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RESIN-BASED SUPPORT FOR REVERSED-PHASE CHROMATOGRAPHY OF PROTEINS

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SUMMARY

A new hydrophilic resin-based support for reversed-phase chromatography, TSKgel Phenyl-5PW RP, was found to be very useful for separating a wide range of proteins. Proteins can be separated under similar elution conditions to those employed on silica-based supports. The new support has advantages such as high chemical stability and a large pore size compared with commercially available silica-based supports.

INTRODUCTION

The reversed-phase chromatography of proteins has advanced considerably in recent years as a result of extensive studies of mobile[1–23] and stationary phases[2,4,6,8,12,14,16–18,24–38], and, rapid separations with high resolution and recovery have become possible by using chemically bonded silica with a large pore size. However, it is usually necessary to employ eluents of low pH (around 2) in order to obtain satisfactory results, although the acidic conditions are harmful to chemically bonded silica. This is a major disadvantage in the current reversed-phase chromatography of proteins.

One solution of this problem is to use chemically stable resin-based supports. It has been reported that low-molecular-weight proteins were successfully separated on porous polystyrene resins[39,40]. However, a decrease in recovery with increasing protein molecular weight[40] and a low resolution in comparison with chemically bonded silica[38] have been found. Therefore, it is likely that there are limitations to the use of polystyrene resins.

As an alternative, a hydrophilic resin-based support has become commercially available recently under the trade-name TSKgel Phenyl-5PW RP (Toyo Soda, Tokyo, Japan). According to the manufacturer, this new support was developed by introducing phenyl groups with an ether linkage into TSKgel G5000PW[41], which is a hydrophilic resin-based material of large pore size (particle diameter 10 μm) for high-performance gel filtration, and has the schematic structure shown in Fig. 1. The surface is covered with many phenyl groups, some hydroxy groups and very small amounts of carboxyl groups (a few microequivalents per millilitre of support). We have evaluated this new support and the results are described in this paper.

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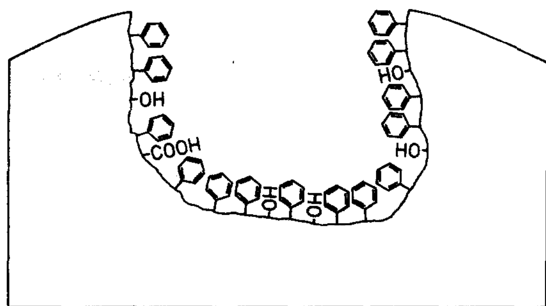

Fig. 1. Schematic structure of TSKgel Phenyl-5PW RP.

EXPERIMENTAL

All chromatographic measurements were performed on 75 × 4.6 mm I.D. stainless-steel columns with a Model SP8700 high-performance liquid chromatograph (Spectra-Physics, San Jose, CA, U.S.A.) equipped with a Model UV-8 variable-wavelength UV detector (Toyo Soda). The proteins were separated using a 60-min linear gradient of acetonitrile from 5 to 80% in 0.05% trifluoroacetic acid (TFA) at a flow-rate of 1 ml/min at 25°C and detected at 220 nm, unless stated otherwise. In some separations the gradient time, gradient shape, flow-rate, TFA concentration, acid component and organic modifier were varied.

All proteins were purchased from Sigma (St. Louis, MO, U.S.A.), except crude human growth hormone which was a gift from Mr. Okamura of our laboratory. Peptides were obtained from the Peptide Institute (Osaka, Japan).

The chemical stability was evaluated by treating the columns with 0.5 *N* sodium hydroxide solution (pH 13.5) or 20% acetic acid (pH 2.0) for 2 weeks at a flow-rate of 0.2 ml/min and comparing the chromatograms of a protein mixture obtained on these columns and a new column.

The pore size was evaluated by electron microscopy with a Model JSM-50A scanning electron microscope (Japan Electron Optics Lab., Tokyo, Japan).

The recovery of proteins was determined from the areas of the eluted peaks. As controls, we used the peak areas obtained when the column was replaced with a 1 mm I.D. empty stainless-steel tube of 1 ml total inner volume and the elution was performed isocratically at the eluent compositions when each protein eluted in gradient elution with a column. Amounts of 20 μg of each protein were injected. The column was washed between each injection of protein with 0.2 *N* sodium hydroxide solution–acetonitrile (40:60, v/v). This mixture is very effective for washing columns, as is shown later. The peak areas were measured with a Model CP-8000 data processor (Toyo Soda).

A 75 × 4.6 mm I.D. TSKgel TMS-250 column packed with trimethylsilyl-bonded silica of average pore diameter 250 Å and particle diameter 10 μm was used for comparison.

RESULTS AND DISCUSSION

Chemical stability

Fig. 2 shows chromatograms of a protein mixture obtained on the new column

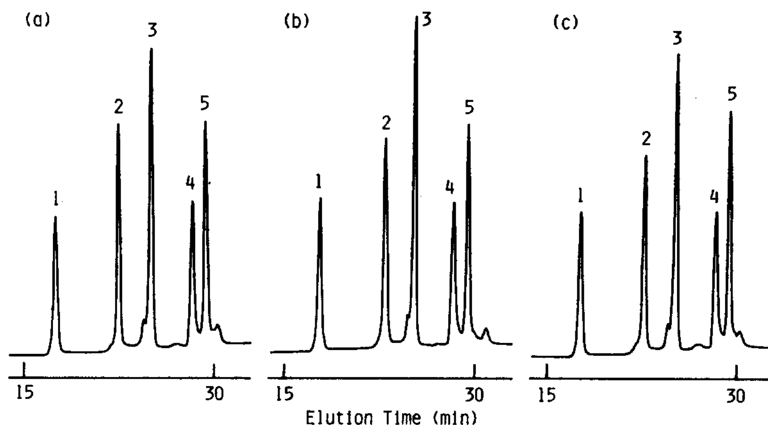

Fig. 2. Chromatograms of a protein mixture obtained on TSKgel Phenyl-5PW RP columns before and after pre-treatment with 0.5 *N* sodium hydroxide solution and 20% acetic acid for 2 weeks at a flow-rate of 0.2 ml/min. Columns: (a) new column; (b) column after pretreatment with 0.5 *N* sodium hydroxide solution; (c) column after pre-treatment with 20% acetic acid. Samples: 1 = ribonuclease; 2 = cytochrome *c*; 3 = lysozyme; 4 = bovine serum albumin; 5 = myoglobin (10 μg each).

and columns pre-treated with 0.5 *N* sodium hydroxide solution or 20% acetic acid. Almost identical chromatograms were observed on the three columns, suggesting that no change in the support characteristics occurred during runs with these alkaline and acidic solutions. Hence the new resin-based support, TSKgel Phenyl-5PW RP, seems to be chemically very stable.

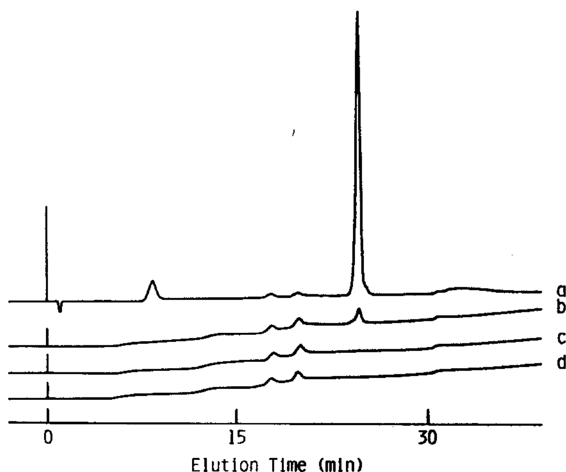

Fig. 3. Column washing procedure for Phenyl-5PW RP. (a) Chromatogram of ovalbumin (50 μg) obtained with a 2-min linear gradient of acetonitrile from 5 to 20% followed by a 32-min linear gradient from 20 to 60% in 0.05% TFA at a flow-rate of 1 ml/min. (b) Chromatogram observed in a blank run with the same gradient after separation (a). A ghost peak is seen at the elution position of ovalbumin. (c) Chromatogram observed in a blank gradient run after separation (a) and washing the column with 0.2 *N* sodium hydroxide solution–acetonitrile (40:60, v/v). The ghost peak is not seen in this instance. (d) Baseline of the gradient elution before separation (a).

In the reversed-phase chromatography of proteins, ghost peaks often appear in successive runs[12]. Because these ghost peaks make it complicated to understand the chromatograms it is highly desirable to eliminate them. However, complete elimination of ghost peaks is time consuming when silica-based supports are used because several blank gradient runs are usually necessary. On the other hand, it is simple with Phenyl-5PW RP owing to its chemical stability. Ghost peaks can be eliminated by washing the column with 0.2 N sodium hydroxide solution–acetonitrile (40:60, v/v), as shown in Fig. 3. The washing was conducted by injecting 100 μl of the mixture five times successively in the final eluent just before re-equilibrating the column with the initial eluent; it took only a few minutes.

Pore size

Fig. 4 shows scanning electron photomicrographs of Phenyl-5PW RP and TMS-250. The bright portion is the resin matrix and dark portion represents pores. Therefore, the pore size of Phenyl-5PW RP can be said to be a few thousand ångstroms, which is much larger than those of commercially available silica-based supports for protein separations (250–330 Å).

Retention and resolution of proteins

Elution volumes of proteins are summarized in Table I, where data obtained on TMS-250 are also included. The elution volumes of proteins on Phenyl-5PW RP and TMS-250 were almost identical. However, there is a tendency for proteins to elute slightly earlier on Phenyl-5PW RP than on TMS-250, so Phenyl-5PW RP is probably slightly less hydrophobic than chemically bonded alkyl silica. The selectivities of Phenyl-5PW RP and TMS-250 were also very similar, although they differed slightly for some pairs of proteins.

The peak widths of proteins are also summarized in Table I. Almost identical peak widths were observed for small proteins on Phenyl-5PW RP and TMS-250, but Phenyl-5PW RP provided narrower peaks than TMS-250 for proteins with relatively

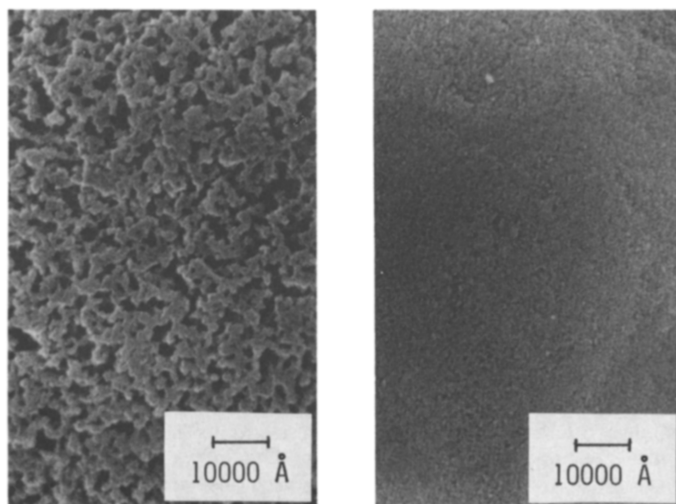

Fig. 4. Scanning electron photomicrographs of Phenyl-5PW RP (left) and TMS-250 (right).

TABLE I

ELUTION VOLUMES AND PEAK WIDTHS OF PROTEINS OBSERVED ON PHENYL-5PW RP AND TMS-250

Protein	*Molecular weight*	*Elution volume (ml)*		*Peak width (ml)*	
		Phenyl-5PW RP	*TMS-250*	*Phenyl-5PW RP*	*TMS-250*
Cytochrome *c*	12 400	22.9	23.4	0.43	0.42
Ribonuclease	13 700	18.1	19.1	0.50	0.43
Lysozyme	14 000	25.5	25.7	0.49	0.48
α-Lactoalbumin	16 000	27.2	27.8	0.39	0.38
Myoglobin	16 900	29.0	29.9	0.46	0.46
Trypsin	23 000	25.6	26.4	0.48	0.42
Trypsinogen	24 000	25.3	26.3	0.56	0.49
Carbonic anhydrase	30 000	29.9	30.9	0.51	0.55
Ovalbumin	43 000	34.6	35.1	0.54	0.63
Bovine serum albumin	67 000	28.0	27.8	0.48	0.66
Conalbumin	70 000	28.2	28.0	0.48	0.86
Transferrin	80 000	27.1	27.0	0.37	0.52
Phosphorylase B	94 000	35.6	35.1	0.71	0.98
Aldolase	158 000	28.3	29.5	0.60	0.73

high molecular weights (>40 000 daltons). Although the peak width tended to increase with increasing protein molecular weight on both supports, the increase was not so significant with Phenyl-5PW RP. Accordingly, it is believed that high resolution can be achieved even for proteins with very high molecular weights on Phenyl-5PW RP.

TABLE II

RECOVERY OF PROTEINS FROM PHENYL-5PW RP

(A) Recovery observed with a 60-min linear gradient of acetonitrile from 5 to 80% in 0.05% TFA at a flow-rate of 1 ml/min; (B) recovery observed as in (A) except the gradient time was 30 min; (C) recovery observed as in (A) except the TFA concentration was 0.02%.

Protein	*Recovery (%)*		
	A	*B*	*C*
Cytochrome *c*	82	86	89
Ribonuclease	94		
Lysozyme	87		
α-Lactoalbumin	84		
Myoglobin	84		
Trypsin	84		
Trypsinogen	78	84	90
Carbonic anhydrase	83	88	90
Ovalbumin	72	88	89
Bovine serum albumin	93		
Conalbumin	93		
Transferrin	89		
Phosphorylase B	78	98	89
Aldolase	86		

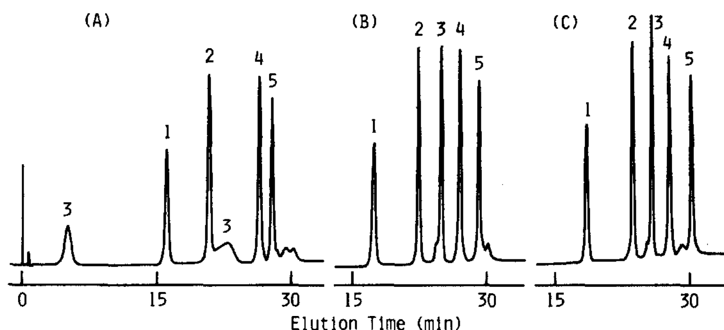

Fig. 5. Effect of TFA concentration on the separation of small proteins on Phenyl-5PW RP. A protein mixture was separated under standard conditions except the TFA concentration was (A) 0.02%, (B) 0.05% or (C) 0.1%. Samples: 1 = ribonuclease; 2 = cytochrome *c*; 3 = lysozyme; 4 = α-lactoalbumin; 5 = myoglobin (10 μg each).

Recovery of proteins

The recovery of proteins is summarized in Table II. Under standard elution conditions (A in Table II), most of the proteins were recovered in high yield (above 80%), although slightly lower recoveries (70–80%) were observed for trypsinogen, ovalbumin and phosphorylase B. These recoveries are comparable to those reported on silica-based supports of large pore size[6,13,16–18,23,25,26,28,30,42]. Furthermore, higher recoveries could be attained by manipulating the elution conditions. An increase in the gradient from 1.25 to 2.5% acetonitrile/min resulted in higher recoveries (B in Table II). Higher recoveries were also observed on decreasing the TFA concentration from 0.05 to 0.02% (C in Table II). Similar effects of variations of gradient and acid concentration have also been observed on silica-based supports[12,17,18,28]. It must be borne in mind, however, that the elution conditions influence not only recovery but also resolution, separation time, etc., as demonstrated in the next section.

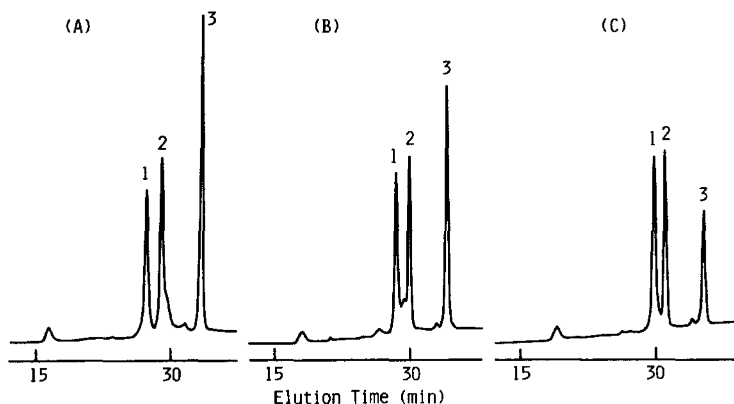

Fig. 6. Effect of TFA concentration on the separation of relatively large proteins on Phenyl-5PW RP. A protein mixture was separated as in Fig. 5. Samples: 1 = aldolase (30 μg); 2 = carbonic anhydrase (5 μg); 3 = ovalbumin (15 μg).

TABLE III

SUITABLE COMPROMISE CONCENTRATIONS OF VARIOUS ACIDS IN PROTEIN SEPARATIONS ON PHENYL-5PW RP

Acid	*Suitable compromise concentration*
TFA	0.05%
HFBA	0.05%
Perchloric acid	5 m*M*
Phosphoric acid	100 m*M*
Methanesulphonic acid	0.05%

Elution conditions in protein separation

Although the effects of elution conditions have already been studied extensively on silica-based supports, we examined the effects of some conditions on Phenyl-5PW RP because these supports differ considerably in both chemical and physical properties.

Fig. 5 shows the effect of TFA concentration on the separation of small proteins. Although proteins were more retained at higher TFA concentrations, the elution patterns were very similar at TFA concentrations of 0.05 and 0.1%. However, at a TFA concentration of 0.02%, lysozyme eluted as two broad peaks and the peak widths or other proteins became slightly greater. It is believed that partial denaturation of proteins is responsible for this behaviour, according to studies of protein denaturation during reversed-phase chromatography by Karger and co-workers[21,43,44]. Fig. 6 shows the effect of TFA concentration on the separation of relatively large proteins. In this instance, the recovery of ovalbumin was considerably affected by TFA concentration. The ovalbumin peak became much smaller when the TFA concentration increased from 0.02 to 0.1%. Consequently, both too low and too high TFA concentrations do not seem to be a good choice for obtaining high resolutions and high recoveries in general. A good compromise seems to be about 0.05%, or a gradient of decreasing TFA concentration from 0.1% in acetonitrile–water (5:95) to 0.02% in acetonitrile–water (60:40) may be better. Similar effects of

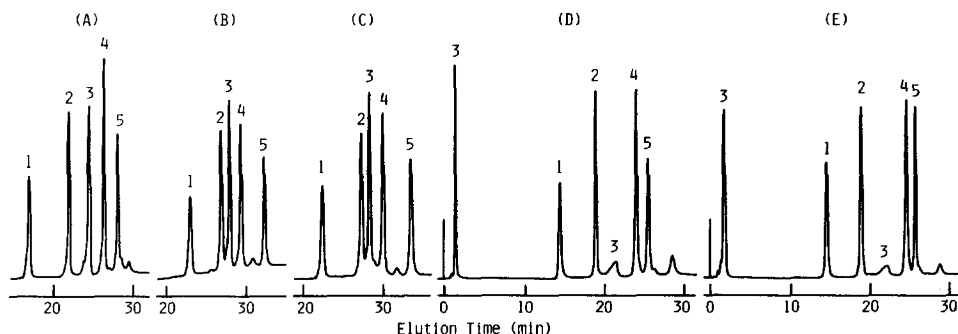

Fig. 7. Effect of type of acid component on the separation of small proteins on Phenyl-5PW RP. A protein mixture was separated under standard conditions except the acid component was (A) 0.05% TFA, (B) 0.05% HFBA, (C) 5 m*M* perchloric acid, (D) 100 m*M* phosphoric acid or (E) 0.05% methanesulphonic acid. Samples as in Fig. 5.

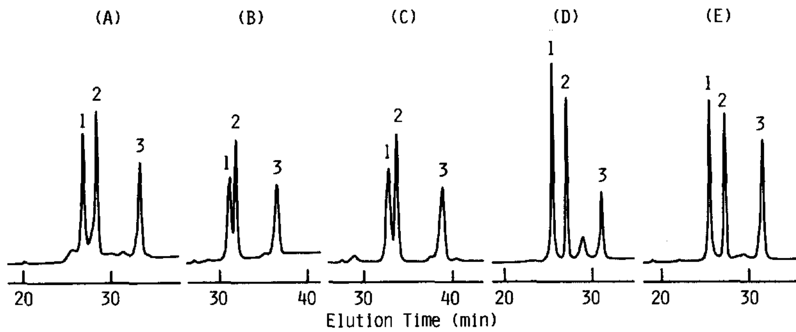

Fig. 8. Effect of type of acid component on the separation of relatively large proteins on Phenyl-5PW RP. A protein mixture was separated as in Fig. 7. Samples as in Fig. 6.

acid concentration to those observed here have been reported on silica-based supports by Cohen *et al.*[17], although salt was included in their eluents.

Heptafluorobutyric acid (HFBA), perchloric acid, phosphoric acid and methanesulphonic acid were also examined as an acid component of the eluent. All these acids had similar concentration effects to those observed with TFA and suitable compromise concentrations are summarized in Table III. With phosphoric acid, the recovery of ovalbumin decreased considerably at concentrations above 50 m*M*, whereas the peak width of myoglobin increased at concentrations below 150 m*M*. Therefore, it is difficult to specify a suitable compromise concentration, although it is given as 100 m*M* in Table III. In addition, the elution volumes of proteins were almost independent of the concentrations of phosphoric acid and methanesulphonic acid. The concentration of methanesulphonic acid also had little influence on the recovery of ovalbumin and the peak sharpness of small proteins. Figs. 7 and 8 show the chromatograms of protein mixtures obtained with the five acids at suitable compromise concentrations. It may be concluded from these results that TFA, which has most commonly been employed in the reversed-phase chromatography of proteins, is not inferior to other acids. However, more hydrophilic acids such as phos-

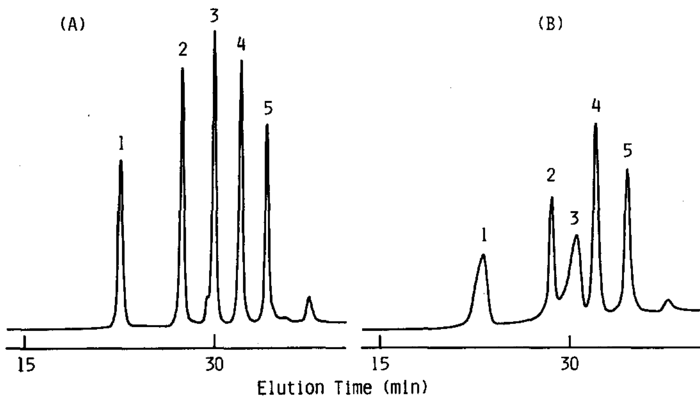

Fig. 9. Effect of type of organic modifier on the separation of proteins on Phenyl-5PW RP. A protein mixture was separated with a 60-min linear gradient of (A) acetonitrile or (B) 2-propanol from 5 to 80% in 0.05% TFA at a flow-rate of 0.5 ml/min. Samples as in Fig. 5.

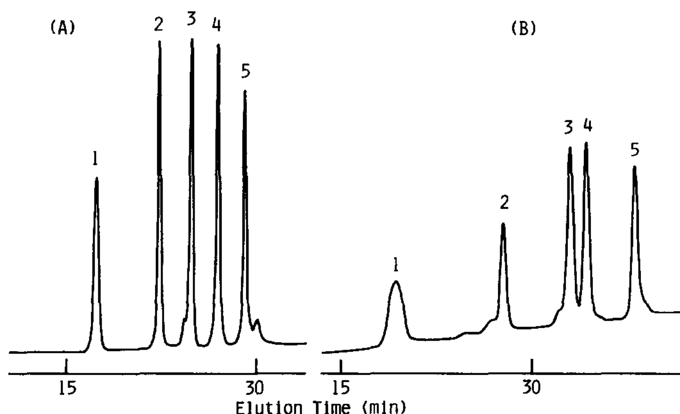

Fig. 10. Effect of type of organic modifier on the separation of proteins on Phenyl-5PW RP. A protein mixture was separated with a 60-min linear gradient of (A) acetonitrile or (B) methanol from 5 to 80% in 0.05% TFA at a flow-rate of 1 ml/min. Samples as in Fig. 5.

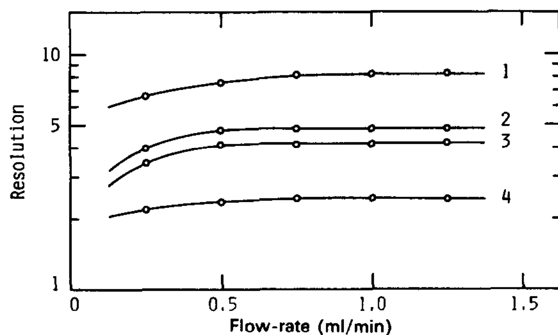

Fig. 11. Effect of flow-rate on resolution in the separation of proteins on Phenyl-5PW RP. Proteins were separated under standard conditions except the flow-rate was varied between 0.25 and 1.25 ml/min. The resolution was calculated from the peak widths and elution volumes of pairs of (1) ribonuclease and cytochrome *c*, (2) cytochrome *c* and lysozyme, (3) lysozyme and α-lactoalbumin and (4) aldolase and carbonic anhydrase.

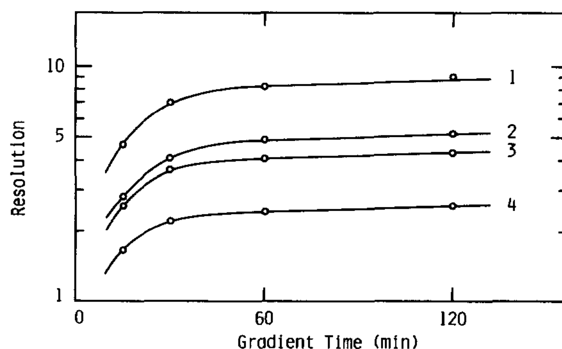

Fig. 12. Effect of gradient time on resolution in the separation of proteins on Phenyl-5PW RP. Proteins were separated under standard conditions except the gradient time was varied between 15 and 120 min. Resolutions as in Fig. 11.

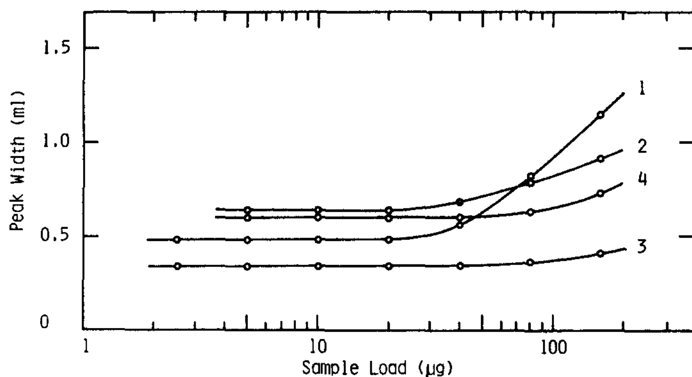

Fig. 13. Effect of sample load on peak width in the separation of proteins on Phenyl-5PW RP. Each protein was separated individually with an injection volume of 20 μl and varying sample concentrations from 0.0125 to 0.8%. Elution conditions as in Fig. 3. Samples: 1 = lysozyme; 2 = ovalbumin; 3 = transferrin; 4 = aldolase.

phoric and methanesulphonic acid may be superior to TFA in some instances, *e.g.*, when protein separation without denaturation is desired. Most of the applied lysozymes were eluted at the void volume and only a small portion of the lysozyme was retained when these hydrophilic acids were employed at concentrations of below 200 m*M* phosphoric acid or below 0.05% methanesulphonic acid. The first peak must represent native lysozyme and the second peak denatured lysozyme[21].

Fig. 9 shows the effect of the type of organic modifier. The retention of proteins did not differ significantly with acetonitrile and 2-propanol on Phenyl-5PW RP, although 2-propanol was more effective than acetonitrile in eluting proteins on silica-based supports[6,13]. On the other hand, the peak sharpness differed appreciably. 2-Propanol gave broader peaks, especially for ribonuclease and lysozyme. It is believed that partial denaturation of the proteins is again responsible for the broad peaks with 2-propanol, at least in part, because the extent of denaturation tends to be reduced with the use of 2-propanol than acetonitrile[21]. Another comparison of organic modifiers is shown in Fig. 10, indicating that acetonitrile is superior to methanol. Therefore, acetonitrile seems to be a good choice for obtaining high resolution.

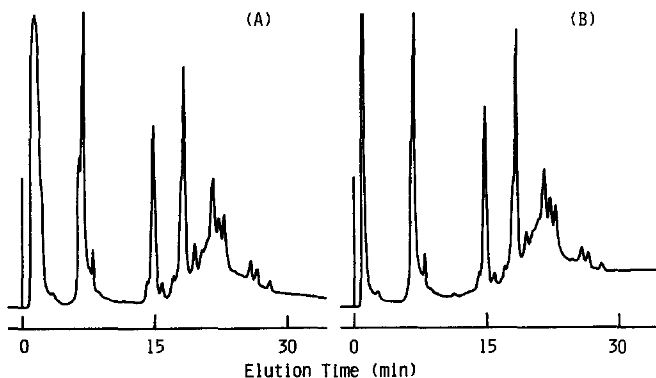

Fig. 14. Chromatograms of crude urease obtained on Phenyl-5PW RP with sample loads of (A) 1 mg and (B) 0.1 mg. Elution conditions as in Fig. 3.

Fig. 11 shows the effect of flow-rate on resolution with a constant gradient time. The resolution increased with increasing flow-rate up to 0.5 ml/min, then became almost constant. Because an increase in flow-rate results in dilution of the sample during separation, too high flow-rates are disadvantageous. Flow-rates of 0.5–1 ml/min seem to be a good choice. A similar effect of flow-rate on resolution was also observed when using 2-propanol instead of acetonitrile, although it was less pronounced.

Fig. 12 shows the effect of gradient time on resolution at constant flow-rate. Higher resolution was obtained with longer gradient times, although the effect of the gradient time decreased at gradient times longer dan 30 min. Consequently, there is no advantage in employing extremely long gradient times (longer than 60 min, corresponding to a gradient of less than 1.25% acetonitrile/min). Longer gradient times result in longer separation times, greater dilution of the sample and lower recoveries of some proteins.

Fig. 13 shows the effect of sample load on peak width. The peak width remained constant at sample loads up to 20–100 μg, depending on the sample, and then increased with further increasing sample load. Accordingly, the maximum sample load that should be used in order to obtain the highest resolution is 20–100 μg. However, this amount is for pure samples, and larger amounts can be applied without any decrease in resolution for crude samples. For example, the same separations were achieved with crude urease with sample loads of up to 1 mg, as shown in Fig. 14. If a slight decrease in resolution is acceptable, larger amounts can be applied. Similar loading capacities have been reported for silica-based supports of large pore size[8,10,18,23,30,42,45,46]. In addition, almost the same results as in Fig. 13 were obtained on TMS-250.

As shown above, the effects of the operational variables in protein separations on Phenyl-5PW RP were usually similar to those observed on silica-based supports.

Applications to the separation of proteins

Fig. 15 shows the separation of crude human growth hormone. This hydrophobic protein could be separated well and almost no ghost peaks appeared in the

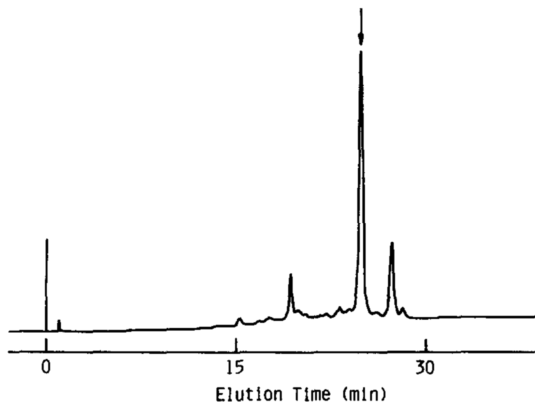

Fig. 15. Separation of crude human growth hormone on Phenyl-5PW RP. Elution conditions as in Fig. 3. The peak arrowed is human growth hormone according to identification from the elution position of a pure sample.

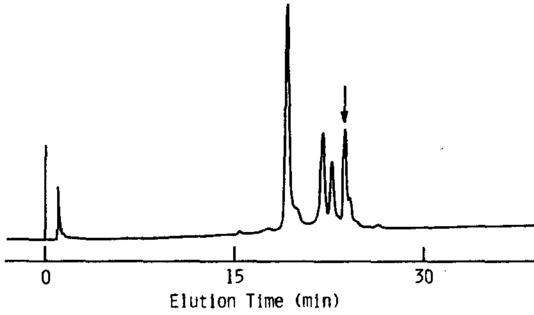

Fig. 16. Separation of crude lactate dehydrogenase on Phenyl-5PW RP. Elution conditions as in Fig. 3. The peak arrowed is lactate dehydrogenase according to identification from the elution position of a pure sample.

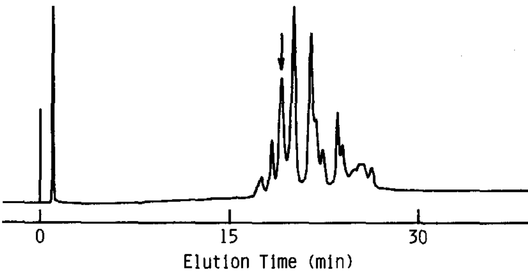

Fig. 17. Separation of crude phosphoglucase isomerase on Phenyl-5PW RP. Elution conditions as in Fig. 3. The peak arrowed is phosphoglucose isomerase according to identification from the elution position of a pure sample.

successive blank gradient run. Examples of separations of large proteins are shown in Figs. 16 and 17. Crude lactate dehydrogenase and phosphoglucose isomerase were separated. Although their molecular weights are approximately 120 000 daltons, they were eluted as sharp peaks. This must be due to the large pore size of Phenyl-5PW RP. A comparison of Phenyl-5PW RP with TMS-250 is shown in Fig. 18. Collagen

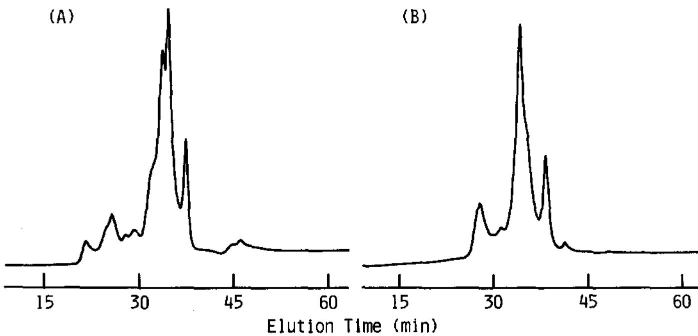

Fig. 18. Separation of collagen (Type VI from human placenta) on (A) Phenyl-5PW RP and (B) TMS-250. Elution was conducted with a 120-min linear gradient of acetonitrile from 5 to 80% in 0.05% TFA at a flow-rate of 1 ml/min.

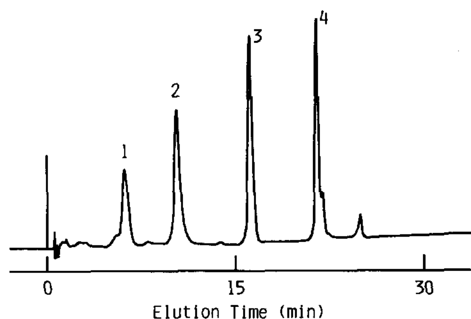

Fig. 19. Separation of peptides on Phenyl-5PW RP. Samples: 1 = aprotinin (20 μg); 2 = adrenocorticotropic hormone (10 μg); 3 = somatostatin (10 μg); 4 = insulin (bovine, 5 μg).

(Type VI from human placenta) was separated on the two columns under the same conditions. Although peaks appeared that were not identified, it can be said that Phenyl-5PW RP afforded a better separation than TMS-250. Again, this is probably due to the larger pore size of Phenyl-5PW RP than TMS-250 because collagen has a high molecular weight of *ca.* 300 000. Fig. 19 shows the separation of peptides, indicating that Phenyl-5PW RP could also separate peptides with fairly high resolution. Although the peaks of peptides became narrower with increasing TFA concentration, the resolution remained almost unchanged because the elution range of peptides was also narrower.

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