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PURIFICATION OF CHONDROITINASE B AND CHONDROITINASE C USING GLYCOSAMINOGLYCAN-BOUND AH-SEPHAROSE 4B*†

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ABSTRACT

Chondroitinase B and chondroitinase C were separated from an extract of *Flavobacterium heparinum* induced with chondroitin 6-sulfate by using column chromatography on hydroxylapatite. Chondroitinase C was eluted together with the activities of hyaluronidase, Δ4,5glycosiduronase, and sulfatase. The latter two activities were eliminated exclusively by passing the crude chondroitinase C fraction through a phosphono-cellulose column pre-equilibrated with 0.07M sodium phosphate buffer (pH 6.8). Chondroitinase C was then purified by affinity chromatography using dermatan sulfate-bound AH-Sepharose 4B coated with the same glycosaminoglycan. Purification of the enzyme was achieved 18-fold and in 73% yield. On the other hand, the activities of Δ4,5glycosiduronase and sulfatase were decreased to 50 and 60%, respectively, as compared with those in the crude chondroitinase B fraction, after passing the fraction through a column of phosphono-cellulose pre-equilibrated with 0.1M sodium phosphate buffer (pH 6.8). The remaining activities of these two enzymes were then eliminated from chondroitinase B by affinity chromatography with heparin-bound AH-Sepharose 4B coated with dermatan sulfate. In the affinity chromatography used in the present study, non-covalent coating of the glycosaminoglycan-bound (covalently) AH-Sepharose 4B with the same or another glycosaminoglycan was found to be important.

INTRODUCTION

Michelacci and Dietrich isolated chondroitinase B and chondroitinase C from an extract of *Flavobacterium heparinum* using agarose preparative electrophoresis[1–3], and Linker and Hovingh, using hydroxylapatite column-chromatography[4,5], purified heparinase and heparitinase from the same microbe induced with heparin.

*Dedicated to Professor Roy L. Whistler.

†Abbreviations: ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; ΔDi-OS, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose; Δ4,5glycosiduronase, a specific hydrolase for the 4-deoxy-α-L-*threo*-hexenopyranosyluronic acid linkage.

In the course of an enzymic study on whale heparin using heparinase[6], we found that chondroitinase B and chondroitinase C were separated in good yields from the foregoing enzyme by column chromatography on hydroxylapatite[7]. Therefore, we applied the same method to the separation of the enzymes from the microbe inducible with chondroitin 6-sulfate. Moreover, as chondroitinase B and chondroitinase C were extremely sensitive to such ion-exchangers as "phosphono-cellulose" (cellulose phosphate), a novel device of affinity chromatography for their purification was achieved. The device was based on the procedure[8] for isolation of antithrombin III by heparin-bound AH-Sepharose 4B, as well as on the fact that some glycosaminoglycans interact[9] with glycosaminoglycan-bound AH-Sepharose 4B.

EXPERIMENTAL

Materials. — Chondroitin 4-sulfate, chondroitin 6-sulfate, chondroitinase ABC, AH-Sepharose 4B, phosphono-cellulose (0.99 meq/g), ΔDi-4S, ΔDi-6S, and ΔDi-OS were obtained from Seikagaku Kogyo Co., Tokyo. Standard specimens of chondroitin 4-sulfate and chondroitin 6-sulfate were kindly supplied by Dr. M. B. Mathews. The supplementary data for the specimens of the unsaturated disaccharide components indicated that the ratios of ΔDi-4S, ΔDi-6S, and ΔDi-OS were 92, 6, and 2%, respectively, in the former, and 15, 74, and 11%, respectively, in the latter. The data were also confirmed in our laboratory. Dermatan sulfate used in the routine assay was a specimen obtained previously from whale mucosa[10]. Porcine mucosal heparin was obtained from Wilson Laboratory, Chicago. Hyaluronic acid from human umbilical cord was prepared by the method of Rodén *et al.*[11]. Water-soluble carbodiimide, 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDC), was purchased from Nakarai Chemicals, Kyoto. Hydroxylapatite was made by the procedure of Bernardi[12]. Other materials were commercial products.

Assay for chondroitinase B, chondroitinase C, and hyaluronidase. — Assay[7] for chondroitinase B, chondroitinase C, or hyaluronidase was performed as follows. An incubation mixture of an enzyme solution (0.02 mL), 0.3M Tris-HCl buffer (pH 8.0) containing 0.25M sodium acetate and 0.1% bovine serum albumin (0.03 mL), and a substrate solution (1.0 mg in 0.1 mL of water) was incubated for 1 h at 37°. Reaction was terminated by heating the mixture for 2 min in a boiling-water bath. To the mixture was added 0.06M hydrochloric acid (2.5 mL), and then the mixture was centrifuged at 10,000 *g* for 15 min. Absorbance at 232 nm of the supernatant was measured against the corresponding blank. Assay for the enzyme in each fraction obtained by column chromatography was performed as follows. An aliquot (0.2–0.4 mL) of each fraction was added to an incubation mixture composed of a substrate (1 mg in 0.1 mL of water) and the foregoing buffer (0.05–0.1 mL) (final concentration of Tris, 0.06M), followed by the same treatment as before. One unit of enzyme was defined as the quantity that catalyzed the decomposition of dermatan sulfate, chondroitin 6-sulfate, or hyaluronic acid to give 1 μmol of unsaturated oligosaccharides per h based on its millimolar absorption coefficient of 5.5. To estimate the chondroiti-

nase B activity in the crude extract, an alternative method was adapted according to the method of Hascall *et al.*[13] as follows[7]. To the incubation mixture obtained by the same procedures as before was added 0.25 mL of 0.04M sodium metaperiodate in 0.08M sulfuric acid, and the mixture was kept for 1 h at 37°. Subsequently, 0.5 mL of 3% sodium arsenite in 0.5M hydrochloric acid was added to the solution with vigorous stirring. The mixture was kept for 30 min at room temperature. Finally, 4 mL of 0.3% aqueous thiobarbituric acid was added to the mixture. The resulting mixture was heated for 15 min in a boiling-water bath and then cooled in ice–water. After 20 min at room temperature, the absorbance at 549 nm was determined against the corresponding blank. A linear relationship between the quantity, up to 0.4 μmol of ΔDi-4S, and its optical density at 549 nm was obtained.

Assay for Δ4,5glycosiduronase. — Assay for Δ4,5-glycosiduronase was performed as follows: A mixture of an enzyme solution (0.4 mL), 0.4M sodium acetate (pH 7.0, 0.1 mL) and a solution of ΔDi-OS (0.06 μmol in 0.06 mL of water) was incubated for 1 h at 37°. After heating the mixture for 3 min in a boiling-water bath, 0.06M hydrochloric acid (2.5 mL) was added to the mixture, and the mixture was centrifuged at 10,000*g* for 15 min. Absorbance at 232 nm of the supernatant was measured against the corresponding blank. In the present study, one unit of Δ4,5glycosiduronase was defined as the quantity that catalyzed the hydrolysis of 1 μmol of ΔDi-OS per h under the foregoing conditions.

Assay for sulfatase. — Assay for sulfatase was performed as follows: A mixture of an enzyme solution (0.4 mL), 0.4M sodium acetate (pH 7.0, 0.1 mL) and a solution of ΔDi-4S (0.2 μmol in 0.05 mL of water) was incubated for 1 h at 37°, and then 0.1 mL of 4.95% aqueous borate was added to the incubation mixture. After heating the mixture for 3 min in a boiling-water bath, the 4-dimethylaminobenzaldehyde reagent (3 mL) was added to the solution, and the mixture was kept for 20 min at 37°, according to the Morgan–Elson procedure of Reissig *et al.*[14]. The same examination without enzyme was performed as a blank. Also, a mixture of a solution of ΔDi-OS (0.1 μmol in 25 μl of water), the same buffer (0.1 mL), and an enzyme solution (0.4 mL) was heated for 3 min in a boiling-water bath, and then treated as a reference by the same procedures as just described. Finally, absorbance at 544 nm of the foregoing assay-solution was measured against the blank. One unit of sulfatase was defined as the quantity that catalyzed the desulfation of 1 μmol of ΔDi-4S per h under these conditions.

Preparation of dermatan sulfate-bound AH-Sepharose 4B and heparin-bound AH-Sepharose 4B. — The coupling reaction of dermatan sulfate (50 mg) or heparin (50 mg) with AH-Sepharose 4B (1.0 g) in the presence of EDC (10 mg) was performed according to the method described by the manufacturer. After the reaction was complete, the suspension was filtered and the residue was washed successively with M sodium chloride (200 mL) and 5mM sodium phosphate buffer (pH 6.8, 300 mL). The resulting dermatan sulfate-bound AH-Sepharose 4B or heparin-bound AH-Sepharose 4B was packed into a column (i.d., 1.0 cm), and the column was kept at 2°. A solution of dermatan sulfate (100 mg in 10 mL of 5mM sodium phosphate buffer,

pH 6.8) was then applied to the column, and washed with 5mM sodium phosphate buffer (pH 6.8, 200 mL). The column thus obtained was denoted dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate or heparin-bound AH-Sepharose 4B coated with dermatan sulfate. The amount of dermatan sulfate covalently binding to AH-Sepharose 4B was estimated to be 39.17 μg/mg of dry gel, and 5.63 mg of this glycosaminoglycan interacted non-covalently with 1 mL of the dermatan sulfate-bound AH-Sepharose 4B. On the other hand, 86.6 μg of heparin bound covalently to 1 mg (dry weight) of AH-Sepharose 4B, and 1 mL of the heparin-bound AH-Sepharose 4B interacted non-covalently with 5.27 mg of dermatan sulfate.

Preparation of crude enzymes. — Culture of *Flavobacterium heparinum* was carried out by the procedures of Yamagata *et al.*[15]. Chondroitin 6-sulfate was added to the medium at 0.1% of the final concentration, and the mixture was incubated for 24 h at 30°. The cells were then harvested, and sonicated under the conditions described by Linker and Hovingh[5]. The resulting extract was treated with protamine sulfate, followed by dilution with 5 volumes of water. Separation and purification of enzymes were performed at 0–4°, unless otherwise indicated.

Digestion of standard chondroitin 4-sulfate and chondroitin 6-sulfate with purified chondroitinase C and chondroitinase ABC. — An aqueous solution (100 μg in 0.1 mL) of standard chondroitin 4-sulfate or chondroitin 6-sulfate was incubated with 3.0 units of chondroitinase ABC for 4 h at 37° according to the method of Saito *et al.*[16]. The incubation mixture was heated for 2 min in a boiling-water bath, and then 2.5 mL of 0.06M hydrochloric acid was added. The mixture was centrifuged at 10,000*g* for 20 min. Absorbance at 232 nm of the supernatant was measured against the corresponding blank. Conversely, standard chondroitin 4-sulfate (300 μg in 0.3 mL of water) or chondroitin 6-sulfate (100 μg in 0.1 mL of water) was incubated with purified chondroitinase C (1 unit in 0.1 mL) in 25 μl of 0.3M Tris-HCl buffer (pH 8.0) containing 0.25M sodium acetate and 0.1% bovine serum albumin for 4 h at 37°. The incubation mixture was treated by the same procedures as before. The optical densities of the digests of chondroitin 4-sulfate and chondroitin 6-sulfate with chondroitinase C were compared with those of the corresponding ones with chondroitinase ABC

RESULTS

Separation of chondroitinase B and chondroitinase C. — The crude enzyme extract (30 mL) described in the experimental section was diluted to 180 mL with water, and then the solution was applied to a column (2.0 × 7.0 cm) of hydroxylapatite. After washing the column with 100 mL of 0.05M sodium phosphate buffer (pH 6.8), a linear gradient-elution with 0–0.3M sodium chloride in the same buffer (600 mL) was performed, followed by elution with 0.5M sodium chloride in the same buffer (200 mL). Elution was effected at a flow rate of 30 mL per h, and 5-mL fractions were collected. The activities of chondroitinase B, chondroitinase C, chondroitinase AC, and hyaluronidase in each fraction were determined as described in the experi-

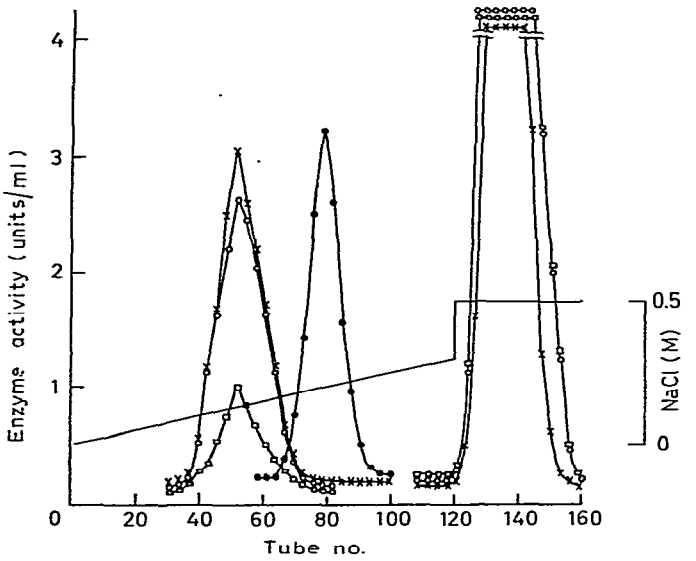

Fig. 1. Hydroxylapatite column chromatography of the crude enzymes from *Flavobacterium heparinum* induced with chondroitin 6-sulfate. Crude enzyme extract (30 mL) was diluted to 180 mL with water, and then the solution was applied to a column (2.0 × 7.0 cm) of hydroxylapatite. A linear gradient-elution with 0–0.3 M sodium chloride in 0.05M sodium phosphate buffer (pH 6.8, 600 mL) was performed, followed by elution with 0.5M sodium chloride in the same buffer (200 mL). Details of the hydroxylapatite column chromatography are described in the text.

□, Chondroitinase A activity; ●, chondroitinase B activity; ○, chondroitinase C activity; ×, hyaluronidase activity.

mental section. As shown in Fig. 1, apart from a large peak of chondroitinase AC eluted with 0.5M sodium chloride, two separate peaks of different enzymes were observed. The peak eluted with the lowest concentration of the salt showed a high activity of chondroitinase C and a low activity of chondroitinase A. The peak eluted with the highest concentration of the salt showed the same degree of activity for each of the two enzymes. These two peaks showed high activities of hyaluronidase.

After purification of the major enzyme in the first peak to be described later, the specificity of the enzyme towards standard specimens of chondroitin 4-sulfate and chondroitin 6-sulfate was identical with that of the enzyme described as chondroitinase C by Michelacci and Dietrich[2,3]. Therefore, it was designated chondroitinase C.

As the middle-peak substance in Fig. 1 was exclusively specific for dermatan sulfate, it may be defined as chondroitinase B[1,2].

The crude chondroitinase C fraction (tube nos. 40–65) and the crude chondroitinase B fraction (tube nos. 70–92) were separately pooled, and then dialyzed overnight against 50 volumes of water at 2°.

The same degree of separation of the chondroitinase C fraction and the chondroitinase B fraction was also achieved by stepwise elution with 0.13 and 0.2M, respectively, sodium chloride in 0.05M sodium phosphate buffer (pH 6.8). The

recoveries of these enzyme activities by the foregoing chromatography was 60–70%.

Purification of chondroitinase C. — The foregoing crude chondroitinase C fraction was contaminated with Δ4,5glycosiduronase and sulfatase to the extent of 7 and 5%, respectively, of the chondroitinase C activity taken as 100%. Moreover, it was found that chondroitinase C, as well as chondroitinase B, was extremely sensitive to such ion-exchangers as phospho-cellulose when adsorbed on the column. Therefore, to remove the contaminants, the crude chondroitinase C fraction (75 mL) was equilibrated with 0.07M sodium phosphate (pH 6.8), and then the solution was passed through a column (1.0 × 5.0 cm) of phospho-cellulose pre-equilibrated with the same buffer. Although the two contaminant enzymes were adsorbed exclusively on the column, chondroitinase C was found in the effluent. The effluent was then dialyzed twice against 50 volumes of 0.05M sodium acetate buffer (pH 7.0), and concentrated to 5 mL through a PM-10 membrane mounted on an Amicon Diafilter. Six units of chondroitinase C were found to be free from the

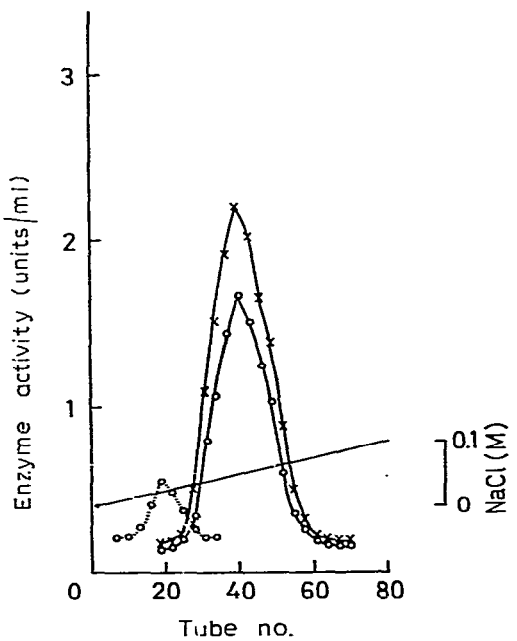

Fig. 2. Affinity chromatography of chondroitinase C on the dermatan sulfate-bound AH-Sepharose 4B. The chondroitinase C fraction obtained by passing through the phospho-cellulose column was equilibrated with 5mM sodium phosphate buffer (pH 6.8), and the solution was applied to a column (1.0 × 5.0 cm) of the dermatan sulfate-bound AH-Sepharose 4B coated with the same glycosaminoglycan. Subsequently, a linear gradient-elution with 0–0.1M sodium chloride in 5mM sodium phosphate buffer (pH 6.8, 400 mL) was performed. Another experiment, using a column (1.0 × 5.0 cm) of the dermatan sulfate-bound AH-Sepharose 4B without coating glycosaminoglycan, was performed by the same procedures as before. The results of the two experiments are shown in this Fig. See details in the text.

○, Chondroitinase C activity; ×, hyaluronidase activity; —○— and —×—, enzyme activities obtained from the coated column; .....○...., enzyme activity obtained from the non-coated column.

activities of Δ4,5glycosiduronase and sulfatase, and no loss of the chondroitinase C activity was observed during the treatment through the column.

As hyaluronidase activity was found in the chondroitinase C fraction, this fraction was subjected to affinity chromatography by using the dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate in an attempt to separate these two enzymes. The foregoing chondroitinase C fraction (124 mL) was equilibrated with 5mM sodium phosphate buffer (pH 6.8), and the solution applied to a column (1.0 × 5.0 cm) of the foregoing gel. Then, a linear gradient-elution with 0–0.1M sodium chloride in 5mM sodium phosphate buffer (pH 6.8, 400 mL) was performed at a flow rate of 30 mL per h. Fractions of 5 mL were collected, and the activities of chondroitinase C and hyaluronidase in each fraction were assayed as described in the experimental section. As shown in Fig. 2, both activities showed identical elution-profiles. At this step, purification of chondroitinase C was achieved 18-fold and in 73% yield.

It should be noted that no release of uronic acid-containing material was observed during the preceding treatment, but the coating material was eluted from the column by a higher concentration (0.6M) of sodium chloride than the previous conditions.

In a comparative study, another experiment was carried out by using the dermatan sulfate-bound AH-Sepharose 4B without coating glycosaminoglycan under the same conditions as before. As shown in Fig. 2, a small peak located around tube no. 20 of the enzyme activity was observed. The amount of the enzyme adsorbed on the latter column was estimated to be less than 10% of that on the former one. It was calculated that the former column adsorbed, at most, 42.9 μg of the enzyme protein per mL of gel. Hence, the coating dermatan sulfate, which was calculated to be 5.63 mg

TABLE I

PURIFICATION OF CHONDROITINASE C AND CHONDROITINASE B

	Total activity (units)[a]	*Specific activity (units/mg of protein)*[b]	*Recovery (%)*
Chondroitinase C			
Hydroxylapatite	136.8	17.6	100.0
Phospho-cellulose	123.1	46.1	90.0
ChsB–ChsB column[c]	99.9	310.1	73.0
Chondroitinase B			
Hydroxylapatite	190.9	20.2	100.0
Phospho-cellulose	181.4	38.9	95.0
Heparin–ChsB column[d]	93.2	154.2	48.8
DEAE-Sephadex A-25	46.6	247.6	24.4

[a]Definition of the activities is described in the experimental section. [b]Protein was assayed by the Lowry method[21]. [c]Dermatan sulfate-bound AH-Sepharose 4B coated with the same glycosaminoglycan. [d]Heparin-bound AH-Sepharose 4B coated with dermatan sulfate.

per mL of gel, on the dermatan sulfate-bound AH-Sepharose 4B, appeared to play an essential role for adsorption of the enzyme protein on the affinity matrix.

The peak fractions (tube nos. 23–45) were pooled, and dialyzed against 50 volumes of 0.05M Tris-HCl buffer (pH 7.0). The non-dialyzable fraction was then concentrated to 5 mL through a PM-10 membrane. The results of the purification of chondroitinase C are summarized in Table I. The final preparation of chondroitinase C was stored at 2° at a protein concentration of 50 μg per mL and it was stable for at least one month.

The purified preparation of chondroitinase C digested the standard chondroitin 4-sulfate and chondroitin 6-sulfate to yield approximately 10 and 85%, respectively, of the unsaturated products, as compared with those obtained from these substrates by chondroitinase ABC digestion, which were determined as described in the experimental section. The results corresponded well to the supplementary data, showing that ΔDi-6S plus ΔDi-OS per total unsaturated disaccharides from the standard specimens of chondroitin 4-sulfate and chondroitin 6-sulfate were 8 and 85%, respectively. The digestion products with the enzyme of chondroitin 4-sulfate and chondroitin 6-sulfate were separated by gel filtration on Sephadex G-50 in M sodium chloride, and the unsaturated disaccharides thus obtained analyzed by paper chromatography according to the method of Saito *et al.*[16]. The results showed that both glycosaminoglycans produced ΔDi-6S and ΔDi-OS, exclusively.

Purification of chondroitinase B. — The crude chondroitinase B fraction (tube nos. 70–92 in Fig. 1) (131 mL) contained 24 and 10% of the activities of Δ4,5glycosiduronase and sulfatase, respectively, as compared with the activity of chondroitinase B taken as 100%. To remove these two contaminants, one half of the fraction was equilibrated with 0.1M sodium phosphate buffer (pH 6.8), and the solution was applied to a column (1.0 × 5.0 cm) of phospho-cellulose pre-equilibrated with the same buffer. The non-adsorbed chondroitinase B fraction still contained 15 and 5% of the activities of Δ4,5glycosiduronase and sulfatase, respectively, as compared with the chondroitinase B activity taken as 100%. This fraction was then dialyzed twice against 50 volumes of 0.05M sodium acetate (pH 7.0), and the non-dialyzable fraction was concentrated to 5 mL through a PM-10 membrane. The chondroitinase B activity was recovered in quantitative yield without any irreversible denaturation in a high concentration of phosphate.

The foregoing concentrate was equilibrated with 5mM sodium phosphate buffer (pH 6.8), and the solution, containing 2.33 mg of protein, was applied to a column (1.0 × 5.0 cm) of the heparin-bound AH-Sepharose 4B coated with dermatan sulfate, which had been previously washed with the same buffer. A linear gradient-elution was performed with 0–0.2M sodium chloride in 5mM sodium phosphate buffer (pH 6.8, 400 mL) at a flow rate of 30 mL per hour. Fractions of 5 mL were collected, and the activities of chondroitinase B, Δ4,5-glycosiduronase, and sulfatase in each fraction were assayed as described in the experimental section, after each had been dialyzed against 50 volumes of 0.05M sodium acetate buffer (pH 7.0), followed by concentration to 1.0 mL through a PM-10 membrane. As shown in Fig. 3, separation of chon-

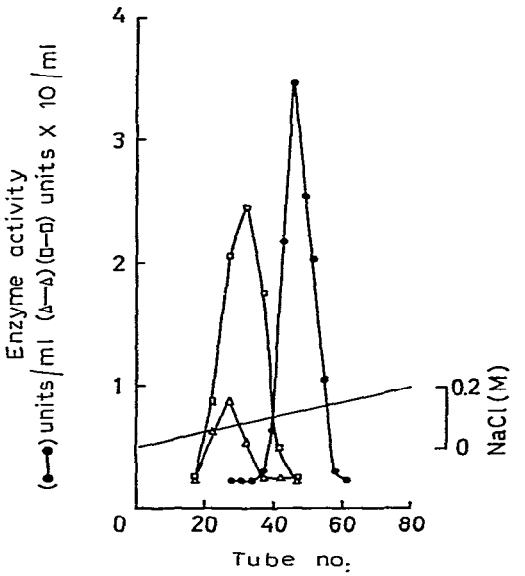

Fig. 3. Separation of chondroitinase B from Δ4,5glycosiduronase and sulfatase by affinity chromatography using the heparin-bound AH-Sepharose 4B coated with dermatan sulfate. The chondroitinase B fraction obtained by passing through the phospho-cellulose column was equilibrated with 5mM sodium phosphate buffer (pH 6.8), and then the solution was applied to a column (1.0 × 5.0 cm) of the heparin-bound AH-Sepharose 4B coated with dermatan sulfate. A linear gradient-elution with 0–0.2 M sodium chloride in 5mM sodium phosphate buffer (pH 6.8, 400 mL) was conducted as described in the text.

●, Chondroitinase B activity; □, Δ4,5glycosiduronase activity; △, sulfatase activity.

droitinase B from the other two enzymes was successful. The fractions (tube nos. 45–60) of chondroitinase B free from the contaminants were pooled and one half (69 mL) of the solution was dialyzed twice against 50 volumes of 0.05M sodium acetate buffer (pH 7.0) containing 10mM 2-mercaptoethanol. The non-dialyzable fraction was concentrated to 5 mL through a PM-10 membrane. The recovery of the enzyme was calculated to be 50%, and the degree of purification was found to be 7.6-fold.

It should be noted that the coating material (dermatan sulfate) on the column was completely eluted by 0.6M sodium chloride. On the other hand, it was found that, in the course of the foregoing affinity chromatography, the degradation products of the coating material with chondroitinase B were eluted together with the enzyme during the linear gradient-elution. Therefore, the foregoing specimen of chondroitinase B contained 9.3 μg of uronic acid per mL, as determined by the carbazole method of Bitter and Muir[17]. Therefore the enzyme-solution (68 mL) was applied to a column (1.0 × 6.0 cm) of DEAE-Sephadex A-25 pre-equilibrated with 0.05M sodium phosphate buffer (pH 6.8) containing 10mM 2-mercaptoethanol, and then washed with the same buffer (10 mL). The effluent and washings were combined, and the solution was dialyzed twice against 50 volumes of 0.05M Tris-HCl buffer (pH 7.0) containing 10mM 2-mercaptoethanol. The non-dialyzable fraction was

concentrated to 5 mL through a PM-10 membrane. The recovery of the enzyme activity was calculated to be 50% of the starting solution. No uronic acid was detected in the final preparation. Some loss of the activity may be ascribed to the denaturation of the enzyme through removal of substrate or products from the enzyme. The results of the purification of chondroitinase B are summarized in Table I. The present specimen of chondroitinase B was stable in the presence of 10mM 2-mercaptoethanol for at least one month.

DISCUSSION

Chondroitinase B and chondroitinase C were separated from the crude extract of *Flavobacterium heparinum* induced with chondroitin 6-sulfate by hydroxylapatite column-chromatography. The recoveries of the activities of these enzymes were estimated to be 60–70% by determination of unsaturated oligosaccharides according to the method of Hascall *et al.*[13]. However, as no specific method for the assay of chondroitinase C activity in the presence of chondroitinase AC is available, precise recovery of the activity remains to be re-evaluated.

It should be noted that chondroitinase B and chondroitinase C were found to be extremely sensitive to such ion-exchangers as phospho-cellulose, when they are adsorbed on the column. Therefore, adsorption of the desired enzymes on the phospho-cellulose column was avoided by increasing the concentration of buffer, which caused the contaminants to be retarded. On the other hand, as no loss of the chondroitinase C activity was observed during treatment through the column, the inhibition of the enzyme with sodium chloride and sodium phosphate as reported by Michelacci and Dietrich[3] might be ascribed to a reversible process.

Purification was successfully achieved by a novel type of affinity chromatography employing glycosaminoglycan-bound AH-Sepharose 4B coated with the same or another glycosaminoglycan. It is unique in two respects. First, the coating glycosaminoglycan on the glycosaminoglycan-bound AH-Sepharose 4B plays an essential role in adsorption of a large amount of enzyme protein on the affinity matrix. Second, as in the case of the purification of chondroitinase B, the substrate of the enzyme, which may have the strongest affinity for the corresponding enzyme, could be used for binding the enzyme protein to the affinity matrix. Moreover, when the column used for affinity chromatography was washed with a high concentration of salt, the contaminants of protein and coating material (or the enzymic digests of the substrate used as the coating material) could be removed for renewal of the glycosaminoglycan-bound AH-Sepharose 4B column. The resulting column could be coated again with the glycosaminoglycan for the next affinity chromatography in an amount sufficient to adsorb the enzyme protein.

We also found that this type of affinity chromatography is useful for purification of heparitinase[18], which is sensitive toward phospho-cellulose, as reported by Linker and Hovingh[4,5]. Moreover, heparinase and chondroitinase AC could also be purified by means of this type of affinity chromatography[18], although these

enzymes have been previously purified mainly by agarose-gel electrophoresis[19,20] and by ion-exchange chromatography[4,5,15].

Michelacci and Dietrich[3], have already reported that a chondroitinase isolated from *F. heparinum* is specific for chondroitin 6-sulfate, based on the analytical data for its digestion products with chondroitin 4-sulfate and chondroitin 6-sulfate. The present data confirm their findings. The activities of chondroitinase C and hyaluronidase were not separated by the methods used in the present study. Co-purification of both activities by column chromatography on hydroxylapatite and phosphocellulose (data are not shown in Results), and by affinity chromatography suggests that a single protein is degrading both chondroitin 6-sulfate and hyaluronic acid. The same result has been reported by Michelacci and Dietrich using an agarose electrophoresis[3]. Furthermore, it was reported by Yamagata *et al.* that the hyaluronidase activity was also found in the preparation of chondroitinase AC isolated from *Flavobacterium heparinum*[15]. On the other hand, the chondroitinase B fraction separated in the present study did not show any hyaluronidase activity, as shown previously[3]. Therefore, it is suggested that chondroitinase C and chondroitinase AC, together with hyaluronidase activity, specifically degrade D-glucuronic acid-containing glycosaminoglycans, whereas chondroitinase B eliminates exclusively the 2-acetamido-2-deoxy-D-galactosyl-L-iduronic acid linkages.

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