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EFFECTS OF MOBILE PHASE AND LIGAND ARM ON PROTEIN RETENTION IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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SUMMARY

The retentive properties of a series of hydrophobic interaction chromatography packings with six different ligand arms (SynChropak Hydroxypropyl, Methyl, Propyl, Butyl, Pentyl, and Benzyl) were investigated with mobile phases of different ionic compositions and pH. Substitution of ammonium acetate for ammonium sulfate resulted in decreased retention for most combinations of proteins and ligands, although the retention of some proteins, such as lysozyme on the pentyl ligand, was unchanged by the salt substitution. Generally, lower pH resulted in reduced retention, but the elution of lysozyme was more affected by pH than that of ovalbumin.

INTRODUCTION

Hydrophobic interaction chromatography (HIC) separates proteins according to their hydrophobicity in a decreasing salt gradient[1–4]. High-performance HIC was modeled after chromatography on agarose and carbohydrate gels containing alkyl chains[5–8]. In these gels, the length of the ligand chains as well as the pH, ionic strength, and buffer composition of the eluents were shown to affect the ability to bind proteins and their resolution. HIC has advantages over reversed-phase chromatography in protein analysis, because it does not denature sensitive biological molecules[9], and small changes in the ligand arm produce different retentive characteristics for proteins[1,2].

In 1977, Melander and Horváth[10] found that the type of salt used in traditional HIC had a profound effect on hydrophobic interactions. They found that the effect of salt type on hydrophobic interactions could be quantified by molal surface tension increments and showed an excellent correlation with the classical lyotropic or salting out series. Von Hippel and Schleich[11] had previously ranked various ions of neutral salts in their order of increasing effectiveness in disrupting the native structure of various macromolecules. They noted that the salting-out effects of ions are independent of the ionic charge, but the order of effectiveness is correlated with the lyotropic or Hofmeister[12] series. This phenomenon was observed in high-performance HIC by Melander *et al.*[13] in 1984. Several research groups have investigated the relationship between protein retention and increased pH on hydrophobic matrices[7,9,14–16], but in

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most instances the HIC columns and the ionic salt composition were different, and the reported results were inconsistent.

We have examined the retentive properties of a series of HIC packings which have six different ligand arms (hydroxypropyl, methyl, propyl, butyl, pentyl, and benzyl). Mobile phases of two ionic compositions were used to investigate the relationship of the nature of the salt and the pH to the retention of proteins by the various ligands on the series of columns.

EXPERIMENTAL

Apparatus

The packings, 6.5-μm particle size and 300-Å pore diameter, were named according to ligand arm, SynChropak Hydroxypropyl, Methyl, Propyl, Benzyl, Butyl, and Pentyl, in 250 × 4.1 mm I.D. columns obtainable from SynChrom (Lafayette, IN, U.S.A.). A Varian Model 5020 gradient high-performance liquid chromatograph (Varian Instruments, Walnut Creek, CA, U.S.A.), equipped with a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.), an ISCO V4 absorbance detector (ISCO, Lincoln, NE, U.S.A.), and a Linear Model 261 recorder (Linear Instruments, Irvine, CA, U.S.A.), were utilized.

Chemicals

Potassium monobasic phosphate (crystals, AR, ACS) were from Mallinckrodt (Paris, KY, U.S.A.); and ammonium acetate and ammonium sulfate (granular, ACS, HPLC-grade) were from J. T. Baker (Phillipsburg, NJ, U.S.A.). With ammonium sulfate from other sources, excessive background absorbance was observed. Dilute hydrochloric acid (37%, NF, AR, ACS) and sodium hydroxide (NF pellets, Mallinckrodt) served for pH adjustment. Just prior to use, mobile phases were filtered through 0.45 μm Nylon-66 filters (Rainin Instrument, Woburn, MA, U.S.A.). Three times crystallized bovine pancreatic proteins, including ribonuclease A (Type I-AS, protease-free), α-chymotrypsin (EC 3.4.21.1) and chymotrypsinogen A, (both Type II, lyophilized, salt-free); and twice crystallized, lyophilized egg proteins, including ovalbumin (Grade V, salt-free) and lysozyme (EC 3.2.1.17, Grade I, dialyzed powder) were from Sigma (St. Louis, MO, U.S.A.).

Conditions

Gradient elution was performed with 4 *M* to 0 *M* ammonium acetate or 2 *M* to 0 *M* ammonium sulfate, both in 0.1 *M* potassium phosphate buffer (pH 5, 6, 6.8, or 8) at a flow-rate of 1 ml/min over a 30-min period. To counteract dead-volume and mixing phenomena, isocratic elution with the final buffer was necessary for some of the proteins. Column re-equilibration required 10 column volumes of the initial high-salt buffer.

RESULTS AND DISCUSSION

Experiments were designed to reveal the effects on the retentive abilities of six HIC ligand arms (hydroxypropyl, methyl, propyl, butyl, pentyl, and benzyl) for ribonuclease, ovalbumin, lysozyme, α-chymotrypsin, and chymotrypsinogen A in gra-

dient elution with two mobile phases of different ionic composition at various pH levels. Because the HIC packings used in this study were synthesized by incorporating ligand arms into a hydrophilic polymeric polyamide bonded phase, it was important to determine whether retention was caused by the ligand arm alone or by differences in the hydrophobic or ionic characteristics of the polymeric matrix. For this study, large batches of 6.5 μm silica were bonded with polymer, and then portions were derivatized with each of six ligand chains. The matrix appears to have no significant cationic character, because ovalbumin had no retention on a propyl column when a typical ion-exchange gradient of sodium acetate at pH 7 was used[17].

Effect of ionic composition on retention

When 2 *M* ammonium sulfate and 4 *M* ammonium acetate were used in the mobile phase, the ammonium sulfate system generally caused greater retention of the five proteins in the test mixture. The major exception to this effect was that at pH 6, 6.8, and 8 the retention times of lysozyme, α-chymotrypsin, and chymotrypsinogen A on the butyl and the pentyl columns were nearly the same with both ammonium salts. Table I shows the effects of both salt and pH on the retention times of the five standard proteins on columns with different ligand chains. Certain trends regarding the salts and ligands are obvious.

Retention from ammonium acetate is less than from ammonium sulfate under most conditions. With ammonium acetate, the maximum protein retention is only 4.2 min on the hydroxypropyl and 10.5 min on the methyl columns, whereas it is 23.3 and 28.2 min, respectively, with ammonium sulfate. With few exceptions, the retention times of ribonuclease and ovalbumin were shorter with ammonium acetate than they were with ammonium sulfate at all pH values. The differences between the salts are minimal for the retention of hydrophobic proteins on the hydrophobic ligands, as illustrated by the retention times of chymotrypsinogen A on the butyl column. Figs. 1a and 1b illustrate the differences between the selectivity of the butyl column for ribonuclease, lysozyme, and chymotrypsinogen A, produced by ammonium sulfate and ammonium acetate at pH 5, 6, 6.8, and 8.

α-Chymotrypsin was not eluted from the pentyl or benzyl columns under most circumstances due to its hydrophobicity and perhaps a special affinity for the benzyl ligand. Chymotrypsinogen A was similarly retained, but the use of ammonium acetate enabled its elution from the benzyl ligand. The retentive properties of the benzyl arm were different for each protein. When ribonuclease was chromatographed in ammonium sulfate, the benzyl arm behaved like the methyl arm, but for ovalbumin, benzyl was like propyl as shown in Fig. 2a. With ammonium acetate, the retention properties of benzyl were intermediate between those of the propyl and butyl ligands for all proteins.

Effect of pH on retention

When ammonium sulfate was used as the salt, ovalbumin was the only protein that showed maximum retention at pH 5, as seen in Fig. 2a. This is probably due to increased hydrophobicity near its p*I* of 4.7 (ref. 9). The other four proteins, which have p*I* values greater than 8, showed an increase in retention at a pH similar to that shown for lysozyme in Fig. 2b. When ammonium acetate was used in the eluent, the pH effects were much more pronounced than in the case of ammonium sulfate, as

TABLE I

EFFECTS OF SALT AND pH ON RETENTION (in min)

Column, 250 × 4.1 mm I.D.; SynChropak HIC, as indicated; flow-rate, 1 ml/min; buffer, 0.1 *M* potassium phosphate; 30-min gradient from 100 to 0% ammonium salt.

Salt	*Ligand*	*Ribonuclease A*				*Ovalbumin*			
		pH 5	*pH 6*	*pH 6.8*	*pH 8*	*pH 5*	*pH 6*	*pH 6.8*	*pH 8*
Ammonium sulfate (2 *M*)	Hydroxypropyl	3.3	3.9	10.4	7.8	11	13.7	15.2	12.8
	Methyl	11.2	13.8	16.9	18.6	18	18.3	19.2	19.2
	Propyl	17.6	19.8	20.9	21.3	23.2	22.2	23.4	21.3
	Butyl	22.6	23.4	23.7	24.3	30.4	28.5	27.2	25.8
	Pentyl	23.7	24.3	26.5	27.6	36.5	30.9	31.8	30
	Benzyl	12.9	14	16.5	20.2	25.2	21	24	25.5
Ammonium acetate (4 *M*)	Hydroxypropyl	2.1	2.4	2.4	2.4	2	2.2	2.2	2.1
	Methyl	2.2	2.2	2.4	2.4	2	2	2.1	2.1
	Propyl	2.4	2.4	3.1	3.5	2.2	2.3	2.4	2.2
	Butyl	2.7	3.9	6	6.4	4.5	7.8	10.5	6.4
	Pentyl	3	3.9	6.1	8.4	12	21.7	21.3	27.3
	Benzyl	2.9	3.2	4.2	3.9	3	3.9	4.8	4.8

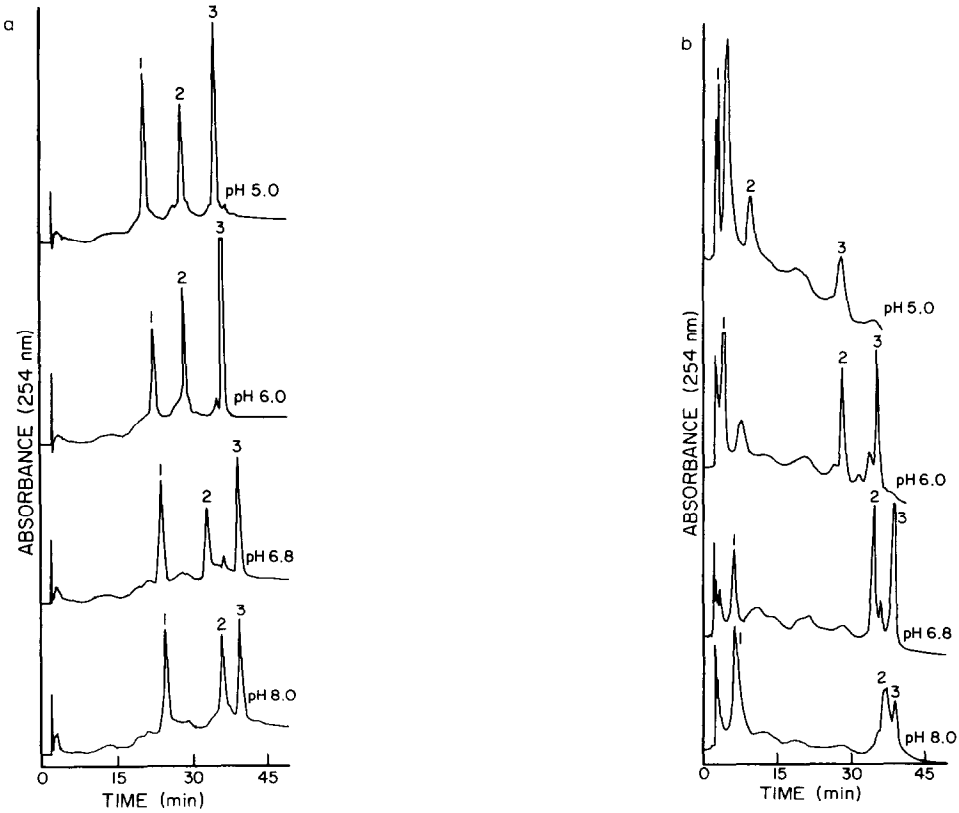

Fig. 1. Effect of mobile phase on selectivity. Column, 6.5 μm SynChropak Butyl, 250 × 4.1 mm I.D. sample mixture, 1 = ribonuclease, 2 = lysozyme, 3 = chymotrypsinogen A; flow-rate, 1 ml/min; mobile phase, 0.1 *M* potassium phosphate; 30-min gradient of (a) 2 *M* to 0 *M* ammonium sulfate at pH 5, 6, 6.8, and 8, and (b) 4 *M* to 0 *M* ammonium acetate at pH 5, 6, 6.8, and 8.

Lysozyme				*α-Chymotrypsin*				*Chymotrypsinogen A*			
pH 5	*pH 6*	*pH 6.8*	*pH 8*	*pH 5*	*pH 6*	*pH 6.8*	*pH 8*	*pH 5*	*pH 6*	*pH 6.8*	*pH 8*
11.5	16.5	17.8	17.4	10.8	19.8	21.9	20.1	15.4	20.9	23.3	21.3
18.9	19.8	22	23.1	18.9	20.7	26.7	24.3	22.3	24.9	28.2	28.2
22.4	25.5	26.6	28.6	25	27.9	30.8	30	27.8	30.6	33.4	33.6
29.5	29.1	33	35.7	34.8	34.8	37.6	37.2	36.3	34.8	39.3	39.3
34.2	33.6	40.2	–	40.2	–	–	–	40.8	–	–	–
23.5	24	25.8	29.1	38.6	–	–	–	30.6			
2.2	3	2.1	3.4	2.2	2.4	3.5	3	2.2	3	4.2	3.3
2.2	2.4	3.6	8.1	2.2	2.8	3.4	4.8	2.2	3.3	5.2	10.5
3.9	14	21	26.7	3	16.2	23.4	27.5	5.4	21.7	27.9	29.5
9.3	27.9	33.9	36.6	11.1	33.3	35.6	37.2	27.6	34.8	38	39
29.5	32.7	40.1	–	38.4	–	–	–	39.9	–	–	–
4.5	13.8	26.4	26.1	3	–	–	–	5.85	26.4	38.4	38.7

seen for lysozyme in Fig. 2c. This effect is probably due, in part, to the incomplete ionization (64%) of ammonium acetate at pH 5 and the concomitant presence of acetic acid and ammonium chloride. At pH 6, ammonium acetate is 95% ionized; therefore, the multiplicity of components is not significant. α-Chymotrypsin will be eluted from the pentyl and benzyl columns only at pH 5, and under normal operating conditions it is not eluted at higher pH values.

Fig. 3a again illustrates the minor effect of pH on the retention on a propyl column when ammonium sulfate is used in the mobile phase, in contrast to the significant increase in retention with pH when ammonium acetate is used, as seen in Fig. 3b.

Effect of pH and ion composition on resolution

Table II shows the effects of pH, salt, and ligand chain on the resolution of each of two protein pairs: ovalbumin and lysozyme, and α-chymotrypsin and chymotrypsinogen A. These resolution values were all calculated from retention times where lysozyme and chymotrypsinogen A are eluted later. Therefore, negative numbers indicate a reversal in elution order. It can be seen that all reversals occurred at pH 5, where the hydrophobicity of ovalbumin increased. For the ovalbumin/lysozyme pair, resolution increased with pH in ammonium sulfate eluents, except on the benzyl column. No trends related to pH can be seen for the α-chymotrypsin/chymotrypsinogen A pair.

When ammonium acetate was used in the eluent, results similar to the ammonium sulfate data were found for the α-chymotrypsin/chymotrypsinogen A resolution. Because ovalbumin was poorly retained with ammonium acetate in the eluent, the resolution of the ovalbumin/lysozyme pair was greater than it was with ammonium sulfate, and an increase in resolution with pH was not uniformly observed.

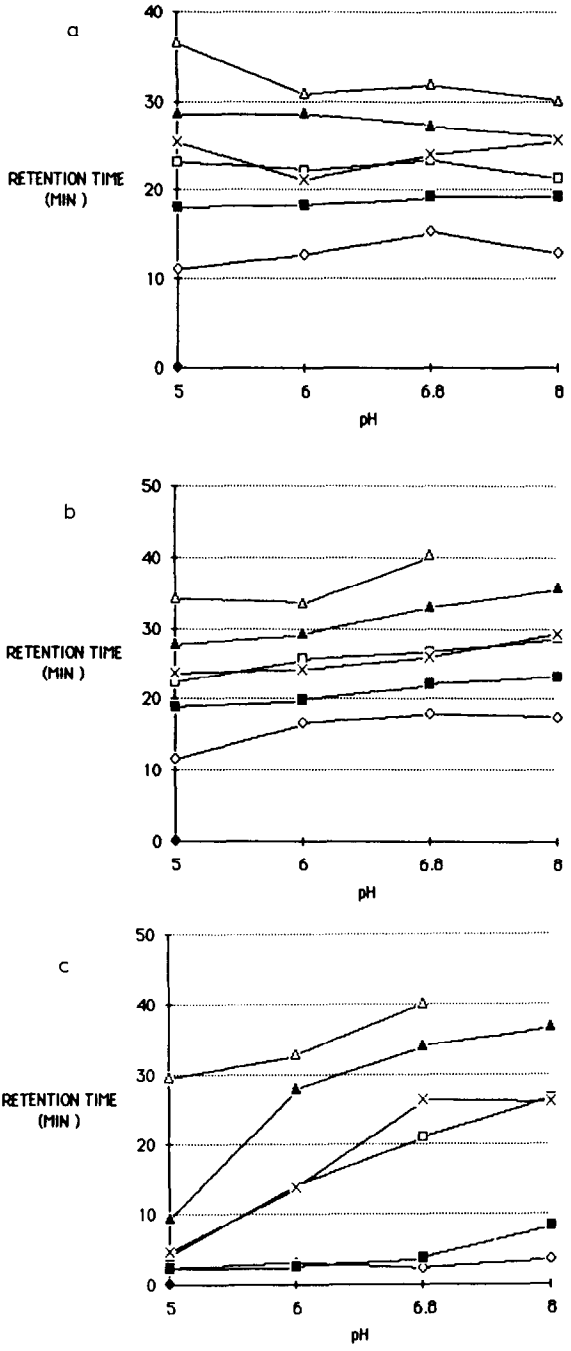

Fig. 2. Effect of pH and ligand on retention. Column, 6.5 μm SynChropak HIC, 250 × 4.1 mm I.D.; ◇ = hydroxypropyl, ■ = methyl, □ = propyl, ▲ = butyl, △ = pentyl, × = benzyl; (a) sample, ovalbumin; other conditions as Fig. 1a; (b) sample, lysozyme; other conditions as Fig. 1a; (c) sample, lysozyme; other conditions as Fig. 1b.

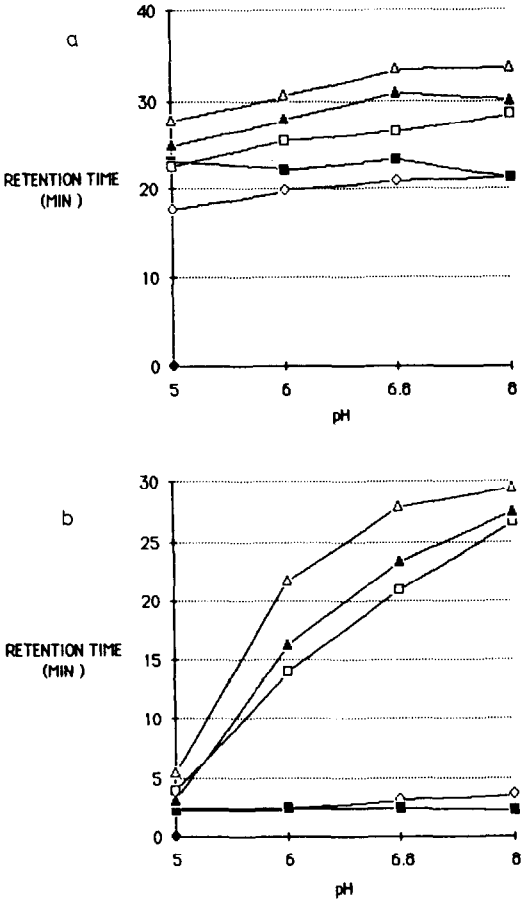

Fig. 3. PH and salt effects on retention of different proteins. Column, 6.5 μm SynChropak Propyl, 250 × 4.1 mm I.D.; sample, protein mixture; ◇ = RNase, ■ = ovalbumin, □ = lysozyme, ▲ = α-chymotrypsin, △ = chymotrypsinogen A; (a) other conditions as Fig. 1a; (b) other conditions as Fig. 1b.

CONCLUSION

It is possible to change the selectivity of HIC for proteins by changing the hydrophobic ligand and/or the salt composition. Very hydrophobic proteins which are highly retained on hydrophobic ligands can be separated on the more hydrophilic matrices, such as hydroxypropyl. Additionally, a salt such as ammonium acetate, which produces less retention, can be substituted for a salt such as ammonium sulfate, or the pH of the buffer system may be altered to aid separation. In like manner, more hydrophilic proteins will have better retentive qualities on hydrophobic ligands, such as pentyl, and salts with high lyotropic qualities should be used in the eluent. Changes in pH do not have as great an effect on protein retention as the ligand chain and nature of the salt, but effects on selectivity are noticeable. The ability to change any

TABLE II

EFFECT OF SALT AND pH ON RESOLUTION

Column, 250 × 4.1 mm I.D. SynChropak HIC, as indicated; flow-rate, 1 ml/min; mobile phase, 0.1 *M* potassium phosphate; 30-min gradient from 100 to 0% ammonium salt.

Salt	*Ligand*	*Ovalbumin/lysozyme*				*α-Chymotrypsin/ chymotrypsinogen A*			
		pH 5	*pH 6*	*pH 6.8*	*pH 8*	*pH 5*	*pH 6*	*pH 6.8*	*pH 8*
Ammonium sulfate (2 *M*)	Hydroxypropyl	0.3	1.2	1.7	2.2	1.6	1.1	1.1	1
	Methyl	0.3	1	2.8	5.5	2.8	3.5	1.8	3.7
	Propyl	−0.4	1.4	1.8	5.4	1.5	1.5	2.9	2
	Butyl	−0.6	0.4	2.9	4.5	0.9	0	0.9	1.1
	Pentyl	−1.3	0.8	2.7	–	0.3	–	–	–
	Benzyl	−0.7	1.5	0.6	1.4	−3.5	–	–	–
Ammonium acetate (4 *M*)	Propyl	1.4	5	9.5	11.4	1.3	1.6	1.3	0.7
	Butyl	2.1	8.4	3.6	5.4	3.6	0.8	1.8	1
	Pentyl	4.2	2	3.6	–	0.8	–	–	–
	Benzyl	1.4	3.5	6.9	6.5	2.4	–	–	–

of the chromatographic variables (ligand, pH, or salt) to alter the selectivity makes HIC a very useful and versatile technique.

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