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Application of a β-cyclodextrin sulfate-immobilized precolumn to selective on-line enrichment and separation of heparin-binding proteins by column-switching high-performance liquid chromatography

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Abstract

A column-switching high-performance liquid chromatography (HPLC) system which consisted of a β-cyclodextrin (β-CD) sulfate-immobilized hydrophilic vinyl–polymer gel precolumn and a reversed-phase analytical column was developed for the selective on-line enrichment and separation of heparin-binding proteins. Of 15 proteins investigated, 10 proteins having heparin-binding activity were retained on the β-CD sulfate precolumn almost quantitatively, in contrast 5 proteins having no heparin-binding activity were not retained. Calibration graphs for basic fibroblast growth factor constructed at various sample volumes were nearly identical, indicating that the protein could be enriched by this system. The system was successfully used for the selective separation of lysozyme in egg white. The β-CD sulfate-immobilized precolumn showed no loss of analytical performance over 2 years during which about 400 samples were analysed.

Keywords: Column switching; Sample preparation; Stationary phases, LC; Proteins; Heparin

1. 1. Introduction

Column switching is a powerful technique in high-performance liquid chromatography (HPLC) for the separation of compounds of interest in various sources. For the analysis of polypeptides and proteins that have a great number of isomers, the technique is an especially good candidate to give better resolution. So far, reversed-phase and immunoaffinity precolumns have been used for the column-switching HPLC analysis of polypeptides and proteins [1–7]. The immunoaffinity method offers highly selective retention, but the preparation of antibody for each analyte is time consuming.

Cyclodextrin (CD) sulfates, obtained by partial sulfation of hydroxyl groups of CDs, show unique biological activities similar and sometimes superior to those of heparin [8–10], and the mixture of insoluble sulfated β-CD polymer and copper-loaded chelating Sepharose has been used as a selective adsorbent for the isolation of heparin-binding proteins, such as fibroblast growth factors (FGFs) [11].

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Recently, we have developed a β-CD sulfate-immobilized hydrophilic vinyl–polymer gel, which was superior in recovery and stability to a heparin-immobilized gel for the high-performance affinity chromatographic separation of heparin-binding proteins such as growth factors, enzymes, coagulation factors and lipoproteins from the other [12]. The excellent characteristics of the β-CD sulfate-immobilized column with regard to separation speed and stability prompted us to investigate the possibility that it might be used as a precolumn in column-switching HPLC.

In this study, we developed a column-switching HPLC system in which proteins retained on the β-CD sulfate-immobilized hydrophilic vinyl–polymer gel precolumn were selectively transferred to and separated on a reversed-phase analytical column. The retention efficiency of various proteins on the β-CD sulfate-immobilized precolumn was examined by using the system. It was also applied to the determination of lysozyme in egg white.

2. Experimental

2.1. Materials

Recombinant human basic FGF (bFGF) was obtained from Scios Nova (CA, USA). Bovine serum albumin (Fraction V) (BSA), bovine serum carbonic anhydrase, bovine pancreas insulin, horse myoglobin and bovine serum thrombin were from Sigma (St. Louis, MO, USA). Bovine plasma fibronectin, chicken egg white lysozyme, chicken egg white ovalbumin and salmon roe protamine sulfate were from Wako (Osaka, Japan). *Pseudomonas* sp. lipoprotein–lipase was from Toyobo (Osaka, Japan). Bovine testes hyaluronidase was from Seikagaku Kougyo (Tokyo, Japan). Bovine serum β-lipoprotein and bovine plasma vitronectin were from Yagai (Yamagata, Japan). Clostridium histolyticum collagenase was from Boehringer Mannheim (Tokyo, Japan). The stock solutions of these proteins were prepared in water and stored at −20°C until use. The sodium salt of β-CD sulfate (Na·β-CD sulfate) was prepared according to the method of Folkman et al. [8]. The average degree of sulfate groups per Na·β-CD sulfate molecule was confirmed to be 10.7 by fast-atom bombardment mass spectrometry and elemental analysis [13]. TSK gel AF-Epoxy Toyopearl 650M and TSKgel AF-Heparin Toyopearl 650M (heparin content, 5 mg/ml gel) were from Tosoh (Tokyo, Japan). Chicken eggs were obtained from a supermarket, and the egg white was diluted 10-fold with water and subjected to the column-switching HPLC system. Other chemicals were of analytical-reagent grade. Deionized water (obtained with a Millipore RO-Q system) was used throughout.

2.2. β-CD sulfate-immobilized precolumn

A β-CD sulfate-immobilized hydrophilic vinyl–polymer gel was prepared by shaking TSK gel AF-Epoxy Toyopearl 650M in alkaline solution containing Na·β-CD sulfate, as described previously [12]. The amount of β-CD sulfate immobilized onto the gel was 1.5 mg/ml gel. This gel was slurry-packed in a stainless steel chromatographic tube (10×6.0 mm I.D.). The column was stored in water at 4°C when not in use.

2.3. Column-switching HPLC

The set-up of the column-switching HPLC system used is shown schematically in Fig. 1. A Model LC-6A pump (P1) (Shimadzu, Kyoto, Japan) was used for sample loading at a flow-rate of 1 ml/min with water (S1) as the eluent. Protein solution was injected onto the β-CD sulfate precolumn (C1) through a Model 7125 injector (Rheodyne, Cotati, CA, USA) equipped with a 0.5-ml loop. After the injection, the precolumn was washed with S1 for 5 min, and then 0.5 ml of 20 m*M* Tris–HCl buffer (pH

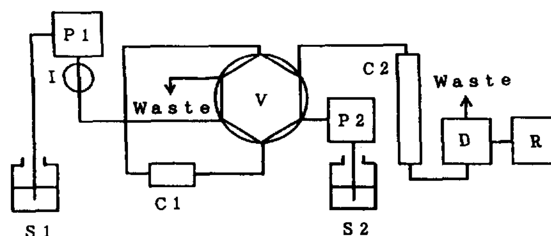

Fig. 1. Schematic diagram of the column-switching HPLC system. P1 and P2=pumps; C1=precolumn; C2=analytical column; I=injector; S1 and S2=eluents; V=switching valve; D=detector; R=integrator.

7.5), containing 60 or 180 mM NaCl, was injected twice at an interval of 2 min to eliminate non-specific adsorption of proteins. The precolumn was further washed with S1 for 5 min. The switching valve, a Model NHV-7000-6M (Nihon Seimitsu Kagaku, Tokyo, Japan), was then changed from 'load' to 'inject'. Another Model LC-6A pump (P2) was used for flushing the retained protein from the precolumn and for successive isocratic elution from an analytical column (C2) (YMC-Pak ODS-AP; 250×4.6 mm I.D.) at a flow-rate of 1 ml/min with acetonitrile–10 mM H_3PO_4 (15:85–45:55, v/v) containing 100–700 mM NaCl (S2) as eluents. This is the so-called back-flushing mode [14]. For the next run, the valve was changed, and C1 was re-equilibrated with S1, for at least 10 min. The column eluent was monitored at 215 nm with an SPD-6A detector (Shimadzu) connected to a Chromatopac C-R6A integrator (Shimadzu). The chromatographic runs were undertaken at room temperature.

The percentage retention of each protein on the β-CD sulfate precolumn was calculated by dividing the peak area obtained with the precolumn by that obtained by injecting 50 μl of solution containing the corresponding amount of the protein directly on to the analytical column.

The deactivated TSKgel AF-Epoxy Toyopearl 650M gel (the gel was treated with 1 M ethanolamine solution) and TSK gel AF-Heparin Toyopearl 650M were slurry-packed in stainless steel chromatographic tubes (10×6.0 mm I.D.). These precolumns were used under the same conditions for comparison with the β-CD sulfate precolumn.

2.4. Measurement of lysozyme activity

Lysozyme activity was measured according to the method of Smolelis and Hartsell [15].

3. Results and discussion

3.1. Optimization of the column-switching HPLC system

A preliminary study showed that certain proteins were non-specifically retained on the deactivated epoxy–gel precolumn. In order to eliminate such non-specific binding, the precolumn was washed with 20 mM Tris–HCl buffer (pH 7.5) containing various concentrations of NaCl. After loading, a sample was examined using lysozyme and bFGF. The non-specific binding was eliminated completely by the washing with the buffer containing NaCl over 60 mM. On the other hand, almost quantitative retention of the protein was obtained at least up to 180 mM when the β-CD sulfate precolumn was used.

The effect of S1 flow-rate on the retention of bFGF on the β-CD sulfate precolumn was examined at two doses, 5 nmol per 50- and 500-μl injection. Almost quantitative retention (99.6–100.5%) was observed at both doses at flow-rates of 0.5–1.5 ml/min. In the following study, a flow-rate of 1 ml/min was used.

3.2. The retention of various proteins

Table 1 shows the retentions of various proteins on the β-CD sulfate and heparin precolumns after washing with 20 mM Tris–HCl buffer (pH 7.5) containing 60 or 180 mM NaCl. All of the heparin-binding proteins, bFGF, collagenase, fibronectin, hyaluronidase, β-lipoprotein, lipoprotein–lipase, lysozyme, protamine, thrombin and vitronectin, were retained almost quantitatively on the β-CD sulfate precolumn after washing with 60 and 180 mM NaCl in 20 mM Tris–HCl buffer (pH 7.5). On the other hand, the retentions of several heparin-binding proteins on the heparin precolumn were decreased markedly at a higher concentration of NaCl. Five proteins having no heparin-binding activity, BSA, carbonic anhydrase, insulin, myoglobin and ovoalbumin were very hardly retained on the β-CD sulfate and heparin precolumns. All these proteins examined showed no or only a little retention to the deactivated epoxy precolumn. These results indicate that the β-CD sulfate precolumn selectively retains the heparin-binding proteins and excels the heparin precolumn in retention efficiency. The higher retention efficiency of the β-CD sulfate precolumn appears to be attributed to its higher charge density, apparently because of spatial constraints imposed on the sulfates by the heptasaccharide ring structure [16].

Table 1
Retention of various proteins on the β-CD sulfate and heparin precolumns

Protein[a]	Retention (%)[b]			
	β-CD sulfate precolumn		Heparin precolumn	
	60 mM[c]	180 mM[c]	60 mM[c]	180 mM[c]
bFGF	101	100	101	99
Collagenase	97	88	78	56
Fibronectin	102	92	102	92
Hyaluronidase	97	90	88	62
β-Lipoprotein	99	96	99	96
Lipoprotein-lipase	98	90	85	60
Lysozyme	100	102	100	101
Protamine	101	101	101	97
Thrombin	99	99	98	100
Vitronectin	98	96	98	94
BSA	5	0	9	0
Carbonic anhzydrase	0	0	0	0
Insulin	0	0	0	0
Myoglobin	2	0	2	0
Ovoalbumin	4	0	9	0

[a] 5 nmol per 50-μl injection.
[b] Calculated on the basis of peak area.
[c] Concentration of NaCl in 20 mM Tris–HCl buffer (pH 7.5).

3.3. Separation of a model protein mixture

Fig. 2 shows the separation of a model protein mixture by the present column-switching HPLC system. The heparin-binding proteins were retained almost quantitatively on the β-CD sulfate and heparin precolumns, and other proteins were completely removed. Better resolution was obtained with the β-CD sulfate precolumn: the peak broadening and tailing observed for the heparin precolumn were clearly suppressed by the use of the β-CD sulfate precolumn (Fig. 2). The resolutions (R_s) of the retained peaks were thus 4.8 and 3.4 for the β-CD sulfate and heparin precolumns, respectively. Similar results were also observed for other heparin-binding proteins: the R_s for β-lipoprotein and thrombin was increased from 3.4 to 4.5 (chromatograms not shown). These phenomena are likely to be attributed to slow dissociation of the solute–ligand complex in the heparin precolumn [17]. Therefore, the β-CD sulfate precolumn is more useful for the selective separation of heparin-binding proteins.

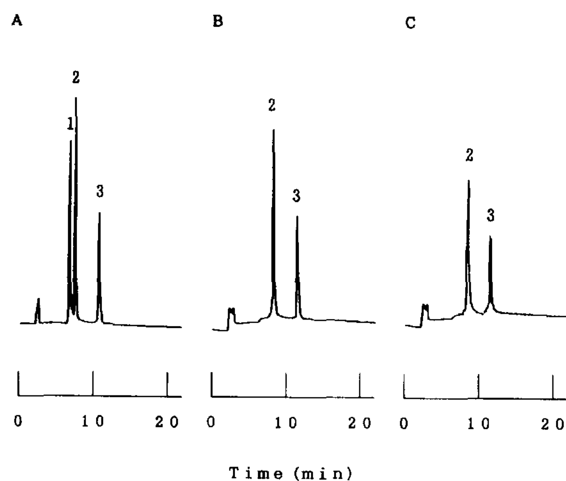

Fig. 2. HPLC separation of a model protein mixture. (A) Without precolumn; (B) with the β-CD sulfate precolumn; (C) with the heparin precolumn. Eluent S2, CH_3CN–10 mM H_3PO_4 (35:65, v/v) containing 500 mM NaCl. Washing solvent, 20 mM Tris–HCl buffer (pH 7.5) containing 180 mM NaCl. Peaks: 1=insulin; 2=lysozyme; 3=bFGF.

3.4. Enrichment

The efficiency of enrichment for lysozyme was evaluated on the basis of the linearity and slope of calibration graphs constructed at three sample vol-

umes (0.6–30 nmol per 50-, 250- and 500-μl injection). The linear relationships (r=0.9991–0.9999) between the peak area and the injected amount and the good reproducibility (relative standard deviations (n=6) for 0.6, 15.3 and 30 nmol=0.3–3.0%) were observed at any sample volume. The slope of the graph decreased with increase in sample volume, but the slope obtained with 500-μl injections was still about 92% of that with 50-μl injections, indicating that the protein could be enriched by this system.

3.5. Selective separation of lysozyme in chicken egg white

Fig. 3 shows the application of the present column-switching HPLC system for the determination of lysozyme in chicken egg white. Lysozyme content was 4.1±0.15% (n=3), and coincided with the report of Canfield and Liu [18]. The lysozyme peak was also confirmed by its enzyme activity [15]: The column effluent between the two vertical lines indicated in Fig. 3 was collected, and the recovery of lysozyme activity was 95%.

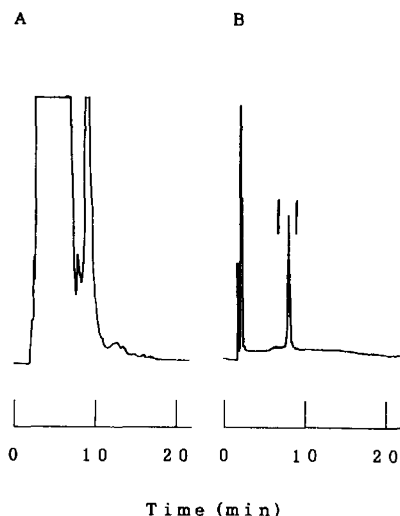

Fig. 3. HPLC separation of lysozyme in chicken egg white. (A) Without precolumn; (B) with the β-CD sulfate precolumn. Eluent S2, CH_3CN–10 mM H_3PO_4 (35:65, v/v) containing 500 mM NaCl. Washing solvent, 20 mM Tris–HCl buffer (pH 7.5) containing 180 mM NaCl. Sample, 50 μl of the diluted chicken egg white solution. The column effluent was collected between the two vertical lines.

3.6. Stability of the β-CD sulfate precolumn

We determined the stability of the β-CD sulfate and heparin precolumns by measuring the retentions of several heparin-binding proteins after various periods of operation. The β-CD sulfate precolumn showed no decrease in retention after about 400 cycles operation during 2 years; the retentions of bFGF, lyozyme and thrombin 2 years after the preparation of the precolumn were 100, 99 and 97%, respectively. On the other hand, the heparin precolumn showed no decrease in retention after about 100 cycles operation during 1 year, but the retentions of the above three proteins after additional 50 cycles operation during 6 months were 78, 65 and 46%, respectively.

4. Conclusions

The β-CD sulfate precolumn was used successfully in the column-switching HPLC system for selective enrichment and separation of heparin-binding proteins. It excels the heparin precolumn in retention efficiency, rapid dissociation of the solute–ligand complex and stability. A preliminary study for determination of lysozyme in egg white showed that the β-CD sulfate precolumn would be useful for on-line pretreatment in the HPLC determination of heparin-binding proteins in biological fluids.

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