Journal of Chromatography, 398 (1987) 327–334
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 572

Note

High-performance ion-exchange chromatography of proteins on non-porous ion exchangers

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(Received March 20th, 1987)

Porous packing materials have been employed with great success in liquid chromatography for a long time. On the other hand, non-porous packings have been examined only recently. Unger and co-workers[1–5] tested non-porous silica packings of mean particle diameter 1.5 μm in affinity, reversed-phase and hydrophobic interaction chromatography of proteins, and found that they are very useful. The usefulness of such materials has also demonstrated by others[6–8].

New non-porous anion and cation exchangers have recently become commercially available under the trade-name of TSKgel DEAE-NPR and SP-NPR (Toyo Soda, Tokyo, Japan). According to the manufacturer, they are prepared by introducing diethylaminoethyl groups or sulfopropyl groups into non-porous spherical hydrophilic resins of 2.5 μm in diameter. Their ion-exchange capacities are *ca.* 0.15 and 0.10 mequiv./ml, and the pK_a values of the ionic groups are around 11.2 and 2.3. The protein adsorption capacities, determined with bovine serum albumin (DEAE-NPR) and haemoglobin (SP-NPR) are *ca.* 5 mg/ml. We have evaluated these ion exchangers for protein separation and the results are now described.

EXPERIMENTAL

Chromatographic measurements were performed at 25°C with a system comprised of a Model CCPM pump and a Model UV-8000 variable-wavelength UV detector operated at 280 nm (Toyo Soda). Proteins were separated on a 35 mm × 4.6 mm I.D. stainless-steel column.

α-Chymotrypsin (bovine pancreas), α-chymotrypsinogen A (bovine pancreas), conalbumin (chicken egg white, iron-free), cytochrome *C* (horse heart), ferritin (horse spleen), γ-globulin (human), haemoglobin (human blood), lysozyme (chicken egg white), ribonuclease A (bovine pancreas), thyroglobulin (bovine), transferrin (human), trypsin inhibitor (soy bean) and trypsinogen (bovine pancreas) were obtained from Sigma (St. Louis, MO, U.S.A.). Ovalbumin (chicken egg) and protein A (*Staphylococcus aureus*) were obtained from Seikagaku (Tokyo, Japan). Bovine serum albumin was from Wako (Osaka, Japan), human haemoglobin standard from Bio-Rad (Richmond, CA, U.S.A.), lipoxidase (soy bean) from P. L. Biochemicals (Milwaukee, WI, U.S.A.) and protein A (recombinant *Escherichia coli* product) from Repligen (Cambridge, MA, U.S.A.).

0021-9673/87/$03.50

RESULTS AND DISCUSSION

Figs. 1 and 2 show separations of protein mixtures obtained on DEAE-NPR and SP-NPR. Proteins were separated very rapidly, in about 5 min, and the resolutions attained were very high. Moreover, even more rapid separations were possible with a slight decrease in resolution by changing the elution conditions, *e.g.*, by decreasing the gradient time. DEAE-NPR and SP-NPR were compared with the porous ion exchangers TSKgel DEAE-5PW and SP-5PW in the separation of proteins. The same protein mixtures were separated under conditions typical to each ion-exchange column, and the results are shown in Figs. 3 and 4. Higher resolution was attained in a much shorter time on DEAE-NPR and SP-NPR than the porous ion exchangers.

The recovery of proteins from DEAE-NPR and SP-NPR columns was examined for a 5-μg injection of each protein. The results are summarized in Tables I and II. All proteins tested were recovered in high yield with sample injections as small as

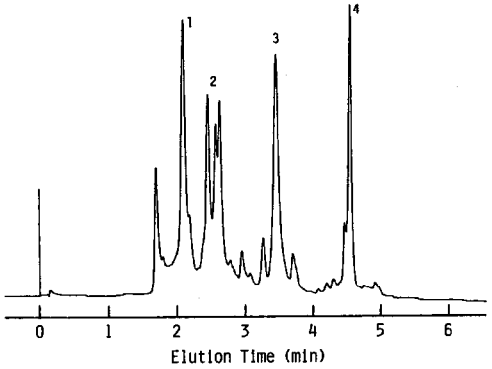

Fig. 1. Separation of a protein mixture on TSKgel DEAE-NPR. A mixture of conalbumin (1), transferrin (2), ovalbumin (3) and trypsin inhibitor (4) was separated with a 10-min linear gradient from 0 to 0.5 *M* sodium chloride in 20 m*M* Tris–HCl buffer (pH 8.0) at a flow-rate of 1.5 ml/min.

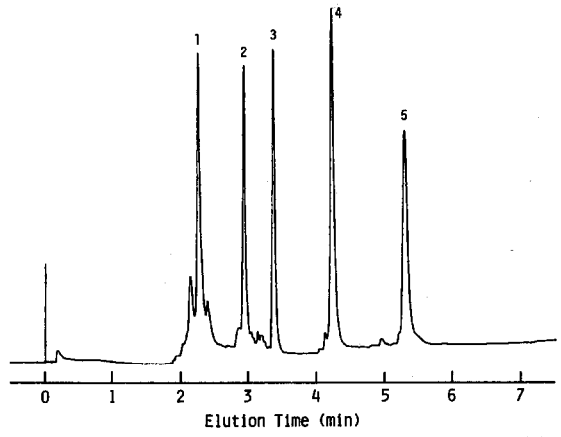

Fig. 2. Separation of a protein mixture on TSKgel SP-NPR. A mixture of trypsinogen (1), α-chymotrypsinogen A (2), ribonuclease A (3), cytochrome *c* (4) and lysozyme (5) was separated with a 10-min linear gradient from 0 to 0.5 *M* sodium sulphate in 20 m*M* acetate buffer (pH 5.0) at a flow-rate of 1.5 ml/min.

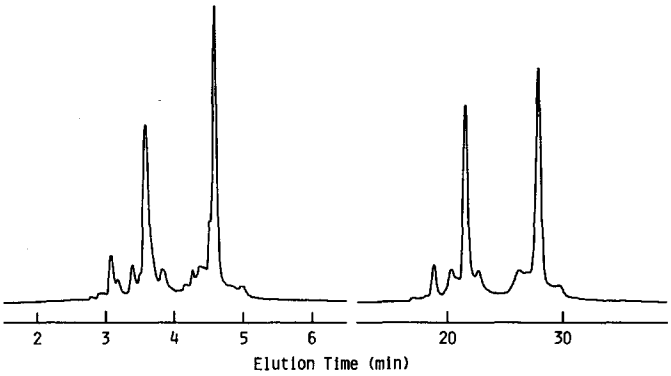

Fig. 3. Comparison of TSKgel DEAE-NPR and DEAE-5PW. A mixture of ovalbumin (first peak) and trypsin inhibitor (second peak) was separated on DEAE-NPR (left) and DEAE-5PW (right). Elution conditions as in Fig. 1 except that the gradient time was 60 min and the flow-rate was 1.0 ml/min on DEAE-5PW. The size of the DEAE-5PW column was 75 mm × 7.5 mm I.D.

5 μg. Especially, the recovery was almost quantitative on DEAE-NPR, although slightly lower recoveries were observed for some proteins from SP-NPR.

The loading capacity was evaluated by separating various sample loads of the individual pure proteins. The peak width is plotted against the sample load in Fig. 5. The peak width is plotted against the sample load in Fig. 5. It remained constant at sample loads up to *ca.* 5 μg, and then increased with further increase in the sample load. Similar results were obtained on SP-NPR. The maximum sample load resulting in the highest resolution is around 5 μg for pure samples. Amounts of crude samples up to 100–200 μg could be applied with little decrease in resolution, as illustrated in Fig. 6.

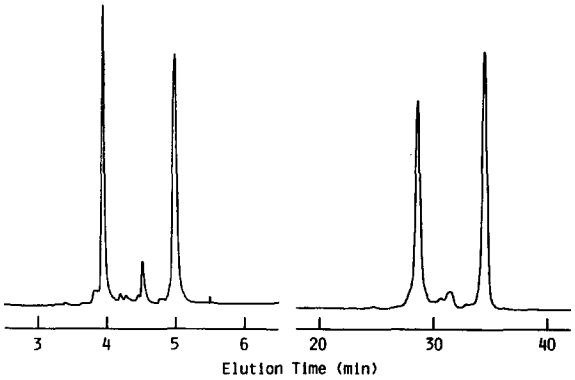

Fig. 4. Comparison of TSKgel SP-NPR and SP-5PW. A mixture of α-chymotrypsinogen A (first peak) and ribonuclease A (second peak) was separated on SP-NPR (left) and SP-5PW (right) with a 10-min (SP-NPR) or 60-min (SP-5PW) linear gradient from 0 to 0.5 *M* sodium chloride in 20 m*M* 2-morpholinoethanesulphonic acid–sodium hydroxide buffer (pH 6.0) at a flow-rate of 1.5 ml/min (SP-NPR) or 1.0 ml/min (SP-5PW). The size of the SP-5PW column was 75 mm × 7.5 mm I.D.

TABLE I

RECOVERY OF PROTEINS FROM TSKgel DEAE-NPR

Each protein (5 μg) was separated on DEAE-NPR under the conditions in Fig. 1. The recovery was determined from the areas of eluted peaks.

Protein	*Recovery (%)*
Thyroglobulin	100
Ferritin	99
γ-Globulin	104
Bovine serum albumin	102
Haemoglobin	91
Ovalbumin	103
β-Lactoglobulin	101
Trypsin inhibitor	98

TABLE II

RECOVERY OF PROTEINS FROM TSKgel SP-NPR

Each protein (5 μg) was separated on SP-NPR with a 10-min linear gradient from 0 to 0.5 *M* sodium chloride in 20 m*M* phosphate buffer (pH 7.0) at a flow-rate of 1.5 ml/min. The recovery was determined from the areas of eluted peaks.

Protein	*Recovery (%)*
Haemoglobin	88
α-Chymotrypsinogen A	95
α-Chymotrypsin	100
Trypsinogen	87
Lysozyme	96
Ribonuclease A	95
Cytochrome *c*	93

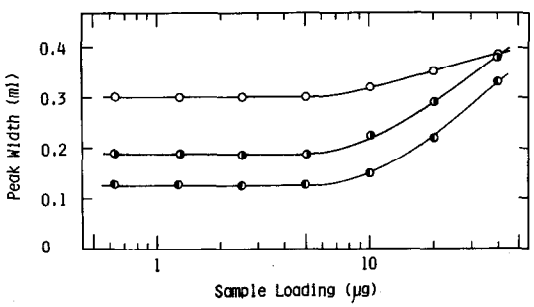

Fig. 5. Dependence of the peak width on sample loading in the separation of proteins on TSKgel DEAE-NPR. Elution conditions as in Fig. 1. Samples: (○) conalbumin; (◑) ovalbumin; (◐) trypsin inhibitor.

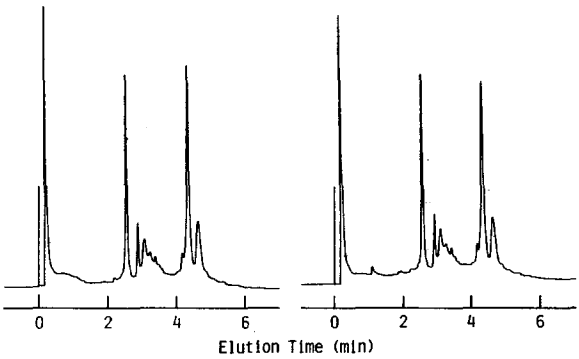

Fig. 6. Chromatograms of a crude sample of lipoxidase obtained with sample loads of 50 μg (left) and 200 μg (right) on TSKgel SP-NPR with a 10-min linear gradient from 0 to 0.5 *M* sodium sulphate in 20 m*M* acetate buffer (pH 4.5) at a flow-rate of 1.5 ml/min.

The effects of flow-rate and gradient time were studied in the separation of proteins on DEAE-NPR and SP-NPR. The dependence of the resolution on flow-rate at a constant gradient time is shown in Fig. 7. Higher resolution was obtained at higher flow-rates although the effect of flow-rate became insignificant at values above 1.0 ml/min. Because an increase in flow-rate results in a slightly shorter separation time and dilution of the sample during separation, flow-rates of 1.0–1.5 ml/min are believed to be a good compromise. The dependence of resolution on gradient time at constant flow-rate is shown in Fig. 8. The resolution increased with increasing gradient time up to 10 min, then became almost constant. Because longer gradient times result in longer separation times and greater dilution of the sample, values of 5–10 min seem to be a good compromise. Similar results were obtained on SP-NPR.

Figs. 9–12 show examples of applications of ion-exchange chromatography on DEAE-NPR and SP-NPR. In the example of clinical analysis in Fig. 9, an human haemoglobin standard was separated on SP-NPR. The peak eluted at *ca.* 1.2 min is

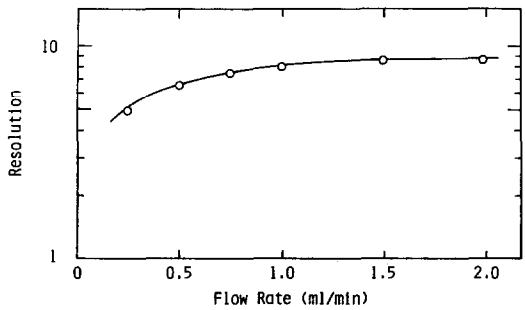

Fig. 7. Dependence of the resolution on flow-rate in the separation of proteins on TSKgel DEAE-NPR. A mixture of ovalbumin and trypsin inhibitor was separated under the conditions in Fig. 1 except that the flow-rate was varied between 0.25 and 2.0 ml/min. The resolution was calculated from the peak widths and elution volumes of the two proteins.

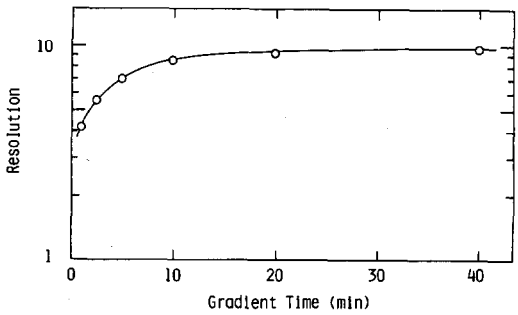

Fig. 8. Dependence of the resolution on gradient time in the separation of proteins on TSKgel DEAE-NPR. A mixture of ovalbumin and trypsin inhibitor was separated under the conditions in Fig. 1 except that the gradient time was varied between 1 and 40 min. Resolutions calculated as in Fig. 7.

believed to be glycosylated haemoglobin (HbA_{1c}) and the largest peak eluted at *ca.* 2.4 min is presumably haemoglobin HbA_0, although they were not confirmed. Therefore, it is possible to quantitate HbA_{1c} in a very short time. Rapid separations like this are very important in the application of high-performance liquid chromatography (HPLC) to routine clinical analyses. Fig. 10 shows an example of a protein purity test. Two samples of protein A were separated on DEAE-NPR. According to the manufacturers they were of high purity; only a single band was seen in sodium-dodecyl sulphate-polyacrylamide gel electrophoresis of the sample from Repligen or in disc gel electrophoresis of the sample from Seikagaku. However, several peaks and shoulders are present on the chromatograms in Fig. 10, suggesting that ion-exchange

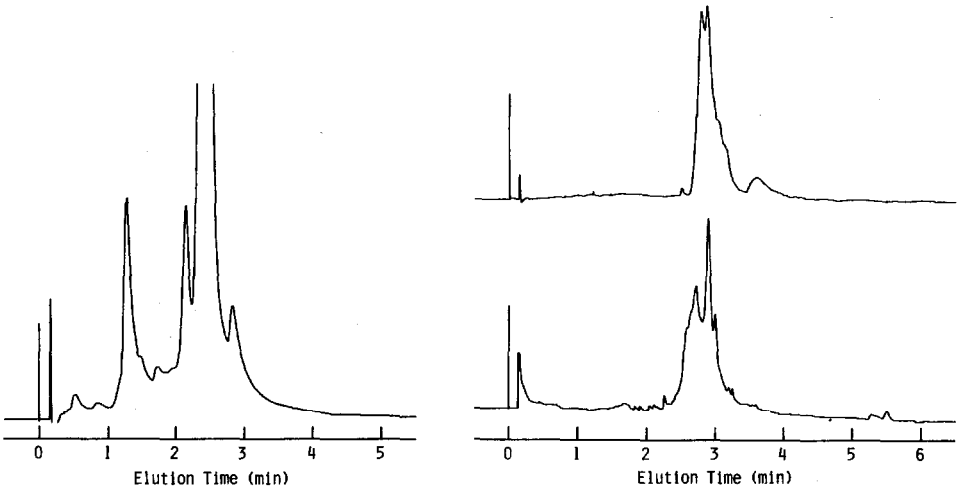

Fig. 9. Separation of a human haemoglobin standard by ion-exchange chromatography on TSKgel SP-NPR using a 3-min linear gradient from 0.05 to 0.2 *M* sodium chloride in 20 m*M* bis-Tris–HCl buffer (pH 6.0) at a flow-rate of 1.5 ml/min and detected at 415 nm.

Fig. 10. Separation of protein A from a recombinant *E. coli* product (upper) and from *Staphylococcus aureus* (lower) by ion-exchange chromatography on TSKgel DEAE-NPR. Conditions as in Fig. 1.

chromatography on DEAE-NPR or SP-NPR can provide higher resolution in protein separation than gel electrophoresis. Fig. 10 also indicates that there is a substantial difference in purity between the two samples. Accordingly, ion-exchange chromatography on DEAE-NPR and SP-NPR should be a good alternative to gel electrophoresis for testing the purity of protein samples. Fig. 11 shows an example of micropreparative separations of proteins. A commercial sample of α-chymotrypsin (5 μg) was separated on DEAE-NPR. Many peaks and shoulders appeared although this sample seemed rather pure when separated on porous ion exchangers. The main peak corresponding to α-chymotrypsin was collected as indicated in the figure. The recovery of enzymatic activity was 80%. α-Chymotrypsin recovered in this fraction was of high purity according to reversed-phase chromatography. Fig. 12 shows the separation of a commercial crude sample of hexokinase (25 μg) on DEAE-NPR. Because hexokinase activity was found in the peak eluted at *ca.* 4.5 min, the peak was fractioned as indicated in the figure. The recovery of enzymatic activity in this fraction was 85%. Its purity was confirmed by reversed-phase chromatography.

As demonstrated above, proteins can be separated very rapidly and with high resolution on DEAE-NPR and SP-NPR. Accordingly, these packings should be useful in particular for applications requiring rapid separations such as clinical and process control analyses. They are also useful for general analyses of proteins like laboratory purity tests. In addition, they should be applicable to very high-molecular-weight proteins owing to the lack of diffusion into and out of their pores. However, the loading capacities of DEAE-NPR and SP-NPR are rather low because their surface areas are small. Consequently, they do not seem suitable for large scale separations. On the contrary, however, proteins can be recovered in high yield even with very small sample loads such as a few micrograms owing to the small surface areas.

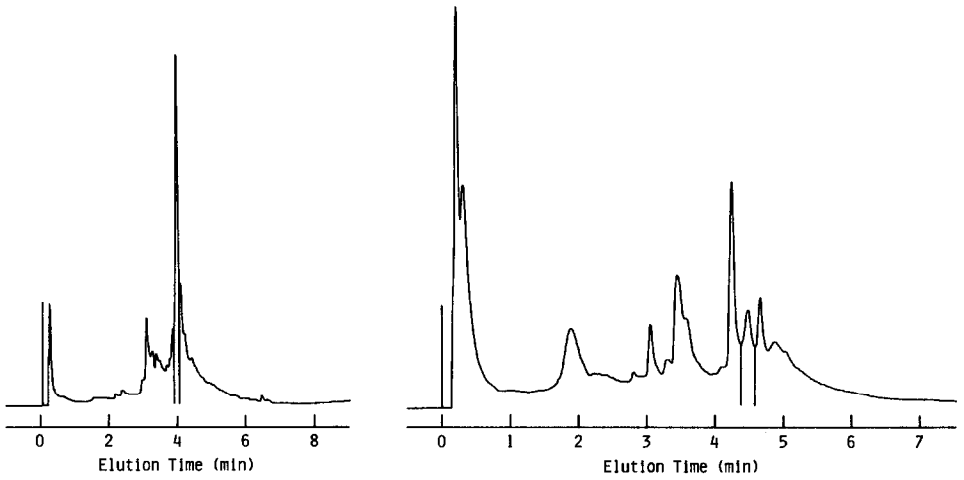

Fig. 11. Separation of α-chymotrypsin (5 μg) by ion-exchange chromatography on TSKgel DEAE-NPR with a 20-min linear gradient from 0 to 0.5 *M* sodium chloride in 20 m*M* 1,3-diaminopropane hydrochloride buffer (pH 10.3) at a flow-rate of 1.5 ml/min.

Fig. 12. Separation of crude hexokinase (25 μg) by ion-exchange chromatography on TSKgel DEAE-NPR. Conditions as in Fig. 1.

Therefore, they are also useful for micropreparative separations of proteins. Although the particles of DEAE-NPR and SP-NPR are 2.5 μm in diameter, the pressure drop of 100–150 bar at a flow-rate of 1.5 ml/min on a 35 mm × 4.6 mm I.D. column presents no problem in practical use. Therefore, ion-exchange chromatography on TSKgel DEAE-NPR and SP-NPR seems to be a new development of HPLC.

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