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Note

New support for hydrophobic interaction chromatography of proteins

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Hydrophobic interaction chromatography has been employed extensively for the separation of proteins since its introduction[1–17]. However, almost all those separations were carried out at low speed using alkyl or aryl derivatives of agarose.

Recently, a new support for hydrophobic interaction chromatography has become commercially available under the trade-name of TSKgel Phenyl-5PW (Toyo Soda, Tokyo, Japan)*. According to the manufacturer, this new support was developed by introducing phenyl groups (*ca.* 0.1 mmol/ml) with ether linkage into TSKgel G5000PW[18], which is a hydrophilic-polymer-based material of large pore size (particle diameter 10 μm) for high-performance gel filtration. Its basic properties and applications are described in this paper.

EXPERIMENTAL

The binding of proteins to TSKgel Phenyl-5PW was investigated by the static method. A 1-ml volume of TSKgel Phenyl-5PW, 10 ml of 1% solution of each protein in 0.1 *M* phosphate buffer (pH 7.0) and 30 ml of 0.1 *M* phosphate buffer (pH 7.0) containing various concentrations of ammonium sulphate were mixed. After the mixture had been left at 25°C for 30 min with occasional swirling, it was filtered through filter-paper. The protein concentration of the filtrate was determined spectrophotometrically at 280 nm. The amount of unadsorbed protein was calculated on the basis of a total volume of protein solution of 40 ml.

The microstructure of TSKgel Phenyl-5PW was observed with a Model JSM-50A scanning electron microscope (Japan Electron Optics Lab., Tokyo, Japan).

The chemical stability was evaluated by measuring the change in ligand (phenyl group) content on treatment of TSKgel Phenyl-5PW with 0.5 *M* sodium hydroxide and 20% acetic acid at 25°C. The ligand content was determined according to the method of Genieser *et al.*[19] with some modifications. We quantified the cleaved ligand by liquid chromatography instead of gas chromatography. Phenol, which is a cleavage product of phenyl groups, was separated by reversed-phase chromatography in water–methanol (50:50) on a TSKgel ODS-120T column (250 × 4.6 mm I.D.) at a flow-rate of 1 ml/min and detected with a UV spectrophotometer at 254 nm.

* Two silica-based supports Butyl-G3000SW and Phenyl-G3000SW were recently described by us[22].

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Chromatographic measurements were carried out on a column (75 × 7.5 mm I.D.) with a high-speed liquid chromatograph Model SP8700 (Spectra-Physics, San Jose, CA, U.S.A.) equipped with a variable-wavelength UV detector Model UV-8 (Toyo Soda). Proteins and nucleic acids were separated by 60-min linear gradient elution with decreasing ammonium sulphate concentration from 1.5–2 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 0.5 or 1 ml/min at 25°C and detected at 280 nm and 260 nm, respectively. In the separation of some enzymes the column effluent was collected and the enzymatic activity was determined.

Lipoxidase was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Crude and purified ferredoxin NADP reductase was a gift from Dr. Shin of Kobe University, Japan. All other proteins and tRNA were purchased from Sigma (St. Louis, MO, U.S.A.). rRNA was obtained from Boehringer (Mannheim, F.R.G.).

RESULTS AND DISCUSSION

Basic properties

Fig. 1 shows the amount of proteins adsorbed on TSKgel Phenyl-5PW in the presence of various concentrations of ammonium sulphate. Proteins began to adsorb on TSKgel Phenyl-5PW at ammonium sulphate concentrations between 0 and 2 *M*, which suggests that many proteins can adsorb first and are then selectively desorbed during chromatography on TSKgel Phenyl-5PW with gradient elution with decreasing ammonium sulphate concentration from 2 *M* to 0. Fig. 1 also shows that 1 ml of TSKgel Phenyl-5PW can absorb a maximum of several tens of milligrams of protein.

Fig. 2 shows the microstructure of TSKgel Phenyl-5PW, illustrating that it is macroreticular and has pores with a diameter of several thousand angstroms into which even very large molecules can easily penetrate. Therefore, TSKgel Phenyl-5PW is applicable to proteins with very high molecular weights. Also, the large pore size

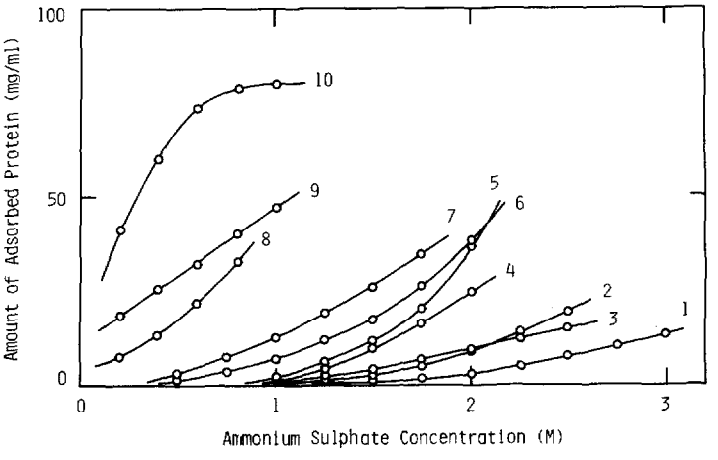

Fig. 1. The amount of proteins adsorbed on TSKgel Phenyl-5PW in 0.1 *M* phosphate buffer (pH 7.0) containing various concentrations of ammonium sulphate. Samples: 1 = cytochrome *c*; 2 = myoglobin; 3 = ribonuclease; 4 = lysozyme; 5 = ovalbumin; 6 = α-chymotrypsin; 7 = α-chymotrypsinogen; 8 = γ-globulin; 9 = thyroglobulin; 10 = ferritin.

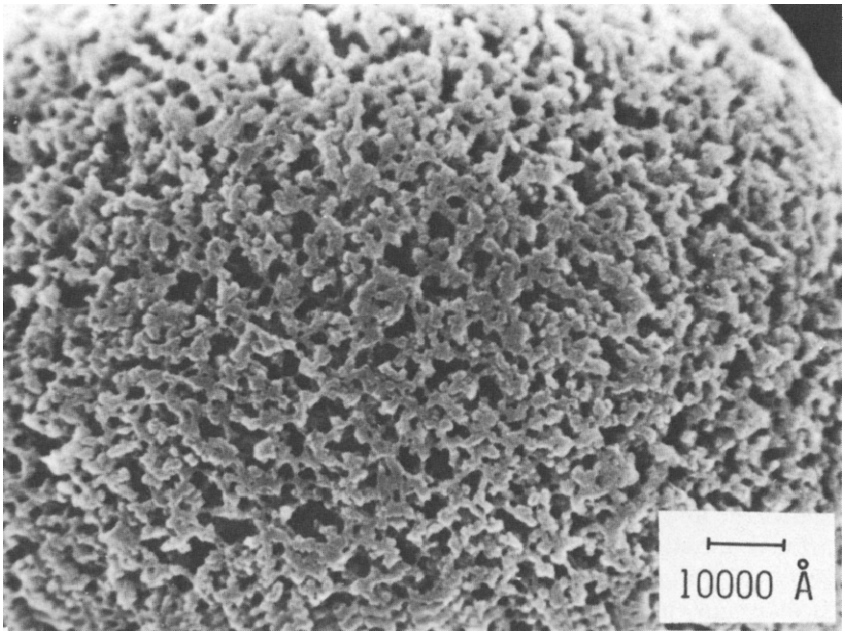

Fig. 2. Scanning electron photomicrograph of TSKgel Phenyl-5PW.

should facilitate high resolution in protein separations, just as in the case of ion-exchange chromatography[20].

Table I shows the change in ligand content of TSKgel Phenyl-5PW on treatment with 0.5 *M* sodium hydroxide and 20% acetic acid at 25°C for 10 days. The ligand content remained unchanged, indicating that TSKgel Phenyl-5PW is chemically very stable. Consequently, TSKgel Phenyl-5PW can be washed with alkaline and acidic aqueous solutions for regeneration.

Applications to the separation of proteins and nucleic acids

Fig. 3 shows a chromatogram of a protein mixture obtained with a 60-min linear gradient elution with decreasing ammonium sulphate concentration from 1.7 *M* to 0. All the proteins were eluted with fairly sharp peaks by the end of the gradient. Since reversed-phase charomatography on a silica-based support also separates pro-

TABLE I

CHANGE IN LIGAND CONTENT OF TSKgel PHENYL-5PW ON TREATMENT WITH 0.5 *M* SODIUM HYDROXIDE AND 20% ACETIC ACID AT 25°C FOR 10 DAYS

Solution	*Ligand content (mmol/ml)*	
	Before treatment	*After treatment*
0.5 *M* NaOH	0.105	0.104
20% CH_3COOH	0.105	0.106

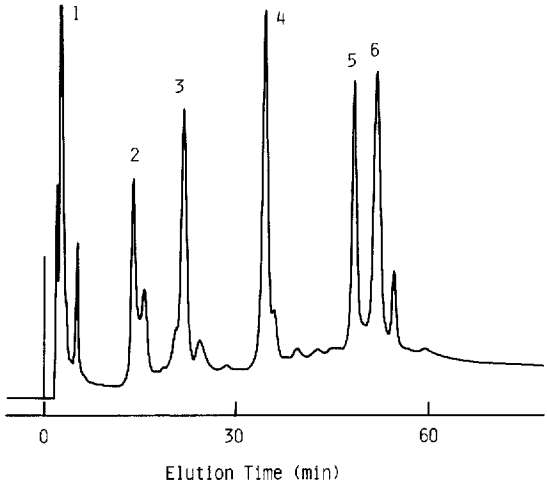

Fig. 3. Chromatogram of a protein mixture obtained by high-performance hydrophobic interaction chromatography on TSKgel Phenyl-5PW with a 60-min linear gradient elution with decreasing ammonium sulphate concentration from 1.7 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min. Peaks: 1 = cytochrome *c*; 2 = myoglobin; 3 = ribonuclease; 4 = lysozyme; 5 = α-chymotrypsinogen; 6 = α-chymotrypsin.

teins according to their hydrophobic properties, the same mixture was separated by this technique and the result is shown in Fig. 4 for comparison. The resolution attained in Figs. 3 and 4 seems to be comparable. However, the elution order of the proteins differed significantly in these two separations.

Commercial lipoxidase, crude and purified ferredoxin NADP reductase and commercial β-amylase (barley) were separated under similar conditions. The results

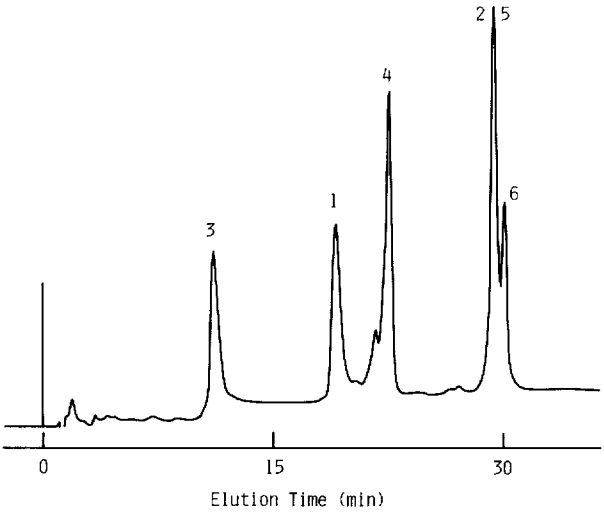

Fig. 4. Chromatogram of a protein mixture obtained by high-performance reversed-phase chromatography on TSKgel TMS-250 with a 40-min linear gradient elution with acetonitrile concentration from 10 to 50% in 0.05% trifluoroacetic acid (pH 2.2) at a flow-rate of 1 ml/min. Peaks as in Fig. 3.

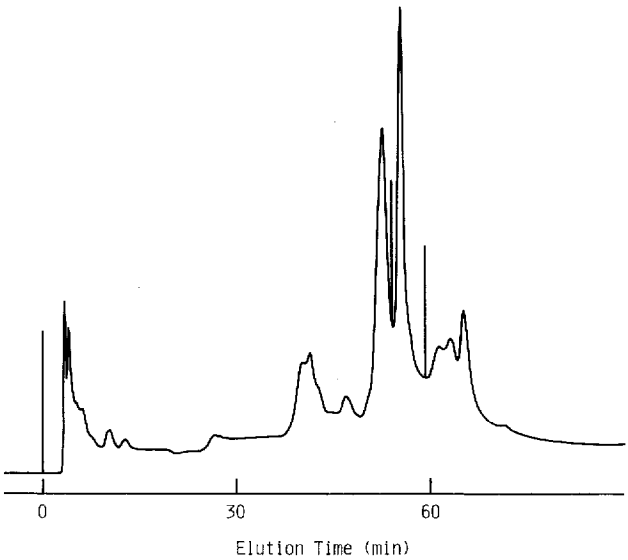

Fig. 5. Chromatogram of commercial lipoxidase (1 mg in 0.1 ml) obtained by high-performance hydrophobic interaction chromatography on TSKgel Phenyl-5PW with a 60-min linear gradient elution with decreasing ammonium sulphate concentration from 1.5 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 0.5 ml/min.

are shown in Figs. 5–7, indicating again that proteins can be separated with high resolution within a reasonable time. Although some component eluted after the end of the gradient (60 min) in the separation of crude samples, the desorption of such components may be promoted by the addition of small quantities of organic solvents or chaotropic agents[1–3,6,7,13,21]. In fact, the separation of β-amylase in Fig. 7 was performed with a final buffer containing 5% 2-propanol and the addition of 2-pro-

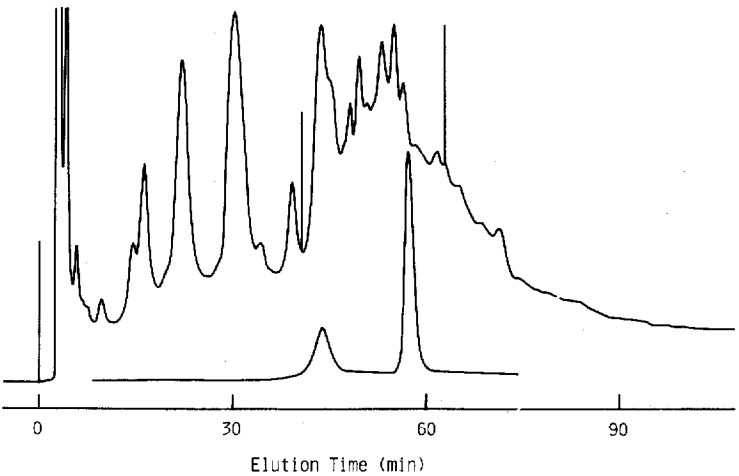

Fig. 6. Chromatograms of crude and purified ferredoxin NADP reductase (3 mg and 0.2 mg, respectively, in 0.1 ml) obtained by high-performance hydrophobic interaction chromatography. Conditions as in Fig. 5.

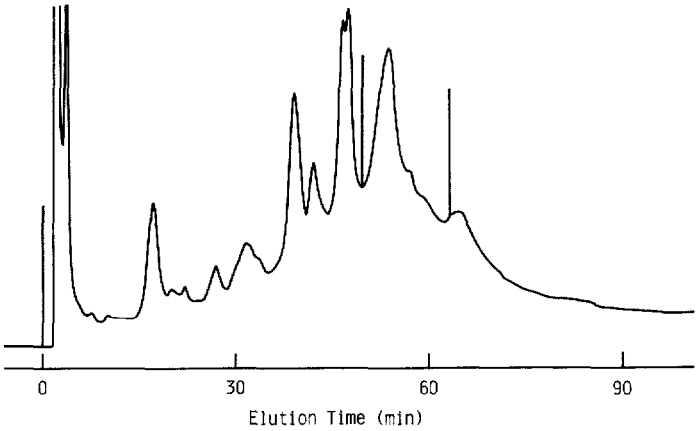

Fig. 7. Chromatogram of commercial β-amylase (1.3 mg in 0.1 ml) obtained by high-performance hydrophobic interaction chromatography with a 60-min linear gradient elution from 0.1 *M* phosphate buffer containing 1.5 *M* ammonium sulphate (pH 7.0) to 0.1 *M* phosphate buffer (pH 7.0) containing 5% 2-propanol at a flow-rate of 1 ml/min.

panol served to promote the desorption of late eluting components. Column effluents between the two vertical lines in these figures, in which each enzyme was shown to be present, were fractionated, and the enzymatic activity was determined. The recovery of enzymatic activity was also determined for α-chymotrypsin and lysozyme in the separations under the same conditions as in Fig. 3. Table II summarizes the recovery of enzymatic activity. A recovery of more than 80% was observed for all enzymes investigated. This is the decisive advantage of separation on TSKgel Phenyl-5PW over reversed-phase chromatography on silica-based supports. Since proteins are separated in buffers containing large quantities of organic solvents at low pH in reversed-phase chromatography, there is always a risk of denaturation.

Fig. 8 shows the dependence of peak width on sample loading in the separation of some proteins. Peak width was constant up to sample loadings of 0.1–0.5 mg and then increased with increasing sample loading. Therefore, sample loading should be kept lower than 0.1–0.5 mg in order to obtain the highest resolution. However, the peak width of a component in a mixture was not affected by the relative proportions of other components, as illustrated in Fig. 9, where two mixtures of myoglobin, ribonuclease, ovalbumin and α-chymotrypsin were separated. The two mixtures con-

TABLE II

RECOVERY OF ENZYMATIC ACTIVITY FROM TSKgel PHENYL-5PW

Enzyme	*Sample loading (mg)*	*Recovery (%)*
Lipoxidase	1.0	89
Ferredoxin NADP reductase	3.0	100
β-Amylase	1.3	80
α-Chymotrypsin	0.4	92
Lysozyme	0.2	90
Lysozyme	0.05	90

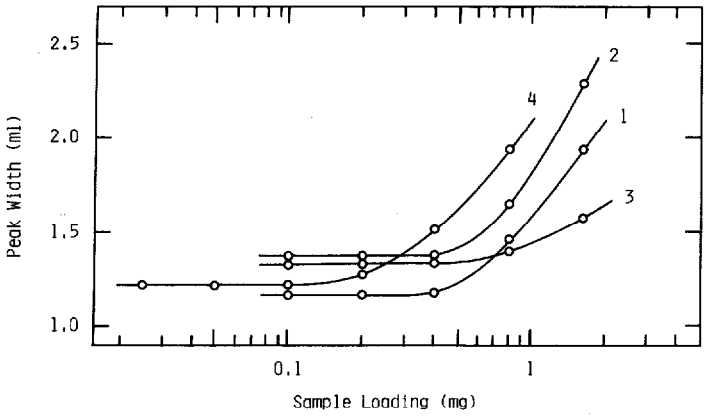

Fig. 8. Dependence of peak width on sample loading in the separation of proteins on TSKgel Phenyl-5PW. Conditions as in Fig. 5. Each protein was separated individually with an injection volume of 0.1 ml and varying sample concentrations from 0.025 to 1.6%. Samples: 1 = myoglobin; 2 = ribonuclease; 3 = ovalbumin; 4 = α-chymotrypsin.

tained the same amounts of ribonuclease and ovalbumin, but different quantities of myoglobin and α-chymotrypsin. Neither peak width nor elution position of ribonuclease and ovalbumin was affected by the increase in loadings of myoglobin and α-chymotrypsin from 0.1 mg to 0.8 mg for each protein. Accordingly, several milligrams of a sample containing many components can be applied without any decrease in resolution.

RNAs could also be separated on TSKgel Phenyl-5PW under conditions similar to those for proteins. Fig. 10 shows a chromatogram of a mixture of 16S and 23S rRNAs. These two rRNAs with rather high molecular weights (560,000 and 1,100,000, respectively) were well separated. Fig. 11 shows a chromatogram of tRNA. Components which are specific for various amino acids were partially separated.

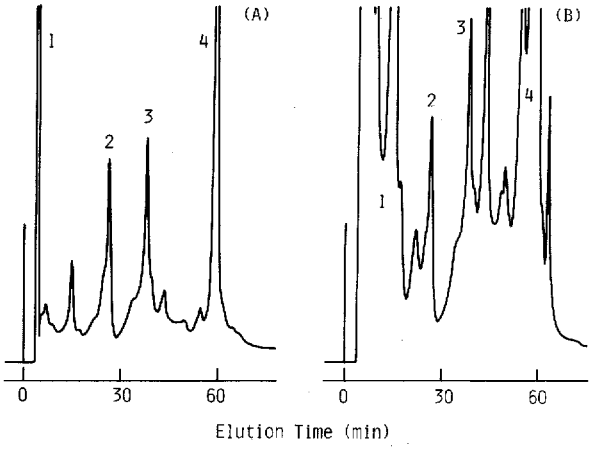

Fig. 9. Chromatograms of two mixtures containing myoglobin (1), ribonuclease (2), ovalbumin (3) and α-chymotrypsin (4) in amounts of 0.1 mg, 0.2 mg, 0.2 mg, 0.1 mg (A) and 0.8 mg, 0.2 mg, 0.2 mg, 0.8 mg (B) obtained on TSKgel Phenyl-5PW. Conditions as in Fig. 5.

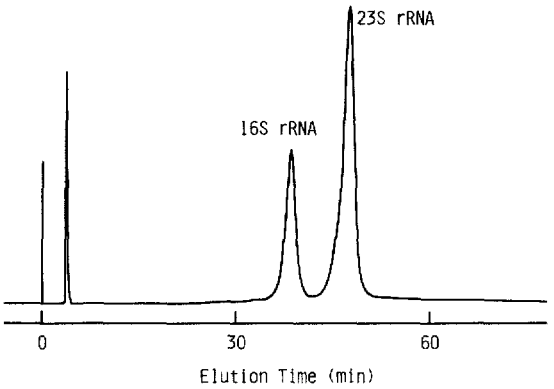

Fig. 10. Chromatogram of a mixture of 16S and 23S rRNAs (0.05 mg in 0.1 ml) obtained by high-performance hydrophobic interaction chromatography on TSKgel Phenyl-5PW with a 60-min linear gradient elution with decreasing ammonium sulphate concentration from 2 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 0.5 ml/min.

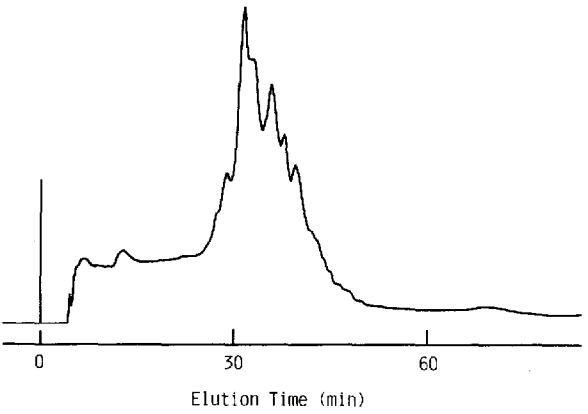

Fig. 11. Chromatogram of tRNA (Type XXI, from *E. coli*, strain W) (0.1 mg in 0.1 ml) obtained by high-performance hydrophobic interaction chromatography on TSKgel Phenyl-5PW. Conditions as in Fig. 10.

However, double-stranded DNA fragments could not be adsorbed on TSKgel Phenyl-5PW under the same conditions as in Figs. 10 and 11.

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