Isolation and Structural Determination of Novel Sulfated Hexasaccharides from Squid Cartilage Chondroitin Sulfate E That Exhibits Neuroregulatory Activities†

Akiko Kinoshita,‡ Shuhei Yamada,‡ Stuart M. Haslam,§ Howard R. Morris,§ Anne Dell,§ and Kazuyuki Sugahara*,‡

Department of Biochemistry, Kobe Pharmaceutical University, Higashinada-ku, Kobe 658-8558, Japan, and Department of Biochemistry, Imperial College, London SW7 2AZ, United Kingdom

Received July 12, 2001; Revised Manuscript Received August 11, 2001

ABSTRACT: Squid cartilage chondroitin sulfate E (CS-E) exhibits various biological activities, including anticoagulant activities, lymphoid regulatory activities, and neuroregulatory activities [Ueoka, C., Kaneda, N., Okazaki, I., Nadanaka, S., Muramatsu, T., and Sugahara, K. (2000) *J. Biol. Chem. 275*, 37407−37413]. These activities are expressed through molecular interactions with specific proteins, including heparin cofactor II, selectins, CD44, chemokines, and the heparin-binding growth factor midkine. Hence, the sugar sequence information is essential for a better understanding of the CS-E functions. Previously, several novel tetrasaccharides containing the unreported 3-*O*-sulfated glucuronic acid (GlcA) were isolated after digestion of squid cartilage CS-E with testicular hyaluronidase. In this study, hexasaccharides were isolated to obtain more detailed sequence information, especially around the GlcA(3-*O*-sulfate) residue, and were characterized by fast atom bombardment mass spectrometry and 500 or 600 MHz ^{1}H NMR spectroscopy. The findings demonstrate one tetrasulfated and five pentasulfated hexasaccharide sequences, five of them being novel. They were composed of three disaccharide building units of either A [GlcA-(β1−3)GalNAc(4-*O*-sulfate)], E [GlcA(β1−3)GalNAc(4,6-*O*-disulfate)], K [GlcA(3-*O*-sulfate)(β1−3)-GalNAc(4-*O*-sulfate)], L [GlcA(3-*O*-sulfate)(β1−3)GalNAc(6-*O*-sulfate)], or M [GlcA(3-*O*-sulfate)(β1−3)GalNAc(4,6-*O*-disulfate)], forming E-A-A, M-A-A, K-L-A, E-E-A, K-K-A, and A-M-A hexasaccharide sequences. The K-L tetrasaccharide sequence is to date unreported. The isolated sequences appear to indicate the occurrence of an unreported GlcA 3-*O*-sulfotransferase specific for chondroitin sulfate. The obtained sequence information will be useful for investigating the structure−function relationship and biosynthesis of CS-E.

Chondroitin sulfate proteoglycans (CS-PGs)[1] are widely found in the extracellular matrixes of various tissues, at the surface of many cell types, and in intracellular secretory granules. They play several key roles in the normal physiology of animal tissues, regulating cell migration, cell recognition and tissue morphogenesis (for reviews, see refs *1* and *2*). Immunological studies have shown developmentally regulated expression of the epitopes in the rodent fetus and in the rat central nervous system (for reviews, see refs *3*−*5*). Some CS epitopes are distributed differentially in distinct tissues and in functionally distinct domains within these tissues (*6*), suggesting that CS chains differing in the degree and profile of sulfation perform distinct functions in development.

Oversulfated CS variants are interesting, since they contain rare structural building units and, hence, may form domain structures interacting with specific protein ligands. These oversulfated CS structures were originally discovered in marine organisms. They include shark cartilage CS-D, king crab cartilage CS-K, hagfish notochord CS-H, and squid cartilage CS-E, which are characterized by the distinct disulfated disaccharide units, GlcA(2S)(β1−3)GalNAc(6S) (D unit; *7*), GlcA(3S)(β1−3)GalNAc(4S) (K unit; *8*), IdoA-(α1−3)GalNAc(4S,6S) (H or iE unit; *9*), and GlcA(β1−3)-GalNAc(4S,6S) (E unit; *10*), where GlcA, IdoA, 2S, 3S, 4S, and 6S represent D-glucuronic acid, L-iduronic acid, and 2-*O*-, 3-*O*-, 4-*O*-, and 6-*O*-sulfate, respectively (*11*). However, these oversulfated units are not limited to marine organisms and have been identified in various tissues and cells from higher vertebrates. D and E units have been found in human rib cartilage in small proportions (*12*), and also in the brains of embryonic chicks (*13*), embryonic rats (*14*), and adult bovine (*15*). Another disaccharide unit, IdoA-GalNAc(4S,-6S), has also been identified in dermatan sulfate (DS) from mammalian liver (*16*) and bovine kidney (*17*). It has been

† The work performed in Kobe was supported in part by the Science Research Promotion Fund from the Japan Private School Promotion Foundation, and Grants-in-Aid for Scientific Research (09558082) and Scientific Research on Priority Areas (10178102) from the Ministry of Education, Science, Sports and Culture of Japan.

* To whom correspondence should be addressed. Telephone: 81-78-441-7570. Fax: 81-78-441-7569. E-mail: k-sugar@kobepharma-u.ac.jp.

‡ Kobe Pharmaceutical University.

§ Imperial College.

[1] Abbreviations: CE, capillary electrophoresis; COSY, correlation spectroscopy; CS, chondroitin sulfate; 1D or 2D, one- or two-dimensional, respectively; FAB-MS, fast atom bombardment mass spectrometry; 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-enepyranosyluronic acid; HexA, hexuronic acid; HexNAc, *N*-acetylhexosamine; HPLC, high-performance liquid chromatography; IdoA, L-iduronic acid. G, U, 2S, 3S, 4S, and 6S represent GalNAc, GlcA, 2-*O*-sulfate, 3-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate, respectively.

10.1021/bi015577n CCC: $20.00 © 2001 American Chemical Society
Published on Web 09/29/2001

proposed to designate this type of DS as DS-E (*18*), since the disaccharide contains GalNAc(4S,6S) linked to IdoA (see above for the iE unit).

CS-E is of particular interest, since it is highly biologically relevant. It exhibits differentiation-associated expression in embryonic chick chondrocytes (*19*), and haemopoietic cells (for a review, see ref *20*) such as macrophages (*21*−*23*), mast cell subsets derived from intestinal mucosa and bone marrow (*24*; for a review, see ref *25*), and related cells (*26*, *27*), and is likely to be associated with specific functions of these cells (see the Introduction in ref *18*). The CS or DS chains with E units on the surfaces of endothelial cells (*28*), macrophages (*21*, *22*), and monocytes (*27*) have been implicated in anticoagulation and in the inhibition of activated properdin in the alternative complement pathway (*29*). Although the PG containing the CS-E or DS-E chain with a unique sequence on the endothelial cell surface has been identified as β-thrombomodulin (*30*), the cell surface PGs containing CS-E/DS-E chains on macrophages and monocytes are unknown. The CS-E or DS-E chains of these putative PG molecules have not yet been characterized.

Squid cartilage CS-E, which mimics heparin under certain circumstances, shows an in vitro anticoagulant property (*31*), primarily by accelerating the heparin cofactor II interaction with thrombin (factor IIa) (*32*). It also mimics the action of the cell surface CS-E or DS-E on the murine bone marrow-derived mast cells to inhibit the function of activated properdin in the alternative complement pathway (*29*). However, the functional domain sequences are unknown for squid cartilage CS-E or the cell surface CS chains mentioned above. Recently, we demonstrated the neurite outgrowth promoting activity of squid cartilage CS-E toward embryonic day 18 rat hippocampal neurons (*33*). Although shark cartilage CS-D also displayed a neurite length promoting effect, this effect was clearly supervened by the CS-E, which displayed a tremendous neurite length promoting effect as shown using quantitative morphometry of neurite lengths (*34*). Interestingly, the activity of the CS-E was not neutralized by mAb 473HD, which blocked the effect of CS-D that contains the functional domain, DSD-1 epitope (*33*−*36*), suggesting that a novel, previously unknown structural domain with neurite outgrowth promoting properties is contained in squid cartilage CS-E. We further showed that CS-E inhibited the neuronal cell adhesion mediated by the neuroregulatory heparin-binding growth factor midkine, through direct molecular interactions with this factor (*14*).

In view of the biological activities of squid cartilage CS-E, we have been conducting systematic structural studies of CS-E as well as DS-E (CS-H), CS-D, and CS−K (reviewed in ref *11*). Previously, several novel tetrasaccharides were isolated after exhaustive digestion of squid cartilage CS-E with testicular hyaluronidase, and we demonstrated the unreported 3-*O*-sulfated GlcA component in the tetrasaccharide sequences (*18*). In this study, to obtain further sequence information adjacent to the unique 3-*O*-sulfated GlcA residue, hexasaccharides were isolated from squid cartilage CS-E and structurally characterized. Preliminary findings were reported in abstract form (*37*).

EXPERIMENTAL PROCEDURES

Materials. Squid cartilage CS-E, six unsaturated standard CS disaccharides, chondroitinases ABC (EC 4.2.2.4) and AC-II (EC 4.2.2.5), chondro-4-sulfatase (EC 3.1.6.9), and chondro-6-sulfatase (EC 3.1.6.10) were purchased from Seikagaku Corp. (Tokyo, Japan). Sheep testicular hyaluronidase (EC 3.2.1.35) was obtained from Sigma. Sephadex G-25 (fine) was from Amersham Pharmacia Biotech (Tokyo, Japan).

Preparation of Oligosaccharide Fractions. A commercial squid cartilage CS-E (100 mg) was exhaustively digested with sheep testicular hyaluronidase, and the digest was fractionated into fractions I−IX by gel filtration on a Bio-Gel P-10 column as described previously (*18*). In this study, fraction VIII, which contains hexasaccharides, was subfractionated by HPLC on an amine-bound silica PA13 column (6.0 mm × 250 mm) (YMC Co., Kyoto, Japan) using a linear NaH_2PO_4 gradient from 16 to 798 mM over a 90 min period at a flow rate of 1.7 mL/min at room temperature. The eluates were monitored by absorbance at 210 nm. Major peaks were purified by rechromatography under the same conditions as the first step and desalted through a column (1.5 cm × 46 cm) of Sephadex G-25 (fine) using distilled water as the eluent. Each peak was quantified by the carbazole method, using GlcA as a standard (*38*).

Capillary Electrophoresis (CE) Analysis. The homogeneity of each purified oligosaccharide fraction was judged by CE as well as by HPLC. The electrophoresis was performed as described previously (*39*), being monitored by absorption at 185 nm.

Fast Atom Bombardment Mass Spectrometry (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 cesium ion gun operated at 20−25 kV as described previously (*18*). Data were obtained and processed using the VG Analytical Opus software. Monothioglycerol was used as the matrix.

^{1}H NMR Spectroscopy (600 or 500 MHz). The purified oligosaccharides for NMR analysis were repeatedly exchanged in ^2H_2O with intermittent lyophilization. The 600 MHz ^{1}H NMR spectra were recorded on a Bruker AM-600 (Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan) at a probe temperature of 26 or 60 °C as reported previously (*40*). The 500 MHz ^{1}H NMR spectra were recorded on a Varian-500 instrument at a probe temperature of 26 or 60 °C as reported previously (*41*). When the available amount of oligosaccharide was small, one- and two-dimensional ^{1}H NMR analyses were carried out on a Varian-500 instrument using a Nano-NMR probe containing 40 μL of the sample solution. The Nano-NMR probe spins samples rapidly at the magic angle to remove the magnetic-susceptibility contributions to the ^{1}H NMR line widths (*42*). The spin rate was typically ∼2 kHz. Proton one-dimensional (1D) NMR spectra were obtained in ∼640 scans using presaturation of the HOD peak. HOHAHA two-dimensional (2D) spectra (*43*) were obtained using a Malcom Levitt-17 spin lock with a duration of 80 ms, a 1.0 s presaturation, 168−320 scans per t_1 data point, and 512 complex data points in t_1; the total measuring time was 15−30 h. COSY spectra (*44*) were obtained using 512 complex t_1 data points having 160−256 scans; measurement times were 7−11 h. HOHAHA spectra were obtained in a phase-sensitive mode using hypercomplex sampling in t_1. All 2D spectra included presaturation of the HOD resonance. Line broadening (0.3 Hz) was applied to 1D spectra, while HOHAHA spectra were

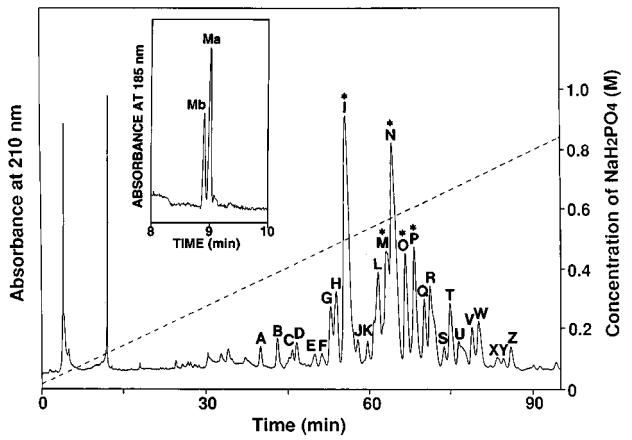

FIGURE 1: Subfractionation of the hexasaccharide fraction by anion-exchange HPLC. The hexasaccharide fraction obtained by gel filtration on a Bio-Gel P-10 column (*18*) was chromatographed on an amine-bound silica PA13 column using a linear salt gradient as described in Experimental Procedures. The linear gradient is indicated by the dashed line. Fractions marked with asterisks were subjected to structural analysis. The inset shows the separation of fraction **M** into subfractions **Ma** and **Mb** by CE.

weighted with Gaussian functions and COSY spectra multiplied by a phase-shifted sine bell function. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone (δ 2.225) in ^2H_2O (*45*).

Digestion of the Isolated Oligosaccharides with Chondroitinases and Sulfatases. Each isolated oligosaccharide (3.0 nmol as GlcA) was digested using 10−20 mIU of chondroitinase ABC or AC-II, chondro-4-sulfatase, or chondro-6-sulfatase as described previously (*41*). Reactions were terminated by boiling for 1 min, and the reaction mixture was analyzed by HPLC as reported previously (*17*, *46*). Eluates were monitored by absorption at both 210 and 232 nm, caused by the *N*-acetyl group of the GalNAc residue and the 4,5-unsaturated bond of the ΔHexA residue, respectively.

RESULTS

Isolation of the Oligosaccharides. A commercial preparation of squid cartilage CS-E was exhaustively digested with sheep testicular hyaluronidase as reported previously (*18*). The digest was size-fractionated by gel filtration on a Bio-Gel P-10 column. A number of peaks were observed when monitored by absorbance at 210 nm, caused primarily by carbonyl groups, and divided into nine fractions (I−IX). The smallest size fraction (IX) contained tetrasaccharides whose structures were determined previously (*18*). In this study, fraction VIII was investigated. It represented approximately 8.1% of the resultant oligosaccharides on the basis of the absorbance at 210 nm and was judged to contain hexasaccharides on the basis of the well-defined mechanism of action of hyaluronidase (*47*, *48*). It was subfractionated into 26 fractions named arbitrarily **A**−**Z** as indicated in Figure 1 by HPLC on an amine-bound silica column. Five major fractions (**I** and **M**−**P**) marked by asterisks were further purified by rechromatography. These individual fractions, except for fraction **M**, gave essentially a single major peak when examined by CE (Table 1). The amounts of the purified oligosaccharides in each fraction from 100 mg of CS-E are summarized in Table 1. The structures of the compounds in fractions **I** and **N**−**P** were analyzed as described below.

Table 1: Quantity and Purity of Each Hexasaccharide Fraction[a]

fraction	yield[b] (nmol)	HPLC (%)	CE (%)
I	800	89	85
M	310	100	62[c]
N	500	100	99
O	220	100	100
P	310	100	100

[a] Quantity is calculated on the basis of the absorption at 210 nm caused by the *N*-acetyl group of the GalNAc residues. Purity of the isolated hexasaccharide fractions was examined by HPLC and CE. [b] Yield is given in nmol obtained from 100 mg CS-E. [c] Fraction **M** was divided into subfractions **Ma** (191 nmol) and **Mb** (119 nmol) in a molar ratio of 62:38.

Fraction **M** was resolved into two subfractions, **Ma** and **Mb**, by CE (Figure 1 inset), and the major component in fraction **Ma** and the minor component in fraction **Mb** accounted for 62 and 38%, respectively, of the components in fraction **M**. However, it was not possible to fractionate fraction **M** preparatively into its subcomponents by CE. Therefore, fraction **M** was divided into four equal parts, **M-1**−**4**, by volume in the order of the elution in the rechromatography on HPLC. When examined by CE (data not shown), each subfraction (**M-1**, **-2**, **-3**, or **-4**) contained components **Ma** and **Mb** in a molar ratio of 100:0, 87:13, 64:36, or 20:80, respectively. The amounts of the oligosaccharides in **M-1**−**4** from 100 mg of CS-E were 84, 50, 65, and 111 nmol, respectively. The molar proportion of the components in fractions **Ma** and **Mb** was calculated to be 62:38, being consistent with the data obtained by CE. Of the four fractions, **M-1** and **-4** only were used for the structural analysis of the components in fractions **Ma** and **Mb**, respectively, as described below.

As described previously (*49*, *50*), oligosaccharides containing GlcA(3S) at the internal position are resistant to the action of chondroitinase AC-II, whereas chondroitinase ABC digestion of such oligosaccharides results in the disappearance of the GlcA(3S)-containing disaccharide units as examined by absorbance at 232 nm on HPLC due to the destruction of the GlcA(3S) structure. Hence, the enzymatic determination of the disaccharide composition and sequential arrangement of the disaccharide units is impossible at present if oligosaccharides contain GlcA(3S) at the internal position. Since some of the oligosaccharides characterized in this study contained GlcA(3S) at the internal position (see below), structural analysis of the component in each fraction was accomplished by ^1H NMR analysis in conjunction with FAB-MS, and enzymatic digestions were used to obtain the supplementary evidence.

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 the case of heparin, heparan sulfate, and CS oligosaccharides (*39*, *49*). In the negative ion mode FAB spectrum, alkali-metal-attached molecular ions of the type $[M + x\text{Na} - (x+1)\text{H}]^-$ (where M represents the fully protonated acid forms of oligosaccharides) were preferentially observed. The most prevalent fragment ions observed corresponded to loss of sulfite from the molecular

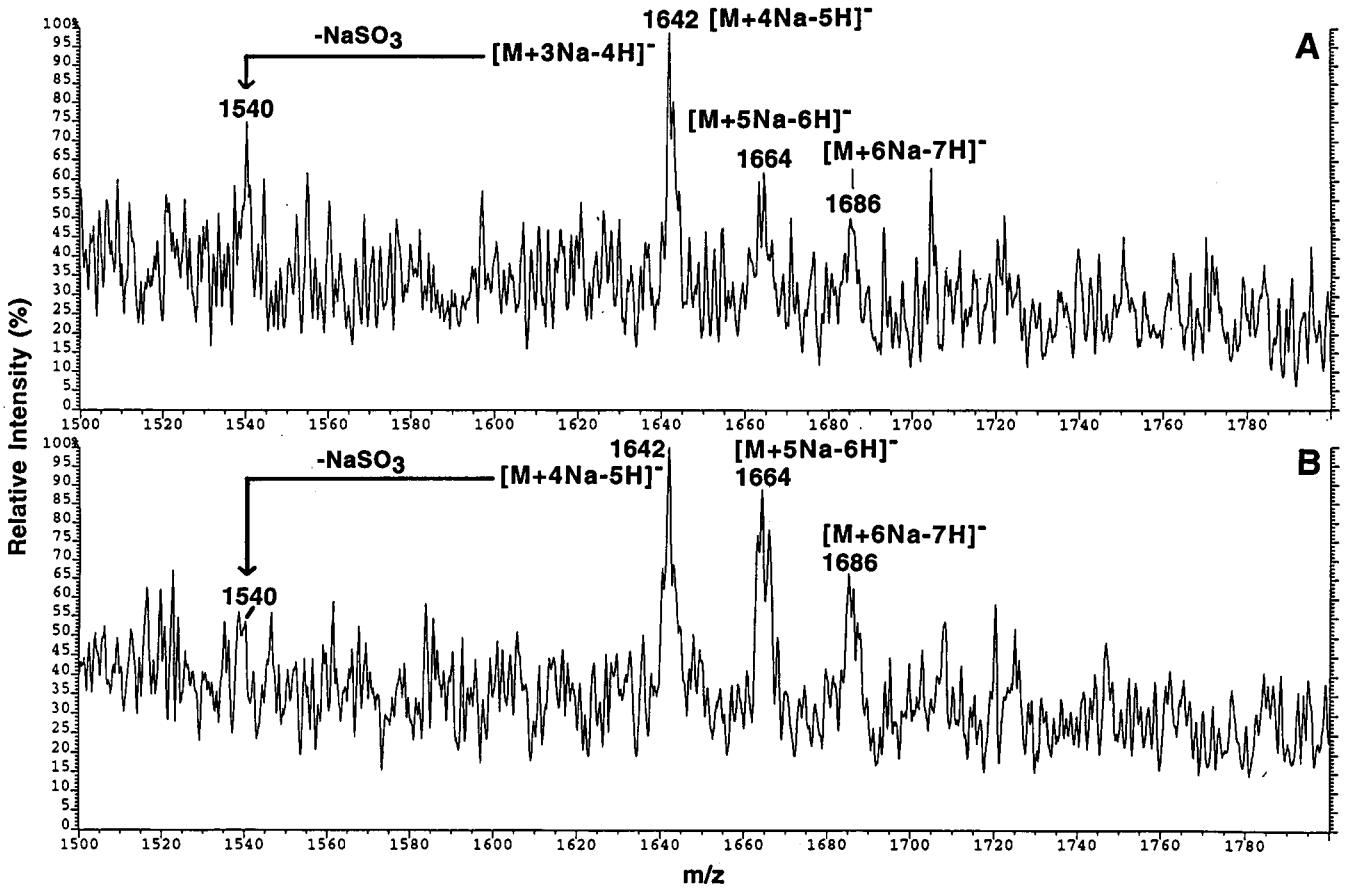

FIGURE 2: Negative ion mode FAB mass spectra of underivatized fractions **M** (A) and **P** (B). Major molecular ion signals were assigned as summarized in Table 2.

Table 2: FAB-MS Analysis of the Five Oligosaccharides Isolated from CS-E of Squid Cartilage

fraction	MW	*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
I	1475	1496	1518	1540	1562	1584		$HexA_3HexNAc_3(OSO_3H)_4$
M	1555	1576	1598	1620	1642	1664	1686	$HexA_3HexNAc_3(OSO_3H)_5$
N	1555			1620	1642	1664		$HexA_3HexNAc_3(OSO_3H)_5$
O	1555		1598	1620	1642	1664		$HexA_3HexNAc_3(OSO_3H)_5$
P	1555				1642	1664	1686	$HexA_3HexNAc_3(OSO_3H)_5$

ions. Representative FAB spectra are shown in Figure 2, and assignments of the molecular ion signals afforded by each of the analyzed fractions are summarized in Table 2.

The molecular ion signal clusters at *m/z* 1576, 1598, 1620, 1642, 1664, and 1686 afforded by fraction **M**, which was unfractionated and hence a mixture of **Ma** and **Mb**, corresponded to $[M + Na - 2H]^-$, $[M + 2Na - 3H]^-$, $[M + 3Na - 4H]^-$, $[M + 4Na - 5H]^-$, $[M + 5Na - 6H]^-$, and $[M + 6Na - 7H]^-$, respectively, of a pentasulfated saturated hexasaccharide $HexA_3HexNAc_3(OSO_3H)_5$ (Figure 2A). The fragment ion signal at *m/z* 1540 corresponded to loss of an SO_3 group from the molecular ion at *m/z* 1620 ($[M + 3Na - 4H]^-$) (Figure 2A). In the negative ion mode FAB-MS spectrum of fraction **P** (Figure 2B), the molecular ion signal clusters at *m/z* 1642, 1664, and 1686 corresponded to $[M + 4Na - 5H]^-$, $[M + 5Na - 6H]^-$, and $[M + 6Na - 7H]^-$, respectively, of a pentasulfated saturated hexasaccharide $HexA_3HexNAc_3(OSO_3H)_5$. Mass spectrometric loss of a sulfite moiety (80 units) from each of the molecular ion signals yielded the cluster of signals at *m/z* 1562, 1584, and 1606, respectively. As shown in Table 2, the molecular ion signal clusters at *m/z* 1496, 1518, 1540, 1562, and 1584 afforded by fraction **I** corresponded to $[M + Na - 2H]^-$, $[M + 2Na - 3H]^-$, $[M + 3Na - 4H]^-$, $[M + 4Na - 5H]^-$, and $[M + 5Na - 6H]^-$, respectively, of a tetrasulfated saturated hexasaccharide $HexA_3HexNAc_3(OSO_3H)_4$. The molecular ion signal clusters at *m/z* 1620, 1642, and 1664 afforded by fraction **N** corresponded to $[M + 3Na - 4H]^-$, $[M + 4Na - 5H]^-$, and $[M + 5Na - 6H]^-$, respectively, of a pentasulfated saturated hexasaccharide $HexA_3HexNAc_3(OSO_3H)_5$. The molecular ion signal clusters at *m/z* 1598, 1620, 1642, and 1664 afforded by fraction **O** corresponded to $[M + 2Na - 3H]^-$, $[M + 3Na - 4H]^-$, $[M + 4Na - 5H]^-$, and $[M + 5Na - 6H]^-$, respectively, of a pentasulfated saturated hexasaccharide $HexA_3HexNAc_3(OSO_3H)_5$. The number of sulfate groups of each fraction determined by FAB-MS analysis is consistent with that estimated from the elution position on HPLC.

Structural Analysis of the Isolated Oligosaccharides. The structure of the major compound in each isolated oligosac-

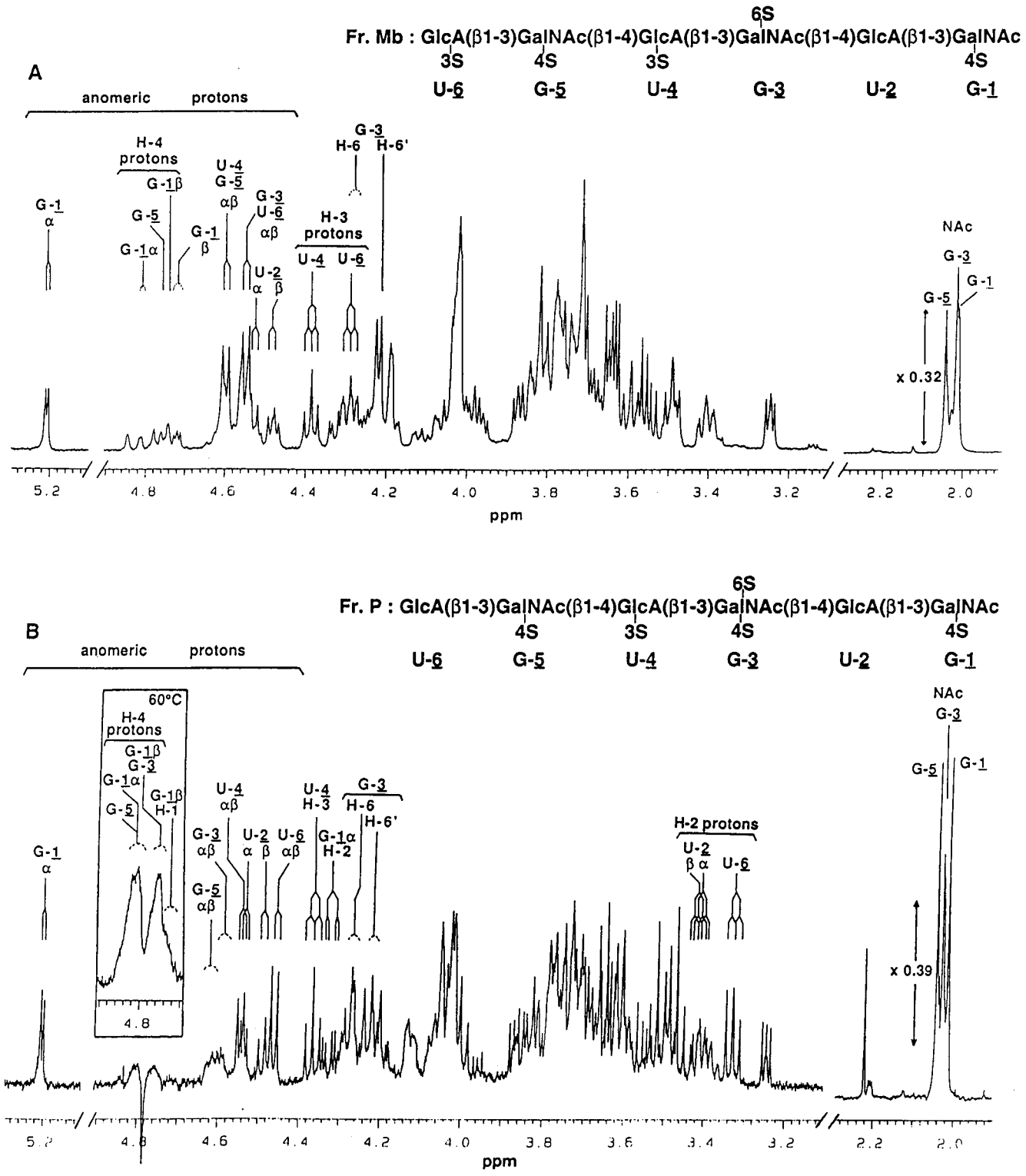

FIGURE 3: Structural-reporter group regions of the 500 MHz 1D ^{1}H NMR spectrum of fraction **Mb** (A) and the 600 MHz 1D ^{1}H NMR spectrum of **P** (B) recorded in ^2H_2O at 26 °C. The numbers and letters in the spectra refer to the corresponding sugar residues in the structures. The inset in panel B is the spectrum recorded at 60 °C to suppress disturbance by the HOD line. U and G stand for GlcA and GalNAc, respectively.

charide fraction was determined by 500 or 600 MHz ^{1}H NMR analysis. The 500 MHz 1D spectrum and 2D HOHAHA spectrum of fraction **M-4** which contains the compound **Mb** as the major component are depicted as representative examples in Figures 3A and 4A, respectively. The 600 MHz 1D and 2D HOHAHA spectra of fraction **P** are also depicted in Figures 3B and 4B, respectively. When the spectra were recorded at 60 °C to suppress the disturbance by the HOD line, weak resonances around 4.8 ppm were recognizable in the 1D spectrum as shown in the inset of Figure 3B.

The resonances were well-resolved in the structural-reporter group regions between 4.4 and 5.3 ppm and ~2.0 ppm, being separated from other signals in the bulk region (3.6−4.3 ppm). The resonances between 4.4 and 5.3 ppm are characteristic of anomeric protons, whereas those at ~2.0

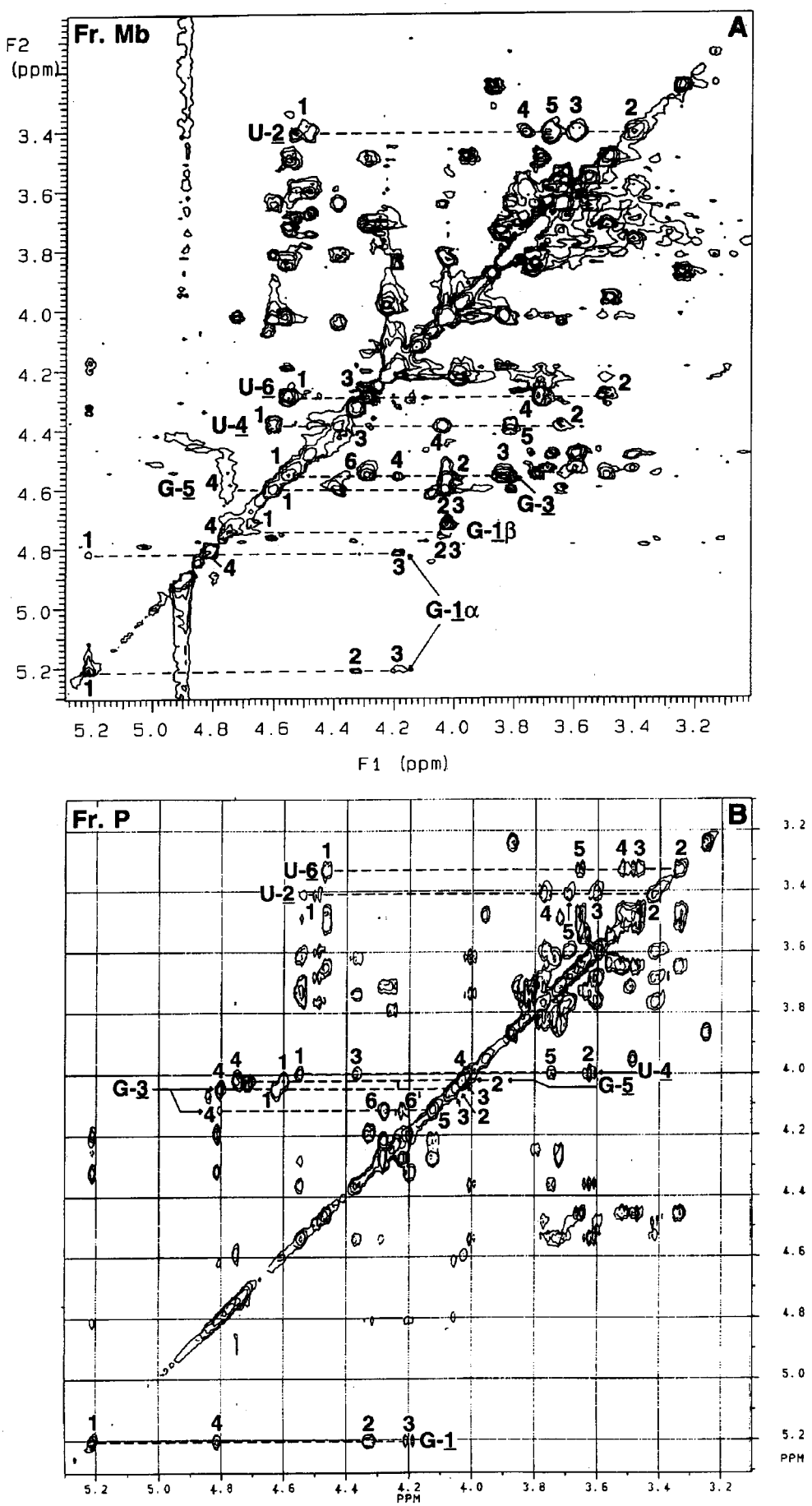

FIGURE 4: 2D HOHAHA spectra of fractions **Mb** (A) and **P** (B) recorded in ^2H_2O at 26 °C. The numbers and letters in the spectra refer to the corresponding sugar residues in the structures. For abbreviations, see the legend of Figure 3.

Table 3: ^{1}H Chemical Shifts of the Constituent Monosaccharides of the Hexasaccharides Isolated from CS-E[a]

		fraction **I** (E-A-A) 6S U-G-U-G-U-G[b] 4S 4S 4S		fraction **Ma** (M-A-A) 6S U-G-U-G-U-G 3S4S 4S 4S		fraction **Mb** (K-L-A) 6S U-G-U-G-U-G 3S4S 3S 4S		fraction **N** (E-E-A) 6S 6S U-G-U-G-U-G 4S 4S 4S		fraction **O** (K-K-A) U-G-U-G-U-G 3S4S3S4S 4S		fraction **P** (A-M-A) 6S U-G-U-G-U-G 4S3S4S 4S	
		α	β	α	β	α	β	α	β	α	β	α	β
GalNAc-**1**	H-1	5.207	4.71	5.209	4.72	5.211	4.72	5.210	4.72[c]	5.208	4.71	5.209	4.72
		(3.0)	(ND[d])	(3.5)	(ND)	(3.5)	(ND)	(3.5)	(ND)	(3.5)	(ND)	(3.0)	(ND)
	H-2	4.327	4.02	4.327	4.01	4.327	4.02	4.327	4.02[c]	4.327	4.02	4.326	4.03
	H-3	4.189	4.04[c]	4.187	4.02	4.18	4.02	4.195	4.07[c]	4.190	4.04[c]	4.197	4.07
	H-4	4.80[c]	4.74[c]	4.81	4.74	4.814	4.74	4.817[c]	4.744[c]	4.809[c]	4.761[c]	4.81[c]	4.76[c]
	H-5	4.25	ND	4.23	ND	4.23	ND	4.25	ND	4.25	ND	4.25	ND
	H-6	3.788	ND	3.78	ND	3.78	ND	3.792	ND	3.78	ND	3.784	ND
	H-6'	3.723	ND	3.71	ND	3.71	ND	3.713	ND	3.71	ND	3.711	ND
	NAc	2.020		2.021		2.021		2.019		2.021		2.019	
GlcA-**2**	H-1	4.521	4.475	4.520	4.474	4.529	4.486	4.539	4.493	4.522	4.478	4.539	4.493
		(8.0)	(7.0)	(8.0)	(8.0)	(8.0)	(8.0)	(8.5)	(8.5)	(8.5)	(8.0)	(8.0)	(8.0)
	H-2	3.397		3.394		3.408		3.412	3.417	3.395		3.414	3.419
	H-3	3.58		3.583		3.596		3.606		3.584		3.604	
	H-4	3.77		3.77		3.75		3.76		3.78		3.770	
	H-5	3.67		3.66		3.68		3.69	3.68	3.67		3.68	
GalNAc-**3**	H-1	4.569		4.569		4.553		4.64[c]		4.578		4.623	
		(7.5)		(8.5)		(8.0)		(ND)		(7.5)		(7.5)	
	H-2	4.028		4.021		4.01		4.06		4.02		4.05	
	H-3	4.04[c]		4.02		3.82		4.08		4.04[c]		4.08[c]	
	H-4	4.74[c]		4.75		4.194		4.791[c]		4.761[c]		4.82[c]	
	H-5	ND		ND		4.12		4.12 - 4.13		ND		4.123	
	H-6	ND		ND		4.28		4.20 - 4.30		ND		4.28	
	H-6'	ND		ND		4.22		4.20 - 4.30		ND		4.22	
	NAc	2.030		2.030		2.016		2.030		2.035		2.033	
GlcA-**4**	H-1	4.470		4.474		4.603		4.476		4.545		4.548	
		(8.0)		(8.0)		(7.5)		(8.0)		(8.0)		(8.0)	
	H-2	3.379		3.386		3.645		3.383		3.622		3.622	
	H-3	3.59		3.583		4.388		3.597		4.371		4.367	
	H-4	3.77		3.77		4.03		3.76		4.006		4.004	
	H-5	3.67		3.66		3.81		3.67		3.73		3.742	
GalNAc-**5**	H-1	4.611		4.617		4.603		4.64[c]		4.606		4.598	
		(7.5)		(7.5)		(7.5)		(ND)		(7.5)		(7.5)	
	H-2	4.076		4.073		4.02		4.06		4.02		4.02	
	H-3	4.09[c]		4.08		4.02		4.08		4.04[c]		4.09[c]	
	H-4	4.83[c]		4.845		4.76		4.840[c]		4.761[c]		4.76[c]	
	H-5	4.126		4.12		ND		4.12 - 4.13		ND		ND	
	H-6	4.278		4.28		ND		4.20 - 4.30		ND		ND	
	H-6'	4.223		4.22		ND		4.20 - 4.30		ND		ND	
	NAc	2.038		2.042		2.048		2.040		2.049		2.047	
GlcA-**6**	H-1	4.465		4.551		4.553		4.466		4.545		4.464	
		(8.0)		(8.0)		(8.0)		(8.0)		(8.0)		(7.5)	
	H-2	3.341		3.497		3.494		3.340		3.488		3.332	
	H-3	3.468		4.289		4.291		3.471		4.288		3.470	
	H-4	3.522		3.71		3.72		3.522		3.71		3.520	
	H-5	3.65		ND		ND		3.65		3.82		3.645	

[a] 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 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) are given in parentheses. [b] G, U, 3S, 4S, and 6S represent GalNAc, GlcA, 3-*O*-sulfate, 4-*O*-sulfate, and 6-*O*-sulfate, respectively. [c] The symbols α and β represent the α- and β-form of each oligosaccharide, respectively, which are in equilibrium in water (also see the text). Consequently, anomeric effects were observed for the H-2−H-6 protons of each GalNAc-**1** residue and the H-1 proton of each GlcA-**2** residue. [d] Values determined at 60 °C. [e] ND, not determined.

ppm are characteristic of the NAc protons of GalNAc. The other proton chemical shifts were assigned using 2D HOHAHA and COSY analyses (*51*), and the NMR data are summarized in Table 3. The types of the three uronic acid residues in each isolated hexasaccharide were identified as GlcA on the basis of the chemical shifts (δ 4.46−4.60) of the anomeric proton signals. Anomeric proton signals of an αIdoA and a βGlcA in oligosaccharides derived from DS and CS are observed around δ 5.0−5.2 and 4.5−4.8, respectively (*41*, *52*). O-Sulfation causes downfield shifts of protons bound to the O-sulfated carbon atoms by approximately 0.4−0.7 ppm (*41*, *51*, *53*). Thus, sulfation positions of the saccharide residues were determined by comparison with the proton signals of nonsulfated saccharide residues (*41*, *51*). Three NAc proton signals observed for each hexasaccharide component were assigned by comparison with those of CS oligosaccharides reported previously (*18*, *41*, *51*).

The data from the FAB-MS analysis indicated that the major compound in fraction **I** had the hexasaccharide structure HexA-GalNAc-HexA-GalNAc-HexA-GalNAc with four sulfate groups. The types of uronic acid and sulfation sites were determined by ^{1}H NMR analysis. 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**αβ, GlcA-**4**αβ, GalNAc-**5**αβ, and GlcA-**6**αβ observed at δ 5.207, 4.71, 4.521, 4.475, 4.569, 4.470, 4.611, and 4.465, respectively, as in the oligosaccharides isolated from various CS variants (*41*). The symbols α and β here

and in Table 3 represent the α- and β-forms of each oligosaccharide, respectively, which are in equilibrium in water; hence, GalNAc-**3**αβ, for example, stands for the GalNAc-**3** residues in both α- and β-forms of the oligosaccharide. In the 1D spectrum, downfield shifts of H-4 of GalNAc-**1**, -**3**, and -**5** as well as H-6 of GalNAc-**5** of approximately 0.6 ppm as well as 0.5 ppm were found, indicating 4-O-sulfation of GalNAc-**1**, -**3**, and -**5** as well as 6-O-sulfation of GalNAc-**5**, respectively. When the spectral data of fraction **I** (Table 3) were compared with those of the previously reported disulfated tetrasaccharide GlcA(β1−3)GalNAc(4S)(β1−4)GlcA(β1−3)GalNAc(4S) (fraction ***h*** in ref *18*) and trisulfated tetrasaccharide GlcA(β1−3)GalNAc-(4S,6S)(β1−4)GlcA(β1−3)GalNAc(4S) (fraction ***n*** in ref *18*), the chemical shifts of the proton signals of the reducing terminal region were very similar to those of fraction ***h***, and those of the signals of the nonreducing terminal region resembled those of fraction ***n***. Hence, the structure of the compound in fraction **I** is as follows: GlcA(β1−3)GalNAc-(4S,6S)(β1−4)GlcA(β1−3)GalNAc(4S)(β1−4)GlcA(β1−3)-GalNAc(4S).

Chondroitinase AC-II digestion of fraction **I** gave rise to a major and a minor peak at the position of ΔHexA-GalNAc-(4S) and GlcA-GalNAc(4S,6S),[2] respectively, as monitored by absorbance at 210 nm, only the former showing absorbance at 232 nm (data not shown). If the parent hexasaccharide in fraction **I** is taken to be 1 mol, the recovery of the unsaturated disaccharide was approximately 2 mol calculated on the basis of the absorbance at 232 nm, indicating that fraction **I** yielded ΔHexA-GalNAc(4S) and GlcA-GalNAc(4S,6S) in a molar ratio of 2:1. These results are in agreement with the structure proposed by FAB-MS and ^{1}H NMR analyses.

Most of the chemical shifts of protons of the major compound in fraction **N** were also 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**αβ, GlcA-**4**αβ, GalNAc-**5**αβ, and GlcA-**6**αβ observed at δ 5.210, 4.72, 4.539, 4.493, 4.64, 4.476, 4.64, and 4.466, respectively. In the 1D spectrum, large downfield shifts of GalNAc-**1** H-4, GalNAc-**3** H-4, H-6, and H-6′, and GalNAc-**5** H-4, H-6, and H-6′ were observed, supporting 4-O-sulfation of GalNAc-**1** and 4,6-O-disulfation of GalNAc-**3** and -**5**. The comparison of the spectral data of fraction **N** (Table 3) with those of the previously reported trisulfated tetrasaccharide GlcA(β1−3)GalNAc(4S,6S)(β1−4)GlcA-(β1−3)GalNAc(4S) (fraction ***n*** in ref *18*) confirmed that this fraction contained a structural element, -GlcA-GalNAc(4-*O*-sulfate), at the reducing terminus. Hence, the following structure is proposed for the compound in fraction **N** and is in agreement with the results obtained from the FAB-MS analysis described above: GlcA(β1−3)GalNAc(4S,6S)(β1−4)GlcA(β1−3)GalNAc(4S,6S)(β1−4)GlcA(β1−3)GalNAc-(4S).

HPLC analysis of the chondroitinase AC-II digest of fraction **N** showed ΔHexA-GalNAc(4S) and ΔHexA-GalNAc-(4S,6S) in a molar ratio of 1:1, as monitored by absorbance at 232 nm. An additional weak UV-absorbing peak at the position of GlcA-GalNAc(4S,6S) was detected when monitored at 210 nm (data not shown). These results indicate that fraction **N** yielded ΔHexA-GalNAc(4S), ΔHexA-GalNAc-(4S,6S), and GlcA-GalNAc(4S,6S) upon chondroitinase AC-II digestion. Since fraction **N** was resistant to chondro-6-sulfatase (data not shown), the unsaturated disaccharide unit containing a 6-O-sulfated GalNAc residue was judged not to be located on the reducing side on the basis of the specificity of the chondro-6-sulfatase (*54*). These results obtained by enzymatic digestions are in agreement with the structure determined by FAB-MS and ^{1}H NMR analyses.

The 1D ^{1}H NMR spectrum (not shown) and the NMR data (Table 3) of fraction **O** were indistinguishable from those of the pentasulfated hexasaccharide GlcA(3S)(β1−3)GalNAc-(4S)(β1−4)GlcA(3S)(β1−3)GalNAc(4S)(β1−4)GlcA(β1−3)GalNAc(4S) isolated from king crab cartilage CS-K (fraction **8** in ref *50*). The deduced composition of the saccharides and sulfates was consistent with that proposed by FAB-MS. Hence, the structure of the compound in fraction **O** is as follows: GlcA(3S)(β1−3)GalNAc(4S)(β1−4)GlcA(3S)(β1−3)GalNAc(4S)(β1−4)GlcA(β1−3)GalNAc-(4S).

HPLC analysis of the chondroitinase AC-II digest of fraction **O** showed a major peak at the elution position of ΔHexA-GalNAc(4S) and a minor one at the position of an authentic tetrasaccharide GlcA(3S)-GalNAc(4S)-GlcA(3S)-GalNAc(4S) as monitored by absorbance at 210 nm (fraction **4** in ref *49*), only the major peak showing absorbance at 232 nm (data not shown). Chondroitinase ABC digestion of fraction **O** yielded a major peak at the position of ΔHexA-GalNAc(4S) and a minor peak at the position of authentic GlcA(3S)-GalNAc(4S) (*49*, *50*) when monitored by absorbance at 210 nm (data not shown), being consistent with the notion that disaccharide units containing GlcA(3S) at the internal positions are destroyed by the action of chondroitinase ABC (*49*, *50*).

The 1D spectrum and 2D HOHAHA spectrum of fraction **P** are presented in Figures 3B and 4B, respectively. The comparison of the spectral data with those of fraction **O** showed no significant differences except for the signals of GalNAc-**3** and GlcA-**6** (Table 3). Large downfield shifts of GalNAc-**3** H-4, H-6, and H-6′ were observed when compared with those of the nonsulfated GalNAc residues found in CS oligosaccharides (*51*), supporting 4,6-O-disulfation of the residue. Upfield shifts of H-2, H-3, and H-4 of GlcA-**6** of 0.17, 0.82, and 0.19 ppm, respectively, were observed when compared with those of fraction **O**, indicating that the C-3 position of the GlcA-**6** residue was not sulfated. These results were consistent with the *m*/*z* value obtained by FAB-MS analysis described above. Hence, the compound in fraction **P** has the following pentasulfated hexasaccharide structure: GlcA(β1−3)GalNAc(4S)(β1−4)GlcA(3S)(β1−3)GalNAc-(4S,6S)(β1−4)GlcA(β1−3)GalNAc(4S).

Chondroitinase AC-II digestion of fraction **P** resulted in mainly two components, a major one that eluted at the position of ΔHexA-GalNAc(4S) and a minor one that eluted in the elution area of tetrasulfated tetrasaccharides[3] when

[2] The elution positions of the rare saturated disaccharides such as GlcA-GalNAc(4S,6S) and GlcA(3S)-GalNAc(4S,6S) were determined using the chondroitinase AC-II digest of the authentic saturated tetrasaccharides isolated previously (*18*).

[3] The elution area of differentially sulfated saturated tetrasaccharides was estimated on the basis of the data obtained previously for authentic tetrasaccharides (*18*, *49*).

monitored by absorbance at 210 nm. When monitored by absorbance at 232 nm, only the major peak was detected (data not shown). Chondroitinase ABC digestion of fraction **P** yielded a major peak at the position of ΔHexA-GalNAc-(4S) and a minor peak at the position of authentic GlcA-GalNAc(4S) (*18*) when monitored by absorbance at 210 nm (data not shown). The failure of detection of a GlcA(3S)-containing unit is in agreement with the notion that disaccharide units containing GlcA(3S) at the internal positions are destroyed by the action of chondroitinase ABC (*49*, *50*).

The comparison of the spectral data of fraction **Ma** (**M-1**) with those of fraction **I** (Table 3) showed no significant differences except for the downfield shifts (0.09−0.82 ppm) of the proton signals belonging to the nonreducing terminal GlcA. The largest downfield shift of 0.82 ppm was observed for the H-3 signal. The results indicate that the major compound in fraction **Ma** had an additional sulfate group on C-3 of the nonreducing terminal GlcA in the structure proposed for the compound in fraction **I**. Hence, the structure of the compound in fraction **Ma** is as follows and in agreement with the results obtained from the FAB-MS analysis of fraction **M**: GlcA(3S)(β1−3)GalNAc(4S,6S)-(β1−4)GlcA(β1−3)GalNAc(4S)(β1−4)GlcA(β1−3)GalNAc-(4S).

Chondroitinase AC-II digestion of fraction **Ma** yielded a major peak at the elution position of ΔHexA-GalNAc(4S) and a minor one that eluted near the elution position of a trisulfated disaccharide when monitored by absorbance at 210 nm, only the former showing absorbance at 232 nm (data not shown). If the parent hexasaccharide in fraction **Ma** is taken to be 1 mol, approximately 2 mol of the unsaturated disaccharide was recovered as calculated on the basis of the absorbance at 232 nm, indicating that fraction **Ma** yielded ΔHexA-GalNAc(4S) and a saturated trisulfated disaccharide in a molar ratio of 2:1. These results obtained by enzymatic digestions are in agreement with the structure proposed by FAB-MS and ^{1}H NMR analyses.

The 1D spectrum and 2D HOHAHA spectrum of fraction **Mb** (**M-4**) are shown in Figures 3A and 4A, respectively, and the NMR data are summarized in Table 3. The chemical shifts of protons belonging to GlcA-**6**, GalNAc-**5**, and GlcA-**4** were very similar to those of the compound in fraction **O**, indicating the presence of the trisulfated trisaccharide sequence of GlcA(3S)(β1−3)GalNAc(4S)(β1−4)-GlcA(3S)(β1−). Compared with the NMR data for the previously reported trisulfated tetrasaccharide GlcA(3S)(β1−3)GalNAc(6S)(β1−4)GlcA(β1−3)GalNAc(4S) (fraction ***l*** in ref *18*), the chemical shifts of the proton signals of the reducing terminal trisaccharide region closely resembled those of fraction ***l***, indicating the presence of the structural element, −3GalNAc(6S)(β1−4)GlcA(β1−3)GalNAc(4S). The estimated number of sulfate groups is consistent with the *m*/*z* value obtained by FAB-MS for fraction **M** described above. Hence, the compound in fraction **Mb** has the following structure: GlcA(3S)(β1−3)GalNAc(4S)(β1−4)-GlcA(3S)(β1−3)GalNAc(6S)(β1−4)GlcA(β1−3)GalNAc-(4S).

Chondroitinase AC-II digestion of fraction **Mb** resulted in mainly two components, a major one that eluted at the position of ΔHexA-GalNAc(4S) and a minor one that eluted in the elution area of tetrasulfated tetrasaccharides, with only the major peak exhibiting absorbance at 232 nm (data not shown). Chondroitinase ABC digestion of fraction **Mb** yielded a major peak at the position of ΔHexA-GalNAc-(4S) and a minor peak at the position of authentic GlcA-(3S)-GalNAc(4S) when monitored by absorbance at 210 nm (data not shown). This result and that from FAB-MS analysis indicated that the compound in fraction **Mb** contains the GlcA(3S)-containing disulfated disaccharide unit at the internal position. Upon successive digestion with chondroitinase AC-II and chondro-6-sulfatase, a presumed tetrasulfated tetrasaccharide peak in the chondroitinase AC-II digest was shifted to a position corresponding to the loss of one sulfate group on HPLC (data not shown), indicating that the reducing GalNAc residue of the tetrasaccharide is monosulfated at its C-6 position. Chondro-6-sulfatase specifically attacks GalNAc(6S) on the reducing end of chondroitin sulfate oligosaccharides (*41*, *54*). These results are in agreement with the structure proposed by ^{1}H NMR analysis.

Three NAc proton signals were observed for the major oligosaccharide component of each isolated fraction. They were assigned by comparison with those of the reported CS oligosaccharides (*18*, *41*, *49*−*51*). In previous ^{1}H NMR studies of saturated CS tetra- and hexasaccharides (*18*, *49*, *50*), the chemical shifts of the NAc proton signals of the reducing terminal GalNAc(4S) and the penultimate GalNAc-(4S) from the nonreducing terminus were observed around δ 2.016−2.024 and 2.039−2.044, respectively, and were influenced by O-sulfation of the neighboring uronic acid residue (*18*, *50*, *51*). On the basis of these observations, the NAc proton signals of the hexasaccharides in the isolated fractions were assigned as shown in Table 3. The NAc proton signals of GalNAc(4S)-**3** were observed at a higher magnetic field than those of GalNAc(4S)-**5**. 3-O-Sulfation of the GlcA-**4** residue resulted in a downfield shift of the NAc proton signal of GalNAc(4S)-**5** by approximately 0.01 ppm.

DISCUSSION

In this study, six sulfated oligosaccharides were isolated from squid cartilage CS-E after digestion with sheep testicular hyaluronidase, which exhibits transglycosylation activity through the reverse reaction of hydrolysis (*55*, *56*). However, these oligosaccharides most likely originated from natural sequences. Although construction of artificial chondro-oligosaccharides has been achieved by taking advantage of the transglycosylation activity of testicular hyaluronidase (*57*), CS chains do not serve as donors or acceptors as efficiently as chondroitin does for the transglycosylation reactions (*57*, *58*). In addition, oversulfated CS variants, CS-D or CS-E, do not serve as donor substrates for the transglycosylation reactions[4] under the established optimum conditions (*57*). Hence, it is unlikely that the isolated hexasaccharides were generated by transglycosylation reactions as discussed for the CS-E tetrasaccharides isolated previously (*18*).

The disaccharide units found at the reducing and nonreducing termini of the isolated oligosaccharides, hence, reflect the cleavage sites of the hyaluronidase rather than the terminal structures generated by the transglycosylation reactions. All the isolated structures contained an A unit [GlcA-

[4] Personal communication from K. Takagaki, Hirosaki University, Hirosaki, Japan.

(β1−3)GalNAc(4S)] at their reducing termini, and any one of the A, E [GlcA(β1−3)GalNAc(4S,6S)], K [GlcA(3S)(β1−3)GalNAc(4S)], and M [GlcA(3S)(β1−3)GalNAc(4S,6S)] units at the nonreducing termini (Table 3). Other oligosaccharides have also been prepared by hyaluronidase digestion (*11*). The structures isolated from king crab cartilage CS-K contained an A, K, or F unit [L-fucose(α1−3)GlcA(β1−3)-GalNAc(4S)] at the reducing termini and an A or K unit, but no F unit, at the nonreducing termini (*49*, *50*). The structures isolated from shark cartilage CS-D contained an A, C, or D unit at both the reducing and nonreducing termini (*11*). These findings together indicate that sheep testicular hyaluronidase has a broad substrate specificity and cleaves most β-1,4-*N*-acetylgalactosaminidic linkages bound to GlcA except for a few. Since no F or E unit has been demonstrated at the nonreducing or reducing termini, respectively (*11*), *N*-acetylgalactosaminidic linkages bound to fucosylated GlcA and 4,6-O-disulfated *N*-acetylgalactosaminidic linkages may be resistant to enzyme action. Thus, the sheep testicular hyaluronidase has now been better characterized as a tool for fragmentation and structural analysis of CS chains.

CS and DS chains that contain E units in mammalian tissues and cells appear to have intriguing biological activities such as anticoagulant activities, lymphoid regulatory activities, and neuroregulatory activities, which include promoting neuronal cell adhesion (*14*) and migration (*59*) as well as neurite outgrowth (*33*, *34*). The biological activities are likely mediated through molecular interactions with specific protein ligands, including anticoagulant factor heparin cofactor II (*32*), leukocyte adhesion molecules (selectins) (*60*), CD44 (*60*), chemokines (*61*), and the growth factor midkine (*14*, *59*). However, the E disaccharide content in mammalian CS chains is usually considerably low (*13*−*15*, *62*), which has hampered analysis of the functional domain sequences of these CS-E or DS-E chains; hence, the sequence information is limited. It has been reported, however, that the GalNAc-(4,6-*O*-disulfate) content of the nonreducing termini of human cartilage aggrecan CS chains increases with age (*63*). Thrombomodulin bears a single CS or DS chain with a unique GalNAc(4S,6S)(β1−4)GlcA(β1−3)GalNAc(4S,6S) sequence at the nonreducing terminus (*30*). In strong contrast to mammalian CS-E chains, squid cartilage CS-E contains a much larger proportion (62%) of E units (*10*, *18*), and mimics or augments some of the activities expressed by mammalian CS-E chains. Therefore, the sequence information for the squid cartilage CS-E chains, which has not yet been vigorously investigated due to the structural complexity, will be useful for a better understanding of the structure−function relationships of CS-E and DS-E chains in mammalian tissues and cells, including neuronal cells.

The isolated oligosaccharides included one tetrasulfated (fraction **I**) and five pentasulfated hexasaccharides (fractions **Ma**, **Mb**, and **N**−**P**). All of them are highly sulfated containing one or two di- or trisulfated disaccharide units, such as the E unit. The other oversulfated components, which are unique and distinct from those found in other ordinary CS variants, included GlcA(3S)-GalNAc(4S), GlcA(3S)-GalNAc(6S), and GlcA(3S)-GalNAc(4S,6S), and are designated here as the K, L, and M disaccharide units, respectively (Table 3 and Figure 5). We previously isolated multiple saturated tetrasaccharides such as A-A, L-A, K-A, E-A, and M-A (*18*) as well as unsaturated tetrasaccharides such as

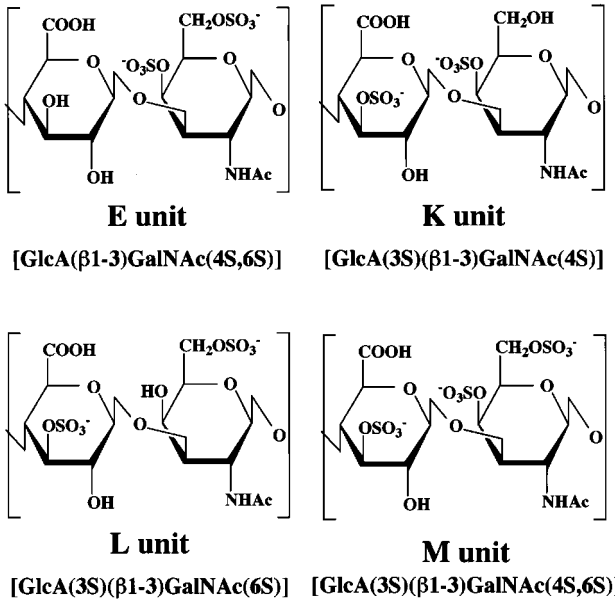

FIGURE 5: Structures of unique sulfated disaccharide units characteristic of CS-E and their terminology. Three unique sulfated disaccharide units, K, L, and M, which were identified in squid carilage CS-E in this and previous work (*18*), along with the conventional E unit are shown.

ΔO-E [ΔHexA-GalNAc-GlcA-GalNAc(4S,6S)], ΔA-A, ΔC-E, ΔE-A, and ΔE-E (the abbreviation Δ denotes unsaturation of the GlcA residue at the nonreducing terminal, and the O represents the nonsulfated disaccharide unit) (*11*). Except for the component (K-K-A) in fraction **O**, which was isolated previously from king crab cartilage CS-K (*50*), the isolated structures in fractions **I** (E-A-A), **Ma** (M-A-A), **Mb** (K-L-A), **N** (E-E-A), and **P** (A-M-A) were novel hexasaccharide sequences, although each di- and tetrasaccharide building unit was isolated previously. While two consecutive E or K units were found, other di- or trisulfated units were found discretely. Interestingly, a K unit was found to be next to an L unit, forming a previously unreported K-L tetrasulfated tetrasaccharide sequence. Although these hexasaccharides represented only small portions [1.3 (**I**), 0.32 (**Ma**), 0.20 (**Mb**), 0.84 (**N**), 0.37 (**O**), and 0.52% (**P**) by weight] of the starting CS-E preparation, such rare sequences may be specifically recognized by functional proteins.

It should be noted, however, that since a panel of larger oligosaccharide fragments was observed on gel filtration chromatography after the hyaluronidase digestion as reported previously (*18*), various biological activities may depend on the sequences composed of the consecutive multiple E units, especially in view of the high content (62%) of E units in the parent CS-E preparation (*18*). It remains to be investigated whether the sequence composed of consecutive multiple E units or the rare sequences shown in this study are present in CS chains in mammalian tissues. Also, in view of the fact that squid cartilage CS-E has a highly negative charge density comparable to that of heparin and that it mimics some of the biological activities of heparin by interacting with heparin-binding proteins such as heparin cofactor II and midkine, it is of interest to perform conformational studies to clarify whether the isolated CS-E oligosaccharides have three-dimensional structures similar to those found in heparin oligosaccharides.

The structures isolated in this study imply some aspects of the biosynthetic pathway of the CS-E structure, although it is largely unknown how the chondroitin polymer backbone is synthesized (*64*) or which sulfotransferases are responsible for the synthesis of GalNAc(4S,6S) and GlcA(3S) (*65*). Although two 6-*O*-sulfotransferases (*66*−*68*) and two 4-*O*-sulfotransferases (*69*, *70*), both of which are specific for CS, have been cloned and a 6-*O*-sulfotransferase specific for DS has been demonstrated (*71*), it is not understood whether they are involved in the generation of E units. Recently, however, a specific 6-*O*-sulfotransferase which transfers the second sulfate to position 6 of both internal and nonreducing terminal GalNAc(4S) residues has been purified from squid cartilage (*72*). In contrast, the 3-*O*-sulfotransferase that produces GlcA(3S) of CS chains remains to be demonstrated. Although a 3-*O*-sulfotransferase that produces GlcA(3S) structure on the HNK-I carbohydrate epitope on glycoproteins has been cloned (*73*, *74*), whether it works on chondroitin or CS chains has not been reported. In the sulfated oligosaccharide sequences isolated in this and previous studies (*18*, *49*, *50*), no GlcA(3S)-GalNAc was found, and the GalNAc residue on the reducing side of the GlcA(3S) residue was always sulfated at its C-4 and/or C-6 positions. The possibility that the GlcA(3S)-GalNAc unit is quickly converted into the K [GlcA(3S)-GalNAc(4S)] or L unit [GlcA(3S)-GalNAc(6S)] cannot be excluded, but is unlikely in view of the strong activity toward chondroitin of the chondroitin 6-*O*-sulfotransferases cloned to date (*66*−*68*) toward nonsulfated chondroitin. Rather, it is most likely that 3-O-sulfation takes place after 4-O-sulfation and/or 6-O-sulfation of the GalNAc residue. Such a 3-*O*-sulfotransferase remains to be demonstrated.

ACKNOWLEDGMENT

We thank Tomoko Hazeki for technical assistance. We also thank Dr. Makiko Sugiura (Kobe Pharmaceutical University) and Masako Akaji (Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan) for recording the NMR spectra.

REFERENCES

1. Poole, A. R. (1986) *Biochem. J. 236*, 1−14.
2. Fransson, L. Å. (1987) *Trends Biochem. Sci. 12*, 406−411.
3. Mark, M. P., Baker, J. R., Kimata, K., and Ruch, J.-V. (1990) *Int. J. Dev. Biol. 34*, 191−204.
4. Lander, A. D. (1993) *Curr. Opin. Nerobiol. 3*, 716−723.
5. Oohira, A., Matsui, F., Tokita, Y., Yamauchi, S., and Aono, S. (2000) *Arch. Biochem. Biophys. 374*, 24−34.
6. Sorrell, J. M., Mahmoodian, F., Schafer, I. A., Davis, B., and Caterson, B. (1990) *J. Histochem. Cytochem. 38*, 393−402.
7. Suzuki, S. (1960) *J. Biol. Chem. 235*, 3580−3588.
8. Seno, N., and Murakami, K. (1982) *Carbohydr. Res. 103*, 190−194.
9. Anno, K., Seno, N., Mathews, M. B., Yamagata, T., and Suzuki, S. (1971) *Biochim. Biophys. Acta 237*, 173−177.
10. Suzuki, S., Saito, H., Yamagata, T., Anno, K., Seno, N., Kawai, Y., and Furuhashi, T. (1968) *J. Biol. Chem. 243*, 1543−1550.
11. Sugahara, K., and Yamada, S. (2000) *Trends Glycosci. Glycotechnol. 12*, 321−349.
12. Iwata, H. (1969) *J. Jpn. Orthop. Assoc. 43*, 455−473.
13. Kitagawa, H., Tsutsumi, K., Tone, Y., and Sugahara, K. (1997) *J. Biol. Chem. 272*, 31377−31381.
14. Ueoka, C., Kaneda, N., Okazaki, I., Nadanaka, S., Muramatsu, T., and Sugahara, K. (2000) *J. Biol. Chem. 275*, 37407−37413.
15. Saigo, K., and Egami, F. (1970) *J. Neurochem. 17*, 633−647.
16. Gregoire, P. E., Dictus-Vermeulen, C., and Ameryckx, J. P. (1972) *Biochim. Biophys. Acta 279*, 102−117.
17. Yoshida, K., Miyauchi, S., Kikuchi, H., Tawada, A., and Tokuyasu, K. (1989) *Anal. Biochem. 177*, 327−332.
18. Kinoshita, A., Yamada, S., Haslam, S. M., Morris, H. R., Dell, A., and Sugahara, K. (1997) *J. Biol. Chem. 272*, 19656−19665.
19. Kim, J. J., and Conrad, H. E. (1982) *J. Biol. Chem. 257*, 1670−1675.
20. Kolset, S. O., and Gallagher, J. T. (1990) *Biochim. Biophys. Acta 1032*, 191−211.
21. Takasu, Y., Hasumi, F., and Mori, Y. (1982) *Biochim. Biophys. Acta 716*, 316−323.
22. Kolset, S. O., Kjellén, L., Seljelid, R., and Lindahl, U. (1983) *Biochem. J. 210*, 661−667.
23. Edwards, I. J., Xu, H., Obunike, J. C., Goldberg, I. J., and Wagner, W. D. (1995) *Arterioscler., Thromb., Vasc. Biol. 15*, 400−409.
24. Enerbäck, L., Kolset, S. D., Kusche, M., Hjerpe, A., and Lindahl, U. (1985) *Biochem. J. 227*, 661−668.
25. Stevens, R. L., and Austen, K. F. (1989) *Immunol. Today 10*, 381−386.
26. Rothenberg, M. E., Caulfield, J. P., Austen, K. F., Hein, A., Edmiston, K., Newburger, P. E., and Stevens, R. L. (1987) *J. Immunol. 138*, 2616−2625.
27. McGee, M. P., Teuschler, H., Parthasarathy, N., and Wagner, W. D. (1995) *J. Biol. Chem. 270*, 26109−26115.
28. Bourin, M.-C., and Lindahl, U. (1993) *Biochem. J. 289*, 313−330.
29. Wilson, J. G., Fearon, D. T., Stevens, R. L., Seno, N., and Austen, K. F. (1984) *J. Immunol. 132*, 3058−3063.
30. Bourin, M.-C., Lundgren-Åkerlund, E., and Lindahl, U. (1990) *J. Biol. Chem. 265*, 15424−15431.
31. Akiyama, F., Seno, N., and Yoshida, K. (1982) *Tohoku J. Exp. Med. 136*, 359−365.
32. Scully, M. F., Ellis, V., Seno, N., and Kakkar, V. V. (1986) *Biochem. Biophys. Res. Commun. 137*, 15−22.
33. Nadanaka, S., Clement, A., Masayama, K., Faissner, A., and Sugahara, K. (1998) *J. Biol. Chem. 273*, 3296−3307.
34. Clement, A. M., Sugahara, K., and Faissner, A. (1999) *Neurosci. Lett. 269*, 125−128.
35. Faissner, A., Clement, A., Lochter, A., Streit, A., Mandl, C., and Schachner, M. (1994) *J. Cell Biol. 126*, 783−799.
36. Clement, A. M., Nadanaka, S., Masayama, K., Mandl, C., Sugahara, K., and Faissner, A. (1998) *J. Biol. Chem. 273*, 28444−28453.
37. Kinoshita, A., and Sugahara, K. (1997) *Seikagaku 69*, 794.
38. Bitter, M., and Muir, H. (1962) *Anal. Biochem. 4*, 330−334.
39. Sugahara, K., Tohno-oka, R., Yamada, S., Khoo, K. H., Morris, H. R., and Dell, A. (1994) *Glycobiology 4*, 535−544.
40. Yamada, S., Oyama, M., Yuki, Y., Kato, K., and Sugahara, K. (1995) *Eur. J. Biochem. 233*, 687−693.
41. Sugahara, K., Shigeno, K., Masuda, M., Fujii, N., Kurosaka, A., and Takeda, K. (1994) *Carbohydr. Res. 255*, 145−163.
42. Barbara, T. M. (1994) *J. Magn. Reson., Ser. A 109*, 265−269.
43. Griesinger, C., Otting, G., Wuthrich, K., and Ernst, R. R. (1988) *J. Am. Chem. Soc. 110*, 7870−7872.
44. Piantini, U., Sorenson, O. W., and Ernst, R. R. (1982) *J. Am. Chem. Soc. 104*, 6800−6801.
45. Vliegenthart, J. F. G., Dorland, L., and Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem. 41*, 209−374.
46. Sugahara, K., Okumura, Y., and Yamashina, I. (1989) *Biochem. Biophys. Res. Commun. 162*, 189−197.
47. Meyer, K. (1971) in *Enzymes* (Boyer, P. D., Ed.) Vol. 5, pp 307−320, Academic Press, New York.
48. Kresse, H., and Glössl, J. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol. 60*, 217−314.
49. Sugahara, K., Tanaka, Y., Yamada, S., Seno, N., Kitagawa, H., Haslam, S. M., Morris, H. R., and Dell, A. (1996) *J. Biol. Chem. 271*, 26745−26754.
50. Kitagawa, H., Tanaka, Y., Yamada, S., Seno, N., Haslam, S. M., Morris, H. R., Dell, A., and Sugahara, K. (1997) *Biochemistry 36*, 3998−4008.

51. Yamada, S., Yoshida, K., Sugiura, M., and Sugahara, K. (1992) *J. Biochem. 112*, 440−447.
52. Sugahara, K., Ohkita, Y., Shibata, Y., Yoshida, K., and Ikegami, A. (1995) *J. Biol. Chem. 270*, 7204−7212.
53. Harris, M. J., and Turvey, J. R. (1970) *Carbohydr. Res. 15*, 57−63.
54. Sugahara, K., and Kojima, T. (1996) *Eur. J. Biochem. 239*, 865−870.
55. Weissman, B. (1955) *J. Biol. Chem. 216*, 783−794.
56. Highsmith, S., Garvin, J. H., Jr., and Chipman, D. M. (1975) *J. Biol. Chem. 250*, 7473−7480.
57. Saitoh, H., Takagaki, K., Majima, M., Nakamura, T., Matsuki, A., Kasai, M., Narita, H., and Endo, M. (1995) *J. Biol. Chem. 270*, 3741−3747.
58. Takagaki, K., Munakata, H., Majima, M., and Endo, M. (1999) *Biochem. Biophys. Res. Commun. 258*, 741−744.
59. Maeda, N., Ichihara-Tanaka, K., Kimura, T., Kadomatsu, K., Muramatsu, T., and Noda, M. (1999) *J. Biol. Chem. 274*, 12474−12479.
60. Kawashima, H., Hirose, M., Hirose, J., Nagakubo, D., Plaas, A. H., and Miyasaka, M. (2000) *J. Biol. Chem. 275*, 35448−35456.
61. Hirose, J., Kawashima, H., Yoshie, O., Tashiro, K., and Miyasaka, M. (2001) *J. Biol. Chem. 276*, 5228−5234.
62. Zou, K., Muramatsu, H., Ikematsu, S., Sakuma, S., Salama, R. H., Shinomura, T., Kimata, K., and Muramatsu, T. (2000) *Eur. J. Biochem. 267*, 4046−4053.
63. Plaas, A. H. K., Wong-Palms, S., Roughley, P. J., Midura, R. J., and Hascall, V. C. (1997) *J. Biol. Chem. 272*, 20603−20610.
64. Sugahara, K., and Kitagawa, H. (2000) *Curr. Opin. Struct. Biol. 10*, 518−527.
65. Habuchi, O. (2000) *Biochim. Biophys. Acta 1474*, 115−127.
66. Fukuta, M., Uchimura, K., Nakashima, K., Kato, M., Kimata, K., Shinomura, T., and Habuchi, O. (1995) *J. Biol. Chem. 270*, 18575−18580.
67. Tsutsumi, K., Shimakawa, H., Kitagawa, H., and Sugahara, K. (1998) *FEBS Lett. 441*, 235−241.
68. Kitagawa, H., Fujita, M., Ito, N., and Sugahara, K. (2000) *J. Biol. Chem. 275*, 21075−21080.
69. Yamauchi, S., Hirahara, Y., Usui, H., Takeda, Y., Hoshino, M., Fukuta, M., Kimura, J. H., and Habuchi, O. (1999) *J. Biol. Chem. 274*, 2456−2463.
70. Hiraoka, N., Nakagawa, H., Ong, E., Akama, T. O., Fukuda, M. N., and Fukuda, M. (2000) *J. Biol. Chem. 275*, 20188−20196.
71. Nadanaka, S., Fujita, M., and Sugahara, K. (1999) *FEBS Lett. 452*, 185−189.
72. Ito, Y., and Habuchi, O. (2000) *J. Biol. Chem. 275*, 34728−34736.
73. Bakker, H., Friedmann, I., Oka, S., Kawasaki, T., Nifant'ev, N., Schachner, M., and Mantei, N. (1997) *J. Biol. Chem. 272*, 29942−29946.
74. Ong, E., Yeh, J. C., Ding, Y., Hindsgaul, O., and Fukuda, M. (1998) *J. Biol. Chem. 273*, 5190−5195.

BI015577N