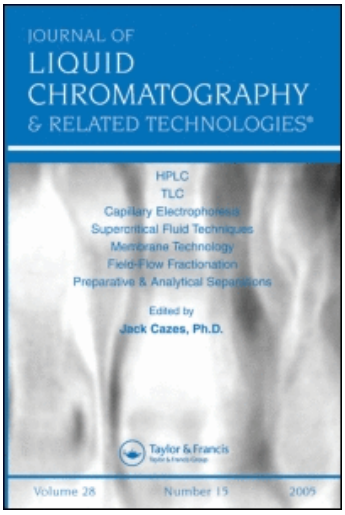

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SALT MEDIATED ELUTION BEHAVIOR OF PROTEINS ON A SILICA-BASED STATIONARY PHASE FOR SIZE EXCLUSION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Salt mediated elution behavior of several proteins on the commercial silica-based size exclusion TSK G 3000 SW column was examined. Depending on the nature and salt concentration, this column exhibits three domains where either sieving effect or electrostatic or hydrophobic interactions are predominant. At sufficiently low salt concentration positively charged proteins were retained by electrostatic interactions, whereas negatively charged proteins were subjected to Donnan exclusion. On the other hand, by using salt with a large molal surface tension increment, at sufficiently high salt concentration proteins were retained by hydrophobic interactions. Under these conditions the hydrophobic parameters, defined as the slope of the linear plot of log K' against salt molality, were

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evaluated and compared to those obtained on two columns for hydrophobic interaction chromatography , prepared by coupling butyl and phenyl glycidil ethers to the TSK G 3000 SW packing material. Proteins in a narrow molecular weight range were separated on the TSK G 3000 SW column according to their isoelectric point by the use of increasing salt gradient.

INTRODUCTION

Besides electrostatic interaction (ion-exchange) chromatography (EIC), where fixed charges on the stationary phase interact with the charged protein molecules (1) and hydrophobic interaction chromatography (HIC), where the corresponding interaction is between hydrophobic moietes (2), electrostatic and hydrophobic interactions are often involved in other non-denaturating modes of protein chromatography, giving rise to mixed mode retention mechanisms. Horvàth and El Rassi have demonstrated that in (HIC) with stationary phases having ionic groups at the surface, electrostatic interactions may effect the retention despite the relatively high salt concentration in the eluent. Mixed mode retention mechanisms have also been observed in biospecific interaction (affinity) chromatography (BIC) (4) and in metal chelate interaction chromatography (MIC) (5).

The nature and concentration of the salt in the eluent effect both electrostatic and hydrophobic interactions and, therefore, it is of fundamental importance to investigate the salt mediated chromatographic

behavior of a given column. A theoretical treatment of the effect of salt on the retention behavior of proteins has been described by Horvàth et al. (6), who also presented the use of commercial ion-exchange stationary phases either in EIC or HIC, depending on the range of the mobile phase salt concentration. The use of ion-exchange stationary phases in dual retention mode was also described by Regnier et al. (7), whereas Karger et al. (8) demonstrated that a given hydrophobic interaction column can be used for the separation of proteins either by size exclusion chromatography (SEC) at low salt concentration or by HIC with decreasing salt gradient.

In SEC, samples are theoretically separated by size and shape, with large excluded molecules eluting first and small, totally included molecules eluting last. In practice, the stationary phase cannot be considered inert over a wide range of experimental conditions because it may exhibit weakly anionic and hydrophobic character (9-11). It is important, therefore, to identify the mode of interaction and either eliminate or take advantage of it when useful. In both cases the appropriate selection of the mobile phase compositions is needed.

In this paper we describe the salt mediated elution behavior of several proteins on a commercial silica-based stationary phase designed to be used in

SEC, which has proven to be a widespread technique for the analytical and preparative scale separation of proteins. The results provide information about the effect of salt concentration and salt molal surface tension increment on the electrostatic and hydrophobic interactions of proteins with this stationary phase.

This information is useful to evaluate the weakly anionic and hydrophobic character of SEC with silica-based, bonded-phase column. Results in this study also suggest that different salts and salt concentrations can be used to control retention and selectivity during SEC with silica-based, bonded phase columns.

EXPERIMENTAL

Materials

Cytochrome c (horse heart), lysozyme (chicken egg white), myoglobin (equine skeletal muscle), ß-lactoglobulin A (bovine milk), α-chymotrypsinogen A (bovine pancreas), conalbumin (chicken egg white), were purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade sodium hydrogen phosphate, ammonium sulfate, sodium chloride, as well as HPLC-grade water were obtained from Carlo Erba (Milan, Italy).

Equipment

The experiments were performed with a Beckman (Fullerton, CA, U.S.A.) Model 342 Liquid Chromatograph, consisting of two Model 114 M solvent delivery pumps, a

Model 420 system controller, a Model 210 sample injection valve with a 20 µl sample loop, a Model 340 dynamically-stirred high pressure mixer and a Model 163 variable wavelenght U.V. detector. Chromatograms were recorded by a Shimadzu (Kyoto, Japan) Model C-R 5A Chromatopac integrator. A Bio Sil TSK 250 (300 x 7.5 mm) column was supplied by Bio-Rad Labs. (Richmond, CA, U.S.A.).

Chromatographic Conditions

Mobile phases were prepared by adding the correct weight of salt and buffer to a volumetric flask containing previously degassed HPLC water, the pH was adjusted to the appropriate value with sodium hydroxide and small amount of degassed HPLC water was added to the mark. All eluents were filtered through a type HA 0.45 µm membrane filter (Millipore, Bedford, MA, U.S.A.) and degassed by sparging with helium. Protein solutions (2-5 mg/ml) were freshly made up in HPLC water. A 20 µl sample injection volume was utilized. Chromatograms were obtained under isocratic conditions at a nominal flow rate of 1.0 ml/min. After each change of mobile phase composition a period of 30 min (+ 0.5 min) was allowed for equilibrating. Proteins were detected by monitoring the column effluent at 280 nm. Exception to these conditions are noted in the paper.

TABLE 1
Proteins Used in This Study

Protein	Symbol	MW	pI*
Cytochrome c	CYT	12.200	10.6
Lysozyme	LYS	14.000	11.0
Myoglobin	MYO	17.500	7.1
ß-Lactoglobulin A	LAC-A	17.500	5.1
α-Chymotrypsinogen A	CHY	25.500	9.5
Conalbumin	CON	93.000	6.0, 6.3 6.6

*From refs. 13-14

RESULTS AND DISCUSSION

A Toya Soda TSK G 3000 SW column (12) (marketed by Bio-Rad as Bio-Sil TSK 250) was chosen for our investigation since it exhibits good resolving power and high mass recovery for proteins. It is frequently applied in biochemical research. This column is packed with a chemically bonded silica-based stationary phase whose precise chemical and physical composition remains proprietary information.

Elettrostatic Interactions

We first investigated the elution behavior of proteins at low ionic strength to evaluate the effect of electrostatic interactions between the G 3000 SW

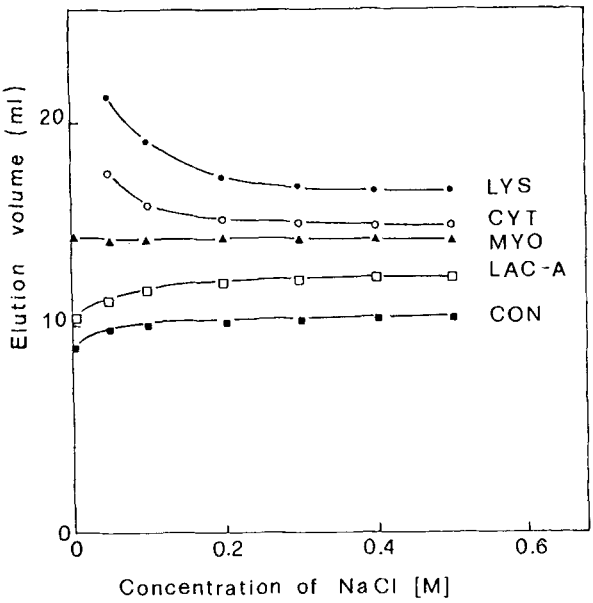

FIGURE 1. Variation of the elution volume of representative proteins as a function of mobile phase salt concentration. Column, TSK G 3000 SW (300 x 75 mm I.D); eluent, sodium chloride in 20 mM phosphate buffer, pH 7.0; flow rate, 1.0 ml/min; temperature, 22 °C; detection, 280 nm.

bonded-phase and charged proteins. The elution volumes of the proteins reported in Table 1 were determined as function of salt concentration in the buffered mobile phase at pH 7.0.

The results for these proteins are reported in Figure 1. It is seen that three types of behavior were observed for the elution volumes of the proteins as the mobile phase salt concentration was decreased below 0.3 M NaCl. The more basic proteins lysozyme and cytocrome

c (pI values 11.0 and 10.6, respectively) showed increasing elution volumes with decreasing mobile phase salt concentration. The opposite trend was exhibited by the acidic proteins conalbumin (pI value 6.0-6.6) and ß-lactoglobulin A (pI value 5.1) which exhibited decreasing elution volumes with decreasing salt concentration. A nearly constant elution volume was displayed by the neutral protein myoglobin (pI value 7.1). The observed behavior of proteins on the G 3000 SW column at low mobile phase salt concentration may be due to the presence of residual charged silanol groups on the surface of the silica-based stationary phase. At low salt concentration, negatively charged proteins would be expected to be partially excluded from the negatively charged porous of the stationary phase and, therefore, they have smaller elution volume than expected on the basis of their molecular size. Since electrostatic interactions decrease with increasing ionic strength, these proteins would gain extended access into the pores as the salt concentration is increased, with the consequent increase in the elution volumes. On the other hand, positively charged proteins would be retained by electrostatic interactions with the negatively charged stationary phase and, therefore, they exhibited opposite behavior relative to the acidic proteins. Neutral proteins would be expected to be the least influenced by a charged stationary phase and, conse-

quently, their elution volumes would not be effected by changes in the ionic strength, as experimentally observed for myoglobin.

The experiments described above were repeated at pH 5.4, so that silanol groups were still ionized and both myoglobin and conalbumin are positively charged. With the pH 5.4 mobile phase conditions both proteins were retained at low salt concentration and both exhibited decreased elution volumes as the salt concentration increased. These results confirmed that electrostatic interaction of positively charged groups on the proteins with the negatively charged stationary phase was mainly responsible for the retention behavior of basic proteins at low ionic strength. The elution volumes of all proteins investigated were essentially independent of mobile phase salt concentration above 0.3 M sodium chloride, indicating that at this mobile phase conditions the interactions of the charged stationary phase with the charged proteins were neutralized to a large extent.

Hydrophobic Interactions

The possible contribution of hydrophobic interactions between the G 3000 SW bonded-phase and proteins was also investigated. According to the theory (15), hydrophobic interactions are expected to be governed by salt mediated changes in the surface tension of the

mobile phase, which depends on the concentration and the molal surface tension increment of the salt. At sufficiently high salt concentration an increase in either quantity will lead to an increase in elution volume which depends on the magnitude of the contact area between the hydrophobic patches at the protein surface and the hydrophobic binding sites on the stationary phase.

We chose to examine the elution behavior of proteins at high salt concentration by using ammonium sulfate which has a higher molal surface tension increment (2160 dyne x g x cm^{-1} x mol^{-1})than sodium chloride (1610 dyne x g x cm^{-1} x mol^{-1}) and, therefore, is more effective in promoting hydrophobic interactions between proteins and silica-based, bonded phase columns.

Upon increasing the concentration of ammonium sulfate above 0.6 M, the elution volumes of proteins increased with salt concentration. The three proteins undergoing the greatest increases were α-chymotrypsinogen A, lysozyme and conalbumin (Figure 2). This finding is consistent with the hydrophobic character of these proteins and agrees with their chromatographic behavior in HIC (16). At salt concentrations higher than 0.8 M, plots of log K' against ammonium sulfate concentration were linear. Slopes were compared to those calculated from retention data obtained on

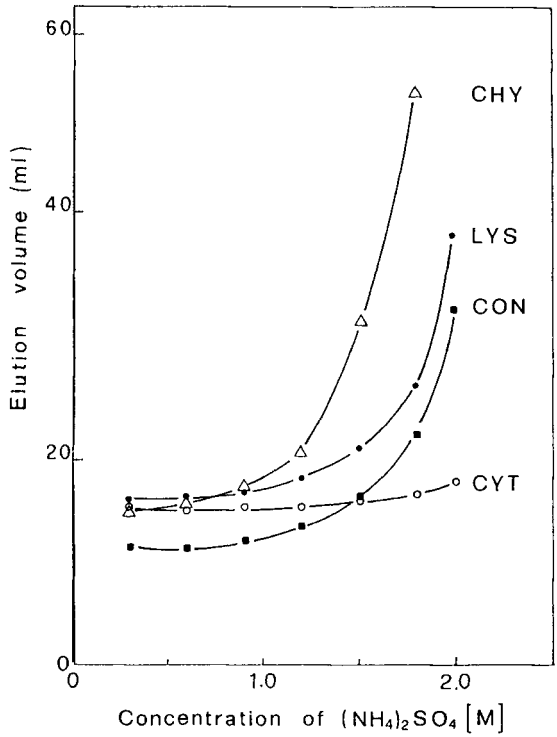

FIGURE 2. Variation of the elution volume of representative proteins as a function of mobile phase salt concentration. Eluent, ammonium sulfate in 100 mM phosphate buffer, pH 6.0. Column and other conditions as in Fig. 1.

two HIC columns synthesized by coupling butyl and phenyl glycidil ethers to the TSK G 3000 SW packing material (17) and are given in Table 2. According to the theoretical treatment developed by Horvàth and coworkers (6), the slope, termed hydrophobic interaction parameter, C, expresses the magnitude of the salt mediated hydrophobic interaction and is expected to

TABLE 2

Hydrophobic Interaction Parameters,C, as Measured by the Limiting Slopes of log K' Against Ammonium Sulfate Concentration in the Mobile Phase (pH 6.0) for Proteins on Different Columns.

Sample		Column G 3000 SW[1]	Butyl G 3000 SW[2]	Phenyl G 3000 SW[2]
CHY	C	0.90	1.35	1.81
	r	0.993	0.997	0.996
LYS	C	0.41	1.10	0.94
	r	0.991	0.996	0.999
CYT	C	0.11	0.97	1.18
	r	0.991	0.999	0.995

[1]Column described in this report
[2]Calculated from ref. 17

increase with the density and size of the hydrophobic binding sites at the stationary phase surface, particularly at low hydrophobic functional group concentrations. Thus, the relative magnitude of the hydrophobic interaction parameter obtained with retention data of the same proteins on the three different columns reveals, as expected, lower density of hydrophobic functions at the surface of the unmodified G 3000 SW bonded-phase than at the surface of the butyl or phenyl G 3000 SW stationary phase.

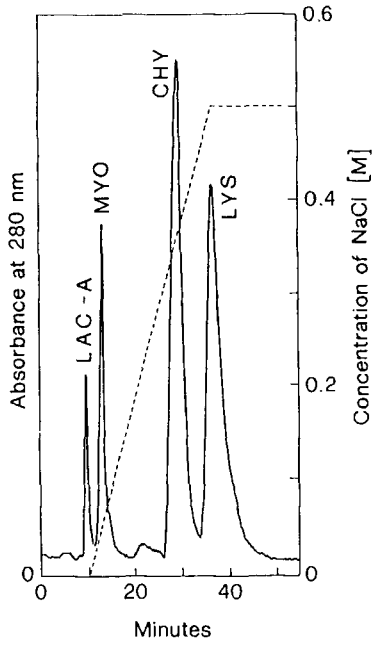

FIGURE 3. Separation of proteins by mixed retention mode HPLC. Eluent A, 20 mM phosphate buffer (pH 7.0); eluent B, 0.5 M sodium chloride in eluent A; 10 min isocratic elution with eluent A followed by 30 min linear gradient from 0 to 100% B; Column and other conditions as in Fig. 1.

These results show that, depending on the nature and range of salt concentrations, the G 3000 SW column exhibits three distinct domains where sieving, electrostatic, or hydrophobic interactions seem to be predominant. This suggests that in principle the G 3000 SW column could be used to elute proteins in each of the three modes, corresponding to the predominant mechanism of separation (SEC, EIC, HIC), by appropriate selection of the composition of the mobile phase.

The use of the G 3000 SW column for the separation of proteins in a narrow molecular weight range under conditions used in electrostatic interaction mode is illustrated by the chromatogram in Figure 3. During the initial isocratic elution step with the mobile phase at pH 7.0 and low ionic strength the negatively charged proteins ß-lactoglobulin A is subjected to Donnan exclusion. Therefore, it is separated from the neutral protein myoglobin which has the same molecular weight, but is not influenced by the charges on the stationary phase. Under these conditions, the positively charged proteins lysozyme and α chymotrypsinogen A are strongly retained by electrostatic interaction and are selectively eluted in the order of increasing pI by the following increasing salt gradient.

CONCLUSIONS

Depending on the salt and salt concentration the TSK G 3000 SW column exhibits three distinct separation mechanisms based either on sieving, electrostatic or hydrophobic interactions. This column behaves as a cation-exchanger at ionic strength values below 0.3 M, independently of the nature and valence of the salt. In contrast, the domain of hydrophobic interactions depends on the concentration and the molal surface tension increment (σ) of the salt. Thus, with sodium chloride, which has a molal surface tension increment

of 1610 dyne x g x cm^{-1} x mol^{-1}, no changes in the elution volumes of proteins were observed with salt concentrations up to 1.8 M. With ammonium sulfate the elution volumes increased with salt concentration above 0.6 M. This indicates that the domain of the predominance of the sieving effect lies in a range that can be enlarged by changing the salt to one with lower molal surface tension increment or restricted by changing the salt to one with greater σ value.

Indeed, it is likely that the separation of proteins under the whole range of salt concentration investigated occurs, to a minor or major extent, according to mixed separation mechanisms rather than solely size exclusion or electrostatic or hydrophobic interactions. However, information about the mobile phase compositions under which each separation mechanism seems to be predominant is useful to characterize the chromatographic behavior of columns and to tailor the selective properties of the stationary phase for a given separation problem.

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