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Electrochromatographic separation of proteins

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Abstract

We have developed a modified electrochromatography system which minimizes Joule heating at electric field strengths up to 125 V/cm. A non-linear equilibrium model is described which incorporates electrophoretic mobility, hydrodynamic flow velocity, and an electrically induced concentration polarization at the surface of the stationary phase. This model is able to provide useful estimates of protein retention time and velocity in a column packed with Sephadex gel and subjected to an electric field. A correlation of electrophoretic mobility of peptide and proteins with respect to their charge, molecular mass, and asymmetry enables the selection of solute target molecules for electrochromatographic separations. Good separation of protein mixtures have been obtained.

1. Introduction

Electrochromatography is a form of gradient liquid chromatography in which an axial electric potential is applied to gel-filtration columns [1–8]. This method combines gel electrophoresis and liquid chromatography to resolve biomacromolecules on the basis of size (M_r), electrophoretic mobility, and solute retention.

Earlier attempts at preparative electrochromatography faced problems due to field-induced dispersion [1,2]. Nonetheless, Nerenberg and Pogojeff [9] were able to partially resolve blood plasma proteins. Early investigations found that the use of high-voltage gradients in electrochromatography and other electrophoretic processes can be limited by heat transfer considerations [10]. Heat causes loss of zone resolution because of heat-induced convection, and possible denaturation of heat-sensitive, biologically active compounds [11].

The superposition of parallel convective and electrical fields suggested by Salak and Roch [3], was used by Luzzio [4] to isolate and purify hepatitis B antigen, and Tsuda [12] to separate nucleotides, pyrimidine bases and their derivatives. O'Farrell's [5] counteracting chromatographic electrophoresis system faced difficulties in sample loading and product extraction [13] which led to the development of new experimental methods and electrokinetic separation mechanisms [6,8,11,13–16].

Preparative continuous zone electrophoresis exhibited by Nath et al. [14] purified a mixture of D- and L-hydroxyisocaproate dehydrogenase. Yoshisato et al. [15] used continuous rotating annular electrophoresis to demonstrate a partial separation of glycine and glutamic acid. Concentration polarization phenomena in electrochromatography described by Rudge et al. [8], have been confirmed by Cole and Cabezas [17],

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who have identified conditions at which these are likely to occur due to electrically driven retention. They also demonstrated the utility of electrochromatography by separating two genetic variants of β-lactoglobulin. Extension of preparative electrophoresis and electrochromatography (EC) to other protein separations can be facilitated by the knowledge of their molecular mass (M_r), charge (q), and electrophoretic mobility (μ), to determine conditions which promote electrokinetic separations.

This work presents a modified electrochromatography system which 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 W/cm^3 in the column which is comparable to capillary electrophoresis. The system presented in this paper 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 estimation of conditions at which electrically induced concentration polarization occurs. Methods and theory for selection of conditions for separation of binary protein mixtures based on their electrophoretic mobility, charge and size are reviewed.

2. Theory

2.1. Electrophoretic mobility

The size, charge, and electrophoretic mobility of proteins are related to their retention behavior in electrochromatography. A correlation was obtained for electrophoretic mobilities (measured experimentally by laser velocimetry and from literature by capillary zone electrophoresis, moving boundary electrophoresis, and microelectrophoresis) with their physicochemical properties and is expressed by [18]:

$$\mu(f/f_0) = 6.048 \cdot 10^{-3} \frac{q}{M_r^{2/3}} - 1.13 \cdot 10^{-5} \quad (1)$$

at 25°C, 100 mM ionic strength, pH range 3.05–11.8, and μ is in cm^2/V s. This correlation fitted 51 measured and literature values over the molecular mass range of 178 to 140 000 for components whose electrophoretic mobilities ranged from $+13.35 \cdot 10^{-5}$ to $-19.7 \cdot 10^{-5}$ cm^2/V·s. This correlation assumes negligible structural changes or isoelectric shifts of proteins due to salt effects. This correlation is based on minimal variation in pK values within amino acids, colloidal stability and invariance in protein size. Eq. 1 provides an estimation of the electrophoretic behavior of proteins in electrochromatography.

2.2. Characterization of electrochromatographic solute velocity

Convective flow, electrophoretic mobility and concentration polarization are the main factors contributing to the solute velocity in electrochromatography [8,19]. 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 (Fig. 1).

The solute velocity in electrochromatography can be represented by [19]:

$$\vec{u} = \sum_{i=1}^{n} \vec{v}_i = \vec{v}_c + \vec{v}_e + \vec{v}_{cp} \quad (2)$$

where $\vec{v}_i$ is a velocity vector component.

Models have incorporated electrical terms into conventional chromatography theory [8,13,20], one of which calculates solute velocities in electrochromatography by using the expression given by [8]:

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

The polarization adsorption function k_p, was expressed in terms of E/v and given by:

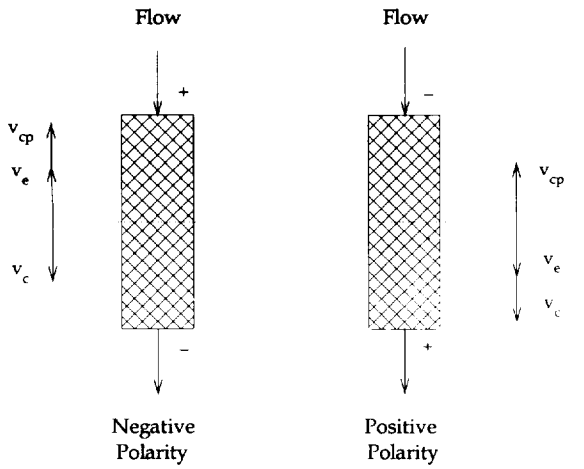

Fig. 1. Schematic diagram of an EC column with a mechanistic hypothesis of solute component velocities for a negatively (net) charged protein. The positive and negative sign refer to the electrodes attached to the column outlets with eluent flow in the downward direction.

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

The values of k_p (or a) and μ values represent unknowns in Eqs. 3 and 4. 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 [8]. The chromatographic velocity for size-exclusion chromatography, electrophoretic velocity, and concentration polarization velocity are expressed by:

$$v_c = \frac{v}{\alpha + (1-\alpha)K_{av}} \tag{5}$$

$$v_e = \mu E \tag{6}$$

$$v_{cp} = \frac{v}{\alpha + (1-\alpha)K_{av}} - \frac{v + \alpha\mu_1 E + (1-\alpha)K_{av}\mu_2 E}{\alpha + (1-\alpha)K_{av} + (1-\alpha)(1-K_{av})k_p} \pm \mu E \tag{7}$$

The velocity component, v_{cp}, is comparable to v_e and represents the concentration polarization velocity effect of different solutes. The values of a for myoglobin and ovalbumin are $13.87 \cdot 10^{-4}$ and $13.13 \cdot 10^{-4}$, respectively. Exclusion coefficients K_{av} in Sephadex G-50 for myoglobin and ovalbumin, determined experimentally, are 0.24 and 0.08 respectively, whereas the extraparticle void fraction, $\alpha = 0.41$ [19]. Eq. 2 and Eq. 7 make it possible to calculate the extent of electrically induced polarization effect and the additional retention of different protein systems. Table 1 summarizes parameters which impact electrochromatography.

3. Experimental

3.1. Materials

Reagent grade Trizma base and glycine were obtained from Sigma (St. Louis, MO, USA) and Fisher Scientific (Fair Lawn, NJ, USA), respectively. A Tris–glycine buffer (3.9 m*M* Tris and 47 m*M* glycine, pH 8.1) was used as the eluent in the EC runs. Myoglobin (Myo), ovalbumin (Oval), and β-lactoglobulin (β-Lac) were obtained from Sigma. Standard protein solutions were prepared in 3.9 m*M* Tris–47 m*M* glycine–0.03 *M* NaCl at concentrations of 2 mg/ml.

Table 1
Summary of experimental separation parameters, associated protein properties, and our selections for electrochromatography

Separation parameter	Protein property	Selection
Porosity, wet bed diameter	Molecular mass	Sephadex G-50, G-100
buffer effectiveness	p*K*, charge, ionic strength, buffer capacity	3.9 m*M* Tris and 47 m*M* glycine
Electrical potential	Charge, zeta potential, electrophoretic mobility	0–150 V/cm
Flow-rate	Solute velocity	0.4–0.7 ml/min

Binary protein mixtures were prepared in a 1:1 volume ratio of individual standard protein solutions to form 0.25 ml of sample. In this work, two protein mixtures, namely, Oval–Myo (over Sephadex G-50, 300 × 15 mm I.D.), and β-Lac-Myo (over Sephadex G-100, 450 × 15 mm I.D.) were used for conducting electrochromatographic separations. Table 2 shows their molecular properties [18,21–23]. Electrophoretic mobilities have been obtained from literature which may reflect divergent pH and ionic strength values. However, we have chosen values consistent with our experimental conditions [8].

3.2. Apparatus and methods

An Ace Glass jacketed column (450 × 15 mm I.D. or 300 × 15 mm I.D.) with Teflon end-caps (Ace Glass, Vineland, NJ, USA) was modified to fit platinum electrodes [8]. It was packed with Sephadex G-100 or Sephadex G-50 (Sigma) size-exclusion gel which was swollen in 3.9 m*M* Tris and 47 m*M* glycine buffer (pH 8.1) for 72 h.

The gravity slurry-sedimentation method typically used for soft gels such as Sephadex was used to pack columns for electrochromatography as described by Basak and Ladisch [19].

The other electrode at the bottom of the column is separated from the column packing by a semi-permeable frit. An EC 650 Power Supply (EC Apparatus, St. Petersburg, FL, USA) 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, Porthsmouth, NH, USA). Eluent passes through tubing submerged in an ice-bucket and is delivered to the column head via a Rabbit-Plus peristaltic pump (Rainin Instrument, Emeryville, CA, USA). A four-way Teflon Rotary valve (Rheodyne, Cotati, CA, USA) serves as an injector connected to the column head. Effluent from the column travels to a UA-5 spectrophotometric absorbance fluorescence detector (ISCO, Lincoln, NE, USA) with a Type 6 optical unit. The electrochromatography apparatus is depicted in Fig. 2.

Table 2
Molecular properties of Myo, Oval, and β-Lac used in this study [18,21–23]

Property	Myo	Oval	β-Lac
M_r	16 900	45 000	18 400
Electrophoretic mobility (10^5) cm^2/Vs	−2.9	−7.2	−11.11
p*I*	7.1	5.08	4.66
Number of residues	153	385	162

Temperature rise in the column due to Joule heating can cause mixing through natural convection in the packed bed. Density changes and convection caused by Joule heating can destroy the separation developed by different migration rates of proteins. The density changes are related to the temperature gradient by [24]:

$$\rho_2 = \rho_1 - \rho_1 \beta \Delta T \quad (8)$$

where ρ_1 is the average fluid density, β is the

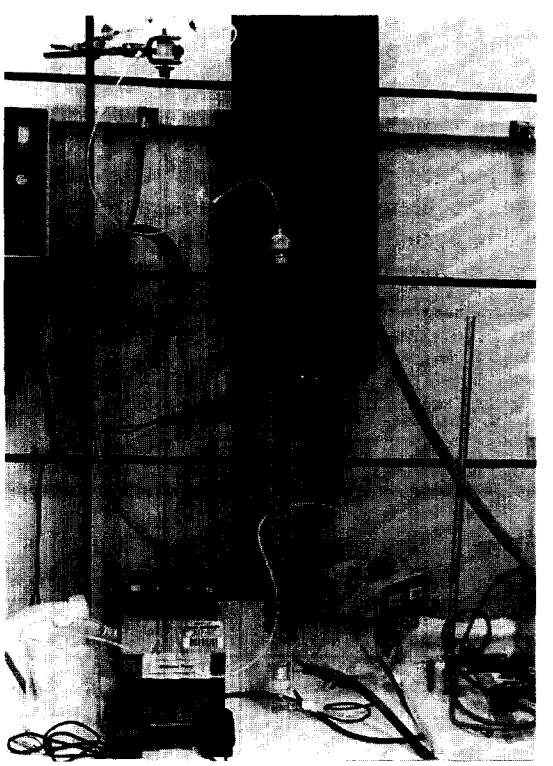

Fig. 2. Electrochromatography apparatus showing a 450 × 15 mm I.D. column packed with Sephadex G-100, having electrodes attached to the outlets.

coefficient of volumetric expansion, and ΔT is a characteristic temperature difference. Basak and Ladisch [19] demonstrated that 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 allows the buffer to dissolve some of the electrolysis gases. Thus, proteins in the column are electrophoresized at 6°C (close to 4°C, where water has a density maximum, so that β is ca. 0). The occurrence of thermal convection depends on the value of the Rayleigh number (Ra), while the amount of thermal convection depends on the Grashof number (Gr). These dimensionless groups are directly proportional to $\beta\Delta T$ [24]. Preliminary experiments show that the temperature gradient in the EC column is negligible [19]. Thus, the EC system presented here has a Ra and Gr of ca. 0, thereby indicating that thermal convection is negligible.

Protein retention time in the absence of an electric field (size-exclusion) 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. Immediately after sample injection, buffer flow-rate was adjusted visually for several minutes to wash the protein band over the bed. The solute velocity in electrochromatography was calculated by:

$$u = L/t_R \tag{9}$$

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 the injector is turned to allow the sample into the column. The measurement of this protein retention time for the case of applied electric fields (EC) 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 such as myoglobin through the glass column or by use of preliminary runs made to determine specific protein retention times.

3.3. Instrumentation

Electrophoretic mobilities of proteins for correlating their physicochemical properties were determined by a Malvern Zetasizer 3 instrument (Malvern Instruments, Southboro, MA, USA) based on laser doppler velocimetry [25]. It is equipped with an optical unit, a digital correlator and a personal computer, and combines photon correlation spectroscopy with laser doppler electrophoresis. This instrument (electrophoretic mobility detection limit = $\pm 1.0 \cdot 10^{-5}$ cm^2/V s) was calibrated for electrophoretic measurements by using the AZ55 Electrophoresis Standard Kit which contained a vial of carboxy-modified hydrophilic polystyrene latex for making up a sample, and buffer tablets to prepare a stock buffer solution (Malvern Instruments). These latex particles of 306 nm diameter, with a discrete zeta potential of −55 mV, enable calibration of the instrument. A Compaq 386, 20 MHz, IBM-compatible personal computer is used to integrate the operation of the optical unit and the signal-processing correlator. Unlike other techniques, this method measures the instantaneous velocity of the proteins rather than the time-averaged velocity.

4. Results and discussion

The electrophoretic mobilities of three proteins, namely, β-Lac (determined experimentally), Oval, and Myo [21,22] were plotted against their charge/mass ratio (Fig. 3). The straight line, which is Eq. 1, correlates the electrophoretic mobility of proteins with their charge and size. The distinct positions of β-Lac, Oval, and Myo in Fig. 3 suggests that they may be separable based on these properties. Binary protein mixtures, namely, Oval–Myo and β-Lac–Myo were selected from this set of proteins for electrochromatographic separations.

Based on our mechanistic hypothesis of electrochromatography (Fig. 1), Oval and β-Lac are larger and electrophoretically faster than Myo. Therefore, electrochromatography with positive polarity, should enhance separation of the

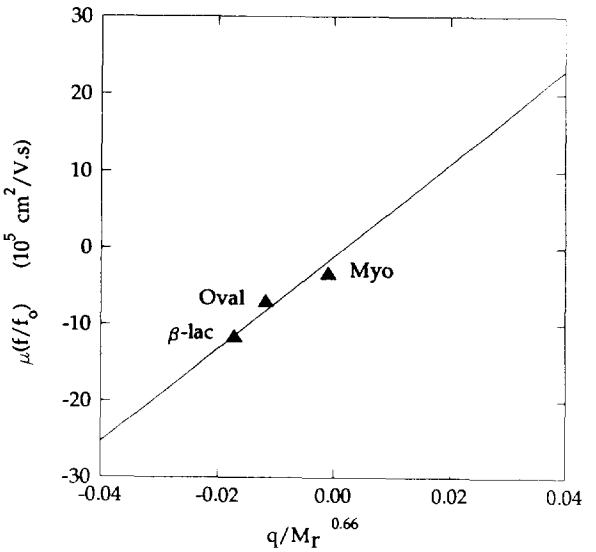

Fig. 3. Correlation of electrophoretic mobility for peptides and proteins with charge-to-size parameter, $q/M_r^{2/3}$ is represented by the solid line. The equation and correlation coefficient by linear regression is: $y = 6.048 \cdot 10^{-3}x - 1.13 \cdot 10^{-5}$; $r = 0.94$. The data points represent the proteins used in this study.

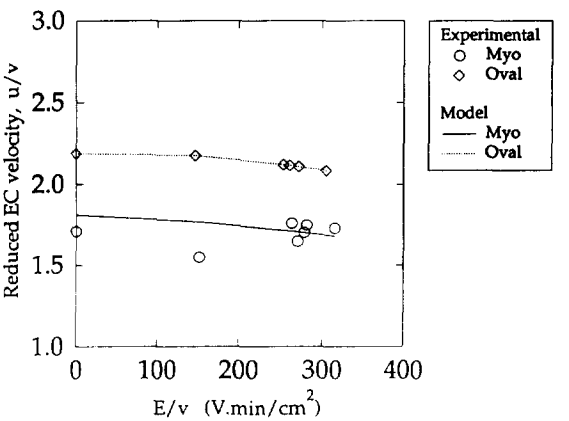

Fig. 4. Reduced electrochromatographic solute velocity of proteins as a function of E/v. All experiments were run in 3.9 mM Tris–47 mM glycine buffer, pH 8.1. Column temperature was held at 6°C, and sample size was 0.25 ml. Eluent velocities range from 18 to 30 cm/h. A Sephadex G-50 packed column (300 × 15 mm I.D.) was used.

above-mentioned binary protein mixtures compared to size-exclusion chromatography. The anticipation of a desirable polarity reduces the number of experiments in obtaining an efficient separation.

An electric field ranging from 0 to 3000 V was applied across a Sephadex G-50 column (300 × 15 mm I.D.) to obtain experimental data for characterizing solute velocities of Oval and Myo at positive polarity. Fig. 4 shows consistency between measured and calculated reduced electrochromatographic solute velocity (u/v), where calculated values of u/v are given by [8,19]:

$$\frac{u}{v} = \frac{[\alpha\mu + (1-\alpha)K_{av}\mu]\dfrac{E}{v} + 1}{\alpha + (1-\alpha)K_{av} + (1-\alpha)(1-K_{av})k_p} \qquad (10)$$

This figure confirms that Oval can be separated from Myo at positive polarity.

4.1. Protein separations

An ovalbumin–myoglobin mixture was partially resolved using size-exclusion chromatography as shown in Fig. 5a. Ovalbumin being bigger and electrophoretically faster, separates from myoglobin under application of positive electrical gradients at different flow-rates (Fig. 5b and c). In electrophoretic systems, such as capillary zone electrophoresis [26] and zonal electrophoresis [24], it has been demonstrated that a higher applied electrical voltage improves the plate count, and thus, performance of separations. Similarly, a higher electrical gradient in electrochromatography (+75 V/cm) gives a better separation of ovalbumin from myoglobin (Fig. 5c). Size-exclusion chromatography in a Sephadex G-100 column was unable to separate β-lactoglobulin from myoglobin (Fig. 6a). The application of a + 62 V/cm electrical gradient resolved β-lactoglobulin from myoglobin (Fig. 6b). Flow-rates were adjusted to get similar retention times.

The number of resolvable components for electrophoresis and liquid chromatography, known as peak capacity, is given by [27]:

$$n_c \approx 0.25(N_{max})^{1/2} \qquad (11)$$

where N_{max} is the highest number of theoretical plates among the species. Under present experimental conditions with observed plate counts of 20–60, it is possible to obtain baseline sepa-

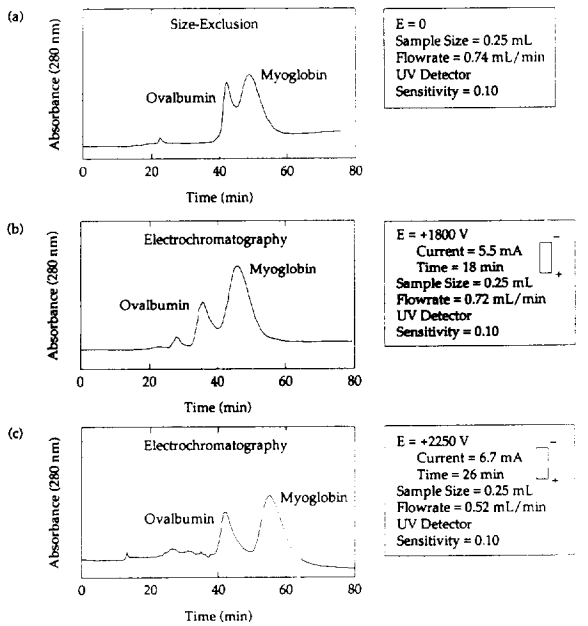

Fig. 5. (a) Partial resolution of ovalbumin from myoglobin by size-exclusion chromatography. (b) Separation of ovalbumin from myoglobin by electrochromatography with positive polarity (+60 V/cm). (c) Separation of ovalbumin from myoglobin by electrochromatography with positive polarity (+75 V/cm). A Sephadex G-50 packed column (300 × 15 mm I.D.) was used.

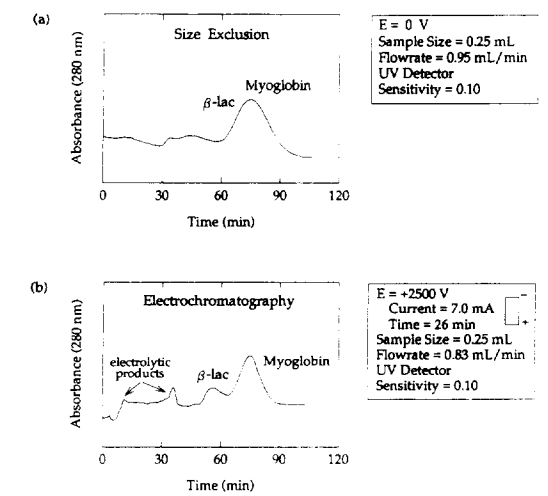

Fig. 6. (a) Single peak of β-lactoglobulin and myoglobin mixture by size-exclusion chromatography. (b) Separation of β-lactoglobulin from myoglobin by electrochromatography with positive polarity (+62 V/cm). A Sephadex G-100 packed column (450 × 15 mm I.D.) was used. Spikes to the left of the protein peaks are due to electrolytic products or gas bubbles in the detector.

ration of two component mixtures. This is consistent with the experimental work presented.

5. Conclusions

The combination of convective (v_c), electrophoretic (v_e) and concentration polarization dependent (v_{cp}) solute velocities results in a mechanistic non-linear equilibrium model. The expression for v_{cp} is based on polarization adsorption function, k_p, which was experimentally determined for proteins used in this study. When k_p is combined with the equilibrium model, protein retention consistent with our experiments is obtained. A correlation relates protein electrophoretic mobility to a size-charge parameter, which enabled selection of target solute molecules for electrochromatography.

This led to specification of conditions for demonstrating separation of binary protein mixtures of ovalbumin–myoglobin and β-lactoglobulin–myoglobin over Sephadex G-100 and G-50 packed columns. Baseline separation of two component mixtures is obtainable in this electrochromatographic system.

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Symbols

a	polarization parameter, $cm^2/V\ s$
c	mobile-phase concentration, mol/cm^3
D	dispersion coefficient, cm^2/s

E electric field strength, V/cm
f/f_o shape factor (asymmetry), dimensionless
Gr Grashof number $= \rho g h^3 \Delta\rho/\eta^2$
k_p polarization adsorption function, cm/s^2
K_{av} protein exclusion coefficient, dimensionless
L length of the packed bed, cm
M_r molecular mass
n gel phase concentration, mol/cm^3
n_o peak capacity
N_{max} maximum theoretical plates
q charge of peptide/protein
Ra Rayleigh number $= \rho g C_p h^3 \Delta\rho/k_T\eta$
t_R retention time, min
u solute velocity, cm/min
$\vec{u}$ vector velocity of solute, cm/min
v superficial velocity, cm/min
$\vec{v}_i$ vector velocity component of solute, cm/min
v_c size-exclusion chromatographic velocity, cm/min
v_{cp} solute velocity due to concentration polarization, cm/min
v_e solute velocity due to electrophoretic mobility, cm/min
α extraparticle void fraction
β coefficient of volumetric expansion, kg m^{-3} $-K$
μ electrophoretic mobility, cm^2/V s
μ_1 electrophoretic mobility of solute in the eluent phase, cm^2/V s
μ_2 electrophoretic mobility of solute in the resin phase, cm^2/V s

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