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SALT-BINDING EFFECTS IN HYDROPHOBIC-INTERACTION CHROMATOGRAPHY

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SUMMARY

Many separations of proteins are performed on hydrophobic interaction columns. Elution is achieved with salt gradients, but anomalous elution behavior is observed often. In this investigation, we show that the binding of salt to proteins may explain these anomalies. Presumably, salt binding alters the number and distribution of protein surface groups, including charged groups. Analysis of retention data was carried out by non-linear regression until the minimum sum of squares was found between the observed capacity factor and that computed from a retention model based upon Wyman's theory of thermodynamic linkage. The relation of retention to salt concentration as described by this model is compared to surface tension models and to relative elution orders based on hydrophobicity scales.

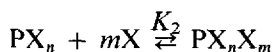
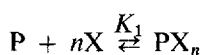
INTRODUCTION

Previous reports from this laboratory have suggested that surface-modified siliceous packings for high-performance liquid chromatography (HPLC) represent a continuum of materials with surface tensions between about 30 mJ/m² and about 55 mJ/m² (refs. 1 and 2). Moreover, adsorption of proteins to these materials was related to the surface tensions of the packing, protein, and mobile phase. Reducing the surface tension of the mobile phase so that it is below that of the protein and higher than that of the packing promoted desorption. This is accomplished commonly in reversed-phase chromatography by the addition of organic modifiers or in hydrophobic interaction chromatography (HIC) by reducing salt concentration^{3,4}. Changes in protein structure by solvent mediated denaturation altered surface tension and, therefore adsorption. However, the influence of specific salt-protein interactions was not considered. In the present investigation the binding of several milk proteins and concomitant effects on retention are evaluated using equations adapted from Wyman's theory of linked functions⁵.

THEORY

Since the retentions of all proteins only varied with ammonium sulfate and not ammonium chloride, it would be prudent to adopt a mechanism involving salt binding

to a protein in order to quantitatively explain the results. Hence, following the concepts of Wyman⁶⁻⁹ and Wyman and Gill¹⁰, we have thermodynamically linked the capacity factor of a protein to the free energy of salt binding. Such methodologies involving the use of thermodynamic linkage of physical and kinetic properties of macromolecules to cosolute binding in combination with non-linear regression analysis have been used by several investigators¹¹⁻¹⁵. With these concepts in mind, we assume a sequential binding model, *i.e.* there are essentially two classes of binding sites on proteins that are responsible for changes in retention and that all sites of one type are saturated before binding to the second type proceeds. Furthermore, we assume that equilibration among the various species is instantaneous and that the observed retention is the resultant of the individual protein species. It should be emphasized that this treatment infers nothing about binding to other site classes, but simply that it is not related to retention. Consider the following sequential equilibria:



where n and m are the number moles of ion X bound at each class of site per mole of protein, P . Then, the fraction, Q , of each species present is

$$Q(P) = \frac{[P]}{[P] + K_1[P][X]^n} \quad (1)$$

$$Q(PX_n) = \frac{K_1[P][X]^n}{[P] + K_1[P][X]^n} - \frac{K_1K_2[P][X]^n[X]^m}{K_1[P][X]^n + K_1K_2[P][X]^n[X]^m} \quad (2)$$

$$Q(PX_nX_m) = \frac{K_1K_2[P][X]^n[X]^m}{K_1[P][X]^n + K_1K_2[P][X]^n[X]^m} \quad (3)$$

Then, since

$$k'_{\text{obs}} = k'_0Q(P) + k'_1Q(PX_n) + k'_2Q(PX_nX_m) \quad (4)$$

where k'_{obs} is the observed capacity factor and k'_0, k'_1, k'_2 are the capacity factors of the respective protein salt species, as shown in Fig. 1. After substitution of eqns. 1-3 into eqn. 4, and rearrangement, the resultant expression links protein retention to salt binding:

$$k'_{\text{obs}} = \frac{k'_0}{1 + K_1[X]^n} + \frac{k'_1K_1[X]^n}{1 + K_1[X]^n} + \frac{(k'_2 - k'_1)K_2[X]^m}{1 + K_2[X]^m} \quad (5)$$

where $[X]$ is the concentration of unbound salt. Here, since the protein concentration is much smaller than the salt concentration in the mobile phase, $[X]$ is taken to be equal to the mobile phase salt concentration.

EXPERIMENTAL

Chromatography was performed at room temperature with a Model 8700XR pumping system, a Model 8750 injection system, a Model 4270 data system, all from Spectra-Physics (San Jose, CA, U.S.A.), and an Isco (Lincoln, NE, U.S.A.) UV detector set at 280 nm. The flow-rate was 1 ml/min.

Two columns were used in this study: (I) Synchropak Propyl (250 × 4.1 mm I.D.) (Synchrom, Lafayette, IN, U.S.A.) and (II) Supelco Hint LC-3 (100 × 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.), both fitted with a guard column.

Buffers for chromatography were prepared with Bio-Rad (Richmond, CA, U.S.A.) grade reagents and water purified with a Continental (San Antonio, TX, U.S.A.) Modulab I system. Composition of the buffers was (A) 0.05 *M* disodium hydrogenphosphate containing 3.75 *M* urea adjusted to pH 6.0 and (B) the same buffer containing 2 *M* ammonium sulphate. These were filtered through a 0.45- μ m biological filter (Alltech Assoc., Deerfield, IL, U.S.A.) before use. The amount of buffer B was varied so that the isocratic composition of the mobile phase was between 0 and 2.0 *M*.

Surface tensions were measured using the du Nouy balance technique. The instrument used was the Fisher Surface Tensiomat, Model 21 (Fisher Scientific, Pittsburgh, PA, U.S.A.). It was important to determine liquid surface tensions immediately before each run.

β -Casein, (β -CS), was isolated from milk¹⁶ or purchased from Sigma (St. Louis, MO, U.S.A.). α -Lactalbumin, (α -LA), and β -lactoglobulin, (β -LG), were from Sigma. All proteins were greater than 90% pure as judged by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on densitometric scan at 550 nm. For HPLC, proteins were dissolved in buffer A (2 mg/ml), filtered through a 0.45- μ m filter and injected (50 or 100 μ l). Elution was carried out isocratically using the solvent delivery system to obtain the desired mobile phase composition.

Capacity factors (k') were calculated from the position of the peak maxima and the void volume which was taken in this study as the solvent peak.

$$k' = \frac{t_R - t_0}{t_0} = \frac{\text{mmol protein in the stationary phase}}{\text{mmol protein in the mobile phase}} \quad (6)$$

The relationships between salt concentration and k' were evaluated using a Gauss-Newton non-linear regression analysis program developed at this laboratory. Eqn. 5 was iterated to minimize the sum of square differences between measured and calculated k' values. Experimental salt values were used and n and m were the adjustable parameters¹⁷.

RESULTS AND DISCUSSION

HPLC has been used as a tool for studying how milk proteins contribute to functional properties of foods^{18,19}. In the latter study, HIC columns were used with ammonium sulfate mobile phases with an without urea as an additional mobile phase modifier. The present evaluation of salt binding effects there focused on similar systems.

Fig. 1 shows the variation of k' of β -LG with HIC column I using an ammonium

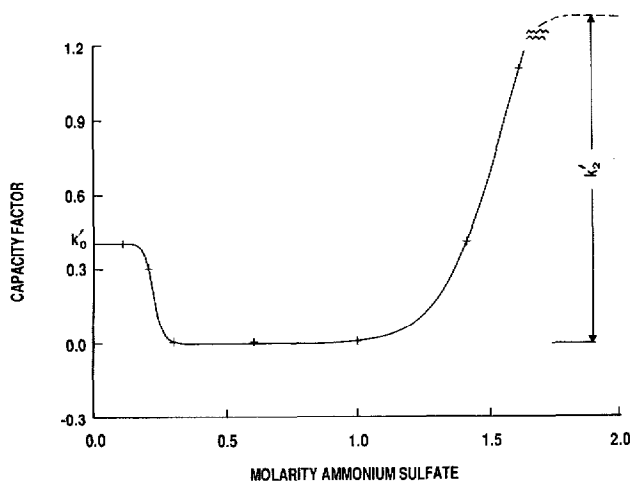


Fig. 1. Effect of ammonium sulfate concentration on retention of β -lactoglobulin. Conditions: column I; mobile phase, buffer A. (+) Data, (—) regression of eqn. 5.

sulfate-urea mobile phase (pH 6) as described in the experimental section. Data points are denoted by (+). Since, retention of proteins in HIC has been related to surface tension of the mobile phase^{1,20}, we determined the surface tensions of the phosphate-urea mobile phases that contained varying amounts of ammonium sulfate. Two fits of the data (+) are given in Figs. 2 and 3. The lines were obtained by the same non-linear regression analysis described in the theoretical section for analysis of the retention data. This analysis indicates that ammonium sulfate binds to urea at either one or three sites. The solution properties of urea are themselves thermodynamically complex because urea forms concentration dependent aggregates²¹. Moreover, some salts have been shown to interact with urea possibly through ion-dipole and dipole-dipole

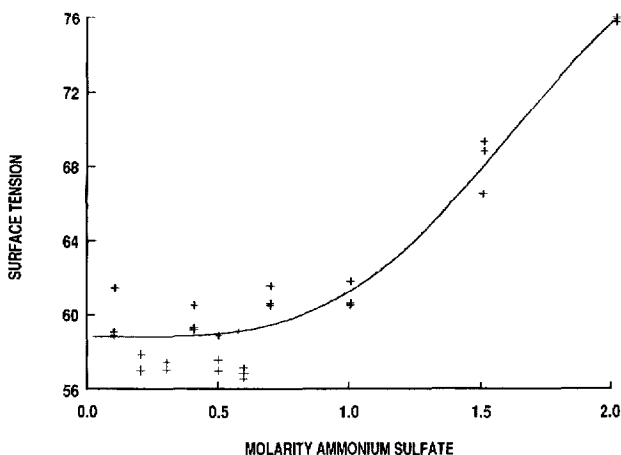


Fig. 2. Effect of ammonium sulfate concentration on surface tension of 3.75 M urea solutions. (+) Measured, (—) line from non-linear regression.

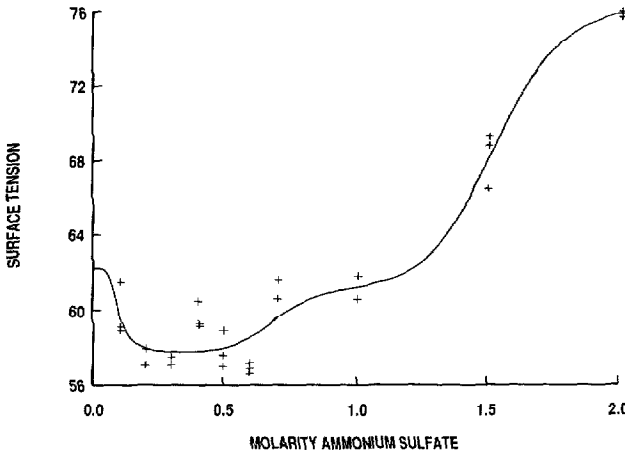


Fig. 3. Effect of ammonium sulfate concentration on surface tension of 3.75 M urea solution with a three binding site model.

interactions²². The lower root-mean-square of the three-site model (1.7 as compared to 1.1) suggests that it is more appropriate. More importantly plots of the deviation was examined for each model (not shown). The plot for the one site model was multi-phasic whereas a random deviation plot was found for the three site model. A random distribution is considered to be an appropriate criterion of goodness of fit to the data²³. Apparently, urea species, under the conditions used in this study, are multimers with three sites that are sequentially saturated with salt. The net result is that a minimum is observed when surface tension of 3.75 M urea is plotted against molarity of added salt. From refs. 1-4 we see that capacity factor is related to the surface tensions of column packing (G_{sv}), protein (G_{pv}), and mobile phase (G_{mv}), by:

$$k' = C \exp \left[\frac{\sqrt{G_{pv}G_{sv}} - (\sqrt{G_{sv}} + \sqrt{G_{pv}}) \sqrt{G_{mv}} + G_{mv}}{RT} \right] \quad (7)$$

Fig. 4 describes some properties of eqn. 7, assuming a G_{pv} of 70 mJ/m², a value that is reasonable for the whey proteins α -LA and β -LG by analogy to blood serum proteins²⁴. A value of 53 mJ/m² was used for the packing. This is a typical value for HIC materials¹. As mobile phase surface tension decreases, k' is predicted to decrease and reach 0 when $G_{mv} = G_{pv}$ as indicated by segment AB. Capacity factor rises again if G_{mv} continues to fall below both G_{pv} and G_{sv} (segment BC). If G_{mv} increases with further reduction in salt concentration as in Fig. 3, then k' is predicted to rise again back along segment BA. As shown clearly in Fig. 5, k' of β -LG does not respond in this manner. Mobile phase surface tension does not uniquely define a single k' , so that the presence of different solute species is indicated at different salt concentrations. G_{pv} is, therefore, a variable also.

The solid line in Fig. 1 represents the excellent fit of the data by non-linear regression with eqn. 5. The root mean square was 0.01 and residual sum of squares was $6 \cdot 10^{-5}$ which demonstrates the credibility of the model for β -LG retention. Plots of protein solubility with varying pH have often been bimodal and have been explained in

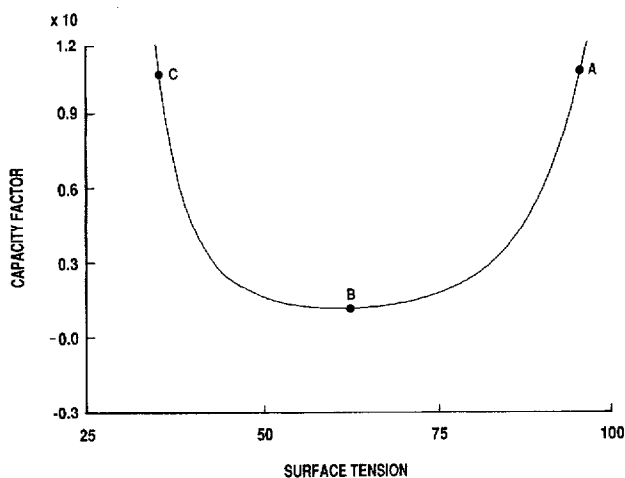


Fig. 4. Calculated curve representing effect of mobile phase surface tension on retention of protein ($G_{pv} = 68$ mJ/m^2); column surface tension ($G_{sv} = 53$ mJ/m^2).

terms of proton binding²⁵. Here, binding of other ions influences retention. When ammonium chloride was substituted for ammonium sulfate, proteins were not retained at concentrations up to 4 *M*, suggesting that sulfate binding is the significant contributor to the retention profile. These data were obtained at pH above the protein's isoelectric points so that proteins had a net negative charge. This does not preclude anion binding, however, since about 80% of the positive sites²⁶ remain. Moreover, direct interactions between large anions and protein amide groups have been reported¹⁶. Cation binding may occur but it is not linked thermodynamically to retention.

The plots from regression analysis of the retention of β -CS and α -LA are given in Figs. 6A and B. As with β -LG, plots of k' do not vary exponentially with salt

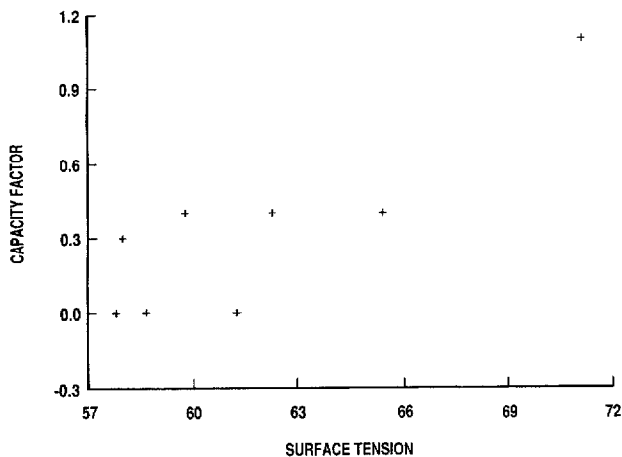


Fig. 5. Effect of surface tension on β -LG retention.

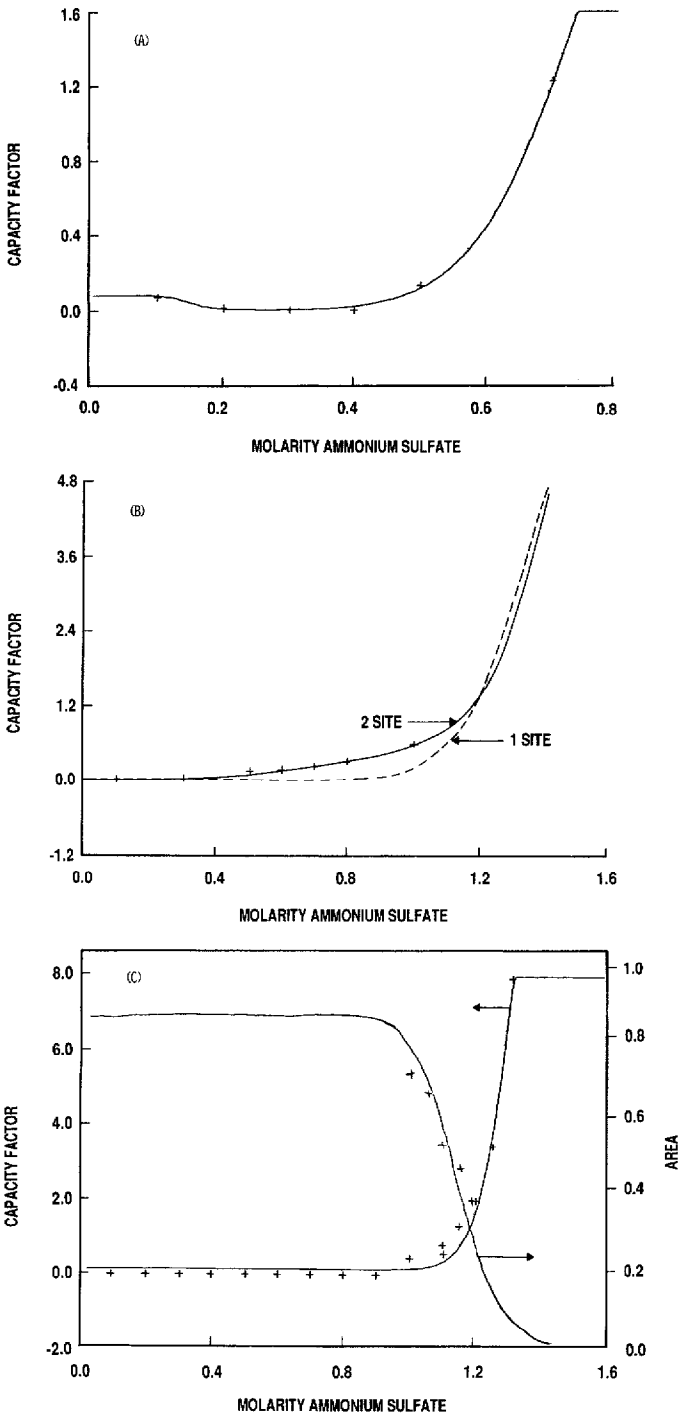


Fig. 6. Effect of ammonium sulfate concentration on retention of: (A) β -casein, (B) α -lactalbumin with (---) one binding site fit, and (—) two binding site fit. Conditions as in Fig. 1. (C) β -Lactoglobulin, conditions as in Fig. 1 except that no urea was used in mobile phase.

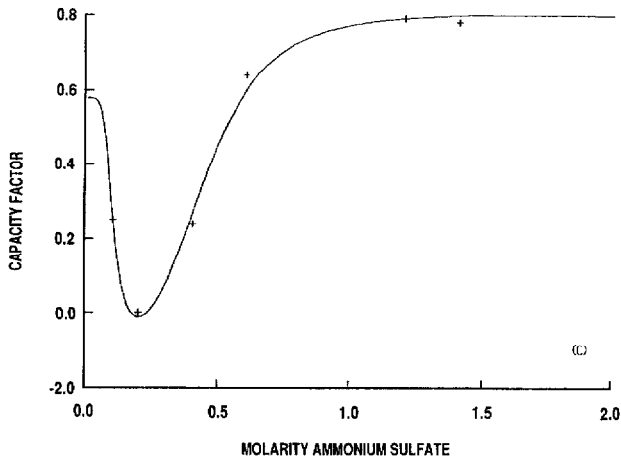
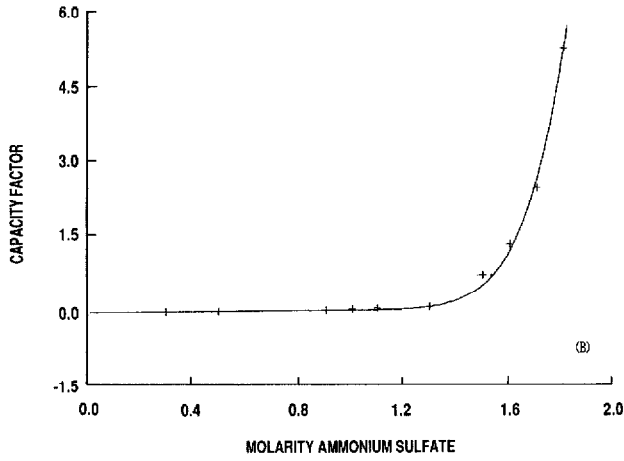
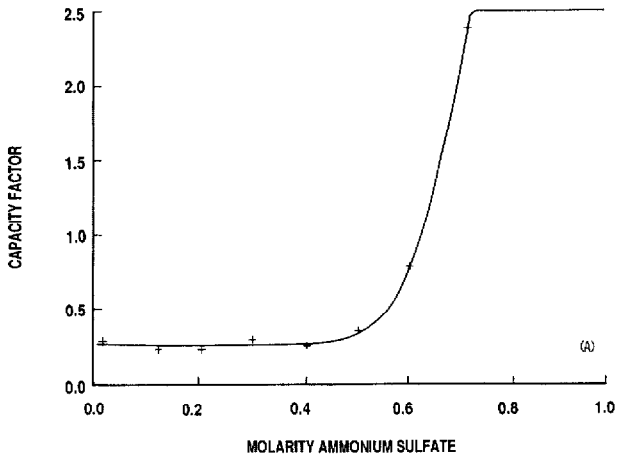


Fig. 7. Effect of ammonium sulfate concentration on retention of: (A) β -casein, (B) β -lactoglobulin, (C) α -lactalbumin. Conditions as in Fig. 1. except column I was used.

TABLE I

SALT BINDING PARAMETERS AS DETERMINED BY NON-LINEAR REGRESSION OF EQN. 5

Protein	K_1 (l/mol)	K_2 (l/mol)	n (mol)	m (mol)	k'_2	RMS
<i>Column I</i>						
β -CS	7.2 ± 1.1	1.4 ± 0.6	8	10	23 ± 6	0.2
	6.8 ± 1.6	1.2 ± 0.04	8	8	38 ± 10	0.1
β -LG	4.6 ± 0.2	0.6 ± 0.02	12	12	1.9 ± 0.3	0.01
	4.5 ± 0.1	0.7 ± 0.00	16	34	1.2 ± 0.0	0.01
α -LA	$(1.1 \pm 0.1)^*$	0.7 ± 0.02	4	12	14 ± 3.0	0.02
<i>Column II</i>						
β -CS	—	1.4 ± 0.0	—	12	5.3 ± 0.6	0.01
β -LG	—	0.5 ± 0.01	—	14	26 ± 7	0.1
α -LA	9.9 ± 0.8	2.2 ± 0.1	4	4	0.9 ± 0.0	0.02

* First site is different than others shown. Single site RMS = 0.2.

concentration. Elution behavior of proteins has often been treated as if there were an exponential dependence of retention on salt concentration^{20,28}. The first binding site of lactalbumin occurs at higher salt concentrations than the first site for either β -LG or β -CS, and is therefore likely to be of a different type. The two site model for α -LA gave a four fold improvement in RMS analysis over the one site model.

All proteins used in this study exhibited a dramatic decrease in peak area with increased retention when they were eluted with sulfate-containing mobile phase that contained no urea. β -LG curves are given in Fig. 6C as an example.

The quantities of urea used in these experiments are insufficient to cause appreciable denaturation of β -LG or α -LA. Molarities $>5 M$ are required to do this^{29,30}. β -CS self aggregates in solution and the micelles increase in size with time. Urea promotes dissociation of these units.

Fig. 7 depicts the retention characteristics of the milk proteins on column II. Again excellent correlation of the data with the salt binding model is observed. However, the proteins exhibited different retention behavior on the two columns as can be seen by comparison of Fig. 7 with Figs. 1 and 6. Retention of proteins is lower on column II with the exception of β -LG. Table I gives values for the salt binding constants (K_1 , K_2), the number of moles bound at each site class that is correlated with retention (n , m) and the estimated retention of the fully saturated salt species (k'_2). These values suggest different interaction modes on the two columns. The support matrix of column II is of the polyol type whereas column I contains amidopropyl functionality. The differences in behavior of the proteins with the two materials could result from sulfate linkages between protein and amide groups. As stated earlier direct interactions between anions and the amide dipole were reported.

Elution order is related to both column binding strength as determined by k' and by the salt binding equilibrium constant (K_1). Significant retention of these milk proteins occurs only when salt binds to the second site class. Thus in conventional HIC high salt mobile phases initially are used followed by a gradient to lower salt. For column I, β -CS has the highest K_2 and k'_2 so it is strongly retained. Salt saturated lactalbumin is more strongly adsorbed to the column than β -LG. Thus their elution order is different than is predicted from considerations of their average hydrophobicities ($H\phi_{ave}$) as defined by Bigelow³¹. These parameters are calculated from

protein amino acid composition and are 1150 and 1230 cal/res, respectively, for α -LA and β -LG³¹. Such anomalies in elution order were observed by others^{19,32} but is explained here through considerations of salt-protein interactions. β -LG has a higher k'_2 on column II but is eluted first because of its smaller salt binding equilibrium constant.

This paper demonstrates that considerations of protein, column, and mobile phase surface tensions indicate a minimum in retention vs. mobile phase surface tension plots when (1) the mobile phase surface tension passes below both the surface tension of the protein and of the column packing, or (2) the mobile phase surface tension passes through a minimum with composition. Moreover, salt binding modulates protein-column packing interactions so that specific salt binding effects cannot be ignored if protein retention in HIC is to be described more adequately. Furthermore the lack of correlation of retention with molecular parameters such as amino acid composition can be explained in terms of salt binding. Research is continuing to evaluate corresponding effects in reversed-phase chromatography.

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