A Novel Pentasaccharide Sequence GlcA(3-sulfate)(β1-3)GalNAc(4-sulfate)(β1-4)(Fucα1-3)GlcA(β1-3)GalNAc(4-sulfate) in the Oligosaccharides Isolated from King Crab Cartilage Chondroitin Sulfate K and Its Differential Susceptibility to Chondroitinases and Hyaluronidase†

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Received November 1, 1996; Revised Manuscript Received January 21, 1997⊗

ABSTRACT: Novel sulfated tetrasaccharide structures containing 3-*O*-sulfated GlcA were isolated recently from king crab cartilage chondroitin sulfate K [Sugahara, K., Tanaka, Y., Yamada, S., Seno, N., Kitagawa, H., Haslam, S. M., Morris, H. R., & Dell, A. (1996) *J. Biol. Chem. 271*, 26745−26754]. In this study, we prepared a series of oligosaccharides from the same source after exhaustive digestion with testicular hyaluronidase and determined the structures of a pentasaccharide, two hexasaccharides, and two heptasaccharides by means of fast atom bombardment mass spectrometry and 500-MHz ^{1}H-NMR spectroscopy. All the oligosaccharides had the following hitherto unreported structures including a novel glucuronate 3-*O*-sulfate: GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(β1-3)GalNAc(4S), GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)-GalNAc(4S), GlcA(3S)(β1-3)GalNAc(4S)(β1-4)(Fucα1-3)GlcA(β1-3)GalNAc(4S), GlcA(3S)(β1-3)-GalNAc(4S)(β1-4)(Fucα1-3)GlcA(β1-3)GalNAc(4S)(β1-4)GlcA(β1-3)GalNAc(4S), and GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)GalNAc(4S)(β1-4)(Fucα1-3)GlcA(β1-3)GalNAc(4S), where 3S or 4S represent 3-*O*- or 4-*O*-sulfate, respectively. Furthermore, the three latter structures contained a novel combination of both 3-*O*-sulfated and 3-*O*-fucosylated GlcA residues. The pentasaccharide with 3-*O*-fucosylated GlcA at the internal position remained totally resistant to chondroitinase AC-II, whereas it was degraded by chondroitinase ABC into a disaccharide unit containing GlcA(3S) derived from the nonreducing side and a trisaccharide unit containing fucose from the reducing side.

There is increasing evidence that chondroitin sulfate (CS[1]) proteoglycans are distributed among various tissues and are involved in cell adhesion and migration, neurite outgrowth, in mediating the effects of growth factors, and other developmental processes [see Poole (1986), Fransson (1987), and Ruoslahti (1988) for reviews]. They are major components of the extracellular tissue matrix, cell surface, and basement membranes and are also found in intracellular granules of certain cells [see Silbert and Sugumaran (1995) for review].

Proteoglycans consist of specific polysaccharide chains, termed GAGs, attached covalently to a core protein. GAG chains of CS proteoglycans consist of a linear polymer structure that possesses repetitive, sulfated disaccharide units containing *N*-acetylgalactosamine (GalNAc) residues and glucuronic acid (GlcA) residues. Because many of the functions of CS proteoglycans are associated with the GAG moieties (Poole, 1986), the structure of the CS chains is of particular interest. However, the relationships between the biological functions of these molecules and their detailed structures have not been elucidated, mainly due to the difficulty in determining the fine structure of sulfated GAGs containing complex modifications such as sulfation and C5 epimerization.

During the last few years we have conducted systematic structural studies of oversulfated CS from cartilages of various animals including shark, squid, and king crab (Sugahara et al., 1994a,b, 1996a,b). Structurally-defined

† The work in Japan was supported in part by the Science Research Promotion Fund from the Japan Private School Promotion Foundation, the Grants-in-aid for Encouragement of Young Scientists 08780602 (to H. K.), for Exploratory Research 08877338 (to K. S.), and for Scientific Research on Priority Areas 05274107 (to K. S.) from the Ministry of Education and Culture of Japan. The Imperial College work was supported by the BBSRC, the Medical Research Council, and the Wellcome Trust (Grant 030825) to H. R. M. and A. D.

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1997.

[1] Abbreviations: COSY, correlation spectroscopy; 1D or 2D, one- or two-dimensional; CS, chondroitin sulfate; FAB-MS, fast atom bombardment mass spectrometry; GAG, glycosaminoglycan; GalNAc, *N*-acetyl-D-galactosamine; GlcA, D-glucuronic acid; HOHAHA, homonuclear Hartmann-Hahn; ΔHexA, 4,5-unsaturated hexuronic acid or 4-deoxy-α-L-threo-hex-4-ene-pyranosyluronic acid; Hex, hexose; HexA, hexuronic acid; HexNAc, *N*-acetylhexosamine; HPLC, high-performance liquid chromatography; NFU, National formulary unit; ΔDi-0S, $\Delta^{4,5}$HexA(α1-3)GalNAc; ΔDi-6S, $\Delta^{4,5}$HexA(α1-3)GalNAc(6-*O*-sulfate); ΔDi-4S, $\Delta^{4,5}$HexA(α1-3)GalNAc(4-*O*-sulfate); Di-4S, GlcA(β1-3)GalNAc(4-*O*-sulfate); ΔDi-diS_D, $\Delta^{4,5}$HexA(2-*O*-sulfate)(α1-3)GalNAc(6-*O*-sulfate); ΔDi-diS_E, $\Delta^{4,5}$HexA(α1-3)GalNAc(4,6-*O*-disulfate); ΔDi-triS, $\Delta^{4,5}$HexA(2-*O*-sulfate)(α1-3)GalNAc(4,6-*O*-disulfate); ΔU, G, U, S, 2S, 3S, 4S, and 6S represent ΔHexA, GalNAc, GlcA, *O*-sulfate, 2-*O*-sulfate, 3-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate, respectively.

S0006-2960(96)02740-7 CCC: $14.00

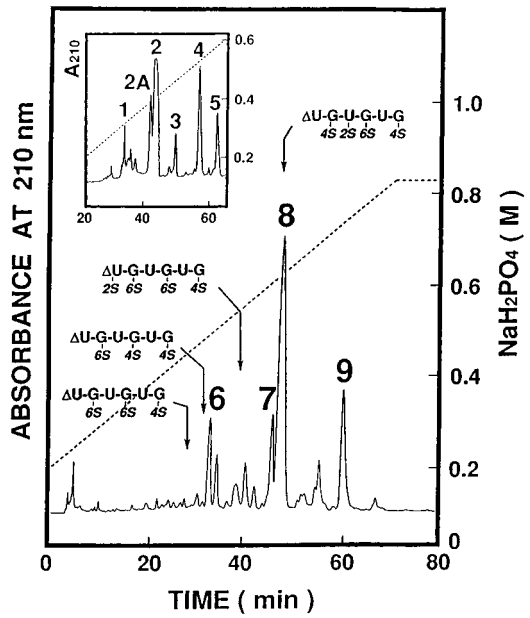


FIGURE 1: Subfractionation of the hexasaccharide fraction by HPLC on an amine-bound silica column. The hexasaccharide fraction obtained by size-fractionation [Figure 2 in Sugahara et al. (1996b)] was chromatographed on an amine-bound silica column using a linear salt gradient, as indicated by the *dashed line*. Also indicated by *arrows* are the elution positions of the authentic sulfated hexasaccharides with the common core saccharide structure ΔHexA(α1-3)GalNAc(*β*1-4)GlcA(*β*1-3)GalNAc(*β*1-4)GlcA(*β*1-3)-GalNAc, which were prepared by chondroitinase ABC digestion of shark cartilage CSD (Sugahara et al., 1996c). Fraction 2A derived from the tetrasaccharide fraction was isolated by HPLC as shown in the inset using the same conditions as described (Sugahara et al., 1996b).

oligosaccharides as well as specific degrading enzymes and monoclonal antibodies are essential tools for microanalyzing biologically active domain structures and antibody epitopes of CS chains. We isolated novel sulfated oligosaccharides containing 3-*O*-sulfated GlcA residues from king crab cartilage CS-K (Sugahara et al., 1996b). This novel structure forms a unique carbohydrate epitope on glycoproteins and glycolipids in nervous tissues, which is recognized by HNK-1 monoclonal antibody (Chou et al., 1986). Immunoblots of the CS proteoglycans of the brain as well as several nervous tissue glycoproteins are intensely stained by the HNK-1 antibody (Gowda et al., 1989). In this study, a pentasaccharide, two hexasaccharides, and two heptasaccharides from CS-K of king crab cartilage were isolated after testicular hyaluronidase digestion to determine the structure of larger oligosacharides. Analyses by FAB-MS and 500-MHz ^{1}H-NMR spectroscopy revealed 3-*O*-sulfated GlcA residues in all these oligosaccharides and 3-*O*-fucosylated GlcA residues in three of them. Furthermore, the present study demonstrated that chondroitinase ABC cleaved a galactosaminidic bond linked to a 3-*O*-fucosylated GlcA residue whereas chondroitinase AC-II did not.

EXPERIMENTAL PROCEDURES

Materials. A CS-K−peptide preparation was purified from king crab cartilage as reported (Seno et al., 1974a). Six unsaturated standard CS disaccharides, chondroitinases ABC (EC 4.2.2.4) and AC-II (EC 4.2.2.5) and chondro-4-*O*-sulfatase (EC 3.1.6.9) were purchased from Seikagaku Corp. (Japan). Sheep testicular hyaluronidase (EC 3.2.1.35)

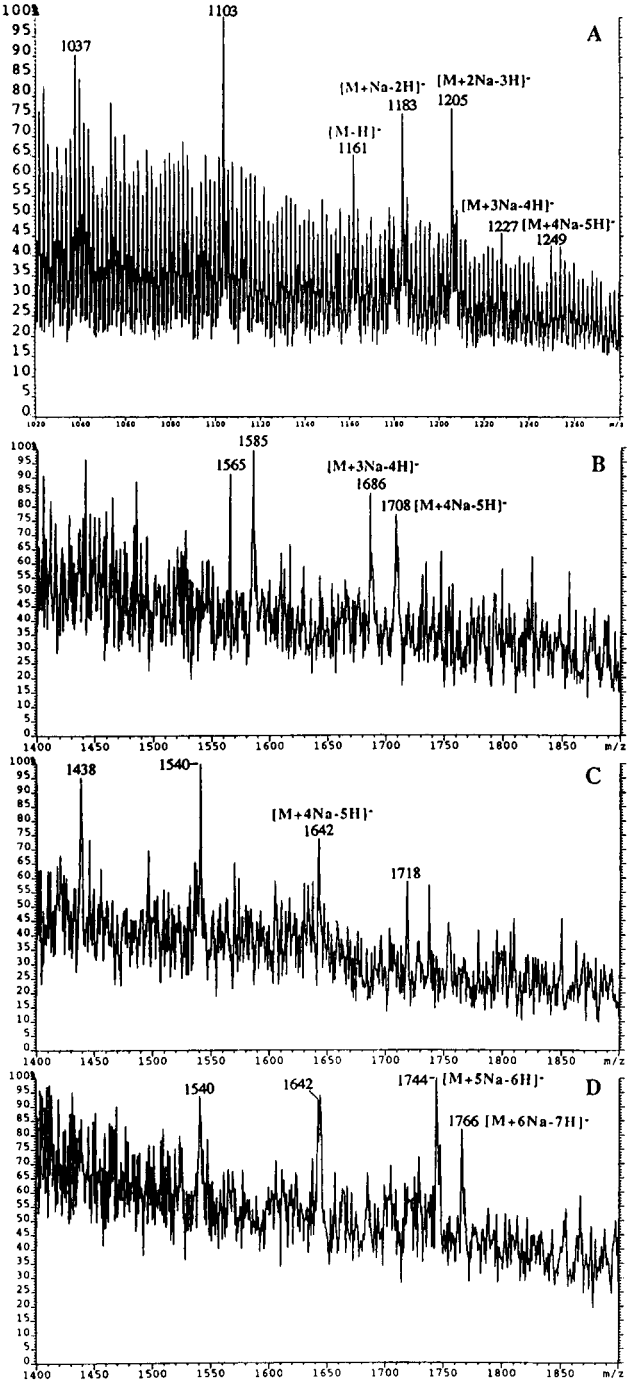


FIGURE 2: Negative FAB mass spectra of underivatized fractions 2A (A), 6 (B), 8 (C), and 9 (D). Major molecular ion signals are assigned as summarized in Table 1. Signals 102 mass units below assigned molecular ions are fragment ions derived from loss of sodium sulfite.

was obtained from Sigma. Bio-Gel P-10 and Sephadex G-25 (fine) were obtained from Bio-Rad and Pharmacia (Sweden).

Preparation of Oligosaccharide Fractions. A CS-K− peptide preparation (20 mg) was digested with 2 mg (approx. 3000 NFU; 1 NFU corresponds to the amount of the enzyme which hydrolyzes 74 μg of hyaluronate/min) of sheep testicular hyaluronidase (Poh et al., 1992; Sugahara et al., 1992) and fractionated by gel filtration on a Bio-Gel P-10 column (1.6 × 95 cm) into tetra-, hexa- and octasaccharide fractions as described [see Figure 2 in Sugahara et al. (1996b)]. Tetra- and hexasaccharide fractions were pooled, concentrated, desalted through a column (1.5 × 46 cm) of

Table 1: FAB-MS Analysis of the Four Oligosaccharides Isolated from CSK of King Crab Cartilage

fraction	yield[a]	*m/z* for $[M - H]^-$	*m/z* for $[M + Na - 2H]^-$	*m/z* for $[M + 2Na - 3H]^-$	*m/z* for $[M + 3Na - 4H]^-$	*m/z* for $[M + 4Na - 5H]^-$	*m/z* for $[M + 5Na - 6H]^-$	*m/z* for $[M + 6Na - 7H]^-$	assignment
2A	322	1161	1183	1205	1227	1249			$DeoxyHex_1HexA_2HexNAc_2(OSO_3H)_3$
6	147				1686	1708			$DeoxyHex_1HexA_3HexNAc_3(OSO_3H)_4$
8	758					1642			$HexA_3HexNAc_3(OSO_3H)_5$
9	218						1744	1766	$HexA_3HexNAc_3(OSO_3H)_6$

[a] Nanomoles obtained from 20 mg of CSK.

Sephadex G-25 (fine), and lyophilized. These fractions were each subfractionated by HPLC on an amine-bound silica column as described (Sugahara et al., 1994a). Each peak was purified by rechromatography under the same conditions as the first step and desalted as described above. Each peak was quantified by the carbazole method using GlcA as a standard (Bitter & Muir, 1962). The homogeneity of each fraction was judged by capillary electrophoresis (Sugahara et al., 1994c) and HPLC.

Digestion of Isolated Oligosaccharides with Chondroitinases ABC, AC-II, or Chondro-4-sulfatase. Oligosaccharides (2 nmol as GlcA) were digested using 5 mIU each of chondroitinases ABC or AC-II as described (Sugahara et al., 1994a). Digestion with chondro-4-sulfatase proceeded under either standard or harsh incubation conditions: the former or latter used 20 or 50 mIU of the enzyme for 10 or 60 min, respectively, basically as described (Sugahara et al., 1994a). Reactions were terminated by boiling for 1 min, and the reaction mixture was analyzed by HPLC as reported (Sugahara et al., 1994a). Eluates were monitored by absorption at both 210 and 232 nm.

FAB-MS. The sugar and sulfate compositions of oligosaccharides were determined by FAB-MS. FAB mass spectra of the oligosaccharide samples were obtained using a VG Analytical ZAB-2SE 2FPD mass spectrometer fitted with a Caesium ion gun operated at 20−25 kV. Data were acquired and processed using the VG Analytical Opus software. Monothioglycerol was used as the matrix.

500-MHz ^{1}H-NMR Spectroscopy. ^{1}H-NMR spectra of oligosaccharides were measured on a Varian-500 at a probe temperature of 15, 26, or 40 °C as reported (Yamada et al., 1992; Sugahara et al., 1996a). Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured relative to acetone (δ 2.225) in ^2H_2O (Vliegenthart et al., 1983). Oligosaccharide samples for NMR analysis were repeatedly exchanged in ^2H_2O with intermediate lyophilization.

RESULTS

Isolation of the Tetrasaccharide Fraction. The CS-K− peptide preparation purified from king crab cartilage was digested with sheep testicular hyaluronidase and the digest was fractionated by gel filtration on a column of Bio-Gel P-10. Three peaks were resolved when monitored by absorbance at 210 nm caused primarily by *N*-acetyl groups and they were assigned to tetra-, hexa-, and octasaccharide fractions as described (Sugahara et al., 1996b). The tetrasaccharide fraction was separated into several fractions by HPLC on an amine-bound silica column, and the structures of the compounds in five (fractions 1−5) of them were determined as sulfated tetrasaccharides as reported (Sugahara et al., 1996b) (also see Figure 1, inset). In this study, fraction 2A (Figure 1, inset), a shoulder peak of fraction 2, and the compounds in the hexasaccharide fraction were studied. Fraction 2A was purified by rechromatography to apparent homogeneity as judged by HPLC and capillary electrophoresis (data not shown), then structurally analyzed. The hexasaccharide fraction was also subfractionated into fractions 6−9 by HPLC on an amine-bound silica column. As shown in Figure 1, fractions 6−8 were eluted at the positions of authentic unsaturated hexasaccharides with three or four sulfate groups, whereas fraction 9 was eluted at higher salt concentrations. They were purified by rechromatography to apparent homogeneity judging by HPLC and capillary electrophoresis (data not shown), then structurally analyzed. The amounts of the purified oligosaccharides in fractions 2A, 6, 8, and 9 from 20 mg of CS-K are summarized in Table 1 with FAB-MS data.

FAB-MS Analysis. FAB-MS analyses of the underivatized oligosaccharide samples in the negative-ion mode defined their molecular weights, from which the composition and the maximum number of *O*-sulfate groups present in each fraction were inferred, as in heparin and heparan sulfate oligosaccharides (Yamada et al., 1993; Sugahara et al., 1994c). In the negative-ion mode FAB spectrum, alkali-metal-attached molecular ions of the type $[M - (x + 1)H + xNa]^-$ (M represents the fully protonated acid forms of oligosaccharides) were preferentially observed. The assignments of the molecular ion signals afforded by each of the analyzed fractions are listed in Table 1. In addition to the molecular ions, major signals were observed at *m/z* 1103 (Figure 2A), *m/z* 1585 (Figure 2B), *m/z* 1438 and 1540 (Figure 2C), and *m/z* 1540 and 1642 (Figure 2D) corresponding to losses of sodium sulphite from molecular ions.

The molecular ion signal clusters at *m/z* 1161, 1183, 1205, 1227, and 1249 afforded by fraction 2A corresponded respectively to $[M - H]^-$, $[M + Na - 2H]^-$, $[M + 2Na - 3H]^-$, $[M + 3Na - 4H]^-$, and $[M + 4Na - 5H]^-$ of a trisulfated pentasaccharide $DeoxyHex_1HexA_2HexNAc_2(OSO_3H)_3$ (Figure 2A). The fraction 6 sample yielded molecular ion signals at *m/z* 1686 and 1708 corresponding to $[M + 3Na - 4H]^-$ and $[M + 4Na - 5H]^-$, respectively, of a tetrasulfated heptasaccharide $DeoxyHex_1HexA_3HexNAc_3(OSO_3H)_4$ (Figure 2B). As shown in Figure 2C, fraction 8 afforded a molecular ion signal at *m/z* 1642, corresponding to $[M + 4Na - 5H]^-$ of a pentasulfated hexasaccharide $HexA_3HexNAc_3(OSO_3H)_5$. Fraction 9 afforded molecular ion signals at *m/z* 1744 and 1766 corresponding to $[M + 5Na - 6H]^-$ and $[M + 6Na - 7H]^-$, respectively, of a hexasulfated hexasaccharide $HexA_3HexNAc_3(OSO_3H)_6$ (Figure 2D). Fraction 7 did not afford molecular ion signals, presumably due to salts which could not be completely removed from the small sample.

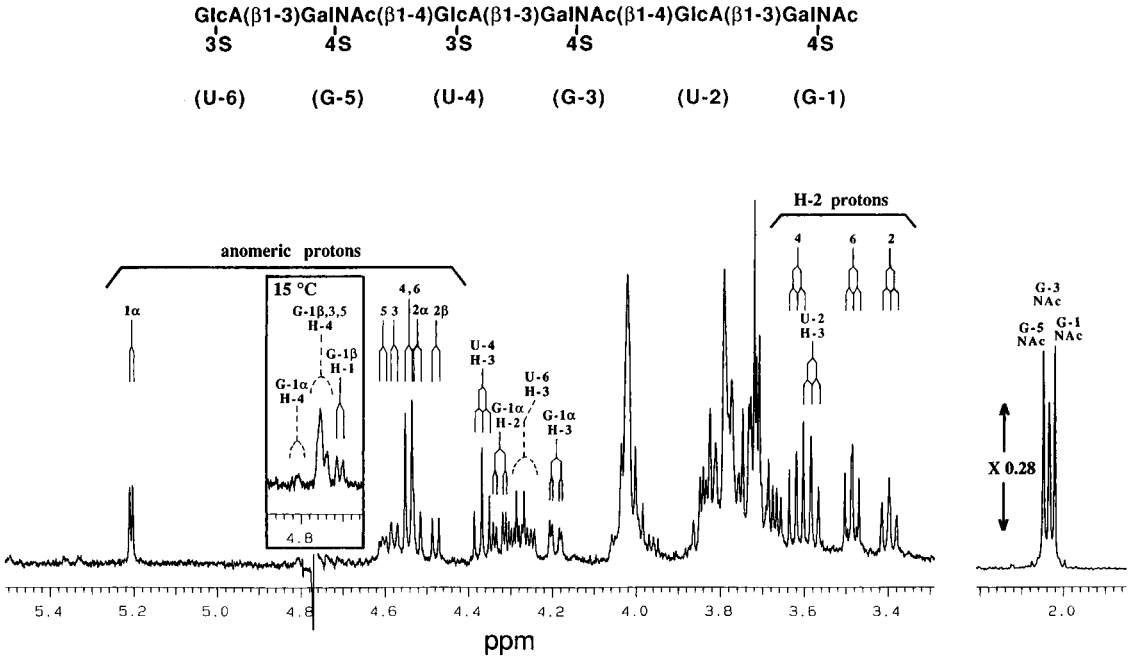


FIGURE 3: Structural-reporter group regions of the 500-MHz ^{1}H-NMR spectrum of fraction 8 recorded in ^2H_2O at 26 °C. The *numbers* and *letters* in the spectrum refer to the corresponding sugar residues in the structure. The inset is the spectrum recorded at 15 °C to suppress disturbance by the HOD line. U and G mean GlcA and GalNAc, respectively.

^{1}H-NMR Analysis. The structure of the major compound in each isolated oligosaccharide fraction was determined by 500-MHz ^{1}H-NMR analysis. The resonances were well resolved in the structural-reporter group region between 4.5 and 5.4 ppm and at around 2.0 ppm, being separated from other signals in the bulk region (3.6−4.4 ppm). The resonances between 4.5 and 5.4 ppm are characteristic of anomeric protons, whereas those at around 2.0 ppm are characteristic of the *N*-acetyl group protons of GalNAc. The other proton chemical shifts were assigned using 2D HOHAHA and COSY analyses (Yamada et al., 1992; Sugahara et al., 1994a).

Data from amino sugar/uronic acid determination and FAB-MS analysis indicated that the major compound in fraction 8 had the hexasaccharide structure HexA-GalNAc-HexA-GalNAc-HexA-GalNAc with five sulfate groups. Types of uronic acid and sulfation site were determined by ^{1}H-NMR analysis. The 1D spectrum of fraction 8 is shown as a representative (Figure 3). Most of the chemical shifts of protons were assigned using 2D HOHAHA and COSY (results not shown), starting with the H-1 resonances of GalNAc-**1**α, GalNAc-**1***β*, GlcA-**2**α, GlcA-**2***β*, GalNAc-**3**α*β*, GlcA-**4**α*β*, GalNAc-**5**α*β*, and GlcA-**6**α*β* at δ 5.208, 4.707, 4.524, 4.479, 4.578, 4.544, 4.605, and 4.544, respectively, as in the oligosaccharides isolated from various CS isoforms (Sugahara et al., 1994a,b). The H-1 resonance of GalNAc-**1***β* was recognizable when recorded at 15 °C to suppress the disturbance by HOD line (Figure 3, inset). The NMR data are summarized in Table 2 with those of fraction 9 and the reference compound R1. Uronic acid residues were identified as GlcA rather than IdoA, based upon chemical shifts of the anomeric proton signals (δ 4.479−4.544), since those of an αIdoA and a *β*GlcA in CS/dermatan sulfate oligosaccharides are observed at around δ 5.0−5.2 and 4.5−4.8 ppm, respectively (Goto & Ogawa, 1994; Sugahara et al., 1994a, 1995).

Sulfation positions of the saccharide residues of the compound in fraction 8 were determined by comparison with the proton signals of nonsulfated saccharide residues (Yamada et al., 1992; Sugahara et al., 1994a), based on the fact that *O*-sulfation causes downfield shifts of protons bound to the *O*-sulfated carbon atoms by approximately 0.4−0.8 ppm (Harris & Turvey, 1970; Yamada et al., 1992). The proton chemical shifts belonging to GlcA-**2**, which were readily distinguished from those of GlcA-**4** and GlcA-**6** based on the doubling of the anomeric proton signal of GlcA-**2** resulting from the anomerization effects by GalNAc-**1**, were very similar to those of the nonsulfated GlcA residues in the various CS oligosaccharides (Yamada et al., 1992; Sugahara et al., 1994a,b). In contrast, H-3 signals of GlcA-**4** and GlcA-**6** were found at δ 4.369 and 4.268, which were approximately 0.8 ppm lower than those (δ 3.45−3.61) of nonsulfated GlcA, indicating 3-*O*-sulfation of these residues. Therefore, we deduced that the compound in fraction 8 contained the following structure with three unidentified sulfate groups on the GalNAc residues:

GlcA(3S)(*β*1-3)GalNAc(S)(*β*1-4)GlcA(3S)
(*β*1-3)GalNAc(S)(*β*1-4)GlcA(*β*1-3)GalNAc(S)

where S represents unidentified *O*-sulfate.

We analyzed the sulfation sites of the GalNAc residues of this compound. All the H-4 resonances of GalNAc-**1**, -**3**, and -**5** of the compound in fraction 8 were observed at δ 4.74−4.81, which were approximately 0.8 ppm lower than those of the nonsulfated GalNAc residues in the authentic CS oligosaccharides (Yamada et al., 1992; Sugahara et al., 1994a), indicating 4-*O*-sulfation of these residues. Since the results from FAB-MS analysis indicated that this fraction contained a hexasaccharide with five sulfate groups, the C6 positions of the GalNAc residues were assumed to be nonsulfated, and H-6,6′ signals of GalNAc-**3** and -**5** were judged to be hidden in the bulk region between 3.6 and 4.4 ppm. On the basis of these results, the following structure is proposed for the compound in fraction 8:

Table 2: [1]H-Chemical Shifts of the Constituent Monosaccharides Belonging to the CS-K Hexasaccharides[a]

	R1[b] 3S 3S U–G–U–G[c] 4S 4S		fraction 8 3S 3S U–G–U–G–U–G 4S 4S 4S		fraction 9 3S 3S 3S U–G–U–G–U–G 4S 4S 4S	
	α	β	α	β	α	β
GalNAc-**1**						
H-1	5.221(3.5)	4.733(8.0)[d]	5.208(3.5)	4.707(8.0)[e]	5.219(4.0)	4.72(ND)[e]
H-2	4.337	4.034	4.327	4.02[e]	4.337	4.03[e]
H-3	4.178	4.06	4.192	3.98−4.04[e]	4.179	3.99−4.04[e]
H-4	4.843[d]	4.760[d]	4.808[e]	4.74−4.76[e]	4.84	4.73−4.76[e]
H-5	4.258	ND[f]	4.257	ND	4.258	3.79−3.86
H-6	3.79	ND	3.792	ND	3.79	3.70−3.79
H-6′	3.71	ND	3.708	ND	3.71	3.70−3.79
NAc	2.035		2.021		2.036	
GlcA-**2**						
H-1	4.635(8.0)	4.581(8.0)	4.524(8.0)	4.479(8.0)	4.638(8.0)	4.589(7.5)
H-2	3.665		3.398		3.673	
H-3	4.396		3.585		4.406	4.400
H-4	4.061		3.780		4.083	4.061
H-5	3.794		3.68		3.816	3.783
GalNAc-**3**						
H-1	4.620(7.0)[d]		4.578(7.5)		4.622(8.5)	
H-2	4.034		4.02		4.02	
H-3	4.04		3.98−4.04[e]		3.99−4.04[e]	
H-4	4.760[d]		4.74−4.76[e]		4.73−4.76[e]	
H-5	ND		ND		3.79−3.86	
H-6	ND		ND		3.70−3.79	
H-6′	ND		ND		3.70−3.79	
NAc	2.055		2.035		2. 051	
GlcA-**4**						
H-1	4.551(8.0)		4.544(8.0)		4.546(7.5)	
H-2	3.491		3.620		3.620	
H-3	4.269		4.369		4.370	
H-4	3.715		4.003		4.006	
H-5	ND		3.73		3.735	
GalNAc-**5**						
H-1	—[g]		4.605(8.0)		4.605(8.0)	
H-2	—		4.02		4.02	
H-3	—		3.98−4.04[e]		3.99−4.04[e]	
H-4	—		4.74−4.76[e]		4.73−4.76[e]	
H-5	—		ND		3.79−3.86	
H-6	—		ND		3.70−3.79	
H-6′	—		ND		3.70−3.79	
NAc	—		2.049		2.051	
GlcA-**6**						
H-1	—		4.544(8.0)		4.546(7.5)	
H-2	—		3.485		3.486	
H-3	—		4.268		4.288	
H-4	—		3.71		3.71	
H-5	—		ND		ND	

[a] [1]H-chemical shifts of the constituent monosaccharides belonging to the isolated CS-K hexasaccharides are shown together with those of the reference compound R1 (Sugahara et al., 1996b). Chemical shifts are given in parts per million downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly in reference to acetone (δ 2.225 ppm) in ^2H_2O at 26 °C. The estimated error for the values to two decimal places was only ±0.01 ppm because of partial overlap of signals. That for the values to three decimal places was ±0.002 ppm. Coupling constants $J_{1,2}$ (in hertz) of GalNAc and GlcA residues. [b] R2: The CS-K tetrasaccharide in fraction 4 in Sugahara et al., 1996b.[c] G, U, 3S, and 4S represent GalNAc, GlcA, 3-*O*-sulfate, and 4-*O*-sulfate, respectively. [d] Values determined at 60 °C. [e] Values determined at 15 °C. [f] ND: not determined. [g] —: Not occurring.

GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(β1-3)GalNAc(4S)

We judged that the major compound in fraction 9 had the hexasaccharide structure HexA-GalNAc-HexA-GalNAc-HexA-GalNAc, with six sulfate groups based on amino sugar/uronic acid determination and FAB-MS analysis. The NMR data of fraction 9 (Table 2) were very similar to those of fraction 8, except for the downfield shifts of H-1, H-2, H-3, H-4, and H-5 of GlcA-**2** by Δ0.11, 0.28, 0.82, 0.29, and 0.12 ppm, respectively, as compared with the compound in fraction 8, indicating that the compound in fraction 9 had the structure with an additional sulfate group on GlcA-**2** C3 of the structure in fraction 8. Thus, the following structure is proposed for the compound in fraction 9:

GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)GalNAc(4S)

The 1D spectrum of fraction 2A is shown in Figure 4, and the NMR data are summarized in Table 3 with those of fractions 6 and 7 and the reference compound R2, GlcA-(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(β1-3)GalNAc(4S). The spectrum of fraction 2A showed an additional H-1 signal at δ 5.400 in the anomeric region, which is characteristic of the anomeric proton signal of an α-L-fucopyranosyl residue (Vieira et al., 1991). The presence of an α-L-Fuc residue was supported by methyl proton signals at δ 1.27 and the

Table 3: ^{1}H-Chemical Shifts of the Constituent Monosaccharides Belonging to the CS-K Oligosaccharides[a]

	R2[b] 3S; U–G–U–G[c]; 4S 4S		fraction 2A 3S Fuc; U–G–U–G; 4S 4S		fraction 6 3S Fuc; U–G–U–G–U–G; 4S 4S 4S		fraction 7 3S 3S Fuc; U–G–U–G–U–G; 4S 4S 4S	
	α	β	α	β	α	β	α	β
GalNAc-**1**								
H-1	5.208(3.0)	4.722(8.5)[d]	5.210 (3.5)	4.714(8.5)[e]	5.210(3.5)	4.709(8.0)[f]	5.211(3.5)	4.71(ND)
H-2	4.328	4.028	4.316	4.02	4.326	4.02	4.32	4.02
H-3	4.185	4.06	4.183	4.02	4.190	ND	4.18	ND
H-4	4.808[d]	4.733[d]	4.812[e]	ND	4.803[e]	ND	4.82	ND
H-5	4.255	ND[g]	4.253	ND	4.25	ND	4.25	ND
H-6	3.788	ND	3.775	ND	3.79	ND	3.78	ND
H-6′	3.708	ND	3.715	ND	3.71	ND	3.70	ND
NAc	2.022		2.027		2.022		2.026	
GlcA-**2**								
H-1	4.522(8.5)	4.477(7.5)	4.513(8.0)	4.471(8.0)	4.527(8.5)	4.477(8.0)	4.51(ND)	4.473(7.5)
H-2	3.395		3.592		3.394		3.59	
H-3	3.585		3.692		3.582		3.69	
H-4	3.786		3.948		3.782		3.95	
H-5	3.669		ND		3.67		ND	
GalNAc-**3**								
H-1	4.576(7.0)		4.534(8.5)		4.572(7.5)		4.53(ND)	
H-2	4.04		4.056		4.03		4.04	
H-3	4.04		3.97		ND		3.94	
H-4	4.790[d]		4.764[e]		4.73−4.81[e]		4.73	
H-5	ND		ND		ND		ND	
H-6	ND		ND		ND		ND	
H-6′	ND		ND		ND		ND	
NAc	2.043		2.054		2.037		2.046[i]	
GlcA-**4**								
H-1	4.547(7.5)		4.529(7.5)		4.453(8.0)		4.53(ND)	
H-2	3.492		3.499		3.558		3.61	
H-3	4.274		4.281		3.69		4.363	
H-4	3.720		3.719		3.94		4.005	
H-5	ND		ND		ND		ND	
GalNAc-**5**								
H-1	—[h]		—		4.527(8.5)		4.60(ND)	
H-2	—		—		4.05		4.02	
H-3	—		—		3.96[e]		ND	
H-4	—		—		4.75[e]		4.71−4.81[d]	
H-5	—		—		ND		ND	
H-6	—		—		ND		ND	
H-6′	—		—		ND		ND	
NAc	—		—		2.056		2.050[i]	
GlcA-**6**								
H-1	—		—		4.527(8.5)		4.54(ND)	
H-2	—		—		3.498		3.485	
H-3	—		—		4.283		4.28	
H-4	—		—		3.71		3.707	
H-5	—		—		ND		ND	
Fuc-**3′** (Fuc-**5′**[j])								
H-1	—		5.400(4.0)		5.395(4.0)		5.400(4.0)	
H-2	—		3.750		3.738		3.74	
H-3	—		3.953		3.952		3.96	
H-4	—		4.026		4.03		4.03	
H-5	—		4.80[e]		4.80[e]		4.78[d]	
CH3	—		1.27		1.282[e]		1.285	

[a] ^{1}H-chemical shifts of the constituent monosaccharides belonging to the CS-K oligosaccharides are shown together with those of the reference compound R2 (Sugahara et al., 1996b). For details, see the legend to Table 2. [b] R1: The CS-K tetrasaccharide in fraction 2 in Sugahara et al., 1996b. [c] G, U, 3S, 4S, and Fuc represent GalNAc, GlcA, 3-*O*-sulfate, 4-*O*-sulfate, and fucose, respectively. [d] Values determined at 60 °C. [e] Values determined at 40 °C. [f] Values determined at 15 °C. [g] ND: not determined. [h] —: Not occurring. [i] Assignments may be interchanged. [j] Fuc-**5′**: fraction 6 contains Fuc-**5′** instead of Fuc-**3′**.

molecular composition afforded by FAB-MS analysis described above. The H-2 to H-4 resonances of the Fuc residue were assigned by connectivities of these signals from the H-1 in the COSY spectrum (Figure 5). In the COSY spectrum recorded at 40 °C, we detected crosspeaks between methyl protons and the H-5 signal of the Fuc residue. This allowed localization of the H-5 resonance at δ 4.80. Compared with the chemical shifts of the protons belonging to the reference compound R2, GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(β1-3)GalNAc(4S), there were no significant differences except for the resonances of H-2, -3, and -4 of GlcA-**2**. This indicated that the compound in fraction 2A had the core trisulfated structure GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(β1-3)GalNAc(4S) with an additional Fuc residue bound to GlcA-**2**. Since the C4 position of GlcA-**2** was substituted by GalNAc-**3**, the Fuc residue (Fuc-**3′**) was assumed to be linked to C2 or C3 of GlcA-**2**. Wang *et al.* (1990) have compiled data on ^{1}H chemical shifts of the

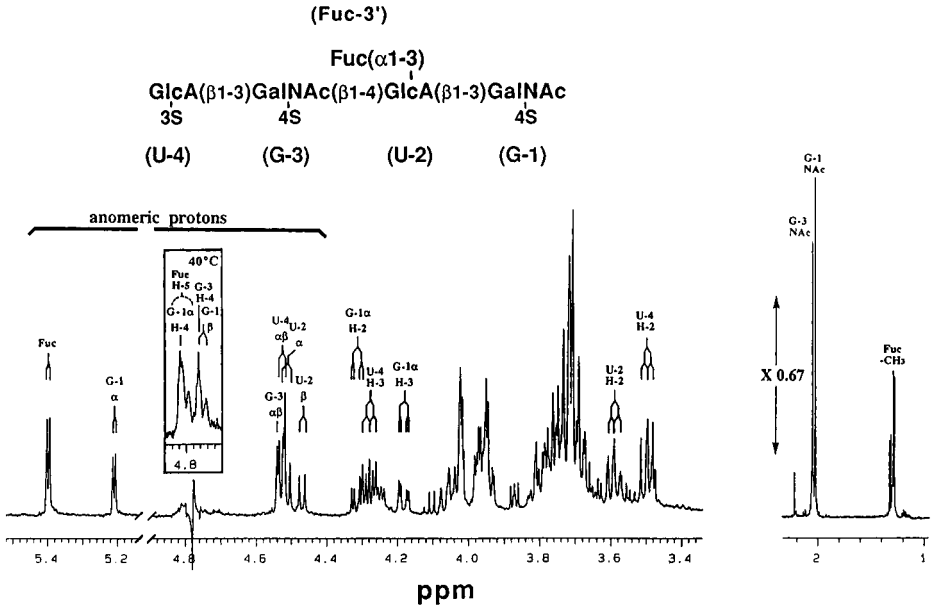


FIGURE 4: Structural-reporter group regions of the 500-MHz ^{1}H-NMR spectrum of fraction 2A recorded in ^2H_2O at 26 °C. The *numbers* and *letters* in the spectrum refer to the corresponding sugar residues in the structure. The inset is the spectrum recorded at 40 °C to suppress disturbance by the HOD line. U and G mean GlcA and GalNAc, respectively.

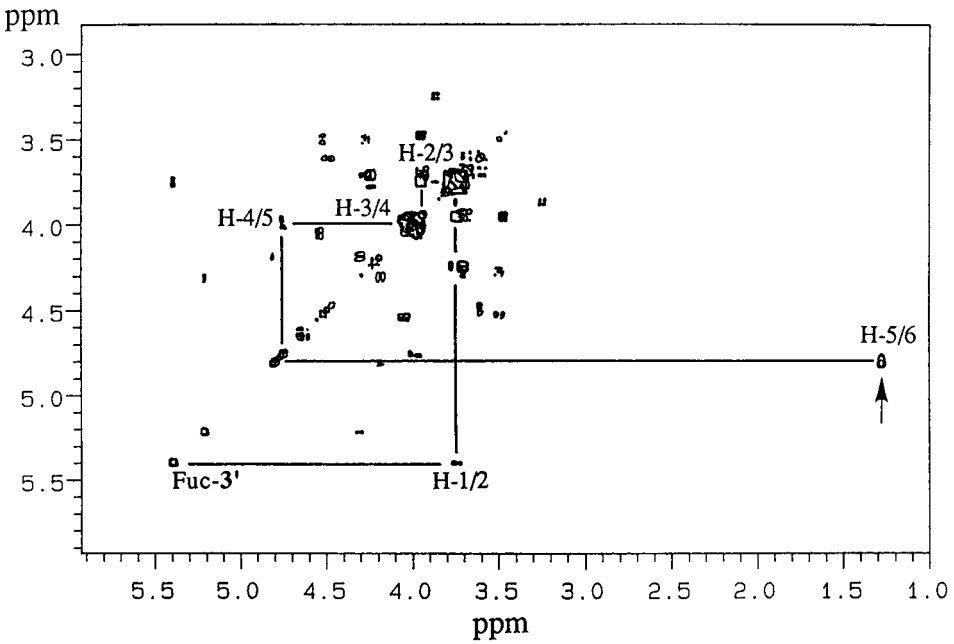


FIGURE 5: Two-dimensional COSY spectrum of fraction 2A recorded at 40 °C. Proton signals of the Fuc were assigned starting with the H-1 resonance at δ 5.400. In the figure the assignment pathway for the Fuc-**3′** residue is drawn (—). The crosspeaks between methyl protons and the H-5 signal of the Fuc residue are indicated by the *arrow*.

structural-reporter groups characteristic of the various types of α-L-fucosyl linkages. In particular, H-5 resonances have been suggested to distinguish between α-L-Fuc residues linked to C2 and C3 of the substituted sugar residue (Chaturvedi & Sharma, 1990). Fuc residues linked through α(1 → 3) and α(1 → 2) give H-5 signals at δ 4.83−4.86 and 4.20−4.35, respectively. Extrapolating this principle to the Fuc branch of the compound in fraction 2A, Fuc-**3′** was judged to be linked to the C3 position of GlcA-**2**. In addition, the ROESY spectrum of this fraction showed connectivities of resonances Fuc-**3′** H-1 to GlcA-**2** H-2 and H-3 (results not shown). The latter crosspeak was more intense than that of the former, indicating that the interproton distance between Fuc-**3′** H-1 and GlcA-**2** H-3 was shorter than that of Fuc-**3′** H-1 and GlcA-**2** H-2. Thus, we concluded that Fuc-**3′** was linked to the C3 position of GlcA-**2**. Therefore, the following structure is proposed for the compound in fraction 2A:

GlcA(3S)(β1-3)GalNAc(4S)(β1-4)(Fucα1-3)
GlcA(β1-3)GalNAc(4S)

Likewise, ^{1}H-NMR data of fractions 6 and 7 are summarized in Table 3. The proton chemical shifts of GalNAc-**1**, GlcA-**2**, and GalNAc-**3** of the compound in fraction 6 were very similar to those of the corresponding sugar residues of the reference compound R2. Those of GlcA-**4**, GalNAc-**5**, GlcA-**6**, and Fuc-**5′** were also analogous to those of GlcA-**2**, GalNAc-**3**, GlcA-**4**, and Fuc-**3′** of the compound in fraction 2A. These data were in good agreement with those

obtained by FAB-MS spectrometery and support the following structure for the compound in fraction 6:

GlcA(3S)(β1-3)GalNAc(4S)(β1-4)(Fucα1-3)
GlcA(β1-3)GalNAc(4S)(β1-4)GlcA(β1-3)GalNAc(4S)

The proton chemical shifts of GalNAc-**1**, GlcA-**2**, GalNAc-**3**, and Fuc-**3′** of the compound in fraction 7 were almost identical to those of the corresponding sugar residues of the compound in fraction 2A. Those of GlcA-**4**, GalNAc-**5** and GlcA-**6** were also in good agreement with those of the corresponding residues of the compound in fraction 9 shown in Table 2. Hence, the following structure is proposed for the compound in fraction 7:

GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)
GalNAc(4S)(β1-4)(Fucα1-3)GlcA(β1-3)GalNAc(4S)

Substrate Specificity Studies of Chondroitinases ABC, AC-II, and Chondro-4-sulfatase Using the Isolated Oligosaccharides. As we described (Sugahara et al., 1996b), chondroitinase ABC digests CS-K tetrasaccharides with GlcA(3S) at the internal position but destroys the disaccharide unit containing GlcA(3S) derived from the reducing side, resulting in only the disaccharide unit from the nonreducing side, whereas these tetrasaccharides remain totally resistant to chondroitinase AC-II. Therefore, we investigated whether the same held true for the hexasaccharides (fractions 7−9) with GlcA(3S) at the internal position(s). In addition, we attempted to clarify whether the oligosaccharides (fractions 2A, 6, and 7) with 3-*O*-fucosylated GlcA at the internal positions were resistant to chondroitinases since Vieira et al. (1991) reported that the presence of fucose branches obstructed the access of chondroitinases ABC and AC. The enzyme digests of fractions 2A and 6−9, which were obtained with chondroitinases ABC, AC-II, or chondro-4-sulfatase, were analyzed by HPLC on an amine-bound silica column.

Although fraction 2A was resistant to chondroitinase AC-II (Figure 6A), it was digested by chondroitinase ABC, yielding a major peak at the elution position between ΔHexA(α1-3)GalNAc(6S) (ΔDi-6S) and ΔHexA(α1-3)-GalNAc(4S) (ΔDi-4S) with a minor one at the position of a saturated disaccharide GlcA(3S)(β1-3)GalNAc(4S) between ΔHexA(2S)(α1-3)GalNAc(6S) (ΔDi-diS_D) and ΔHexA(α1-3)GalNAc(4S, 6S) (ΔDi-diS_E) when monitored by absorbance at 210 nm (Figure 6B). Only the major peak showed strong absorbance at 232 nm (Figure 6C). The data are summarized in Table 4 with those obtained from the other fractions. Thus, the major and minor peaks detected by absorbance at 210 nm were judged to be an unsaturated trisaccharide derived from the reducing side and a saturated disaccharide derived from the nonreducing side of the parent pentasaccharide, respectively, like the tetrasaccharides derived from CS-D (Sugahara et al., 1996a) and CS-K (Sugahara et al., 1996b). The results indicated that chondroitinase ABC, but not AC-II, indeed cleaved a hexosaminidic bond linked to a 3-*O*-fucosylated GlcA residue, in contrast to the previous report (Vieira et al., 1991). Fraction 2A was resistant to chondro-4-sulfatase even under harsh incubation conditions (Sugahara et al., 1994a) although it consisted of two GalNAc(4S) residues (Table 4), indicating

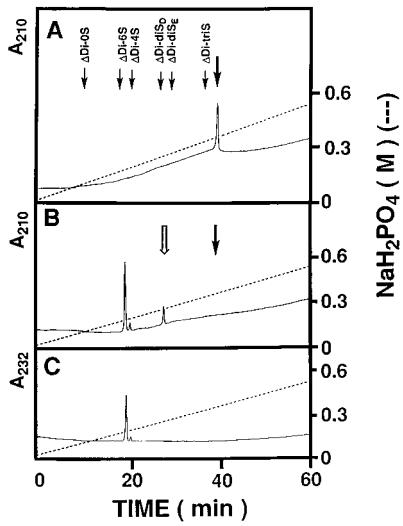


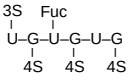
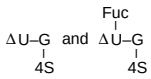
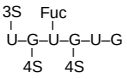
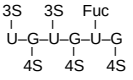
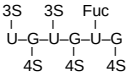
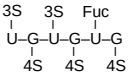
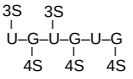
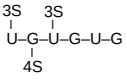
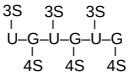
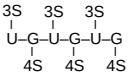
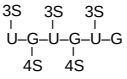
FIGURE 6: HPLC analysis of the chondroitinase AC-II and ABC digests of fractions 2A. The pentasaccharide fraction 2A (2 nmol) was digested with chondroitinase AC-II or ABC and analyzed by HPLC on an amine-bound silica column (panel A, chondroitinase AC-II digest; panels B and C, chondroitinase ABC digest). Fractions were monitored by absorbance at 210 (panels A and B) and 232 nm (panel C). The elution positions of CS-derived authentic unsaturated disaccharides are indicated in the top panel. The *bold arrows* indicate the elution positions of the corresponding intact oligosaccharides. The *open arrow* indicates the elution position of the authentic disaccharide GlcA(3S)(β1-3)GalNAc(4S). For disaccharide abbreviations, see the title page.

that 3-*O*-fucosylation of a GlcA residue rendered the adjacent GalNAc(4S) residues resistant to chondro-4-sulfatase digestion.

When digested with chondroitinase AC-II, fraction 6 yielded a major peak at the elution position of ΔDi-4S and a minor one at the position of GlcA(3S)(β1-3)GalNAc-(4S)(β1-4)(Fucα1-3)GlcA(β1-3)GalNAc(4S) (fraction 2A) (Table 4) when monitored by absorbance at 210 nm. Only the major peak showed absorbance at 232 nm (data not shown), confirming that the resulting pentasaccharide with 3-*O*-fucosylated GlcA at the internal position was resistant to chondroitinase AC-II. As predicted from the susceptibility of fraction 2A to chondroitinase ABC, fraction 6 was sensitive to chondroitinase ABC digestion, yielding two major peaks at the elution position of ΔDi-4S and at that between ΔHexA(α1-3)GalNAc(6S) (ΔDi-6S) and ΔHexA-(α1-3)GalNAc(4S) (ΔDi-4S), as well as a minor one at the position of authentic GlcA(3S)(β1-3)GalNAc(4S) (Table 4) when monitored by absorbance at 210 nm. Only the major peaks, ΔDi-4S and putative Fuc(α1-3)ΔHexA(α1-3)GalNAc-(4S), showed absorbance at 232 nm (data not shown). Fraction 6 was only partially sensitive to chondro-4-sulfatase action, releasing the sulfate group on the reducing GalNAc alone (Table 4).

Fraction 7 was totally resistant to chondroitinase AC-II, as predicted from the observations that chondroitinase AC-II did not cleave a hexosaminic bond linked to a GlcA(3S) residue (Sugahara et al., 1996b) nor a 3-*O*-fucosylated GlcA residue (see above). Chondroitinase ABC digestion of fraction 7 gave rise to a major peak at the elution position between ΔHexA(α1-3)GalNAc(6S) (ΔDi-6S) and ΔHexA-(α1-3)GalNAc(4S) (ΔDi-4S) and a minor one at the position of authentic GlcA(3S)(β1-3)GalNAc(4S) when monitored by absorbance at 210 nm (Table 4), only the major peak showing absorbance at 232 nm (data not shown). No peak cor-

Table 4: Enzymatic Action of Chondroitinases ABC or AC-II or Chondro-4-sulfatase for the CS-K Oligosaccharides[a]

fractions	structures proposed based on NMR analysis[b]	enzymes	reaction products: saturated oligosaccharides	reaction products: unsaturated oligosaccharides
2A		AC-II		—[c]
		ABC		
		chondro-4-sulfatase		
6		AC-II		
		ABC		
		chondro-4-sulfatase		
7		AC-II		—
		ABC		
		chondro-4-sulfatase		
8		AC-II		
		ABC		
		chondro-4-sulfatase		
9		AC-II		—
		ABC		ND[d]
		chondro-4-sulfatase		

[a] Each oligosaccharide was digested with chondroitinases ABC or AC-II or chondro-4-sulfatase and the digest was analyzed by HPLC as described under Experimental Procedures. Saturated and unsaturated oligosaccharides were monitored by absorbance at 210 and 232 nm, respectively. [b] G, U, 3S, 4S, and Fuc stand for GalNAc, GlcA, 3-*O*-sulfate, 4-*O*-sulfate and Fucoseα(1-3), respectively. [c] —, not occurring because the oligosaccharide was resistant to chondroitinase AC-II. [d] ND, not detected.

responded to ΔHexA(3S)(α1-3)GalNAc derived from the internal disaccharide unit. These results are consistent with the findings that chondroitinase ABC cleaved a hexosaminidic bond linked to a 3-*O*-fucosylated GlcA residue (see above) and specifically destroyed the disaccharide units containing an internal GlcA(3S) residue (Sugahara et al., 1996b). Fraction 7 was totally resistant to chondro-4-sulfatase digestion (Table 4) as predicted from the resistance of fractions 2A and 6 to the enzyme.

Chondroitinase AC-II digestion of fraction 8 yielded a major peak at the elution position of ΔDi-4S and a minor one at the position of authentic GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)GalNAc(4S) [fraction 4 in Sugahara et al. (1996b)]. When monitored by absorbance at 210 nm (Table 4), only the major peak absorbed at 232 nm (data not shown). In contrast, fraction 9 was totally resistant to chondroitinase AC-II, being consistent with the finding that chondroitinase AC-II cannot cleave a hexosaminic bond linked to a GlcA(3S) residue (Sugahara et al., 1996b). Chondroitinase ABC digestion of fraction 8 yielded a major peak at the position of ΔDi-4S and a minor peak at the position of authentic GlcA(3S)(β1-3)GalNAc(4S) when monitored by absorbance at 210 nm, while only a single peak was detected at the position of authentic GlcA(3S)(β1-3)GalNAc(4S) in the digest of fraction 9 (Table 4). These results are in agreement with the notion that disaccharide

units containing GlcA(3S) at the internal positions are destroyed by the action of chondroitinase ABC (Sugahara et al., 1996b). Two sulfate groups of fraction 8 and one sulfate group of fraction 9 were lost upon chondro-4-sulfatase digestion as judged by HPLC (Table 4), being consistent with the finding that 3-*O*-sulfation of a GlcA residue renders the adjacent GalNAc(4S) residue resistant to chondro-4-sulfatase (Sugahara et al., 1996b).

DISCUSSION

The penta-, hexa- and heptasaccharides isolated in this study contained 3-*O*-sulfated GlcA, some containing additional 3-*O*-fucosylated GlcA at the internal positions, and they are all hitherto unreported, although GlcA(3S)-containing tetra- and pentasaccharides were isolated from king crab cartilage CS-K (Sugahara et al., 1996b). To our knowledge, this is the first description of CS with nonsulfated fucose branches. Vieira et al. (1991) have reported that the connective tissues of a sea cucumber contain large amounts of CS with disaccharide branches composed of sulfated fucose on GlcA C3 as well as ester sulfates at the C3 positions of some nonfucosylated GlcA residues. A preponderance of a disaccharide unit Fuc(3,4diS)(α1-2)Fuc(4S) was proposed, although extreme heterogeneity was suggested for the positions of the glycosidic linkage and the sites of sulfation in the fucose branches, according to methylation analysis and NMR spectroscopy (Vieira et al., 1991). Considering the structural similarity of sea cucumber CS and king crab cartilage CS-K, both of which contain 3-*O*-sulfated GlcA and 3-*O*-fucosylated GlcA, it remains to be determined whether or not larger CS-K oligosaccharide fractions contain branched disaccharide units of sulfated fucose residues. Another fucose-rich GAG has been identified in squid ink, but the fucose residues were found inside the nonsulfated polysaccharide chain (Takaya et al., 1994). A linear trisaccharide repeating unit -6GalNAc(α1-3)GlcA(β1-3)Fucα1- reported originally (Takaya et al., 1994) was recently revised to be -3GlcA(β1-4)(GalNAcα1-3)Fucα1- based on ^{1}H-NMR spectroscopy and MS (Takaya et al., 1996). Some other CS containing branches have been identified in invertebrates and microorganisms. CS containing small amounts of glucose branches substituted at GalNAc C6 has been found in squid cartilage (Habuchi et al., 1977). In addition, nonsulfated chondroitin containing β-fructose linked to GlcA C3 has been isolated from the capsular polysaccharide of a uropathogenic *Escherichia coli* (Rodriguez et al., 1988). Although the biological relevance of the above described unique marine GAGs containing fucose or branches is presently unclear, it has been proposed that the branches protect the polysaccharides from enzymatic degradation (Vieira et al., 1991).

Although squid CS is degraded by prolonged incubation with chondroitinase ABC (Habuchi et al., 1977), *E. coli.* chondroitin containing β-fructose is totally resistant to chondroitinase ABC digestion (Rodriguez et al., 1988). In this regard, Vieira et al. (1991) described that the presence of fucose branches and 3-*O*-sulfation of GlcA obstructs the access of the chondroitinases to the CS core of the sea cucumber polysaccharides. However, the GalNAc linkage bound to 3-*O*-fucosylated GlcA in the oligosaccharides in fractions 2A, 6, and 7 was degraded by chondroitinase ABC, whereas it was resistant to chondroitinase AC-II. Although the reason for the apparent discrepancy in the susceptibility to chondroitinase ABC is unknown, the resistance of the fucose-branched CS from the sea cucumber to chondroitinase ABC may be due to the sulfation of fucose or additional fucosylation of the fucose linked to GlcA, or both.

Characterization of chondro-4-sulfatase using the novel oligosaccharides revealed a unique substrate specificity. This enzyme preferentially works on a GalNAc(4S) residue at the reducing end but also affects internal GalNAc(4S) residues under harsh conditions, unless the GalNAc(4S) residues bear another sulfate group on the C6 position (Seno et al., 1974b; Sugahara et al., 1994a; Sugahara & Kojima, 1996). In addition, we recently demonstrated that the enzyme removed a sulfate group only from the reducing GalNAc(4S) of tetrasaccharides with an internal GlcA(3S) residue such as GlcA(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)GalNAc(4S) (Sugahara et al., 1996b). The 3-*O*-sulfation of a GlcA residue seems to render the GalNAc(4S) residue linked to the GlcA(3S) residue resistant to chondro-4-sulfatase. This property of the enzyme was confirmed using hexasaccharides in fractions 8 and 9 in this study (see Table 4). In contrast, the enzyme did not remove any sulfate group from the fucosylated oligosaccharides such as GlcA(3S)(β1-3)GalNAc(4S)(β1-4)(Fucα1-3)GlcA(β1-3)GalNAc(4S) in fraction 2A and GlcA(3S)(β1-3)GalNAc(4S)(β1-4)GlcA(3S)(β1-3)GalNAc(4S)(β1-4)(Fucα1-3)GlcA(β1-3)GalNAc(4S) in fraction 7 (see Table 4). The enzyme removed a sulfate group only from the reducing GalNAc(4S) of the heptasaccharide GlcA(3S)(β1-3)GalNAc(4S)(β1-4)(Fucα1-3)GlcA(β1-3)GalNAc(4S)(β1-4)GlcA(β1-3)GalNAc(4S) in fraction 6 (see Table 4). These results indicate that the 3-*O*-fucosylation of a GlcA residue renders both adjacent GalNAc(4S) residues resistant to chondro-4-sulfatase.

There were 3-*O*-fucosylated GlcA residues in the isolated oligosaccharides only in the internal position, in contrast to the GlcA(3S) residues, which were found on the nonreducing termini. This suggests that testicular hyaluronidase cleaves the *N*-acetylgalactosaminidic linkage in GalNAc(4S)(β1-4)-GlcA(3S), but not in GalNAc(4S)(β1-4)(Fucα1-3)GlcA (Seno & Murakami, 1982). Taken together with all above results, chondroitinases ABC, AC-II, and testicular hyaluronidase exhibit distinct substrate specificities, although they all cleave the *N*-acetylgalactosaminidic linkage in the GalNAc(β1-4)GlcA structure.

GlcA(3S) has been demonstrated in a glycolipid isolated from human peripheral nerves using the mouse monoclonal antibody HNK-1 raised against human natural killer cells (Chou et al., 1986; Ariga et al., 1987). The GlcA(3S)-containing carbohydrates are temporally and spatially regulated molecules present in the nervous system during its development (Chou et al., 1991) and implicated in cell−cell adhesion and recognition of neurons and astrocytes (Künemund et al., 1988). Similar epitopic structures occur on CS proteoglycans of mammalian tissues (Margolis et al., 1987). It is of interest to investigate whether fucose-containing CS occurs on proteoglycans in the nervous system. The oligosaccharides isolated in this study are potentially useful tools for elucidating the occurrence and function of such putative polysaccharide structures.

ACKNOWLEDGMENT

We thank Yukiko Iwamoto for excellent technical assistance.

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BI962740J