Journal of Chromatography, 298 (1984) 407–418
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,834

OPERATIONAL VARIABLES IN HIGH-PERFORMANCE HYDROPHOBIC INTERACTION CHROMATOGRAPHY OF PROTEINS ON TSKGEL PHENYL-5PW

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(Received April 16th, 1984)

SUMMARY

The effect of operational variables on retention, recovery and resolution was investigated in hydrophobic interaction chromatography of proteins on TSKgel Phenyl-5PW with a linear gradient comprising a decreasing salt concentration. Sodium sulphate was more effective in retaining proteins than ammonium sulphate or potassium phosphate. The effect of the eluent pH on retention was not as great as reported on other supports. The addition of an organic solvent or chaotropic agent promoted the desorption of proteins. High recovery was observed in most separations where the flow-rate, gradient time, initial ammonium sulphate concentration, isopropanol concentration in the final buffer and eluent pH were varied. The resolution was affected to different extents by the column length, flow-rate, gradient time and addition of organic solvent or chaotropic agent in the final buffer.

INTRODUCTION

Hydrophobic interaction chromatography has become increasingly popular for the separation and purification of proteins. Although it was originally a slow technique, rapid separations have become possible by the introduction of a new support, TSKgel Phenyl-5PW (Toyo Soda, Tokyo, Japan). It has already been shown that proteins can be separated with high resolution without denaturation on this support[1]. However, since it is very important to understand the effect of the operational variables in order to obtain optimum separations, we have evaluated further the TSKgel Phenyl-5PW with respect to various operational variables.

EXPERIMENTAL

Chromatographic measurements were carried out 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). The proteins were usually separated on a TSKgel Phenyl-5PW column (75 × 7.5 mm I.D.) by a 30- or 60-min linear gradient comprising a decreasing concentration of ammonium

0021-9673/84/$03.00

sulphate, 1.5–2.0 *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 were detected at 280 nm. However, the column, eluent pH, type of salt, initial salt concentration, gradient time and flow-rate were all varied. In addition, an organic solvent or chaotropic agent was added in the final buffer in some experiments.

Lipoxidase was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Crude ferredoxin NADP reductase was a gift from Dr. Shin of Kobe University, Japan. All other proteins were purchased from Sigma (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Retention (adsorption and desorption)

Retention was investigated with different types of salts in the initial buffer, different eluent pH values and addition of different organic solvents or chaotropic agents in the final buffer.

Table I shows the effect of the type of salt. Since strongly anti-chaotropic ions seemed to be effective in retaining proteins on other supports[2,3], ammonium sulphate, sodium sulphate and potassium phosphate were compared. Sodium sulphate was most effective, and ammonium sulphate and potassium phosphate were comparable. Therefore, sodium sulphate seems more suitable than ammonium sulphate, which has commonly been utilized in conventional hydrophobic interaction chromatography. However, because the solubility of sodium sulphate in water is limited (to 1.5 *M* at 25°C) and significantly depends on the temperature, sodium sulphate is not appropriate when high concentrations of salt are required. In such cases, ammonium sulphate would be better. Ammonium sulphate is not suitable at alkaline pH because pH control is difficult owing to loss of ammonia. Even at pH 8, the stability was not high.

Fig. 1 shows the effect of eluent pH. The elution volumes of proteins varied with pH, but only slightly. Although it has been reported on other supports that proteins were more strongly retained at lower pH[3–6], such definite trend was not observed on TSKgel Phenyl-5PW. Accordingly, the optimal pH to retain or desorb

TABLE I

EFFECT OF SALT TYPE ON THE ELUTION VOLUMES OF PROTEINS IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY ON A TSKGEL PHENYL-5PW COLUMN

Proteins were separated at a flow-rate 1 ml/min with a 30-min linear gradient of ammonium sulphate, sodium sulphate or potassium phosphate concentration decreasing from 1.5 to 0 *M* in 0.1 *M* phosphate buffer (pH 7.0).

Protein	*Elution volume (ml)*		
	Ammonium sulphate	*Sodium sulphate*	*Potassium phosphate*
Cytochrome *c*	2.70	5.35	2.55
Myoglobin	8.07	15.18	7.50
Ribonuclease	13.50	18.72	13.41
Lysozyme	20.10	24.99	20.70
α-Chymotrypsinogen	27.18	29.58	26.70
α-Chymotrypsin	30.06	32.28	29.49

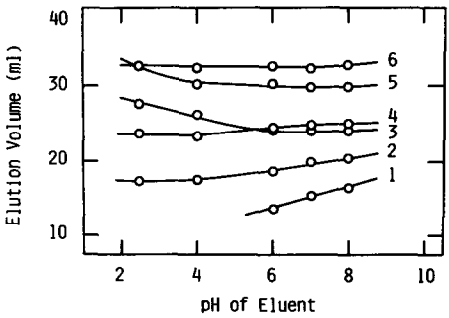

Fig. 1. Dependence of elution volume on the eluent pH in hydrophobic interaction chromatography on a TSKgel Phenyl-5PW column. Proteins were separated at a flow-rate of 1 ml/min with a 30-min linear gradient of ammonium sulphate concentration from 2 *M* to 0. Samples: 1 = myoglobin; 2 = ribonuclease; 3 = ovalbumin; 4 = lysozyme; 5 = α-chymotrypsinogen; 6 = α-chymotrypsin.

proteins cannot be specified. However, it is often possible to change the selectivity for some components in a sample by manipulating the eluent pH. For example, lysozyme and ovalbumin were separated at pH < 4 although they were eluted together at pH > 6. α-Chymotrypsinogen and α-chymotrypsin were separated at pH > 4, but not at pH 2.4.

Table II shows the effect of the type of organic solvent added in the final buffer in low concentration to promote desorption of proteins. Isopropanol was most effective and the elution volumes of proteins were reduced by 10–20% upon addition of 5% isopropanol. Fig. 2 shows the dependence of elution volume on isopropanol concentration in the final buffer. The elution volume decreased continuously with increasing isopropanol concentration up to 7%. However, the elution volumes of myoglobin, ribonuclease and lysozyme slightly increased upon further increasing the isopropanol concentration to 10%. Therefore, addition of larger concentrations of organic solvent seems to promote adsorption rather than desorption. This may be reason why the *n*-butanol was less effective than isopropanol (Table II) at the 5%

TABLE II

EFFECT OF ORGANIC SOLVENT ON THE ELUTION VOLUMES OF PROTEINS IN HYDROPHOBIC INTERACTION CHROMATOGRAPHY ON A TSKGEL PHENYL-5PW COLUMN

Proteins were separated at a flow-rate of 1 ml/min with a 30-min linear gradient from 0.1 *M* phosphate buffer containing 2 *M* ammonium sulphate (pH 7.0) to 0.1 *M* phosphate buffer (pH 7.0) containing 5% organic solvent.

Protein	*Elution volume (ml)*					
	Buffer	*Methanol*	*Ethanol*	*Isopropanol*	*n-Butanol*	*Acetonitrile*
Cytochrome *c*	7.95	8.16	8.10	5.73	8.25	8.43
Myoglobin	15.54	15.15	14.55	11.85	*	15.15
Ribonuclease	19.80	18.90	18.15	14.70	18.06	18.84
Lysozyme	24.69	23.10	21.90	18.18	22.35	22.50
α-Chymotrypsinogen	29.67	28.80	27.90	25.14	26.40	28.50
α-Chymotrypsin	32.13	30.15	27.90	25.14	28.95	28.50

* Myoglobin was not eluted.

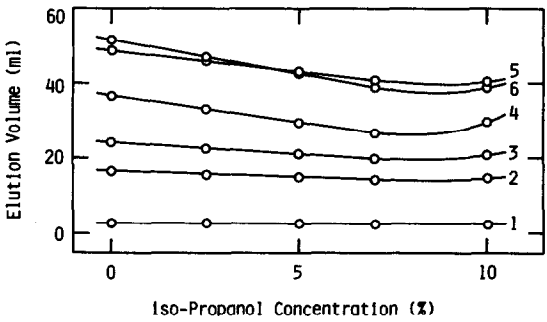

Fig. 2. Dependence of elution volume on the concentration of isopropanol in the final buffer in hydrophobic interaction chromatography on a TSKgel Phenyl-5PW column. Proteins were separated at a flow-rate of 1 ml/min with a 60-min linear gradient from 0.1 *M* phosphate buffer containing 1.8 *M* ammonium sulphate (pH 7.0) to 0.1 *M* phosphate buffer (pH 7.0) containing 0, 2.5, 5, 7 or 10% isopropanol. Samples: 1 = cytochrome *c*; 2 = myoglobin; 3 = ribonuclease; 4 = lysozyme; 5 = α-chimotrypsinogen; 6 = α-chimotrypsin.

level. Fig. 2 also indicates that the elution volumes of different proteins are affected to different extents by the addition of organic solvent. This means that the selectivity varies with the concentration of organic solvent. Therefore, it should be possible to improve the separation of a sample or some components in a sample by adjusting the concentration of organic solvent.

Fig. 3 shows the effect of chaotropic agents in desorbing proteins. 2 *M* Urea and 1 *M* guanidine hydrochloride had similar effects to 5% isopropanol, although they resulted in slightly different elution patterns. Ferrodoxin NADP reductase activity was recovered in more than 90% yield in all separations in Fig. 3. Accordingly,

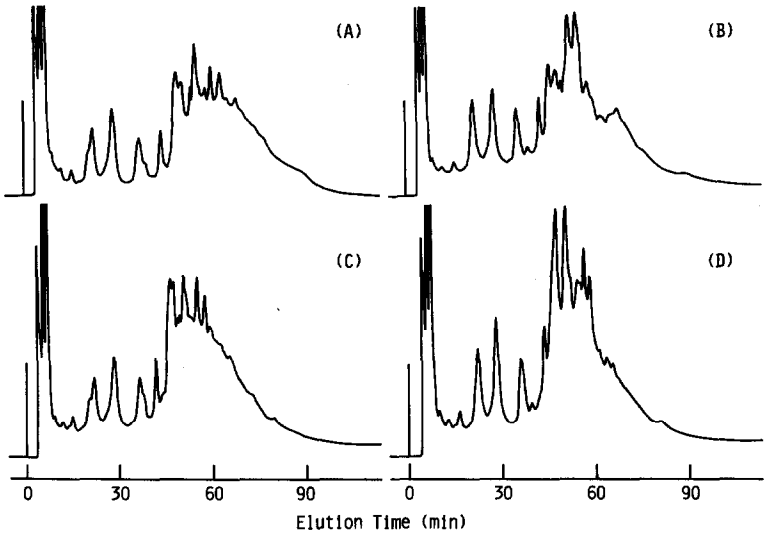

Fig. 3. Chromatograms of crude ferredoxin NADP reductase obtained on a TSKgel Phenyl-5PW column at a flow-rate of 0.5 ml/min with a 60-min linear gradient from 0.1 *M* phosphate buffer containing 1.5 *M* ammonium sulphate (pH 7.0) to 0.1 *M* phosphate buffer (pH 7.0) (A) or 0.1 *M* phosphate buffer (pH 7.0) containing 5% isopropanol (B), 2 *M* urea (C) or 1 *M* guanidine hydrochloride (D).

TABLE III

RECOVERY OF PROTEINS FROM A TSKGEL PHENYL-5PW COLUMN AT VARIOUS FLOW-RATES

Proteins (each 0.5 mg) were adsorbed in 0.1 *M* phosphate buffer containing 1.8 *M* ammonium sulphate (pH 7.0) and desorbed with a 60-min linear gradient of ammonium sulphate concentration decreasing to 0 at a flow-rate of 0.25, 0.5 or 1 ml/min. Column effluents of 20 ml containing all components in the samples were collected and the protein contents were determined by spectrophotometry at 225 nm. The same method was employed also in Tables IV–VII.

Protein	*Recovery (%)*		
	0.25 ml/min	*0.5 ml/min*	*1.0 ml/min*
Cytochrome *c*	98	98	96
Myoglobin	97	78	66
Ribonuclease	95	95	92
Lysozyme	101	104	97
Ovalbumin	97	96	88
α-Chymotrypsinogen	94	92	89
Trypsin inhibitor	95	97	89
α-Chymotrypsin	94	88	78

the addition of small quantities of organic solvent or chaotropic agent in the eluent could be expected to promote desorption or improve separation without causing denaturation of proteins.

Recovery

Recovery was investigated under various flow-rates, gradient times, initial ammonium sulphate concentrations, concentrations of isopropanol in the final buffer and eluent pH values. The results are summarized in Tables III–VII. Very high recoveries were observed in most cases. However, the recoveries of myoglobin and α-

TABLE IV

RECOVERY OF PROTEINS FROM A TSKGEL PHENYL-5PW COLUMN WITH VARIOUS GRADIENT TIMES

Proteins (each 0.5 mg) were adsorbed in 0.1 *M* phosphate buffer containing 1.8 *M* ammonium sulphate (pH 7.0) and desorbed with a 1-, 30- or 60-min linear gradient of ammonium sulphate concentration decreasing to 0 at a flow-rate of 1 ml/min.

Protein	*Recovery (%)*		
	1 min	*30 min*	*60 min*
Cytochrome *c*	101	96	96
Myoglobin	101	84	66
Ribonuclease	104	98	92
Lysozyme	92	95	97
Ovalbumin	110	106	88
α-Chymotrypsinogen	99	92	89
Trypsin inhibitor	100	95	89
α-Chymotrypsin	109	89	78

TABLE V

RECOVERY OF PROTEINS FROM A TSKGEL PHENYL-5PW COLUMN AT VARIOUS INITIAL AMMONIUM SULPHATE CONCENTRATIONS

Proteins (each 0.5 mg) were adsorbed in 0.1 *M* phosphate buffer containing 0.5, 1.0, 1.8 or 2.5 *M* ammonium sulphate (pH 7.0) and desorbed with a 60-min linear gradient of ammonium sulphate concentration decreasing to 0 at a flow-rate of 1 ml/min.

Protein	*Recovery (%)*			
	0.5 M	*1.0 M*	*1.8 M*	*2.5 M*
Cytochrome *c*	99	100	96	78
Myoglobin	98	95	66	70
Ribonuclease	101	99	92	98
Lysozyme	99	96	97	100
Ovalbumin	97	83	88	86
α-Chymotrypsinogen	93	92	89	101
Trypsin inhibitor	98	72	89	94
α-Chymotrypsin	97	70	78	90

chymotrypsin tended to decrease slightly at higher flow-rates, with longer gradient times and with the addition of more than 7% isopropanol. Furthermore, some proteins were recovered in slightly low yields at certain initial ammonium sulphate concentrations, which seem to correspond to such concentrations as cause intermediate retardation of the proteins. At initial ammonium sulphate concentrations where the proteins were not retained or were retained strongly, the recoveries were very high.

Resolution

Resolution was investigated with respect to the dependence on column length, flow-rate, gradient time, initial ammonium sulphate concentration and addition of organic solvent or chaotropic agent in the final buffer.

TABLE VI

RECOVERY OF PROTEINS FROM A TSKGEL PHENYL-5PW COLUMN AT VARIOUS ISOPROPANOL CONCENTRATIONS IN THE FINAL BUFFER

Proteins (each 0.5 mg) were adsorbed in 0.1 *M* phosphate buffer containing 1.8 *M* ammonium sulphate (pH 7.0) and desorbed with a 60-min linear gradient to 0.1 *M* phosphate buffer (pH 7.0) containing 0, 2, 5, 7 or 10% isopropanol at a flow-rate of 1 ml/min.

Protein	*Recovery (%)*				
	0%	*2%*	*5%*	*7%*	*10%*
Cytochrome *c*	96	101	100	96	97
Myoglobin	66	79	79	40	25
Ribonuclease	92	97	98	100	95
Lysozyme	97	95	102	96	100
Ovalbumin	88	94	92	93	90
α-Chymotrypsinogen	89	105	101	93	90
Trypsin inhibitor	89	93	92	90	90
α-Chymotrypsin	78	91	92	95	84

TABLE VII

RECOVERY OF PROTEINS FROM A TSKGEL PHENYL-5PW COLUMN AT VARIOUS pH VALUES

Proteins (each 0.5 mg) were adsorbed in 0.1 *M* phosphate buffer containing 1.8 *M* ammonium sulphate (pH 5.0, 7.0 or 8.0) and desorbed with a 60-min linear gradient of ammonium sulphate concentration decreasing to 0 at a flow-rate of 1 ml/min.

Protein	*Recovery (%)*		
	pH 5.0	*7.0*	*8.0*
Cytochrome *c*	100	96	95
Myoglobin	—*	66	75
Ribonuclease	95	92	99
Lysozyme	98	97	100
Ovalbumin	88	88	95
α-Chymotrypsinogen	94	89	94
Trypsin inhibitor	90	89	93
α-Chymotrypsin	90	78	86

* Recovery was not determined due to low solubility of myoglobin at pH 5.0.

Fig. 4 shows the dependence of resolution on column length. A slightly higher resolution was attained with a two-column system. This is because the peaks became narrower with longer columns. The two-column system resulted in 10–20% narrower peaks than the one-column system. A three-column system yielded narrower (5–10%) peaks than the two-column system. The separation time increased only slightly with the longer columns.

Figs. 5 and 6 show the effect of flow-rate at constant gradient time. A slightly higher resolution was achieved at lower flow-rates. This flow-rate dependence of resolution was almost negligible at short gradient times and became pronounced as the gradient time increased. The dilution of proteins during separation decreased almost in proportion to the flow-rate, while the separation time increased only slightly with decreasing flow-rate, as is seen from Fig. 7.

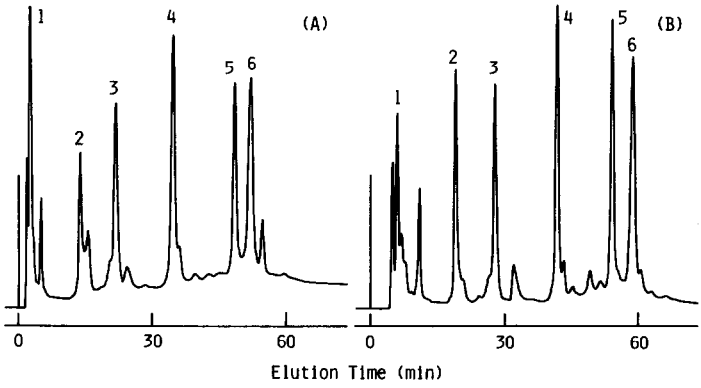

Fig. 4. Chromatograms of a protein mixture obtained on column systems consisting of one (A) or two (B) TSKgel Phenyl-5PW columns at a flow-rate of 1 ml/min with a 60-min linear gradient of ammonium sulphate concentration from 1.7 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). Samples as in Fig. 2.

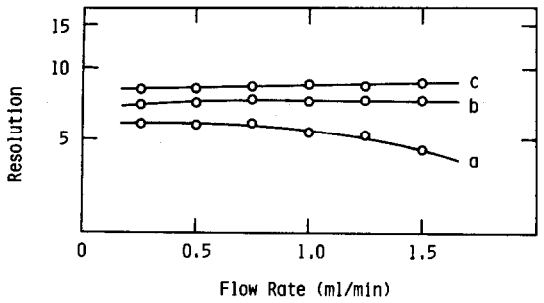

Fig. 5. Dependence of resolution on the flow-rate at constant gradient time (60 min) in hydrophobic interaction chromatography on a TSKgel Phenyl-5PW column. Proteins were separated at flow-rates of 0.25–1.5 ml/min with a 60-min linear gradient of ammonium sulphate concentration from 1.8 *M* to 0. The resolution was calculated from the peak widths and elution volumes of the pairs myoglobin and ribonuclease (a), ribonuclease and lysozyme (b) and lysozyme and α-chymotrypsinogen (c).

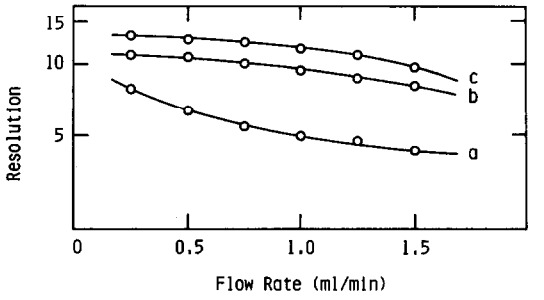

Fig. 6. Dependence of resolution on the flow-rate at constant gradient time (240 min) in hydrophobic interaction chromatography on a TSKgel Phenyl-5PW column. Proteins were separated at flow-rates of 0.25–1.5 ml/min with a 240-min linear gradient of ammonium sulphate concentration from 1.8 *M* to 0. Resolutions as in Fig. 5.

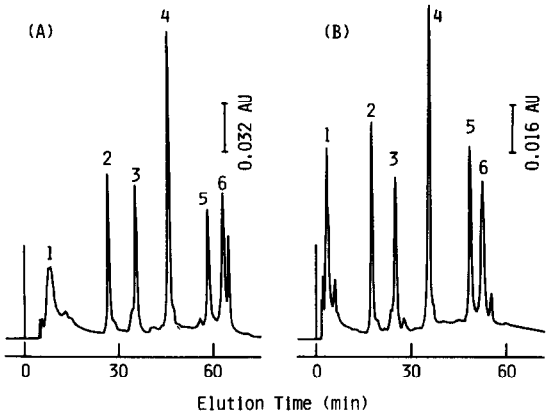

Fig. 7. Chromatograms of a protein mixture obtained on a TSKgel Phenyl-5PW column at a flow-rate of 0.5 ml/min (A) or 1 ml/min (B) with a 60-min linear gradient of ammonium sulphate concentration from 1.8 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). Samples as in Fig. 2.

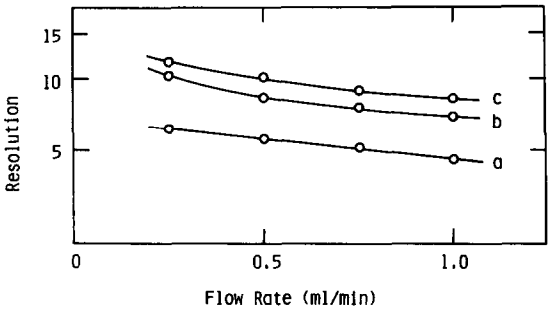

Fig. 8. Dependence of resolution on the flow-rate at constant gradient volume (60 ml) in hydrophobic interaction chromatography on a TSKgel Phenyl-5PW column. Proteins were separated at flow-rates of 0.25–1 ml/min with a linear gradient of ammonium sulphate concentration from 1.8 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). Resolutions as in Fig. 5.

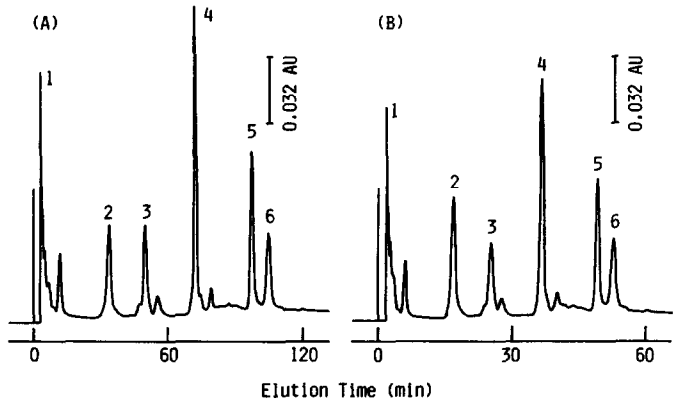

Fig. 9. Chromatograms of a protein mixture obtained on a TSKgel Phenyl-5PW column at a flow-rate of 0.5 ml/min (A) or 1 ml/min (B) with a linear gradient of ammonium sulphate concentration from 1.8 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). The gradient volume was 60 ml in each case. Samples as in Fig. 2.

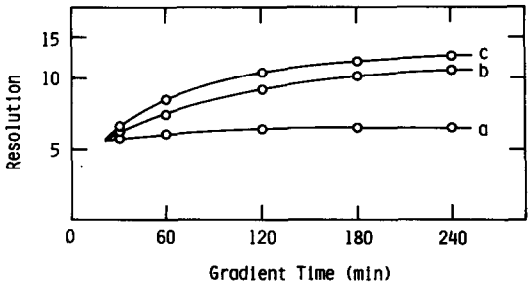

Fig. 10. Dependence of resolution on the gradient time in hydrophobic interaction chromatography on a TSKgel Phenyl-5PW column. Proteins were separated at a flow-rate of 0.5 ml/min with 30–240 min linear gradients of ammonium sulphate concentration from 1.8 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). Resolutions as in Fig. 5.

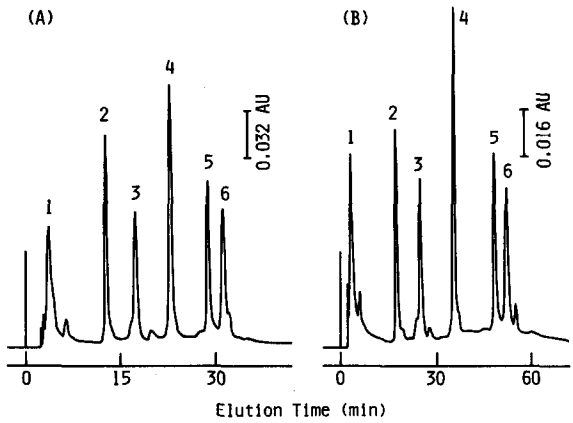

Fig. 11. Chromatograms of a protein mixture obtained on a TSKgel Phenyl-5PW column at a flow-rate of 1 ml/min with a 30-min (A) or 60-min (B) linear gradient of ammonium sulphate concentration from 1.8 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). Samples as in Fig. 2.

Fig. 8 shows the effect of flow-rate at constant gradient volume. The resolution slightly increased with decreasing flow-rate. However, the separation time became longer almost in inverse proportion to the flow-rate, as is seen from Fig. 9. Dilution of the proteins decreased with flow-rate.

Fig. 10 shows the effect of the gradient time on resolution. Higher resolution was attained with increasing gradient time. However, this effect became insignificant at gradient times longer than 120 min. Also, the effect of gradient time tended to be less pronounced at higher flow-rates. The separation time became longer and the dilution of proteins increased almost in proportion to the gradient time, as is seen from Fig. 11.

Figs. 12 and 13 show the effect of the initial ammonium sulphate concentration. The resolution varied with the initial ammonium sulphate concentration al-

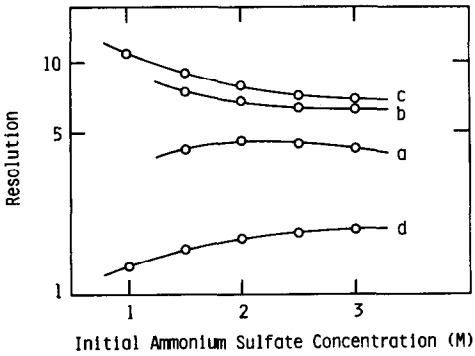

Fig. 12. Dependence of resolution on the initial ammonium sulphate concentration in hydrophobic interaction chromatography on a TSKgel Phenyl-5PW column. Proteins were separated at a flow-rate of 1 ml/min with a 60-min linear gradient of ammonium sulphate concentration from 1–3 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). Resolutions as in Fig. 5; d is for the pair α-chymotrypsinogen and α-chymotrypsin.

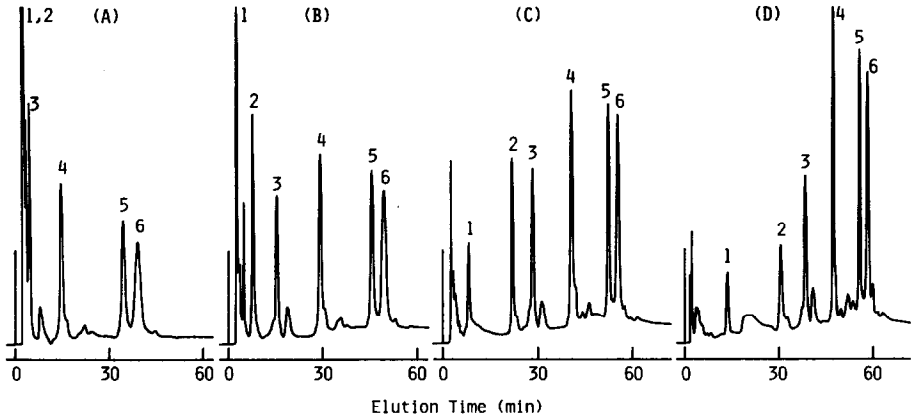

Fig. 13. Chromatograms of a protein mixture obtained on a TSKgel Phenyl-5PW column at a flow-rate of 1 ml/min with a 60-min linear gradient of ammonium sulphate concentration from 1.0 *M* (A), 1.5 *M* (B), 2.0 *M* (C) and 2.5 *M* (D) to 0 in 0.1 *M* phosphate buffer (pH 7.0). Samples as in Fig. 2.

though there was no definite trend. Therefore, it can be concluded that the optimum initial ammonium sulphate concentration depends on the sample. When proteins were separated at different initial ammonium sulphate concentrations but with a constant gradient of decreasing ammonium sulphate concentration (1 *M* in 30 min), only a parallel shift of the whole chromatogram was observed except for components eluting very early (Fig. 14).

Fig. 15 shows the effect of addition of a chaotropic agent in the final buffer. Peaks eluted at around 50 min were better separated upon addition of 2 *M* urea in the final buffer because they became narrower. The addition of an organic solvent in the final buffer was also effective in obtaining narrower peaks (Fig. 16). Therefore, the addition of organic solvents or chaotropic agents in the final buffer can be expected to improve separations. However, since such additions sometimes results in changes in relative elution positions, inferior separations for some samples or some components in a sample may be obtained.

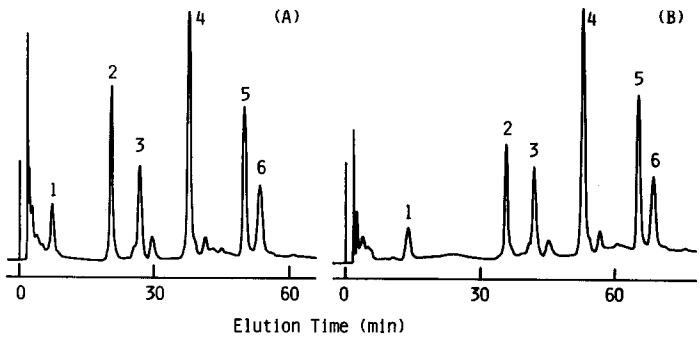

Fig. 14. Chromatograms of a protein mixture obtained on a TSKgel Phenyl-5PW[1]column at a flow-rate of 1 ml/min with a 60-min (A) or 75-min (B) linear gradient of ammonium sulphate concentration from 2 *M* (A) or 2.5 *M* (B) to 0 in 0.1 *M* phosphate buffer (pH 7.0). Samples as in Fig. 2.

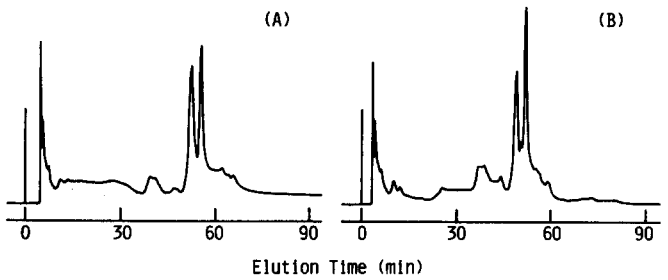

Fig. 15. Chromatograms of commercial lipoxidase obtained on a TSkgel Phenyl-5PW column at a flow-rate of 0.5 ml/min with a 60-min linear gradient from 0.1 *M* phosphate buffer containing 1.5 *M* ammonium sulphate (pH 7.0) to 0.1 *M* phosphate buffer (pH 7.0) (A) or to 0.1 *M* phosphate buffer containing 2 *M* urea (pH 7.0) (B).

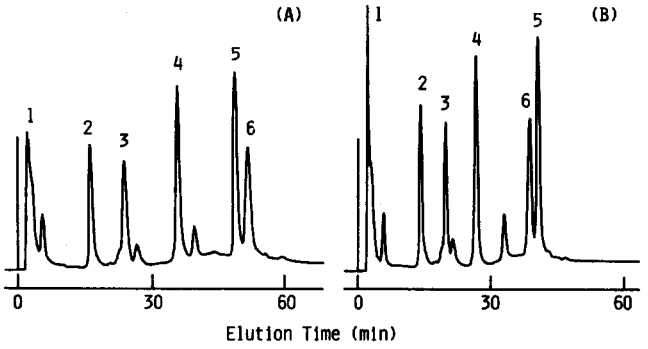

Fig. 16. Chromatograms of a protein mixture obtained on a TSKgel Phenyl-5PW column at a flow-rate of 1 ml/min with a 60-min linear gradient from 0.1 *M* phosphate buffer containing 1.8 *M* ammonium sulphate (pH 7.0) to 0.1 *M* phosphate buffer (pH 7.0) (A) or to 0.1 *M* phosphate buffer (pH 7.0) containing 7% isopropanol (B). Samples as in Fig. 2.

According to the described results, higher resolution can be obtained by using longer columns, lower flow-rates, longer gradient times, properly adjusting the initial salt concentration and adding an organic solvent or chaotropic agent in small quantities in the final buffer, although the effect of all these variables is small.

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