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PREPARATION OF PHENYL 4-DEOXY-α- AND β-L-*threo*-HEX-4-ENOPYRANOSIDURONIC ACIDS AND DETERMINATION OF THE ANOMERIC SPECIFICITY OF THE Δ4,5-GLYCOSIDURONASE INDUCED FROM *Flavobacterium heparinum* WITH HEPARIN AND CHONDROITIN SULFATE*

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ABSTRACT

To investigate the anomeric specificity of the Δ4,5-glycosiduronase induced from *Flavobacterium heparinum* with heparin and chondroitin sulfate, phenyl 4-deoxy-α- and β-L-*threo*-hex-4-enopyranosiduronic acids were chemically synthesized and then digested with the purified enzymes. Only the α-L anomer of the unsaturated uronic acid was degraded by the purified enzymes induced from the microbe with heparin or chondroitin sulfate. It was also confirmed that the Δ4,5-glycosiduronase induced with heparin and that induced with chondroitin sulfate hydrolyzed exclusively 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose and 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose, respectively. It was concluded that the purified preparation of Δ4,5-glycosiduronase induced with heparin was specific for the (1→4)-α-L-*threo*-4-enopyranosyluronic acid linkage, whereas that of the Δ4,5-glycosiduronase induced with chondroitin sulfate was specific for the (1→3)-α-L-*threo*-4-enopyranosyluronic acid linkage.

INTRODUCTION

Since the first description of the β-elimination reaction of 4-substituted hexuronic acids by Kiss[1], various reports concerning the preparation and characterization of 4,5-unsaturated hexuronic acid derivatives have appeared[2]. Moreover, oligosaccharides bearing an unsaturated hexuronic acid residue at their non-reducing end have also been prepared from such hexuronic acid-containing poly-

*Compounds: 4-deoxy-α-L-*threo*-hex-4-enopyranosiduronic acid (**1**); 4-deoxy-β-L-*threo*-hex-4-enopyranosiduronic acid (**2**); 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose (**3**); 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose (**4**); 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose (**5**); and 2-deoxy-2-sulfoamino-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-glucose (**6**).

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saccharides as heparin, chondroitin sulfates, and pectin by chemical and enzymic methods[2]. However, there are few reports describing the enzymic degradation of this 4,5-unsaturated hexuronic acid residue.

Yamagata *et al.* reported the purification and characterization of a Δ4,5-glycosiduronase from extracts of *Flavobacterium heparinum* induced with chondroitin sulfate[3], and Warnick and Linker described the purification of the Δ4,5-glycosiduronase obtained from the same microbe induced with heparin[4]. Subsequently, the latter investigators suggested that the enzyme induced with chondroitin sulfates was mainly specific for the (1→3)-linked 4,5-unsaturated uronic acid, whereas the enzyme induced with heparin that hydrolyzes the uronic acid bond of the disaccharides obtained from heparin and heparan sulfate appeared[5] to be specific for the (1→4) linkage. In order to confirm this assumption and to determine the anomeric specificity of the Δ4,5-glycosiduronase, we prepared phenyl α- and β-L-*threo*-hex-5-enopyranosiduronic acids and examined their enzymic digestion with the crude and partially purified Δ4,5-glycosiduronase obtained from *F. heparinum* induced with either heparin or chondroitin sulfate.

EXPERIMENTAL

Materials. — Agar slants of *Flavobacterium heparinum*, which had been subcultured in either heparin-containing or chondroitin 6-sulfate-containing medium, were supplied by Prof. A. Linker, Salt Lake City and Seikagaku Kogyo Co., Ltd., Tokyo, respectively. Hydroxylapatite was made by the procedure of Bernardi[6]. Phosphocellulose, *p*-nitrophenyl β-D-glucosiduronic acid and unsaturated disaccharide **4** were supplied by Seikagaku Kogyo Co., Ltd. Unsaturated disaccharide **3** was prepared by the method previously described[7]. Other chemicals were commercial products.

General methods. — Proton magnetic resonance spectra (Fig. 1) for compound **1** and **2** (Fig. 2) were recorded at 100 MHz with a Varian HA-100 spectrometer. T.l.c. was performed on silica gel (Wakogel B-10, Wako Chemical Co., Ltd., Osaka). Optical rotations were determined with a Jasco-DIP-4S (Japan Spectroscopic Co., Ltd., Tokyo) polarimeter. Melting points are not corrected.

*Phenyl (4-deoxy-α-*L*-threo-hex-4-enopyranosid)uronic acid* (**1**). — To a solution of methyl (phenyl 2,3,4-tri-*O*-acetyl-β-D-galactopyranosid)uronate[8] (576 mg) in 10 mL of dry benzene was added 0.36 mL of 1,5-diazabicyclo[5.4.0]undec-5-ene (DBU), and the mixture was stirred overnight at room temperature. The mixture was then diluted with 50 mL of ether and the solution was washed with water, dried ($MgSO_4$), and evaporated *in vacuo*. The residue was purified by preparative t.l.c. on silica gel with 4:1 (v/v) benzene–ether as eluent to give purified material showing a single spot, R_F 0.48 (t.l.c.) in 70% yield as a viscous oil; $[\alpha]_D^{25}$ −75.1° (*c* 0.8, methanol).

Anal. Calc. for $C_{17}H_{18}O_8$: C, 58.28; H, 5.18. Found: C, 58.50; H, 5.50.

To the eno-ester (338 mg) in methanol (20 mL) was added NaOH (240 mg) and

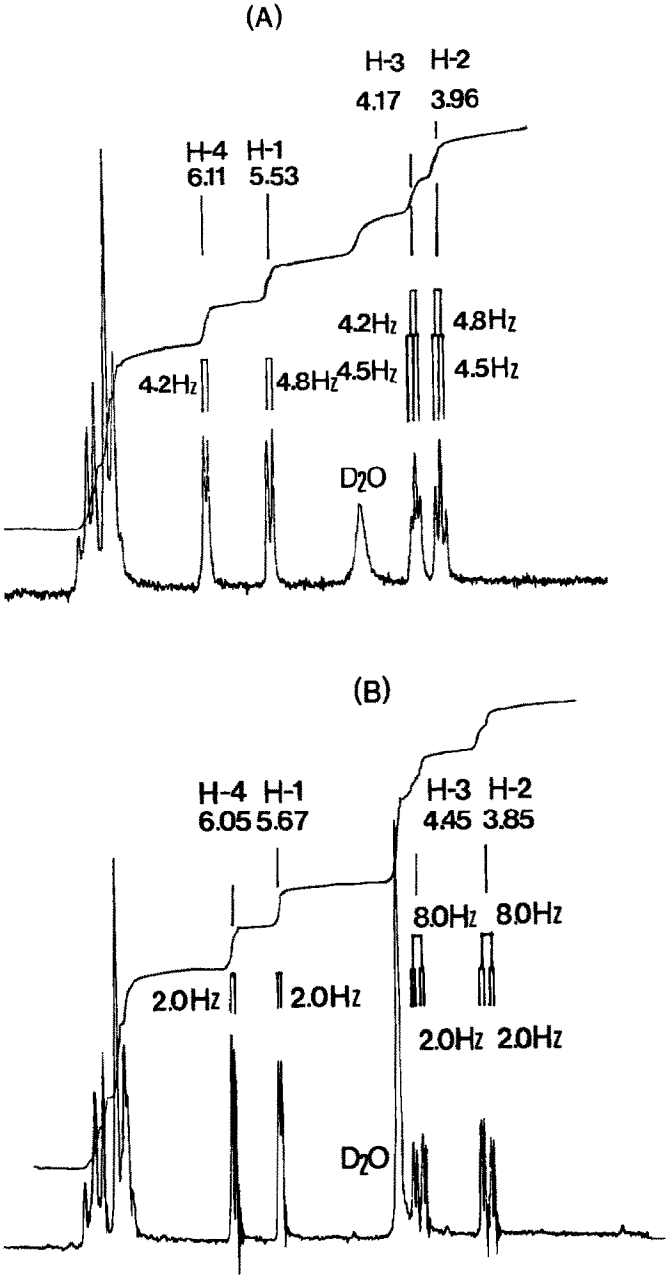

Fig. 1. Proton magnetic resonance spectra of phenyl α- (**1**, A) and β- (**2**, B) L-*threo*-4-hexenopyranosiduronic acids, recorded at 100 MHz, using sodium 4,4-dimethyl-4-silapentane-1-sulfonate in deuterium oxide as the internal standard.

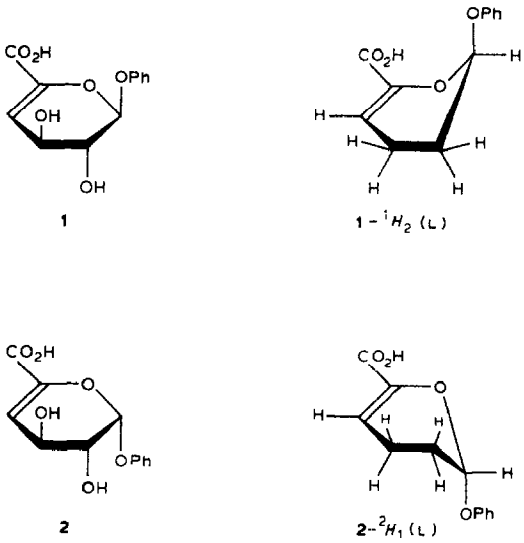

Fig. 2. Structures of phenyl α-L-*threo*-hex-4-enopyranosiduronic acid (**1**) and its β-anomer (**2**) and their conformations.

the mixture was stirred overnight at room temperature. The mixture was concentrated to low volume *in vacuo* at 40°, and a solution of the residue in water (10 mL) was deionized on a column (2.0 × 5.0 cm) of Dowex 50 (H^+) and then evaporated to dryness *in vacuo* at 40°. Purification of the product by preparative t.l.c. on silica gel with 10:10:1 (v/v) ether–benzene–acetic acid gave an amorphous powder, yield 80%, that showed a single spot (R_F 0.50, analytical t.l.c. same solvent); $[\alpha]_D^{22}$ −139.7° (*c* 0.43, methanol).

The final preparation failed to crystallize. Treatment with cyclohexylamine (20 μL) and addition of ether gave cyclohexylammonium (phenyl 4-deoxy-α-L-*threo*-hex-4-enopyranosiduronate (**1a**), m.p. 156–158° (decomp.).

Anal. Calc. for $C_{18}H_{24}NO_6$: C, 61.70; H, 6.90; N, 4.00. Found: C, 61.40; H, 7.19; N, 3.71.

*Phenyl (4-deoxy-β-*L-threo-*hex-4-enopyranosid)uronic acid* (**2a**). — To a solution of methyl (phenyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosid)uronate[9] (240 mg) in 10 mL of dry benzene was added DBU (0.28 mL) and the mixture was stirred overnight at room temperature, and then the product was treated as in the preceding experiment. The crude product was purified by preparative t.l.c. on silica gel with 4:1 (v/v) benzene–ether to give pure material showing a single spot, R_F 0.56 as a viscous oil, yield 67%, $[\alpha]_D^{22}$ +337.3° (*c* 0.38, methanol).

Anal. Calc. for $C_{17}H_{18}O_8$: C, 58.28; H, 5.18. Found: C, 58.23; H, 5.29.

To a solution of the eno-ester (118 mg) in methanol (10 mL) was added 80 mg of sodium hydroxide and the mixture was stirred overnight at room temperature. After treatment by the same procedure as just described, the crude product

obtained was purified by preparative t.l.c. on silica gel with 10:10:1 (v/v) ether–hexane–acetic acid to give pure material showing a single spot, R_F 0.58 (t.l.c.) in 60% yield as an amorphous powder; $[\alpha]_D^{22}$ +301.3° (*c* 0.23, methanol). Treatment of this preparation with cyclohexylamine (20 μL) and addition of ether gave a crystalline solid; recrystallization from ethanol–ether gave compound **2**, m.p. 196–198° (decomp.).

Anal. Calc. for $C_{18}H_{24}NO_6$: C, 61.70; H, 6.90; N, 4.00. Found: C, 61.74; H, 7.21; N, 3.69.

Assay for Δ4,5-glycosiduronase. — The Δ4,5-glycosiduronase activity on compound **1** was assayed by incubating the enzyme (up to 0.5 mL) and substrate for 1 h at 37° in 0.1M sodium acetate (pH 7.0), the substrate concentration being 10mM. The liberated phenol was then determined by the method described by Asp[10] against a known amount of phenol dissolved in the same mixture. One unit of Δ4,5-glycosiduronase was defined as 1 μmol of phenol liberated per h. In the time-course study of hydrolysis, the substrate concentration was 5mM. To achieve sufficient sensitivity for the assay following chromatography, the incubation time was extended to 10–15 h. The Δ4,5-glycosiduronase activity on the naturally occurring disaccharides was determined by incubating enzyme and substrate (disaccharides **3** or **4**, at final concentrations of 1.14 mM at 37° in 0.1M NaOAc (pH 7.0) for the times indicated in Figs. 6–8. The reaction was then terminated by adding 0.06M HCl (2 mL) and the absorption at 232 nm (or 235 nm) was measured. The molar absorption coefficient of **4** at 232 nm has been reported[3] to be 5,700 (3) and that[11] of **3** at 235 nm to be 5,700.

Assay for β-D-glucosiduronase. — Assay for β-D-glucosiduronase activity on *p*-nitrophenyl β-D-glucosiduronic acid was conducted by incubating the enzyme and substrate (final concentration, 10mM) at 37° in 0.1M NaOAc (pH 7.0) for 3 h. The mixture was then supplemented with 0.1M Na_2CO_3 (2 mL) and the absorption at 420 nm was measured. The molar absorption-coefficient of *p*-nitrophenol under these conditions has been reported[12] to be 13,200.

Assay for mucopolysaccharidase. — Assays for the mucopolysaccharidase activities on heparin, heparan sulfate, dermatan sulfate, and chondroitin 4-sulfate was performed as previously described[13,14].

Preparation of Δ4,5-glycosiduronase from F. heparinum *induced with chondroitin 6-sulfate.* — Crude extracts of *F. heparinum* induced with chondroitin 6-sulfate were obtained by the method described previously[14]. The crude extract (80 mL) was applied to a column (2.0 × 7.0 cm) of hydroxylapatite that was eluted with a linear gradient of 0–0.5M NaCl in 0.05M sodium phosphate buffer (pH 6.8, 1 L). Fractions (tubes nos. 80–120 in Fig. 3) containing the Δ4,5-glycosiduronase and β-D-glycosiduronase activities were pooled and dialyzed against 50 vol. of 0.01M sodium phosphate buffer (pH 6.8). The dialyzate was then applied to a column (1.2 × 7.0 cm) of phosphocellulose and eluted with a linear gradient of 0–0.2M NaCl in 0.01M sodium phosphate buffer (pH 6.8, 400 mL). Fractions (tubes no. 113–135 in Fig. 4) having Δ4,5-glycosiduronase activity were pooled and

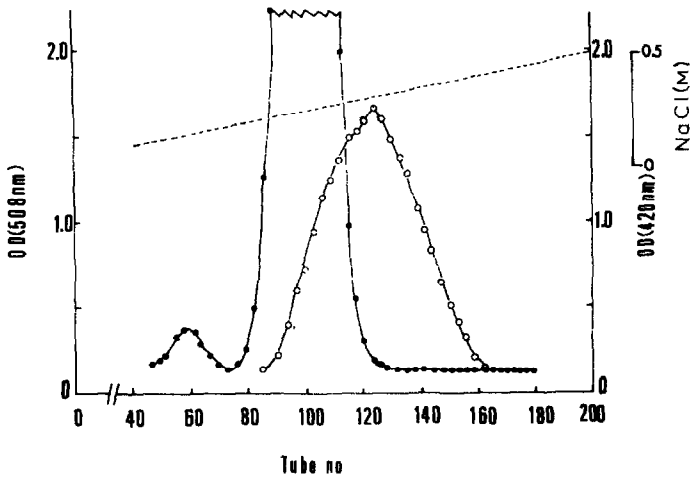

Fig. 3. Hydroxylapatite column chromatography of the crude enzymes from *Flavobacterium heparinum* induced with chondroitin 6-sulfate. The crude enzyme extract (80 mL) was diluted to 300 mL with water, applied to a column (2.0 × 7.0 cm) of hydroxylapatite, and then subjected to linear gradient-elution with 0–0.5M sodium chloride in 0.05M sodium phosphate buffer (pH 6.8, 1 L). Details of the hydroxylapatite column chromatography and assay of Δ4,5-glycosiduronase on 1, and β-D-glucosiduronase on D-nitrophenyl β-D-glucosiduronic acid are described in the text. ● Δ4,5-glycosiduronase activity measured at 508 nm. ○ β-D-glucosiduronase activity measured at 420 nm.

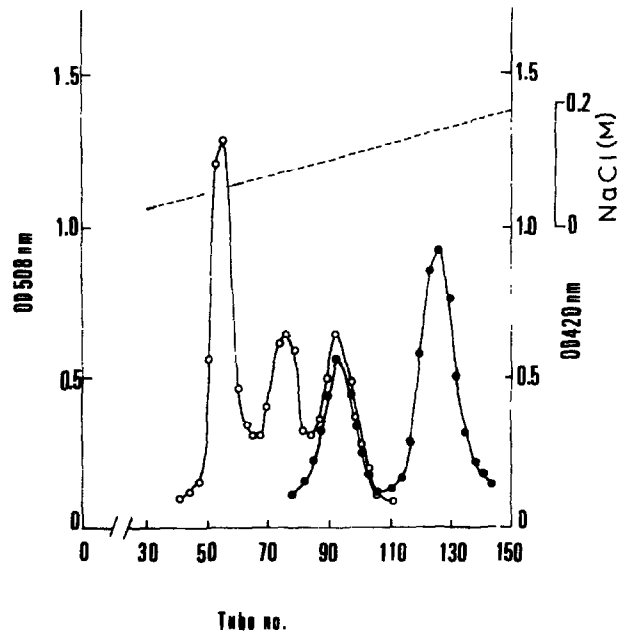

Fig. 4. Column chromatography on phosphocellulose of the crude Δ4,5-glycosiduronase induced with chondroitin 6-sulfate. The Δ4,5-glycosiduronase fractions obtained by hydroxylapatite column chromatography were dialyzed against 0.01M sodium phosphate (pH 6.8). The dialyzate was then applied to a column (1.2 × 7.0 cm) of phosphocellulose that was eluted with a linear gradient of 0–0.2M sodium chloride in 0.01M sodium phosphate buffer (pH 6.8, 400 mL), as described in the text. ● Δ4,5-glycosiduronase activity measured at 508 nm. ○ β-D-glucosiduronase activity measured at 420 nm.

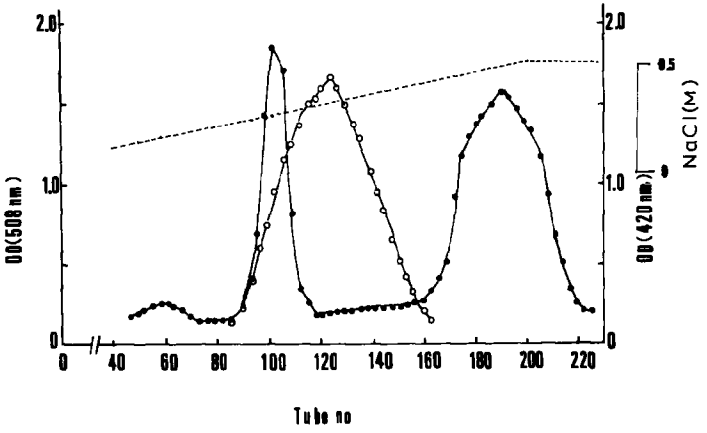

Fig. 5. Column chromatography on hydroxylapatite of the crude enzymes from *Flavobacterium heparinum* induced with heparin. The crude enzyme extract (80 mL) was diluted to 300 mL with water, and the solution was then applied to a column (2.0 × 7.0 cm) of hydroxylapatite. Elution with a linear gradient of 0–0.5M sodium chloride in 0.05M sodium phosphate buffer (pH 6.8, 1 L) was then followed by elution with 0.5M sodium chloride in the same buffer (200 mL). ● Δ4,5-glycosiduronase activity measured at 508 nm. ○ β-D-glucosiduronase activity measured at 420 nm.

dialyzed against 50 vol. of 0.01M Tris–HCl buffer (pH 7.2) containing 0.01M NaCl. To the dialyzate was then added 1 mg of bovine serum albumin and the solution was concentrated to 5 mL through a PM-10 membrane mounted in an Amicon Diafilter.

Preparation of Δ4,5-glycosiduronase from F. heparinum *induced with heparin.* — A crude extract of *F. heparinum* induced with heparin was obtained by the method described by Linker and Hovingh[15]. Subsequently, the crude enzyme (80 mL) was applied to a column (2.0 × 7.0 cm) of hydroxylapatite that was eluted with a linear gradient of 0–0.5M NaCl in 0.05M sodium phosphate buffer (pH 6.8, 1 L) under the conditions described previously[13]. Fractions (tubes no. 169–214 in Fig. 5) showing Δ4,5-glycosiduronase activity were pooled and dialyzed against 50 vol. of 0.01M sodium phosphate buffer (pH 6.8). The dialyzate was then applied to a column (1.2 × 7.0 cm) of phosphocellulose pre-equilibrated with the buffer and eluted with a linear gradient according to the method described by Hovingh and Linker[5]. Fractions exhibiting only Δ4,5-glycosiduronase activity were pooled and dialyzed overnight against 50 vol. of 0.01M Tris–HCl buffer (pH 7.2) containing 0.01M NaCl. To the dialyzate was then added 1 mg of bovine serum albumin and the solution was concentrated to 5 mL through a PM-10 membrane.

RESULTS AND DISCUSSION

*N.m.r. studies on phenyl α- and β-*L-threo*-hex-4-enopyranosyliduronic acids* (**1** and **2**). — The compounds of **1** and **2** were examined by ^{1}H-n.m.r. spectroscopy. In the spectrum (Fig. 1A) of the α-L-anomer **1**, triplets at 4.17 and 3.96 p.p.m.

were assigned to H-3 and H-2, respectively, with a coupling ($J_{2,3}$) of 4.5 Hz. The H-1 signal was observed at 5.53 p.p.m. as a sharp doublet (splitting 4.8 Hz). These spectral data are comparable to those of the derivatives of methyl (*α*-L-*threo*-hex-4-enopyranosyl)uronate described by Alföldi *et al.*[16]. In the spectrum of the 2,6,2′-trisulfated disaccharide **6** reported by Perlin *et al.*[17], the H-1 signal of the unsaturated uronic acid moiety appears as a quartet with a smaller spacing (~1 Hz, not observed in our spectrum of **1**), that was attributed to long-range coupling between H-1 and H-3. This coupling indicates that the anomeric C–O-bond of the unsaturated uronic acid residue of the disaccharide is axially oriented. The relatively small, major splitting found for the H-1, H-2, and H-3 (2.6–3.2 Hz) also suggested that these protons adopt equatorial or quasiequatorial orientations; the conformation of the unsaturated uronic acid moiety of the naturally occurring trisulfated disaccharide **6** as well as that of the monosulfate **5** was therefore assumed to be $^1H_2$(L). However, the $J_{2,3}$ coupling observed in the spectrum of **6** appears slightly higher than that expected between the equatorial and quasiequatorial protons H-2 and H-3 of the uronic acid moiety, as the dihedral angle between these protons in the typical $^1H_2$(L) conformation is 90°. Therefore, it may be assumed that the $^1H_2$(L) conformation is slightly deformed by interaction between the functional groups at C-1 and C-3. However, the $J_{2,3}$ value of 4.5 Hz found in the spectrum of **1** is considerably higher than expected for orientations between equatorial and quasiequatorial, although it is much lower than that expected between axial and quasiaxial protons. These data, together with the absence of long-range coupling between H-1 and H-3 in this spectrum, indicate that, because of strong interaction between functional groups, the $^1H_2$(L) conformation of **1** may also be considerably deformed, or the equilibrium between the $^1H_2$(L) and $^2H_1$(L) conformations may shift from $^1H_2$(L) to $^2H_1$(L).

In the spectrum (Fig. 1B) of the *β*-L-anomer **2**, the signals for H-3 and H-2 are observed at 4.45 (d.d.) and 3.85 p.p.m. (d.d.), respectively, with couplings of 2.0 and 8.0 Hz. The latter coupling ($J_{2,3}$) is typical for axial or quasiaxial H-2 and H-3 protons. The $J_{3,4}$ coupling of 2.0 Hz, also indicates the quasiaxial orientation of H-3, as the torsional angle of the allylic proton and the alkene plane is assumed to be ~90°, for which the coupling is small[18]. These results suggest that the conformation of **2** is $^2H_1$(L) (2B in Fig. 2).

Enzyme preparation of Δ4,5-glycosiduronase from F. heparinum *induced with heparin and chondroitin sulfate.* — Separation of Δ4,5-glycosiduronase from the crude extract of *F. heparinum* induced with chondroitin 6-sulfate was attempted by column chromatography on hydroxylapatite. As can be seen in Fig. 3, the peak substance having Δ4,5-glycosiduronase activity was partially coeluted together with the activity of *β*-D-glucosiduronase, the existence of which has never been demonstrated in this microbe. These activities were, however, separated by column chromatography on phosphocellulose. Fig. 4 shows two peaks having Δ4,5-glycosiduronase and three peaks having *β*-D-glucosiduronase activity. One of these peaks exhibits both activities. Therefore, the peak substance (tubes no. 115–135)

exhibiting Δ4,5-glycosiduronase activity alone was collected and used for the determination of anomeric specificity. The final preparation of this Δ4,5-glycosiduronase prior to the addition of albumin exhibited a specific activity of 53.0 U/mg protein and no mucopolysaccharidase activity on heparin, heparan sulfate, dermatan sulfate, or chondroitin 4-sulfate. As the crude enzymes contained several types of Δ4,5-glycosiduronase, the extent of purification and recovery were not determined.

Separation of Δ4,5-glycosiduronase from the crude extract of *F. heparinum* induced with heparin was then performed by column chromatography on hydroxylapatite. As shown in Fig. 5, a different peak (tubes no. 170–215) having Δ4,5-glycosiduronase activity from that induced with chondroitin sulfate (tubes no. 88–110) was noted. The former peak fraction (tubes no. 170–215) contained chondroitinase AC. Accordingly, these activities were separated by column chromatography on phosphocellulose according to the method described by Hovingh and Linker[5]. The Δ4,5-glycosiduronase thus obtained exhibited a specific activity of 7.3 U/mg protein and no mucopolysaccharidase activity on chondroitin 4-sulfate, dermatan sulfate, heparin, or heparan sulfate.

Enzymic cleavage of phenyl hex-4-enuronic acids and unsaturated disaccharides derived from heparin and chondroitin sulfate. — Unsaturated glycosides **1** and **2** were digested with a crude extract of *F. heparinum* induced with either

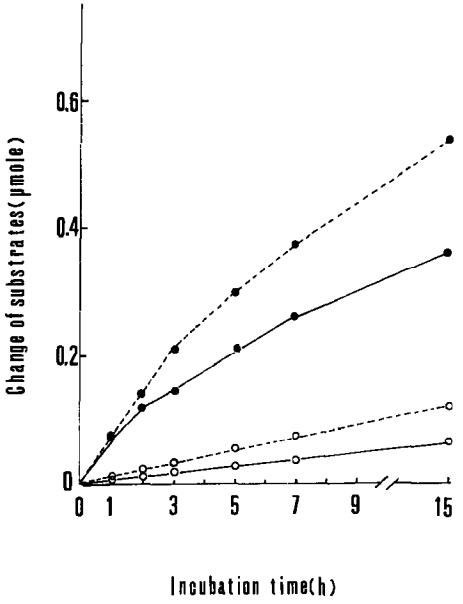

Fig. 6. Enzymic hydrolysis of phenyl 4-deoxy-α- and β-L-*threo*-hex-4-enopyranosiduronic acids (**1** and **2**) with the crude enzyme induced with chondroitin 6-sulfate and heparin. Compounds **1** and **2** at a final concentration of 5mM were incubated with crude enzymes induced with chondroitin 6-sulfate (——) and heparin (-----) for the time indicated in the figure, and the liberated phenol was assayed by the method described in the text. ● **1**; ○ **2**.

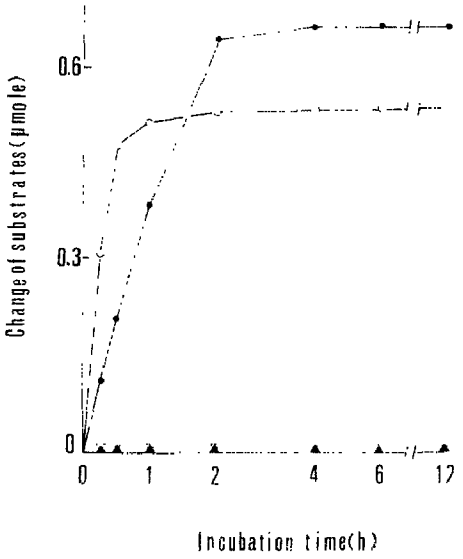

Fig. 7. Enzymic hydrolysis of phenyl 4-deoxy-α- and β-L-*threo*-hex-4-enopyranosiduronic acids (**1** and **2**), together with the naturally occurring disaccharides **3** and **4**, by the purified Δ4,5-glycosiduronase induced with chondroitin 6-sulfate. Compounds **1** and **2** at a final concentration of 5mM, together with **3** and **4** at a final concentration of 1.14mM, were incubated with the purified Δ4,5-glycosiduronase induced with chondroitin sulfate for the time indicated in the figure, and the extent of hydrolysis was measured by the methods described in the text. ● **1**, ▲ **2**; ○ **4**; ▽ **3**

heparin or chondroitin 6-sulfate. The substrate (6mM) in 0.5 mL of 0.1M sodium acetate (pH 7.0) was added to 0.1 mL of the crude enzyme extract from the microbe, and the solution was incubated at 37°. The time course of hydrolysis was measured as described in Methods. As shown in Fig. 6, the α-L anomer **1** was cleaved at a much higher rate than the β-L anomer **2** with both enzymes induced with heparin and chondroitin 6-sulfate.

In order to determine whether or not these anomers were hydrolyzed by the same enzyme, they were subjected to enzymic digestion with the purified Δ4,5-glycosiduronases. Thus, compounds **1** and **2** (final concentration of 5mM) were incubated with 0.1 mL of Δ4,5-glycosiduronase induced with chondroitin 6-sulfate at 37°. Fig. 7 shows the time course of hydrolysis of **1** exhibiting a hyperbolic curve, whereas **2** is not digestible by this enzyme. These results indicate that a Δ4,5-glycosiduronase specific for **1** could be separated from the enzyme(s) acting on both α and β anomers existing in the crude enzyme-preparation. The latter activity, with broad specificity, was found in tubes no. 50–70 on hydroxylapatite column chromatography (Fig. 3). Because of the low activity, further investigation was not undertaken.

The next study on enzymic hydrolysis with the purified Δ4,5-glycosiduronase was performed on naturally occurring disaccharides. The incubation conditions

were as already described, the substrate concentration being 1.14mM. As shown in Fig. 7, the disaccharide **4** derived from chondroitin sulfate was completely hydrolyzed, whereas the disaccharide **3** from heparin was not digested at all. It is also notable that the naturally occurring disaccharide **4** was hydrolyzed at a higher rate than **1**. It may thus be concluded that the Δ4,5-glycosiduronase induced with chondroitin sulfate is specific for the (1→3)-α-L-*threo*-4-enopyranosyluronic acid linkage.

Next conducted was a study on the enzymic cleavage of **1** and **2** with the purified Δ4,5-glycosiduronase induced with heparin. Thus, **1** and **2** (6mM) in 0.5 mL of 0.1M sodium acetate buffer (pH 7.0) were separately incubated with 0.1 mL of Δ4,5-glycosiduronase at 37°. As shown in Fig. 8, the unsaturated disaccharide **3** and **1** were both hydrolyzed by the Δ4,5-glycosiduronase. In contrast, the unsaturated disaccharide **4** and **2** were not cleaved at all. The inertness of the β-L anomer toward Δ4,5-glycosiduronase was not attributable to the $^2H_1$(L) conformation, as neither incubation with the enzyme (0.1 mL) at the elevated temperature of 45° for 1–3 h nor incubation with a large amount of enzyme (0.5 mL) for 15 h caused any hydrolysis of this substrate. Therefore, it could be concluded that the purified preparation of Δ4,5-glycosiduronase induced with heparin was specific for the (1→4)-α-L-*threo*-4-enopyranosyluronic acid linkage. This conclusion was

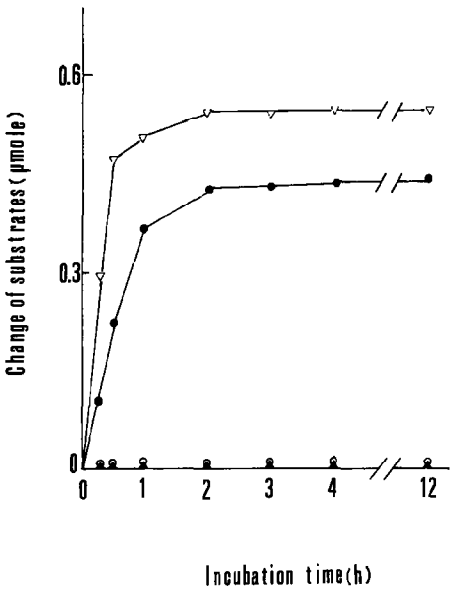

Fig. 8. Enzymic hydrolysis of phenyl 4-deoxy-α- and β-L-*threo*-hex-4-enopyranosyluronic acids, together with the naturally occurring disaccharides **3** and **4**, by the purified Δ4,5-glycosiduronase induced with heparin. Compounds **1** and **2** at a final concentration of 5mM, together with **4** and **3** at a final concentration of 1.14mM, were separately incubated with the purified Δ4,5-glycosiduronase induced with heparin for the time indicated in the figure, and the extent of hydrolysis was measured by the methods described in the text. ● **1**; ▲ **2**; ○ **4**; ▽ **3**.

further extended to the assumption that, since **3** was obtained in yields of 87.8, 72.1, and 49.0% from digests of preparations (BL-1.0-1, BL-1.0-2, and BL-1.0-3) of bovine lung heparan sulfate with the mixed enzymes of purified heparinase, heparitinase 1, and heparitinase 2, respectively, most of the uronic acid linkages of the glycosaminoglycan are assumed to involve α-L-iduronic acid or β-D-glucuronic acid[19].

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