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Effects of stationary phase ligand density on high-performance ion-exchange chromatography of proteins

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ABSTRACT

Cation-exchange matrices with ligand densities from 10 to 500 μmol/g were prepared by reaction of diglycolic anhydride with diol-bonded silica. Lysozyme and cytochrome *c* were isocratically eluted from these columns under various conditions. The data was used to examine a retention model of proteins in which the slope (*Z* number) of a plot of log *k'* *vs.* log (1/sodium ion activity) was assumed to represent the number of points of binding between the protein and the matrix. As expected from the model, the *Z* number increased as the ligand density of the matrix increased. However, many qualitative and quantitative deviations from the model were also found, some of which may have been due to heterogeneity of the distribution of the ion-exchange sites on the matrix. An interesting observation was a change in elution order of lysozyme and cytochrome *c* as the ligand density changed; however, this may not have much practical benefit dual to the large band-broadening observed at low ligand densities.

INTRODUCTION

Ion-exchange chromatography is a powerful separation tool for the isolation of proteins. This method has seen rapid growth over the past decade [1,2] and numerous stationary phase materials have been developed to optimize the chromatographic performance [3–8]. The main advantage is the high recovery of proteins in terms of both mass and biological activity. Although the methodology has been frequently applied and the experimental conditions (*i.e.*, mobile phase velocity, pore diameter, column length, salt composition, pH, temperature, loading capacity, etc.) have been extensively investigated [9–13], little is known about the underlying retention mechanism and the factors that contribute to the selectivity.

In a proposed retention model for ion-exchange chromatography of proteins, Kopaciewicz *et al.* [14] suggested that as a protein comes into contact with the chromatographic stationary phase, only a fraction of the protein surface covers the binding area and that the retention is exponentially related to a parameter called the *Z* number, which is the number of binding sites on a protein surface interacting with the stationary phase. Since the number and distribution of charged groups on the surface of a macromolecule are fixed as well as its chromatographic binding domain, it follows that the *Z* number and retention should be mainly dependent on the stationary phase ligand density. Consequently, the ligand density should have a profound effect on the binding mechanism and chromatographic behavior of macromolecules. For example, in hydrophobic interaction chromatography, the protein retention was found to increase with an increase in ligand density [15]. There have been very few published reports describing the effect of ligand density on the performance of ion-exchange chromatography of proteins. This has primarily been due to the unavailability of supports covering a sufficiently wide range of ligand density.

We have developed a technique [16] for the

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preparation of silica-based supports of variable ligand density by means of controlled reaction of anhydrides with diol-bonded silica. By using this technique, surface ligand densities ranging from a few percent to complete monolayer coverage have been obtained. In this study, cation-exchange supports of different ligand densities were prepared by reaction with diglycolic anhydride. Chromatographic behavior of proteins was subsequently examined as a function of ligand density. The Z number, based on a displacement model proposed by Boardman and Partridge in 1955 [17], was used to characterize retention. The equilibrium of sodium ions, Na^+, and proteins, P^{n+}, between the resin (R) and mobile phase (m) can be represented by the equation:

$$Z\,Na_R + P_m \rightleftharpoons P_R + Z\,Na_m \quad (1)$$

where Z is the number of sodium ions displaced when one protein is adsorbed. The mass action expresses the equilibrium as:

$$\frac{[P_R][Na_m]^Z}{[P_m][Na_R]^Z} = K \quad (2)$$

where K is the equilibrium constant; P_R and P_m are proteins in resin phase and mobile phase; Na_m and Na_R are sodium ions in mobile phase and resin phase, respectively. The equation can be further reduced to the following form:

$$\log k' = Z \log 1/[Na] + \text{constant}\,(I) \quad (3)$$

By plotting log capacity factor (k') *vs.* log 1/[Na], Z is thus obtained. The retention model has been widely applied to various high-performance liquid chromatographic (HPLC) systems. For example, Parenter and Wetlaufer [18] were able to demonstrate using cation-exchange chromatography that denatured α-chymotrypsinogen A exhibited a larger Z value than native protein even though it had a shorter retention time. The same authors also measured the log–log slope to fit a gradient elution retention model [19], which made it possible to relate the gradient and isocratic elution retention data. Melander *et al.* [20] used the log–log plot to fit a retention model that takes into account both the ionic and hydrophobic interaction. They also used the same plot to probe the ligand density [20]. The displacement model was evaluated in this study by using protein retention as a function of ligand density in conjunction with Z number.

EXPERIMENTAL

Reagents

Cytochrome *c* (equine) and lysozyme (egg) were from Sigma (St. Louis, MO, USA). Diglycolic anhydride (DGA), benzylamine, and diglycolic acid were from Aldrich (Milwaukee, WI, USA). Nucleosil 300–5 (surface area: 100 m^2/g) was from Alltech (Deerfield, IL, USA). Tetrahydrofuran (THF), ethanol and diglycolic acid were purified as described previously [16].

Instrumentation

A Model 344 gradient liquid chromatograph (Beckman, Berdeley, CA, USA) was used. Absorbance was monitored at either 280 nm (proteins and acetone) or 262 nm (benzylamine) by a V^4 variable-wavelength absorbance detector (ISCO, Lincoln, NE, USA). Data were collected and processed on an Apple IIe computer via an ADALAB interface board (Interactive Microware, State College, PA, USA).

Methods

All of the carboxylate cation-exchange supports were prepared according to a previously published procedure [16]. The ligand density was quantitated by a ferric hydroxamate ester assay [21]. Briefly, samples containing 0.2–1 μmol of ester in 0.5 ml of distilled ethanol were treated with 0.25 ml of alkaline hydroxylamine reagent and sonicated for 5 min. After 1 h of reaction at room temperature, 4.0 ml of ferric reagent was added followed by 2 min of sonication and an additional 5-min reaction at room temperature. The silica was removed by centrifugation prior to the final absorbance measurement at 530 nm. Dimethyl diglycolate was used as standard.

The carboxylated support was suspended in 0.5 *M* sodium sulfate and packed into 100 × 4.1 mm I.D. columns at 5000 p.s.i. using upward-flow method [22].

Chromatography

The weak mobile phase (A) was 0.01 *M* sodium phosphate (pH 6.0) and the strong mobile phase (B) was 0.01 *M* sodium phosphate–0.2 *M* sodium sulfate (pH 6.0). The sodium concentration was adjusted by premixing A and B in the desired ratio. Chromatography was performed isocratically at room temper-

ature using a flow-rate of 1 ml/min. Aliquots of 10 μl of either a 5 mg/ml protein solution or 10 μM benzylamine solution were injected. In the experiments where k' was measured, the void volume was individually determined for each column by injecting 10 μl of 1% acetone. The experimental error of the Z number measurement was estimated to be $\pm 5\%$ to $\pm 10\%$ depending on the magnitude of the Z number.

The protein adsorption capacities were determined by continuously applying 0.5 mg/ml lysozyme to 6.2 × 2.1 mm I.D. columns. The breakthrough curves were corrected for the void volume.

The statistical moments were found by using the modified $B/A_{0.5}$ method, where $B/A_{0.5}$ is the peak width ratio at half height [23].

The activity coefficient of sodium ions was not constant over the concentration range studied. This was especially true at high salt concentrations (*e.g.* when $\%B > 50\%$), where a two-fold change in %B led to a 15% change in the activity coefficient [24]. To avoid errors due to this, the activity of the ions was used in all calculations.

RESULTS AND DISCUSSION

Ligand quantitation and lysozyme binding capacity

The ligand density was quantitated by means of assay of the ester groups formed when diglycolic anhydride reacted with the diol groups of the matrix [16]. To confirm the assay, k' was measured as a function of ligand density using benzylamine as the probe molecule (eluted isocratically at pH 6.0). Since the k' value of a monovalent molecule like benzylamine should be linearity dependent on the number of ligands in the column, the linearity of a plot of k' *vs.* ligand density serves to confirm the validity of the ligand density measurements. A linear relationship was observed (Fig. 1) except at the highest ligand density, indicating that the results of ester assay were consistent with retention, at least in the low to intermediate ligand density region of primary interest.

The lysozyme binding capacity *vs.* ligand density was measured using breakthrough curves. Blank columns containing either no packing material or diol-bonded silica were used to detect the possible presence of nonspecific adsorption. The ion-exchange capacity so obtained was virtually negligible.

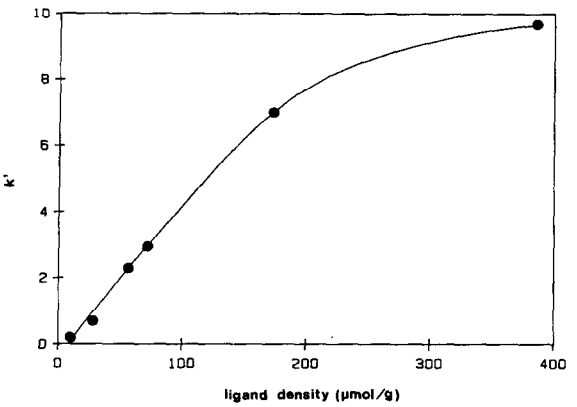

Fig. 1. Ligand density dependence of k' of benzylamine obtained with phosphate buffer, pH 6.0.

This was probably due to the hydrophilic nature of diol phase and the complete coverage of silanol sites. For carboxylated supports, lysozyme capacity was measured after previously saturating the column several times to remove any irreversible adsorption sites. Fig. 2 presents the lysozyme capacity as a function of ligand density. The shape of the curve was substantially different from that seen for a small molecule (Fig. 1). The slope of the curve changed sharply at a ligand density of approximately 70 μmol/g. Below that point, the binding capacity increased sharply, while above that point the curve flattened. A calculation of the average distance between the ligands (Table I) reveals that at ligand density of 72 μmol/g, the distance between two nearest ligands (15 Å) was less than the diameter of

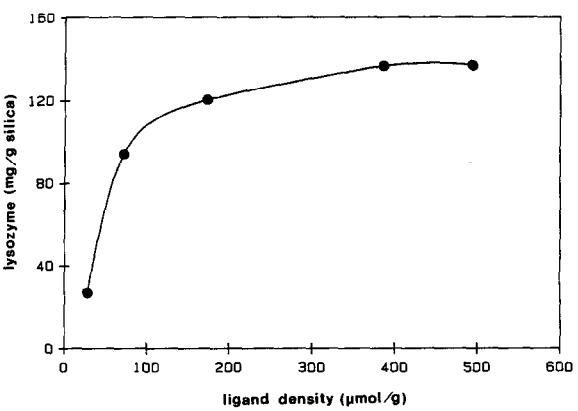

Fig. 2. Ion-exchange capacity for lysozyme as a function of the ligand density.

TABLE I

DISTANCE BETWEEN TWO NEAREST LIGANDS AS A FUNCTION OF LIGAND DENSITY

Ligand Density (μmol/g)	Distance between two nearest ligands (Å)[a]
10	41
28	24
72	15
173	9.8
386	6.6
494	5.8

[a] Calculated based on: $S = (A/CN)^{1/2}$, where S is the average distance between two nearest ligands, C is the ligand density, N is Avogadro's number and A is the surface area given by manufacturer.

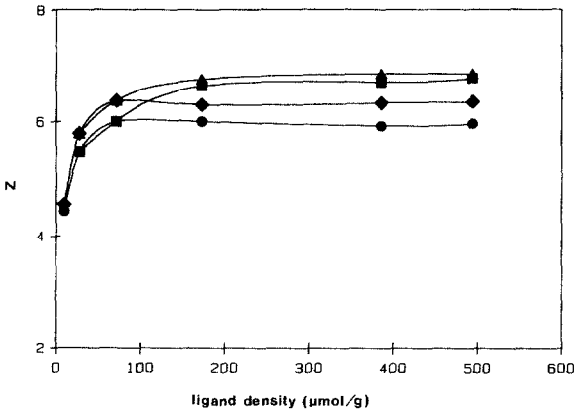

Fig. 3. The Z number of cytochrome c (●, ■) and lysozyme (▲, ◆) on various ligand density supports, as measured in the low ionic strength range (▲, ■) and high ionic strength range (◆, ●). For high ionic strength, columns of 100 × 4.1 mm I.D. were used. Other conditions refer to Experimental section.

lysozyme (*ca.* 20 Å), enabling the protein to form multiple bonds with the stationary phase. Below 28 μmol/g, the two closest ligands averaged 24 Å or more apart, limiting the protein binding to a single ligand. It appeared from Fig. 2 that once the stationary phase ligand density passed the threshold of multiple binding (*ca.* 70 μmol/g) further increases in ligand density did not have much effect on the binding capacity. The maximum binding capacity in Fig. 2 was approximately 28% of a theoretical monolayer of lysozyme. A similar relationship between the protein binding capacity and ligand density was also observed by Alpert and Regnier [25] when hemoglobin was bound to polyethyleneimine-coated (CPE) silica.

Z number and stationary phase heterogeneity

The Z numbers of lysozyme and cytochrome c, based on the model proposed by Boardman and Partridge [17], were measured as a function of ligand density and ionic strength, as shown in Fig. 3. The Z number initially increased rapidly with ligand density, but leveled off in the intermediate ligand density region, in a manner similar to that observed in the lysozyme binding capacity measurement of Fig. 2.

The lowest value of the Z number was about 4 at the lowest ligand density (Fig. 3). One would have expected the Z number to decline to 1 at low ligand density, *i.e.*, the protein should have been able to interact with only one ion-exchange site when the spacing of the sites was larger than the diameter of the protein. Possible reasons for this discrepancy include (a) the irregular and microporous surface of the support, formed from 1–100 nm silica spheres [26], which could interact with several sides of the protein molecule at once and thus increase the apparent protein size, and (b) a heterogeneity in ion-exchange site distribution which could result in clusters of sites, with the Z number primarily determined by the stronger multivalent interactions.

It is noted that the Z numbers obtained in this study were typically non-integer values. This was probably a result of (a) partial ionization of various basic groups in the proteins, which created varying number of charges among individual molecules, giving rise to statistically averaged fractional values; and (b) heterogeneous distribution of ligands on silica surface, leading to an averaging of Z values of protein binding to different locations. Non-integer Z values have been observed with other proteins, *e.g.*, β-lactoglobulin on an anion-exchange column [14].

Z number and ionic strength

Although it is a common observation that an increase in salt concentration would decrease protein retention, it was not clear whether the process involved any change in Z value. In this study, the Z number was measured in different regions of ionic strength, as shown in Fig. 3 and Table II. A

TABLE II

Z NUMBER AS A FUNCTION OF IONIC STRENGTH[a]

%B[a]	μ(ionic strength)[b]	Z(lysozyme)	Z(cytochrome c)
60–80	0.37–0.48	6.34	5.93
30–60	0.19–0.37	6.86	6.71
25–35	0.16–0.22	8.59	7.72

[a] Range of %B used in the Z measurement.

[b] Obtained from a 386 μmol/g column at 1.0 ml/min. $\mu = 1/2\sum[i]Z_i^2$, where [i] is the molar concentration of an ion, and Z_i is the charge of that ion.

moderate increase in the Z number was observed at various ligand densities when the ionic strength was decreased (Fig. 3), especially on high ligand density columns where a strong mobile phase is usually applied, suggesting that the Z number is ionic strength dependent.

Z number and retention

Although for individual proteins and stationary phases the Z number is determined from the slope of a log–log plot, the model does not predict how the retention (k') should relate to the Z number across a range of matrices of different ligand density. As shown in Fig. 4 for lysozyme and cytochrome c, retention clearly increased with an increase in the Z number, but other parameters must also have affected retention since the curves for the two proteins were not superimposable. Thus, the number of apparent points of interaction between the protein and the stationary phase must not be the only factor affecting the strength of those interactions.

Additional evidence that the Z number is only one component of retention is indicated by the log–log plot shown in Fig. 5 for lysozyme and cytochrome c on three stationary phases of different ligand densities. Although the slopes, *i.e.*, Z number, of these plots are all similar, note that there is an elution order reversal of the two proteins as the ligand density increased. At a ligand density of about 70 μmol/g, both proteins coeluted. Above this density, lysozyme eluted first; below this density, cytochrome c eluted first. The elution order reversal is better represented by the intercept term (I) of eqn. 3, as shown by a plot of I *vs.* Z (Fig. 6).

The divergence between the Z and retention (k') was also observed by Parenter and Wetlaufer [18] in a denaturation study of α-chymotrypsinogen A, where the denatured protein was found to exhibit a larger Z value but a much shorter retention time.

Band broadening and resolution

In principle, ligand density changes would provide a very useful means of altering selectivity, as shown in Fig. 6. However, it was observed that band-broadening was large at low ligand density, as shown in Fig. 7 for cytochrome c. The medium-to-high density phases exhibited much less broadening.

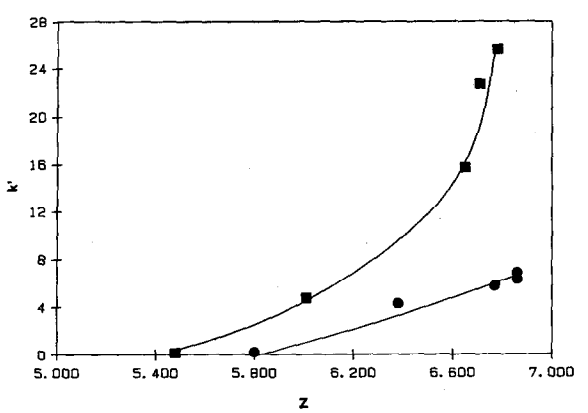

Fig. 4. Capacity factors obtained from isocratic elution of varying ligand density matrices with 50% B *vs.* the Z number measured in the %B range of 30% to 60% for cytochrome c (■) and lysozyme (●).

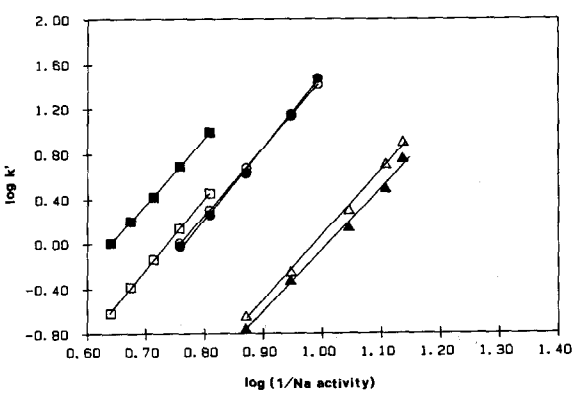

Fig. 5. A plot of the logarithm of capacity factor *vs.* logarithm of the reciprocal of sodium activity for cytochrome c (▲, ○, ■) and lysozyme (△, ●, □) on supports of ligand density 28 μmol/g (△, ▲), 72 μmol/g (○, ●) and 386 μmol/g (□, ■). Comparison of k' values at different ligand densities shows elution order reversal.

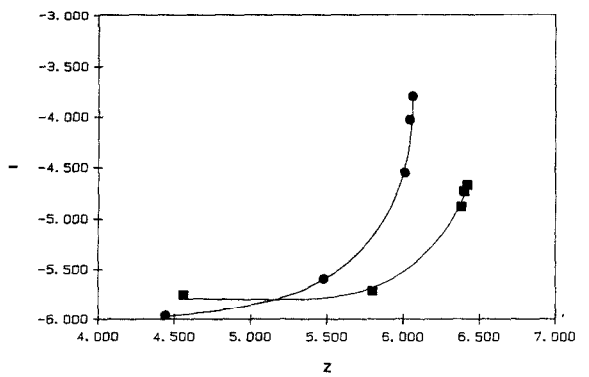

Fig. 6. Intercept I as a function of the Z number for cytochrome c (●) and lysozyme (■).

Lysozyme behaved in a similar manner. The problem was not a result of poor column packing since benzylamine yielded low values for the plate height on both low and high ligand density phases. The possibility of overloading at low ligand density, which could lead to severe tailing, was ruled out by the observation that the elution profile was not affected by a reduction of sample size. Factors which might have affected the band-broadening include heterogeneity of ligand distribution, which presumably is more severe at low ligand density, or changes in the adsorption/desorption rate constants due to changes in the electrical double layer thickness. In any case, the higher ligand density matrices exhibited the best chromatographic resolution.

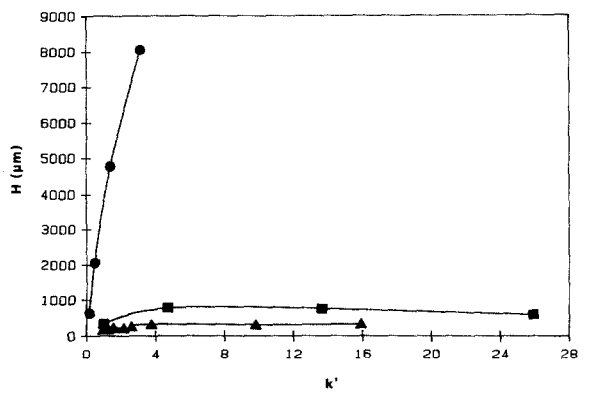

Fig. 7. Plate height *vs.* capacity factor for cytochrome c on supports of ligand density 28 μmol/g (●), 72 μmol/g (■) and 386 μmol/g (▲). The k' was varied by varying elution salt concentration. The data were derived from those in Fig. 5 by measuring plate height at each k' obtained.

TABLE III

EFFECT OF PHOSPHATE *VS.* ACETATE BUFFER ON CAPACITY FACTOR OF CYTOCHROME c

%B	k'(phosphate)	k'(acetate)	% change in k'
30	477	612	21.9
40	79.5	115	30.9
50	33.6	37.4	10.1
60	11.2	16.2	30.5

Phosphate bound to cytochrome c

Phosphate is known to bind to cytochrome c and alter its retention on cation exchangers [27]. To characterize the chromatographic behavior of cytochrome c in the presence of phosphate, phosphate and acetate buffers of the same concentration and pH were used to measure the Z number and k' of cytochrome c at a ligand density of 386 μmol/g. It was found that the Z numbers obtained from these different buffers were essentially the same (phosphate, 6.71; acetate, 6.69) but the k' value was markedly greater in the case of acetate, as shown in Table III. The fact that the Z number did not change with k' further demonstrates that there is no inherent correlation between protein retention (k') and the Z number, even for a given protein.

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