Journal of Chromatography, 359 (1986) 331–337
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 803

OPTIMIZATION OF PREPARATIVE HYDROPHOBIC INTERACTION CHROMATOGRAPHIC PURIFICATION METHODS

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SUMMARY

The chromatographic behavior of five proteins on hydrophobic interaction matrices having six different ligand arms was investigated using gradient elution with ammonium sulfate and ammonium acetate buffers at two pH values. The nature of the mobile phase and/or the ligand chain arm of the matrix was found to have substantial effect on the resolution, retention, and selectivity. Ovalbumin was moderately or highly retained with ammonium sulfate on all columns; however, with ammonium acetate, ovalbumin was not retained on SynChropak Hydroxypropyl and Propyl columns.

Chromatographic conditions developed for analytical hydrophobic interaction chromatography columns containing 6.5-μm packings were adapted to preparative columns packed with 30-μm SynChroprep packings for the separation of serum components. Dynamic load capacities were 4–13 mg of ovalbumin per ml of column volume.

INTRODUCTION

Reversed-phase chromatography is a popular method for purifying proteins by hydrophobic retention, but due to the use of acidic eluents containing organic solvents, many proteins are denaturated during the chromatographic run. An alternative method which separates proteins according to their hydrophobicity is hydrophobic interaction chromatography (HIC), which employs salt gradients of decreasing ionic strength. In studies using HIC agarose and carbohydrate gels, the length of the ligand chains used to derivatize the supports was seen to affect the resolution and the ability to bind proteins[1–5]. During the past three years, various high-performance packing materials[6–10] and methods[6–14] have been introduced for the analysis and the purification of proteins by HIC. The effect of the nature of the bonded phase[6–12], mobile phase[6–14], pH[8,11,12], and temperature[9] on the separation has been examined. The technique has also been used for the purification of gram quantitities of proteins[10].

This report examines the effect of the hydrophobic ligand on retention, mobile phase selection, and load capacity. Both 6.5-μm and 30-μm supports are investigated

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to see whether data obtained under analytical conditions can be used for the optimization of preparative purification of proteins.

EXPERIMENTAL

Chemicals

Potassium phosphate (monobasic) was purchased from Mallinckrodt, Inc. (Paris, KY, U.S.A.). Tris(hydroxymethyl)aminomethane (Tris) and sodium acetate were from Aldrich (Milwaukee, WI, U.S.A.). Ammonium acetate and ammonium sulfate were HPLC-grade from J. T. Baker (Phillipsburg, NJ, U.S.A.). With ammonium sulfate from other sources, excessive background absorbance was observed. Ovalbumin, lysozyme, ribonuclease A, α-chymotrypsin, and chymotrypsinogen A were purchased from Sigma (St. Louis, MO, U.S.A.). Human serum was Monitrol I-X from American Scientific Products (McGaw Park, IL, U.S.A.).

Apparatus

The 6.5-μm SynChropak Hydroxypropyl, Propyl, Methyl, Butyl, Pentyl, and Benzyl columns (250 × 4.1 mm I.D.) and 30-μm SynChroprep Hydroxypropyl, Propyl, Methyl, Butyl, Pentyl, and Benzyl packings (columns, 250 × 10 mm I.D.) were from SynChrom (Linden, IN, U.S.A.). All of these packings have 300-Å pores; their names denote the ligand arm. A Varian Model 5000 gradient high-performance liquid chromatograph (Varian, Walnut Creek, CA, U.S.A.) with a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) and a Chem Research Model 2020 UV detector (ISCO, Lincoln, NE, U.S.A.) with a Linear Model 1200 recorder (Linear Instruments, Irvine, CA, U.S.A.) were used for this study. A System 42 preparative chromatograph with 50-ml heads (Gilson Medical Electronics, Middleton, WI, U.S.A.) with a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) and a Model UA-5 detector and recorder (ISCO) were also used.

Methods

The dynamic load capacity is defined to be the amount of ovalbumin that produces a peak having a width at half-height that is 70% greater than that of a 40-mg sample on a 250 × 10 mm I.D. column with a 120-min reversed salt gradient from 2 *M* to 0 *M* ammonium sulfate[10,16]. The absolute load capacity is the total amount of ovalbumin that can be bound on a column of a given size when the displacement mode of chromatography is used[10,16]. Sample is repeatedly injected onto a column which is run isocratically with 2 *M* ammonium sulfate. Absolute load is achieved when the breakthrough peak has a constant area.

RESULTS AND DISCUSSION

Effect of ligand arm on retention

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 that retention was caused by the ligand arm alone and not by differences in the hydrophobic or ionic characteristics of the polymeric matrix. In this study, large batches of 6.5-μm and 30-μm matrix were bonded with polymer, after which,

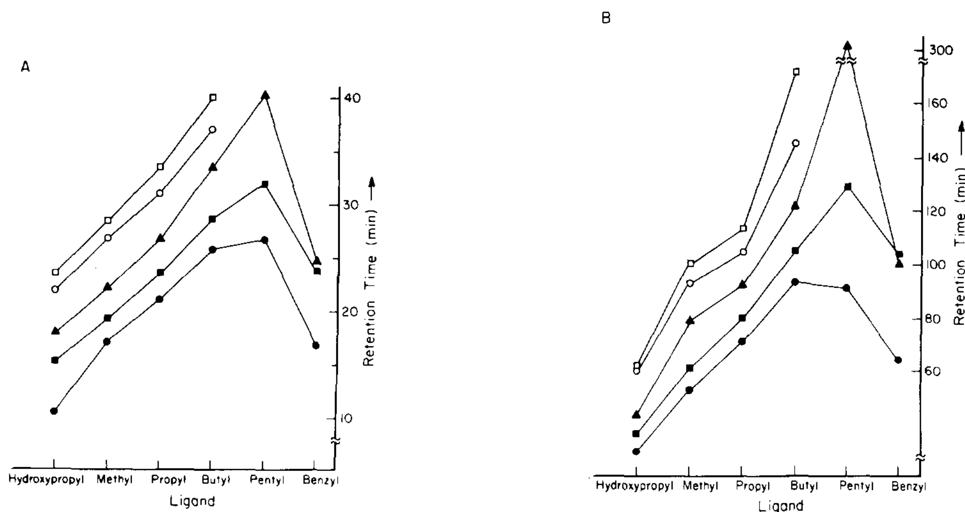


Fig. 1. Effect of ligand arm on retention. (A) Column, 6.5-μm SynChropak HIC, 250 × 4.1 mm I.D.; mobile phase, 0.1 *M* potassium phosphate (pH 6.8); 30-min gradient from 2 *M* to 0 *M* ammonium sulfate; flow-rate, 1 ml/min; t_0, 2.1–2.4 min. (B) Column, 30-μm SynChroprep HIC, 250 × 10 mm I.D.; mobile phase, 0.1 *M* potassium phosphate (pH 6.8); 120-min gradient from 2 *M* to 0 *M* ammonium sulfate; flow-rate, 1.5 ml/min; t_0, 9–9.6 min. ● = Ribonuclease, ■ = ovalbumin, ▲ = lysozyme, ○ = α-chymotrypsin, and □ = chymotrypsinogen A.

portions were derivatized with each of six ligand chains. In order to test for possible ionic interactions, ovalbumin was chromatographed on a Propyl column with a 0–0.5 *M* sodium acetate gradient in 0.02 *M* Tris at pH 7[15]. Because ovalbumin was not retained under these conditions typically used in ion-exchange chromatography, the support is believed to have no significant cationic character.

Fig. 1. illustrates that the nature of the ligand arm had a significant and similar effect on retention of the proteins on both 6.5-μm and 30-μm packings. Generally, the 6.5-μm packings were more retentive, which may be due to their higher surface area or to slight differences between the polymeric layers on the two sets of packings. It can be seen that the least hydrophobic hydroxypropyl ligand would be most useful for the chromatography of very hydrophobic proteins, whereas the pentyl arm would be used to retain hydrophilic proteins.

Effect of mobile phase on retention

The effects of pH and salt on retention were examined for the hydroxypropyl, propyl, and pentyl ligand arms because they were the least, intermediate, and most hydrophobic columns in the study. As can be seen in Table I, effects of both salt and pH were different for each protein on each column. For example, when ammonium sulfate was used as the salt, the retention time for ribonuclease was unaffected by an increase in pH on the propyl column, whereas the retention time of ovalbumin decreased, and the retention time of lysozyme increased. The retention of ribonuclease was inversely affected by an increase in pH on the hydroxypropyl column, while ovalbumin and lysozyme both increased in retention time. The retention of all three proteins increased with increased pH on the pentyl column. When ammonium acetate

TABLE I

EFFECTS OF SALT AND pH ON RETENTION TIME

No = no elution before 60 min; 6.5-μm SynChropak columns, 250 × 4.1 mm I.D.; buffer, 0.1 *M* potassium phosphate; 30-min gradient; flow-rate, 1 ml/min.

Salt	*Protein*	*Retention time (min)*					
		Column					
		Hydroxypropyl (t_0 = 2.2 min)		*Propyl (t_0 = 2.1 min)*		*Pentyl (t_0 = 2.1 min)*	
		pH 6	*pH 6.8*	*pH 6*	*pH 6.8*	*pH 6*	*pH 6.8*
Ammonium sulfate (2 *M*)	Ribonuclease A	13.7	10.4	20.9	20.9	24.3	26.5
	Ovalbumin	13.7	15.2	24.9	23.4	30.7	31.8
	Lysozyme	16.5	17.8	26.1	26.6	33.5	40.2
	α-Chymotrypsin	19.8	21.9	28.4	30.8	No	No
	Chymotrypsinogen A	20.9	23.3	31.4	33.4	No	No
Ammonium acetate (4 *M*)	Ribonuclease A	2.4	2.4	2.4	3.1	3.9	6.1
	Ovalbumin	2.2	2.2	2.3	2.4	21.7	21.3
	Lysozyme	3	4.1	14	21	32.7	40.1
	α-Chymotrypsin	2.4	3.5	16.2	23.4	No	No
	Chymotrypsinogen A	3	4.2	21.7	27.9	No	No

was used to form the gradient, none of the proteins were significantly retained at either pH on the hydroxypropyl column. Ribonuclease and ovalbumin were not retained at all on the propyl column in either of the ammonium acetate buffers, but ovalbumin was moderately retained on the pentyl column. Lysozyme was retained on the propyl and pentyl columns in ammonium acetate. Fig. 2 illustrates the dramatic differences between the selectivities caused by two different salts on the packings with the pentyl ligand.

Capacities of the columns for protein purification

The dynamic and absolute load capacities of the 30-μm packings for ovalbumin can be seen in Table II. No large differences based on ligand chain length were observed, with the exception of hydroxypropyl, which had low capacities. When amounts of ovalbumin exceeding 80 mg were injected onto the hydroxypropyl column, a non-retained peak appeared. This may be a denatured protein peak since such peaks have been seen to appear in reversed-phase chromatography[17]. When columns having the least hydrophobic ligands, *i.e.* methyl and hydroxypropyl, were loaded, an initial ammonium sulfate concentration of 1 *M* was not adequate to induce a strong interaction and therefore the absolute loading procedure resulted in the bleeding of sample from the column after small quantities were applied. Dynamic load capacities on the 6.5-μm columns ranged from 1 to 10 mg/ml.

Although a previous study[10] showed that gram quantities of protein could be purified on 30-μm HIC packings, complex protein mixtures of biological origin had not been run on the columns. Fig. 3 shows the chromatogram of serum proteins on

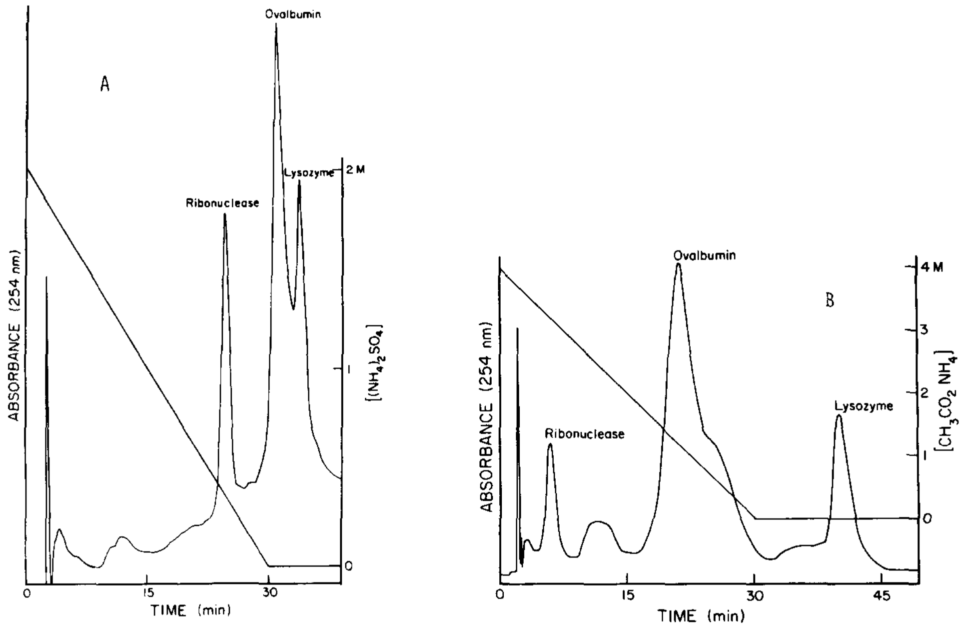


Fig. 2. Effect of mobile phase on selectivity. Column, 6.5-µm SynChropak Pentyl, 250 × 4.1 mm I.D.; flow-rate, 1 ml/min; mobile phase, 0.1 *M* potassium phosphate; 30-min gradient of (A) 2 *M* to 0 *M* ammonium sulfate (pH 6.0), or (B) 4 *M* to 0 *M* ammonium acetate (pH 6.8).

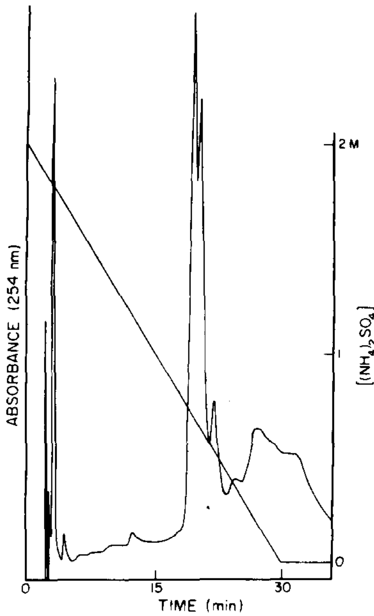
a column containing 6.5-µm SynChropak Propyl. Fig. 4 illustrates the separation of the same serum sample on a column containing 30-µm SynChroprep Propyl, with a fourfold decrease in mobile phase velocity and increase in gradient time with respect to the conditions used with the analytical columns, as were earlier specified for such columns[10,16]. An increase in injected volume of serum to 1 ml still gave chromatograms (Fig. 5) similar to those obtained with the analytical column.

TABLE II

LOAD CAPACITIES OF 30-µm HIC COLUMNS

Column, SynChroprep HIC, 250 × 10 mm I.D.; mobile phase, 0.1 *M* potassium phosphate (pH 6.8); 120-min gradient from 2 *M* to 0 *M* ammonium sulfate for dynamic load; flow-rate, 1.5 ml/min.

Ligand arm	*Load capacity (mg ovalbumin/ml column material)*	
	Dynamic	*Absolute*
Hydroxypropyl	3.8	7.1
Methyl	12.7	38.3
Propyl	8.7	68.9
Butyl	10.7	68.9
Pentyl	8.7	43.4
Benzyl	7.6	30.6



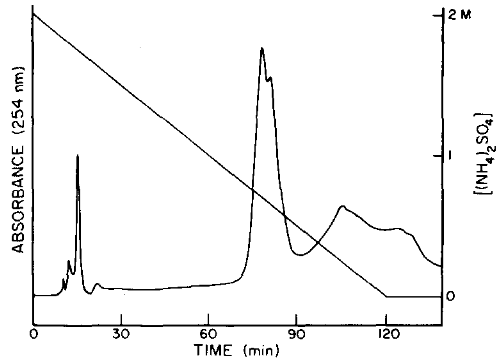


Fig. 3. Resolution of serum components on a 6.5-μm HIC column. Column, SynChropak Propyl, 250 × 4.1 mm I.D.; other conditions as in Fig. 1A; sample volume, 20 μl.

Fig. 4. Resolution of serum components on a 30-μm HIC support. Column, SynChroprep Propyl, 250 × 10 mm I.D.; other conditions as in Fig. 1B; sample volume, 100 μl.

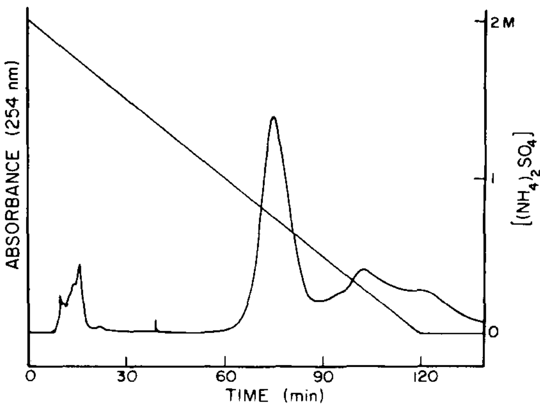


Fig. 5. Analysis of 1 ml of serum on a HIC support. Column, SynChroprep Propyl, 250 × 10 mm I.D.; other conditions as in Fig. 1B; sample volume, 1 ml.

CONCLUSIONS

By changing the hydrophobic ligand of the stationary phase, it is possible to vary the selectivity for protein separation by HIC. Each ligand examined in this study had different retentive properties, with hydroxypropyl being the least retentive and

pentyl being the most retentive. The effect of changing the nature and pH of the buffer used in the gradient elution was different for each of the five proteins in the study when they were chromatographed on supports having ligands such as hydroxypropyl, propyl and pentyl. The 30-μm HIC columns had dynamic load capacities for ovalbumin of 4–13 mg/ml, which, combined with their good resolution and selectivities, should make them valuable for preparative protein purification.

ACKNOWLEDGEMENT

This investigation was supported in part by a SBIR grant from the National Institutes of Health.

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