*Journal of Chromatography*, 360 (1986) 260–265
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 584

## Note

# New resin-based hydrophilic support for high-performance hydrophobic interaction chromatography

YOSHIO KATO*, TAKASHI KITAMURA and TSUTOMU HASHIMOTO
*Central Research Laboratory, Toyo Soda Mfg. Co., Ltd., Tonda, Shinnanyo, Yamaguchi 746 (Japan)*
(Received February 18th, 1986)

Hydrophobic interaction chromatography has been employed extensively for protein separations since its introduction. Although it was originally a slow technique, rapid separations have been made possible by the introduction of microparticulate rigid supports. TSKgel Phenyl-5PW (Toyo Soda, Tokyo, Japan), which is a resin-based weakly hydrophobic support prepared by introducing phenyl groups into TSKgel G5000PW, is the first commercial high-performance support. Many proteins have been successfully separated on Phenyl-5PW[1–5]. On the other hand, silica-based hydrophilic supports chemically bonded with oligoethylene glycol have also been demonstrated to be useful for hydrophobic interaction chromatography of proteins[6–8]. Accordingly, we prepared a resin-based hydrophilic support by introducing oligoethylene glycol into G5000PW, which is now commercially available under the trade-name of TSKgel Ether-5PW, and evaluated it comparatively with Phenyl-5PW for the separation of proteins. The results are described in this note.

## EXPERIMENTAL

Chromatographic measurements were carried out on analytical columns of 75 × 7.5 mm I.D. 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), exept for large-scale separations. Proteins were separated by 60-min linear gradient elution with decreasing ammonium sulphate concentration from 1.1–2 $M$ to zero in 0.1 $M$ phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min at 25°C and detected at 280 nm. The large-scale separations were performed on a preparative column (200 × 55 mm I.D.) with a high-speed liquid chromatograph Model HLC-837 (Toyo Soda) equipped with UV-8. The analytical and preparative columns were packed with particles of 10 and 20 $\mu$m in diameter, respectively.

Human serum was purchased from Miles Labs. (Elkhart, IN, U.S.A.). All other proteins were purchased from Sigma (St. Louis, MO, U.S.A.).

## RESULTS AND DISCUSSION

A protein mixture was separated on Phenyl-5PW and Ether-5PW under the same conditions to compare the retention and resolution. The chromatograms are

0021-9673/86/$03.50 © 1986 Elsevier Science Publishers B.V.

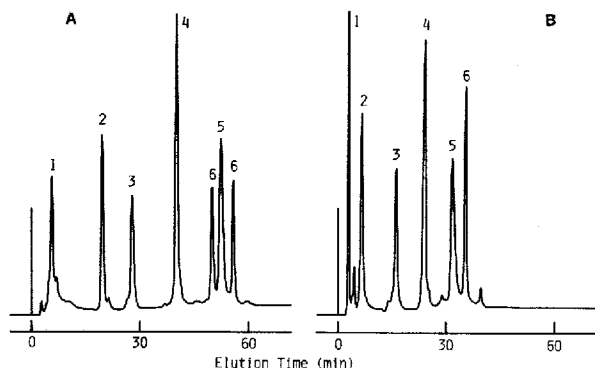


Fig. 1. Chromatograms of a protein mixture obtained on Phenyl-5PW (A) and Ether-5PW (B) with a 60-min linear gradient of ammonium sulphate from 2 $M$ to zero in 0.1 $M$ phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min. Samples: 1 = cytochrome $c$; 2 = myoglobin; 3 = ribonuclease; 4 = lysozyme; 5 = α-chymotrypsin; 6 = α-chymotrypsinogen.

shown in Fig. 1. Proteins were eluted earlier on Ether-5PW than on Phenyl-5PW. This means that Ether-5PW requires a higher salt concentration than Phenyl-5PW to retain proteins to the same extent. The resolution was calculated from the peak widths and elution volumes for the pairs of proteins eluted adjacently. The results are summarized in Table I. Ether-5PW provided higher resolution for some pairs (myoglobin/ribonuclease and α-chymotrypsin/α-chymotrypsinogen), whereas Phenyl-5PW provided higher resolution for other pairs (ribonuclease/lysozyme and lysozyme/α-chymotrypsin). On average, however, slightly higher resolution was obtained on Phenyl-5PW than on Ether-5PW. This seems to be a general fact because proteins were eluted in a wider range of elution volume when they were separated on Phenyl-5PW, although the peak widths were similar on both supports.

Although satisfactory results have been obtained for most proteins on Phenyl-5PW so far, it has been reported that bovine serum albumin and β-lactoglobulin were eluted as broad peaks under typical elution conditions[3]. We also noticed that α-lactoalbumin was not eluted from the column and that α-amylase was recovered

TABLE I

COMPARISON OF RESOLUTION IN THE SEPARATION OF PROTEIN PAIRS ON TSKgel PHENYL-5PW AND ETHER-5PW

| *Protein pair* | *Resolution* | |
|---|---|---|
| | *Phenyl-5PW* | *Ether-5PW* |
| Myoglobin/ribonuclease | 5.8 | 7.7 |
| Ribonuclease/lysozyme | 8.6 | 6.2 |
| Lysozyme/α-chymotrypsin | 7.5 | 4.6 |
| α-Chymotrypsin/α-chymotrypsinogen | 2.0 | 2.4 |
| Average | 6.0 | 5.2 |

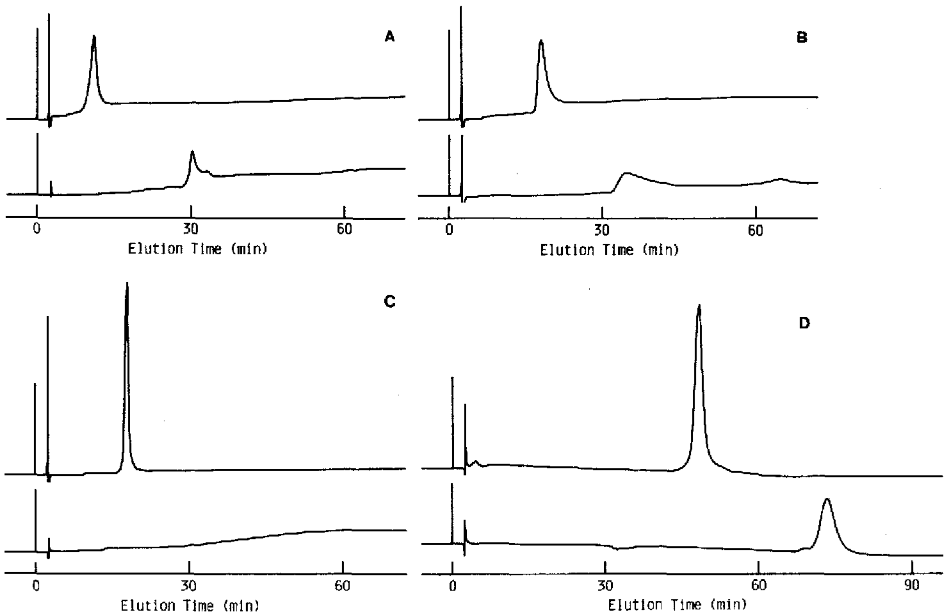


Fig. 2. Chromatograms of bovine serum albumin (A), β-lactoglobulin (B), α-lactoalbumin (C) and α-amylase (D) obtained on Ether-5PW (upper curves) and Phenyl-5PW (lower curves) with a 60-min linear gradient of ammonium sulphate from 1.5 *M* to zero (A), from 2 *M* to zero (B), from 1.5 *M* to zero (C) or from 1.1 *M* to zero (D) in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min. The recoveries of α-amylase activity were 104% and 60%, respectively, on Ether-5PW and Phenyl-5PW.

in low yield under typical elution conditions on Phenyl-5PW. Ether-5PW was examined for the separation of these proteins. Good results were obtained as shown in Fig. 2. Bovine serum albumin and β-lactoglobulin were eluted as fairly sharp peaks. α-Lactoalbumin was eluted from the column, and yet as a very sharp peak. α-Amylase was recovered quantitatively. Goheen and Engelhorn[3] suggested that the broad peaks observed on Phenyl-5PW resulted from partial denaturation of proteins during the separation. Consequently, it is supposed that the partial denaturation of proteins was

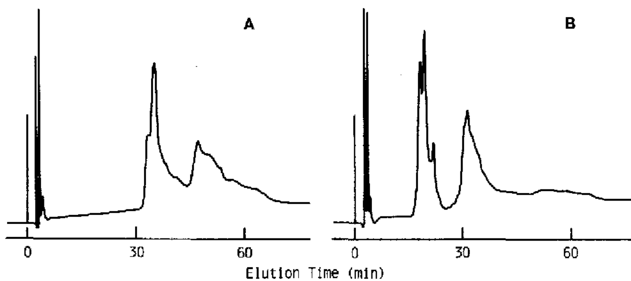


Fig. 3. Chromatograms of human serum obtained on Phenyl-5PW (A) and Ether-5PW (B) with a 60-min linear gradient of ammonium sulphate from 1.7 *M* to zero in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min.

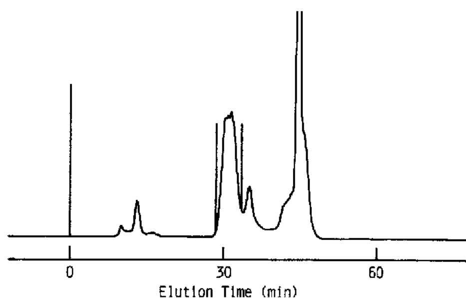


Fig. 4. Purification of human serum albumin on the Ether-5PW preparative column (200 × 55 mm I.D.). A 16-ml sample of human serum containing *ca.* 1.2 g of proteins was separated with a 36-min linear gradient of ammonium sulfate from 1.7 *M* to 0.68 *M* followed by step gradient to zero in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 40 ml/min.

suppressed in the separation on Ether-5PW owing to the hydrophilic properties of the support.

Fig. 3 also shows a comparison between Ether-5PW and Phenyl-5PW. Human serum was separated on the two columns. Column effluents were fractioned and subjected to immunoelectrophoresis. In the chromatogram obtained on Ether-5PW, two peaks appearing at elution times of 18–20 min were identified as albumin, a peak at *ca.* 22 min was transferrin, and the broad peak at 28–40 min was confirmed to be $\gamma$-globulin. The broad peak of $\gamma$-globulin should be indicative that the components of $\gamma$-globulin were partially separated. In the case of Phenyl-5PW, albumin was eluted as a tailing peak and was not separated well from other proteins.

The large-scale separation of human serum was performed on the Ether-5PW preparative column to purify albumin. The separation of 16 ml of human serum (containing *ca.* 1.2 g proteins) is shown in Fig. 4. In the preliminary experiments with various sample loadings, almost identical patterns were observed for up to 16 ml of human serum. The albumin peak was collected between the two vertical lines. The recovery of albumin was determined by immunodiffusion. A 92% albumin was recovered in the fraction. The purity was examined by immunoelectrophoresis (Fig. 5). Only a single band corresponding to albumin is seen on the pattern of the fraction, indicating that very pure albumin was obtained in one step of hydrophobic interaction chromatography on Ether-5PW.

Fig. 6 shows a separation of 1.5 g of crude $\alpha$-amylase (type VI-A from porcine pancreas) on the Ether-5PW preparative column. The $\alpha$-amylase crude sample could be applied up to 1.5 g without loss of separation efficiency. The peak corresponding

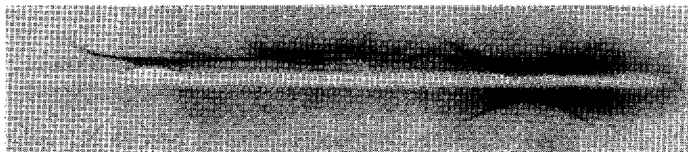

Fig. 5. Immunoelectrophoresis patterns of the albumin fraction (lower) and the original human serum (upper).

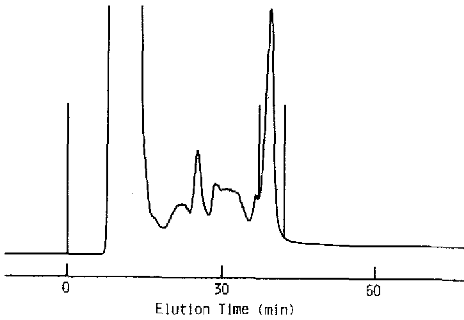


Fig. 6. Purification of α-amylase on the Ether-5PW preparative column (200 × 55 mm I.D.). A 1.5-g sample of crude α-amylase was separated with a 36-min linear gradient of sodium sulphate from 0.66 $M$ to zero in 0.1 $M$ phosphate buffer (pH 7.0) at a flow-rate of 40 ml/min.

A
0 30 60
Elution Time (min)

B
0 15 30
Elution Time (min)

C
0 10 20 30
Elution Time (min)

D
0 15 30 45
Elution Time (min)

Fig. 7. Chromatograms of the α-amylase fraction (lower curves) and the α-amylase original crude sample (upper curves) obtained by hydrophobic interaction chromatography (A), reversed-phase chromatography (B), gel filtration (C) and ion-exchange chromatography (D). Hydrophobic interaction chromatography was performed on a TSKgel Ether-5PW column (75 × 7.5 mm I.D.) with a 60-min linear gradient of sodium sulphate from 1.1 $M$ to zero in 0.1 $M$ phosphate buffer (pH 7.0). Reversed-phase chromatography was performed on a TSKgel Phenyl-5PW RP column (75 × 4.6 mm I.D.) with a 2-min linear gradient of acetonitrile from 5% to 20% followed by a 48-min linear gradient of acetonitrile from 20% to 80% in 0.05% trifluoroacetic acid. Gel filtration was performed on a TSKgel G3000SW column (600 × 7.5 mm I.D.) in 0.05 $M$ phosphate buffer containing 0.2 $M$ sodium chloride (pH 7.0). Ion-exchange chromatography was performed on a TSKgel DEAE-5PW column (75 × 7.5 mm I.D.) with a 60-min linear gradient of sodium chloride from zero to 0.5 $M$ in 0.02 $M$ Tris–HCl buffer (pH 8.0). The flow-rate was 1 ml/min in all these separations.

to α-amylase between the two vertical lines was collected. The recovery of enzymatic activity in the fraction was 90%. Te purity of the fraction was examined by high-performance liquid chromatography (Fig. 7). One major peak and several small peaks are seen in each chromatogram of the fraction. Because the major peaks showed enzymatic activity (except in the case of reversed-phase chromatography), they must correspond to α-amylase. This means that α-amylase of high purity was obtained.

The above two separations were completed within 1 h including regeneration of the column with the initial eluent. Therefore, the samples could be applied at intervals of 1 h. Because the sample loadings were 1.2 g and 1.5 g per injection, substantially large amounts of samples (*ca.* 30 g) can be treated in a day by repeated injections.

As demonstrated above, a wide range of proteins can be separated successfully under mild elution conditions with high resolution and recovery on TSKgel Ether-5PW. The Ether-5PW was especially useful for unstable proteins that were partially denatured in the separation on weakly hydrophobic suport (TSKgel Phenyl-5PW) under typical elution conditions. It was also useful for fairly hydrophobic proteins, such as α-amylase. However, it seems that the Ether-5PW provides slightly lower resolution than the Phenyl-5PW, for proteins which can be separated well on both supports. Moreover, the Ether-5PW requires a higher salt concentration than the Phenyl-5PW to retain proteins.

## REFERENCES

1 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 292 (1984) 418.
2 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 298 (1984) 407.
3 S. C. Goheen and S. C. Engelhorn, *J. Chromatogr.*, 317 (1984) 55.
4 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 333 (1985) 202.
5 H. Ichinose, K. Kojima, A. Togari, Y. Kato, S. Parvez, H. Parvez dand T. Nagatsu, *Anal. Biochem.*, 150 (1985) 408.
6 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1985) 519.
7 J.-P. Chang, Z. E. Rassi and Cs. Horváth, *J. Chromatogr.*, 319 (1985) 396.
8 N. T. Miller, B. Feibush, K. Corina, S. P. Lee and B. L. Karger, *Anal. Biochem.*, 148 (1985) 510.