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ANALYSIS OF PROTEINS WITH NEW, MILDLY HYDROPHOBIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY PACKING MATERIALS

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SUMMARY

A series of packings that had alkyl and aryl groups incorporated into a hydrophilic polymeric matrix were developed for hydrophobic interaction chromatography. Using an inverse salt gradient of ammonium sulfate or sodium chloride, a series of proteins were purified and the activity of trypsin was monitored using post-column detection. Retention times of proteins generally increased in the following order of ligands: hydroxypropyl, propyl, benzyl, isopropyl, phenyl and pentyl.

INTRODUCTION

Over the past few years, reversed-phase supports have been well characterized for small-molecule and peptide analyses. Proteins have also been purified by reversed-phase high-performance liquid chromatography (HPLC), but the procedures have been limited for many by the denaturing qualities of the eluting solvents[1]. Although ion-exchange and gel-permeation HPLC have become popular for protein purification, it is desirable to purify enzymes without denaturation by hydrophobic interaction chromatography (HIC). The latter employs a high initial salt concentration to enhance hydrophobic interaction between the solute and the support, followed by a decrease in salt to release the solute. Because proteins retain their structures under such conditions, it is especially suitable for studies where enzymatic activity is of prime importance. Traditionally, this mode of interaction was utilized on agarose[2,3] or semi-rigid methacrylate gels[4] derivatized with hydrophobic chains. There have also been a limited number of reports describing supports which implement this method for HPLC[5–8].

This paper will discuss supports suitable for high-performance HIC of proteins. These supports were prepared by covalently attaching alkyl and phenyl moieties to a hydrophilic polymeric matrix bound to macroporous silica. Proteins are eluted from these supports when decreasing aqueous salt gradients are applied, and the enzymatic activity of trypsin is retained. The effect of ligand length on resolution and selectivity for proteins will be discussed below.

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EXPERIMENTAL

Chemicals

Sodium chloride, ammonium sulfate, and potassium phosphate (monobasic) were purchased from Mallinckrodt (Paris, KY, U.S.A.). Sodium sulfate, cytochrome *c*, ribonuclease A, ovalbumin, lysozyme, α-chymotrypsin, chymotrypsinogen A, trypsin, trypsinogen, bovine serum albumin (BSA), and benzoyl-DL-arginine *p*-nitroanilide (BAPNA) were purchased from Sigma (St. Louis, MO, U.S.A.). trifluoroacetic acid was from Pierce (Rockford, IL, U.S.A.).

Apparatus

SynChropak RP-P, RP-8, RP-4, Isopropyl, Propyl, Hydroxypropyl, Benzyl and Phenyl columns, 250 × 4.1 mm I.D. (particle size 6.5 μm), and a SynChropak PCR column, 400 × 5 mm I.D. were obtained from SynChrom (Linden, IN, U.S.A.). A Varian Model 5000 gradient high-performance liquid chromatograph with a Valco Model CV-6-UHPa-N-60 injection valve (Varian, Walnut Creek, CA, U.S.A.) and an AN-203 UV detector (Anspec, Ann Arbor, MI, U.S.A.) or a Chem Research Model 2020 multiple-wavelength detector (Instrumentation Specialties, Lincoln, NE, U.S.A.) with a Linear Model 1200 recorder (Linear Instruments, Irvine, CA, U.S.A.) were used for the analyses.

Methods

The buffers were prepared by adding the appropriate amount of ammonium sulfate or sodium chloride to 0.02 *M* potassium phosphate (monobasic) in distilled water and adjusting the pH to 7 with sodium hydroxide.

Post-column reaction

SynChropak PCR is a macroparticulate non-porous column packing with a hydrophilic layer that is essentially nonreactive to enzymes and substrates[9]. A SynChropak PCR column was connected to the exit of an analytical column with a tee, into which a solution of the substrate was pumped at 1 ml/min. The effluent of the reactor was monitored at 410 nm. All reactions were performed at room temperature. The reactor volume was 3.7 ml.

The substrate for trypsin was BAPNA, which is hydrolyzed by trypsin to produce *p*-nitroaniline[10]. By heating and stirring, 0.79 mmoles of BAPNA were dissolved in 0.1 *M* potassium phosphate, pH 7. BAPNA remained in solution after cooling to room temperature. It remained colorless throughout the heating and chromatography until hydrolysed by trypsin.

RESULTS AND DISCUSSION

Reversed-phase chromatography

The effect of chain length on protein retention in reversed-phase chromatography was observed by analyzing ovalbumin and BSA on three columns with 300Å supports and carbon chain lengths of 18, 8 and 4. The data, as seen in Table I, show few differences in the retention times for ovalbumin or BSA on the different supports. This lack of discrimination, in addition to the deleterious effects of acids and organic

TABLE I

COMPARISON OF REVERSED-PHASE SELECTIVITY

Conditions: 30-min gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in 2-propanol; flow-rate 1 ml/min; absorbance 254 nm. Column dimensions 250 × 4.1 mm I.D.

Column	*Retention time (min)*	
	Ovalbumin	*BSA*
SynChropak RP-4	18.6	15.7
SynChropak RP-8	19.3	15.6
SynChropak RP-P (C-18)	20.7	16.9

eluents on many proteins, suggested that alternative methods of hydophobic chromatography should be sought.

Hydrophobic interaction chromatography

In order to insure that any interaction with this series of packings would be totally hydrophobic, a hydrophilic polymer was developed which gave no separation

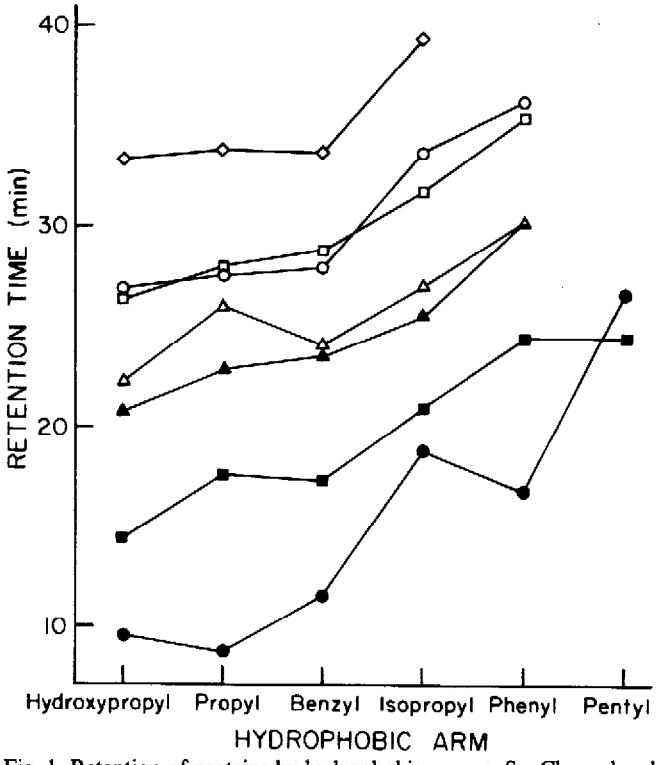

Fig. 1. Retention of proteins by hydrophobic groups. SynChropak columns as indicated (250 × 4.1 mm I.D.), flow-rate: 1 ml/min, pressure: 80 atm, 30-min gradient from 2.1 *M* to 0.1 *M* $(NH_4)_2SO_4$ in 0.02 *M* KH_2PO_4, pH 7. Proteins: ◇, chymotrypsinogen A; ○, α-chymotrypsin; □, trypsinogen; △, lysozyme; ▲, ovalbumin; ■, ribonuclease A; ●, cytochrome *c*.

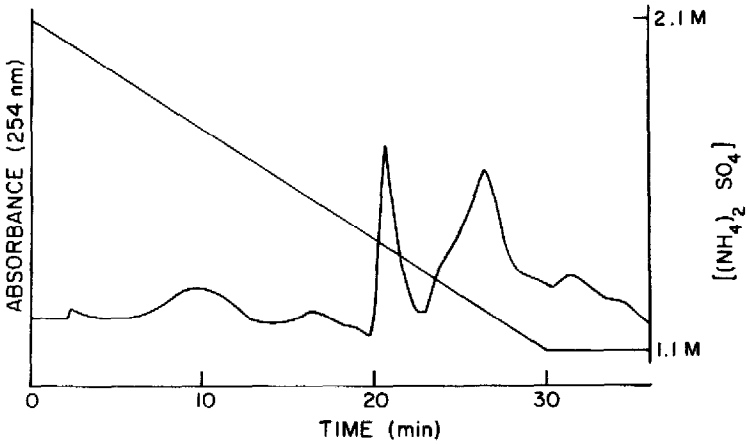

Fig. 2. Analysis of BSA by HIC. Column: SynChropak Propyl (250 × 4.1 mm I.D.), flow-rate: 1 ml/min, pressure: 80 atm, 30-min gradient from 2.1 *M* to 0.1 *M* $(NH_4)_2SO_4$ in 0.02 *M* KH_2PO_4, pH 7.

except by size when proteins were analyzed with a buffer of 0.1 *M* phosphate, pH 7. Insertion of hydrophobic alkyl or aryl groups into the polymer resulted in packings which bound proteins when a buffer of 1 *M* or 2 *M* salt was used; the proteins were released as the salt concentration gradually decreased. Fig. 1 illustrates the retention times for a series of seven proteins on six different hydrophobic supports when a reverse gradient of ammonium sulfate is used. It can be seen that very small changes, such as propyl to isopropyl, result in very large changes in retention times for some proteins. This is in contrast to reversed-phase HPLC, where large changes in chain length had little effect on retention. The pentyl packing material bound most proteins irreversibly when these aqueous salt solutions were used. The phenyl ligand strongly

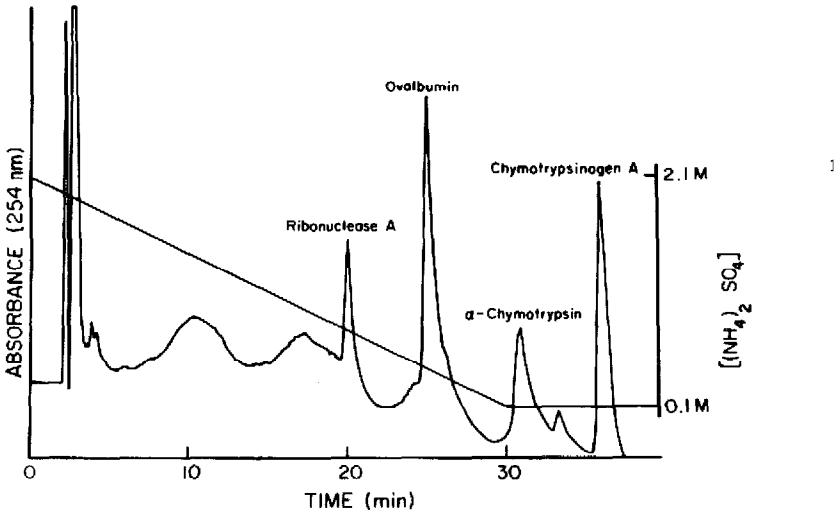

Fig. 3. Analysis of a protein mixture by HIC. Column: SynChropak Isopropyl (250 × 4.1 mm I.D.), flow-rate: 1 ml/min, pressure: 80 atm, 30-min gradient from 2.1 *M* to 0.1 *M* $(NH_4)_2SO_4$ in 0.02 *M* KH_2PO_4, pH 7.

TABLE II

THE EFFECT OF SALT ON PROTEIN RETENTION

Conditions: SynChropak columns as stated; 30-min gradient as shown; flow-rate: 1 ml/min; absorbance at 254 nm.

	Propyl			*Isopropyl*	
	NaCl 4 *M* 0.1 *M*	$(NH_4)_2SO_4$		$(NH_4)_2SO_4$	
		2 *M*–0.1 *M*	1 *M*–0.1 *M*	2 *M*–0.1 *M*	1 *M*–0.1 *M*
Cytochrome *c*	4.6	8.6	–	18.6	2.1
Ovalbumin	4.0	18.5	4.4	25.1	6.0
BSA	25.0	20.7, 26.4	–	33.9	–
Lysozyme	31.4	26.0	9.5	26.6	9.5
α-Chymotrypsin	30–32	27.5	18.9	30.9	19.2
Ribonuclease A		16.3	2.6	20.1	3.9
Chymotrypsinogen A		25.2	23.9	36.0	30.3

retained proteins; when the more electron-rich benzyl ligand was substituted, the weaker pi–pi interactions caused earlier elution times. Fig. 1 illustrates that for some proteins, there are trends in the retention caused by different hydrophobic ligands. These differences in retention could also be caused by changes in ligand density, but the differences in selectivities must be due to the nature of the hydrophobic group.

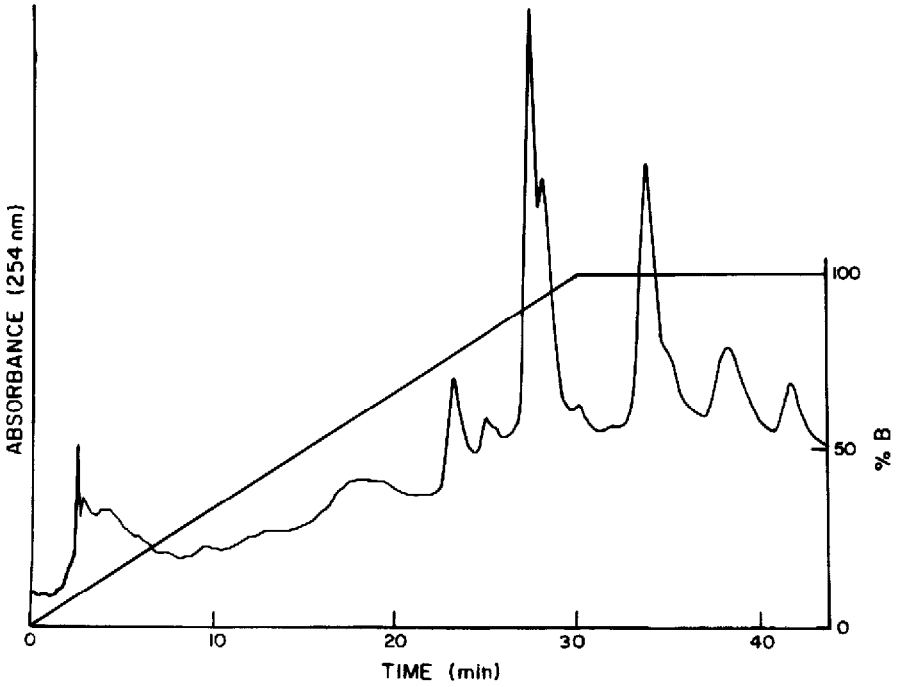

Fig. 4. Analysis of soybean trypsin inhibitor. Column: SynChropak Propyl (250 × 4.1 mm I.D.), flow-rate: 1 ml/min, pressure: 80 atm, 30-min gradient from 4 *M* to 0 *M* NaCl in 0.1 *M* KH_2PO_4, pH 7.

Data for BSA and trypsin have been omitted from the graph because these proteins are split into multiple peaks when certain packings are used. This behavior of BSA is a good illustration of their selectivity. On the phenyl and benzyl columns, it is eluted as one broad peak; however, on the three propyl modifications, multiple peaks are observed. Fig. 2 shows the best resolution of the BSA constituents on these columns; the multiple peaks are probably multiple forms of the protein, such as the monomer and dimer. An example of the separation of a protein mixture obtained by HIC on an isopropyl column is seen in Fig. 3.

Salt effect

Three different salts were used for elution of proteins from propyl columns. A comparison of the data for sodium chloride and ammonium sulfate is seen in Table II. The third salt, sodium sulfate, was not used extensively because of solubility problems. Ammonium sulfate, which causes strong salting out of proteins, was chosen for the remaining studies, because it gave greater retention of less hydrophobic proteins in comparable concentrations and because it is less injurious to the stainless-steel components of the chromatograph. Sodium chloride gradients, however, produced nicely shaped peaks as can be seen for soybean trypsin inhibitor in Fig. 4.

Table II also illustrates the influence of the initial salt concentration on the retention. As would be expected, for highly retained proteins, such as chymotrypsinogen A, a lower initial salt concentration has little effect on retention time. Conversely, less hydrophobic proteins have greatly reduced retention when 1 *M* salt is

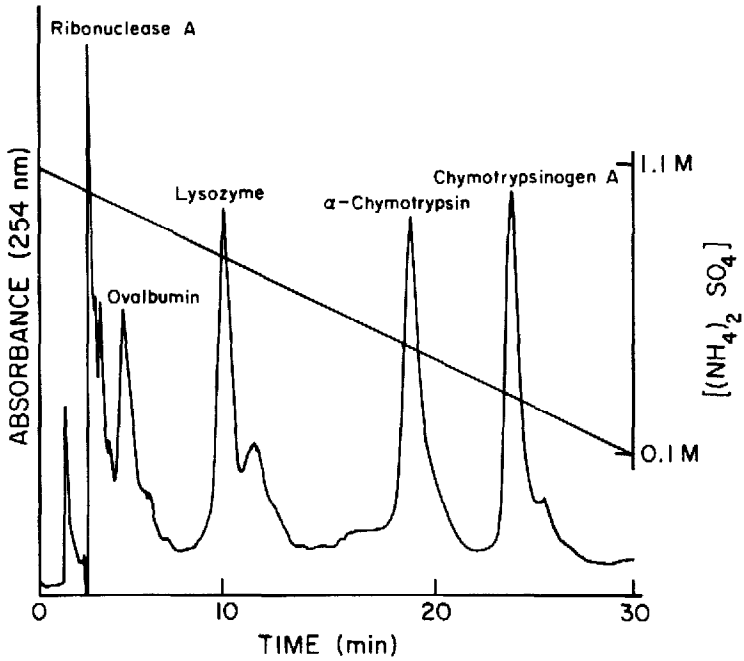

Fig. 5. Analysis of a protein mixture by HIC. Column: SynChropak Propyl (250 × 4.1 mm I.D.), flow-rate: 1 ml/min, pressure: 80 atm, 30-min gradient from 1.1 *M* to 0.1 *M* $(NH_4)_2SO_4$ in 0.02 *M* KH_2PO_4, pH 7.

TABLE III

ANALYSIS OF TRYPSIN ACTIVITY

Conditions: SynChropak columns as stated; 30-min gradient from 2 to 0.1 *M* $(NH_4)_2SO_4$ in 0.02 *M* KH_2PO_4, pH 7; flow-rate 1 ml/min; detection by post-column reaction with BAPNA as described in text.

	Retention time (min) of peaks showing activity			
	A	*B*	*C*	*D*
Hydroxypropyl	24.2	26.4	28.8	31.2
Propyl	29.5	33.0	34.8	37.2
Isopropyl	29.3	32.6	34.8	37.6
Benzyl	26.7	28.7	31.2	34.4
Phenyl	37.1	45.0		

used instead of 2 *M*. Fig. 5 illustrates the separation of five proteins by the use of the milder 1 *M* initial salt concentration.

Enzyme activity

One major consideration in enzyme purification is retention of enzymatic activity throughout the procedure. A commercial trypsin sample was analyzed on each of the columns and enzymatic activity was detected by means of a post-column reaction system. All columns, except the pentyl column, resolved trypsin into 2–4 peaks of activity, as seen in Table III. Multiple forms, including α- and β-trypsin, have been

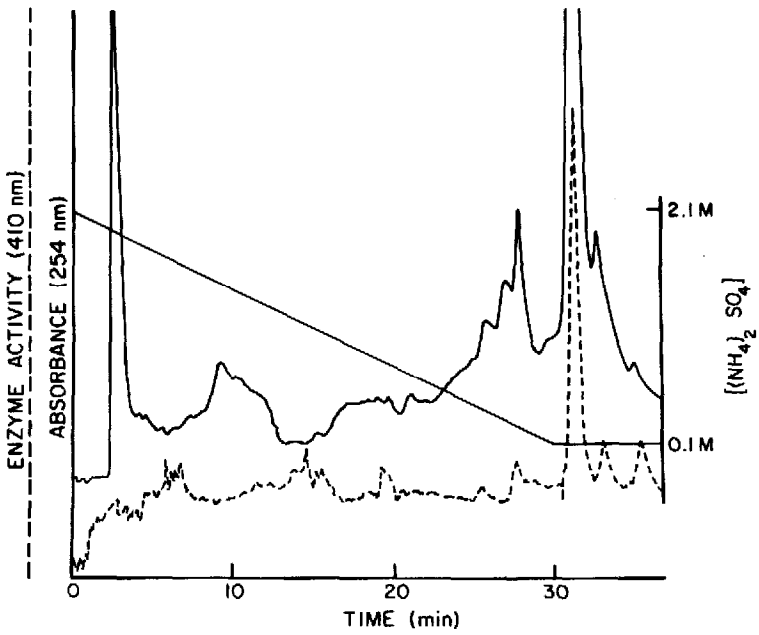

Fig. 6. Purification of trypsin by HIC. Column: SynChropak Propyl (250 × 4.1 mm I.D.), flow-rate: 1 ml/min, pressure: 80 atm, 30-min gradient from 2.1 *M* to 0.1 *M* $(NH_4)_2SO_4$ in 0.02 *M* KH_2PO_4, pH 7. Detection by post-column reaction, using BAPNA as substrate, as described in Experimental.

observed previously by HIC on Spheron P300[11–12]. A comparison of the activity profile *versus* the crude protein separation of trypsin, detected at 254 nm is seen in Fig. 6 for the Propyl column.

CONCLUSIONS

From the data presented here, it can be seen that HIC is a suitable method for protein purification, and could become a popular method for enzyme analysis. It separates proteins according to their hydrophobic character by the use of descending salt gradients under nondenaturing conditions. The elution order is not identical with that of reversed-phase columns; BSA and ovalbumin, for example, reverse their order when chromatographed on the basis of the two mechanisms. This is probably due to the tertiary structure that is maintained in HIC but disrupted in reversed-phase HPLC, which results in fewer external amino acids partaking in the HIC separations[14]. Small changes in the alkyl or aryl residues result in substantial changes in retention and selectivity, and enzymes such as trypsin retain activity throughout the analyses. These two qualities suggest the great potential of HIC for protein analysis.

REFERENCES

1 F. E. Regnier, *Methods Enzymol.*, 91 (1983) 137.
2 S. Hjertén, *J. Chromatogr.*, 87 (1973) 325.
3 J. Porath, L. Sundberg, N. Fornstedt and J. Olsson, *Nature (London)*, 245 (1973) 465.
4 P. Štrop, F. Mikeš and Z. Chytilová, *J. Chromatogr.*, 156 (1978) 239.
5 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 266 (1983) 49.
6 F. E. Regnier and J. Fausnaugh, *LC Magazine*, 1 (1983) 402.
7 D. L. Gooding and K. M. Gooding, *Pittsburgh Conference, Atlantic City, NJ*, 1983, No. 560A.
8 S. Gupta and F. E. Regnier, *First International Symposium on HPLC of Proteins and Peptides, Washington, D.C., 1981*, Paper No. 213.
9 T. D. Schlabach, S. H. Chang, K. M. Gooding and F. E. Regnier, *J. Chromatogr.*, 134 (1977) 91.
10 B. J. Haverbach, B. Dyce, H. Bundy and H. A. Edmonson, *Am. J. Med.*, 29 (1960) 424.
11 P. Štrop and D. Čechová, *J. Chromatogr.*, 207 (1981) 55.
12 P. Štrop, D. Čechová and V. Tomášek, *J. Chromatogr.*, 259 (1983) 255.
13 K. M. Gooding and M. N. Schmuck, *J. Chromatogr.*, 266 (1983) 633.
14 F. E. Regnier, *LC Magazine*, 1 (1983) 350.