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# CHROMATOGRAPHY OF PROTEINS ON HYDROPHOBIC INTERACTION AND ION-EXCHANGE CHROMATOGRAPHIC MATRICES: MOBILE PHASE CONTRIBUTIONS TO SELECTIVITY

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## SUMMARY

The effect of mobile phase pH on the retention characteristics of eleven proteins was examined in hydrophobic interaction chromatography (HIC) on a SynChropak propyl stationary phase. Selectivity was shown to change with eluent pH. The effect of the displacing salt on the separation of proteins on a weakly hydrophobic weak-anion-exchange chromatography (AEC) packing was examined. Some differences in selectivity were observed when sodium sulfate was used as the displacing salt, compared to that observed with sodium chloride in the eluent. It was demonstrated that these AEC packings exhibited both electrostatic and hydrophobic properties, depending upon the type and concentration of salt used in the mobile phase. The addition of 20% ethylene glycol to the mobile phase was shown to reduce the hydrophobic interactions. The application of weakly hydrophobic weak-cation-exchange packings to HIC of proteins was demonstrated. Elution of such columns with descending sodium sulfate gradients was found to provide a selectivity different from that observed with a propyl stationary phase. Manipulation of mobile phases was shown to provide useful selectivity as a result of the combination of electrostatic and hydrophobic contributions to the separation process.

## INTRODUCTION

Hydrophobic interaction chromatography (HIC) is a valuable technique for the separation of proteins under non-denaturing conditions. In HIC, the protein is adsorbed from solutions of salts high on the lyotropic series (such as sodium sulfate or ammonium sulfate) at high concentrations[1]. Protein separations are accomplished by elution with gradients from high salt (typically $> 1\ M$) to low salt concentrations. The technique is valuable for preparative or analytical separations on high-performance liquid chromatographic (HPLC) columns. Column packings feature weakly hydrophobic surfaces, such as propyl, butyl, phenyl, poly(ethylene glycol), or poly(alkyl aspartamide)[2–7]. Selectivity in HIC is influenced by many factors: the type of salt used

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to induce hydrophobic interactions[8,9], addition of surfactants[10], and temperature[6,11] have been shown to influence the separation of proteins.

Kennedy *et al.*[12] reported the synthesis of a weak-anion-exchange chromatographic sorbent, and examined its application to the HIC separation of proteins. They demonstrated that polyethyleneimine, adsorbed, on silica, exhibits both hydrophobic and electrostatic character and, when applied to the separation of proteins under HIC conditions, exhibited unique selectivity. This selectivity was attributed to secondary electrostatic interactions of the charged solute with the charged anion-exchange matrix. Separations accomplished on matrices operating by multiple independent separation mechanisms were termed "multimodal chromatography". Chromatogaphy in which simultaneous mechanisms contribute to the separation was referred to as "mixed mode chromatography". A combination of solute–chromatographic surface interactions, working in concert with size-exclusion effects, has been identified in liquid chromatography[13–16]. When these individual phenomena function independently, the retention process can be expressed by the equation

$$k'_T = k'_{EI} + k'_{HI} + k'_{HB} + k'_{SE} + k'_{SI} + \ldots\ldots k'_{XI} \quad (1)$$

where $k'_T$ is the total capacity factor of the solute, $k'_{EI}$ is the electrostatic interaction contribution, $k'_{HI}$ is the contribution from hydrophobic interactions, $k'_{HB}$ is the contribution from hydrogen bonding, $k'_{SE}$ is the size-exclusion contribution (which is usually so small that it is ignored), $k'_{SI}$ is from specific interactions, and $k'_{XI}$ is from other contributions to retention. Cooperative adsorption, such as simultaneous electrostatic and hydrophobic interactions, is well known in chromatography, *e.g.*, affinity chromatography[17].

The chemical composition of both the stationary and mobile phases contributes to the retention mechanism of a solute on a column. Although most systems have the potential for interacting with solutes in multiple modes, mobile phases are usually chosen which eliminate some modes while accentuating others. Mixed-mode chromatography can provide unique advantages for the separation of proteins and nucleic acids. Bischoff and McLaughlin[14] obtained excellent resolution of oligonucleotides and tRNA on a $C_{18}$ reversed-phase HPLC support, coated with methyltrioctylammonium chloride. The chromatographic matrix exhibited both ionic and hydrophobic character when eluted with ascending ammonium acetate gradients. Kenley *et al.*[18] made use of multiple interactions for analysis of the decapeptide nafarelin and the polymer poly(lactide–co-glycolide), formulated in a controlled-release pharmaceutical formulation. We have examined the contribution of hydrophobic and electrostatic properties to the separation of polypeptides on HIC and weak-ion-exchange chromatography (IEC) matrices. The contribution of mobile phase composition, including pH and choice of eluting salt, to selectivity in HIC, IEC, and multimodal chromatography was studied.

## EXPERIMENTAL

### *Materials*

Vydac 101 TPB 5.5-$\mu$m (spherical, 300 Å) silica was obtained from the

Separations Group (Hesperia, CA, U.S.A.); glutaric anhydride and diisopropylethylamine were from Aldrich (Milwaukee, WI, U.S.A.); polyethyleneimine and diglycidylglycerol were from Polysciences (Warrington, PA, U.S.A.); proteins were from Sigma (St. Louis, MO, U.S.A.); ethylene glycol was from Fisher Scientific (Fair Lawn, NJ, U.S.A.); analytical-reagent-grade granular anhydrous sodium sulfate was from Mallinckrodt (Paris, KY, U.S.A.); ammonium sulfate was from Bio-Rad Labs. (Richmond, CA, U.S.A.). Analytical-reagent-grade dibasic sodium hydrogenphosphate, monobasic potassium phosphate, sodium chloride, and tris(hydroxymethyl)-aminomethane (Tris) were used.

*Apparatus*

Separations were achieved on a Varian 5500 liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.), equipped with a Spectroflow 773 detector (Kratos, Ramsey, NJ, U.S.A.) or on a Beckman Model 322 liquid chromatograph, equipped with Model 114M pumps and Model 152 detector (Beckman Instruments, Fullerton, CA, U.S.A.). Detection was at 254 nm. SynChropak propyl (100 × 4.6 mm I.D.), SynChropak AX300 (100 × 4.6 mm I.D.), and SynChropak CM300 (100 × 4.6 mm I.D.) columns were obtained from SynChrom (Linden, IN, U.S.A.). Synthesized sorbents were packed into 50 × 4.1 mm I.D. columns, using a high-pressure slurry technique[19].

*Methods*

Polypeptides were prepared in the low-salt eluent at the following concentrations: ferritin, myoglobin, and cytochrome *c* at 3 mg/ml; lysozyme, ovalbumin, and bovine serum albumin at 20 mg/ml; all other protein were prepared at 10 mg/ml. Proteins were injected with a 20 $\mu$l loop.

*Anion-exchange chromatography.* A flow-rate of 1 ml/min was used. Separations were carried out as follows: eluent A, 10 mM Tris–HCl (pH 8.0); eluent B, 0.5 *M* displacing salt (the type is indicated in the paper) in 10 m*M* Tris–HCl (pH 8.0); gradient profile, 20-min linear gradient from 100% A (0% B) to 0% A (100% B). Deviations from these conditions are indicated in the paper.

*Hydrophobic-interaction chromatography.* A flow-rate of 1 ml/min was used. Separations were carried out as follows: eluent A, displacing salt in 20 mM phosphate buffer (the type of salt, salt concentration, and the pH are indicated in the paper); eluent B, 20 m*M* phosphate buffer (pH indicated below) gradient profile, linear 20-min gradient from 100% A (0% B) to 0% A (100% B). Deviations from these conditions are indicated in the paper.

*Retention vs. ionic strength.* Isocratic retention measurements were carried out as follows: eluents A and B were as described for HIC. A flow-rate of 1 ml/min was used. The column was equilibrated with the appropriate mixture of eluents A and B, and the retention of the protein was determined. The mobile phase composition was altered as incremental mixtures of eluents A and B and the ionic strength at each data point was calculated[20].

*Cation-exchanger synthesis.* Silica (1 g) was suspended in 10 ml of methanol containing 1% (w/v) polyethyleneimine-18 (PEI-18), filtered, and the adsorbed coating was cross-linked, using 10 ml of 5% (w/v) diglycidylglycerol in methanol[21]. The material was filtered, washed with methanol, and dried under vacuum at room

temperature. After drying, 0.7 g of PEI-18 coated silica was suspended in a solution of 4 ml of dry dimethylformamide, 250 μl dry, redistilled diisopropylethylamine (DIEA), and 200 mg of glutaric anhydride. The reaction was allowed to proceed overnight at 60°C. The product was filtered on a sintered-glass funnel, washed with methanol, triethylamine, water, and finally methanol. The sorbent was vacuum-dried at room temperature and stored in a dessicator.

## RESULTS AND DISCUSSION

The effect of mobile phase pH on protein retention characteristics was examined on a SynChropak propyl chromatographic matrix, eluted with a gradient from high to low salt concentrations of ammonium sulfate. The results are summarized in Fig. 1. The change in selectivity with change in pH was related to the isoelectric points (p*I*) of the proteins[22–24]. No general relationship between p*I* and retention behavior in HIC was observed. Srinivasan and Ruckenstein[25] ascribed the pH effect on protein retention to a number of factors including: (1) electrostatic double-layer interactions between protein and adsorbent, (2) intramolecular electrostatic interactions, and (3) pH-induced conformational changes. As in previous reports[3,9,26], the above results demonstrate that manipulation of pH can be useful for obtaining selective separations of proteins in HIC.

The separation of proteins on a weak anion exchanger, SynChropak AX300, at pH 8 was examined, using two different eluting salts. AEC was performed under gradient conditions from low to high salt concentrations. Protein retention at pH 8 in AEC with sodium chloride was compared with retention in AEC with sodium sulfate, a salt known to induce hydrophobic interactions[1]. The results are summarized in Table I.

Retention with sodium chloride as the displacing salt may be interpreted as representing predominantly electrostatic interactions between the positively charged

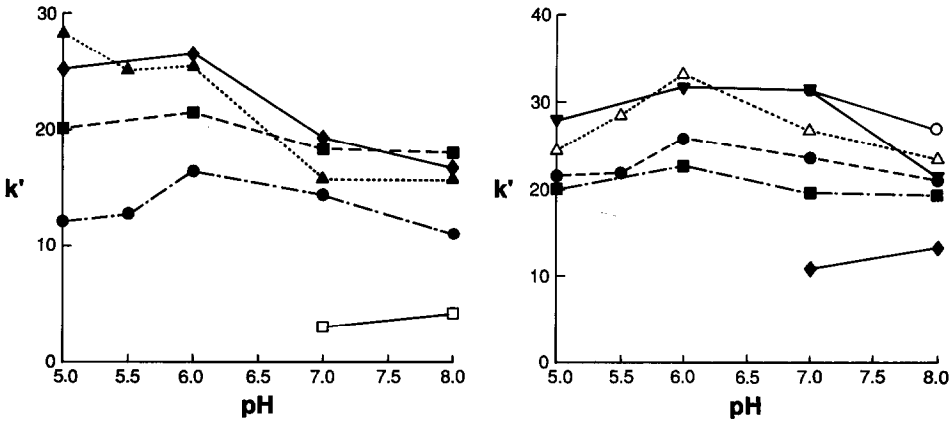


Fig. 1. HIC of eleven proteins on SynChropak propyl as a function of pH. Eluent A, 2 *M* ammonium sulfate in 100 m*M* phosphate buffer; eluent B, 100 m*M* phosphate buffer; linear gradient, 100% A (0% B) to 0% A (100% B) over 30 min. Left: (◆—◆) ovalbumin, (▲...▲) bovine serum albumin, (■——■) conalbumin, (●—·—●) ribonuclease, (□—□) cytochrome *c*. Right: (▼—▼) β-glucosidase, (△...△) α-chymotrypsinogen, (●---●) α-chymotrypsin, (■—·—■) lysozyme, (○—○)ferritin, (◆—◆) myoglobin.

TALBE I

ANION-EXCHANGE CHROMATOGRAPHY OF PROTEINS AT pH 8 ON AN AX300 COLUMN

| *Protein* | *pI*** | *Retention time (min)* | |
|---|---|---|---|
| | | *Eluting salt* | |
| | | *Sodium chloride** | *Sodium sulfate*** |
| Conalbumin | 6.3 | 10.33 | 6.08 |
| α-Chymotrypsinogen | 9.2 | 11.74 | 6.19 |
| α-Chymotrypsin | 8.4 | 12.83 | 7.74 |
| β-Glucosidase | 7.3 | 13.94 | 9.19 |
| Ovalbumin | 4.7 | 14.08 | 9.15 |
| Soy trypsin inhibitor | 4.5 | 21.16 | 17.27 |
| Bovine serum albumin | 5.1 | 21.21 | 8.06 |
| Ferritin | 4.4 | 22.11 | 16.26 |

* Gradient (20 min) from 0.02 to 0.50 *M* sodium chloride in 10 m*M* Tris–HCl.
** Gradient (20 min) from 0.01 to 0.25 *M* sodium sulfate in 20 m*M* Tris–HCl.
*** Refs. 22–24.

protein and the negative anion-exchange matrix. Acidic proteins, such as ferritin, soy trypsin inhibitor, bovine serum albumin, and ovalbumin (all of which have p*I* values below 5) were well retained at pH 8. When sodium sulfate was used as the displacing salt, a different selectivity was observed. The order of soy bean trypsin inhibitor and ferritin was reversed from that observed with sodium chloride. Bovine serum albumin was eluted much earlier than the other proteins when sodium sulfate was used. These differences in elution order were postulated to result from hydrophobic contributions

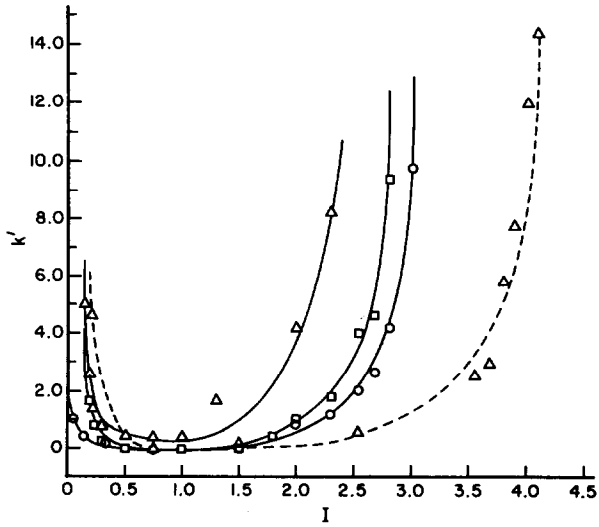


Fig. 2. Protein retention (capacity factor, *k'*) on AEC matrix as a function of ionic strength (*I*) at (○—○) ovalbumin, (□—□) bovine serum albumin, (△—△) chymotrypsinogen. (——) Sodium sulfate, (---) sodium chloride.

to retention, induced by sodium sulfate. Although electrostatic interactions still predominated, the column also exhibited hydrophobic properties. The resultant chromatographic process ("mixed mode chromatography") contributed to later elution of some proteins relative to others. In general, retention was reduced with sodium sulfate as the displacing salt. Thus, selectivity and retention time may be manipulated in AEC by the choice of eluting salt.

Protein retention on an AEC matrix as a function of displacing salt concentration was examined for three proteins; ovalbumin, bovine serum albumin, and α-chymotrypsinogen. The results are summarized in Fig. 2. At low salt concentations, proteins were retained on the basis of electrostatic interactions with the AEC matrix. As the ionic strength of the mobile phase was increased, retention decreased. At an ionic strength of 0.5, little or no retention was observed. As the ionic strength was further increased, retention was induced as a result of hydrophobic interactions. α-Chymotrypsinogen was the most hydrophobic of the three proteins, as evidenced by its retention at an ionic strength of 2 with sodium sulfate as the eluting salt. When sodium chloride was used as the displacing salt, α-chymotrypsinogen was not retained until the ionic strength was nearly 3.5. At low ionic strengths ($<0.5$), protein retention was different for the two salts. The data indicate that electrostatic and hydrophobic interactions may be controlled by the choice of eluting salt. Application of a salt high on the lyotropic series, such as sodium sulfate, to ion-exchange chromatography of hydrophobic proteins was shown to provide selectivity as a result of the combination of hydrophobic and electrostatic interactions with the weakly hydrophobic ion-exchange stationary phase.

The application of weak-cation-exchange packings to chromatography of

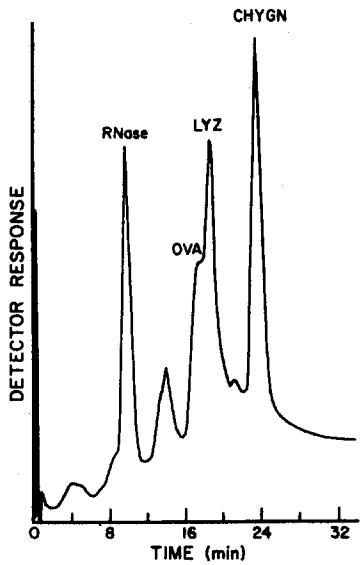


Fig. 3. HIC of proteins on a synthesized weak cation exchanger. Eluent A, 1.5 *M* sodium sulfate in 20 m*M* phosphate (pH 5.5); eluent B, 20 m*M* phosphate (pH 5.5); linear gradient, 100% A (0% B) to 0% A (100% B) over 20 min; flow-rate, 1.5 ml/min. Peaks: RNase = ribonuclease, OVA = ovalbumin, LYZ = lysozyme, CHYGN = α-chymotrypsinogen.

proteins under HIC conditions was examined. The packing used for this application was prepared by bonding carboxyl ligands to a polyethyleneimine-coated silica. The separation of a mixture of proteins with a descending sodium sulfate gradient at pH 5.5 on the cation-exchange chromatographic (CEC) packing is shown in Fig. 3. Elution of CEC matrices under HIC condtions is termed CEC–HIC. The results demonstrate that CEC matrices may be applied to the separation of proteins under HIC conditions. Hydrophobic interactions were induced when the column was eluted with gradients from high to low salt concentrations of a salt high on the lyotropic series. The selectivity in CEC–HIC was different from that observed at pH 5.5 on the SynChrom propyl matrix. Although ribonuclease emerged first and α-chymotrypsinogen emerged last from both columns, the order of elution of ovalbumin and lysozyme from the CEC–HIC column was reversed from that observed on the propyl column.

The elution order of ovalbumin and lysozyme on the cation exchanger eluted under CEC–HIC conditions was examined as a function of pH, and the results were compared to those from a SynChropak propyl packing. The results, summarized in Fig. 4, showed improved resolution of the two proteins on the CEC–HIC matrix. The data suggest that the enhanced selectivity of the CEC–HIC matrix was due to a

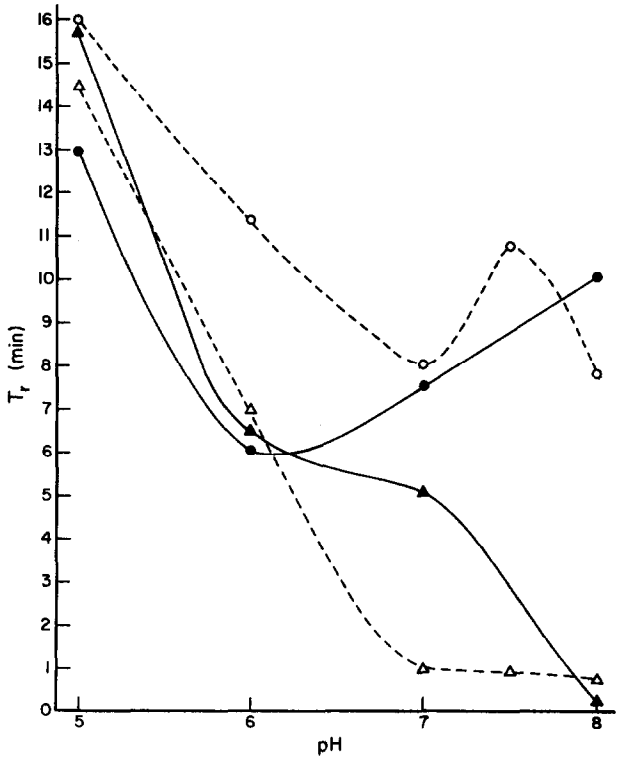


Fig. 4. Retention of ovalbumin (△, ▲) and lysozyme (○, ●) in HIC on a SynChropak propyl column (——) and on a cation-exchange chromatography SynChropak CM300 column (----). Eluent A, 1.5 *M* sodium sulfate in 20 m*M* phosphate; eluent B, 20 m*M* phosphate; linear gradient, 100% A (0% B) to 0% A (100% B) over 20 min.

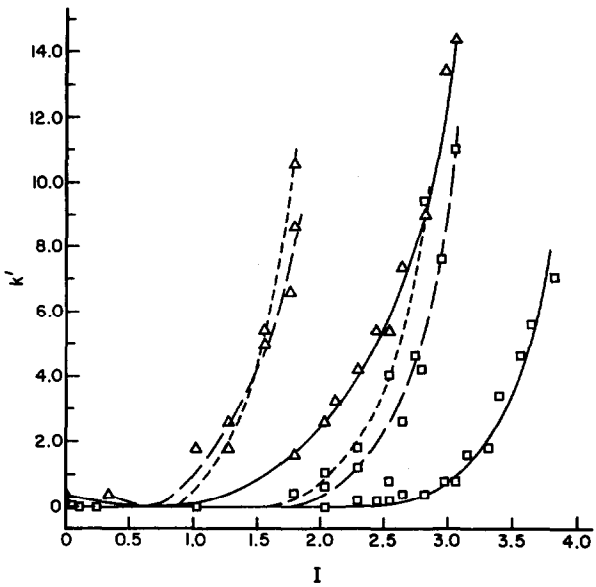


Fig. 5. Effect of ethylene glycol on protein retention on an anion-exchange chromatography column at pH 7.5 as a function of the ionic strength of sodium sulfate. (□) Bovine serum albumin, (△) α-chymotrypsinogen. (---) No ethylene glycol added, (——) 10% ethylene glycol added, (——) 20% ethylene glycol added.

combination of hydrophobic and electrostatic interactions of the proteins with the sorbent surface. As the pH of the mobile phase was increased above the p*I* of ovalbumin (p*I* 4.7) the electrostatic repulsion between the negatively charged protein and the negatively charged functional groups on the stationary phase became more and more important. Ovalbumin emerged earlier from the CEC–HIC column than lysozyme owing to electrostatic repulsion between the charged protein and the charged stationary phase.

Theory suggests that electrostatic interactions between protein and adsorbent are annulled when eluents of high ionic strength are used[25,27], although such interactions may still be operating over short distances ($< 4$ Å)[28] when the protein is in intimate contact with the sorbent surface. In contrast, hydrophobic interactions, or Lifshitz–van der Waals interactions, are long-range in nature, operating at distances up to 100 Å[25,29]. Experimental evidence suggests that even at high salt concentrations electrostatic interactions may affect the chromatographic process. Fausnaugh and Regnier[30] demonstrated that groups of hydrophobic amino acids, *i.e.* "greasy" patches, on the lysozyme surface interacted with the HIC matrix surface. When ionizable histidine was in the vicinity of the hydrophobic patch, retention was markedly affected by pH changes of the mobile phase. The mixed-mode separation of nucleic acid species on the tetraalkyl ammonium matrix reported by Bischoff and McLaughlin[14] was accomplished with eluents containing as much as 2.5 *M* ammonium acetate. The results of the present study suggest that electrostatic interactions can contribute to the chromatographic retention mechanism at high ionic strengths, such as those employed in HIC.

Retention characteristics of bovine serum albumin and α-chymotrypsinogen on an AEC matrix, eluted at pH 7.5 with sodium sulfate in the presence of ethylene glycol (an additive used to reduce protein interactions with the HIC stationary phase) were also examined (Fig. 5). The data showed that addition of of 20% ethylene glycol resulted in substantial reduction of protein retention compared to results in the absence of ethylene glycol. α-Chymotrypsinogen, the more strongly retained of the pair, required a sodium sulfate ionic strength ($I$) of 2.25 to produce a capacity factor ($k'$) of 4 in contrast to $I < 1.5$ to induce similar retention in the absence of ethylene glycol. Elution of bovine serum albumin was similarly hastened by the addition of ethylene glycol. A sodium sulfate ionic strength of 3.5 was required to provide a retention equivalent to that produced at $I = 2.5$ in the absence of ethylene glycol. The results demonstrate that hydrophobic interactions contribute to protein retention when weakly hydrophobic anion-exchange sorbents are eluted with sodium sulfate as the displacing salt.

Ethylene glycol is frequently added to mobile phases in HIC to reduce the magnitude of hydrophobic interactions between the protein and the sorbent[25]. The effect of organic solvents on interactions in HIC was discussed by Srinivasan and Ruckenstein[25]. In contrast to the structure-making salts (those high on the lyotropic series[1]), ethylene glycol is structure-breaking. The dielectric constant and the surface tension of the medium are reduced, and consequently, the van der Waals interactions between the protein and the sorbent are reduced. When ethylene glycol is present at sufficiently high concentrations, the protein is preferentially hydrated, and the native structure is often stabilized[31,32]. Such effects begin to occur at ethylene glycol concentrations $\geqslant 20\%$. In contrast, organic solvents such as ethanol or methanol show a marked tendency to denature proteins[33].

## CONCLUSIONS

Manipulation of mobile phase pH was shown to affect selectivity in the separation of proteins on a SynChropak propyl HIC stationary phase. Ion-exchange chromatography of proteins on a weak anion exchanger having weak hydrophobic effects was shown to be affected by the choice of eluting salt. Elution by sodium sulfate as the displacing salt resulted in an elution order different from that observed with sodium chloride. Protein retention studies under isocratic conditions showed that these matrices exhibited both electrostatic and hydrophobic properties. The latter could be reduced by addition of 20% ethylene glycol to the eluent. Elution of weak cation-exchange packings under HIC conditions was shown to make both electrostatic and hydrophobic contributions to the separation process and provided a selectivity different from that observed with a propyl matrix. Judicious selection of mobile phases and salt gradient were shown to provide useful selectivity in the chromatography of proteins.

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