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## Note

# High-performance hydrophobic interaction chromatography of proteins on a pellicular support based on hydrophilic resin

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Although totally porous supports have mainly been employed in high-performance liquid chromatography (HPLC), pellicular supports which were once explored in the early stages of HPLC[1,2] but did not come into general use, have been drawing attention during the last few years, especially in the separation of biopolymers. Since Unger and co-workers[3,4] showed that pellicular supports of small particle diameter (1.5 μm) are very useful for rapid separations of proteins, various types of pellicular supports based on non-porous silicas[5–12], synthetic hydrophilic resins[13–20], cross-linked polystyrenes[21,22] and agarose[23–25] have been prepared and examined for the separation of biopolymers. We have also been investigating pellicular supports based on hydrophilic resin. Ion-exchange and reversed-phase chromatography on such supports have already been reported[17,19,21,26]. Hydrophobic interaction chromatography of proteins has now been studied on a newly developed support, which was prepared by chemically bonding butyl groups on the surface of non-porous spherical synthetic hydrophilic resin of 2.5 μm particle diameter and has become commercially available recently under the trade-name TSKgel Butyl-NPR (Tosoh, Tokyo, Japan). The results are described in this paper.

## EXPERIMENTAL

Chromatographic measurements were carried out with a system consisting of a Model CCPM double-plunger pump and a Model UV-8000 variable-wavelength UV detector operated at 280 nm (Tosoh). Proteins were separated at 25°C on a 35 mm × 4.6 mm I.D. stainless-steel column of Butyl-NPR by a linear gradient of decreasing ammonium sulphate concentration from 1.8, 2.0 or 2.3 *M* to zero in 0.1 *M* phosphate buffer (pH 7.0) or 20 m*M* Tris–HCl buffer (pH 7.5) at a flow-rate of 1.0 ml/min except in the study of operational variables.

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

## RESULTS AND DISCUSSION

Fig. 1 shows an example of a protein separation. Proteins were usually separated in less than 10 min with high resolution. The resolution attainable on Butyl-NPR in

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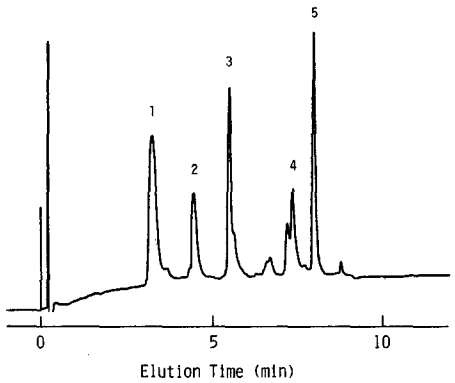


Fig. 1. Separation of a protein mixture on TSKgel Butyl-NPR. A mixture of (1) myoglobin (4 μg), (2) ribonuclease (4 μg), (3) lysozyme (1.5 μg), (4) α-chymotrypsin (3 μg) and (5) α-chymotrypsinogen (2 μg) was separated with a 12-min linear gradient of ammonium sulphate from 2.3 to 0 *M* in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 1.0 ml/min.

5–10 min was comparable to that obtained on totally porous supports in 30–60 min. This means that the separation time can be reduced to approximately one fifth with comparable resolution by using Butyl-NPR in place of totally porous supports. Such short separation times should be a great advantage in the separation of proteins. In hydrophobic interaction chromatography, some proteins tend to be denatured while they are adsorbing on the surface of the support in the column[27]. Accordingly, the short separation time is advantageous for preventing denaturation of proteins. In addition, proteins were recovered almost quantitatively even with small sample injections, *e.g.*, 2 μg (see Table I) and yet in small volumes, *ca.* 0.2 ml under typical elution conditions as in Fig. 1. The recovery of enzymatic activity was also high, as expected (see Table II). Therefore, Butyl-NPR is very useful for the micropreparative separation of proteins, just like other pellicular supports[17,26]. In contrast, Butyl-NPR does not seem to be useful for large scale separations because the loading capacity is fairly low. When pure samples of proteins were separated with various sample loads, the peak widths remained constant at sample loads up to 1–2 μg, and then increased with further increase in the sample load. Consequently, the maximium sample load that can be applied to the column without a decrease in resolution is only 1–2 μg for

TABLE I

RECOVERY OF PROTEINS FROM TSKgel Butyl-NPR

Each protein (2 μg) was separated on Butyl-NPR under the conditions in Fig. 1. The recovery was determined from the areas of the eluted peaks.

| *Protein* | *Recovery (%)* | *Protein* | *Recovery (%)* |
|---|---|---|---|
| Ribonuclease | 90 | α-Chymotrypsin | 95 |
| Lysozyme | 102 | α-Chymotrypsinogen | 98 |
| Myoglobin | 96 | Ovalbumin | 92 |
| Trypsin inhibitor | 84 | | |

TABLE II

RECOVERY OF ENZYMATIC ACTIVITY FROM TSKgel Butyl-NPR

A crude sample of peroxidase (25 μg) was separated with a 10-min linear gradient of ammonium sulphate from 1.8 to 0 *M* in 0.1 *M* phosphate buffer (pH 7.0). A pure sample of lactate dehydrogenase (2.5 μg) was separated with a 10-min linear gradient of ammonium sulphate from 2 to 0 *M* in 0.1 *M* phosphate buffer (pH 7.0). The flow-rates were 1.0 ml/min.

| *Enzyme* | *Recovery of enzymatic activity (%)* |
|---|---|
| Peroxidase | 81 |
| Lactate dehydrogenase | 90 |

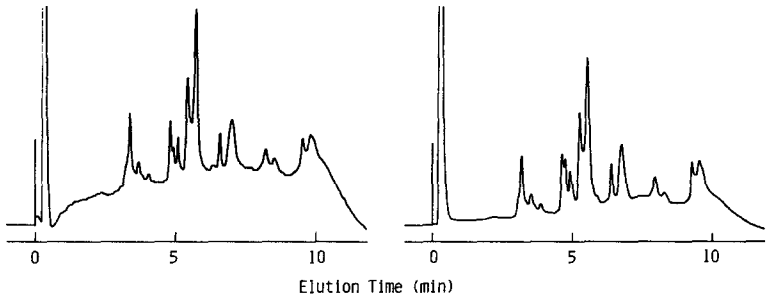


Fig. 2. Chromatograms of a crude sample of phosphoglucose isomerase obtained with sample loads of 25 μg (left) and 100 μg (right) on TSKgel Butyl-NPR with a 10-min linear gradient of ammonium sulphate from 1.8 to 0 *M* in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 1.0 ml/min.

pure samples. In the separation of crude samples, the maximum sample load was 10–100 μg, depending on the purity of the sample. Almost identical separations were obtained at sample loads up to 100 μg in the separation of a crude sample of phosphoglucose isomerase, as shown in Fig. 2, although the peaks became broader and the resolution decreased at sample loads above 100 μg.

Fig. 3 shows an example of the application of hydrophobic interaction

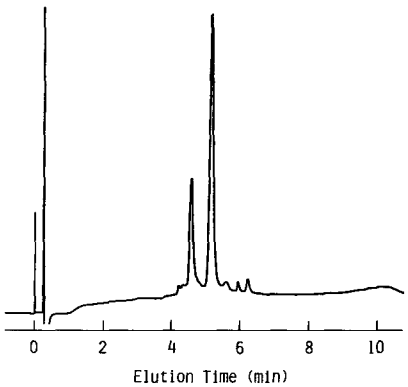


Fig. 3. Separation of trypsin (10 μg) by hydrophobic interaction chromatography on TSKgel Butyl-NPR with a 10-min linear gradient of ammonium sulphate from 2 to 0 *M* in 20 m*M* Tris–HCl buffer (pH 7.5) at a flow-rate of 1.0 ml/min.

chromatography on Butyl-NPR. A commercial sample of trypsin was separated under typical conditions. Several peaks were obtained. The two large peaks were collected and the enzymatic activity was examined. Both of the peaks exhibited trypsin activity. The two peaks were also examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis after reduction. A single band was observed at a position corresponding to a molecular weight of about 23 000 for the largest peak, and two bands were observed at molecular weights of about 10 000 and 13 000 for the second largest peak. Accordingly, the largest and second largest peaks are considered to be β-trypsin and α-trypsin, respectively. β-Trypsin is converted into α-trypsin by cleaving the molecular chain at a single position. However, the two chains of α-trypsin are connected with six disulphide bonds and the structures of α- and β-trypsins are very similar. Therefore, these two types of trypsins differ only slightly in their chromatographic behaviour. However, it was possible to separate them completely in about 6 min. It has been reported that α- and β-trypsin could be separated by reversed-phase chromatography on a pellicular support[26], but they were separated better by hydrophobic interaction chromatography than by reversed-phase chromatography.

The effects of flow-rate and gradient steepness of ammonium sulphate were studied. The same protein mixture as in Fig. 1 was separated under the same conditions except that the flow-rate was varied between 0.5 and 1.5 ml/min or the gradient time was varied between 5 and 25 min. The dependence of resolution on flow-rate at a constant gradient time is shown in Fig. 4. A higher resolution was obtained at higher flow-rates, although this effect of flow-rate was not very significant and particularly at flow rates above 1.0 ml/min the resolution was almost constant. Although the separation time becomes slightly shorter as the flow-rate increases, the samples become more diluted and the pressure drop becomes higher almost proportionately with the increase in flow-rate. Consequently, flow-rates of *ca.* 1.0 ml/min seem to be a good compromise. The dependence of resolution on gradient time at a constant flow-rate is shown in Fig. 5. The resolution increased with increasing gradient time up to about 15 min and then became almost constant. Because longer gradient times result in longer separation times and greater dilution of the sample during separation,

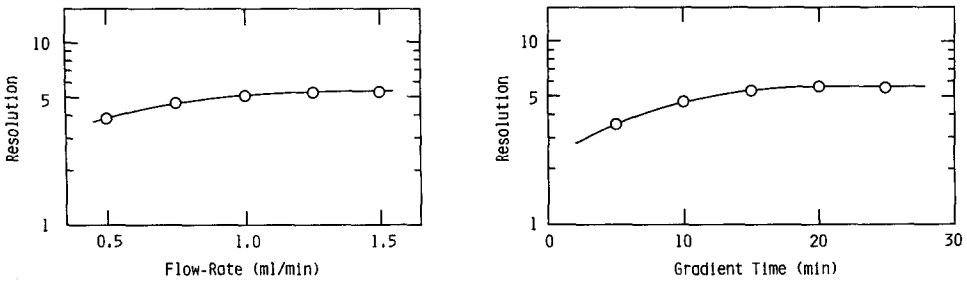


Fig. 4. Dependence of resolution on flow-rate in the separation of proteins on TSKgel Butyl-NPR. The same protein mixture as in Fig. 1 was separated under the same conditions except that the flow-rate was varied between 0.5 and 1.5 ml/min. The resolution was calculated for a pair of ribonuclease and lysozyme from the peak widths and elution volumes of the two proteins.

Fig. 5. Dependence of resolution on gradient time in the separation of proteins on TSKgel Butyl-NPR. The same protein mixture as in Fig. 1 was separated under the same conditions except that the gradient time was varied between 5 and 25 min. Resolutions calculated as in Fig. 4.

gradient times of *ca.* 15 min corresponding to a gradient steepness of about 150 m*M* ammonium sulphate/min are considered to be a good compromise.

As demonstrated above, Butyl-NPR is very useful for the rapid separation of proteins with high resolution by hydrophobic interaction chromatography. The separation time is typically 5–10 min. This short separation time should be advantageous for the separation of some proteins that are easily denatured. Butyl-NPR is particularly useful for the micropreparative separation of proteins owing to the high recovery even with very small sample injections, although it does not seem to be suitable for large-scale separations owing to the low loading capacity.

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