THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 39, Issue of September 29, pp. 22914–22923, 1995

Printed in U.S.A.

Structure Determination of the Octa- and Decasaccharide Sequences Isolated from the Carbohydrate-Protein Linkage Region of Porcine Intestinal Heparin*

(Received for publication, April 18, 1995, and in revised form, July 18, 1995)

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Previously we isolated a tetrasaccharide-serine and a hexasaccharide-serine from the carbohydrate-protein linkage region of porcine intestinal heparin after digestion with a mixture of *Flavobacterium* heparinase and heparitinases I and II (Sugahara, K., Yamada, S., Yoshida, K., de Waard, P., and Vliegenthart, J.F.G. (1992) *J. Biol. Chem.* 267, 1528–1533). In this study four longer carbohydrate sequences (I–IV) attached to Ser or a dipeptide (Ser-Gly or Gly-Ser), which accounted for at least 18.2% of the total linkage region, were isolated from the same heparin preparation after digestion with heparinase only. IV was successfully isolated only after subsequent digestion with glycuronate-2-sulfatase. Their structures were determined by chemical and enzymatic analyses and ^{1}H NMR spectroscopy and found to be the following octa- and decasaccharide sequences attached to Ser in a molar ratio of 1.1:2.3:1.0:1.3: ΔHexA(2S)α1–4GlcN(*N*S,6S)α1–4GlcAβ1–4GlcNAcα1–4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser (I), ΔHexA(2S)α1–4GlcN(*N*S,6S)α1–4IdoAα1–4GlcNAcα1–4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser (II), ΔHexA(2S)α1–4GlcN(*N*S,6S)α1–4IdoAα1–4GlcNAcα1–4GlcAβ1–4GlcNAcα1–4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser (III), ΔHexAα1–4GlcN(*N*S,6S)α1–4IdoAα1–4GlcNAc(6S)α1–4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser (IV) (ΔHexA, GlcA, IdoA, and GlcN represent 4,5-unsaturated hexuronic acid, D-glucuronic acid, L-iduronic acid, and D-glucosamine, whereas 2S, 6S, and *N*S stand for 2-sulfate, 6-sulfate, and *N*-sulfate, respectively). I and II contained 1 mol of Gly in addition to Ser. The four structures indicate that sulfation in heparin chains takes place on the monosaccharide residues located in closer vicinity to the core protein than found for heparan sulfate chains and that there exist at least several heparin subclass chains with different linkage region structures. The significance of the isolated structures is discussed in relation to the biological functions and the biosynthetic mechanisms of heparin.

Heparin exerts a variety of biological activities such as inhibition of blood coagulation (Marcum and Rosenberg, 1989), modulation of cellular proliferation (Clowes and Karnovsky, 1977; Thornton *et al.*, 1983), potentiation of angiogenesis (Folkman and Ingber, 1989), and interactions with acidic and basic fibroblast growth factors (Maciag *et al.*, 1984; Shing *et al.*, 1984; Klagsbrun and Shing, 1985). Some of these activities seem to reside within the complex fine structure of heparin. It is generally accepted that heparin expresses most of these activities by mimicking *in vitro* the physiological activities of heparan sulfate through its structure, similar to that of heparan sulfate. However, the structure-function relationships of heparin/heparan sulfate are not fully understood.

The basic polymeric common structure of heparin and heparan sulfate is an alternating repeat sequence of →4GlcAβ/IdoAα1→4GlcNα1→,[1] which can be variably sulfated (for reviews see Rodén (1980), Gallagher and Lyon (1989), and Lindahl (1989)). Heparin contains more sulfate and IdoA but less *N*-acetyl groups and GlcA as compared with heparan sulfate. Sulfate groups can be located at C-2 of hexuronic acid and C-2, C-3, and/or C-6 of glucosamine residue and add the structural complexity to the carbohydrate backbone to form various active domain structures responsible for a number of biological activities. Recent structural studies of the binding domains to antithrombin III (for review see Lindahl (1989)) and basic fibroblast growth factor (Habuchi *et al.*, 1992; Turnbull *et al.*, 1992; Tyrrell *et al.*, 1993; Maccarana *et al.*, 1993) are the best known examples showing the relationships between the fine structure and biological functions.

Heparin and heparan sulfate are synthesized on the specific serine residues of the core polypeptides through the unique carbohydrate-protein linkage region, -3Galβ1–3Galβ1–4Xylβ1-*O*-Ser, which is also shared by chondroitin sulfate and

* This work was supported in part by the Science Research Promotion Fund from Japan Private School Promotion Foundation, a grant from Takeda Science Foundation, Grants-in-Aid for Scientific Research on Priority Areas 05274107 from the Ministry of Education, Science and Culture of Japan. The Utrecht part of this investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: GlcA, D-glucuronic acid; TOCSY, total correlation spectroscopy; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; MLEV, Malcom Levitt; HexA, hexuronic acid; HPLC, high performance liquid chromatography; ΔHexA, 4,5-unsaturated hexuronic acid or 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid; IdoA, L-iduronic acid; GlcN, D-glucosamine; ΔDiHS-0S, $\Delta^{4,5}$HexAα(1–4)GlcNAc; ΔDiHS-6S, $\Delta^{4,5}$HexAα(1–4)GlcNAc(6-sulfate); ΔDiHS-NS, $\Delta^{4,5}$HexAα(1–4)GlcN(*N*-sulfate); ΔDiHS-diS_1, $\Delta^{4,5}$HexAα(1–4)GlcN(*N*, 6-disulfate); ΔDiHS-diS_2, $\Delta^{4,5}$HexA(2-sulfate)α(1–4)GlcN(*N*-sulfate); ΔDiHS-triS, $\Delta^{4,5}$HexA(2-sulfate)α(1–4)GlcN(*N*, 6-disulfate); *N*S, *N*-sulfate; 2S, 2-*O*-sulfate; 3S, 3-*O*-sulfate; 6S, 6-*O*-sulfate.

dermatan sulfate. It has not been clarified yet how these glycosaminoglycans diverge in biosynthesis into different structures from the same trisaccharide sequence. The biosynthetic sorting mechanism of glucosaminoglycans (heparin/heparan sulfate) and galactosaminoglycans (chondroitin sulfate and dermatan sulfate) has been an enigma. Although unique modifications by phosphorylation and sulfation of the linkage trisaccharide sequence have been demonstrated (Oegema *et al.*, 1988; Fransson *et al.*, 1985; Sugahara *et al*., 1988, 1991, 1992b; de Waard *et al*., 1992), no evidence has been presented for the involvement of these modifications in the biosynthetic sorting mechanism. The importance of the amino acids near the heparan sulfate attachment site has also been pointed out (Zhang and Esko, 1994), but whether peptide sequences are the primary determinants for heparan sulfate synthesis remains to be determined. Differences between biosynthesis of heparin and heparan sulfate are not well understood either. Although they share a number of common structural features, there are several structural differences. Heparan sulfate has a long nonsulfated sequence consisting of at least eight repeating units (-4GlcAβ1–4GlcNAcα1-)$_8$ in the vicinity of the linkage region (For review see Gallagher and Lyon (1989); Lyon *et al.* (1994)), whereas heparin has a shorter nonsulfated sequence and appears to be modified by sulfation near the linkage region (For review see Lindahl (1989); Rosenfeld and Danishefsky (1988); Sugahara *et al*. (1992a)). However, the exact modified structure of this region of a heparin chain has not been investigated in detail yet.

We have been analyzing the structure of the carbohydrate-protein linkage region of various sulfated glycosaminoglycans to investigate the structure-function relationships and the biosynthetic mechanisms of these glycosaminoglycans (Sugahara *et al*., 1988, 1991, 1992a, 1992b, 1994, 1995; de Waard *et al*., 1992). Previously we isolated two glycoserines, ΔHexAα1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser (glycoserine I) and ΔHexAα1–4GlcNAc(6S)α1–4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser (glycoserine II) from the linkage region of heparin after exhaustive digestion with a mixture of heparinase and heparitinases I and II (Sugahara *et al.*, 1992a). In the present study we isolated and characterized larger glycoserines and glycopeptides after digestion with only heparinase in order to investigate the structure beyond the above tetra- and hexasaccharide sequences.

EXPERIMENTAL PROCEDURES

Materials—Stage 14 heparin was purchased from American Diagnostica (New York) and purified by DEAE-cellulose chromatography as previously reported (Sugahara *et al.*, 1992a), and the preparation should have been devoid of heparan sulfate. Cellulofine gels, heparinase (EC 4.2.2.7), and purified heparitinases I (EC 4.2.2.8) and II (no EC number) were obtained from Seikagaku Corp., Tokyo. $\Delta^{4,5}$-Glycuronate-2-sulfatase (EC 3.1.6.-), abbreviated as 2-sulfatase, was purified from *Flavobacterium heparinum* (McLean *et al.*, 1984). Six standard unsaturated disaccharides were prepared from heparin as previously reported (Yamada *et al.*, 1992).

Fragmentation of Heparin and Size Fractionation of the Fragments—The purified heparin (140 mg) was digested by 5 IU of heparinase in a total volume of 10 ml of 30 mM acetate-NaOH buffer, pH 7.0, containing 3 mM $Ca(OAc)_2$ and 1% bovine serum albumin. When the reaction reached a plateau after 7 h as monitored by absorption at 232 nm, it was terminated by heating at 100 °C for 2 min. The digest was adjusted to 0.2 M NaCl and fractionated on a column (3 × 96 cm) of Cellulofine GCL-90-m equilibrated with 0.2 M NaCl. Elution was performed with the same solution at a flow rate of 30 ml/h. Fractions (7.5 ml) were collected and monitored by absorption at 232 nm. Eluates were separated into fractions a–d as shown in Fig. 1. The separated fractions were concentrated, desalted by gel filtration on a column (0.8 × 58 cm) of Cellulofine GCL-25-m, and lyophilized.

HPLC and Capillary Electrophoresis—Fractionation and analysis of unsaturated oligosaccharides were carried out by HPLC on an amine-bound silica PA03 column using a linear gradient of NaH_2PO_4 at a flow rate of 1 ml/min basically as described previously (Sugahara *et al.*, 1992b). Eluates were monitored by absorption at 232 nm. The separated fractions were concentrated and desalted through a column of Sephadex G-25. Capillary electrophoresis was carried out to examine the purity of each isolated fraction using a fused silica capillary in a Waters capillary ion analyzer (Sugahara *et al*., 1994). A new capillary (60 cm total length, 75 μm internal diameter; Millipore Corp.) was activated by sequential washes with 0.1 M sodium hydroxide, distilled water, and 25 mM sodium phosphate buffer, pH 3.0, before use. The electrophoretic fractions were detected by absorption at 185 nm caused by OH groups, since that of the test compounds (the linkage hexasaccharide alditols) was at least 5-fold higher than that obtained at 232 nm. Samples (1 nmol/10 μl), prepared in distilled water, were injected using hydrostatic pressure to give a total volume of 24 nl of an injected solution. The electrophoresis was performed using constant voltage of 15 kV for a period of 20 min. Negative polarity power supply was used.

Quantification of the Linkage Region Glycoserines—The content of the linkage region in fractions a–d was quantified by exhaustive digestion with a mixture of heparinase and heparitinases I and II followed by determination of the resultant glycoserine I by HPLC on an amine-bound silica column as described previously (Sugahara *et al.*, 1992a). The glycoserine II content was below the detection limit of HPLC; the molar ratio of glycoserines I and II in the heparin preparation used was 96:4 (Sugahara *et al*., 1992a).

Digestion of Fraction b-10 with 2-Sulfatase and Subfractionation of the Digest—Fraction *b*-10 (120 nmol) was incubated with 60 mIU of 2-sulfatase in a total volume of 150 μl of 6.7 mM CH_3COONa, pH 6.5, containing 0.05% bovine serum albumin at 37 °C for 1 h. The reaction was terminated by boiling for 1 min, and the digest was fractionated by HPLC on an amine-bound silica column. Otherwise the HPLC conditions were the same as described above.

Digestion of the Isolated Oligosaccharides with Heparinase, Heparitinases, or 2-Sulfatase—Oligosaccharides (0.5 nmol) were digested using 2 mIU of heparinase at 37 °C for 20 min in a total volume of 40 μl of 100 mM acetate-NaOH buffer, pH 7.0, containing 3 mM $Ca(OAc)_2$. For exhaustive heparitinase I digestion, an incubation was conducted using 2.4 mIU of the enzyme and 0.3 nmol of each substrate at 37 °C for 150 min in a total volume of 30 μl of 20 mM acetate-NaOH buffer, pH 7.0, containing 2 mM $Ca(OAc)_2$. For partial heparitinase I digestion, an incubation was carried out using 1 mIU of the enzyme and 0.5 nmol of an oligosaccharide at 37 °C for 5 (for fraction b-5) or 60 min (for fraction b-6) in a total volume of 50 μl of the buffer described above. Heparitinase II digestion was performed using 0.5 mIU of the enzyme and 0.5 nmol of a substrate at 37 °C for 20 min in a total volume of 50 μl of the buffer described above for heparitinase I. 2-Sulfatase digestion was carried out using 5 mIU of the enzyme and 1.0 nmol of a substrate at 37 °C for 60 min in a total volume of 65 μl of the buffer described above for 2-sulfatase. Enzymatic reactions were terminated by boiling for 1 min, and the digests were analyzed by HPLC on an amine-bound silica column as reported (Sugahara *et al.*, 1992b).

^{1}H NMR Spectroscopy—Oligosaccharides for NMR analysis were fully sodiated by cation-exchange chromatography through a column of Dowex 50-X8 (Na^+ form) (7 × 18 mm) and then repeatedly exchanged in ^2H_2O with intermediate lyophilization.

NMR spectra of fractions b-5 and b-6 were recorded on a Bruker AMX-500 or AMX-600 spectrometer (Department of NMR spectroscopy, Utrecht University) operated at a probe temperature of 292, 295, or 300 K. One-dimensional spectra and a double quantum-filtered correlation spectroscopy spectrum of fraction b-5 were recorded as described previously (Piantini *et al*., 1982; Derome and Williamson, 1990; Hård *et al*., 1992). Two-dimensional NOESY experiments were performed with a mixing time of 150–200 ms (Jeener *et al.*, 1979). Two-dimensional TOCSY spectra were recorded using a clean-MLEV-17 spin-lock pulse sequence of 100 ms, preceded by a 2.5-ms trim-pulse (Braunschweiler and Ernst, 1983; Bax and Davis, 1985; Griesinger *et al.*, 1988). In all two-dimensional experiments the HO^{2}H resonance was presaturated during the relaxation delay and additionally during the NOE-mixing time in NOESY-experiments. Phase-sensitive detection was achieved by the time-proportional phase increment method (Marion and Wüthrich, 1983). Two-dimensional spectra were recorded with 300–512 t_1 experiments, and 80–160 free induction decays of 2048 or 4096 data points were collected per t_1 increment. Data sets were processed using the Bruker UXNMR software package. In short, time domain data were zero-filled, multiplied by a phase-shifted sine bell function, and after Fourier transformation base line-corrected with fifth order polynomal fits.

NMR spectra of fraction b-10S-II were recorded on a Varian VXR-500 spectrometer (Kobe Pharmaceutical University) at a probe temperature of 299 K as previously reported (Yamada *et al.*, 1992).

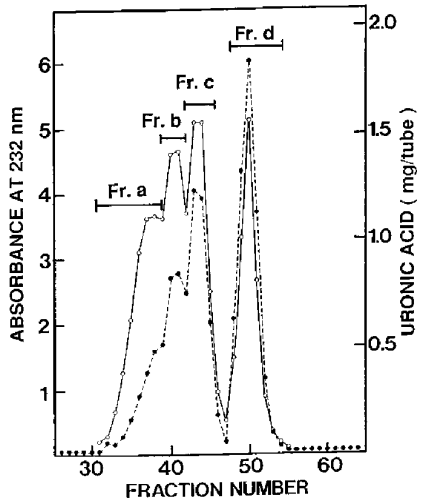

FIG. 1. **Fractionation of the heparinase digest by gel filtration.** Purified stage 14 heparin was exhaustively digested by heparinase and gel-filtrated on a column of Cellulofine GCL-90-m. Fractions were monitored by absorption at 232 nm (●) and the carbazole reaction (○) and pooled as indicated.

Other Analytical Methods—Uronic acid was determined by the carbazole method (Bitter and Muir, 1962). Unsaturated uronic acid was spectrophotometrically quantified based upon an average millimolar absorption coefficient of 5.5 at 232 nm (Yamagata *et al.*, 1968). Amino acids and amino sugars were quantified after acid hydrolysis in 6 M HCl at 110 °C for 20 h and 3 M HCl at 100 °C for 16 h, respectively, using a Beckman 6300E amino acid analyzer (Sugahara *et al.*, 1987).

RESULTS

Isolation of the Linkage Glycoserines—Heparinase specifically cleaves the glucosaminide linkage in -GlcN(*N*S)α1–4IdoA(2S)- (Linker and Hovingh, 1984; Linhardt *et al.*, 1990; Yamada *et al.*, 1994), and heparinase-resistant structures containing a few or several *N*-acetylglucosamine residues have been claimed to occur in the vicinity of the linkage region (Lindahl, 1966; Rosenfeld and Danishefsky, 1988). Therefore, to isolate linkage region fragments larger than the previously isolated glycoserines I and II, stage 14 heparin purified from porcine intestine, which contains Ser as the predominant amino acid (Lindahl and Rodén, 1972), was exhaustively digested with heparinase only. A heparinase digest was fractionated by gel filtration into fractions a–d as indicated in Fig. 1. The recoveries of serine in fractions a, b, c, and d were 67, 24, 2, and 1%, respectively, of that contained in the starting heparin. Fractions c and d mainly contained tetra- and disaccharides, respectively, which were derived from the repeating disaccharide region as characterized by HPLC (data not shown). The oligo- or polysaccharides attached to serines or peptides were recovered in fractions a and b. The amounts of the linkage region structure in fractions a and b were quantified by exhaustive digestion with a mixture of heparin lyases followed by HPLC analysis, which demonstrated (per 100 mg of heparin) 1.67 and 0.60 μmol of glycoserine I in fractions a and b, respectively. They represented 91 and 93% of Ser in the fractions a and b, respectively. Glycoserine II was not detected and seems to be below the detection limit. Fraction a contained dermatan sulfate as a contaminant, which was revealed by the GalN content accounting for approximately 5% (w/w) of the purified heparin preparation. Trials of obtaining appreciable amounts of linkage oligosaccharides from fraction a by HPLC have been unsuccessful. Low recoveries were probably due to strong retention of larger sulfated oligosaccharides on the HPLC column. In this study we demonstrated four linkage structures in fraction b.

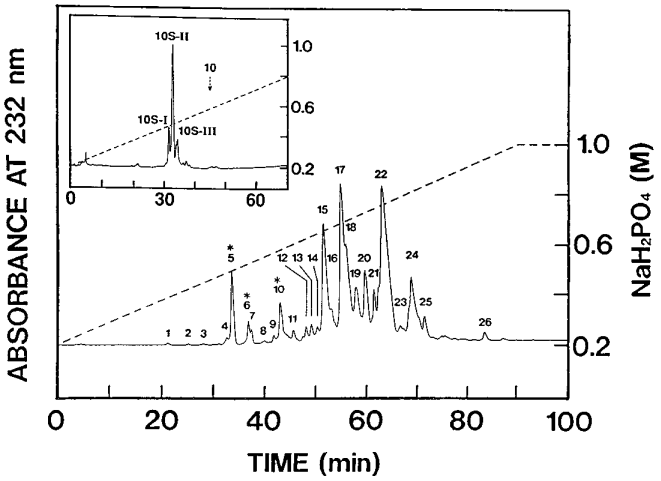

FIG. 2. **Subfractionation of fraction b by HPLC on an amine-bound silica column.** Oligosaccharide fraction b obtained by gel filtration was subfractionated into fractions b-1 to b-26 on an amine-bound silica column using a linear gradient of NaH_2PO_4 from 0.2 to 1.0 M over 90 min. Fractions b-5, b-6, and b-10, which contained Ser, are marked by *asterisks*. Fraction b-10 was digested by 2-sulfatase, and the digest, designated fraction b-10S, was separated into three subfractions, b-10S-I, -II, and -III, by HPLC as shown in the *inset* using the same conditions. The elution position of the parent fraction b-10 is indicated by an *arrow*.

Fraction b was subfractionated by HPLC on an amine-bound silica column, and peaks were designated fractions b-1 to b-26 as shown in Fig. 2. Nine major fractions, 5, 6, 10, 15, 17, 19, 20, 22, and 24, were purified by rechromatography, and the amino acid analysis was performed for each fraction. Based upon the detected serine, approximately 41, 12, and 28% of the linkage region in fraction b was recovered in fractions b-5, -6, and -10, which accounted for 3.6, 1.1, and 2.7% of the applied ΔHexA, respectively. Preparative HPLC yielded 239, 71, and 166 nmol (as Ser) of fractions b-5, -6, and -10, respectively, per 100 mg of the purified heparin. No appreciable amount of serine was recovered in the other peaks, most of which contained unsaturated hexasaccharides derived from the repeating disaccharide region as judged from the ratio of uronic acid and GlcN to ΔHexA. Structural studies of these hexasaccharides are in progress.

The separated fractions b-5, -6, and -10 gave a single symmetrical peak upon HPLC. In the preliminary experiments their sensitivity to 2-sulfatase was examined to evaluate their purity and structural characteristics. 2-Sulfatase acts only on the 4,5-unsaturated hexuronic acid 2-sulfate structure at the nonreducing end of a saccharide chain (McLean *et al.*, 1984). Fractions b-5, -6, and -10 were all sensitive to the enzyme, as expected from the linkage specificity of heparinase (see above), indicating that the major compound in each fraction has a sulfate group at the C-2 position of the ΔHexA (not shown). After 2-sulfatase digestion, fractions b-5 and -6 gave a single peak, which eluted approximately 10 min earlier than the corresponding parent compound on HPLC (not shown), supporting the homogeneity of fractions b-5 and b-6. In contrast, a 2-sulfatase digest of fraction b-10, designated fraction b-10S, gave three peaks, fractions b-10S-I, -II and -III, in a molar ratio of 21:58:21, which eluted approximately 8 min earlier than the parent fraction (Fig. 2, *inset*), indicating that fraction b-10 was a mixture of at least three components. Since it was not possible to resolve fraction b-10 into its subcomponents preparatively, it was first digested with 2-sulfatase, and then the digest was fractionated by HPLC. The major peak b-10S-II, the yield of which was about 90 nmol from 100 mg of the starting heparin, was isolated and subjected to structural analysis.

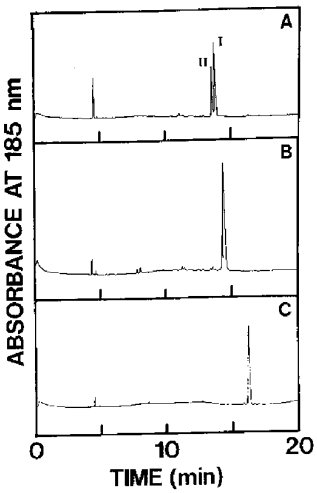

FIG. 3. **Capillary electrophoresis of the isolated linkage region fractions.** Fraction b-5 (*A*), b-6 (*B*) or b-10S-II (*C*) was subjected to electrophoresis as described under "Experimental Procedures." The peak at around 4 min is presumably due to a non-carbohydrate contaminant.

Fractions 10S-I and -III were not analyzed due to their limited amounts.

Characterization of Fraction b-5—Capillary electrophoresis resolved fraction b-5 into two fractions, b-5-II and b-5-I, in a molar ratio of 1.0:2.2 (Fig. 3*A*), indicating that it contains at least two components. However, it was not separable on a large scale into each component and was therefore analyzed without further purification.

As shown in Table I, chemical analysis showed that fraction b-5 contained ΔHexA, HexA, GlcN, Ser, and Gly in a molar ratio of 1.00:1.73:1.96:1.11:0.93. The disaccharide analysis of fraction b-5, carried out by exhaustive heparitinase I digestion followed by HPLC on an amine-bound silica column, gave rise to equimolar amounts of ΔDiHS-0S, ΔDiHS-triS, and a component that eluted shortly before ΔDiHS-0S, corresponding to glycoserine I derived from the carbohydrate-protein linkage region of heparin (Sugahara *et al.*, 1992a) (Fig. 4*B*). These results indicate that fraction b-5 most likely contained two isomeric octasaccharide-peptides, each of which was composed of 1 mol each of the nonsulfated and the trisulfated disaccharide units and HexA1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser (Gly).

Heparitinase II digestion of fraction b-5 yielded equimolar amounts of two components, namely ΔDiHS-triS and a component that eluted near the elution position of the nonsulfated hexasaccharide, ΔHexAα1–4GlcNAcα1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl, derived from the carbohydrate-protein linkage region of bovine kidney heparan sulfate (Sugahara *et al.*, 1994) (Fig. 4*C*). The results are summarized in Table II and are consistent with the previous observation that the hexasaccharide structure is resistant to heparitinase II (Fig. 5) (Sugahara *et al.*, 1994). Together the above results indicate that the two components in fraction b-5 share the common structure, ΔHexA(2S)α1–4GlcN(*N*S,6S)α1–4HexA1–4GlcNAcα1–4HexA1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser(Gly) with only a subtle difference between them.

Characterization of Fraction b-6—Fraction b-6 gave a single peak on HPLC (not shown) and is more than 85% pure as judged by capillary electrophoresis (Fig. 3*B*). Chemical analysis showed that fraction b-6 contained ΔHexA, HexA, GlcN, and Ser in a molar ratio of 1.00:3.17:2.46:1.13 (Table I). The disaccharide analysis of fraction b-6 was performed by heparitinase I digestion followed by HPLC. Exhaustive heparitinase I digestion gave rise to three unsaturated components, ΔDiHS-0S, ΔDiHS-triS and glycoserine I, in a molar ratio of 2:1:1, based upon peak areas (Table II). The results indicate that fraction b-6 contains a decasaccharide-serine composed of 2 mol of the nonsulfated disaccharide units, 1 mol each of the trisulfated disaccharide unit and glycoserine I.

TABLE I
Chemical composition of the isolated linkage regions

Components	Molar ratio		
	Fraction b-5	Fraction b-6	Fraction b-10S-II
ΔHexA	1.00	1.00	1.00
HexA[a]	1.73	3.17	1.91
GlcN[b]	1.96	2.46	2.38
Ser	1.11	1.13	1.40
Gly	0.93	0.18	0.02
Other Amino Acids	0.68	0.46	<0.01

[a] Values have been corrected by subtracting 1.00 and thus do not include ΔHexA.

[b] Values have been corrected for the degradation (5.3%) during acid hydrolysis, which was determined using authentic glycoserine I.

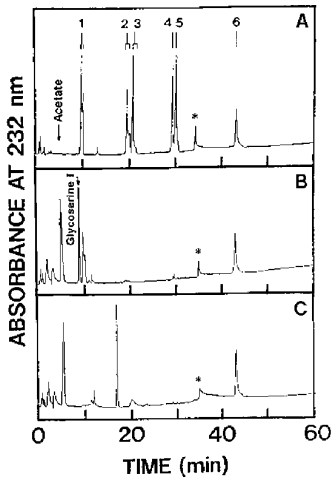

FIG. 4. **HPLC analysis of the enzyme digests of fraction b-5.** Fraction b-5 was digested by heparitinase I (*panel B*) or by heparitinase II alone (*panel C*) as described under "Experimental Procedures." The digest was subjected to HPLC on an amine-bound silica column using a linear gradient of NaH_2PO_4 from 16 to 530 mM over 60 min. Elution positions of the standard disaccharides isolated from heparin/heparan sulfate are indicated in *panel A*. *1*, ΔDiHS-0S; *2*, ΔDiHS-6S; *3*, ΔDiHS-NS; *4*, ΔDiHS-diS_1; *5*, ΔDiHS-diS_2; *6*, ΔDiHS-triS. Glycoserine I is the tetrasaccharide-serine ΔHexAα1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser reported previously (Sugahara *et al.*, 1992a). The peak marked by an *asterisk* at around 35 min is often observed upon high sensitivity analysis and is due to an unknown substance eluted from the column resin.

Heparitinase II digestion of fraction b-6 produced mainly two components (Table II), ΔDiHS-triS and one that eluted approximately 6 min later than ΔHexAα1–4GlcNAcα1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl derived from heparan sulfate (Sugahara *et al.*, 1994), and which therefore was assumed to be a nonsulfated octasaccharide-serine ΔHexAα1–4GlcNAcα1–4HexA1–4GlcNAcα1–4HexA1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser. When exhaustively digested using 6 times the amount of enzyme used under the standard conditions, fraction b-6 gave rise to equimolar amounts of ΔDiHS-0S, ΔDiHS-triS, and a presumably nonsulfated hexasaccharide-serine (data not shown). These results indicate that the major compound in fraction b-6 is a trisulfated decasaccharide-serine ΔHexA(2S)α1–4GlcN(*N*S,6S)α1–4HexA1–4GlcNAcα1–4HexA1–4GlcNAcα1–4HexA1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser.

Characterization of Fraction b-10S-II—Fraction b-10S-II gave a single symmetrical peak both on HPLC (data not shown) and on capillary electrophoresis (Fig. 3*C*), indicating its homogeneity. Chemical analysis showed that fraction b-10S-II contained ΔHexA, HexA, GlcN, and Ser in a molar ratio of 1.00: 1.91:2.38:1.40 (Table I). Upon HPLC analysis of the

TABLE II
Enzymatic analysis of the isolated linkage fractions

After each fraction was incubated with heparitinase I or II, the reaction products were analyzed by HPLC. Recoveries of the disaccharides calculated based on absorbance at 232 nm are shown in parentheses. The absorptions obtained with intact compounds are taken as 100%.

Digestion Fraction b-5	Fraction b-6	Fraction b-10S-II
Heparitinase I		
ΔDiHS-0S (107%)	ΔDiHS-0S (195%)	ΔDiHS-6S (88%)[a]
ΔDiHS-triS (89%)	ΔDiHS-triS (91%)	ΔDiHS-diS_1 (94%)
Nonsulfated tetrasaccharide linkage component (97%)	Nonsulfated tetrasaccharide linkage component (90%)	Nonsulfated tetrasaccharide linkage component (92%)
Heparitinase II		
ΔDiHS-triS (106%)	ΔDiHS-triS (148%)	ΔDiHS-diS_1 (136%)[a]
Nonsulfated hexasaccharide linkage component (103%)	Nonsulfated octasaccharide linkage component (101%)	Monosulfated hexasaccharide linkage component (111%)

[a] A few minor products were also observed.

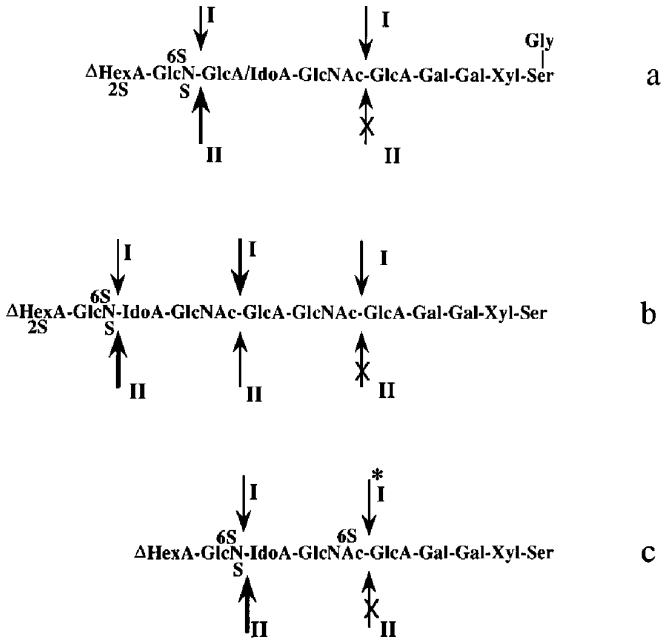

FIG. 5. **Specificities of heparitinases I and II.** Enzymatic action of heparitinases I and II on the isolated linkage compounds are shown by *arrows* with the Roman numerals *I* and *II above* and *below* each structure, respectively. A *thick arrow* shows a preference for the indicated linkage over the other(s). *a*, the octasaccharide-peptides in fractions b-5-I and b-5-II; *b*, the decasaccharide-serine in fraction b-6; *c*, the octasaccharide-serine in fraction b-10S-II. *, it is noted that this linkage in the octasaccharide-serine in fraction b-10S-II was cleaved by heparitinase I, whereas the corresponding linkage in glycoserine II ΔHexA-GlcNAc(6S)-GlcA-Gal-Gal-Xyl-Ser was not (Sugahara *et al.* (1992a), and see "Discussion").

heparitinase I digest of fraction b-10S-II, three major UV-absorbing peaks of glycoserine I, ΔDiHS-6S, and ΔDiHS-diS_1 were observed in a molar ratio of 1.00:0.96:1.02 with a few minor peaks (Table II), indicating that the major compound in fraction b-10S-II was a trisulfated octasaccharide-serine composed of equimolar amounts of the above three components.

When digested with heparitinase II, this compound yielded equimolar amounts of ΔDiHS-diS_1 and the component eluted at the position of glycoserine II (Sugahara *et al.*, 1992a) (Table II), indicating that the disulfated disaccharide unit, ΔDiHS-diS_1, was located at the nonreducing terminus. Thus, the structure of the compound in fraction b-10S-II is ΔHexAα1–4GlcN(*N*S,6S)α1–4HexA1–4GlcNAc(6S)α1–4HexA1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser.

^{1}H NMR Spectroscopy—The structures in fractions b-5 and b-6 were determined using one- and two-dimensional ^{1}H correlation spectroscopy, TOCSY, and NOESY spectra. The anomeric region of the one-dimensional ^{1}H NMR spectrum (Fig. 6*A*) of fraction b-5 shows two sets of signals differing in intensity, reflecting the presence of two compounds in an approximately 2:1 ratio. Resonances stemming from the protons of the core monosaccharide residues Xyl-**1**, Gal-**2**, Gal-**3**, and GlcA-**4** were readily assigned by their characteristic TOCSY patterns on the H-1 tracks (Fig. 7*A*), and the close resemblance of their chemical shifts with those of the common core region GlcA-Gal-Gal-Xyl-Ser (Table III) (van Halbeek *et al.*, 1982). The presence of this partial structure is corroborated by NOEs between GlcA-**4** H-1 and Gal-**3** H-3, between Gal-**3** H-1 and Gal-**2** H-3, and between Gal-**2** H-1 and Xyl-**1** H-4 (Fig. 7*B*). The presence of a single series of proton resonances stemming from this partial structure indicates that both compounds contain this common core region. Almost identical sets of signals are observed for the terminal ΔHexA residue (Fig. 6*A*), the chemical shifts being indicative of sulfation at the 2-position (Table III) (Horne and Gettins, 1991), showing that both structures contain a terminal ΔHexA(2S) residue.

The similar intensities of the resonances at δ 5.377, δ 5.335, and δ 4.948 suggest that they belong to the major compound (b-5-I). The resonance at δ 5.377 stems from the anomeric proton of an *N*,6-disulfated glucosamine residue (Fig. 6*A*). This assignment is based on the upfield shift out of the bulk region of the H-2 resonance (δ 3.279) reflecting *N*-sulfation (Horne and Gettins, 1991; Yamada *et al.*, 1994), and the downfield shifts out of the bulk region of the H-5 (δ 3.985) and the hydroxymethyl-proton signals (δ 4.203, 4.353), indicating sulfation at the 6-position (Fig. 6A) (Horne and Gettins, 1991; Sugahara *et al.*, 1992a; Yamada *et al.*, 1994). The presence of an NAc resonance (δ 2.034), and the characteristic TOCSY pattern observed on the H-1 track (Fig. 7*A*) show that the signal at δ 5.335 stems from the anomeric proton of a nonsulfated glucosamine residue (Sugahara *et al.*, 1994). The presence of an iduronic acid residue is deduced from the small coupling constant (2 Hz) observed on the signal at δ 4.948 and its characteristic TOCSY pattern observed on the H-1 track (Fig. 7*A*, Table III) (Sugahara *et al.*, 1994). The sequence of the major compound b-5-I is unequivocally established by NOEs between ΔHexA(2S)-**8** H-1 and GlcN(*N*S,6S)-**7** H-4 and H-6, between GlcN(*N*S,6S)-**7** H-1 and IdoA-**6** H-3 and H-4, between IdoA-**6** H-1 and GlcNAc-**5** H-4, and between GlcNAc-**5** H-1 and GlcA-**4** H-4 (Fig. 7*B*).

The minor compound (b-5-II) also contains an *N*,6-disulfated and a nonsulfated glucosamine residue as judged from the TOCSY patterns on their H-1 tracks at δ 5.566 and δ 5.359, respectively (Fig. 7*A*) (Sugahara *et al.*, 1994; Yamada *et al.*, 1994). NOEs between GlcNAc H-1 and GlcA-**4** H-4 and the one between ΔHexA(2S)-**8** H-1 and GlcN(*N*S,6S) H-4 and H-6, locate these glucosamine residues at positions **5** and **7** in the oligosaccharide sequence. The resonance at δ 4.510 ppm, dis-

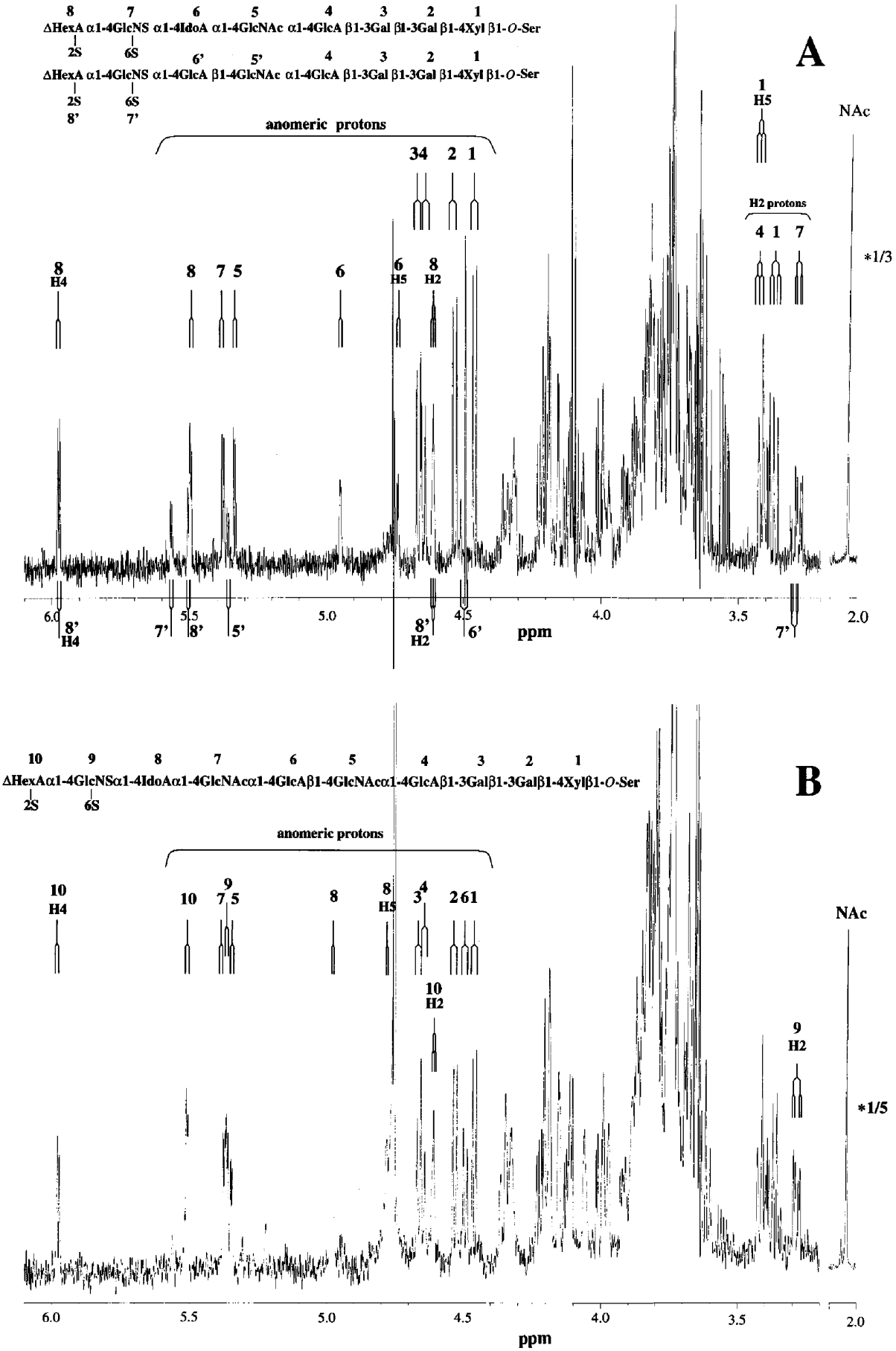

FIG. 6. **One-dimensional 600-MHz ^1H NMR spectra of the structures in fractions b-5 and b-6, recorded in $^2H_20$ at 300 K.** The *Arabic numerals* in the spectra refer to the corresponding residues in the structures. *A*, fraction b-5; *B*, fraction b-6.

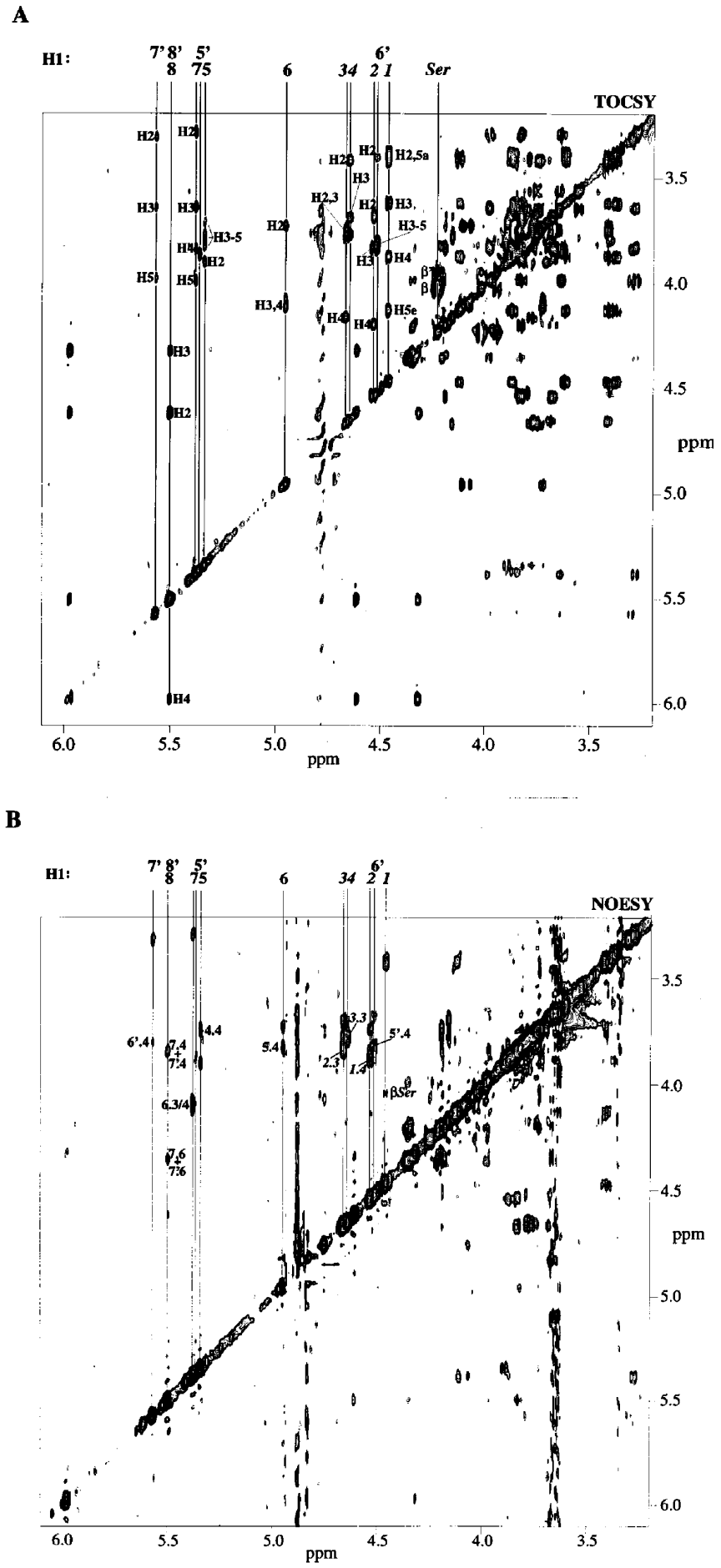

FIG. 7. **Two-dimensional TOCSY (*A*) and NOESY (*B*) spectra of the structures in fraction b-5 recorded at 300 and 292 K, respectively.** Anomeric protons are indicated by *vertical lines*, and the *number* at the *top* corresponds to the monosaccharide number. Resonances of the core region overlap and are indicated in *italics*. Cross-peaks on the H-1 tracks are labeled by proton number in the TOCSY spectrum. In the NOESY spectrum only trans-glycosidic NOEs on the H-1 tracks are labeled. The *double digit number* represents the monosaccharide unit followed by the proton number.

TABLE III

^{1}H chemical shifts of structural reporter groups of the monosaccharide constituents of the linkage glycoserines

^{1}H chemical shifts of structural reporter groups of the monosaccharide constituents of the four linkage oligosaccharides attached to serine are shown together with those of a reference compound (Sugahara et al., 1992a). Chemical shifts were determined from well-resolved signals in one-dimensional ^{1}H NMR spectra or in case of overlap from cross-peaks in two-dimensional correlation spectroscopy, TOCSY, or NOESY[a] spectra. They are given in ppm by reference to internal acetone (δ 2.225) in ^2H_2O (Vliegenthart *et al.*, 1983) at 300 K (b-5 and b-6) or 299 K (b-10S-II), except where noted.[a] Coupling constants $J_{1,2}$ (in Hz) of GlcA and IdoA residues are given in parentheses.

Residue	Reporter group	*R*	b-5-I	b-5-II	b-6	b-10S-II
Ser	Hα	3.90	4.226	4.226	4.14	ND[b]
	Hβ	3.90	4.013	4.013	3.92	ND
	Hβ′	4.14	3.942	3.942	3.92	ND
Xyl-1	H1	4.438	4.458	4.458	4.458	4.466
	H2	3.349	3.363	3.363	3.350	3.374
	H3	3.605	3.612	3.612	3.605	3.616
	H4	3.861	3.869	3.869	3.863	3.871
	H5ax	3.400	3.406	3.406	3.392	3.409
	H5eq	4.108	4.123	4.123	4.110	4.113
Gal-2	H1	4.533	4.531	4.531	4.529	4.533
	H2	3.669	3.671	3.671	3.670	3.664
	H3	3.828	3.828	3.828	3.818	3.829
	H4	4.193	4.188	4.188	4.186	4.187
Gal-3	H1	4.658	4.663	4.663	4.661	4.663
	H2	3.739	3.737	3.737	3.74	3.739
	H3	3.782	3.768	3.768	3.763	3.79
	H4	4.162	4.155	4.155	4.154	4.155
GlcA-4	H1	4.642	4.645 (8.0)	4.645 (8.0)	4.647 (8.0)	4.647 (8.0)
	H2	3.418	3.403	3.403	3.403	3.420
	H3	3.667	3.680	3.680	3.672	3.676
	H4	3.75	3.73[a]	3.74[a]	3.714[a]	3.75
GlcN-5	H1	5.406	5.335	5.359	5.347	5.358
	H2	3.941	3.895	3.863	3.893	3.913
	H3	3.799	3.775	ND	3.81	3.775
	H4	3.853	3.818[a]	3.82[a]	3.798[a]	3.73
	H5	4.015	3.707	ND	3.736	3.996
	H6	4.446	ND	ND	ND	ND
	H6′	4.170	ND	ND	ND	ND
	NAc	2.044	2.034	2.034	2.035	2.036
HexA-6[c]	H1	—[d]	4.948 (2.0)	4.510 (8.0)	4.489 (8.0)	5.023 (2.5)
	H2	—	3.720	3.393	3.345	3.783
	H3	—	4.107	3.79	3.668	4.126
	H4	—	4.066	3.784[a]	3.744[a]	4.051
	H5	—	4.754	ND	ND	ND
GlcN-7	H1	—	5.377	5.566	5.375	5.350
	H2	—	3.279	3.298	3.847	3.272
	H3	—	3.636	3.634	ND	3.676
	H4	—	3.83[a]	3.83[a]	3.80[a]	3.849
	H5	—	3.985	3.977	ND	4.028
	H6	—	4.353	4.335	ND	ND
	H6′	—	4.203	4.182	ND	ND
	NAc	—	—	—	2.035	—
IdoA-8	H1	—	—	—	4.982 (2.0)	—
	H2	—	—	—	3.745	—
	H3	—	—	—	4.111	—
	H4	—	—	—	4.060	—
	H5	—	—	—	4.775	—
GlcN-9	H1	—	—	—	5.364	—
	H2	—	—	—	3.282	—
	H3	—	—	—	3.661	—
	H4	—	—	—	3.840[a]	—
	H5	—	—	—	3.977	—
	H6	—	—	—	4.352	—
	H6′	—	—	—	4.196	—
	NAc	—	—	—	—	—
ΔHexA	H1	5.158	5.495	5.503	5.509	5.137
	H2	3.813	4.61	4.61	4.609	3.761
	H3	4.236	4.32	4.32	4.325	4.267
	H4	5.808	5.973	5.971	5.975	5.782

[a] Chemical shifts were determined from NOESY spectra recorded at 292 K (b-5) or 295 K (b-6).
[b] ND, not determined.
[c] HexA-6 is IdoA in b-5-I and b-10S-II; GlcA in b-5-II and b-6.
[d] —, not occurring.

playing a coupling constant of 8 Hz, and its characteristic TOCSY-pattern on the H-1 track show the presence of a GlcA residue (Sugahara *et al.*, 1994; Yamada *et al.*, 1994). The NOE between GlcN(*N*S,6S)-**7** H-1 and GlcA H-4 and the one between GlcA H-1 and H-4 of GlcNAc-**5** locate this residue at position **6** in the sequence. The resonance of the anomeric proton of GlcN(*N*S,6S)-**7** (δ 5.566) has shifted downfield by Δδ 0.19 with respect to the corresponding signal of the major compound, in agreement with the previously reported observation that the GlcN(*N*S,6S) H-1 reports on the identity of the preceding hex-

uronic acid (Horne and Gettins, 1991). In conclusion, the two compounds in fraction b-5 are as follows: b-5-I (ΔHexA(2S)α1–4GlcN(*N*S,6S)α1–4IdoAα1–4GlcNAcα1–4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser) and b-5-II (ΔHexA(2S)α1–4GlcN(*N*S,6S)α1–4GlcAβ1–4GlcNAcα1–4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser).

The one-dimensional ^{1}H NMR spectrum of fraction b-6 is shown in Fig. 6*B*. The TOCSY spectrum of fraction b-6 (not shown) is very similar to that of fraction b-5, especially with respect to the signals stemming from the core region, GlcA-Gal-Gal-Xyl-*O*-Ser and those of the terminal ΔHexA(2S) residue (Table III), showing the presence of these structural elements (van Halbeek *et al*., 1982; Horne and Gettins, 1991; Sugahara *et al*., 1992a; Yamada *et al*., 1994). Characteristic TOCSY patterns on the H-1 tracks at δ 5.364, δ 5.375, and δ 5.347 led to identification of one *N*,6-disulfated and two nonsulfated glucosamine residues (Sugahara *et al*., 1994; Yamada *et al*., 1994). Furthermore, both an IdoA and a GlcA residue are present based on their characteristic chemical shifts (Sugahara *et al*., 1994; Yamada *et al*., 1994) determined from the TOCSY pattern and intraresidue NOEs. The sequence of this decasaccharide was elucidated by a series of trans-glycosidic H-1 to H-4 NOEs, an NOE between ΔHexA H-1 and H-6 of GlcN(*N*S,6S), and one between GlcN(*N*S,6S) H-1 and IdoA H-3, showing that fraction b-6 contains the following structure: b-6, ΔHexA(2S)α1–4GlcN(*N*S,6S)α1–4IdoAα1–4GlcNAcα1–4GlcAβ1–4GlcNAcα1–4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser.

Fraction b-10S-II was analyzed by one-dimensional, TOCSY, and correlation spectroscopy spectra (not shown), and the NMR data are summarized in Table III. Based on these NMR data the following trisulfated octasaccharide-serine is proposed for the structure of the compound in fraction b-10S-II: b-10S-II, ΔHexAα1–4GlcN(*N*S,6S)α1–4IdoAα1–4GlcNAc(6S)α1–4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser.

Since fraction b-10S-II was isolated after 2-sulfatase digestion as described above, the major compound in the parent fraction b-10 contains the following structure: b-10, ΔHexA(2S)α1–4GlcN(*N*S,6S)α1–4IdoAα1–4GlcNAc(6S)α1–4GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-*O*-Ser.

DISCUSSION

In this study we identified one deca- and three octasaccharide structures in the linkage-derived fractions b-5-I, -5-II, -6, and -10, which accounted for at least 7.4, 3.5, 3.2, and 4.1 mol % of the total linkage region of porcine intestinal heparin, respectively. Since they were prepared using only heparinase, they represent most likely the molecules from which glycoserines I (a tetrasaccharide-serine) and II (a hexasaccharide-serine) were previously produced by exhaustive digestion with a mixture of heparinase and heparitinases I and II (Sugahara *et al*., 1992a). Presumably, glycoserine I had been produced partly from the compounds corresponding to fractions b-5-I, b-5-II, and b-6, whereas glycoserine II had been produced at least from the compound corresponding to fraction b-10 (see Fig. 5 for the enzyme specificities).

Although the majority of the linkage structures with even longer sequences were recovered in fraction a and remain to be investigated, the molecules isolated in this study contain hitherto unreported structural features in the extended region beyond the sequences found in glycoserines I and II. They share the trisulfated disaccharide unit in common at the nonreducing termini, indicating that the trisulfated unit characteristic of heparin begins emerging even in the second and the third disaccharide units from the carbohydrate attachment site. The GlcNAc in the first disaccharide unit can be 6-sulfated as in the compound in fraction b-10, but it cannot be *N*-sulfated. The sugar residue at the first uronic acid position is always GlcA but never IdoA in these four molecules, which is in contrast to the recent finding of both GlcA and IdoA at this position in dermatan sulfate from bovine aorta (Sugahara *et al*., 1995). The second and the third uronic acid could be IdoA when located adjacent to the trisulfated disaccharide unit as in the compounds in fractions b-5-I, b-10, and b-6. However, the uronic acid next to the trisulfated disaccharide unit was not always IdoA but could be GlcA as in the compound in fraction b-5-II. The above structural characteristics of these four molecules may have some implications in the expression of biological functions and in the biosynthetic mechanisms of heparin.

The trisulfated disaccharide unit characteristic of heparin and heparan sulfate were demonstrated to begin appearing nearer the linkage region in heparin as compared with heparan sulfate. A heparin chain with an IdoA-containing segment closer to the linkage region would be more flexible and mobile around the core protein due to the specific conformational properties of sulfated or nonsulfated iduronic acid, which appears to be present in dynamic equilibrium of different conformations (Casu, 1989). In contrast, heparan sulfate has a long nonsulfated stretch of more than eight repeating disaccharide units, which are assumed to contain only GlcA (Gallagher and Lyon, 1989; Lindblom *et al*., 1991; Lyon *et al*., 1994) and therefore would be rather rigid in the proximal portion to the linkage region but plastic in the distal portion.

The present study indicates that there are at least four subclasses of heparin chains different in structure of the linkage region and/or in length of the nonsulfated sequence proximal to the protein core. It is likely that there exist other subclass chains in fraction a, and it is possible that different chains have different patterns of modification. It remains to be determined whether biologically active domain structures such as the binding domains to the antithrombin III and basic fibroblast growth factor are found on a specific subclass chain and where along a heparin chain they are embedded. Since the linkage region is first constructed in biosynthesis, differences in the structure of the linkage region may influence that of the repeating disaccharide region to be synthesized thereafter. It should be noted that the anticoagulant-conferring area appears to occur about 20 disaccharide units away from the linkage region (Rosenfeld and Danishefsky, 1988). The observed heterogeneity in the linkage region also raises questions of whether the different chains are derived from different core proteins and whether they come from identical or different sites of a single core protein. Answers to these questions require further investigation.

The present work provided some useful information about the substrate specificities of heparitinases I and II, which are essential tools for structural studies of heparin/heparan sulfate. Previously, heparitinase I was shown to cleave glucosaminidic linkages bound to nonsulfated GlcA and IdoA except for two glucosaminidic linkages: the one linked to the GlcA residue substituting the 3-sulfated glucosamine residue in the antithrombin III-binding sequence (Yamada *et al*., 1993) and the one linked to the GlcA residue located between the Gal and the 6-sulfated GlcNAc found in glycoserine II (Sugahara *et al*., 1992a). In this study, however, all the glucosaminidic linkages in the four molecules including the octasaccharide in fraction b-10S-II were cleaved by this enzyme (Fig. 5*c*). It may be that the enzyme acts on the glucosaminidic linkage in the sequence -GlcNAc(6S)-GlcA-Gal- when it is located in an octasaccharide (b-10S-II) but not in a hexasaccharide (glycoserine II). Digestability of the glucosaminidic linkages in fraction b-6 suggests some linkage preference of this enzyme. Although all of the three glucosaminidic linkages were cleaved by the enzyme under harsh conditions, only the middle linkage was cleaved

under milder conditions for partial digestion (see "Experimental Procedures"), yielding a tetrasaccharide and a linkage hexasaccharide-serine but no disaccharides, as illustrated in Fig. 5*b*. The enzyme appears to prefer the linkage between the two nonsulfated disaccharide units to the other two, consistent with the notion that the enzyme acts on the relatively low sulfated region of a heparan sulfate chain (Linhardt *et al.*, 1990). Heparitinase I digestion of fraction b-5 under limited conditions resulted in both di- and tetrasaccharides, suggesting the comparable sensitivity of the two glucosaminidic linkages to this enzyme as illustrated in Fig. 5*a*. Heparitinase II has been demonstrated to have a broad specificity (Linhardt *et al.*, 1990; Nader *et al.*, 1990; Yamada *et al.*, 1994, 1995) acting on every α-glucosaminidic bond in heparin except for the two unique hexosaminidic bonds: the one in the structure -4GlcNAc(6S)α1–4GlcAβ1–4GlcN(*N*S,3S,6S) found within the antithrombin III-binding domain (Yamada *et al.*, 1993) and the one in the structure -4HexA1–4GlcNAcα1–4GlcAβ1–3Galβ1- of the carbohydrate-protein linkage region (Fig. 5) (Sugahara *et al.*, 1994). In this study it was demonstrated that this enzyme cleaves the hexosaminidic linkage adjacent to a trisulfated disaccharide unit more preferentially than the one next to a nonsulfated disaccharide unit (Fig. 5*b*).

Acknowledgment—We thank Dr. Makiko Sugiura (Kobe Pharmaceutical University) for recording the NMR spectrum of fraction b-10S-II.

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