and stationary phase, since the Sephadex held about 50% of 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 $\alpha$ 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 $\alpha = 0$, there is no concentration polarization (i.e., $a = 0$). As $\alpha$ increases, the concentration polarization effect increases. The exponential form of $k_p$ captures the rapid increase in concentration polarization, and thereby the decrease in electrolymographic solute velocity with increasing electric field strengths.

Rudge et al. (1993) incorporated electrophoretic mobilities of solutes from the literature into these equations by assuming $\mu_1$ and $\mu_2$ 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 (1976) and reviewed by Dees (1987). Fujii and Zylkens (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 x 450 mm) column for 45 min to obtain experimental data for characterizing solute velocities of $\alpha$-lact and Bhb. Figures 4a and 4b show consistency between measured and calculated reduced electrolymographic solute velocity ($u/V$), where calculated values of $u/V$ are given by

$$
u = \frac{(\alpha \mu + (1 - \alpha)K_{eq} \mu E/V + 1)}{\alpha + (1 - \alpha)K_{eq} + (1 - \alpha)X1 - K_{eq}X_p} \tag{5}$$

and $u$ is given by Eq. 2, while $v$ is the superficial velocity. The proteins $\alpha$-lact and Bhb have values of $k_p$ determined from Eq. 4 where $\alpha = 0.0029$ and 0.0036, respectively. The polarization adsorption functions are valid for $E/V$ in the range of +100 V/min/cm² to -170 V/min/cm².

The two proteins $\alpha$-lact 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 $\alpha$-lact 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 electrolymographic velocity that is lower than the convective velocity, and decreases with increasing positive electric field strength. This provides evidence to the existence of electrically induced concentration polarization phenomena 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 $\alpha$-lact and Bhb is due to their sizes, caus-
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 Sephads G-75 (regular gel, 40–120-μm dry bed diameter) with respect to the proteins myoglobin (17,800 Da) and BSA (MW 68,000 Da). However, Sephads G-25 (exclusion limit = 5.000 Da), a coarse gel with 100–300-μm dry bed diameter and lower porosity, did not show concentration polarization. The data for Sephads 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 Sephads G-25 indicate that dispersion, usually associated with a larger particle size, can mask such an effect.

Electrophoretic separations α-Lactalbumin and bovine hemoglobin were partly sepa-

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

<table>
<thead>
<tr>
<th>Component</th>
<th>α cm²/V-s</th>
<th>E V/cm</th>
<th>Field time min</th>
<th>Eluent Flow Rate ml/min</th>
<th>t₁ Calc. min⁻¹</th>
<th>t₁ Meas. min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bhb</td>
<td>0.0036</td>
<td>-100.00</td>
<td>45</td>
<td>0.55</td>
<td>125</td>
<td>116</td>
</tr>
<tr>
<td>α-lac</td>
<td>0.0029</td>
<td>-100.00</td>
<td>45</td>
<td>0.55</td>
<td>142</td>
<td>141</td>
</tr>
<tr>
<td>Bhb</td>
<td>0.0036</td>
<td>+11.1</td>
<td>45</td>
<td>0.63</td>
<td>65</td>
<td>67</td>
</tr>
<tr>
<td>α-lac</td>
<td>0.0029</td>
<td>+11.1</td>
<td>45</td>
<td>0.63</td>
<td>81</td>
<td>82</td>
</tr>
</tbody>
</table>

**Assumptions**: $\mu_1 = \mu_2; \beta = 0.30 \text{ cm/min}; \alpha = 0.35 \text{ cm/min}$. 

**Figure 7.** (a) Separation of α-lactalbumin from bovine hemoglobin by electrophoresis 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 electrophoresis with positive polarity. Sephads G-100 packed column (15 × 450 mm) was used.
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_m) compared to BSA. On the other hand, a positive polarity would tend to bring the electrophoretically faster α-lac closer to BSA.

A separation of α-lac and BSA 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 η_n aligns itself with η_p making the use of negative polarity preferable (Figure 3).

Partial resolution of α-lac from BSA 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^-3 and 4.2 × 10^-3 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 6a 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 Eq. 1 and 3. Since Figure 7a has a k_E/μ of ~272 V·min/cm, which is outside the limits of polarization adsorption functions defined for BSA and α-lac, we extrapolated the results in Figure 4 to determine the electrophoretic solute velocity. Good agreement can be seen between the measured and calculated retention time.

Figure 9. (a) Separation of bovine serum albumin from myoglobin by electrophoresis with positive polarity; (b) single peak of α-lactalbumin and myoglobin by size-exclusion chromatography.

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

Size-exclusion elution profiles of BSA-myoglobin and α-lactalbumin-myglobin 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 ~467 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_p) 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_p (difference in v_p 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 × 500 mm) was used.

Conclusions

The combination of convective (ξ_c), electrophoretic (ξ_e), and concentration polarization dependent (ξ_p) solute velocities results in a mechanistic nonlinear equilibrium model. When ξ_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 β-lactoglobulin, 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.

Acknowledgments

This work was supported by the Merck Company Foundation, NSF grant BCS 8912150, NSF Equipment grant CTS 8906628, and a Purdue University Fellowship (1991–1993). We thank Dr. Ayj Velayudhan for suggesting the representation of solute velocity as the sum of convective, electrochromatographic, and concentration polarization velocities. We thank Dr. Karen Kahlmann and Manish Galai for helpful comments and review of this manuscript. One of the authors (S. K. B.) is a Merck Fellow.

Notation

E = electric field strength, V/cm
ι = time, min
v = vector velocity of solute, cm/min
γ = vector velocity component of solute, cm/min
r = axial distance, cm

Greek letters

α = extraparticle void fraction
μ = electrophoretic mobility, cm²/V·s

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2506 November 1995 Vol. 41, No. 11 AIChe Journal


Manuscript received Aug. 11, 1994, and revision received Dec. 27, 1994.