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# Note

## Reversed-phase high-performance liquid chromatography of proteins and peptides on a pellicular support based on hydrophilic resin

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Totally porous supports have commonly been employed in high-performance liquid chromatography (HPLC). On the other hand, pellicular supports, which were once explored in the early stages of HPLC but did not come into general use, have been attracting attention during the last few years particularly in the separation of biopolymers. Since Unger and co-workers[1–3] showed that pellicular supports of small particle diameter (1.5 μm) are very useful for rapid separations of proteins, various types of pellicular supports have been prepared and some of them have become commercially available. TSKgel Octadecyl-NPR is one such support for reversed-phase chromatography. It was prepared by chemically bonding octadecyl groups on the surface of non-porous spherical hydrophilic resin of diameter 2.5 μm and has become commercially available recently (Tosoh, Tokyo, Japan). We have evaluated it for the separation of proteins and peptides and the results are presented in this paper.

## EXPERIMENTAL

Chromatographic measurements were performed at 25°C on a 35 mm × 4.6 mm I.D. stainless-steel column with a system consisting of a Model CCPM double plunger pump and a Model UV-8000 variable-wavelength UV detector (Tosoh). Proteins were usually separated with a 10-min linear gradient of acetonitrile from 15 to 80% in 0.05% trifluoroacetic acid (TFA) at a flow-rate of 1.5 ml/min and detected at 220 nm. Peptides were usually separated with a 10-min linear gradient of acetonitrile from 0 to 80% in 100 m*M* perchloric acid at a flow-rate of 1.5 ml/min and detected at 215 nm. Some separations, however, were performed under slightly different conditions. In the study of operational variables, the type and concentration of acid component, flow-rate and gradient of acetonitrile were varied over a wide range. (Acids were always added at the same concentrations to both initial and final eluents in our experiments. Therefore, the acid concentrations mentioned in this paper are those in mixtures of water and acetonitrile.)

All proteins were purchased from Sigma (St. Louis, MO, U.S.A.): bovine serum

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albumin, cytochrome C, α-lactoalbumin, lysozyme, myoglobin, ovalbumin, ribonuclease, transferrin and trypsin, product numbers A-0281, C-2506, L-5385, L-6876, M-0630, A-5378, R-5125, T-2252 and T-8642, respectively. Insulin was also obtained from Sigma (product number I-5500). All other peptides were purchased from the Peptide Institute (Osaka, Japan).

## RESULTS AND DISCUSSION

Fig. 1 shows the separation of a protein mixture. As exemplified here, proteins were separated rapidly, in 5–6 min, with high resolution. The same protein mixture was also separated on a totally porous support, TSKgel Phenyl-5PW RP[4], and the result was compared with that in Fig. 1. Octadecyl-NPR provided a higher resolution in a much shorter time (*ca.* 20%) than the totally porous support. The peak of ovalbumin in Fig. 1 is broader than the peaks of other proteins and some shoulders are observed. Similar patterns were observed for ovalbumin even under different conditions (see Fig. 4). Although the reason for this is not clear, one possibility is that some components existed in the ovalbumin sample that were partially separated. If this is so, it is also an indication of very high resolution in the separation of proteins on Octadecyl-NPR because such a partial separation of ovalbumin components by reversed-phase chromatography has not been reported previously.

Fig. 2 shows the separation of a peptide mixture. It was also possible to separate peptides, except small ones, in about 5 min with high resolution. However, small peptides with molecular weights below *ca.* 1000 were eluted as slightly broad peaks, probably because the support of Octadecyl-NPR is resin-based and has very small pores, although it is stated to be non-porous. If small molecules enter such very small pores, the diffusion rate there should be slow, which results in broad peaks. Such broad peaks have also been observed in the separation of other types of small molecules such as nucleotides on non-porous resin-based supports[5].

The recovery from the Octadecyl-NPR column was examined for some proteins and peptides. The recovery was estimated from the areas of the eluted peaks. As controls, we used peak areas observed when the column was replaced with an empty

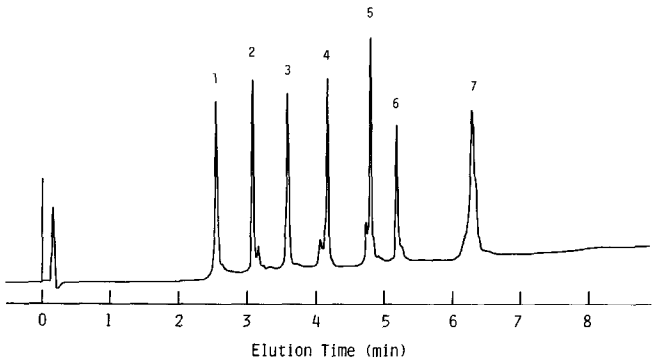


Fig. 1. Separation of a protein mixture on TSKgel Octadecyl-NPR. A mixture of (1) ribonuclease, (2) insulin, (3) cytochrome C, (4) lysozyme, (5) α-lactoalbumin, (6) myoglobin and (7) ovalbumin was separated with a 10-min linear gradient of acetonitrile from 15 to 80% in 0.05% TFA at a flow-rate of 1.5 ml/min.

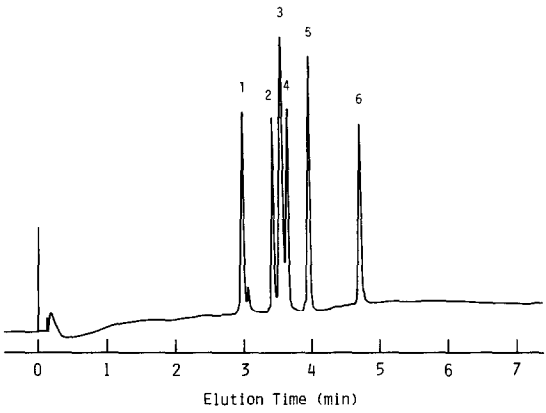


Fig. 2. Separation of a peptide mixture on TSKgel Octadecyl-NPR. A mixture of (1) α-endorphin, (2) bombesin, (3) γ-endorphin, (4) angiotensin, (5) somatostatin and (6) calcitonin was separated with a 10-min linear gradient of acetonitrile from 0 to 80% in 0.2% TFA at a flow-rate of 1.5 ml/min.

1 mm I.D. stainless-steel tube of 1 ml total inner volume. The results obtained with sample injections of 0.5 μg are summarized in Table I. Most proteins and peptides were recovered almost quantitatively. A recovery of more than 85% was achieved even with sample injections of 50 ng, as shown in Table II. The recovery of myoglobin and somatostatin was investigated with sample injections of 12.5–400 ng. Both were recovered quantitatively even with the smallest sample injection (12.5 ng). In addition, the peak volumes were usually very small (50–100 μl) in the separations on Octadecyl-NPR, as can be seen from Figs. 1 and 2. Accordingly, Octadecyl-NPR should be useful for the separation of small amounts of proteins and peptides. Such high recoveries are considered to be due to the small surface area of the support of Octadecyl-NPR. This is one of the advantages of pellicular supports.

TABLE I

RECOVERY OF PROTEINS AND PEPTIDES FROM TSKgel Octadecyl-NPR WITH A SAMPLE INJECTION OF 0.5 μg

Proteins were separated with an 8-min linear gradient of acetonitrile from 20 to 80% in 0.05% TFA at a flow-rate of 1.5 ml/min. Peptides were separated with a 10-min linear gradient of acetonitrile from 0 to 80% in 100 m*M* perchloric acid at a flow-rate of 1.5 ml/min.

| *Protein* | *Recovery (%)* | *Peptide* | *Recovery (%)* |
|---|---|---|---|
| Cytochrome C | 95 | Leu-enkephalin | 99 |
| Ribonuclease | 94 | Oxytocin | 92 |
| Lysozyme | 104 | Bradykinin | 89 |
| Myoglobin | 97 | Angiotensin I | 103 |
| Ovalbumin | 74 | Bombesin | 95 |
| Bovine serum albumin | 96 | Somatostatin | 103 |
| Transferrin | 101 | γ-Endorphin | 102 |
| | | Glucagon | 98 |
| | | Insulin | 102 |

TABLE II

RECOVERY OF PROTEINS AND PEPTIDES FROM TSKgel Octadecyl-NPR WITH A SAMPLE INJECTION OF 50 ng

Proteins were separated with a 10-min linear gradient of acetonitrile from 15 to 80% in 5 m*M* perchloric acid at a flow-rate of 1.5 ml/min. Peptides were separated under the conditions in Table I.

| *Protein* | *Recovery (%)* | *Peptide* | *Recovery (%)* |
|---|---|---|---|
| Cytochrome C | 96 | Leu-enkephalin | 94 |
| Ribonuclease | 92 | Oxytocin | 88 |
| Lysozyme | 94 | Angiotensin I | 90 |
| Ovalbumin | 89 | γ-Endorphin | 85 |
| Bovine serum albumin | 104 | | |

Ovalbumin is a typical sample whose recovery is low in reversed-phase chromatography, and it was recovered in a slightly low yield (74%) also on Octadecyl-NPR, as shown in Table I. However, the recovery of ovalbumin depended considerably on the elution conditions and it was possible to attain a higher recovery by manipulating the elution conditions. For example, the recovery of ovalbumin increased on decreasing the concentration of the acid component in the eluent, by using phosphoric acid in place of TFA or by employing a steeper gradient of acetonitrile.

The loading capacity was evaluated by separating some pure and crude samples with various sample loads. The peak width remained constant at sample loads up to 0.5–1 μg and then increased with further increase in sample load in the separation of pure samples. Consequently, the maximum sample load resulting in the highest resolution is only 0.5–1 μg for pure samples. On the other hand, the maximum sample load for crude samples was higher. Such samples could be applied in amounts up to tens of micrograms with little decrease in resolution. These maximum sample loads are significantly lower (*ca.* 1–5%), than those on totally porous supports. This is the greatest disadvantage of pellicular supports. Accordingly, Octadecyl-NPR does not seem suitable for large-scale separations.

Octadecyl-NPR is chemically stable and hence it can be operated over a wide pH range. This is advantageous because separations of proteins and peptides are sometimes improved by changing the eluent pH, owing to the different selectivities at different pH. Fig. 3 shows separations of peptides at acidic, neutral and alkaline pH. The best separation was achieved at akaline pH for this sample, although alkaline pH is not always optimum. All five components were separated at pH 9.3, whereas angiotesin III, angiotensin II and α-endorphin were eluted together at pH 1.0 and γ-endorphin and angiotensin I were not separated well at pH 7.0.

The effects of the type and concentration of the acid component of eluent, flow-rate and gradient of organic solvent (acetonitrile) were studied.

Proteins were separated at various concentrations of TFA, perchloric acid or phosphoric acid. The peaks of the proteins became broader as the acid concentration decreased in the ranges below 0.02% (TFA), 2 m*M* (perchloric acid) or 50 m*M* (phosphoric acid). In addition, lysozyme was eluted as two peaks at such low acid concentrations. In contrast, the recovery of ovalbumin decreased greatly as the acid

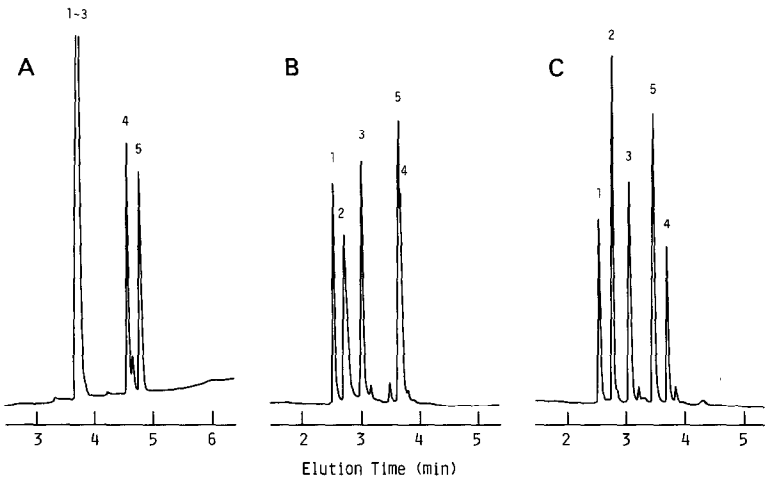


Fig. 3. Separation of a peptide mixture on TSKgel Octadecyl-NPR at acidic, neutral and alkaline pH. A mixture of (1) angiotensin II, (2) angiotensin III, (3) α-endorphin, (4) γ-endorphin and (5) angiotensin I was separated with 10-min linear gradient of acetonitrile from 0 to 60% in (A) 100 m*M* perchloric acid of pH 1.0, (B) sodium phosphate of pH 7.0 and (C) sodium phosphate of pH 9.3 at a flow-rate of 1.5 ml/min. In B and C, the initial eluent was 10 m*M* sodium phosphate and the final eluent was 0.5 m*M* sodium phosphate–acetonitrile (40:60).

concentration increased in the ranges above 0.1% (TFA), 10 m*M* (perchloric acid) or 300 m*M* (phosphoric acid). Therefore, concentrations of around 0.05%, 5 m*M* and 100–200 m*M* seem to be the best compromise for TFA, perchloric acid and phosphoric acid, respectively, in the separation of proteins. However, when some proteins are recovered in low yield, lower concentrations of acids may be better in order to obtain higher recoveries.

TFA provided the narrowest peaks for proteins among the three types of acids at their optimum concentrations, as exemplified in Fig. 4. In contrast, perchloric acid and phosphoric acid provided more stable baselines than TFA. Consequently, TFA is better than perchloric acid or phosphoric acid for achieving high resolution, whereas perchloric acid or phosphoric acid is better than TFA when a high sensitivity of detection is required in the separation of small amounts of proteins.

TFA, perchloric acid and heptafluorobutyric acid (HFBA) were explored for the separation of peptides. The peaks became broader and the resolution decreased as the acid concentration decreased in the ranges below 0.1% (TFA), 50 m*M* (perchloric acid) and 0.02% (HFBA) also in the separation of peptides, in particular for peptides that were eluted early. At higher acid concentrations, the peak widths and resolution were almost constant and the recovery was also unchanged. However, because there is no benefit in employing unnecessarily high acid concentrations, concentrations of *ca.* 0.2%, 100 m*M* and 0.05% seem to be the best choice for TFA, perchloric acid and HFBA, respectively, in the separation of peptides.

The peak widths observed with the three types of acids were very similar, but the selectivity differed to some extent with the three types of acids. Accordingly, the best acid to obtain the highest resolution varies depending on the sample to be separated.

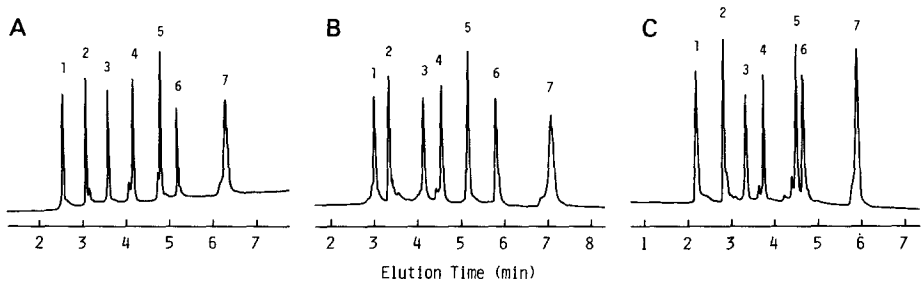


Fig. 4. Effect of type of acid component of the eluent on the separation of proteins on TSKgel Octadecyl-NPR. The same protein mixture as in Fig. 1 was separated with 10-min linear gradients of acetonitrile from 15 to 80% in (A) 0.05% TFA, (B) 5 m*M* perchloric acid and (C) 100 m*M* phosphoric acid at a flow-rate of 1.5 ml/min.

However, perchloric acid is better than TFA or HFBA for obtaining a stable baseline, just as in the separation of proteins.

The effect of flow-rate on resolution was similar in the separation of proteins and peptides with a constant gradient time. The resolution increased with increasing flow-rate up to 1.0–1.5 ml/min, then became almost constant at higher flow-rates. Although the separation time becomes slightly shorter as the flow-rate increases, the samples are more diluted and the pressure drop becomes higher almost proportionally with the increase in flow-rate. Accordingly, flow-rates of 1.0–1.5 ml/min seem to be a good compromise.

A similar tendency was also observed for the effect of the gradient of acetonitrile on resolution in the separation of proteins and peptides. A higher resolution was achieved with decreasing gradient of acetonitrile down to about 5%/min, whereas the resolution was almost constant at gradients shallower than about 5%/min. Because the shallower gradient results in a longer separation time and greater dilution of the sample, gradients of acetonitrile of *ca.* 5%/min seem to be a good compromise.

Fig. 5 shows an example of the application of reversed-phase chromatography

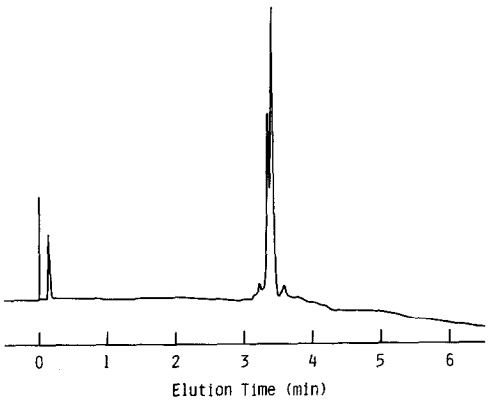


Fig. 5. Separation of trypsin (1 μg) on TSKgel Octadecyl-NPR with a 10-min linear gradient of acetonitrile from 25 to 80% in 0.05% TFA at a flow-rate of 1.5 ml/min.

on Octadecyl-NPR. A commercial sample of trypsin was separated. Two peaks appeared on the chromatogram and were collected and subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis after reduction. Two bands were seen at positions corresponding to molecular weights of *ca.* 10 000 and 13 000 in the gel electrophoresis pattern of the first fraction, whereas a single band was seen at a position corresponding to a molecular weight of *ca.* 23 000 in the pattern of the second fraction. Accordingly, the two peaks are assumed to be α-trypsin (first peak) and β-trypsin (second peak). When this sample was separated on a totally porous support, only a single peak appeared on the chromatogram.

As demonstrated, proteins and peptides can be separated very rapidly with high resolution by reversed-phase chromatography on Octadecyl-NPR. The separation time is typically *ca.* 5 min. Consequently, Octadecyl-NPR should be very useful in particular for applications that require rapid separations such as clinical analysis and monitoring of purification or reaction (such as tryptic digest) processes. It is also useful for general analyses of proteins and peptides such as laboratory purity tests. However, the loading capacity of Octadecyl-NPR is fairly low because its surface area is small. Therefore, it does not seem suitable for large-scale separations. In contrast, proteins and peptides are recovered in high yield even with sample injections of very small amounts (low- and sub-microgram levels) owing to the small surface area. Accordingly, Octadecyl-NPR is also useful for micropreparative separations of proteins and peptides. Some pellicular supports for the reversed-phase chromatography of proteins and peptides have been reported during the last few years[2,6–11]. However, they were all laboratory-made and were not commercially available. In contrast, Octadecyl-NPR is commercially available and therefore there is no difficulty in its practical application.

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