Carbohydrate Research, 88 (1981) 291–303
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

PURIFICATION OF HEPARINASE AND HEPARITINASE BY AFFINITY CHROMATOGRAPHY ON GLYCOSAMINOGLYCAN-BOUND AH-SEPHAROSE 4B*

NOBORU OTOTANI, MASAKI KIKUCHI, AND ZENSAKU YOSIZAWA
Department of Biochemistry, Tohoku University School of Medicine, Sendai 980 (Japan)
(Received January 31st, 1980; accepted for publication in revised form, March 15th, 1980)

ABSTRACT

Heparinase and heparitinase were separated from an extract of *Flavobacterium heparinum*, induced with heparin by using column chromatography on hydroxylapatite. As the heparinase preparation contained chondroitinases B and C, chondroitinase B was removed by rechromatography on a hydroxylapatite column. Chondroitinase C was then eliminated by column chromatography on *O*-phosphono("phospho")-cellulose. The heparinase preparation thus obtained was free from sulfoamidase for 2-deoxy-2-sulfoamino-D-glucose (GlcN-2S), sulfatase for 2-amino-2-deoxy-6-*O*-sulfo-D-glucose (GlcN-6S), as well as $\Delta_{4,5}$glycosiduronase for the unsaturated disaccharides obtained from heparin. The remaining sulfatase for 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulfate (ΔUA-2S) in the heparinase preparation was removed by affinity chromatography with dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate. The heparitinase preparation separated by column chromatography on hydroxylapatite was purified by affinity chromatography with heparin-bound AH-Sepharose 4B coated with heparin. Sulfatase for 2-amino-2-deoxy-6-*O*-sulfo-D-glucose (GlcN-6S) and $\Delta_{4,5}$glycosiduronase for the unsaturated disaccharides obtained from heparin were removed by this chromatography. Sulfatase for 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid 2-sulfate (ΔUA-2S) remaining in the heparitinase preparation was finally removed by column chromatography on hydroxylapatite. The recoveries of the purified preparations of heparinase and heparitinase were estimated to be 39 and 50%, respectively, from the crude enzyme fractions obtained by the first column chromatography on hydroxylapatite. The purified heparinase and heparitinase were free from all enzymes that

*Abbreviations: GlcN-2S, 2-deoxy-2-sulfoamino-D-glucose; GlcN-6S, 2-amino-2-deoxy-6-*O*-sulfo-D-glucose; ΔUA, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; ΔUA-2S, 4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid; ΔUA→Ph, phenyl 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; ΔUA→GlcN-2S, 2-deoxy-2-sulfoamino-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; ΔUA-2S→GlcN-2S, 2-deoxy-2-sulfoamino-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; $\Delta_{4,5}$ glycosiduronase, a specific hydrolase for the 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid linkage.

could degrade the sulfated unsaturated disaccharides produced from heparin with heparinase.

INTRODUCTION

Korn and Payza found a heparin-degrading enzyme in an extract of *Flavobacterium heparinum* induced with heparin[1]. This enzyme was later found to cleave eliminatively the 2-amino-2-deoxy-α-D-glucosyl-hexuronic acid linkages in heparin, yielding oligosaccharides having an α,β-unsaturated hexuronic acid at their non-reducing end[2]. Linker and Hovingh fractionated the crude enzyme by gel filtration on Sephadex G-200 and separated this eliminase from the $\Delta_{4,5}$glycosiduronase acting on the unsaturated oligosaccharides obtained from heparin[2]. Subsequently, they separated the eliminase acting on heparin (heparinase) from that acting on heparan sulfate (heparitinase) by column chromatography on hydroxylapatite. These enzymes were then purified by column chromatography on phospho-cellulose to remove sulfatase, sulfoamidase, and $\Delta_{4,5}$glycosiduronase acting on the sulfated, unsaturated oligosaccharides from heparin[3,4]. Moreover, they suggested the presence of sulfatase acting on ΔUA-2S in the heparinase preparation[3,4].

Dietrich *et al.* also purified enzymes designated as heparinase, heparitinase I, and heparitinase II by preparative electophoresis on agarose[5,6]. Sulfatase for GlcN-6S and ΔUA-2S, and sulfoamidase for GlcN-2S were also isolated[5,6]. The methods used by the foregoing authors for the purification of heparinase and heparitinase were considered unsatisfactorily, because heparitinase is unstable to such ion-exchangers as "phospho-cellulose" (cellulose phosphate), and the electrophoretic procedure appears unsuitable for the preparation of large amounts of these enzymes.

Recently, we purified chondroitinases B and C from an extract of *Flavobacterium heparinum* induced with chondroitin 6-sulfate by using a novel type of affinity chromatography with glycosaminoglycan-bound AH-Sepharose 4B coated with glycosaminoglycan[7,8]. This method was found useful for the purification of heparinase and heparitinase, offering the great advantages of high recovery and selectivity. This paper reports methods for purification of heparinase and heparitinase, together with some findings on the interaction of the enzyme proteins with glycosaminoglycan-bound AH-Sepharose 4B. A part of this study was reported preliminarily[9].

EXPERIMENTAL

Materials. — Heparin from porcine mucosa was purchased from Wilson Laboratory, Chicago. Heparan sulfate was separated by column chromatography on Dowex 1 (Cl^- form) from bovine lung by-products, kindly supplied by Dr. L. L. Coleman of the Upjohn Co., Kalamazoo, Michigan. Heparan sulfate eluted from the column with 1.25M sodium chloride was used. Hyaluronic acid from human umbilical cord[10] and dermatan sulfate from whale mucosa[11] were prepared according to the

references cited. Chondroitin 4-sulfate, chondroitin 6-sulfate, AH-Sepharose 4B, phospho-cellulose, sulfoethyl-cellulose and ΔDi-6S were purchased from Seikagaku Kogyo Co., Tokyo. *p*-Nitrocatechol sulfate was supplied by Nakarai Chemicals, Kyoto. 2-Amino-2-deoxy-6-*O*-sulfo-D-glucose (GlcN-6S), 2-deoxy-2-sulfoamino-D-glucose (GlcN-2S), 2-deoxy-2-sulfoamino-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose (ΔUA-2S→GlcN-2S) and 2-deoxy-2-sulfoamino-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose (ΔUA→GlcN-2S) were prepared from heparin as reported previously[12,13]. The method of preparation of phenyl 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid (ΔUA→Ph) will be published elsewhere. Hydroxylapatite was made by the procedure of Bernardi[14]. Heparin-bound AH-Sepharose 4B coated with heparin, and dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate, were prepared as reported previously[7,8]. Other materials were commercial products.

Assay for mucopolysaccharidases. — Assays for chondroitinases B, C, and A or hyaluronidase were performed as reported previously[8]. Assays for heparinase and heparitinase were conducted by the procedure of Linker and Hovingh[4]. In the present study, one unit of each enzyme is defined as the quantity that catalyzes the eliminative cleavage of the corresponding substrate to give 1 μmol of unsaturated oligosaccharides, based on a molecular absorption-coefficient of 5,500 at 235 nm, per h.

Assay for sulfatases. — Sulfatases were assayed by two methods. In the first, a non-specific assay was performed with *p*-nitrocatechol sulfate as substrate as follows. A mixture of enzyme solution (1 mL), 0.7M sodium acetate (pH 7.0, 0.15 mL), and a solution of *p*-nitrocatechol sulfate (1.6 μmol in 0.05 mL of water) was incubated for 15 h at 30°. Subsequently, 2 mL of 2.5M sodium hydroxide was added to the incubation mixture, and the absorbance at 515 nm of the solution was determined against a corresponding blank. The second assay, more specific than the foregoing one, was performed with GlcN-2S as substrate for sulfamidase, GlcN-6S as substrate for one sulfatase, and ΔUA-2S→GlcN-2S as substrate for a second sulfatase. The assay conditions for these enzymes were set according to the methods of Dietrich *et al.*[5,15]. Assay for these enzymes was performed as follows. For sulfoamidase, a mixture of enzyme solution (0.2 mL), 0.7M sodium acetate (pH 7.0, 0.05 mL) containing 0.01M magnesium chloride, and a solution of GlcN-2S (0.7 μmol in 0.1 mL of water) was incubated for 15 h at 25°. The incubation mixture was then diluted to 0.5 mL with distilled water, and GlcN released into the solution was determined by the method of Good and Bessman[16]. For action of sulfatase on ΔUA-2S, a mixture of enzyme solution (1.0 mL), 0.7M sodium acetate (pH 7.0, 0.15 mL), and a solution of ΔUA-2S→GlcN-2S (0.2 μmol in 0.05 mL of water) was incubated for 15 h at 30°. The ΔUA in the resulting ΔUA→GlcN-2S was determined by a modification[8] of the method of Hascall *et al.*[17]. For action of sulfatase on GlcN-6S, a mixture of enzyme solution (1.0 mL), 0.7M sodium acetate (pH 7.0, 0.15 mL), and a solution of GlcN-6S (0.7 μmol in 0.1 mL of water) was incubated for 15 h at 42°. The sulfate liberated after incubation was determined by the method of Waheed and van Etten[18].

Details of the specific assay-methods used for these sulfatases and sulfoamidases in the present investigation will be described elsewhere.

Assay for $\Delta_{4,5}$glycosiduronase. — Assays for $\Delta_{4,5}$glycosiduronase were performed in two ways. Firstly, a non-specific method was performed with ΔUA→Ph as a substrate as follows. A mixture of enzyme solution (1.0 mL), 0.7M sodium acetate (pH 7.0, 0.15 mL), and a solution of ΔUA→Ph (1 μmol in 0.1 mL of water) was incubated for 15 h at 30°. The liberated phenol was then determined according to the method of Asp[19]. Secondly, a method more specific than the foregoing one was performed with ΔUA→GlcN-2S from heparin and ΔDi-6S from chondroitin 6-sulfate as follows. A mixture of enzyme solution (1.0 mL), 0.7M sodium acetate (pH 7.0, 0.15 mL), and a solution of ΔUA→GlcN-2S (0.5 μmol in 0.1 mL of water) or ΔDi-6S (0.5 μmol in 0.1 mL of water) was incubated for 15 h at 30°. The mixture was then heated for 2 min in a boiling-water bath. After cooling and addition of 2.5 mL of 0.06M hydrochloric acid, the mixture was centrifuged at 10,000*g* for 15 min. Absorbance at 235 nm of the supernatant solution was measured against the corresponding blank. $\Delta_{4,5}$Glycosiduronase degraded these substrates to give an α-keto acid and phenol or sulfated 2-amino-2-deoxy-D-hexose, resulting in the loss of absorbance at 235 nm. The enzyme activity is therefore expressed as the quantity of the degraded substrate, which may be calculated from the decrease in absorbance at 235 nm and its molecular coefficient of 5,500.

Preparation of crude enzyme extracts. — *Flavobacterium heparinum* was cultured in a medium composed of polypeptone (1.5%), meat extracts (0.45%), yeast extracts (1.0%), malt extracts (1.0%) and sodium chloride (0.15%) for 18 h at 30°. Heparin solution (200 mg per L of the medium) was then added to the culture, and the mixture was incubated for 6 h at 30°. The cells were then harvested, sonicated, and extracted according to the method of Linker and Hovingh[4]. Separation and purification of enzymes was performed at 0–4°, unless otherwise indicated.

RESULTS

Purification of heparinase. — Crude enzyme extracts from *F. heparinum* induced with heparin were subjected to column chromatography on hydroxylapatite as described in Fig. 1. Heparinase was separated from heparitinase. The activities of chondroitinases A, B, and C, and hyaluronidase were, however, found in the heparinase fraction.

To remove chondroitinase B, the crude heparinase fraction (tubes no. 62–92) was pooled, and then dialyzed overnight against 70 volumes of distilled water. The dialyzate was equilibrated with 0.05M sodium phosphate buffer (pH 6.8), and the solution was subjected to rechromatography on the same size column of hydroxylapatite by the same procedures as the foregoing one, except for replacement of a linear gradient-elution with 0–0.3M sodium chloride in the same buffer (800 mL) from that with 0–0.5M of the salt. The activities of heparinase and chondroitinase B were found in tube nos. 100–120 and 125–140, respectively (data not shown).

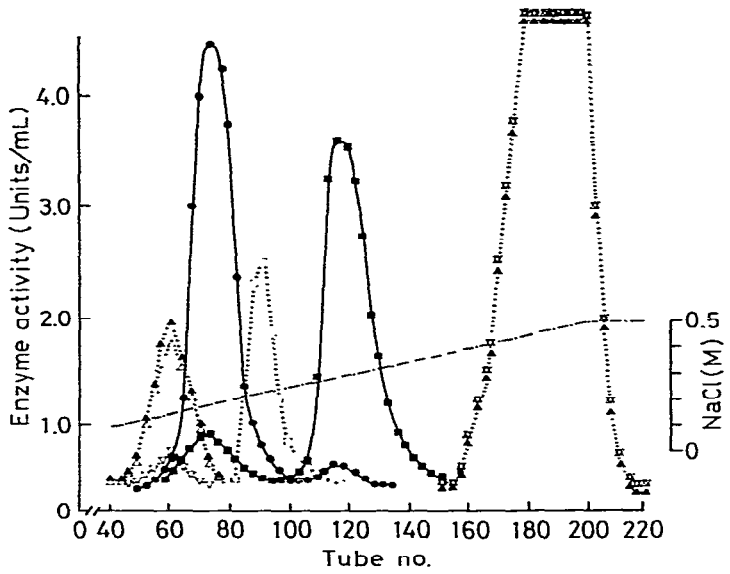

Fig. 1. Column chromatography on hydroxylapatite of the crude enzymes from *Flavobacterium heparinum* induced with heparin. Crude enzyme extract (80 mL) was diluted to 400 mL with distilled water, and then the solution was applied to a column (2.0 × 7.0 cm) of hydroxylapatite pre-equilibrated with 0.05M sodium phosphate buffer (pH 6.8). After washing the column with 100 mL of the same buffer, a linear gradient-elution was performed with 0–0.5M sodium chloride in the same buffer (1.0 L) at a flow rate of 30 mL/h, followed by a elution with 0.5M sodium chloride in the same buffer (100 mL). Fractions of 5 mL were collected and the activities of chondroitinases A, B, and C, heparinase, heparitinase and hyaluronidase in each fraction were determined as described in the Experimental section. ·▽·, Chondroitinase A activity; ·□·, chondroitinase B activity; ·△·, chondroitinase C activity; ·▲·, hyaluronidase activity; —●—, heparinase activity; —■—, heparitinase activity.

To remove the chondroitinase C activity, the heparinase fractions (tubes no. 100–120) obtained by the foregoing rechromatography was pooled, and then dialyzed against 50 volumes of 0.01M sodium phosphate buffer (pH 6.8). The dialyzate was subjected to column chromatography on phospho-cellulose as shown in Fig. 2. Chondroitinase C was completely separated from heparinase.

The heparinase fraction thus obtained, however, contained four enzymes acting on the sulfated, unsaturated oligosaccharides from heparin. These enzymes were found to be two kinds of $\Delta_{4,5}$glycosiduronase, a sulfatase for ΔUA-2S and a sulfatase for GlcN-6S. Although sulfoamidase was isolated from the crude extracts of *F. heparinum* by Dietrich[15], it was not present in this preparation, but was found in tubes no. 1–20 of the first hydroxylapatite column chromatography. The distribution of $\Delta_{4,5}$glycosiduronase and sulfatase in the eluates were determined as described in Fig. 2 with ΔUA→Ph and *p*-nitrocatechol sulfate, respectively.

The $\Delta_{4,5}$glycosiduronase activity on ΔUA→Ph was detected in two portions, tubes no. 51–70 and tubes no. 81–90, as shown in Fig. 2. These enzyme activities were identified by more-specific assay methods with ΔDi-6S from chondroitin 6-sulfate and ΔUA→GlcN-2S from heparin. ΔDi-6S (0.4 μmol) and ΔUA→GlcN-2S

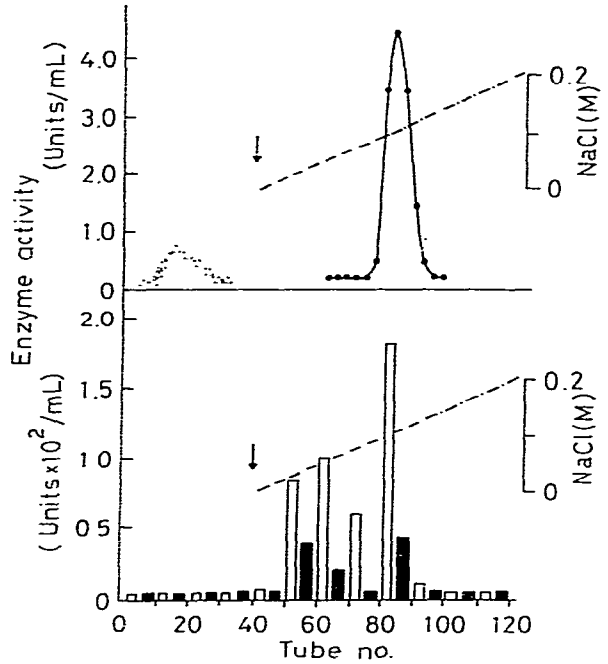

Fig. 2. Column chromatography of heparinase on phospho-cellulose. The heparinase fraction (tubes no. 100–120) obtained by rechromatography on a column of hydroxylapatite was dialyzed against 0.01M sodium phosphate buffer (pH 6.8). The dialyzate was applied to a column (1.2 × 7.0 cm) of phospho-cellulose pre-equilibrated with the same buffer, and then eluted with 200 mL of 0.05M sodium phosphate buffer (pH 6.8). Subsequently, a linear gradient-elution with 0–0.2M sodium chloride in the same buffer (400 mL) was performed at a flow rate of 30 mL/h. Fractions of 5 mL were collected, and the activities of heparinase and chondroitinase C in each fraction were determined as described in the Experimental section. The arrow shows the start of the linear gradient-elution. On the other hand, every 50 mL of the effluents (ten tubes) was separately pooled, and then dialyzed overnight against 50 volumes of 0.02M Tris-HCl buffer (pH 7.2) containing 0.01M mercaptoethanol. Each dialyzate was concentrated to 5 mL through a PM-10 membrane mounted on an Amicon DiaFlo-filter, and assayed for the activities of sulfatase and $\Delta_{4,5}$glycosiduronase as described in the Experimental section. —●—, heparinase activity; ·△·, chondroitinase C activity; □, sulfatase activity; ■, $\Delta_{4,5}$glycosiduronase activity.

(0.4 μmol) were separately incubated with the enzymes (0.5×10^{-2} activity unit on ΔUA→Ph) as described in the Experimental section. The results showed that 0.11 μmol of the former was degraded exclusively by the enzyme in tubes no. 81–90, whereas 0.30 μmol of the latter was digested exclusively by the enzyme in tubes no. 51–70. No activity of $\Delta_{4,5}$glycosiduronase was found in other fractions. These findings indicated that the activity in tubes no. 51–70 was specific for ΔUA→GlcN-2S and that in tubes no. 81–90 for ΔDi-6S.

On the other hand, the sulfatase activity on *p*-nitrocatechol sulfate was detected in tubes no. 51–70 and tubes no. 71–90. The results of assay for these sulfatases by more-specific methods with GlcN-6S (0.5 μmol) and ΔUA-2S→GlcN-2S (0.5 μmol) showed that 0.25 μmol of sulfate was liberated exclusively from the former by the

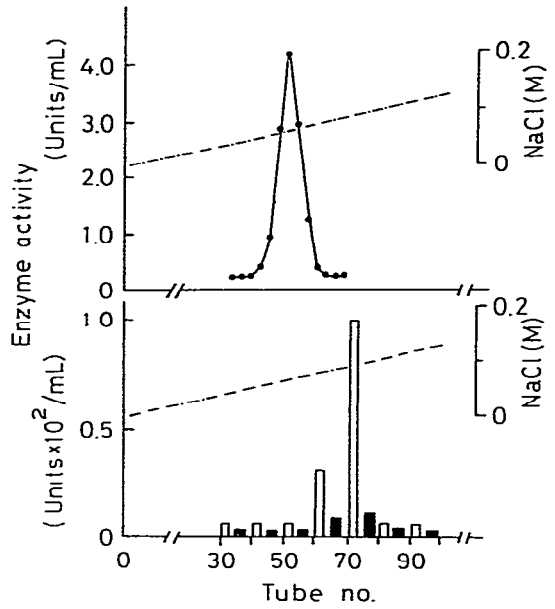

Fig. 3. Affinity chromatography of heparinase on dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate. The heparinase fraction (tubes no. 80–90 in Fig. 2) was pooled, and then dialyzed against 50 volumes of 5mM sodium phosphate buffer (pH 7.5). The dialyzate was applied to a column (1.0 × 5.0 cm) of dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate, which had been pre-equilibrated with the same buffer. Subsequently, a linear gradient-elution was performed with 0–0.2M sodium chloride in the same buffer (800 mL) at a flow rate of 30 mL/h. Fractions of 5 mL were collected, and the heparinase activity was determined as described in Fig. 2. In addition, the activities of sulfatase and $\Delta_{4,5}$glycosiduronase were also determined as described in Fig. 2. —●—, heparinase activity; □, sulfatase activity; ■, $\Delta_{4,5}$glycosiduronase activity.

enzyme (1×10^{-2} activity unit on *p*-nitrocatechol sulfate) in tubes no. 51–70 and 0.23 μmol of ΔUA→GlcN-2S was solely detected after digestion of the latter by the enzyme (1×10^{-2} activity unit on *p*-nitrocatechol sulfate) in tubes no. 71–90. No activity of sulfatase was found in other fractions. These findings indicated that the activity in tubes no. 51–70 was specific for GlcN-6S and that in tubes no. 71–90 for ΔUA-2S.

To remove the activities of sulfatase and $\Delta_{4,5}$glycosiduronase in the heparinase fraction, affinity chromatography with dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate was performed as described in Fig. 3. The heparinase activity showed a symmetrical elution-profile, and was separated from the activities of sulfatase and $\Delta_{4,5}$glycosiduronase. By this affinity chromatography, purification of heparinase was achieved 1.9-fold and in 80% recovery. The final preparation of heparinase (tube no. 45–57 in Fig. 3) was dialyzed overnight against 50 volumes of 0.02M Tris-HCl buffer (pH 7.2) containing 0.01M 2-mercaptoethanol. The dialyzate was concentrated to a protein concentration of 50–100 μg per mL and stored at 4° until used. The results of the purification of heparinase is summarized in Table I. Purification of heparinase was achieved 70.5-fold and in 39% recovery from the crude

TABLE I

PURIFICATION OF HEPARINASE AND HEPARITINASE

	Total activity (units)[a]	*Specific activity (units/mg of protein)*[b]	*Recovery (%)*
Heparinase			
Hydroxylapatite	318.2	29.3	100.0
Hydroxylapatite (Re)[c]	193.6	74.5	60.8
Phospho-cellulose	154.9	1107.0	48.6
DS-DS column[d]	124.0	2066.0	39.0
Heparitinase			
Hydroxylapatite	197.2	12.7	100.0
HP-HP column[e]	123.3	112.1	62.5
Hydroxylapatite (Re)[c]	98.6	230.0	50.0

[a]Definition of the activities is described in the Experimental section. [b]Protein was assayed by the Lowry method[25]. [c]Rechromatography. [d]Dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate. [e]Heparin-bound AH-Sepharose 4B coated with heparin.

heparinase fraction obtained by the first column chromatography on hydroxylapatite. The purified heparinase preparation was free from all enzymes that could degrade the sulfated unsaturated disaccharides produced from heparin with heparinase.

This heparinase preparation degraded the heparin preparation from porcine mucosa to give sulfated di-, tetra-, and oligo-saccharides in 37, 28, and 35% yields, respectively. The ΔUA in these products amounted to 60% of the total uronic acids in the heparin preparation. This enzyme degraded a certain portion of the heparan sulfate preparation from bovine lung, as shown in Fig. 1. The nature of these products will be described elsewhere.

Purification of heparitinase. — The crude heparitinase fraction obtained by column chromatography on hydroxylapatite was substantially free from other mucopolysaccharidases, except for a low activity of heparinase, as shown in Fig. 1. However, sulfatases for GlcN-6S and ΔUA-2S, and $\Delta_{4,5}$glycosiduronase for the unsaturated disaccharides from heparin were detected in this fraction. These sulfatases also exhibited activity on *p*-nitrocatechol sulfate, as already mentioned. Although phosphocellulose column chromatography was performed according to the method of Linker and Hovingh[4] to remove these contaminants from the heparitinase preparation, the recovery was very low (15%). Therefore, we employed affinity chromatography with heparin-bound AH-Sepharose 4B coated with heparin for the purification of heparitinase, as described in Fig. 4.

A symmetrical elution-profile was obtained. Purification of heparitinase was achieved 8.8-fold and in 62.5% recovery by this affinity chromatography. The non-adsorbed fractions were combined (Fr. NA), and aliquots (50 mL) of every ten tubes of the foregoing eluates were separately pooled, and then each pooled fraction was assayed for the activities of sulfatase and $\Delta_{4,5}$glycosiduronase, as described in Fig. 2.

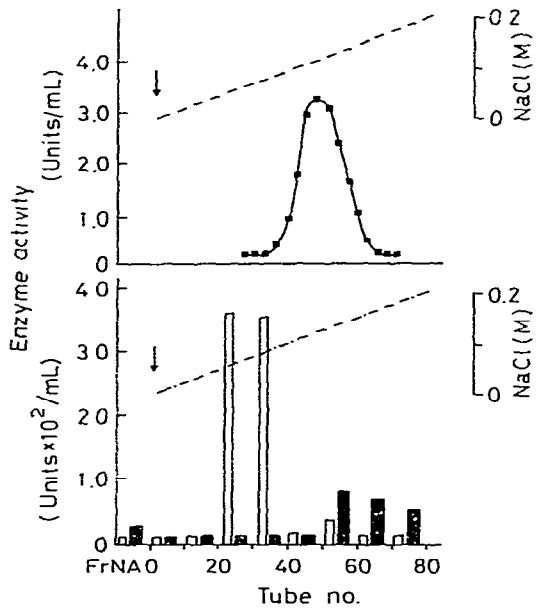

Fig. 4. Affinity chromatography of heparitinase on heparin-bound AH-Sepharose 4B coated with heparin. The crude heparitinase fraction (tubes no. 108–136 in Fig. 1) was pooled, and then dialyzed overnight against 50 volumes of distilled water. The dialyzate was equilibrated with 5mM sodium phosphate buffer (pH 6.8), and the solution was applied to a column (1.0 × 5.0 cm) of heparin-bound AH-Sepharose 4B coated with heparin, which had been previously washed with the same buffer. Subsequently, the column was washed with 100 mL of the same buffer, and then a linear gradient-elution was performed with 0–0.2M sodium chloride in the same buffer (400 mL) at a flow rate of 30 mL/h. Fractions of 5 mL were collected, and the heparitinase activity was determined as described in the Experimental section. In addition, the activities of sulfatase and $\Delta_{4,5}$glycosiduronase were also determined as described in Fig. 2. The arrow shows the start of the linear gradient-elution. —■—, heparitinase activity; □, sulfatase activity; $\Delta_{4,5}$glycosiduronase activity.

Both activities of sulfatase with *p*-nitrocatechol sulfate and $\Delta_{4,5}$glycosiduronase with ΔUA→Ph were separated into two peaks. The results obtained by more-specific assay methods indicated that the sulfatase found in tubes no. 21–40 was specific for GlcN-6S, whereas that in tubes no. 51–60 for ΔUA-2S. $\Delta_{4,5}$Glycosiduronase found in the non-adsorbed fraction exhibited activity against the unsaturated disaccharides from heparin and that in tubes no. 51–80 against ΔDi-6S from chondroitin 6-sulfate. The degrees of degradation of these substrates with these enzymes were similar to those described in Fig. 2. By this affinity chromatography, heparitinase was separated from sulfatase for GlcN-6S and $\Delta_{4,5}$glycosiduronase for the unsaturated disaccharides from heparin, but was still contaminated with some sulfatase activity on ΔUA-2S and the $\Delta_{4,5}$glycosiduronase activity on ΔDi-6S.

To remove these contaminants, the heparitinase preparation was purified by a second column chromatography on hydroxylapatite. The heparitinase fraction (tubes no. 40–63 in Fig. 4) was pooled, and then dialyzed overnight against 50 volumes

of 0.05M sodium phosphate buffer (pH 6.8). The dialyzate was applied to a column (1.2 × 7.0 cm) of hydroxylapatite pre-equilibrated with the same buffer. The column was then washed with 100 mL of 0.15M sodium chloride in the same buffer, and then a linear gradient-elution was performed with 0.15–0.35M sodium chloride in the same buffer (400 mL). Heparitinase was eluted in tubes no. 40–67, whereas the activities of sulfatase and $\Delta_{4,5}$glycosiduronase were found exclusively in tubes no. 20–30 (data not shown). The final preparation of heparitinase (tubes no. 40–67) was dialyzed overnight against 50 volumes of 0.02M Tris-HCl buffer (pH 7.2) containing 0.01M mercaptoethanol, and the dialyzate was concentrated to a protein concentration of 50–100 μg per mL, and stored at 4° until use. The results of the purification of heparitinase are summarized in Table I. The recovery of the enzyme activity was calculated to be 50% and the degree of purification was found to be 18.1-fold from the crude heparitinase preparation obtained by the first column chromatography on hydroxylapatite.

The purified heparitinase degraded the heparan sulfate preparation from bovine lung to give the products, including non- and mono-sulfated disaccharides, having 2-acetamido-2-deoxy-D-glucose residues at the reducing ends, plus oligosaccharide(s). The ΔUA in these products amounted to be 42% of the total uronic acid in the heparan sulfate preparation. This enzyme degraded a certain proportion of the heparin preparation from porcine mucosa as shown in Fig. 1. The nature of these products will be described elsewhere.

Interaction of mucopolysaccharidases with glycosaminoglycan-bound AH-Sepharose 4B coated with glycosaminoglycan. — Although heparinase was adsorbed onto dermatan sulfate-bound AH-Sepharose 4B uncoated with glycosaminoglycan, the amount was less than 10% of that adsorbed on the column coated with dermatan sulfate. The same results were obtained by affinity chromatography of heparitinase with heparin-bound AH-Sepharose 4B coated or uncoated with heparin. These results indicated that non-covalent coating of glycosaminoglycan-bound AH-Sepharose 4B with glycosaminoglycan is essential for binding of the enzyme protein to the matrix, as reported previously[7,8].

To examine the capacity of neutral salts in the elution of enzyme protein from the matrix, a column of dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate was used with heparinase in 5mM sodium phosphate buffer (pH 6.8). A linear gradient-elution was performed with 0–0.2M lithium chloride (or sodium chloride or potassium chloride) in the same buffer (400 mL). The heparinase was eluted by 0.095M lithium chloride (or 0.12M sodium chloride, or 0.075M potassium chloride). The results showed that the capacity of the neutral salts in the elution of the enzyme protein was KCl > LiCl > NaCl, indicating that the Hofmeister series[20] was not applicable to the present affinity chromatography. On the other hand, glycosaminoglycan could also elute the enzyme protein from the matrix. That is, a 0.5% solution of dermatan sulfate in 5mM sodium phosphate buffer (pH 6.8) eluted heparinase from dermatan sulfate-bound AH-Sepharose 4B coated with

dermatan sulfate, and a 0.4% solution of heparin in the same buffer was able to elute heparitinase from heparin-bound AH-Sepharose 4B coated with heparin.

On the other hand, sodium phosphate buffer (5mM), adjusted to pH 10, did not elute any heparinase from the matrix of dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate. Conversely, the heparinase preparation equilibrated with 5mM sodium phosphate buffer (pH 10) was not adsorbed onto a column of sulfoethyl-cellulose pre-equilibrated with the same buffer.

DISCUSSION

Heparinase and heparitinase were successfully separated from an extract of *Flavobacterium heparinum* induced with heparin by using column chromatography on hydroxylapatite as reported by Linker and Hovingh[4]. Low activities of heparitinase and heparinase detected in the heparinase and heparitinase fractions, respectively, suggested that heparan sulfate and heparin used in the present study might contain some heparin-like and heparan sulfate-like segments, respectively, as stated by Linker and Hovingh[4]. On the other hand, certain portions of chondroitinases B and C were eluted in the heparinase fraction: chondroitinase B was removed by rechromatography on a hydroxylapatite column. Chondroitinase C was then eliminated by column chromatography on phospho-cellulose, as low affinity of this enzyme to the ion-exchanger was found previously[8]. All enzymes that could degrade the sulfated, unsaturated disaccharides produced from heparin with heparinase were completely removed from the heparinase and heparitinase fractions by affinity chromatography with glycosaminoglycan-bound AH-Sepharose 4B coated with glycosaminoglycan and by column chromatography on phospho-cellulose and/or hydroxylapatite.

The degradation products of the heparin preparation from porcine mucosa with the purified heparinase in the present study were similar to those reported by Linker and Hovingh[21], indicating that the specificity of the present heparinase is consistent with that of heparinase reported previously[3-6], but differed from that[6,22,23] of heparitinase II. On the other hand, heparitinase obtained by Hovingh and Linker[3] and heparitinase I obtained by Dietrich *et al.*[5,6] was reported to be specific for non- and mono-sulfated 2-acetamido-2-deoxy-α-D-glucosyl linkages of heparan sulfates[3,6], as found with the purified heparitinase preparation in the present study. In addition, the heparitinase I obtained by Dietrich *et al.*[5,6] was reported to be specific for 2-amino-2-deoxy-α-D-glucosyl-D-glucuronic acid linkages[23], which is not contradictory to the finding on the specificity of the present heparitinase. As *N*-, *O*-disulfated or -trisulfated disaccharides have not yet been detected in the degradation products of heparan sulfate with the present heparitinase, it is not certain whether the present heparitinase preparation contained[5,6] heparitinase II or tetrasaccharidase[24].

The specificity of sulfoamidase and sulfatases found in the present study were as follows. Sulfoamidase acted on GlcN-2S, ΔUA→GlcN-2S and *p*-nitrocatechol sulfate, but was without activity on GlcN-6S. Sulfatase obtained from the heparitinase fraction, which was mainly found in tubes no. 21–40 in Fig. 4, acted specifically on

GlcN-6S. The other sulfatase separated from the heparinase fraction, which was mainly found in tubes no. 61–80 in Fig. 3, acted specifically on ΔUA-2S. Some activity of this sulfatase also contaminated the heparitinase fraction and was removed by the second column chromatography on hydroxylapatite. Linker and Hovingh already suggested the presence of this sulfatase activity in the preparation obtained by column chromatography on phospho-cellulose[4].

The purification of heparinase and heparitinase was achieved with good recoveries by using a new type of affinity chromatography reported in previous papers[7,8]. It should be noted again that this type of affinity chromatography may be applicable to the purification of other enzymes that have high affinity to uronic acid-containing glycosaminoglycans.

To elute the enzyme proteins from glycosaminoglycan-bound AH-Sepharose 4B coated glycosaminoglycan, it was necessary to use a solution of neutral salt. In addition, it was found that heparinase was not separated from sulfatase acting on ΔUA-2S by affinity chromatography, unless the pH of the sodium phosphate buffer (5mM) was elevated from 6.8 to 7.5. However, in the absence of neutral salt, more alkaline sodium phosphate buffer (5mM, pH 10) than the foregoing one did not elute the enzyme protein from the matrix. Conversely, the enzyme protein equilibrated with the same alkaline buffer was not adsorbed onto a column of sulfoethyl-cellulose. At pH 10, it was assumed that few cationic charges on the protein might be available for binding to cation-exchangers. Nevertheless, the enzyme protein did bind to dermatan sulfate-bound AH-Sepharose 4B coated with dermatan sulfate at pH 10. It is suggested, therefore, that hydrogen-bond and/or some other interactions may be more effective than ionic ones in the binding of the enzyme protein to the matrix. Under neutral conditions, the ionic interaction between the enzyme protein and the matrix may also be important in their binding.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan. We thank Dr. T. Okuyama and Dr. L. L. Coleman for supplying the materials. We are also indebted to Dr. A. Linker for providing a culture of *F. heparinum*.

REFERENCES

1 E. D. KORN AND A. N. PAYZA, *J. Biol. Chem.*, 223 (1956) 859–864.
2 A. LINKER AND P. HOVINGH, *J. Biol. Chem.*, 240 (1965) 3724–3728.
3 P. HOVINGH AND A. LINKER, *J. Biol. Chem.*, 245 (1970) 6170–6175.
4 A. LINKER AND P. HOVINGH, *Methods Enzymol.*, 28 (1972) 902–911.
5 C. P. DIETRICH, M. E. SILVA, AND Y. M. MICHELACCI, *J. Biol. Chem.*, 248 (1973) 6408–6415.
6 M. E. SILVA AND C. P. DIETRICH, *Biochem. Biophys. Res. Commun.*, 56 (1974) 965–972.
7 N. OTOTANI AND Z. YOSIZAWA, *J. Biochem. (Tokyo)*, 84 (1978) 1005–1008.
8 N. OTOTANI AND Z. YOSIZAWA, *Carbohydr. Res.*, 70 (1979) 295–306.
9 N. OTOTANI AND Z. YOSIZAWA, *Proc. Int. Symp. Glycoconjugates*, 5 (1979) 388–389.

10 L. RODÉN, J. R. BAKER, J. A. CIFONELLI, AND M. B. MATHEWS, *Methods Enzymol.*, 28 (1972) 73–140.
11 N. OTOTANI AND Z. YOSIZAWA, *Carbohydr. Res.*, 64 (1978) 143–153.
12 N. OTOTANI, K NAKAMURA, AND Z. YOSIZAWA, *J. Biochem. (Tokyo)*, 75 (1974) 1283–1289.
13 N. OTOTANI AND Z. YOSIZAWA, *J. Biochem. (Tokyo)*, 76 (1974) 545–551.
14 G. BERNARDI, *Methods Enzymol.*, 22 (1971) 326–327.
15 C. P. DIETRICH, *Biochem. J.*, 111 (1969) 91–95.
16 T. A. GOOD AND S. P. BESSMAN, *Anal. Biochem.*, 9 (1964) 253–262.
17 V. C. HASCALL, R. L. RIOLO, J. HAYWARD, JR., AND C. C. REYNOLDS, *J. Biol. Chem.*, 247 (1972) 4521–4528.
18 A. WAHEED AND R. L. VAN ETTEN, *Anal. Biochem.*, 89 (1978) 550–560.
19 N.-G. ASP, *Anal. Biochem.*, 40 (1971) 281–286.
20 P. H. VON HIPPEL AND T. SCHEICH, in S. N. TIMASHEFF AND C. D. FASMAN (Eds.), *Structure and Stability of Biological Macromolecules*, Marcel Dekker, New York, 1969, pp. 417–574.
21 A. LINKER AND P. HOVINGH, in N. M. MCDUFFIE (Ed.), *Heparin: Structure, Cellular Functions and Clinical Applications*, Academic Press, New York, 1979, pp. 3–24.
22 M. E. SILVA AND C. P. DIETRICH, *J. Biol. Chem.*, 250 (1975) 6841–6846.
23 C. P. DIETRICH, H. B. NADER, D. M. COHEN, AND Y. M. MICHELACCI, in ref. 21, pp. 39–49.
24 A. LINKER AND P. HOVINGH, *Fed. Proc.*, 36 (1977) 43–46.
25 O. H. LOWRY, N. J. ROSENBROUGH, A. L. PARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.