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Note

High-performance affinity chromatography of proteins on TSKgel Heparin-5PW

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Heparin is a general ligand which interacts with various substances. Therefore, immobilized heparin has been widely used as an adsorbent of biological substances[1–8]. However, most separations have been performed at low speed using agarose derivatives. If this aspect were to be improved, this technique would become much more useful.

Very recently, a new support for high-performance affinity chromatography (HPAC) has become commercially available under the trade-name of TSKgel Heparin-5PW (TOSOH, Tokyo, Japan). According to the manufacturer, it is prepared by introducing heparin from porcine intestinal mucosa into TSKgel G5000PW[9], which is an hydrophilic polymer-based material of large pore size employed for high-performance gel filtration. Its basic properties and application to the purification of proteins and enzymes are described in this paper.

EXPERIMENTAL

Materials

Human antithrombin III was obtained from Green Cross (Osaka, Japan), Boc-Val-Pro-Arg-4-methyl-coumaryl-7-amide (MCA) from Peptide Institute (Osaka, Japan) and lipoprotein lipase (Pseudomonas) from Toyobo (Osaka, Japan). All other proteins were from Sigma (St. Louis, MO, U.S.A.).

Measurement of antithrombin III activity

The antithrombin III activity was measured with a Testzym AT-III kit (Daiichi Pure Chemicals, Tokyo, Japan).

Measurement of thrombin activity

Thrombin activity was measured with Boc-Val-Pro-Arg-MCA as a substrate essentially according to the method of Morita *et al.*[10].

Measurement of lipoprotein lipase activity

Lipoprotein lipase activity was measured according to the method of Okuda and Fujii[11].

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Determination of adsorption capacity for human antithrombin III

The adsorption capacity of TSKgel Heparin-5PW for human antithrombin III was determined by the amount of human antithrombin III adsorbed on 10 mm × 4.6 mm I.D. column equilibrated with 0.02 *M* Tris–HCl buffer (pH 7.5) containing 0.15 *M* sodium chloride. The equilibrated column was saturated with human antithrombin III, dissolved in the buffer used for the equilibration. The excess of human antithrombin III was removed with the same buffer. Desorption of human antithrombin III was achieved by 0.02 *M* Tris–HCl buffer (pH 7.5) containing 1.5 *M* sodium chloride. The amount of protein in the eluate was calculated from the volume and the absorption at 280 nm.

Affinity chromatography on TSKgel Heparin-5PW

A TSKgel Heparin-5PW column (75 mm × 7.5 mm I.D.) with a particle size of 10 μm and an average pore size of 1000 Å (Tosoh) was used for all HPAC experiments. All chromatographic measurements were performed at 25°C with a CCPM pump (TOSOH) equipped with a variable-wavelength UV detector Model UV-8000 (TOSOH) operated at 280 nm.

RESULTS AND DISCUSSION

The TSKgel Heparin-5PW had the following properties. The amount of heparin covalently bound to TSKgel G5000PW was 5 mg per ml wet gel according to elemental analysis. The adsorption capacity for human antithrombin III was 2.5 mg per ml wet gel. This value was not changed after 6 months of use, indicating that the background bleed of heparin from the column was negligible.

Heparin binds selectively to many blood proteins such as thrombin, plasmin and antithrombin III[3,12]. Fig. 1 shows the purification of human antithrombin III

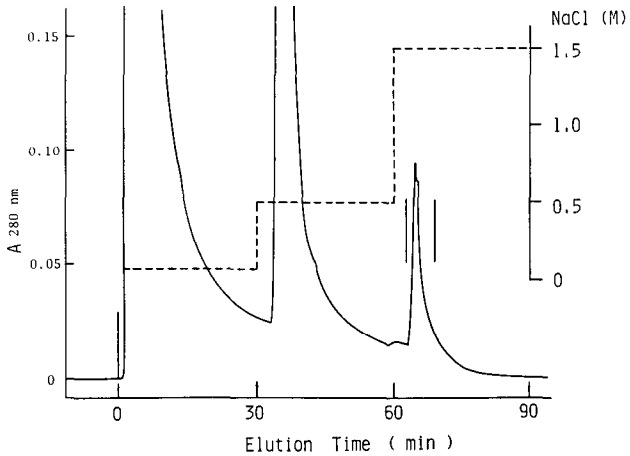

Fig. 1. Chromatogram of human plasma on TSKgel Heparin-5PW. Eluents: A, 0.02 *M* Tris–HCl + 0.15 *M* sodium chloride (pH 7.5); B, 0.02 *M* Tris–HCl + 1.5 *M* sodium chloride (pH 7.5); gradient, dotted line; flow-rate, 1.0 ml/min. Sample: human plasma (0.5 ml). The column effluent was collected between the two vertical lines.

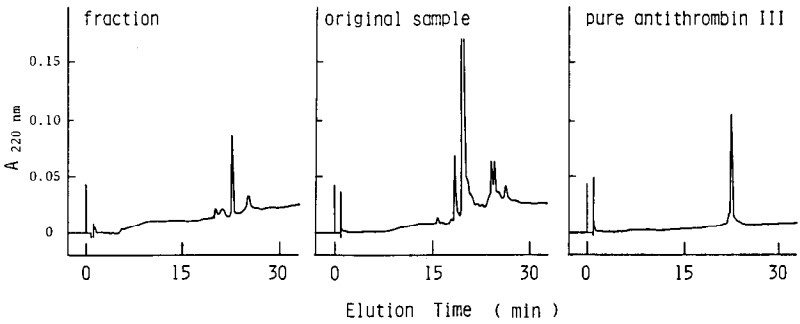

Fig. 2. Reversed-phase chromatograms of the fraction (100 μl), the original sample (human plasma, 2 μl) and pure antithrombin III (20 μl, 500 μg/ml). The samples were separated on TSKgel Phenyl-5PW RP (75 mm × 4.6 mm I.D.) with a 2-min linear gradient from 5 to 20% acetonitrile followed by a 48-min linear gradient from 20 to 80% acetonitrile in 0.05% trifluoroacetic acid at a flow-rate of 1 ml/min at 25°C, and detected at 220 nm.

from human plasma on TSKgel Heparin-5PW. The peak eluted at *ca.* 65 min was confirmed to be antithrombin III by the activity test. The column effluent was collected between the two vertical lines indicated in Fig. 1. The recovery of antithrombin III activity was quantitative. The fraction was subjected to reversed-phase chromatography to check the purity. Fig. 2 shows the reversed-phase chromatograms of the fraction, the original sample and pure antithrombin III obtained by TSKgel Phenyl-5PW RP. The fraction contains small amounts of impurities in addition to antithrombin III.

Fig. 3 shows a separation of commercial bovine thrombin on TSKgel Heparin-5PW which reveals many impurities. The peak eluted at *ca.* 20 min was confirmed to be thrombin by a thrombin activity test. The column effluent was collected between the two vertical lines indicated in Fig. 3. The recovery of thrombin activity

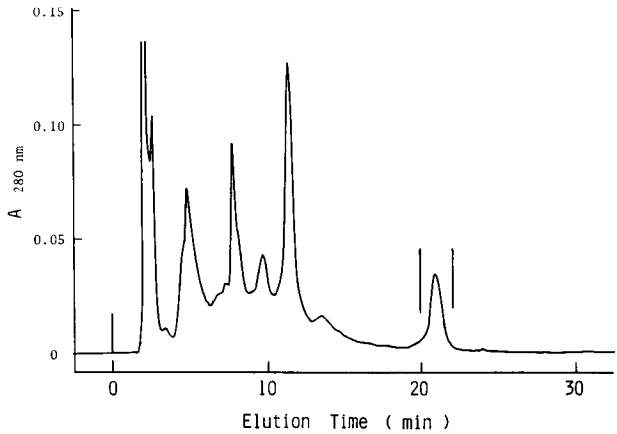

Fig. 3. Chromatogram of bovine thrombin (100 μl, 20 mg/ml) on TSKgel Heparin-5PW with a 30-min linear gradient from 0 to 1.0 *M* sodium chloride in 0.02 *M* phosphate buffer (pH 7.5) at a flow-rate of 1 ml/min. The column effluent was collected between the two vertical lines.

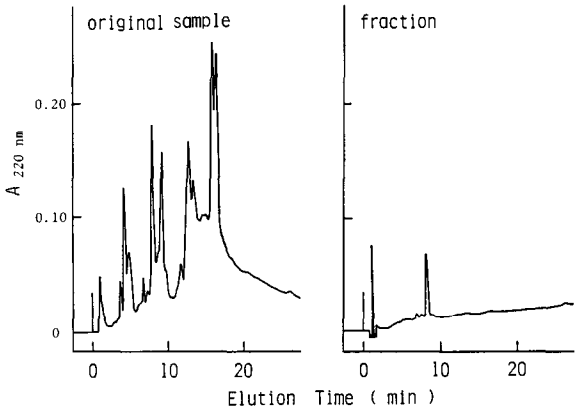

Fig. 4. Reversed-phase chromatograms of the original sample (10 μl, 20 mg/ml) and the fraction (100 μl). Conditions as in Fig. 2.

was 87%. The fraction was subjected to reversed-phase chromatography to check the purity. Fig. 4 shows the reversed-phase chromatograms of the fraction and the original sample. Fig. 4 indicates that the purity of thrombin is fairly high. Figs. 1 and 3 indicate that TSKgel Heparin-5PW was successfully used for the purification of antithrombin III and thrombin under the same conditions as for the conventional immobilized heparin adsorbents.

Fig. 5 shows a separation of commercial lipoprotein lipase on TSKgel Heparin-5PW. Three isozymes can be recognized, eluted at *ca.* 2, 7 and 14 min, respectively, by an enzymatic activity test. Each column effluent was collected between the two vertical lines indicated in Fig. 5. The total recovery of lipoprotein lipase activity was quantitative. Although the exact interaction between heparin and lipoprotein lipase is not known, some workers[13–15] have suggested that intravenous injections of heparin in the rat, pig and man cause the release of two lipoprotein lipases which differ ion their sensitivity to sodium chloride.

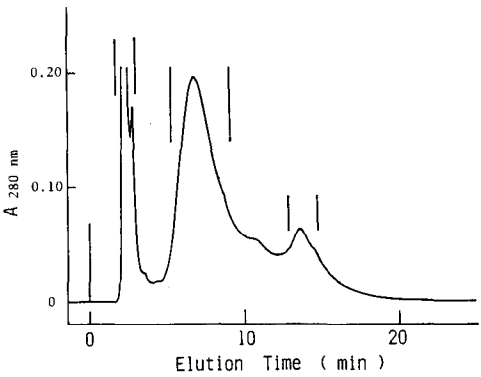

Fig. 5. Chromatogram of commercial lipoprotein lipase (100 μl, 20 mg/ml) on TSKgel Heparin-5PW with a 60-min linear gradient from 0 to 1.0 *M* sodium chloride in 0.02 *M* phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min. Each column effluent was collected between the two vertical lines.

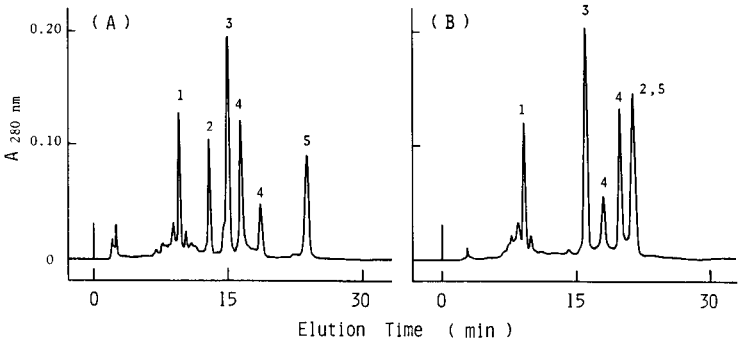

Fig. 6. Chromatograms of standard protein mixtures on (A) TSKgel SP-5PW and (B) TSKgel Heparin-5PW with a 60-min linear gradient from 0 to 1.0 *M* sodium chloride in 0.02 *M* phosphate buffer (pH 7.0) at a flow-rate of 1 ml/min. Samples: 1 = trypsinogen (0.2 mg); 2 = ribonuclease (0.4 mg); 3 = α-chymotrypsinogen A (0.2 mg); 4 = cytochrome *c* (0.2 mg); 5 = lysozyme (0.1 mg). Injection volume: 100 μl.

Fig. 6 shows the separation of standard mixtures containing trypsinogen, ribonuclease, α-chymotrypsinogen A, cytochrome *c* and lysozyme on TSKgel SP-5PW (cation exchanger) and Heparin-5PW under the same conditions. As heparin is polyanionic, it interacts with many basic proteins like a cation exchanger. Fig. 6 indicates that the elution order of ribonuclease on Heparin-5PW is different from that on SP-5PW, probably due to enzyme–inhibitor interactions[16].

The purification of RNA polymerase on TSKgel Heparin-5PW has been reported in detail elsewhere[17].

As demonstrated above, TSKgel Heparin-5PW was useful for the purification of proteins. Proteins which have affinity towards heparin were separated rapidly with high resolution and recovery under the same conditions as with the conventional immobilized heparin adsorbents.

REFERENCES

1 P. W. Gentry and B. Alexander, *Biochem. Biophys. Res. Commun.*, 50 (19973) 500.
2 K. Fujikawa, A. R. Thompson, M. E. Legaz, R. G. Meyer and E. W. Davie, *Biochemistrry*, 12 (1973) 4938.
3 M. Miller-Andersson, H. Borg and L. Andersson, *Thromb. Res.*, 5 (1974) 439.
4 W. Kisiel and E. W. Davie, *Biochemistry*, 14 (1975) 44928.
5 T. Olivecrona and T. Egerud, *Biochem. Biophys. Res. Commun.*, 43 (1973) 524.
6 S. Sakamoto, M. Skamoto, P. Goldhaber and M. J. Glimcher, *Arch. Biochem. Biophys.*, 188 (1978) 439.
7 N. Ototani and Z. Yoshizawa, *J. Biochem. (Tokyo)*, 84 (1978) 1005.
8 D. P. Buhrer and A. A. Gottlieb, *Anal. Biochem.*, 87 (1978) 411.
9 T. Hashimoto, H. Sasaki, M. Aiura and Y. Kato, *J. Polym. Sci., Polym. Phys. Ed.*, 16 (1978) 1789.
10 T. Morita, H. Kato, S. Iwanaga, T. Kimura, T. Takada and S. Sakakibara, *J. Biochem. (Tokyo)*, 82 (1977) 1495.
11 H. Okuda and S. Fujii, *J. Biochem. (Tokyo)*, 64 (19678) 344.
12 J. Ehrlich and S. S. Stivala, *J. Pharm. Sci.*, 62 (1973) 517.
13 C. J. Fielding, *Biochim. Biophys. Acta*, 280 (1972) 569.
14 H. Jansen and W. C. Hulsmann, *Biochim. Biophys. Acta*, 369 (1974) 387.
15 C. H. Ehnhoim, H. Greten and W. V. Brown, *Biochim. Biophys. Acta*, 369 (1974) 68.
16 R. F. Cox, *Eur. J. Biochem.*, 39 (1973) 49.
17 J. M. Egly, *EMBO J.*, in press.