Journal of Chromatography, 333 (1985) 202–210
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 966

Note

Preparative high-performance hydrophobic interaction chromatography of proteins on TSKgel Phenyl-5PW

YOSHIO KATO*, TAKASHI KITAMURA and TSUTOMU HASHIMOTO
Central Research Laboratory, Toyo Soda Mfg. Co., Ltd., Tonda, Shinnanyo, Yamaguchi (Japan)
(Received June 13th, 1985)

Hydrophobic interaction chromatography has become increasingly popular for the separation and purification of proteins. Although it was originally a slow technique, rapid separations are now possible by the use of microparticulate supports[1–11]. TSKgel Phenyl-5PW (Toyo Soda, Tokyo, Japan) is one such support and it has been reported that proteins can be separated rapidly and with high resolution without denaturation on analytical columns (75 × 7.5 mm I.D.) of this support[3,5,6,8]. In this paper, the results of protein separations on the Phenyl-5PW preparative column (150 × 21.5 mm I.D.) are described. The resolution, sample loading capacity and applications to enzyme purifications have been investigated.

EXPERIMENTAL

All chromatographic separations were carried out at 25°C 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) operated at 280 nm except in reversed-phase chromatography where it was operated at 220 nm.

Lipoxidase was purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). All other proteins were from Sigma (St. Louis, MO, U.S.A.).

The resolution of the Phenyl-5PW preparative column was studied for a mixture of myoglobin, ribonuclease, lysozyme, α-chymotrypsinogen A and α-chymotrypsin with a linear gradient of ammonium sulphate concentration decreasing from 1.8 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). The flow-rate and gradient time were varied to investigate the effects of these parameters.

The sample loading capacity of the Phenyl-5PW preparative column was studied by separating crude samples of lipoxidase, phosphoglucose isomerase and lactate dehydrogenase at various loadings. The sample concentrations were kept constant (*ca.* 20 mg/ml) and the injection volumes were varied. Lipoxidase was separated with a 120-min linear gradient of ammonium sulphate from 1.5 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). Phosphoglucose isomerase was separated with a 60-min linear gradient of ammonium sulphate from 1.5 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). Lactate dehydrogenase was separated with a 120-min linear gradient of ammonium

0021-9673/85/$03.30

sulphate from 1.8 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0). The flow-rate was 4 ml/min.

The crude samples of lipoxidase (200 mg), phosphoglucose isomerase (100 mg) and lactate dehydrogenase (54 mg) were separated under the same conditions as above and the peaks corresponding to each enzyme were fractionated. The fractions were examined for the recovery of enzymatic activity and purity. The enzymatic activity was determined according to the procedures described in ref. 12. The purity was tested by high-performance liquid chromatography (HPLC). Hydrophobic interaction chromatography was performed on an analytical Phenyl-5PW column with 60-min linear gradients of the same eluents as in the preparative separations. Reversed-phase chromatography was performed on a TSKgel Phenyl-5PW RP column (75 × 4.6 mm I.D.) with a 2-min linear gradient from 5 to 20% acetonitrile followed by a 48-min linear gradient from 20 to 80% acetonitrile in 0.5% trifluoroacetic acid (TFA). 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 from 0 to 0.5 *M* sodium chloride in 0.02 *M* Tris–HCl buffer (pH 8.0). The flow-rate was 1 ml/min in all these separations.

RESULTS AND DISCUSSION

Resolution

Fig. 1 shows the dependence of resolution on the flow-rate at a constant gradient time of 60 min. The effect of flow-rate was small in the range studied (2–8 ml/min). The dilution of samples during separation decreased almost in proportion to the flow-rate, while the separation time increased slightly with decreasing flow-rate. Accordingly, a flow-rate around 4 ml/min seems to be a good choice for the Phenyl-5PW preparative column.

Fig. 2 shows the dependence of resolution on the gradient time at a constant flow-rate of 4 ml/min. The resolution greatly increased with increasing gradient time up to 60 min. However, this effect was insignificant at gradient times longer than 120

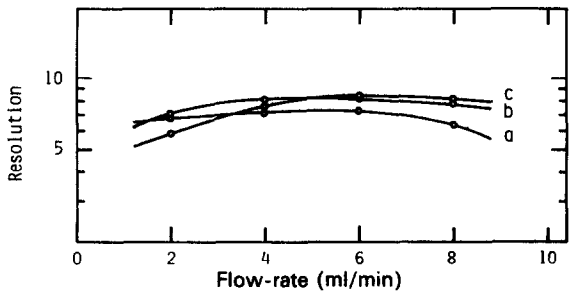

Fig. 1. Dependence of resolution on the flow-rate at a constant gradient time (60 min) in hydrophobic interaction chromatography on a Phenyl-5PW preparative column. The resolution was calculated from the peak widths and elution volumes of the pairs, myoglobin and ribonuclease (a), ribonuclease and lysozyme (b) and lysozyme and α-chymotrypsinogen A (c).

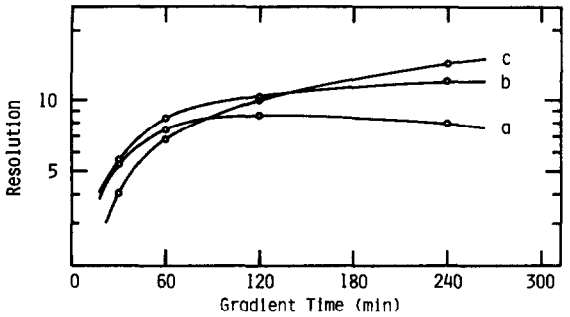

Fig. 2. Dependence of resolution on the gradient time at a constant flow-rate (4 ml/min) in hydrophobic interaction chromatography on a Phenyl-5PW preparative column. Resolutions as in Fig. 1.

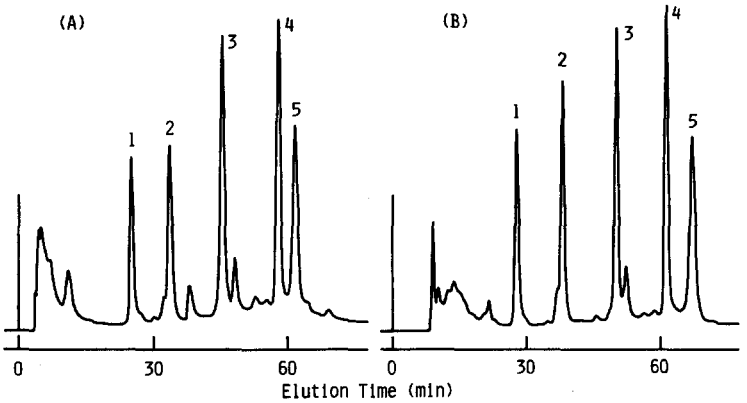

Fig. 3. Chromatograms of a protein mixture obtained on Phenyl-5PW analytical (A) and preparative (B) columns. The separation on the analytical column was performed with a 60-min linear gradient of ammonium sulphate concentration from 1.8 *M* to 0 in 0.1 *M* phosphate buffer (pH 7.0) at a flow-rate of 0.5 ml/min. In the separation on the preparative column, the flow-rate was 4 ml/min and the other conditions were as on the analytical column. Peaks: 1 = myoglobin; 2 = ribonuclease; 3 = lysozyme; 4 = α-chymotrypsinogen A; 5 = α-chymotrypsin.

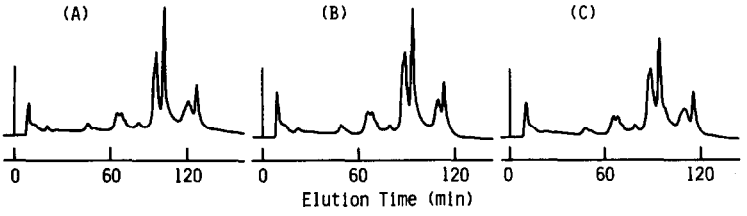

Fig. 4. Chromatograms of lipoxidase obtained on the Phenyl-5PW preparative column with sample loadings of 50 (A), 100 (B) and 200 mg (C).

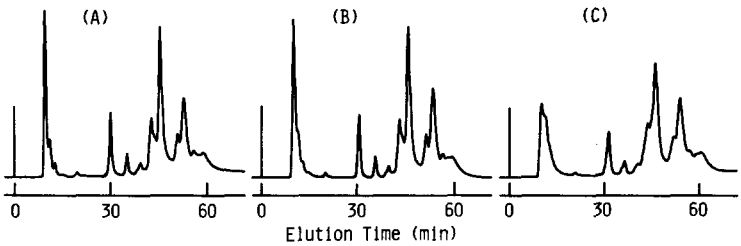

Fig. 5. Chromatograms of phosphoglucose isomerase obtained on the Phenyl-5PW preparative column. Sample loadings as in Fig. 4.

min. Because the separation time became longer and the dilution of samples increased almost in proportion to the gradient time, gradient times of 60–120 min are believed to be a good compromise.

Fig. 3 shows the chromatograms of a protein mixture obtained on analytical and preparative columns. As exemplified here, the separation efficiency attained on the preparative column at flow-rates of around 4 ml/min and gradient times of 60–120 min was equivalent to that attained on the analytical column under the appropriate conditions, although the support in the preparative column has a particle diameter 13 μm, larger than that in the analytical column (10 μm). Therefore, it is easy to scale-up separations obtained on the analytical column.

Sample loading capacity

Figs. 4 and 5 show chromatograms of lipoxidase and phosphoglucose isomerase obtained with various sample loadings. In the separation of lipoxidase, almost identical patterns were observed with sample loadings up to 200 mg. Phosphoglucose isomerase could be applied up to 100 mg without loss of separation effciency. In the case of lactate dehydrogenase, almost the same separations were obtained up to 50 mg (data not shown). Consequently, the maximum sample loadings of the Phenyl-5PW preparative column which permit the highest resolution are 50–200 mg depending on the sample. If a slight decrease in resolution is acceptable, much more sample can be applied.

Applications to enzyme purification

Fig. 6 shows a separation of 200 mg crude lipoxidase. The peak corresponding to lipoxidase between the two vertical lines was fractionated. The recovery of enzy-

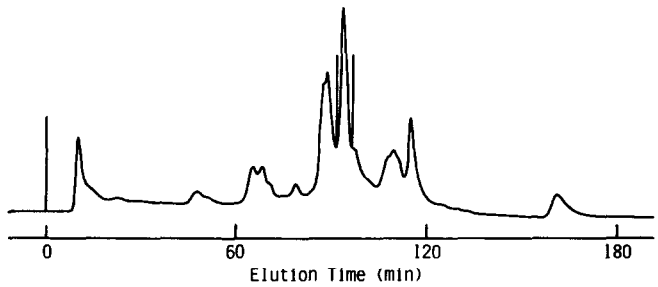

Fig. 6. Separation of 200 mg lipoxidase on the Phenyl-5PW preparative column.

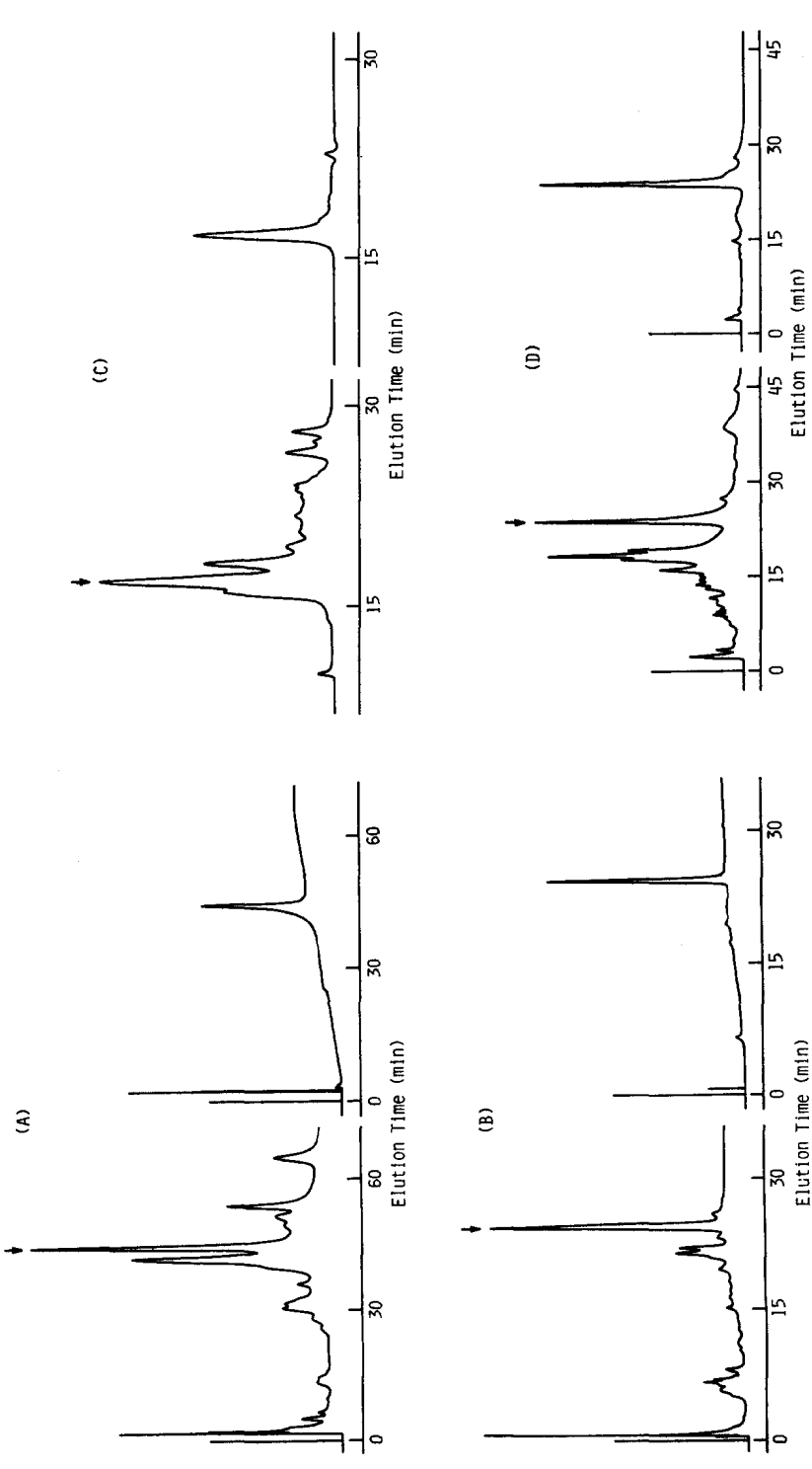

Fig. 7. Chromatograms of crude lipoxidase sample (left) and the fraction in Fig. 6 (right) obtained by hydrophobic interaction chromatography (A), reversed-phase chromatography (B), gel filtration (C) and ion-exchange chromatography (D). Peaks indicated by arrows in the chromatograms of the crude sample correspond to the main peaks of the fraction.

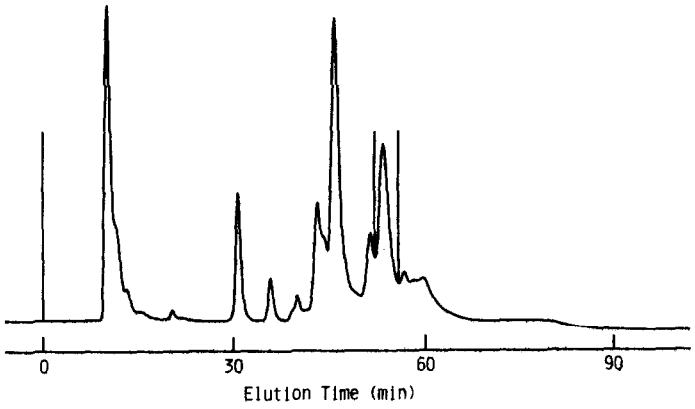

Fig. 8. Separation of 100 mg phosphoglucose isomerase on the Phenyl-5PW preparative column.

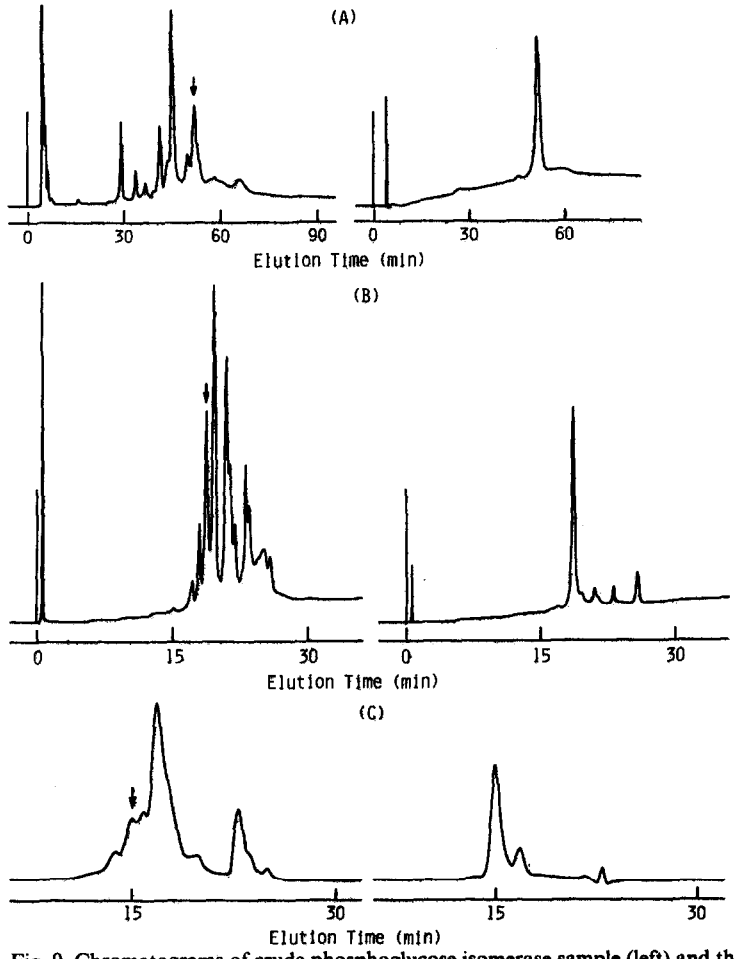

Fig. 9. Chromatograms of crude phosphoglucose isomerase sample (left) and the fraction in Fig. 8 (right) obtained by hydrophobic interaction chromatography (A), reversed-phase chromatography (B) and gel filtration (C). Peaks indicated by arrows in the chromatograms of the crude sample correspond to the main peaks of the fraction.

matic activity in the fraction was 86%. The degree of purification based on the specific activity was 5.6-fold. The results of purity tests of the fraction by HPLC are shown in Fig. 7. One major peak and several very 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 lipoxidase. This means that lipoxidase of high purity was obtained from commercial lipoxidase containing large amounts of impurities.

Fig. 8 shows a separation of 100 mg crude phosphoglucose isomerase. Enzymatic activity was found in several peaks: of the applied activity, 70% was found in the peak between the two vertical lines and 26% in three small peaks eluted between 55 and 60 min. The degree of purification was 3.7-fold for the fraction between the two vertical lines. Fig. 9 shows the results of purity tests of this fraction. The main peaks were confirmed to correspond to phosphoglucose isomerase by enzymatic activity test. This indicates that pure phosphoglucose isomerase was obtained.

Fig. 10 shows a separation of 54 mg crude lactate dehydrogenase. The peak corresponding to lactate dehydrogenase between the two vertical lines was fractionated. The recovery of enzymatic activity in the fraction was 93%. The degree of purification was 2.6-fold. The results of purity tests on the fraction are shown in Fig. 11. Lactate dehydrogenase of high purity was obtained.

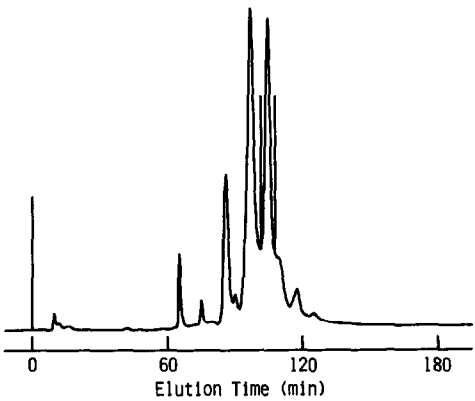

Fig. 10. Separation of 54 mg lactate dehydrogenase on the Phenyl-5PW preparative column.

As demonstrated above, a fairly high degree of purification was achieved with high yield in one hydrophobic interaction chromatography step on the Phenyl-5PW preparative column. Since this separation is rapid, it should also be useful for unstable samples. The sample loading capacity was 50–200 mg per injection. Substantially large amounts of samples can be treated by repeated injections, typically, *ca.* 1 g of crude sample in a day. Consequently, this hydrophobic interaction chromatography should be very useful for the purification of proteins.

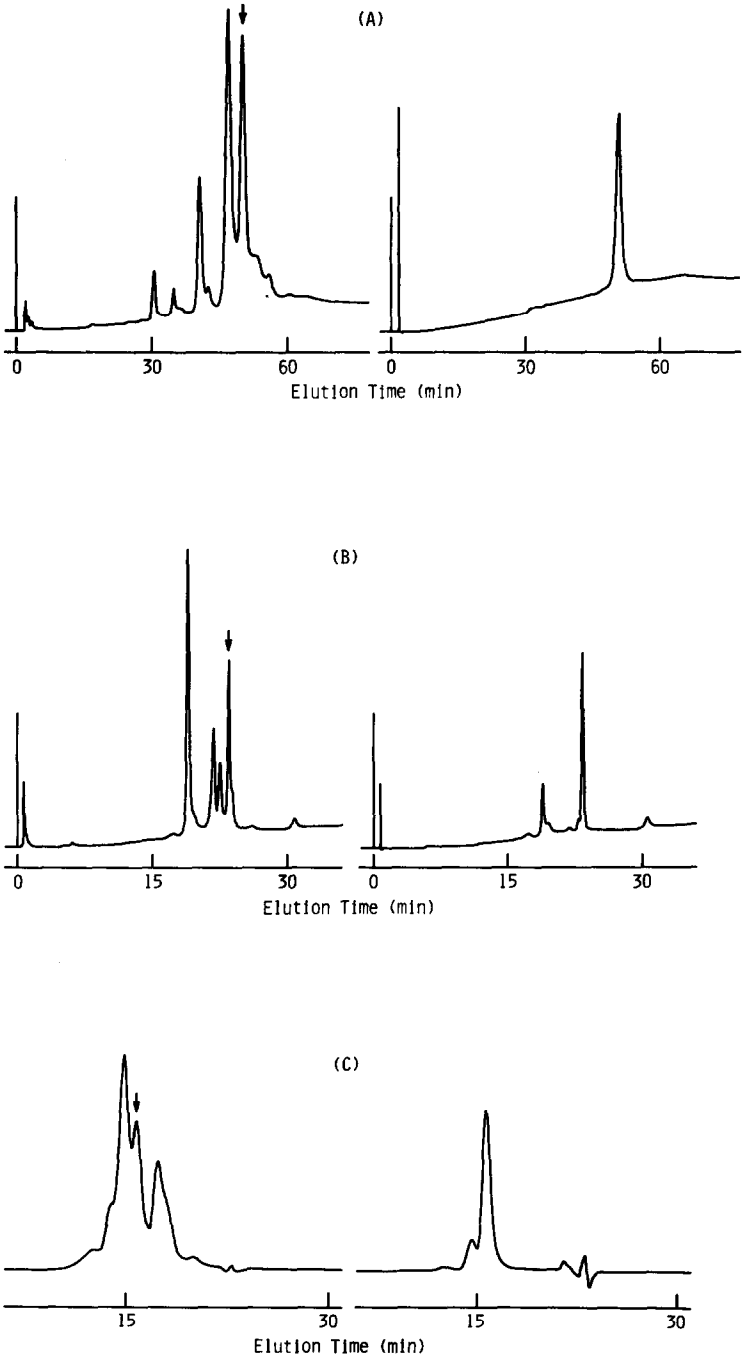

Fig. 11. Chromatograms of crude lactate dehydrogenase sample (left) and the fraction in Fig. 10 (right) obtained by hydrophobic interaction chromatography (A), reversed-phase chromatography (B) and gel filtration (C). Peaks indicated by arrows in the chromatograms of the crude sample correspond to the main peaks of the fraction.

REFERENCES

1 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 266 (1983) 49.
2 F. E. Regnier and J. Fausnaugh, *LC, Liq. Chromatogr. HPLC Mag.*, 1 (1983) 402.
3 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 292 (1984) 418.
4 D. L. Gooding, M. N. Schmuck and K. M. Gooding, *J. Chromatogr.*, 296 (1984) 107.
5 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 298 (1984) 407.
6 S. C. Goheen and S. C. Engelhorn, *J. Chromatogr.*, 317 (1984) 55.
7 W. R. Melander, D. Corradini and Cs. Horváth, *J. Chromatogr.*, 317 (1984) 67.
8 J. L. Fausnaugh, L. A. Kennedy and F. E. Regnier, *J. Chromatogr.*, 317 (1984) 141.
9 J. L. Fausnaugh, E. Pfannkoch, S. Gupta and F. E. Regnier, *Anal. Biochem.*, 137 (1984) 464.
10 N. T. Miller, B. Feibush and B. L. Karger, *J. Chromatogr.*, 316 (1985) 519.
11 J.-P. Chang, Z. E. Rassi and Cs. Horváth, *J. Chromatogr.*, 319 (1985) 396.
12 H. U. Bergmeyer, M. Grassl and H.-E. Walter, in H. U. Bergmeyer (Editor), *Methods of Enzymatic Analysis*, Vol. II, Verlag Chemie, Weinheim, 3rd ed., 1983, Ch. 2.2.1, p. 126.