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Chondroitinase ABC-resistant sulfated trisaccharides isolated from digests of chondroitin/dermatan sulfate chains

Kazuyuki Sugahara [a,*], Yuki Takemura [a], Makiko Sugiura [b], Yuniko Kohno [c], Keiichi Yoshida [c], Kyoko Takeda [a], Kay Hooi Khoo [d], Howard R. Morris [d] and Anne Dell [d]

[a] *Department of Physiological Chemistry,* [b] *NMR-Laboratory, Kobe Women's College of Pharmacy, Higashinada, Kobe 658 (Japan)*
[c] *Tokyo Research Institute of Seikagaku Corp., Higashiyamato, Tokyo 189 (Japan)*
[d] *Department of Biochemistry, Imperial College, London (United Kingdom)*

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ABSTRACT

Four kinds of sulfated trisaccharides resistant to chondroitinase ABC were isolated after chondroitinase B or ABC treatment of dermatan sulfate or various chondroitin sulfate isomers, respectively. Their composition was determined by chemical analysis and fast atom bombardment-mass spectrometry. Their structures were characterized by chondroitinase ACII digestion in conjunction with HPLC, and 500-MHz one- and two-dimensional ^{1}H NMR spectroscopy. All the four trisaccharides have in common the core saccharide sequence, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-D-Glc*p*A. A monosulfated component isolated from shark scapular cartilage chondroitin sulfate C or bovine aorta dermatan sulfate was elucidated as α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-D-Glc*p*A or α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^--(1 → 4)-D-Glc*p*A, respectively. A disulfated component obtained from shark scapular cartilage chondroitin sulfate C or squid cartilage chondroitin sulfate E was identified as α-L-$\Delta^{4,5}$Hex*p*A2SO_3^--(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-D-Glc*p*A or α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^- 6SO_3^--(1 → 4)-D-Glc*p*A, respectively. These trisaccharides are derived from the reducing termini of the parent polysaccharides. Some of the trisaccharides could be derived from the reducing termini exposed by the peeling reaction during the alkaline treatment while some others may represent the cleavage sites exposed by tissue endo-β-D-glucuronidase(s), indicating the presence of such enzyme(s) which may release chondroitin/dermatan sulfate fragments from proteoglycans.

INTRODUCTION

Chondroitin sulfate (CS) [†]/dermatan sulfate (DS) proteoglycans are found in various body fluids, extracellular matrices, and also on cell surfaces, and play

* Corresponding author.

† Abbreviations used are: L-$\Delta^{4,5}$Hex*p*A, 4,5 unsaturated L-hexuronic acid; CS, chondroitin sulfate; CSA, CSC, CSD or CSE, chondroitin sulfate A, C, D or E; DS, dermatan sulfate; HA, hyaluronan; HS, heparan sulfate; Hep, heparin; 2-sulfatase, $\Delta^{4,5}$-hexuronate-2-*O*-sulfatase; 4- or 6-sulfatase, chondro-4- or -6-sulfatase; FAB-MS, Fast Atom Bombardment-Mass Spectrometry; 2D, two-dimensional; COSY, correlation spectroscopy; HOHAHA, homonuclear Hartmann–Hahn.

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important roles in various cellular recognition systems[1–3]. The primary backbone structure of a CS or a DS glycosaminoglycan chain is composed of a repeating disaccharide unit, →4)-β-D-Glc*p*A-(or α-L-Ido*p*A)-(1→3)-β-D-Gal*p*NAc-(1→, which is variably sulfated, while the linkage region between the carbohydrate and protein moieties has a unique tetrasaccharide structure, →4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Xyl*p*-(1→, which is covalently attached to a Ser residue of a core polypeptide of CS/DS proteoglycans. These polysaccharides can be decomposed using chondroitinase ABC, which has been widely used as a tool for structural studies of CS/DS glycosaminoglycans[4]. This enzyme degrades in an eliminative fashion both CS and DS glycosaminoglycans yielding a variety of sulfated unsaturated disaccharides originating from the repeating disaccharide region and hexasaccharides derived from the carbohydrate-protein linkage region[5–10].

We obtained several sulfated tri- and tetra-saccharides resistant to highly purified chondroitinase ABC, which is commercially available as a "protease-free" enzyme, from DS and several CS isomers, and elucidated their structures. In the preceding paper[11] the structures of the tetrasaccharides were reported while those of the trisaccharides are described in this article. These trisaccharides are resistant to the conventional chondroitinase ABC as well. The structures of the trisaccharides indicate that they are derived from the reducing ends of polysaccharides, at least some of which seem to have been released endoglycosidically by tissue endo-β-D-glucuronidase from proteoglycans or free glycosaminoglycan chains.

EXPERIMENTAL

Most of the materials and methods used in this report including preparation of the trisaccharides are described in detail in the first article of this series[11]. Chondroitinase B, chondro-4- and -6-sulfatases, which are abbreviated as 4- and 6-sulfatase, respectively, were purchased from Seikagaku Corp., Tokyo.

$\Delta^{4,5}$-Hexuronate-2-*O*-sulfatase, abbreviated as 2-sulfatase, was purified from *Flavobacterium heparinum*[12].

Preparation of peptidodermatan sulfate from bovine aorta.—Minced bovine aorta (14 kg) was treated in 1.4 L of boiling water for 30 min. After Actinase E digestion of the tissue peptidoglycans were precipitated with EtOH as previously reported[13]. The peptidoglycans were fractioned by anion exchange chromatography on HPA-11 resin (Mitsubishi Kasei Corp., Tokyo). The fractions eluted stepwise with 0.5, 0.75, 1.5, and 2.5 M NaCl contained predominantly HA, HS, DS, and DS/Hep, respectively, as examined by cellulose acetate strip electrophoresis. The 1.5 M NaCl fraction containing mainly DS was treated with DNase I and RNase A to remove contaminating nucleic acids[13], and peptidoglycans (27 g) were recovered by EtOH precipitation. This peptidoglycan fraction which contained small proportions of HS and CS was further fractionated by the second cycle of anion exchange chromatography. The 1.5 M NaCl fraction containing 1.79 g of polysaccharides was

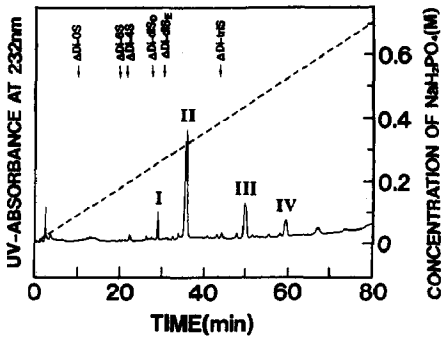

Fig. 1. HPLC analysis of the oligosaccharides from bovine aorta dermatan sulfate. The oligosaccharide fraction was chromatographed on an amine-bound silica column using a linear gradient of 16 to 800 mM NaH_2PO_4 over a 90 min period, otherwise as indicated in the Experimental. The elution positions of authentic unsaturated CS disaccharides are indicated by arrows.

purified by EtOH fractionation. The fraction precipitated at 25–36% EtOH and designated as L25% fraction contained 688 mg of purified DS peptidoglycans. This fraction (100 mg) contained 1.12 and 1.95 μmol of Ser and Gly, respectively, as major amino acids.

Preparation of trisaccharides.—A monosulfated trisaccharide (DS fraction I) and its desulfated counterpart (DS fraction I_0) were prepared from bovine aorta DS peptidoglycan preparation (L25% fraction) as follows. Bovine aorta DS peptidoglycan preparation (50 mg) was digested with 0.1 unit of chondroitinase B in a total volume of 4 mL of 0.05 M Tris · HCl buffer, pH 8.0, containing 60 mM sodium acetate and 100 μg/mL of bovine serum albumin as a stabilizer for 8 h at 37°C; an additional 0.01 unit of the enzyme was added after 6 h. The digest was chromatographed[11] on a column of Sephadex G-15. Two UV-absorbing discrete peaks corresponding to 10.5 and 33.3 μmol of L-$\Delta^{4,5}$Hex*p*A were observed in the included volume (data not shown). The faster-eluting peak I represented oligosaccharides and glycopeptides, while the slower-eluting peak II contained disaccharides. Disaccharide analysis by HPLC of peak II showed that the molar ratio of ΔDi-0S/ΔDi-6S/ΔDi-4S/ΔDi-diS_B * was 1:1:87:11. When peak I was subfractionated by HPLC four major UV-absorbing peaks were observed as shown in Fig. 1. Fraction II resulted in two moles of ΔDi-4S upon chondroitinase ACII digestion (data not shown), indicating that it is a tetrasaccharide, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^-. Fractions III and IV have not been characterized yet. Chemical analysis of fraction I indicated that

* Abbreviations for disaccharide fragments: ΔDi-0S, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-D-Gal*p*NAc; ΔDi-UA2S, α-L-$\Delta^{4,5}$Hex*p*A2SO_3^--(1 → 3)-D-Gal*p*NAc; ΔDi-4S, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^-; ΔDi-6S, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-D-Gal*p*NAc6SO_3^-; ΔDi-diS_B, α-L-$\Delta^{4,5}$Hex*p*A2SO_3^--(1 → 3)-D-Gal*p*NAc4SO_3^-; ΔDi-diS_D, α-L-$\Delta^{4,5}$Hex*p*A2SO_3^--(1 → 3)-D-Gal*p*NAc6SO_3^-; ΔDi-diS_E, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^- 6SO_3^-; ΔDi-triS, α-L-$\Delta^{4,5}$Hex*p*A2SO_3^--(1 → 3)-D-Gal*p*NAc4SO_3^- 6SO_3^-.

it is an unsaturated trisaccharide. Although sulfate analysis was not carried out for this sample the elution position on HPLC suggests that it is monosulfated. This fraction was resistant to both the conventional and the highly purified chondroitinase ABC. The total yield of DS fraction I was 770 nmol per 100 mg of the peptidoglycans. Since the average molecular weight of the starting DS preparation is estimated to be at least 10 000 by gel filtration, DS fraction I occupies more than 8% of the reducing termini of the glycosaminoglycan chains. The desulfated counterpart (DS fraction I_0) of DS fraction I was prepared by 4-sulfatase treatment of DS fraction I.

Two trisaccharides (CSC fractions 9 and 14-2) were obtained as byproducts when hexasaccharides derived from the carbohydrate–protein linkage region were prepared by chondroitinase ABC digestion of CSC peptidoglycans from shark scapular cartilage as previously reported[9]. Subfractionation of fraction 14 into fractions 14-1 and 14-2 by HPLC was achieved as previously reported[14]. The isolated CSC fractions 9 and 14-2 were resistant to both the conventional and the highly purified chondroitinase ABC. The yields of CSC fractions 9 and 14-2 were 99 and 45 nmol, respectively, per 100 mg of the CS peptidoglycans. They represent 8.0 and 3.6% of the total oligosaccharides (1235 nmol) isolated from the carbohydrate–protein linkage region fraction[9,14]. These trisaccharides were found to be nonreduced even though sodium borohydride was present during the alkaline treatment. It may be due to the low borohydride concentration (20 mM).

Three trisaccharides (CSE fractions 1, 2, and 7a) were obtained as byproducts when tetrasaccharides were prepared by exhaustive digestion of commercial squid cartilage CSE glycosaminoglycans with a highly purified chondroitinase ABC as reported in the first article of this series[11]. The isolated three fractions were resistant to both the conventional and the highly purified enzyme. Their yields were 1460, 770, and 6130 nmol per 100 mg of the polysaccharides, respectively. Most of the glycosaminoglycan chains are calculated to have these trisaccharides at the reducing ends since the average molecular weights were estimated to be at least 10 000 by gel filtration (data not shown). The origin of the trisaccharides isolated in this study is summarized in Table I.

Permethylation.—Hakomori permethylation[15] and the subsequent Sep-Pak® clean-up procedures of the permethylated samples were carried out as described previously[16]. The monosulfated trisaccharide was eluted in the 35% MeCN fraction from the Sep-Pak® cartridge (Waters, Ltd.), whilst disulfated trisaccharide was collected in the 15% MeCN fraction. The resulting Sep-Pak® purified permethyl derivative was loaded onto the monothioglycerol matrix for FABMS analysis.

Peracetylation.—Perdeuterioacetylation was performed by incubating the sample with 1:5 1-methylimidazole–hexadeuteroacetic anhydride (30 μL) at room temperature for 1 h. The reagents were then dried down under a stream of N_2 and samples were redissolved in MeOH for aliquoting into the monothioglycerol matrix.

FABMS.—The negative-ion FAB-spectra of oligosaccharide samples were ob-

tained using a VG Analytical ZAB-HF mass spectrometer fitted with an M-Scan FAB gun operated at 10 kV. Spectra were recorded on oscillographic chart paper and manually counted. In some of the experiments, data were acquired using a ZAB-2SE FPD mass spectrometer fitted with a Cs-ion gun operated at 25 kV. Data acquisition and processing were performed using the VG Analytical Opus® software.

Enzyme treatments of the isolated oligosaccharides.—Chondroitinase ACII digestion of mono- or di-sulfated trisaccharides was carried out using 0.5 nmol of each isolated oligosaccharide and 3.5 or 15 mU of the enzyme, respectively, in a total volume of 40 μL of 0.05 M sodium acetate buffer, pH 6.0 at 37°C for 10 min as previously described[11]. Digestions with chondroitinase ABC and sulfatases were carried out as described in ref 11.

HPLC.—Fractionation of the oligosaccharides and analysis of enzyme digests of the isolated trisaccharides were carried out by HPLC[13,17]. Chromatography was performed on a 4.6 × 250 mm amine-bound silica PA03 column (YMC Co., Kyoto) using a linear gradient of 16 to 530 mM NaH_2PO_4 over a 60 min period at a flow rate of 1.0 mL/min at room temperature unless otherwise indicated.

500-MHz ^1H NMR spectroscopy.—Tetrasaccharides were repeatedly exchanged in D_2O with intermediate lyophilization. The 500-MHz ^1H NMR spectra were determined in D_2O, using a Varian VXR-500 spectrometer at a probe temperature of 26°C. Chemical shifts, given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, were measured from internal acetone at δ 2.225 (refs 18,19). The 2D HOHAHA spectrum was measured with 128 t_1 increments and each t_1 increment consisted of 512 data points from 48 transients. A spinlock mixing pulse of 80 ms was used. Data sets were multiplied by Gaussian function and zero-filled to 512 data points in F1 prior to Fourier transformation.

RESULTS

Preparation of the trisaccharides.—During the course of the preparation of the tetrasaccharides by chondroitinase ABC digestion of various CS isomers three unidentified oligosaccharides, CSE fractions 1, 2, and 7a, which were resistant to chondroitinase ABC were obtained[11]. The sugar analysis (Table I) and the elution positions on HPLC indicate that both CSE fractions 1 and 2 are monosulfated trisaccharides and fraction 7a is a disulfated trisaccharide. Fractions corresponding to CSE fractions 1 and 2 were also obtained from CSA and CSC glycosaminoglycans[11]. Likewise, two other unsaturated oligosaccharides (CSC fractions 9 and 14-2) which had been isolated from the shark scapular cartilage CSC peptidoglycan preparation[9] turned out to be a mono- and a di-sulfated trisaccharide, respectively (Table I). Another unsaturated oligosaccharide (DS fraction I) isolated from the bovine aorta DS peptidoglycan preparation is a monosulfated trisaccharide. In order to examine the relative elution positions of the trisaccharides isolated from various CS/DS glycosaminoglycans and peptidoglycans these trisaccharides and

TABLE I

Chemical composition of the isolated oligosaccharides

Fraction	Origin	Composition (molar ratios) [a]			
		D-Gal*p*N	L-$\Delta^{4,5}$Hex*p*A	D-Glc*p*A	Sulfate
CSC fraction 9	Shark cartilage	1.05	1.00	2.34	0.75
CSC fraction 14-2	Shark cartilage	0.98	1.00	1.86	1.93
DS fraction I	Bovine aorta	0.82	1.00	1.75	n.d. [b]
CSE fraction 1	Squid cartilage	0.85	1.00	1.52	n.d.
CSE fraction 2	Squid cartilage	0.81	1.00	1.66	n.d.
CSE fraction 7a	Squid cartilage	0.94	1.00	1.96	n.d.

[a] The values are expressed by molar ratio to the L-$\Delta^{4,5}$Hex*p*A determined by UV absorption. D-Gal*p*N was determined using an amino acid analyzer and was not corrected for degradation during acid hydrolysis. D-Glc*p*A was determined by the carbazole reaction and includes L-$\Delta^{4,5}$Hex*p*A. Sulfate was determined by ion chromatography. [b] n.d., Not determined.

the desulfated counterpart (DS fraction I_0) of DS fraction I were mixed and co-chromatographed on HPLC. Their elution positions, as compared with those of the authentic CS disaccharides, reflect the number of uronic acid residues and the sulfate groups contained (Fig. 2).

Characterization of the oligosaccharides by FABMS.—The negative-ion FAB-spectrum of underivatised CSC fraction 9 (Fig. 3A) afforded a molecular ion signal at *m/z* 634 corresponding to $[M - H]^-$ of a monosulfated unsaturated trisaccharide $\Delta HexA_1HexA_1HexNAc_1$. After Hakomori permethylation[15], which retains the sulfate substituent[20], the $[M - H]^-$ molecular ion was observed at *m/z* 760 (Fig. 3B), consistent with the assigned saccharide composition and one degree of sulfation.

In comparison, the negative-ion FAB-spectrum of underivatised CSC fraction 14-2 (Fig. 4A) suggested that fraction 14-2 probably contained the same trisaccharide substituted with two sulfates, giving $[M - H]^-$ and $[M - 2H + Na]^-$ molecular

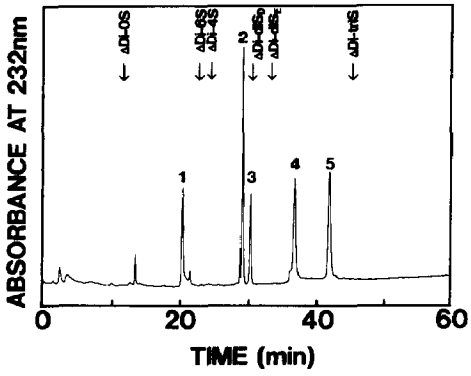

Fig. 2. HPLC of the isolated sulfated trisaccharides. A prepared mixture of the trisaccharides was chromatographed on an amine-bound silica column. 1, DS fraction I_0 (0.5 nmol); 2, CSC fraction 9 (0.8 nmol); 3, DS fraction I (0.48 nmol); 4, CSC fraction 14-2 (0.6 nmol); 5, CSE fraction 7a (1.0 nmol). The elution positions of authentic unsaturated CS disaccharides are indicated by arrows.

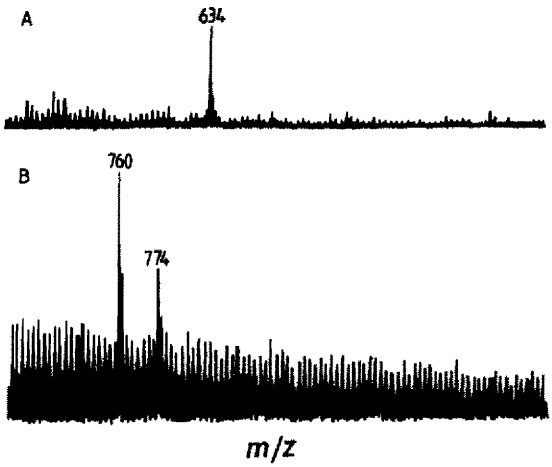

Fig. 3. Negative-ion FAB-spectra of underivatised (A) and permethylated (B) CSC fraction 9. The signal at m/z 774 corresponds to $[M-H]^-$ of a fully *O*- and *N*-methylated $\Delta HexA_1HexA_1HexNAc_1$ bearing an additional methyl group, probably attached to carbon.

ion signals at m/z 714 and 736, respectively. Facile mass spectrometric cleavage of a sulfite moiety from the disulfated trisaccharide gave rise to the intense fragment ion signal at m/z 634. This assignment was further corroborated by negative-ion FAB analysis of the permethyl derivative (Fig. 4B) which afforded an intense molecular ion signal at m/z 848 corresponding to $[M-2H+Na]^-$ of disulfated $\Delta HexA_1HexA_1HexNAc_1$. The loss of a sodium sulfite moiety (102 Da) through mass spectrometric cleavage yielded the signal at m/z 746. Significantly, the absence of a signal at m/z 760 (monosulfated $\Delta HexA_1HexA_1HexNAc_1$, cf. Fig. 3B) provided evidence for fraction 14-2 containing only a disulfated trisaccharide.

The negative-ion FAB-spectrum of underivatised DS fraction I afforded an intense $[M-H]^-$ molecular ion signal at m/z 634 (Fig. 5). The absence of a signal at m/z 714 or 736 (molecular ion signals for disulfated $\Delta HexA_1HexA_1HexNAc_1$,

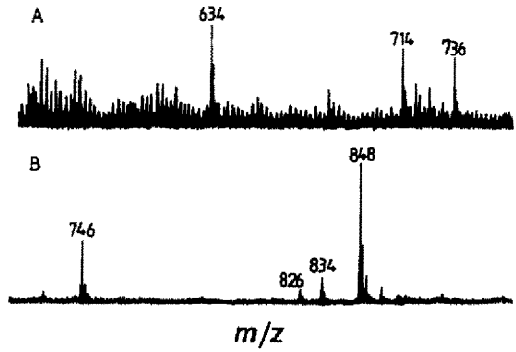

Fig. 4. Negative-ion FAB-spectra of underivatised (A) and permethylated (B) CSC fraction 14-2. The signals at m/z 834 and 826 correspond to undermethylation and loss of methanol, respectively.

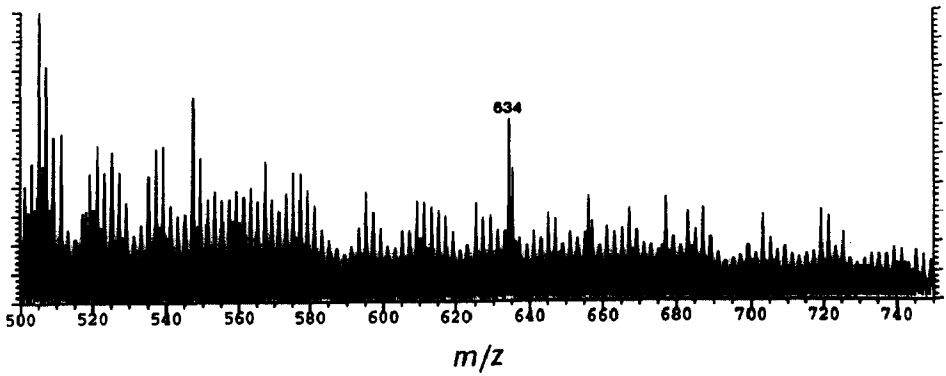

Fig. 5. Negative-ion FAB-spectrum of underivatised DS fraction I.

cf. Fig. 4A) was indicative of DS fraction I being a monosulfated $\Delta HexA_1HexA_1HexNAc_1$.

The underivatised CSE fraction 7a did not afford a good quality negative FAB-spectrum although a very weak signal could be observed at m/z 736. To enhance the signal to noise ratio, both permethyl- and perdeuterio-acetyl derivatives of this fraction were examined. Previous experiments have established that sulfated tetrasaccharides from Hep can be fully deuterioacetylated using the 1-methylimidazole catalyst conditions[21] without losing the labile sulfate substituents. The resulting perdeuterioacetyl derivatives afford negative-ion FAB-spectra of significantly enhanced quality as well as being compatible with further Hakomori permethylation. The perdeuterioacetylated CSE fraction 7a yielded a molecular ion signal at m/z 939 and a signal at 80 Da lower (m/z 859) (data not shown), corresponding respectively to the $[M-H]^-$ of a disulfated $\Delta HexA_1HexA_1HexNAc_1$ and its fragment ion resulting from loss of a sulfite moiety. After permethylation, the sample yielded an $[M-H]^-$ molecular ion signal at m/z 848, thus providing further corroborative evidence for CSE fraction 7a being a disulfated $\Delta HexA_1HexA_1HexNAc_1$.

Enzymatic characterization of the oligosaccharides.—DS fraction I was resistant to 2-sulfatase or 6-sulfatase, but was sensitive to 4-sulfatase and converted to a presumed desulfated counterpart, DS fraction I_0, as demonstrated by HPLC (data not shown). Chondroitinase ACII digestion of DS fraction I resulted in ΔDi-4S, which was further converted to ΔDi-0S by the 4-sulfatase digestion, suggesting that DS fraction I is sulfated at the C-4 position of the internal D-Gal*p*NAc residue. The sensitivity to chondroitinase ACII indicates that the uronic acid at the reducing end is D-Glc*p*A. The unsaturated uronic acid formed by chondroitinase ACII digestion was not detected since it is labile and decomposed into an α-keto acid[22]. Thus the following structures are proposed.

TABLE II

Action of chondroitinase ACII and various sulfatases on the trisaccharides isolated from chondroitin/dermatan sulfate

Fraction	Structure [a]	Enzyme action [b]			
		2-Sulfatase	4-Sulfatase	6-Sulfatase	Chondroitinase ACII
CSC Fr. 9	ΔU-G(6S)-U	−	−	−	+
CSC Fr. 14-2	ΔU(2S)-G(6S)-U	+	−	−	+
DS Fr. I	ΔU-G(4S)-U	−	+	−	+
CSE Fr. 7a	ΔU-G(4,6S)-U	−	−	−	+

[a] Abbreviations: ΔU, L-Δ^{4,5}Hex*p*A; U, D-Glc*p*A; G, D-Gal*p*NAc; 2S, 2-sulfate; 4S, 4-sulfate; 6S, 6-sulfate. [b] Each trisaccharide was digested with 2-, 4-, or 6-sulfatase, and the digest was analyzed by HPLC as described in the Experimental.

DS fraction I:

α-L-Δ^{4,5}Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO$_3^-$-(1 → 4)-D-Glc*p*A

DS fraction I_0:

α-L-Δ^{4,5}Hex*p*A-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-D-Glc*p*A

CSC fraction 9 was digested with chondroitinase ACII yielding ΔDi-6S (data not shown), which indicates that it is sulfated at the C-6 position of the internal D-Gal*p*NAc residue. Although CSC fraction 9 was resistant to 6-sulfatase (Table II) it is probably because the 6-*O*-sulfated D-Gal*p*NAc residue is covered with uronic acid at the reducing end. CSC fraction 14-2 was digested by chondroitinase ACII giving rise to ΔDi-diS_D and also digested by 2-sulfatase yielding quantitatively a compound with the same retention time as that of CSC fraction 9. It was resistant to 4- or 6-sulfatase before and after 2-sulfatase digestion. These results, summarized in Table II, indicate that CSC fraction 14-2 bears two sulfate groups, one at the C-2 position of L-Δ^{4,5}Hex*p*A at the nonreducing end and the other at the C-6 position of the internal D-Gal*p*NAc residue. Thus the following structures are proposed.

CSC fraction 9:

α-L-Δ^{4,5}Hex*p*A-(1 → 3)-β-D-Gal*p*NAc6SO$_3^-$-(1 → 4)-D-Glc*p*A

CSC fraction 14-2:

α-L-Δ^{4,5}Hex*p*A2SO$_3^-$-(1 → 3)-β-D-Gal*p*NAc6SO$_3^-$-(1 → 4)-D-Glc*p*A

Fractions 1 and 2 obtained from CSE as well as from CSA and CSC glycosaminoglycans were identical with CSC fraction 9 and DS fraction I, respectively, as characterized by chondroitinase ACII digestion and ^{1}H NMR spectroscopy (data not shown). It should be noted that CSE fraction 1 contained a minor contaminant (32%) resistant to chondroitinase ACII and remains to be character-

ized. CSE fraction 7a was digested with chondroitinase ACII yielding ΔDi-diS$_E$ as demonstrated by HPLC (data not shown), which indicates that the parent compound contained two sulfate groups at both C-4 and C-6 positions of the internal D-Gal*p*NAc residue. The compound was resistant to all the three sulfatases tested including 4- and 6-sulfatases. It has been demonstrated that the sulfate group on the C-6 position of the D-Gal*p*NAc residue interferes with the removal of a sulfate group from the C-4 position of the D-Gal*p*NAc residue by the action of 4-sulfatase[23]. The following structure is proposed for this fraction.

CSE fraction 7a:

α-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^-6SO_3^--(1 → 4)-D-Glc*p*A

500-MHz ^{1}H NMR spectroscopy.—The above 5 trisaccharides were characterized by 500-MHz ^{1}H NMR spectroscopy. The one-dimensional spectrum of DS fraction I measured at 26°C is shown in Fig. 6B. The resonances between δ 4.5 and 5.3 ppm are characteristic of anomeric protons, whereas those at around δ 6.0 and 2.1 ppm are characteristic of the H-4 proton of L-$\Delta^{4,5}$Hex*p*A[7,9] and the acetamido group protons of D-Gal*p*NAc, respectively. The other proton chemical shifts were assigned using 2D HOHAHA and COSY spectra. Beginning at δ 5.966 for the H-4 proton of L-$\Delta^{4,5}$Hex*p*A-3, a cross-peak showing connectivity to the H-3 resonance at δ 3.940 was found in the COSY spectrum (data not shown). Continuation of this process allowed localization of the H-2 and H-1 resonances as indicated in the 2D HOHAHA spectrum (Fig. 7). Starting with the H-1 resonance of the D-Gal*p*NAc-2 of the α anomer at δ 4.627 or of the β anomer at δ 4.622, the H-2, H-3, and H-4 resonances were identified at δ 4.090, 4.151, and 4.628, respectively. The anomerization effect resulted in resonances at δ 4.627 (α H-1) and δ 4.622 (β H-1) of the second constituent D-Gal*p*NAc-2. Starting with the H-1 signal at δ 5.210 for α-D-Glc*p*A, the H-2, H-3, H-4, and H-5 signals were identified. The remaining β-anomeric proton signals at δ 4.639 for the H-1 of this residue led to localization of H-2, H-3, and H-4 proton signals as indicated in the 2D HOHAHA spectrum. H-2 and H-3 proton signals at around δ 3.3 and 3.6 are structural reporter groups of nonsulfated β-D-Glc*p*A[7,9]. The NMR data are summarized in Table III. The one-dimensional spectrum of DS fraction I_0 measured at 26°C is shown in Fig. 6A. The H-1, H-2, H-3, and H-4 resonances of the three monosaccharide components and the H-5 resonances of the reducing D-Glc*p*A-1 were readily identified in the 2D HOHAHA spectrum with the aid of a COSY spectrum (not shown) as described for DS fraction I. The NMR data are summarized in Table III with those of the reference compound R1 which is the D-1 component [α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Xyl*p*-(1 → O^3)-L-Ser] isolated from rat chondrosarcoma CSA proteoglycans[7]. The chemical shifts of protons belonging to D-Gal*p*NAc-2 and L-$\Delta^{4,5}$Hex*p*A-3 were similar to those for the corresponding residues of the D-1

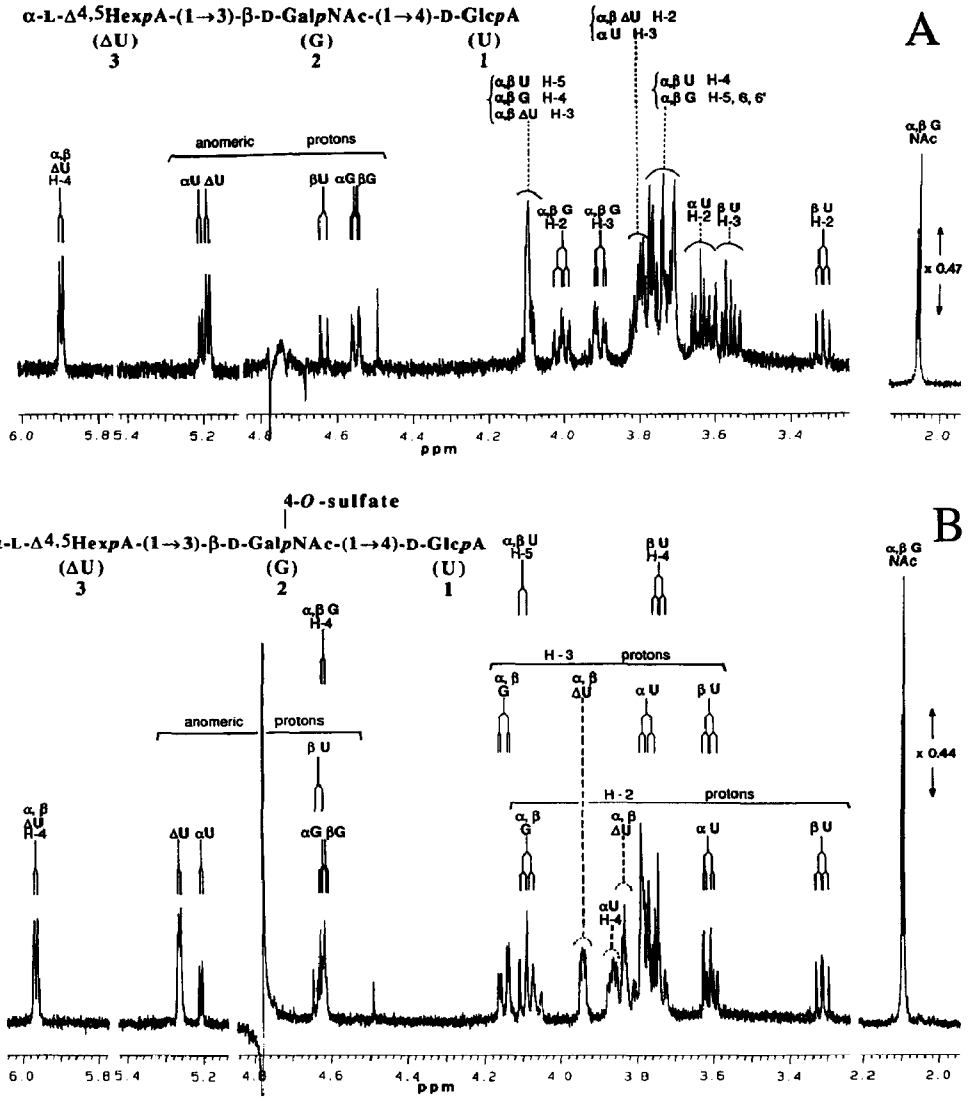

Fig. 6. 500-MHz ^{1}H NMR spectra of DS fraction I_0 (A) and DS fraction I (B) recorded in D_2O at 26°C. The letters in the spectra refer to the corresponding residues in the structure.

component, indicating the presence of the nonsulfated α-L-$\Delta^{4,5}$HexpA-(1 → 3)-D-GalpNAc structure in DS fraction I_0. When the spectra of DS fractions I and I_0 were compared, it was found that the H-4 proton signal of D-GalpNAc-2 in the former shifted downfield ($\Delta\ \delta$ 0.528) as compared with that of D-GalpNAc-2 in the latter, being consistent with 4-sulfation of D-GalpNAc-2 in the former. Thus, the structure of DS fraction I is α-L-$\Delta^{4,5}$HexpA-(1 → 3)-β-D-GalpNAc4SO_3^--(1 → 4)-D-GlcpA and that of DS fraction I_0 is α-L-$\Delta^{4,5}$HexpA-(1 → 3)-β-D-GalpNAc-(1 → 4)-D-GlcpA.

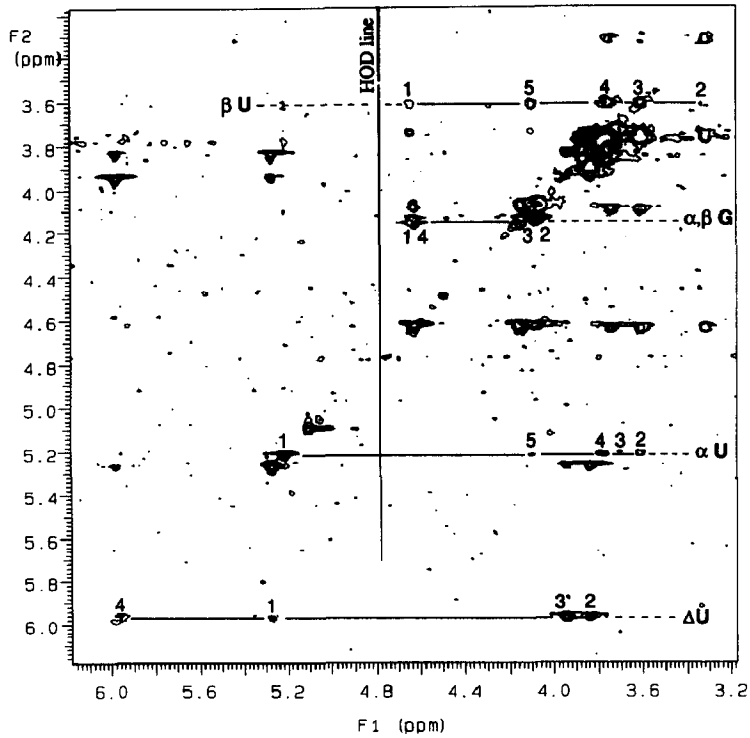

Fig. 7. Two-dimensional HOHAHA spectrum of DS fraction I recorded in D_2O at 26°C.

The one-dimensional spectrum of CSC fraction 9 measured at 26°C is shown in Fig. 8A. The H-1, H-2, H-3, and H-4 resonances of the three monosaccharide components and the H-5 resonances of the reducing D-Glc*p*A-1 were readily identified in the 2D HOHAHA spectrum with the aid of COSY spectrum (not shown). Although no cross-peak between H-4 and H-5 was observable owing to the weak coupling between them, H-6 and H-6′ resonances characteristic of 6-*O*-sulfated β-D-Gal*p*NAc residue were found (see compound 7 in ref 9) at δ 4.234 and 4.223, respectively. The NMR data are summarized in Table III. The spectral data were very similar to those of DS fraction I_0 except for the large downfield shift (Δ δ 0.52–0.53) of the H-6 and H-6′ of D-Gal*p*NAc-2 (Table III), indicating that the compound in CSC fraction 9 has the same trisaccharide core structure with DS fraction I_0, but with an additional sulfate group on the C-6 position of D-Gal*p*NAc-2, being consistent with the structure, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-D-Glc*p*A, proposed above based on the enzyme digestion.

The one-dimensional spectrum of CSC fraction 14-2 measured at 26°C is shown in Fig. 8B. The proton chemical shifts were assigned using 2D HOHAHA and COSY spectra (data not shown). The NMR data are summarized in Table III with those of the reference compound R2 (CSD fraction 7b, ref 11), α-L-$\Delta^{4,5}$Hex*p*A2SO_3^--(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*-

TABLE III

^{1}H Chemical shifts of the trisaccharides together with those of the disaccharide portion at the nonreducing end of the reference compounds, R1, R2, and R3 [a]

H atom [b]	Chemical shifts							
	R1 [c]	DS Fr. I_0	DS Fr. I	CSC Fr. 9	CSC Fr. 14-2	R2 [d]	CSE Fr. 7a	R3
Glc*p*A-1								
H-1α		5.205	5.210	5.205	5.205		5.209	
H-1β		4.633	4.639	4.636	4.637		4.644	
H-2α		3.622	3.612	3.622	3.626		3.625	
H-2β		3.313	3.314	3.330	3.331		3.339	
H-3α		3.770	3.773	3.784	3.786		3.74	
H-3β		3.597	3.608	3.60	3.60		3.620	
H-4α		3.74 [e]	3.87	3.74	3.73		3.80	
H-4β		3.74	3.743	3.74	3.73		3.730	
H-5		4.082	4.100	4.082	4.094		4.095	
Gal*p*NAc-2								
H-1α		4.555	4.627	4.581	4.594		4.680	
H-1β	4.538	4.548	4.622	4.575	4.590	4.589	4.672	4.662
H-2	4.002	3.995	4.090	4.02	4.002	4.02	4.101	4.104
H-3	3.901	3.906	4.151	3.946	3.968	3.966	4.194	4.19
H-4	4.098	4.100	4.628	4.174	4.033	3.976	4.680	4.688
H-5	n.d. [f]	3.7	n.d.	4.02	4.01	4.00	4.21	4.20
H-6	n.d.	3.7	n.d.	4.234	4.216	4.20	4.290	4.278
H-6′	n.d.	3.7	n.d.	4.223	4.205	4.20	4.268	4.258
NAcα		2.055	2.093	2.051	2.085		2.091	
NAcβ	2.057	2.061	2.099	2.055	2.089	2.087	2.091	2.100
$\Delta^{4,5}$Hex*p*A-3								
H-1	5.184	5.184	5.265	5.181	5.519	5.519	5.263	5.267
H-2	3.793	3.799	3.834	3.784	4.467	4.466	3.826	3.824
H-3	4.093	4.083	3.940	4.105	4.182	4.18	3.953	3.950
H-4	5.896	5.897	5.966	5.879	6.032	6.031	5.957	5.964

[a] R1, D-1 component, [α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-β-D-Xyl*p*-(1 → O^3)-L-Ser], isolated from rat chondrosarcoma CSA proteoglycans[7]; R2, CSD fraction 7b, α-L-$\Delta^{4,5}$Hex*p*A2SO_3^--(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc6SO_3^-, isolated from shark cartilage CSD glycosaminoglycans[11]; R3, CSE fraction 9, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^- 6SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^-, isolated from squid cartilage CSE glycosaminoglycans[11]. [b] Listed by successive monosaccharide residues, numbered as in Figs. 6, 8, and 9. [c] Spectrum determined at 22°C. [d] Spectrum determined at 15°C. [e] The estimated error for the values to two decimal places is ±0.01 ppm due to the partial overlap of signals. That for the values to three decimal places is ±0.001 ppm. [f] n.d., Not determined.

NAc6SO_3^-. The chemical shifts of the protons of D-Gal*p*NAc-2 and L-$\Delta^{4,5}$Hex*p*A-3 are comparable with those of the protons belonging to the corresponding disaccharide at the nonreducing end of the CSD fraction 7b, indicating the presence of the disaccharide structure, α-L-$\Delta^{4,5}$Hex*p*A2SO_3^--(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → , at the nonreducing end of CSC fraction 14-2. Thus, the structure of CSC fraction 14-2 is α-L-$\Delta^{4,5}$Hex*p*A2SO_3^--(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-D-Glc*p*A.

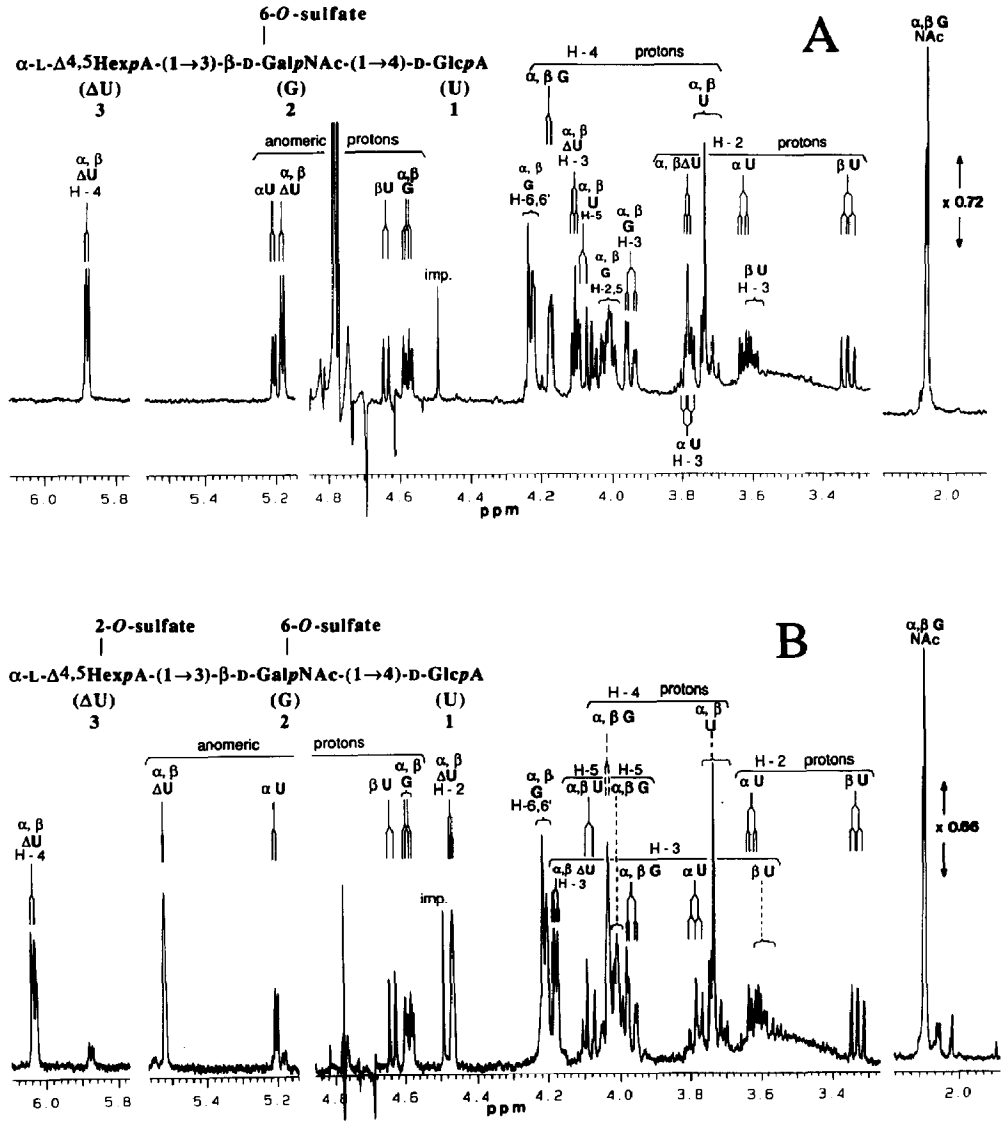

Fig. 8. 500-MHz ^{1}H NMR spectra of CSC fraction 9 (A) and CSC fraction 14-2 (B) recorded in D_2O at 26°C. The letters on the spectra refer to the corresponding residues in the structure.

The one-dimensional ^{1}H NMR spectrum of CSE fraction 7a measured at 26°C is shown in Fig. 9. The proton chemical shifts were assigned using COSY and 2D HOHAHA spectra (not shown). The NMR data are summarized in Table III with those of the reference compound R3 (CSE fraction 9, ref 11), α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^-6SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^-. The chemical shifts of the protons of D-Gal*p*NAc-2 and L-$\Delta^{4,5}$Hex*p*A-3 in CSE fraction 7a are comparable with those of the protons belonging to the disaccharide structure at the nonreducing end of CSE fraction 9, indicating the presence of the

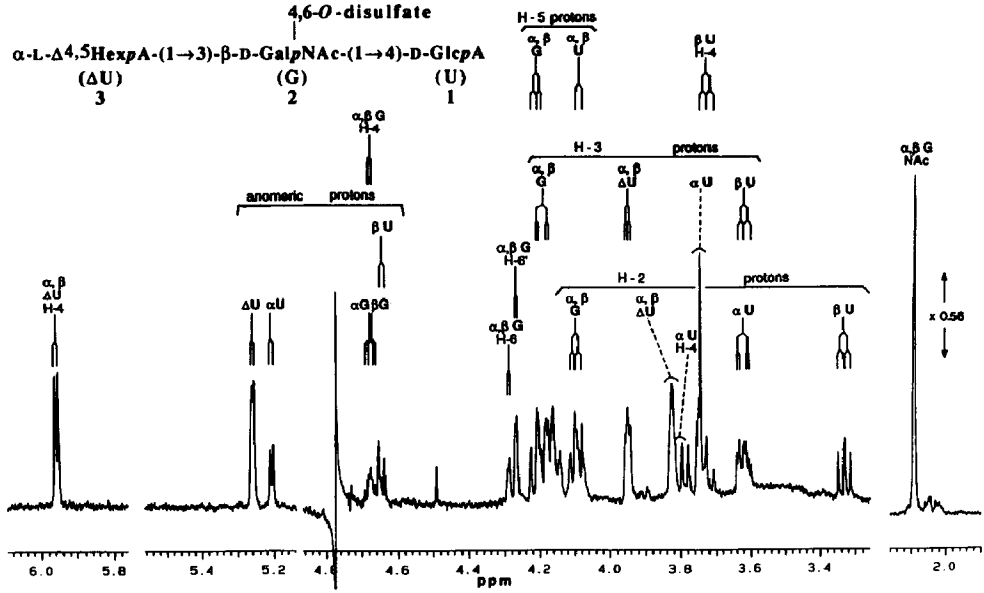

Fig. 9. 500-MHz ^{1}H NMR spectrum of CSE fraction 7a recorded in D_2O at 26°C. The letters on the spectrum refer to the corresponding residues in the structure.

disaccharide structure, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^-6SO_3^--(1 → , at the nonreducing end. Thus, the disulfated trisaccharide structure, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^-6SO_3^--(1 → 4)-D-Glc*p*A, was confirmed for CSE fraction 7a.

DISCUSSION

In the present study the four unsaturated trisaccharides isolated from DS and various CS isomers were characterized. They were resistant to chondroitinase ABC and share the common trisaccharide sequence, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-D-Glc*p*A, but have different sulfation patterns. Their structures suggest that they are derived from the reducing termini of the parent glycosaminoglycan chains. Such trisaccharides have never been reported so far to our knowledge.

The preparation procedure of the commercial CSE glycosaminoglycans from squid cartilage and the purification method of the trisaccharides from the CSC peptidoglycans of shark scapular cartilage included alkaline treatment. There is a possibility, therefore, that degradation of the carbohydrate–protein linkage region by the peeling reaction[24] may lead to the exposure of the reducing D-Glc*p*A during alkaline treatment. Most of the CSE chains are calculated to have trisaccharides at the reducing ends (see Experimental). The commercial CSA or CSC glycosaminoglycan preparation is calculated to contain 2000–4000 or 1250–2500 nmol of glycosaminoglycan chains per 100 mg, respectively, based on the molecular

weight described in the manufacturer's specification for an A isomer (M_r, 25 000–50 000) or C isomer (M_r, 40 000–80 000). The amounts of α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-D-Glc*p*A and α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^--(1 → 4)-D-Glc*p*A isolated from CSA or CSC in the present study (cf. ref 11) are comparable with the number of the glycosamioglycan chains.

In contrast, however, such trisaccharides were also isolated from the samples prepared without alkaline treatment. The isolation of DS fraction I from the DS peptidoglycan preparation did not involve alkaline treatment. Recently we also isolated α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-D-Glc*p*A and α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^--(1 → 4)-D-Glc*p*A in significant amounts as byproducts while isolating the carbohydrate–protein linkage region from a peptidoglycan fraction of whale cartilage CSA without using alkaline treatment[25]. This was achieved by HPLC fractionation of a chondroitinase ABC digest of the minor glycopeptide fraction II which is illustrated in Fig. 1A, ref 8. Thus it is possible that the trisaccharide found in DS fraction I and these two monosulfated trisaccharides are derived from the reducing termini of free DS and CS glycosaminoglycan chains, respectively, which may have been released from proteoglycans endoglycosidically by tissue endo-β-D-glucuronidase. Thus the present study may also indicate the presence of an endo-β-D-glucuronidase in aorta and in cartilage.

Several endoglycosidases acting on glycosaminoglycans have been reported. Endo-β-D-glucuronidases specific for glucosaminoglycans (Hep, HS, and HA) have been well documented. One such example is an endo-β-D-glucuronidase demonstrated in mouse mastcytoma, which is involved[26] in the intracellular post-synthetic fragmentation of macromolecular Hep. A lysosomal type of endo-β-D-glucuronidase which abolishes the anticoagulant activity of Hep has been purified from human platelets[27]. Another type of endo-β-D-glucuronidase which is specific for HS and seems to be involved in the vascular invasion of melanoma cells has been purified from mouse and human melanoma[28,29] and an enzyme with a similar substrate specificity has been purified from rat liver[30]. Although an endo-type hyaluronidase from leech, *Hirudo medicinalis*, is an endo-β-D-glucuronidase, it is specific for HA[31], but not for CS/DS. Well-known testicular-type hyaluronidase which acts on both HA and CS, is an endo-β-D-hexosaminidase[32]. An endo-β-D-glucuronidase specific for galactosaminoglycans (CS/DS) has not been reported. It should be noted, however, that Takagaki et al.[33,34] recently purified from rabbit liver a unique lysosomal endo-β-D-glucuronidase which is specific for the β-D-Glc*p*A-(1 → 3)-D-Gal*p* linkage in the carbohydrate–protein linkage region, but does not cleave the β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc linkage in the repeating disaccharide region of CS proteoglycans. It remains to be seen whether some of the trisaccharides isolated in the present study are derived from the cleavage sites generated by such an endo-β-D-glucuronidase or by another, hitherto unreported, endo-β-D-glucuronidase specific for the β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc linkage in the repeating disaccharide region of CS/DS glycosaminoglycan chains.

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