Carbohydrate Research, 255 (1994) 145–163
Elsevier Science B.V., Amsterdam

Structural studies on the chondroitinase ABC-resistant sulfated tetrasaccharides isolated from various chondroitin sulfate isomers

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(Received March 1st, 1993; accepted August 30th, 1993)

ABSTRACT

Various commercially available chondroitin sulfates, including an A isomer from whale cartilage, C and D isomers from shark cartilage, and an E isomer from squid cartilage, were exhaustively digested with a commercial highly purified *Proteus vulgaris* chondroitinase ABC. Gel chromatography of all digests yielded a disaccharide and an oligosaccharide fraction which was resistant to the enzyme digestion and which accounts for 20–31 mol% of the produced total oligosaccharides. Variably sulfated tetrasaccharides were isolated from the oligosaccharide fraction of each chondroitin sulfate isomer by HPLC, then characterized chemically and enzymatically. One disulfated and three trisulfated components were also characterized by 500-MHz one- and two-dimensional ^{1}H NMR spectroscopy. The structures of one tetrasulfated, four trisulfated, and five disulfated tetrasaccharides with the common core structure, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc, were determined. All isolated tetrasaccharides were resistant to the highly purified enzyme, but susceptible to the conventional, commercial chondroitinase ABC. The former was also inactive towards α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc isolated from chondroitin, β-D-Glc*p*A-(1 → 3)-β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Glc*p*NAc from hyaluronan, and α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^--(1 → 4)-α-L-Ido*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^- from dermatan sulfate. These results indicate that, unlike the conventional enzyme, highly purified chondroitinase ABC cannot degrade tetrasaccharides irrespective of their sulfation profiles. The enzymatic action is size-dependent.

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SSDI 0008-6215(93)E0340-7

INTRODUCTION

Chondroitinase ABC has been widely used as a powerful tool for structural studies of chondroitin sulfate (CS) */dermatan sulfate (DS) glycosaminoglycans since its discovery by Yamagata et al.[1]. This enzyme degrades in an eliminative fashion both CS and DS glycosaminoglycans, yielding a variety of unsaturated disaccharides originating from the repeating disaccharide region and hexasaccharides derived from the carbohydrate–protein linkage region[2–7]. Recently a highly purified enzyme has become commercially available, and the deglycosylated core protein has been obtained from proteoglycans using it[3]. Thus chondroitinase ABC is an essential tool for characterizing both the carbohydrate and protein moieties of CS/DS proteoglycans, the importance of which, in various cellular recognition systems has become more evident[8–10].

During the structural studies of sulfated glycosaminoglycans[5,6] we found that the highly purified preparation of chondroitinase ABC, which is purified by a different method from that used for the conventional enzyme and which is commercially available as a "protease-free" preparation, could not degrade certain sulfated oligosaccharides, unlike the conventional product. We therefore obtained from several CS isomers, a series of sulfated tri- and tetra-saccharides which are resistant to highly purified chondroitinase ABC. We systematically elucidated their structures in order to characterize the substrate specificity of this enzyme. We describe the structures of the tetrasaccharides here and those of the trisaccharides will be reported in the second article of this series[11].

EXPERIMENTAL

Materials.—The following materials and enzymes were purchased from Seikagaku Corp., Tokyo: sodium salt glycosaminoglycan preparations (super special grade) of whale cartilage CSA, shark cartilage CSC and CSD, squid cartilage CSE, pig skin DS, squid cartilage Chn, eight unsaturated disaccharides derived from CS, chondroitinase ACII (EC 4.2.2.5), conventional and highly purified chondroitinase ABC (EC 4.2.2.4) (the latter being available as a "protease-free" preparation), chondro-4-sulfatase (EC 3.1.6.9, abbreviated as 4-sulfatase), and chondro-6-sulfatase (EC 3.1.6.10, abbreviated as 6-sulfatase). $\Delta^{4,5}$-Hexuronate-2-*O*-sulfatase (2-sulfatase) purified from *Flavobacterium heparinum*[12] was provided by Dr. Keiichi Yoshida, Seikagaku Corp. The tetrasaccharide β-D-Glc*p*A-(1 → 3)-β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Glc*p*NAc was isolated from HA as previously reported[13]. Similarly, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc or α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^--(1 → 4)-α-L-Ido*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^- were isolated by HPLC as a major component from squid cartilage Chn or pig skin DS glycosaminoglycans, respectively, after

* Abbreviations used: 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; Chn, chondroitin; DS, dermatan sulfate; HA, hyaluronan; 2-sulfatase, $\Delta^{4,5}$-hexuronate-2-*O*-sulfatase; 4- or 6-sulfatase, chondro-4- or -6-sulfatase; 2D, two-dimensional; COSY, correlation spectroscopy; HOHAHA, homonuclear Hartmann–Hahn.

digestion with highly purified chondroitinase ABC essentially as described for the CS tetrasaccharides below.

Preparation of tetrasaccharide fractions.—A commercial preparation (50 mg) of CSA, CSC, CSD, CSE, or Chn was digested, respectively, with 0.9 unit of highly purified chondroitinase ABC in a total volume of 2.0 mL of 0.05 M Tris · HCl buffer, pH 8.0, containing 60 mM sodium acetate and 100 μg/mL of bovine serum albumin. The digestion was carried out for 22 h at 37°C, then an additional 0.1 unit of the enzyme was added after 18 h to complete the digestion. The reaction reached a plateau during this period as monitored by UV absorption at 232 nm. The mixture was adjusted to pH 6.5 with 1 M AcOH and boiled at 100°C for 1 min to terminate the reaction. The digest was gel filtered on a column of Sephadex G-15 with 0.25 M NH_4HCO_3–7% 1-propanol as the eluent. The digest of Chn and all four CS preparations resulted in two major peaks, the presumed tetrasaccharide (fraction I) and disaccharide peaks (fraction II). Both fractions were desalted by repeated evaporation with added water, dissolved in water, and subfractionated by HPLC.

Chondroitinase or sulfatase digestion of the oligosaccharides.—Enzyme digestion proceeded using 0.5 nmol of each isolated oligosaccharide and the indicated amount of the enzyme in a total volume of 40 μL of the appropriate buffer at 37°C for 10 min unless otherwise indicated. Chondroitinase ABC digestion was accomplished with 5 mU of either the conventional, or the highly purified enzyme in the buffer described above[1]. Chondroitinase ACII digestion was performed using 2.5 mU of the enzyme in 0.05 M sodium acetate buffer, pH 6.0 as described[1]. 2-Sulfatase digestion was carried out for 20 min using 1 mU of the enzyme in 10 mM imidazole–HCl buffer, pH 6.5 as described[12]. Chondro-4- or -6-sulfatase digestion used 20 mU of the enzyme in 34 mM Tris · HCl, pH 7.5, containing 34 mM sodium acetate and 0.01% (w/v) bovine serum albumin[1,14]. After incubation the mixtures were boiled at 100°C for 2 min, cooled to room temperature, mixed with 360 μL of 16 mM NaH_2PO_4, and analyzed by HPLC.

Successive enzyme digestion of CSD fraction 8a with 2-sulfatase and chondroitinase ACII.—CSD fraction 8a (1 nmol) was first incubated with 2 mU of 2-sulfatase in a total volume of 30 μL of 10 mM imidazole–HCl buffer, pH 6.5 at 37°C for 20 min, and boiled at 100°C for 2 min. One half of the sample was analyzed by HPLC while the other half was mixed with 35 μL of water and 50 μL of 0.25 M sodium acetate buffer, pH 6.0, containing 15 mU of chondroitinase ACII and incubated at 37°C for 30 min. The reaction was terminated by boiling for 2 min and the mixture was analyzed by HPLC.

HPLC.—The tetrasaccharide fraction from gel filtration was subfractionated and the enzyme digests of all oligosaccharides were analyzed by HPLC as previously reported for the separation of CS disaccharides[15,16]. Chromatography was performed on a 4.6 × 250 mm amine-bound silica PA03 column (YMC Co., Kyoto) using a linear gradient from 16 to 530 mM NaH_2PO_4 over a 60 min period at a flow rate of 1.0 mL/min at room temperature. Isocratic conditions (250 mM

NaH_2PO_4) were also used to separate fraction 4bc into subfractions 4b and 4c which eluted in that order with retention times of ~ 20 min. Samples dissolved in 16 mM NaH_2PO_4 were treated with a C3HV membrane filter (Millipore) and injected. Eluates were monitored by UV absorbance at 232 nm.

Analytical methods.—Oligosaccharides produced by chondroitinases were quantified based upon the UV absorbance ($\epsilon_{232} = 5500\ M^{-1}\ cm^{-1}$) caused by the $\Delta^{4,5}$ sites of the uronic acid at the nonreducing ends[1]. Uronic acid was determined by the carbazole method[17] using D-Glc*p*A as the standard. Amino sugars were analyzed with an amino acid analyzer after hydrolysis in 6 M HCl at 100°C for 3 h[18].

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 15 or 26°C. Chemical shifts, given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, were measured from acetone at δ 2.225 (refs 13,19).

RESULTS

Isolation of the chondroitinase ABC-resistant oligosaccharides.—Commercial CS isomers, the A from whale cartilage, C or D from shark cartilage, E or Chn from squid cartilage, were exhaustively digested with a commercial highly purified chondroitinase ABC as described in the Experimental. After gel permeation chromatography on Sephadex G-15, each digest yielded the UV absorbing fractions I and II, which contained mainly unsaturated tetrasaccharides and disaccharides, respectively, as judged by the ratios of total uronic acid to L-$\Delta^{4,5}$Hex*p*A. The molar ratios of fractions I : II produced from A, C, D, E isomers, or Chn were 20 : 80, 22 : 78, 23 : 77, 31 : 69, and 21 : 79, respectively, as judged by UV absorption at 232 nm. Fraction I from each CS isomer was not digested by highly purified chondroitinase ABC, but was completely degraded mainly into disaccharides *, by the conventional commercial enzyme as examined by HPLC. The disaccharide composition of the enzyme digests of each fraction I and the disaccharide fraction II from the CS isomers were analyzed by HPLC. The results summarized in Table I reveal no common characteristics in the disaccharide composition among the resistant oligosaccharide fractions.

We determined whether the oligosaccharides resistant to the highly purified enzyme had any particularly sulfated fine structure resulting from the combination

* Abbreviations for the 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^-.

TABLE I

Disaccharide composition of the tetrasaccharide fraction I and disaccharide fraction II obtained by gel filtration of highly purified chondroitinase ABC digests of various chondroitin sulfate isomers [a]

Disaccharide	Composition (mol%)							
	CSA		CSC		CSD		CSE	
	Fr. I	Fr. II	Fr. I	Fr. II	Fr. I	Fr. II	Fr. I	Fr. II
ΔDi-0S	[b]	2.2	1.5	8.0	2.0	4.8		
ΔDi-4S	64.7	67.6	16.8	8.4	32.6	20.8	21.7	27.9
ΔDi-6S	20.8	28.8	59.8	73.0	35.9	46.6	6.1	15.1
ΔDi-diS$_B$								
ΔDi-diS$_D$	9.8	1.4	9.1	10.6	18.1	26.2	1.7	1.0
ΔDi-diS$_E$	1.6		2.9		1.7	1.6	47.3	56.0
ΔDi-triS			1.8				2.9	
Unidentified	3.1		8.1		9.7		20.3	

[a] The disaccharide fraction II and conventional chondroitinase ABC digest of tetrasaccharide fraction I from each CS isomer were analyzed by HPLC as described in the Experimental. [b] No appreciable amount.

of the disaccharide units. Various discrete tetrasaccharide structures were isolated from the fraction I of each CS isomer by HPLC on an amine-bound silica column. The chromatograms varied considerably as shown in Fig. 1, reflecting the differences in the oligosaccharide composition. Fraction I from the A, C, D, or E isomer was separated into 6, 8, 7, or 10 subfractions, respectively, as shown in Fig. 1. The fractions were named after the structures of the oligosaccharide components were determined. Thus, fractions obtained from different isomers, but having the same name contained oligosaccharides with the same structure. Each fraction was desalted through Sephadex G-25, further purified to homogeneity by rechromatography and structurally analyzed. Fraction 4bc was separated into two subfractions, 4b and 4c, which accounted for 80 and 20%, respectively, when rechromatographed under isocratic conditions (see Experimental). The amounts of the purified fractions obtained from 10 mg of the polysaccharides are summarized in Table II.

Enzymatic characterization of the isolated tetrasaccharides.—The sugar composition of the isolated fractions is summarized in Table III. The results indicate that fractions 3b, 4a, 4bc, 5, 6, 7b, 7c, 8a, 9, and 10 contain tetrasaccharides, while fractions 1, 2, and 7a contain trisaccharides. In this study the tetrasaccharide fractions were further analyzed while the trisaccharides, which are derived from the reducing ends of the polysaccharides, will be described in the second article of this series[11]. All isolated tetrasaccharide fractions were resistant to highly purified chondroitinase ABC, indicating that this was not due to inhibition by a particular oligosaccharide. All the tetrasaccharide fractions were sensitive to both conventional chondroitinase ABC and chondroitinase ACII, quantitatively yielding two disaccharide units as determined by HPLC. The results indicate that the internal

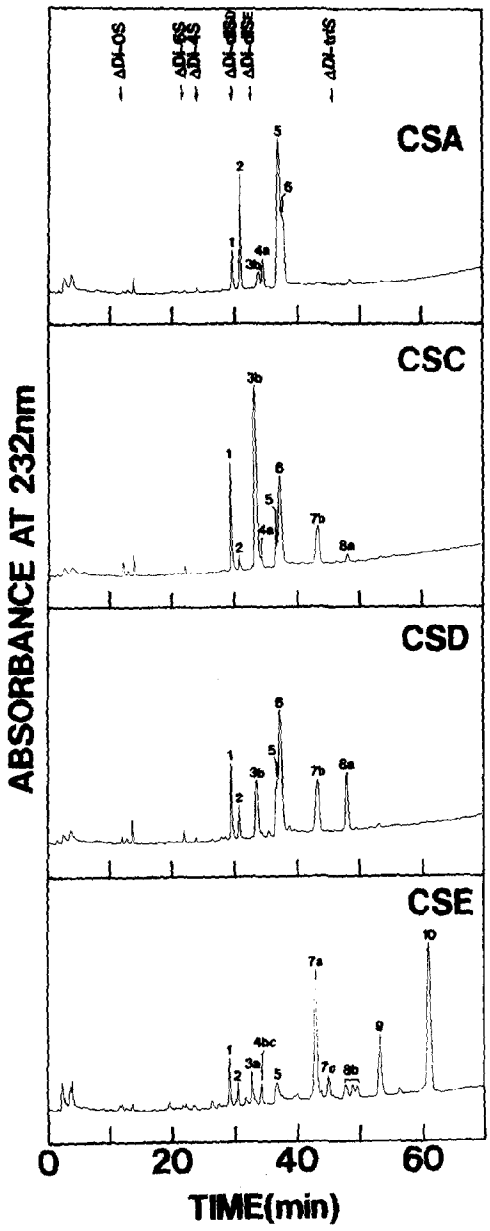

Fig. 1. HPLC fractionation of the tetrasaccharide fractions prepared from various CS isomers. Each tetrasaccharide fraction corresponding to 4.5 to 5.0 nmol of L-$\Delta^{4,5}$Hex*p*A was chromatographed on an amine-bound silica column using a salt gradient (see Experimental). The successive panels represent the separation profiles of the oligosaccharide fractions prepared from the indicated isomers, by digestion with highly purified chondroitinase ABC. The elution positions of authentic unsaturated CS disaccharides are indicated in the top panel. Equivalent fractions in each preparation are represented by the same fraction numbers.

TABLE II

The oligosaccharide fractions isolated from chondroitin sulfate isomers by digestion with commercial highly purified chondroitinase ABC

Fraction	Amounts (nmol/10 mg of polysaccharide)			
	CSA	CSC	CSD	CSE
1	151	321	212	146
2	430	66	121	77
3a	[a]			93
3b	135	920	332	
4a	183	23		
4b				64
4c				18
5	1097	105	323	81
6	423	592	727	
7a				613
7b		242	378	
7c				109
8a		58	310	
8b				244
9				363
10				794
Total	2419	2327	2403	2602

[a] No appreciable amount.

TABLE III

Sugar composition of the oligosaccharides prepared from various chondroitin sulfate isomers

Fraction	Composition (molar ratios) [a]		
	D-Gal*p*N	L-$\Delta^{4,5}$Hex*p*A	D-Glc*p*A
CSD fraction 1	0.85	1.00	1.52
CSD fraction 2	0.81	1.00	1.66
CSE fraction 3a	0.64	1.00	0.79
CSD fraction 3b	2.32	1.00	1.90
CSA fraction 4a	2.25	1.00	1.92
CSE fraction 4bc	1.92	1.00	1.74
CSD fraction 5	2.05	1.00	1.92
CSC fraction 6	2.04	1.00	1.63
CSE fraction 7a	0.94	1.00	1.96
CSD fraction 7b	2.38	1.00	1.78
CSE fraction 7c	1.61	1.00	1.87
CSD fraction 8a	2.13	1.00	2.10
CSE fraction 8b	n.d. [b]	1.00	n.d.
CSE fraction 9	1.88	1.00	2.19
CSE fraction 10	2.08	1.00	2.26

[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. For any given fraction, values are shown for only one chondroitin sulfate isomer, but these are representative of the corresponding fraction from the other isomers. [b] n.d., Not determined.

TABLE IV

Disaccharide composition of the tetrasaccharides isolated from various chondroitin sulfate isomers [a]

Fraction	Disaccharides formed (% recovery) [b,c]
Fraction 1	n.d. [d]
Fraction 2	n.d.
CSE fraction 3a	resistant
CSD fraction 3b	ΔDi-6S (204%)
CSA fraction 4a	ΔDi-4S (81%) + ΔDi-6S (108%)
CSE fraction 4b	ΔDi-4S (84%) + unidentified substance (100%)
CSE fraction 4c	ΔDi-0S (100%) + ΔDi-diS$_E$ (85%)
CSD fraction 5	ΔDi-4S (244%)
CSC fraction 6	ΔDi-4S (100%) + ΔDi-6S (93%)
CSE fraction 7a	n.d.
CSD fraction 7b	ΔDi-6S (93%) + ΔDi-diS$_D$ (102%)
CSE fraction 7c	ΔDi-6S (74%) + ΔDi-diS$_E$ (64%) + undigested (48%)
CSD fraction 8a	ΔDi-4S (86%) + ΔDi-diS$_D$ (87%)
CSE fraction 8b	n.d.
CSE fraction 9	ΔDi-4S (120%) + ΔDi-diS$_E$ (120%)
CSE fraction 10	ΔDi-diS$_E$ (220%)

[a] Each tetrasaccharide was digested with chondroitinase ACII, and the digest was identified and quantified by HPLC as described in the Experimental. Similar results were obtained using a conventional commercial chondroitinase ABC instead of chondroitinase ACII. [b] See footnote in the Results section for key to the abbreviations defining the disaccharides. [c] The per cent recovery was calculated based upon the peak area on HPLC. [d] n.d., Not determined.

uronic acid in each component is D-Glc*p*A, but not L-Ido*p*A. The results are summarized in Table IV.

After incubation with chondroitinase ACII the oligosaccharides in fractions 3b, 5, or 10 yielded about twice as much of the single disaccharide species, ΔDi-6S, ΔDi-4S, or ΔDi-diS$_E$, respectively (data not shown). Therefore, the parent tetrasaccharides in fractions 3b, 5 and 10 have the following structures.

Fraction 3b:

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

Fraction 5:

α-L-Δ^{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$_3^-$

Fraction 10:

α-L-Δ^{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^-$6SO$_3^-$

Chondroitinase ACII digestion of the other fractions yielded two disaccharide species in about equal quantities (Table IV). The sequential arrangement of the two disaccharide units in each parent tetrasaccharide was determined. An example of the enzymatic sequence analysis of fraction 8a derived from CSD is presented in Fig. 2. When digested with chondroitinase ACII alone, fraction 8a yielded equal amounts of ΔDi-4S and ΔDi-diS$_D$ (Fig. 2A). After 2-sulfatase digestion the peak shifted to a position corresponding to the loss of one sulfate group (Fig. 2B). This was confirmed by sequential digestion of fraction 8a with 2-sulfatase and chon-

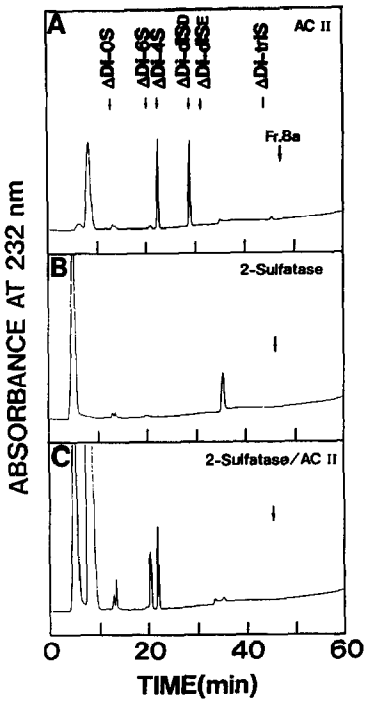

Fig. 2. Identification of fraction 8a by enzymatic digestion in conjunction with HPLC. Fraction 8a isolated from CSD was digested with either chondroitinase ACII (A), 2-sulfatase (B), or successively with 2-sulfatase and chondroitinase ACII (C), then the digests were analyzed by HPLC as described in the Experimental. The elution positions of authentic unsaturated CS disaccharides are indicated in the top panel, and that of the intact fraction 8a is indicated by an arrow in each panel.

droitinase ACII, which demonstrated equal proportions of ΔDi-6S and ΔDi-4S (Fig. 2C). The results altogether indicate that L-$\Delta^{4,5}$Hex*p*A at the nonreducing terminus of the parent tetrasaccharide is sulfated at the C-2 position. Thus we propose the following structure for the tetrasaccharide in fraction 8a.

Fraction 8a:

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*NAc4SO$_3^-$

Likewise, fraction 7b isolated from CSD was degraded by chondroitinase ACII into equal amounts of ΔDi-6S and ΔDi-diS$_D$ (Table IV). One sulfate group was lost upon 2-sulfatase digestion as analyzed by HPLC (Table V). Successive digestions of fraction 7b with 2-sulfatase and then chondroitinase ACII yielded twice as much ΔDi-6S as the parent tetrasaccharide (data not shown). We therefore propose the following structure for the tetrasaccharide in fraction 7b.

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$_3^-$

TABLE V

Action of various sulfatases on the disaccharides and tetrasaccharides isolated from chondroitin sulfate

Fraction	Structure [a]	Enzymatic action [b]		
		2-Sulfatase	4-Sulfatase	6-Sulfatase
ΔDi-4S	ΔU-G(4S)	−	+ [c]	−
ΔDi-6S	ΔU-G(6S)	−	−	+
ΔDi-diS$_B$	ΔU(2S)-G(4S)	+	+	−
ΔDi-diS$_D$	ΔU(2S)-G(6S)	+	−	+
ΔDi-diS$_E$	ΔU-G(4,6S)	−	−	+
ΔDi-triS	ΔU(2S)-G(4,6S)	+	−	+
Fraction 3b	ΔU-G(6S)-U-G(6S) [d]	−	−	+
Fraction 4a	ΔU-G(4S)-U-G(6S)	−	+	+
Fraction 4b	unidentified	−	+	−
Fraction 4c	ΔU-G-U-G(4,6S)	−	−	+
Fraction 5	ΔU-G(4S)-U-G(4S)	−	+ +	−
Fraction 6	ΔU-G(6S)-U-G(4S)	−	+	−
Fraction 7b	ΔU(2S)-G(6S)-U-G(6S)	+	−	+
Fraction 7c	ΔU-G(6S)-U-G(4, 6S)	−	−	+
Fraction 8a	ΔU(2S)-G(6S)-U-G(4S)	+	+	−
Fraction 9	ΔU-G(4,6S)-U-G(4S)	−	+	−
Fraction 10	ΔU-G(4,6S)-U-G(4, 6S)	−	−	+

[a] Abbreviations: ΔU, L-$\Delta^{4,5}$Hex*p*A; U, D-Glc*p*A; G, D-Gal*p*NAc; 2S, 2-sulfate; 4S, 4-sulfate; 6S, 6-sulfate. [b] Each tetrasaccharide was digested with 2-, 4-, or 6-sulfatase, and the digest was analyzed by HPLC as described in the Experimental. [c] + or + +, one or two sulfate groups were removed. [d] Of the two possible 4- or 6-sulfate groups, those underlined were removed.

The sequential arrangement of the disaccharide units in the tetrasaccharides in fractions 4a, 6, and 9 were determined using 4- and 6-sulfatases. The substrate specificity of these enzymes was initially examined using authentic unsaturated disaccharides and the above tetrasaccharides. The results are summarized in Table V. The 4-sulfatase removed two sulfate groups from fraction 5, indicating that it can remove sulfates from both the penultimate D-Gal*p*NAc residue substituted by L-$\Delta^{4,5}$Hex*p*A and the D-Gal*p*NAc at the reducing end. Since it removed a sulfate from ΔDi-diS$_B$, it is assumed that a 2-sulfate group on L-$\Delta^{4,5}$Hex*p*A at the nonreducing end does not interfere with the enzymatic removal of the 4-sulfate group from the neighboring D-Gal*p*NAc residue. However, 4-sulfatase hardly acted on ΔDi-diS$_E$ or CSE fraction 10, suggesting steric interference by the 6-sulfate group on the 4,6-disulfated D-Gal*p*NAc residue. The 6-sulfatase removed only one sulfate from fractions 3b and 10. It also removed one sulfate from fraction 7b, but not from fraction 8a. These data indicate that 6-sulfatase acts on the sulfate group on C-6 of the reducing D-Gal*p*NAc residue, but not the penultimate 6-sulfated D-Gal*p*NAc residue substituted by L-$\Delta^{4,5}$Hex*p*A. The resistance of the 6-sulfate group on the penultimate D-Gal*p*NAc residue is not due to the substitution of the neighboring L-$\Delta^{4,5}$Hex*p*A by 2-sulfate group because the enzyme acted on ΔDi-diS$_D$ yielding ΔDi-UA2S, as checked by HPLC (data not shown). Thus, 6-sulfatase removes only a sulfate group on C-6 of the D-Gal*p*NAc

residue at the reducing end of the di- and tetra-saccharides. It remains to be determined if this applies to oligosaccharides larger than tetrasaccharides.

Both fractions 4a and 6 were degraded by chondroitinase ACII into equal amounts of ΔDi-4S and ΔDi-6S. The former was sensitive to 6-sulfatase while the latter was resistant (Table V). Based upon the enzyme specificity mentioned above, it is assumed that a D-Gal*p*NAc6SO_3^- residue is located in the reducing side disaccharide unit of fraction 4a, but in the nonreducing side disaccharide unit of fraction 6. Thus the following structures are proposed for the tetrasaccharides in these fractions, and the structure of fraction 6 has been confirmed by 500-MHz ^{1}H NMR as described below.

Fraction 4a:

α-L-Δ^{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*NAc6SO_3^-

Fraction 6:

α-L-Δ^{4,5}Hex*p*A-(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^-

Fraction 9 derived from CSE was degraded by chondroitinase ACII into equal amounts of ΔDi-4S and ΔDi-diS_E (Table IV), and was resistant to 6-sulfatase (Table V), suggesting that the 4,6-disulfated D-Gal*p*NAc is not located at the reducing end, but at the penultimate position. The following structure is proposed.

Fraction 9:

α-L-Δ^{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^-

A major component in fraction 7c derived from CSE was degraded by chondroitinase ACII into equal amounts of ΔDi-6S and ΔDi-diS_E (Table IV), and was sensitive to 6-sulfatase (Table V). Successive digestions of this fraction with 6-sulfatase and then chondroitinase ACII resulted in equal amounts of ΔDi-4S and ΔDi-6S as determined by HPLC (data not shown). The results suggest that the 6-sulfatase removed a 6-sulfate group from the 4,6-disulfated D-Gal*p*NAc residue located at the reducing end of the tetrasaccharide. Thus the following structure is proposed. A minor component resistant to chondrotinase ACII has not yet been characterized.

Fraction 7c:

α-L-Δ^{4,5}Hex*p*A-(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^-6SO_3^-

Fraction 4c derived from CSE yielded ΔDi-0S and ΔDi-diS_E upon chondroitinase ACII digestion. It was sensitive to 6-sulfatase (Table V), indicating that the 4,6-disulfated D-Gal*p*NAc residue is located at the reducing end of the tetrasaccharide. Thus the following structure is proposed.

Fraction 4c:

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

Chondroitinase ACII digestion of fraction 4b derived from CSE resulted in ΔDi-4S and an unsaturated component which eluted near ΔDi-6S, but which was resistant to 6-sulfatase. It was also resistant to 2-sulfatase, although ΔDi-UA2S eluted near ΔDi-6S. The structure of this substance remains to be identified.

500 MHz ^1H NMR spectroscopy.—The tetrasaccharides in fractions 6, 7b, 8a, and 9 were characterized by 500-MHz ^1H NMR spectroscopy. The one-dimensional spectrum of CSC fraction 6 measured at 26°C is shown in Fig. 3A. The resonances between δ 4.4 and 5.3 ppm are characteristic of anomeric protons, whereas those at around δ 5.9 and 2.0 ppm are characteristic of the H-4 proton of L-$\Delta^{4,5}$Hex*p*A^{4,6} and the acetoamide group protons of D-Gal*p*NAc, respectively. The other proton chemical shifts were assigned using 2D HOHAHA and COSY spectra. Beginning at δ 5.877 for the H-4 proton of L-$\Delta^{4,5}$Hex*p*A-4, a cross-peak showing connectivity to the H-3 resonance at δ 4.105 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. 4A). Starting with the H-1 resonance of the D-Gal*p*NAc-1 of the α anomer at δ 5.212, the H-2, H-3, and H-4 resonances were identified at δ 4.328, 4.191, and 4.810, respectively, in the 2D HOHAHA spectrum. The weak H-4 resonance along the cross-section is more clearly observed above it as indicated by the dotted vertical line in the 2D HOHAHA spectrum, and is recognizable also in the one-dimensional spectrum recorded at 15°C to suppress the disturbance by HOD line (inset, Fig. 3A). The chemical shift of the H-4 proton is comparable[20] with that (δ 4.685) of the H-4 proton belonging to the D-Gal*p*NAc of the α anomer of α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-D-Gal*p*NAc4SO$_3^-$, indicating the presence of the 4-sulfated D-Gal*p*NAc structure at the reducing end of CSC fraction 6. The H-1 and H-4 resonances of the β-D-Gal*p*NAc-1 were found at δ 4.712 and δ 4.743, respectively, which are consistent with the 4-sulfation of the D-Gal*p*NAc-1. The anomerization effect resulted in resonances at δ 4.530 (α H-1) and δ 4.483 (β H-1) of the second constituent D-Glc*p*A-2. In the 2D HOHAHA spectrum, the indicated assignment pathway, starting at the H-1 signal for α-D-Glc*p*A or β-D-Glc*p*A, leads to the H-2, H-3, H-4, and H-5 signals of each D-Glc*p*A, respectively. H-2 and H-3 proton signals at around δ 3.4 and 3.6 are structural reporter groups of nonsulfated β-D-Glc*p*A^{4,6}. No anomerization effects were observed for D-Gal*p*NAc-3. Starting with the remaining anomeric proton signals at δ 4.593 for the H-1 of this residue, H-2, H-3, and H-4 proton signals were localized as indicated in the 2D HOHAHA spectrum. Although no cross-peak between H-4 and H-5 could be seen owing to the weak coupling between them, H-6 and H-6′ resonances characteristic of a 6-sulfated β-D-Gal*p*NAc residue (see compound 7 in ref 6) were found both at δ 4.22. The NMR data are summarized in Table VI with those of the reference compound, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc6SO$_3^-$-(1 → 4)-β-D-Glc*p*A-(1 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Xyl-ol. The chemical shifts of protons belonging to the nonreducing trisaccharide portion of the tetrasaccharide in CSC fraction 6 were similar to those of the corresponding portion of the reference

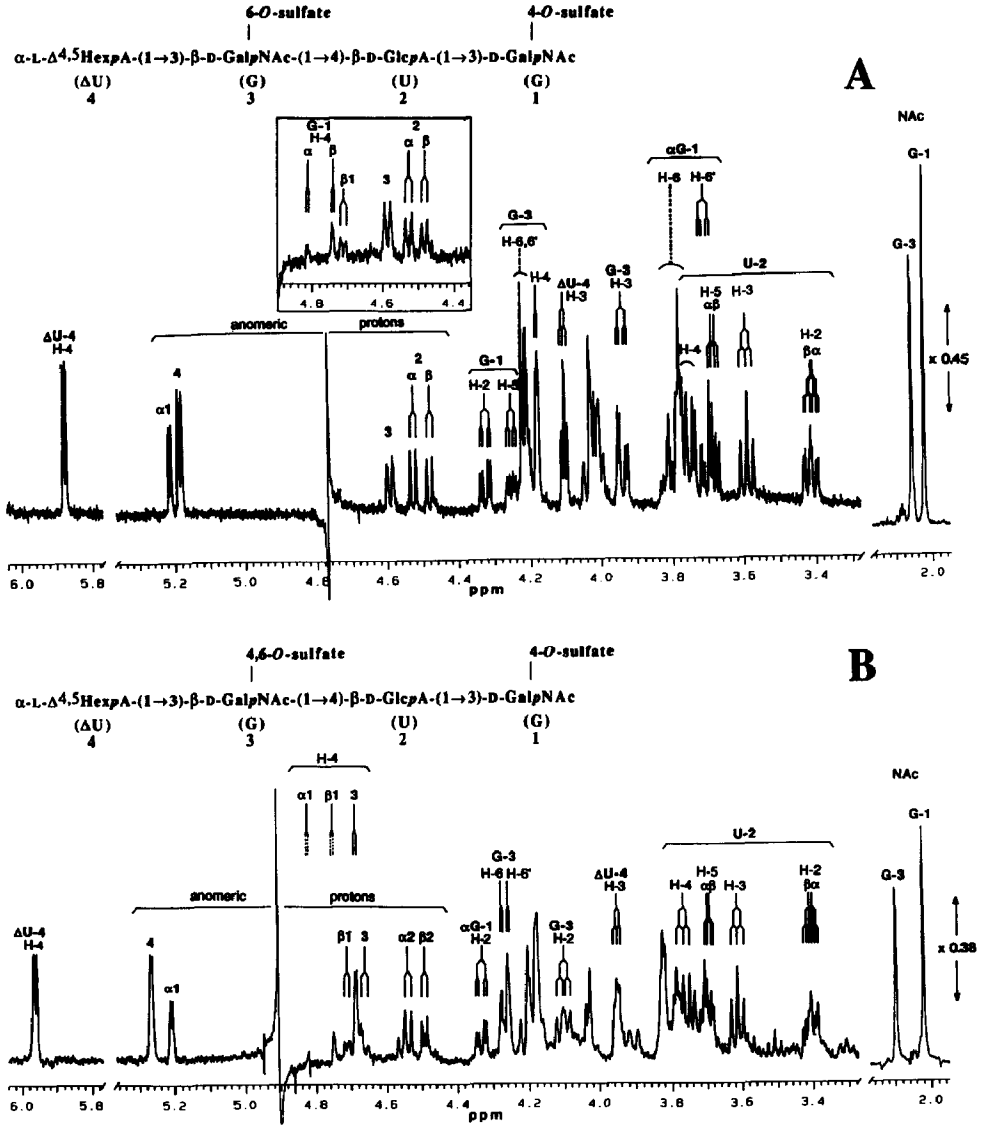

Fig. 3. 500-MHz ^{1}H NMR spectra of CSC fraction 6 (A) and CSE fraction 9 (B) recorded in D_2O at 26 and 15°C, respectively. The inset in A is the spectrum of CSC fraction 6 recorded at 15°C. The numbers and letters in the spectra refer to the corresponding residues in the structures. Abbreviations: G, D-Gal*p*NAc; U, D-Glc*p*A; Δ*U*, L-$\Delta^{4,5}$Hex*p*A.

compound, indicating the presence of the trisaccharide structure, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → , in CSC fraction 6. Thus, the NMR data are consistent with the structure proposed above based upon the enzyme digestion.

The one dimensional spectrum of CSE fraction 9 measured at 15°C is shown in Fig. 3B, and the NMR data are summarized in Table VI. The proton chemical

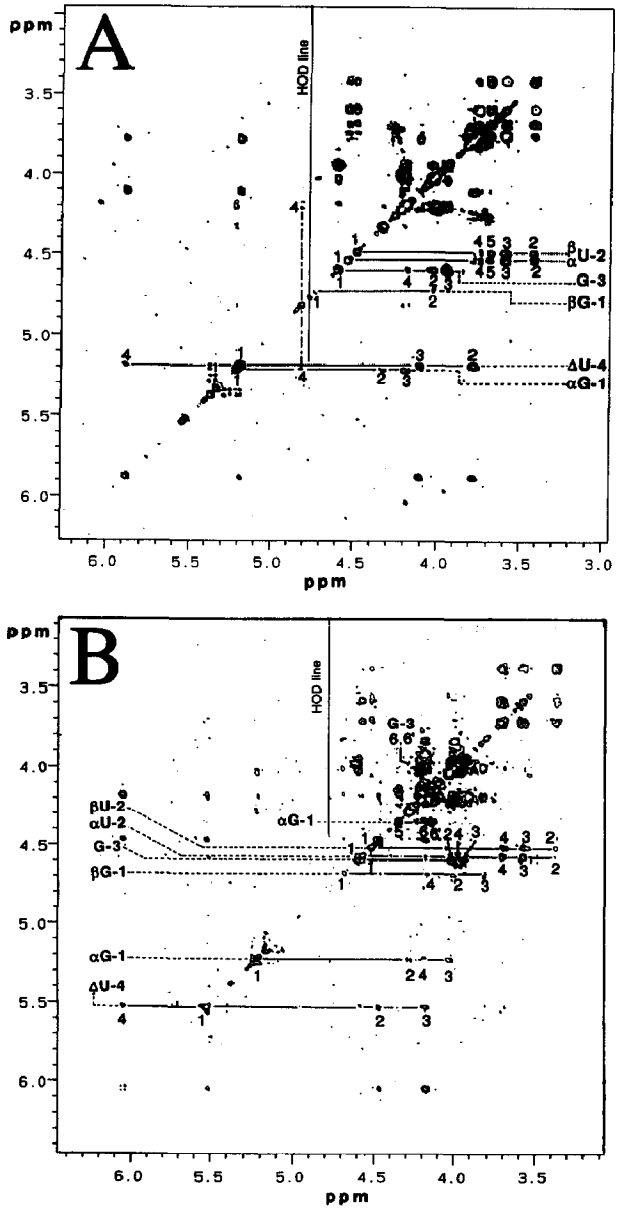

Fig. 4. Two-dimensional HOHAHA spectra of CSC fraction 6 (A) and CSD fraction 7b (B) recorded in D_2O at 26°C. For abbreviations, see the legend to Fig. 3.

shifts were assigned using COSY and 2D HOHAHA spectra recorded at 26°C (not shown) as described for CSC fraction 6. The spectral data were very similar to those of CSC fraction 6 except for the large downfield shift (Δ δ 0.508) of the H-4 of D-Gal*p*NAc-3 (Table VI), indicating that the compound in CSE fraction 9 has the same tetrasaccharide core structure with CSC fraction 6, α-L-$\Delta^{4,5}$Hex*p*A-(1 →

TABLE VI

^{1}H Chemical shifts of the sulfated tetrasaccharides together with those for reference compound R (fraction 7 in ref 6)

H atom [a]	R [b]	Chemical shifts [c]			
		CSC Fr. 6	CSE Fr. 9	CSD Fr. 7b	CSD [d] Fr. 8a
Gal*p*NAc-1					
H-1α		5.212	5.208	5.216	5.211
H-1β		4.712 [e]	4.718	4.684	4.711 [e]
H-2α		4.328	4.334	4.283	4.328
H-2β		4.020	n.d. [f]	4.01	4.03
H-3α		4.191	4.180	4.03	4.19
H-3β		n.d.	n.d.	3.840	n.d.
H-4α		4.810	4.820	4.184	4.79
H-4β		4.743 [e]	4.750	4.17	4.745 [e]
H-5α		4.254	n.d.	4.348	4.258
H-5β		3.82	n.d.	3.937	n.d.
H-6α		3.79	n.d.	4.19	3.80
H-6β		n.d.	n.d.	4.21	n.d.
H-6′α		3.71	n.d.	4.14	3.74
H-6′β		n.d.	n.d.	4.13	n.d.
NAc		2.018	2.020	2.015	2.018
Glc*p*A-2					
H-1α		4.530	4.540	4.568	4.531
H-1β	4.677	4.483	4.494	4.510	4.486
H-2α		3.411	3.411	3.386	3.413
H-2β	3.470	3.416	3.422	3.386	3.418
H-3	3.636	3.591	3.614	3.59	3.590
H-4	3.74	3.76	3.768	3.72	3.765
H-5α		3.689	3.698	n.d.	3.693
H-5β	3.74	3.680	3.692	n.d.	3.684
Gal*p*NAc-3					
H-1	4.568	4.593	4.662	4.589	4.607
H-2	4.032	4.03	4.104	4.02	4.03
H-3	3.947	3.941	4.19	3.966	3.966
H-4	4.178	4.180	4.688	3.976	3.978
H-5	4.010	n.d.	4.20	4.00	4.00
H-6	4.232	4.22	4.278	4.20	4.20
H-6′	4.220	4.22	4.258	4.20	4.20
NAc	2.052	2.056	2.100	2.087	2.089
ΔHex*p*A-4 [g]					
H-1	5.179	5.184	5.267	5.519	5.521
H-2	3.780	3.77	3.824	4.466	4.466
H-3	4.109	4.105	3.950	4.18	4.18
H-4	5.881	5.877	5.964	6.031	6.031

[a] Listed by successive monosaccharide residues, numbered as in Figs. 3 and 5. [b] R, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Xyl-ol. The data for the nonreducing trisaccharide portion are presented. [c] Chemical shifts in D_2O as solvent, in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, measured relative to acetone (δ 2.225) at 26°C (CSC fraction 6, CSD fractions 7b and 8a) or 15°C (CSE fraction 9 and the reference compound). [d] The estimated error for the values to two decimal places is only $\pm$0.01 ppm because of partial overlap of signals. That for the values to three decimal places is $\pm$0.001 ppm. [e] Values determined at 15°C. [f] n.d., Not determined. [g] $\Delta^{4,5}$Hex*p*A.

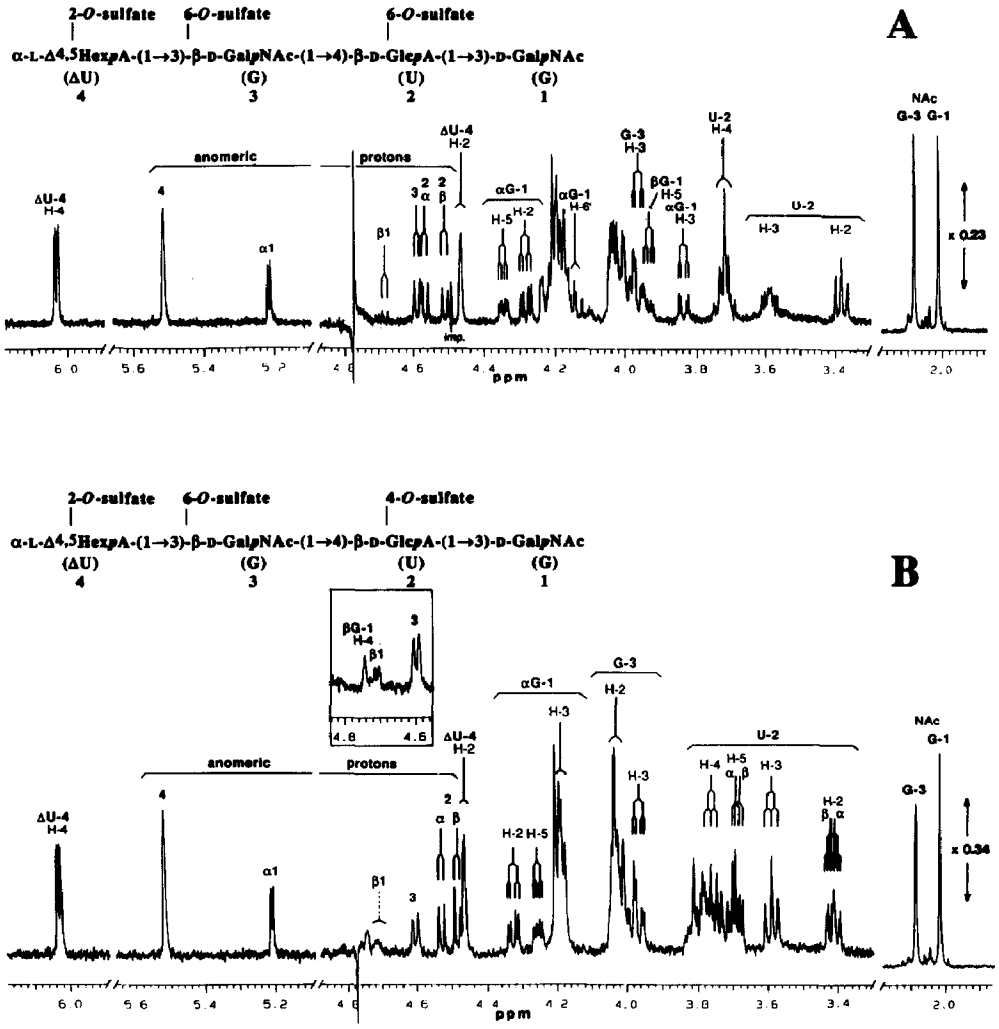

Fig. 5. 500-MHz ^{1}H NMR spectra of CSD fractions 7b (A) and 8a (B) recorded in D_2O at 26°C. The inset in B is the spectrum of CSD fraction 8a recorded at 15°C. The numbers and letters in the spectra refer to the corresponding residues in the structures. For abbreviations, see the legend to Fig. 3.

3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^-, but with an additional sulfate group on the C-4 position of D-Gal*p*NAc-3, being consistent with the structure proposed above on the basis of the enzyme digestion.

The one-dimensional spectrum of CSD fraction 7b measured at 26°C is shown in Fig. 5A, and the NMR data are summarized in Table VI. The H-1, H-2, H-3 and H-4 resonances of the four monosaccharide components were readily identified as indicated in the 2D HOHAHA spectrum (Fig. 4B) with the aid of COSY spectrum (not shown). The H-2 proton signal of L-$\Delta^{4,5}$Hex*p*A-4 shifted downfield (Δ δ 0.642) as compared with that of L-$\Delta^{4,5}$Hex*p*A-4 in CSE fraction 9, and the chemical shifts of protons belonging to this residue were similar[20] to those for the

2-sulfated L-$\Delta^{4,5}$Hex*p*A residue of α-L-$\Delta^{4,5}$Hex*p*A2SO_3^--(1 → 3)-D-Gal*p*NAc6SO_3^-, indicating the presence of the 2-sulfated L-$\Delta^{4,5}$Hex*p*A structure in CSD fraction 7b. Chemical shifts of H-4 protons of D-Gal*p*NAc-1 and D-Gal*p*NAc-3 indicate that the C-4 positions of both D-Gal*p*NAc residues are not sulfated. Rather H-5, H-6, and H-6′ proton signals of D-Gal*p*NAc-1 of the α anomer were found at δ 4.348, 4.19, and 4.14, indicating the 6-sulfation of D-Gal*p*NAc-1 although no cross-peak was observed between H-4 and H-5 protons in the 2D HOHAHA spectrum, owing to the weak coupling between them. The resonances at δ 4.00, 4.20, and 4.20 are most likely the H-5, H-6, and H-6′ proton signals of D-Gal*p*NAc-3, and are indicative of 6-sulfation of this residue.

The one-dimensional ^{1}H NMR spectrum of CSD fraction 8a measured at 26°C is shown in Fig. 5B, and the NMR data are summarized in Table VI. The proton chemical shifts were assigned using COSY and 2D HOHAHA spectra recorded at 26°C (not shown) as described for CSD fraction 7b. The chemical shifts of protons belonging to D-Glc*p*A-2, D-Gal*p*NAc-3, and L-$\Delta^{4,5}$Hex*p*A-4 were very similar to those of CSD fraction 7b, indicating that they share the trisaccharide structure, 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 → , on the nonreducing side. However, when compared with CSD fraction 7b, a large downfield shift of the H-4 and a large upfield shift of H-6 and H-6′ of D-Gal*p*-NAc-1 were observed (Table VI), indicating that D-Gal*p*NAc-1 in CSD fraction 8a bears a 4- instead of a 6-sulfate group. Thus the tetrasaccharide structure, 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*NAc4SO_3^-, was confirmed.

DISCUSSION

Previously we found that highly purified chondroitinase ABC did not completely degrade CS oligosaccharides into disaccharides[5,6], which prompted us to investigate the structure of the resistant oligosaccharides. In this study we systematically isolated a number of CS tetrasaccharides, which remained undigested after exhaustive incubation using a highly purified enzyme. The structures of these tetrasaccharides were determined by HPLC in conjunction with enzymatic digestion using chondroitinase ACII and various sulfatases, and also by 500-MHz ^{1}H NMR spectroscopy. The structures include one tetrasulfated, four trisulfated, and five disulfated tetrasaccharides. Among them, the structures corresponding to fractions 3b, 5, 6, and 9 have been previously isolated from CS after testicular hyaluronidase digestion, but as saturated tetrasaccharides with a common core carbohydrate sequence β-D-Glc*p*A-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc[14,21,22]. Fractions 4a, 4c, 7b, 8a, and 10 have not been previously isolated as discrete structures. The structures include a variety of sulfated tetrasaccharides combining two units of nonsulfated, 4-sulfated, 6-sulfated, 4,6-disulfated, or 2,6-disulfated disaccharide, indicating that the resistant nature of these tetrasaccharides to the enzyme does not correlate with the sulfation profile of the

oligosaccharides, but rather correlates with their size. Highly purified chondroitinase ABC did not act on the following tetrasaccharides either (data not shown); α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc isolated from Chn, β-D-Glc*p*A-(1 → 3)-β-D-Glc*p*NAc-(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Glc*p*NAc isolated from HA, and α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^--(1 → 4)-α-L-Ido*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^- isolated from DS. It is most likely that the highly purified enzyme only acts on penta- or larger oligosaccharides, but not on tetra- or tri-saccharides. Preparation of larger oligosaccharides to prove this hypothesis is in progress. An extra protein band (M_r 100000) on SDS-PAGE was clearly observed for the conventional chondroitinase ABC in addition to that (M_r 98000) observed for the highly purified enzyme (data not shown). The results may indicate that the latter component cannot degrade tetrasaccharides, while the former is an isomer of the latter which can degrade tetrasaccharides into disaccharides. Investigations along this line are also in progress.

In this study substrate specificities of chondro-4- and -6-sulfatases were demonstrated. The former can act on 4-sulfated D-Gal*p*NAc-containing disaccharide units on both the reducing and nonreducing sides of tetrasaccharides. In contrast, the latter enzyme acts upon the 6-sulfated unit on the reducing side, but not upon that on the nonreducing side of tetrasaccharides. It has been reported[14] that 4-sulfatase removes both sulfate groups of the saturated tetrasaccharide, β-D-Glc*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc4SO_3^- while 6-sulfatase does not remove either of the two sulfate groups from the saturated tetrasaccharide, β-D-Glc*p*A-(1 → 3)-β-D-Gal*p*NAc6SO_3^--(1 → 4)-β-D-Glc*p*A-(1 → 3)-D-Gal*p*NAc6SO_3^-. It is possible that the saturated tetrasaccharide has a different conformation from that of its unsaturated counterpart, so that it is not recognized by 6-sulfatase. These specific enzymes would be useful for characterizing sulfated oligosaccharides. It remains to be tested whether 4-sulfatase removes 4-sulfate groups in the internal disaccharide units of larger oligosaccharides, and whether 6-sulfatase removes a 6-sulfate group from the reducing GalNAc of larger oligosaccharides.

It has become evident that sulfated glycosaminoglycans including CS and DS play important roles in various cellular recognition systems[8–10]. However, the complex sulfated structures of these glycosaminoglycans have hampered the structural studies. We have been accumulating ^{1}H NMR data on sulfated oligosaccharides to construct a data base. However, so far the ^{1}H NMR characterization of CS oligosaccharides has been limited to disaccharides[20] and oligosaccharides derived from the carbohydrate–protein linkage region[4–6,23]. In this study, the 500-MHz ^{1}H NMR spectra of four fractions, 6, 7b, 8a, and 9, isolated from the repeating disaccharide region were recorded. These data will be useful for characterizing larger oligosaccharides.

The unsaturated tetrasaccharides, α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^--(1 → 4)-β-D-Glc*p*A2SO_3^--(1 → 3)-D-Gal*p*NAc6SO_3^- and α-L-$\Delta^{4,5}$Hex*p*A-(1 → 3)-β-D-Gal*p*NAc4SO_3^-6SO_3^--(1 → 4)-β-D-Glc*p*A2SO_3^--(1 → 3)-D-Gal*p*NAc6SO_3^-,

isolated from shark fin cartilage CS after chondroitinase ACI digestion have been successfully used for inhibition studies of monoclonal antibodies against CS to determine their epitopes[24]. It will be of interest to evaluate the biological activities of the oligosaccharides isolated in this study.

ACKNOWLEDGMENTS

We thank Dr. Makiko Sugiura (Kobe Women's College of Pharmacy) for measuring the NMR spectra of the oligosaccharides. This work was supported in part by Japanese Private School Promotion Funds, a grant from the Nakatomi Foundation, and Grants-in-Aid for Scientific Research (04807162) and Scientific Research on Priority Areas (05274226) from the Ministry of Education, Science, and Culture of Japan.

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