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HIGH-PERFORMANCE HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PROTEINS

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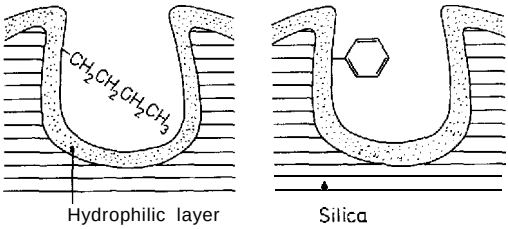
SUMMARY

High-performance hydrophobic interaction chromatography of proteins was investigated on Butyl-G3000SW and Phenyl-G3000SW prepared by coupling butyl and phenyl groups with G3000SW, which is a support for high-performance gel chromatography. It was possible to separate proteins with high efficienty under mild eluting conditions such as isocratic elution with 0 to 2 *M* ammonium sulphate in 0.1 M phosphate buffer (pH 6.0) and linear gradient elution with ammonium sulphate concentration decreasing from 1.5 or 2 *M* to 0 in 0.1 *M* phosphate buffer (pH 6.0). The recovery of proteins or enzymatic activity was almost quantitative.

INTRODUCTION

Hydrophobic interaction chromatography has been employed for the separation of proteins and especially in recent years it has been applied to many kinds of protein&'*. Alkyl or aryl derivates of agarose have been mainly used for these separations. Since this type of column material cannot withstand high pressure, hydrophobic interaction chromatography had to be performed at low presure until now.

We prepared rigid supports for use in hydrophobic interaction chromatography by coupling butyl and phenyl groups to TSK-GEL G3000SW, which is a silica-based column material of 250 Å mean pore diameter for high-performance gel chromatography. These matrices are believed to have the following schematic structures and differ from now commercially available silica-based reversed-phase matrices in having the hydrophilic layer on the surface of silica.



These supports are investigated for the separation of proteins in this paper.

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EXPERIMENTAL

Butyl and phenyl groups were introduced into G3000SW of 10-μm particle diameter (Toyo Soda, Tokyo, Japan) by the reaction between hydroxyl groups on the support and butyl or phenyl glycidyl ethers according to the method of Hjertén et *al.*[11]. The ligand content was estimated from the carbon content of the matrix, determined by elemental analysis before and after the reaction. Butyl-G3000SW and Phenyl-G3000SW with ligand contents of, respectively, 0.12 and 0.14 mmol/g (corresponding to 38 and 44 μmol/ml) were employed in the following experiments.

Chromatographic measurements were carried out at 25°C with a high-speed liquid chromatograph Model HLC-803C equipped with gradient generator Model GE-2 and variable-wavelength UV detector (Toyo Soda). The detector was operated at 280 nm. Columns of 15 x *0.6* cm I.D. packed with the above mentioned materials were used. These columns were prepared by the slurry packing method at flow-rates of *ca.* 5 ml/min. A 0.1 *M* phosphate buffer (pH 6.0) was employed for both the preparation of the slurry and packing. Capacity factors and recoveries were determined for commercial proteins listed in Table I by isocratic elution with 0 – 2 *M* ammonium sulphate concentrations in 0.1 *M* phosphate buffer (pH 6.0) at a flow-rate of 1 ml/min. Proteins (0.5 mg each) were applied to the columns equilibrated with 0.1 *M* phosphate buffer (pH 6.0) containing 2 *M* ammonium sulphate (initial buffer). The proteins should be bound by the column materials in this initial buffer, as can be seen from Figs. 1 and 2. After elution with the initial buffer for 2 min, the bound proteins were eluted with a 1-min linear gradient from the initial buffer to 0.1 *M* phosphate buffer. The column effluent was collected for 25 min from the start of gradient elution and the proteins in the effluent were determined spectrophotometrically at 215 nm. The recovery of enzymatic activity was evaluated for α-chymotrypsin. α-Chymotrypsin (0.4 mg) was chromatographed with a 60-min linear gradient of ammonium sulphate concentration from 1.5 *M* to 0 in 0.1 *M* phosphate buffer (pH 6.0) at a flow-rate of 1 ml/min. The column effluent corresponding to the peak of α-chymotrypsin was fractioned and subjected to the determination of enzymatic activity, which was performed using N-benzoyl-L-tyrosine ethyl ester as substrate[12]. A mixture of the eight proteins in Table I (0.04 mg

TABLE I

RECOVERY OF PROTEINS FROM BUTYL-G3000SW AND PHENYL-G3000SW

Sample no.	*Protein*	*Molecular weight*	*Source**	*Recovery (%)*	
				Butyl-G3000S W	*Phenyl-G3000S W*
1	Cytochrome c	12,400	A	95	93
2	Ribonuclease	13,700	B	96	108
3	Lysozyme	14,300	B	101	99
4	Bovine serum albumin	67,000	A	105	100
5	Ovalbumin	43,000	C	99	101
6	a-Chymotrypsin	25,000	A	94	102
7	a-Chymotrypsinogen	25,700	A	88	95
8	Myoglobin	16,900	A	94	98

* A, Sigma (St. Louis, MO, U.S.A.); B, P-L Biochemicals (Milwaukee, WI, U.S.A.); C, Seikagaku Kogyo (Tokyo, Japan).

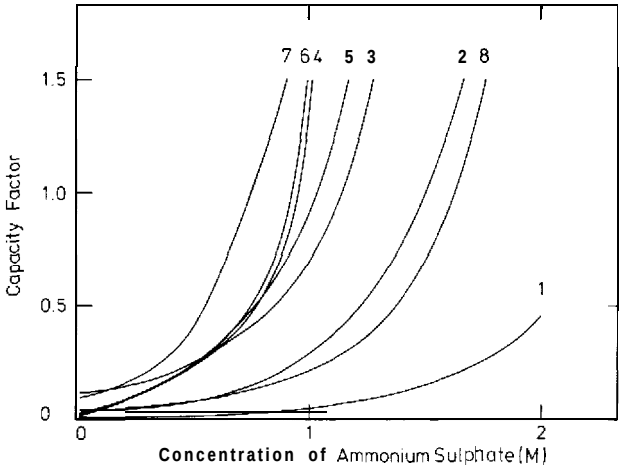

Fig. 1. Dependence of capacity factors on ammonium sulphate concentration in the hydrophobic interaction chromatography of proteins on Butyl-G3000SW in 0.1 M phosphate buffer (pH 6.0) containing 0 – 2 M ammonium sulphate. Samples as 'in Table I.

each protein in 0.1 ml initial buffer) was separated with 60 or 120 min linear gradient elution of ammonium sulphate concentrations from 1.5 or 2 A4 to 0 in 0.1 *M* phosphate buffer (pH 6.0) at a flow-rate of 1 or 0.5 ml/min.

RESULTS AND DISCUSSION

Figs. 1 and 2 show the dependence of capacity factors on ammonium sulphate concentration in chromatography on Butyl-G3000SW and Phenyl-G3000SW. The void volume for each protein was determined for a 15 x 0.6 cm I.D. column, packed with

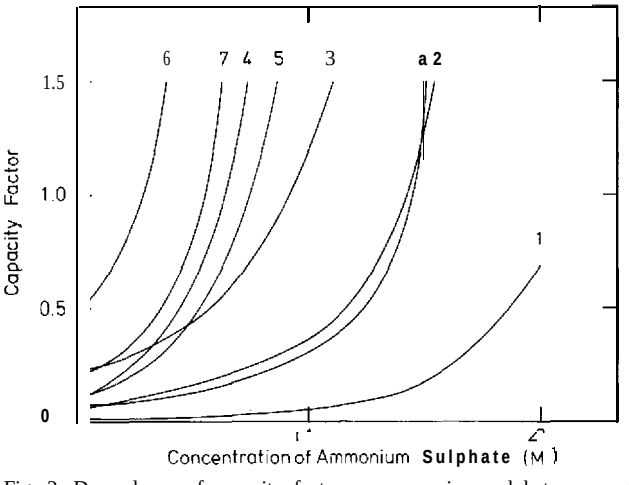

Fig. 2. Dependence of capacity factor on ammonium sulphate concentration in hydrophobic interaction chromatography of proteins on Phenyl-G3000SW in 0.1 M phosphate buffer (pH 6.0) containing Cl – 2 M ammonium sulphate. Samples as in Table I.

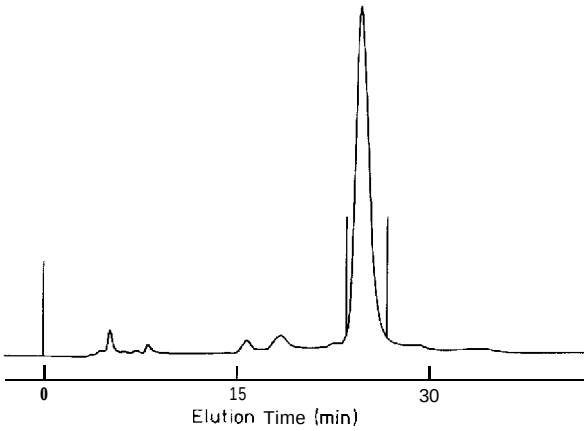

Fig. 3. Separation of α-chymotrypsin (0.4 mg in 0.1 ml) on Phenyl-G3000SW with a 60 min linear gradient of ammonium sulphate concentrations from 1.5 *M* to 0 in 0.1 *M* phosphate buffer (pH 6.0) at a flow-rate of 1 ml/min.

unmodified G3000SW in 0.1 *M* phosphate buffer (pH 6.0), where the protein-matrix interaction is assumed to be minima. The capacity factors increased from almost zero to a very large value with the increase in ammonium sulphate concentration from 0 to 2 *M,* suggesting that proteins can be absorbed and desorbed under very mild conditions. Therefore, proteins can be expected to seperate without risk of denaturation.

The recovery of all proteins investigated (Table I) was almost quantitative. Fig. 3 shows the chromatogram of a-chymotrypsin obtained with Phenyl-G30OOSW. The column effluent between the two vertical lines in the figure was collected. The recovery of enzymatic activity in the fraction was 95%. The same experiment was also performed on Butyl-G3000SW, resulting in 100% recovery of enzymatic activity.

Figs. 4 and 5 show chromatograms of a mixture of the eight proteins obtained

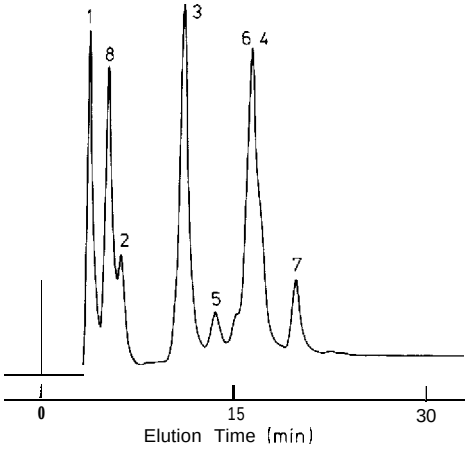

Fig. 4. Separation of a mixture of cytochrome-C (1), ribonuclease (2), lysozyme (3), bovine serum albumin (4), ovalbumin (5), α-chymotrypsin (6), α-chymotrypsinogen (7) and myoglobin (8) (0.04 mg each in 0.1 ml) on Butyl-G3000SW. Conditions as in Fig. 3.

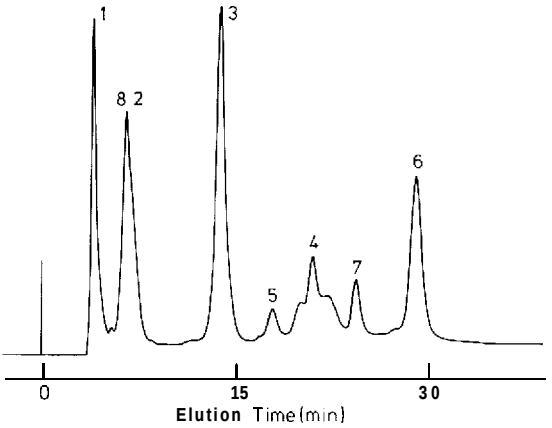

Fig. 5. Separation of a protein mixture on Phenyl-G3000SW. Conditions as in Fig. 3. Samples as in Fig. 4.

on Butyl-G3000SW and Phenyl-G3000SW with a 60 min linear gradient of ammonium sulphate concentration from 1.5 *M* to 0. The separation of proteins is much better than in ordinary hydrophobic interaction chromatography. In Fig. 5, peaks appearing on both sides of bovine serum albumin are due to impurities. Elution patterns were similar on both Butyl-G3000SW and Phenyl-G3000SW as a whole. However, some proteins such as α-chymotrypsin behaved a little differently on the two columns. This suggests that some interactions, e.g. charge transfer interaction, may contribute to the hydrophobic interaction in the separation on these columns. Fig. 6 shows a chromatogram of the protein mixture obtained on Phenyl-G3000SW with a longer gradient time and at a higher ammonium sulphate concentration in the initial buffer. Components eluted early were better separated than in Fig. 5. This is probably due to the increase in ammonium sulphate concentration in the initial buffer rather than to the longer gradient time because the separation of components eluted late was not so greatly improved. Fig. 7 shows a chromatogram of the protein mixture obtained under the same conditions as in Fig. 6 except that the flow-rate was 0.5 ml/min. The separation of proteins

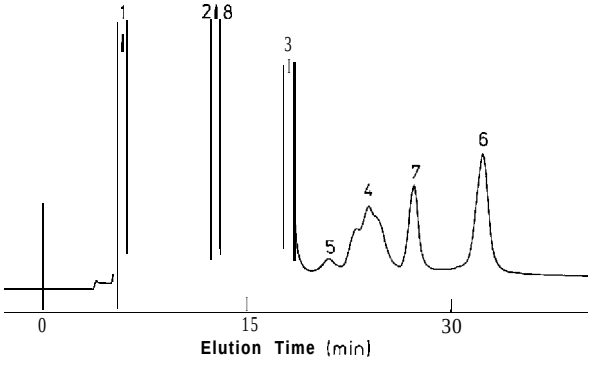

Fig. 6. Separation of a protein mixture on Phenyl-G3000SW with a 120 min linear gradient of ammonium sulphate concentrations from 2 *M* to 0 in 0.1 *M* phosphate buffer (pH 6.0) at a flow-rate of 1 ml/min. Samples as in Fig. 4.

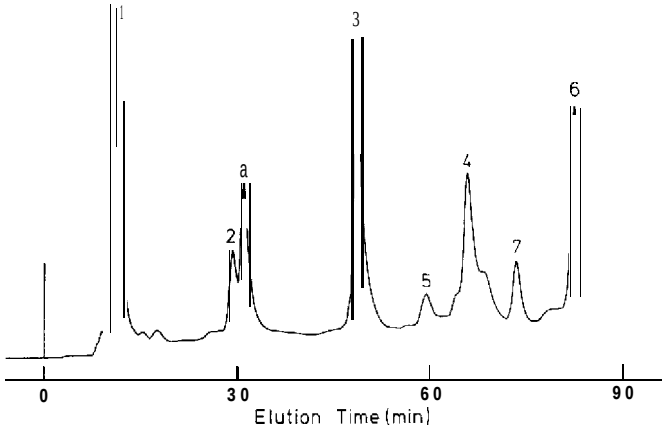

Fig. 7. Separation of a protein mixture on Phenyl-G3000SW. Conditions as in Fig. 6 except for the flow-rate which was 0.5 ml/min. Samples as in Fig. 4.

was improved by lowering the flow-rate although the separation time was more than doubled.

Reversed-phase groups, such as C_{18}, has been another approach to the separation of proteins according to their hydrophobic properties. High-performance reversed-phase chromatography has been extensively investigated in these days. However, sice proteins are separated in buffers containing large quantities of organic solvents at low pH, there is always a risk of denaturation. In addition, proteins are very often recovered in low yield. In contrast, the high-performance hydrophobic interaction chromatography described here was performed with almost quantitative recovery of both protein mass and enzymatic activity owing to the mild eluting conditions, and hence it can be expected to become one of the useful methods for the separation and purification of proteins. The separation of proteins exemplified in Figs. 4-7 may not be so good as that attainable by reversed-phase chromatography. However, it should be possible to increase the seperation efficiency by optimizing the operational variables.

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