Novel Sulfated Octa- and Decasaccharides from Squid Cartilage Chondroitin Sulfate E: Sequencing and Application for Determination of the Epitope Structure of the Monoclonal Antibody MO-225†

Sarama Sathyaseelan Deepa,[‡,§] Kittiwan Kalayanamitra,[‡,§,||] Yumi Ito,[§] Prachya Kongtawelert,[||] Shigeyuki Fukui,[⊥] Shuhei Yamada,[§,#] Tadahisa Mikami,[§] and Kazuyuki Sugahara*[,§,#]

Department of Biochemistry, Kobe Pharmaceutical University, Higashinada-ku, Kobe 658-8558, Japan, Thailand Excellence Center for Tissue Engineering, Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand, Department of Biotechnology, Faculty of Engineering, Kyoto Sangyo University, Kyoto 603-8558, Japan, and Laboratory of Proteoglycan Signaling and Therapeutics, Graduate School of Life Science, Hokkaido University, Sapporo 001-0021, Japan

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ABSTRACT: A mixture of octa- and decasaccharides obtained by the digestion with the hyaluronidase of chondroitin sulfate E derived from squid cartilage was subfractionated into 20 and 23 different components, respectively, by anion-exchange HPLC. MALDI-TOF/MS was used to assign the sugar and sulfate composition of the putative octa- and decasaccharides, and a disaccharide composition analysis revealed the building blocks to be A- [GlcUAβ1−3GalNAc(4S)], C- [GlcUAβ1−3GalNAc(6S)], and E- [GlcUAβ1−3GalNAc(4S,6S)] units, where 4S and 6S represent 4-*O*- and 6-*O*-sulfate, respectively. The sequences of these octa- and decasaccharides were determined at low picomole amounts by a combination of enzymatic digestions with chondroitinases in conjunction with anion-exchange HPLC. Sequencing revealed that each fraction is a mixture of a major component together with one to three minor components, reflecting the heterogeneity of the parent polysaccharide. Among the 11 different octasaccharide sequences reported here, 8 are novel, while all of the 6 decasaccharide sequences are novel, and this is the first report of the sequencing of CS oligosaccharides longer than octasaccharides. The reactivity of the monoclonal antibody MO-225 with octa- and decasaccharides tested with an oligosaccharide microarray revealed that a CS-E decasaccharide is the minimal requirement for antibody recognition. Among the 6 decasaccharides, only E-E-E-E-C was recognized by MO-225, suggesting the requirement of a C-unit at the reducing end and also the importance of chain length, which in turn may indicate the importance of the conformation acquired by this specific sequence for antibody recognition.

Chondroitin sulfate proteoglycans (CS-PGs)[1] are widely distributed in the extracellular matrix and at the cell surface in mammalian tissues, participating in biological processes including cell migration, differentiation, proliferation, cell−cell recognition, and tissue morphogenesis (for reviews, see refs *1*−*4*), often through their GAG chains. The CS chains are composed of repeating disaccharide units consisting of glucuronic acid (GlcUA) and *N*-acetylgalactosamine (GalNAc) with different sulfation patterns forming a variety of disaccharide units. Among the variant forms, the oversulfated CS chains CS-E and CS-D having E-units [GlcUAβ1−3GalNAc(4S,6S)] and D-units [GlcUA(2S)β1−3GalNAc-(6S)], respectively, where 2S, 4S, and 6S stand for 2-*O*-, 4-*O*-, and 6-*O*-sulfate, are of special interest in view of their biological activities such as neurite outgrowth-promoting activity (reviewed in refs *3* and *5*) and the ability to bind various heparin- (Hep-) binding growth factors (*6*).

Differential expression of CS-PGs during the development of the rat central nervous system has been reported (*7*), and immunological studies using monoclonal antibodies have revealed that CS isoforms differing in the position and degree of sulfation perform distinct functions in development (*8*).

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* Corresponding author. Tel: 81-(11)-706-9054. Fax: 81-(11)-706-9056. E-mail: k-sugar@sci.hokudai.ac.jp.

‡ These authors contributed equally to this work.

§ Kobe Pharmaceutical University.

|| Chiang Mai University.

⊥ Kyoto Sangyo University.

Hokkaido University.

[1] Abbreviations: PG, proteoglycan; GAG, glycosaminoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; mAb, monoclonal antibody; DPPE, L-α-dipalmitoylphosphatidylethanolamine; MALDI-TOF/MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; GlcUA, D-glucuronic acid; GalNAc, *N*-acetyl-D-galactosamine; HexUA, hexuronic acid; ΔHexUA, 4-deoxy-L-threo-hex-4-enepyranosyluronic acid; HPLC, high-performance liquid chromatography; 2AB, 2-aminobenzamide; ΔO, ΔHexUAα1-3GalNAc; ΔC, ΔHexUAα1-3GalNAc(6-*O*-sulfate); ΔA, ΔHexUAα1-3GalNAc(4-*O*-sulfate); ΔD, ΔHexUA(2-*O*-sulfate)α1-3GalNAc(6-*O*-sulfate); ΔE, ΔHexUAα1-3GalNAc(4,6-*O*-disulfate); 4S, 4-*O*-sulfate; 6S, 6-*O*-sulfate.

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CS-PGs and dermatan sulfate PGs (DS-PGs) play important roles in neural development by regulating neuronal adhesion and migration, the formation of neurites, and axonal guidance (for reviews, see refs *9* and *10*). Small yet significant amounts of E- and D-units have been detected in the bovine (*11*), mouse (*12*−*14*), rat (*15*), chick (*16*), and embryonic pig (*17*, *18*) brain. These oversulfated structures form unique functional domains capable of making specific interactions with protein ligands to regulate their functions (*5*, *17*). The presence of oversulfated E-units in appreciable proportions has been reported for the brain CS-PG appican (14.3%) (*19*) and the CS chains of syndecan-1 and -4 derived from normal murine mammary gland epithelial cells (7% and 9%, respectively) (*20*).

Squid and shark cartilages are rich sources of CS-E and CS-D, respectively, and various functions in vitro have been demonstrated for these chains. The neuronal cell adhesion and migration mediated by growth factor midkine (MK) is inhibited by CS-E (*15*, *21*). Various Hep-binding growth factors, which are expressed in the brain during development, exhibit strong binding to CS-E (*6*). The binding of proinflammatory molecules such as L- and P-selectins and various chemokines to PG-M/versican is inhibited by E-unit-containing tetrasaccharides obtained from CS-E (*22*). CS-E is a potent inhibitor of herpes simplex virus 1 infections (*23*, *24*), and neurite outgrowth-promoting activity of CS-D (*12*, *25*) and CS-E (*26*) has been documented (for review, see ref *3*).

We have been systematically investigating in detail the structure of various CS isoforms, particularly CS-E and CS-D, in view of the presence of oversulfated units. The isolation and sequencing of oligosaccharides from shark cartilage CS-D (*25*, *27*, *28*), squid cartilage CS-E (*29*, *30*), hagfish notochord CS-H (*31*), and king crab CS-K (*32*, *33*) have been accomplished. In view of the biological activities exhibited by oversulfated CS isoforms, elucidation of the minimal structural units will be essential to study CS−protein interactions and for drug development aiming at therapeutic applications.

The monoclonal antibody (mAb) MO-225 was developed against PG-M from the chick embryonic limb bud (*34*), and the A-D tetrasaccharide sequence present in CS-D and CS-C is the critical requirement for the antibody to bind (*35*) (Deepa et al., unpublished results).[2] In contrast, CS-E from squid cartilage, which does not contain any D-units, binds MO-225 (*34*), though the reason for this seemingly unrelated action is not known. In the present study, we have isolated and structurally characterized octa- and decasaccharides from squid cartilage CS-E and examined the reactivity of MO-225 with these structurally defined oligosaccharides using an oligosaccharide microarray (*36*) to demonstrate the epitope's structure.

EXPERIMENTAL PROCEDURES

Materials. Squid cartilage CS-E (superspecial grade), CS disaccharide standards, chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4), chondroitinase AC-I from *Flavobacterium heparinum* (EC 4.2.2.5), chondroitinase AC-II from *Arthrobacter aurescens* (EC 4.2.2.4), and the monoclonal antibody MO-225 were purchased from Seikagaku Corp., Tokyo, Japan. Bovine albumin (fraction V, chemical grade) was from Serological Proteins, Inc., Kankakee, IL. Peroxidase-conjugated goat anti-mouse IgG + IgM (H + L) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Sheep testicular hyaluronidase (EC 3.2.1.35) and 2,5-dihydroxybenzoic acid were obtained from Sigma. The $(Arg\text{-}Gly)_{15}$ was custom-made by Peptide Institute, Inc., Osaka, Japan. Bio-Gel P-10 resin and nitrocellulose membrane (Trans-Blot Transfer membrane, 0.45 μm) were obtained from Bio-Rad Laboratories, Hercules, CA.

Preparation of Oligosaccharide Fractions by Digestion of CS-E. A commercial preparation of squid cartilage CS-E (89 mg) was digested with 10 mg (approximately 15000 National formulary units) of sheep testicular hyaluronidase in a total volume of 3.0 mL of 50 mM sodium phosphate buffer, pH 6.0, containing 150 mM NaCl (1 National formulary unit corresponds to the amount of the enzyme that hydrolyzes 74 μg of hyaluronate/min) (*37*, *38*) at 37 °C for 22 h. More enzyme (approximately 3000 and 6000 NFU, respectively) was added at 22 and 26 h, and the digestion was continued until 45 h to confirm a complete digestion. Proteins were precipitated by the addition of 0.66 mL of 30% cold trichloroacetic acid (TCA) and removed by centrifugation at 2500 rpm for 10 min. After recovery of the supernatant, the precipitate was washed with 1.0 mL of 5% TCA. The combined supernatant was extracted with ether to remove TCA, and the aqueous phase was neutralized with 1 M Na_2CO_3. The sample was fractionated on a Bio-Gel P-10 column (1.6 × 95 cm), using 1 M NaCl containing 10% ethanol as an eluent. The eluate was monitored by measuring the absorbance at 210 nm, which is mainly due to the carbonyl groups. Fractions I−IX were pooled separately (see Figure 1), concentrated, and desalted by gel filtration through a Sephadex G-25 (fine) column (1.5 × 46 cm) using distilled water as the eluent, with monitoring at 210 nm. Fractions corresponding to oligosaccharides were pooled, dried, and quantified by the carbazole reaction using GlcUA as a standard (*39*).

Subfractionation of the Putative Octa- and Decasaccharide Fractions by Anion-Exchange HPLC. The putative octasaccharide fraction IX, obtained by gel filtration on a Bio-Gel P-10 column, was subfractionated by anion-exchange HPLC on an amine-bound silica PA-03 column using a linear NaH_2PO_4 gradient from 0.2 to 1 M over a 120 min period at a flow rate of 1 mL/min at room temperature, and the eluate was monitored at 210 nm. Likewise, the decasaccharide fraction VIII was subfractionated using a linear gradient from 0.6 to 1 M over a 115 min period. The separated peaks were collected and desalted individually as described above, except for fractions VIIIk, VIIIl, and VIIIm, for which a PD-10 column was used, followed by quantification using the carbazole reaction.

Enzymatic Digestion of Oligosaccharides. For determination of the disaccharide composition, each oligosaccharide was digested with 5 mIU of chondroitinase ABC in a total volume of 30 μL in a 50 mM Tris-HCl buffer, pH 8.0, containing 60 mM sodium acetate at 37 °C. Reactions were terminated by boiling for 1 min. The samples were dried, and the liberated unsaturated disaccharides were labeled with a fluorophore, 2-aminobenzamide (2AB) (*40*). After removal of the excess 2AB reagent by paper chromatography using Whatman 3MM paper in a solvent system of butanol−ether−

[2] S. S. Deepa, S. Yamada, S. Fukui, and K. Sugahara, submitted for publication.

water (4:1:1 v/v/v) (*41*), the samples were analyzed by HPLC on an amine-bound silica PA-03 column (4.6 × 250 mm; YMC Co., Kyoto, Japan) using a linear gradient of NaH_2PO_4 at a flow rate of 1 mL/min (*41*). The eluted peaks were compared with the elution positions of authentic standards for identification.

The sequencing of octa- and decasaccharides was achieved by enzymatic analysis using chondroitinases AC-II and ABC in conjunction with HPLC. The sequences of octa- and decasaccharides were determined by the exosequencing method as described previously (*41*).

Delayed Extraction Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (DE MALDI-TOF/MS). DE MALDI-TOF/MS of octa- and decasaccharides was carried out in the linear mode using a Voyager DE-RP/Pro (PerSeptive Biosystems, Framingham, MA). An aqueous solution of $(Arg\text{-}Gly)_{15}$ (10 pmol/μL) was first mixed with 10 pmol of each octa- or decasaccharide (*42*) and then with 1 μL of the matrix (2,5-dihydroxybenzoic acid) solution. The mixture was placed on the probe surface, dried under a stream of air, and used for the measurement of the spectrum.

Analysis of the Interaction of MO-225 with DPPE-Derivatized CS-E Oligosaccharides Immobilized on the Nitrocellulose Membrane (36). The individual CS-E octa- or decasaccharide fractions were conjugated with L-α-dipalmitoylphosphatidylethanolamine (DPPE) to generate neoglycolipids (*43*) and were immobilized on a nitrocellulose membrane, and the reactivity with MO-225 was tested as described previously (Deepa et al., unpublished results).[2]

RESULTS

Previously we isolated and sequenced several octasaccharides from shark cartilage CS-C, which were recognized by commercially available mAbs raised against vertebrate CS-PGs. MO-225 is an antibody developed against the PG from limb buds of chick embryos, and the requirement of an A-D unit for its reactivity is well documented (*34*, *35*) (Deepa et al., unpublished results).[2] Hence, it is intriguing that CS-E devoid of D-units cross-reacts with MO-225. In this study, we have purified and sequenced octa- and decasaccharides from squid cartilage CS-E and tried to identify the sequence that binds MO-225.

Preparation and Isolation of Octa- and Decasaccharides from CS-E. A commercial preparation of squid cartilage CS-E (89 mg) was exhaustively digested with sheep testicular hyaluronidase to generate saturated oligosaccharides. It should be noted that the CS-E preparation contained no detectable IdoUA as examined by 500 MHz ^{1}H NMR spectroscopy (see Supporting Information). The hyaluronidase digest was size-fractionated by gel filtration on a Bio-Gel P-10 column, and the separated fractions were designated as I−XI, based on elution from the column by measuring the absorbance at 210 nm, caused primarily by the carbonyl groups (Figure 1). According to previous chromatographic data (*29*), fraction IX is a putative octasaccharide, and fraction VIII is a putative decasaccharide accounting for 11.9% and 10.9%, respectively, of all oligosaccharides, based on the absorbance at 210 nm. A portion of the putative octasaccharide fraction IX (1 mg) was subfractionated by anion-exchange HPLC on an amine-bound silica column into fractions IXa−IXt (Figure 2), while the putative decasaccharide fraction VIII (3.25 mg) was subfractionated into 23 different fractions designated VIIIa−VIIIw (Figure 3). Major fractions were desalted by gel filtration on a Sephadex G-25 column and quantified by the carbazole method. The total recovery of the putative octasaccharides (fraction IX) and decasaccharides (fraction VIII) after fractionation by HPLC and subsequent desalting was ~57% and 50%, respectively, and the yields of the isolated oligosaccharides are listed in Table 1. The apparent purity of each oligosaccharide fraction was checked by anion-exchange HPLC after labeling the oligosaccharides individually with the fluorophore 2AB as described in Experimental Procedures (data not shown). The results indicated that the purity of the isolated oligosaccharides varied from 72% to 98% due to the presence of contaminating minor compounds

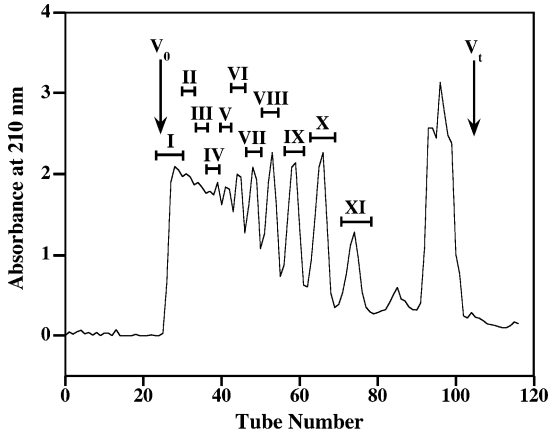


FIGURE 1: Gel filtration column chromatography of the hyaluronidase digest of squid cartilage CS-E on Bio-Gel P-10. A commercial preparation of squid cartilage CS-E was exhaustively digested with sheep testicular hyaluronidase as described under Experimental Procedures. The digest was fractionated on a Bio-Gel P-10 column (1.6 × 95 cm) using 1 M NaCl/10% ethanol as the eluent, collecting 2 mL fractions. The absorbance at 210 nm was measured, and separated fractions were pooled as indicated by horizontal bars. Fractions XI−IV correspond to tetra- to octadecasacharides based on a previously reported profile (*29*), while fractions I−III are oligosaccharides larger than an octadecasaccharide. V_0, void volume; V_t, total volume.

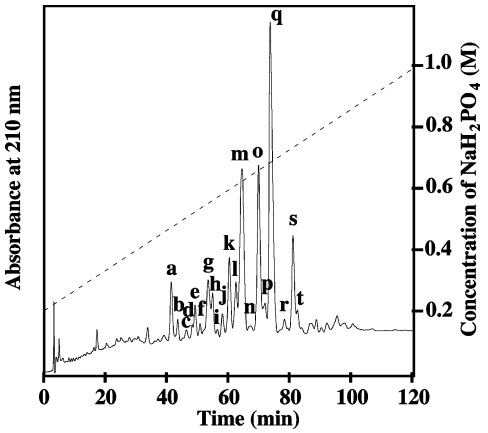


FIGURE 2: Subfractionation of the CS-E octasaccharide fraction (IX) by HPLC on an amine-bound silica column. The octasaccharide fraction (IX) obtained by size fractionation on a Bio-Gel P-10 column (Figure 1) was separated into subfractions on an amine-bound silica column with a linear gradient of NaH_2PO_4 from 0.2 to 1 M over 120 min, as indicated by the dashed line.

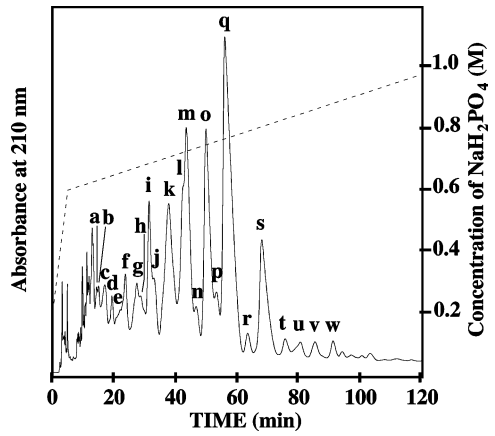


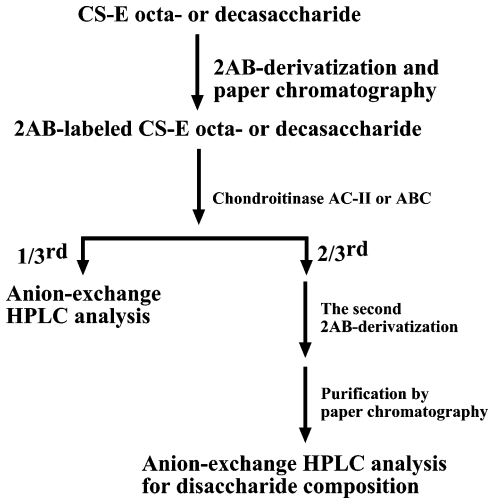
FIGURE 3: Subfractionation of the CS-E decasaccharide (VIII) fraction by HPLC on an amine-bound silica column. The decasaccharide fraction obtained by size fractionation on a Bio-Gel P-10 column (Figure 1) was separated into subfractions on an amine-bound silica column with a linear gradient of NaH_2PO_4 from 0.6 to 1 M over 115 min (from 5 to 120 min), as indicated by the dashed line.

in each individual preparation, except for fraction IXk which was only 47% pure.

Sequencing of Fraction VIIIo. All the isolated fractions were sequenced by the strategy outlined in Scheme 1. Among the oligosaccharide fractions only VIIIo, which is a putative decasaccharide, contained a reactive sequence for MO-225; hence the sequencing of the major component in VIIIo is described in detail. Fraction VIIIo was found to be 85−89% pure by enzymatic digestion and HPLC analysis, and the present sequencing strategy could successfully sequence this fraction.

The sugar composition and number of sulfate groups of the major compound in fraction VIIIo were determined by MALDI-TOF/MS by making a noncovalent complex with the basic peptide $(Arg\text{-}Gly)_{15}$ (*42*). The molecular mass of the detected decasaccharide was calculated to be 2636.1 by subtracting the measured *m*/*z* value of the protonated peptide (*m*/*z* 3218) from that of the protonated peptide/decasaccharide complex (*m*/*z* 5854.1) (Figure 4), which corresponds to a nonasulfated decasaccharide (Table 2). The disaccharide composition of fraction VIIIo was analyzed by digestion with

Scheme 1: Strategy for the Exosequencing of CS-E Octa- and Decasaccharides[a]



[a] Individual octa- or decasaccharide fractions (300 pmol) were derivatized with 2AB at the reducing termini. The excess 2AB reagent was removed by paper chromatography, and the 2AB-derivatized octa- or decasaccharide fractions were digested individually with chondroitinase ABC or AC-II. Aliquots from these digests were analyzed for 2AB-labeled unsaturated disaccharide or tetrasaccharide products by anion-exchange HPLC to identify the reducing terminal disaccharide unit in each octasaccharide (step 1), while the rest of each digest was further labeled with 2AB, purified by paper chromatography, and analyzed by anion-exchange HPLC to identify the nonreducing terminal and internal disaccharide units (step 2) (see Experimental Procedures).

chondroitinase ABC, and the resultant disaccharides were tagged with 2AB at the reducing terminus. Anion-exchange HPLC of the digest identified 2AB-labeled E [GlcUA-GalNAc(4S,6S)], ΔC [ΔHexUA-GalNAc(6S)], and ΔE [ΔHexUA-GalNAc(4S,6S)] as major products in a molar ratio of 1.0:0.60:3.0 (Table 1), where ΔHexUA represents 4,5-unsaturated hexuronic acid, being consistent with the results obtained by the MALDI-TOF/MS analysis. Thus, the major compound in fraction VIIIo consists of one C-unit and four E-units, one of the E-units being located at the nonreducing terminal and the others at the internal or reducing terminal positions. In addition to these major disaccharide units, a small proportion of ΔA (6.9%) was also detected (Table 1), presumably representing an impurity,

Table 1: Disaccharide Composition of the Isolated CS-E Oligosaccharides[a]

fraction	yield[b] (nmol)	apparent purity[c] (%)	disaccharides formed[d] (mol %) saturated GlcUA-GalNAc backbone	disaccharides formed[d] (mol %) unsaturated ΔHexUA-GalNAc backbone
IXk	60	47	C (16.2) + E (6.0) + A (3.2)	ΔE (49.6) + ΔC (15.8) + ΔA (9.2)
IXl	66	72	E (29.6) + C (12.5)	ΔE (28.5) + ΔA (20.6) + ΔC (8.8)
IXm	280	79	E (29.1) + C (2.6)	ΔE (39.9) + ΔA (25.4) + ΔC (3.0)
IXo	437	94	E (23.9)	ΔE (52.9) + ΔC (17.6) + ΔA (5.6)
IXq	863	98	E (24.8)	ΔE (53.9) + ΔA (20.3) + ΔC (1.0)
IXs	171	93	E (24.6) + A (1.4)	ΔE (69.8) + ΔA (4.2)
VIIIm	240	83	E (15.9) + C (7.9)	ΔE (47.6) + ΔA (23.8) + ΔC (4.8)
VIIIo	212	89	E (20.1)	ΔE (60.9) + ΔC (12.1) + ΔA (6.9)
VIIIq	427	97	E (20.5)	ΔE (63.7) + ΔA (15.8)
VIIIs	158	81	E (17.4) + C (2.3)	ΔE (74.9) + ΔA (3.1) + ΔC (2.3)

[a] Each oligosaccharide fraction was digested with chondroitinase ABC, and the released disaccharides were tagged with the fluorophore 2AB and subjected to anion-exchange HPLC on an amine-bound silica column. The identity of each peak was confirmed by comparing with the elution position of authentic 2AB-labeled disaccharides. [b] The amount of octa- and decasaccharide in each fraction obtained from 89 mg of CS-E was quantified by the carbazole method. [c] The apparent purity of each fraction was assessed by anion-exchange HPLC after 2AB labeling on an amine-bound silica column and was calculated on the basis of the peak area. [d] The molar ratio of the disaccharides was calculated in percentage terms from the total area of the peaks obtained by digestion with chondroitinase ABC on an amine-bound silica column.

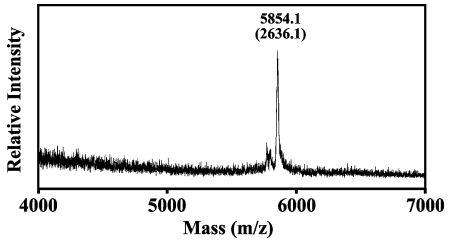


FIGURE 4: DE MALDI-TOF mass spectrum of the protonated complex of fraction VIIIo. Fraction VIIIo was mixed with the basic peptide $(Arg\text{-}Gly)_{15}$, and a DE MALDI-TOF mass spectrum of the protonated complex was recorded as described in Experimental Procedures. Signals of the noncovalent complex of the peptide and saccharides were observed. Shown in parentheses is the mass of the saccharide after subtraction of the peptide mass.

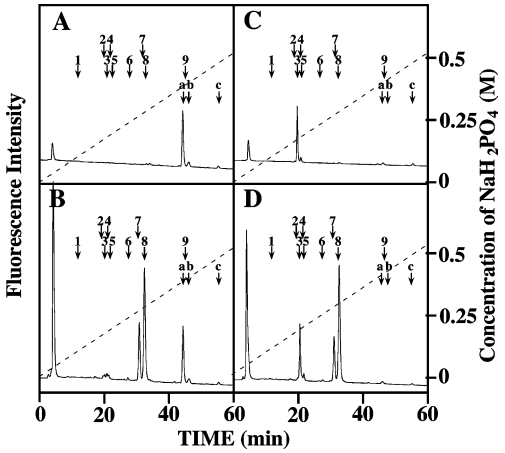


FIGURE 5: Sequencing of fraction VIIIo by enzymatic analysis. The 2AB-derivatized fraction VIIIo was digested with chondroitinase ABC (A) or AC-II (C), and each digest was analyzed by anion-exchange HPLC on an amine-bound silica column. The chondroitinase ABC digest in panel A and the AC-II digest in panel C were further labeled with 2AB and analyzed by HPLC on the same column, and the results are shown in panels B and D, respectively. The elution positions of authentic 2AB-labeled disaccharides are indicated by numbered arrows: 1, ΔDi-0S; 2, Di-6S; 3, ΔDi-6S; 4, Di-4S; 5, ΔDi-4S; 6, ΔDi-diS_D; 7, Di-diS_E; 8, ΔDi-diS_E; 9, ΔDi-triS. The elution positions of 2AB-labeled authentic tetrasaccharides are indicated by arrows marked by letters: a, ΔE-C (this sequence was identified for the first time in this study based on the sequences of the major compounds in fractions IXo and VIIIo determined by ^{1}H NMR spectroscopy); b, ΔE-A (*44*); c, ΔE-E (*44*).

containing an E-unit at the nonreducing terminal and a ΔE-unit at the internal position and/or reducing terminal.

For sequence determination, fraction VIIIo was first derivatized with 2AB at the reducing terminus of the constituent oligosaccharides, and the labeled chains were purified by paper chromatography to remove the derivatization reagents. Anion-exchange HPLC of a chondroitinase AC-II digest of the purified 2AB-labeled VIIIo showed ΔC to be the predominant 2AB-labeled product (85.7%) derived from the reducing terminal of the major compound together with small proportions of 2AB-labeled ΔA (8.4%) probably derived from minor components containing an A-unit at the reducing end and 2AB-labeled ΔE-C (1.3%) and ΔE-E (4.6%), which are presumably from products of incomplete digestion of the major and minor decasaccharides, respectively (Figure 5C and Table 3). A chondroitinase ABC digest of the 2AB-labeled VIIIo showed a major peak at the elution position of the 2AB-labeled ΔE-C tetrasaccharide derived from the reducing end accounting for 85.6% of the total peak area along with three minor tetrasaccharides, ΔE-A-2AB (9.3%), ΔE-E-2AB (3.3%), and ΔA-A-2AB (1.8%) (Figure 5A and Table 3) derived from probable minor components, suggesting that the major compound in fraction VIIIo is approximately 85% pure. These results suggest that the major compound in fraction VIIIo has the decasaccharide sequence E-E-E-E-C since the reducing terminal residue is a C-unit, as shown by digestion with chondroitinase AC-II. The digestion with chondroitinase ABC indicates the presence of at least three other minor decasaccharide components with the E-A, E-E, and A-A tetrasaccharide sequences on their reducing terminal sides, which together account for 14% of all the decasaccharides in fraction VIIIo.

The proposed sequence of the major compound in fraction VIIIo was confirmed below by tagging the released disaccharide with 2AB followed by anion-exchange HPLC. A portion of each digest of the 2AB-labeled VIIIo obtained with chondroitinase AC-II or ABC was derivatized again with 2AB to tag the resultant disaccharides derived from the nonreducing terminal and internal positions of the parent decasaccharides, and the 2AB-labled di- and tetrasaccharides were analyzed by anion-exchange HPLC after purification

Table 2: MALDI-TOF/MS Analysis of CS-E Octa- and Decasaccharides and Their Deduced Sugar and Sulfate Composition[a]

	oligosaccharides			
fraction	complex observed mass[b] (*m*/*z*)	observed mass[c] (*m*/*z*)	theoretical mass[d] (*m*/*z*)	deduced sugar and sulfate composition
IXk[e]	5234.9	2016.8	2014	$HexUA_4HexNAc_4(OSO_3H)_6$
IXl[e]	5233.7	2015.7	2014	$HexUA_4HexNAc_4(OSO_3H)_6$
IXm	5232.8	2014.8	2014	$HexUA_4HexNAc_4(OSO_3H)_6$
IXo	5310.4	2092.4	2094	$HexUA_4HexNAc_4(OSO_3H)_7$
IXq	5311.9	2093.9	2094	$HexUA_4HexNAc_4(OSO_3H)_7$
IXs	5394.0	2176.0	2174	$HexUA_4HexNAc_4(OSO_3H)_8$
VIIIm[e]	5777.7	2559.7	2553	$HexUA_5HexNAc_5(OSO_3H)_8$
VIIIo	5854.1	2636.1	2633	$HexUA_5HexNAc_5(OSO_3H)_9$
VIIIq	5854.1	2636.1	2633	$HexUA_5HexNAc_5(OSO_3H)_9$
VIIIs	5853.5	2635.5	2633	$HexUA_5HexNAc_5(OSO_3H)_9$
	5935.3	2717.3	2713	$HexUA_5HexNAc_5(OSO_3H)_{10}$

[a] Each octa- and decasaccharide fraction (10 pmol) was mixed with an equimolar amount of a basic peptide $(Arg\text{-}Gly)_{15}$ followed by a matrix 2,5-dihydroxybenzoic acid, and the spectrum was measured in the positive ion mode. [b] *m*/*z* value of the protonated 1:1 complex of the octa- or decasaccharide and the basic peptide $(Arg\text{-}Gly)_{15}$. [c] Observed mass of the octa- or decasaccharide obtained by subtracting the *m*/*z* value of the protonated peptide from that of the protonated 1:1 complex. [d] The theoretical masses calculated for the deduced structures. [e] An additional signal for the protonated 1:1 complex was observed, presumably due to a contaminant.

Table 3: Reducing Terminal Di- and Tetrasaccharides Released from 2AB-Derivatized Octa- or Decasaccharides by Digestion with Chondroitinase AC-II or ABC[a]

fraction	chondroitinase AC-II (mol %)	chondroitinase ABC (mol %)
IXk	**ΔC (75.3)**,[b] ΔA (2.3), ΔE-A (22.4)	**ΔE-C (60.8)**, ΔE-A (22.6), ΔC-C (16.6)
IXl	**ΔC (33.8)**, ΔA (4.9), **ΔA-C (39.6)**, ΔE-C (8.5), ΔE-A (8.0)	**ΔA-C (78.7)**, ΔE-C (8.5), ΔA-A (8.0), ΔE-A (4.8)
IXm	**ΔA (58.7)**, **ΔE-A (26.4)**, ΔA-A (12.1), ΔC-A (2.8)	**ΔA-A (56.5)**, **ΔE-A (35.1)**, ΔC-A (8.4)
IXo	**ΔC (87.4)**, ΔA (1.8), ΔE-A (4.5), ΔE-C (3.2), ΔE-E (3.1)	**ΔE-C (89.4)**, ΔE-A (6.9), ΔE-E (3.7)
IXq	**ΔA (79.7)**, ΔC (1.9), ΔE-A (18.4)	**ΔE-A (98.5)**, ΔE-C (1.5)
IXs	ΔE (6.8), **ΔE-E (87.4)**, ΔE-A (5.8)	**ΔE-E (94.1)**, ΔE-A (5.9)
VIIIm	**ΔA (82.6)**, ΔC (7.7), ΔE-A (9.7)	**ΔA-A (58.4)**, **ΔE-A (34.9)**, ΔC-A (6.7)
VIIIo	**ΔC (85.7)**, ΔA (8.4), ΔE-E (4.6), ΔE-C (1.3)	**ΔE-C (85.6)**, ΔE-A (9.3), ΔE-E (3.3), ΔA-A (1.8)
VIIIq	**ΔA (75.7)**, ΔC (5.1), ΔE-A (19.2)	**ΔE-A (96.4)**, ΔE-C (3.6)
VIIIs	**ΔE (29.9)**, ΔA (19.5), ΔC (13.1), **ΔE-E (37.5)**	**ΔE-E (79.5)**, ΔE-A (20.5)

[a] Each octa- or decasaccharide fraction was labeled with 2AB at the reducing terminus and was digested separately with chondroitinase AC-II or ABC. Each digest was analyzed by anion-exchange HPLC on an amine-bound silica column to determine the reducing terminal sequences of the major and minor components. [b] Di- or tetrasaccharide units in bold letters indicate the major component released by each digestion.

Table 4: Deduced Novel Octa- and Decasaccharide Sequences Isolated from Squid Cartilage CS-E

fraction	proposed structure (%)[a]	fraction	proposed structure (%)[a]
IXk	C-E-E-C (47)	IXs	E-E-E-E (90−92)[b]
IXl	E-E-A-C (73−79)		E-E-E-A (6)
	E-E-A-A (8)		E-C-E-E (1−2)
	C-E-E-C (8.5)		A-E-E-E (1−2)
	C-E-E-A (4.8)	VIIIm	E-E-E-A-A (58)
IXm	E-E-A-A (57)		C-E-E-E-A (35)
	C-E-E-A (35)		E-E-E-C-A (7)
	E-E-C-A (8)	VIIIo	E-E-E-E-C (86)
IXo	E-E-E-C (89)[b]		E-E-E-E-A (9)
	E-E-E-A (7)		E-E-E-A-A (2)
	E-A-E-E (4)	VIIIq	E-E-E-E-A (95−96)
IXq	E-E-E-A (98)[b]		E-E-E-E-C (3−5)
	E-E-E-C (2)	VIIIs	E-E-E-E-E (79)
			E-E-E-E-A (21)

[a] Numbers in parentheses represent the percentage of the corresponding sequence in each fraction. [b] These sequences have been reported (*45*).

by paper chromatography (Scheme 1). The 2AB-labeled chondroitinase AC-II digest contained 2AB derivatives of E, ΔC, and ΔE at a ratio, based on peak area, of 1.0:0.92:2.8 with minor di- and tetrasaccharides derived presumably from contaminants (Figure 5D and Supporting Information, Table S1). The 2AB-labeled chondroitinase ABC digest contained 2AB derivatives of E, ΔE, and ΔE-C at a ratio of 1.0:2.2:0.97 (Figure 5B) with several minor components derived from contaminating oligosaccharides (Supporting Information, Table S1). Taken together, it was concluded that the sequence of the major compound in VIIIo representing 85% of all the decasaccharides in the fraction is E-E-E-E-C [GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(6S)] (Table 4), which is consistent with the proposed sequence. The structure was confirmed by ^{1}H NMR spectroscopy (Supporting Information, Figure S1 and Table S2).

The three minor decasaccharides, which constitute 14% of all the decasacharides, have ΔE-A (9.3%), ΔE-E (3.3%), and ΔA-A (1.8%) as their reducing terminal ends (Table 3). Since only an E-unit was released from the nonreducing terminal by digestion with chondroitinases, all of the decasaccharides in fraction VIIIo have an E-unit at the nonreducing terminus. Since digestion of the 2AB-labeled VIIIo with chondroitinase ABC released ΔE as the predominant unsaturated disaccharide (Supporting Information, Table S1), all of the decasaccharides in fraction VIIIo contain ΔE-units in their internal positions. Hence, the sequences of the minor components in fraction VIIIo were deduced as E-E-E-E-A (9.3%) and E-E-E-A-A (1.8%), which were found to be the major components in fractions VIIIq and VIIIm, respectively (see below).

Using the same strategy, fractions IXk, IXl, IXm, IXo, IXq, and IXs, as well as VIIIm, VIIIq, and VIIIs, were also sequenced. Di- and tetrasaccharide sequences released by chondroitinases AC-II and ABC from the reducing termini of the octa- and decasaccharides in each fraction are summarized in Table 3. Digestion of the 2AB-labeled oligosaccharides with chondroitinase AC-II mainly generated an unsaturated 2AB-labeled disaccharide from the reducing terminus, along with some undigested 2AB-labeled tetrasaccharides. In contrast, digestion of the 2AB-labeled oligosaccharide with chondroitinase ABC generated unsaturated 2AB-labeled tetrasaccharides for all of the octa- and decasaccharide fractions.

Sequencing of Fraction IXk. The data obtained from the MALDI-TOF/MS analysis suggested that the major compound in fraction IXk is a hexasulfated octasaccharide (Table 2). A disaccharide analysis of the chondroitinase ABC digest after 2AB labeling identified 2AB derivatives of three saturated disaccharides, C, E, and A, in a molar ratio of 64:24:12 released from the nonreducing terminal, indicating the presence of at least three compounds in fraction IXk. A chondroitinase AC-II digest of 2AB-labeled IXk revealed ΔC-2AB (75.3%), while a chondroitinase ABC digest revealed ΔE-C-2AB (60.8%), to be the major product (Table 3), indicating an E-C tetrasaccharide sequence at the reducing terminus of the major component. In view of the findings that the total number of sulfate groups is 6 and ΔE is the major unit derived from the internal disaccharide (Table 1), it was deduced that the major compound of IXk, which accounts for approximately 47% (Table 1) of the fraction, is C-E-E-C [GlcUAβ1−3GalNAc(6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(6S)]. The minor components, which contain E-A (22.6%) and C-C (16.6%), respectively, at the reducing terminus, are assumed to have C-E-E-A, E-A-E-A, and E-E-C-C sequences. Another possible minor component such as A-E-E-C may also be present, which corresponds to the difference in the mole percentage of the major component (47%, Table 1) and ΔE-C (60.8%, Table

3). However, identification is difficult due to the mixed nature of the fraction and has to await the isolation of each sequence.

Sequencing of Fraction IXl. The major compound in fraction IXl is a hexasulfated octasaccharide as evidenced by the results of MALDI-TOF/MS (Table 2). A disaccharide analysis of the chondroitinase ABC digest after 2AB labeling identified 2AB derivatives of E, ΔA, and ΔE as major products in a molar ratio of 1.0:0.70:0.96 (Table 1). In addition, a significant proportion (30% when the total proportion of C- and E-units was taken as 100%) of the C-unit was also released from the nonreducing terminal, indicating the presence of another compound in fraction IXl. Anion-exchange HPLC of the chondroitinase ABC digest of the 2AB-labeled IXl showed the 2AB derivative of ΔA-C to be the major product (78.7%), while chondroitinase AC-II released a ΔC-unit (33.8%) from the reducing terminus along with undigested ΔA-C (39.6%), suggesting that fraction IXl is 73−79% pure (Table 3). 2AB labeling and HPLC of the chondroitinase ABC digest showed E, ΔE, and ΔA-C as major products in a molar ratio of 1.4:1.1:1.0 (Supporting Information, Table S1), suggesting the sequence E-E-A-C for the major compound in fraction IXl.

In addition to the major product, chondroitinase ABC released ΔA-A (8%), ΔE-A (4.8%), and ΔE-C (8.5%) from the reducing terminus, indicating the presence of three minor compounds in fraction IXl. The minor di- and tetrasacccharides released by the chondroitinases are listed in Supporting Information, Table S1. On the basis of these results, the sequence of the major compound in fraction IXl, which is 73−79% pure, is E-E-A-C [GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S)β1−4GlcUAβ1−3GalNAc(6S)] (Table 4). The sequence of one of the minor components could be E-E-A-A (8%), which is the major compound in fraction IXm (see below), while the other two minor compounds have the sequence C-E-E-C (8.5%) and C-E-E-A (4.8%) in view of the presence of a "C" unit at the nonreducing end and a total of six sulfate groups. The possibility of E-C-E-C and E-C-E-A cannot be excluded.

Sequencing of Fraction IXm. The results of MALDI-TOF/MS indicated a molecular mass of 2014.76 for the major compound, which corresponded to a hexasulfated octasaccharide (Table 2). A disaccharide composition analysis of the chondroitinase ABC digest identified 2AB-labeled E, ΔA, and ΔE as major products in a molar ratio of 1.0:0.87:1.4 (Table 1). In addition to the E-unit, an appreciable proportion (8.2% when the total proportion of C- and E-units was taken as 100%) of the C-unit was released from the nonreducing terminus (Table 1), indicating that fraction IXm contains a mixture of at least two compounds. Anion-exchange HPLC of the chondroitinase AC-II digest of the 2AB-labeled IXm showed 2AB-labeled ΔA (58.7%), while that of the chondroitinase ABC digest showed ΔA-A-2AB (56.5%), to be the major tetrasaccharide product released from the reducing terminus (Table 3), suggesting that the apparent purity of fraction IXm is approximately 57−59%. Derivatization with 2AB of the chondroitinase AC-II digest of the 2AB-labeled IXm showed 2AB derivatives of E, ΔE, and ΔA units as major products in a ratio of 1.0:1.3:0.7 (Supporting Information, Table S1), while 2AB labeling of the chondroitinase ABC digest by HPLC generated 2AB derivatives of E, ΔE, and ΔA-A in a ratio of 1.0:1.3:0.9 (Supporting Information, Table S1). While the ratio of the di- and tetrasacchrides generated by chondroitinase ABC agrees with the proposed sequence, the ratio of the disaccharides generated by chondrotinase AC-II is only roughly consistent with the proposed sequence. This could be partly due to minor components and also due to a possible lower 2AB-labeling efficiency (*14*). Since ΔA-A was the major tetrasaccharide released from the reducing terminus, the E-unit was the major disaccharide released from the nonreducing terminal, and the ΔE-unit was the major disaccharide released from the internal position, the sequence of the major compound in fraction IXm, which accounts for ∼56% of all the octasaccharides, was concluded to be E-E-A-A [GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S)β1−4GlcUAβ1−3GalNAc(4S)]. This sequence was confirmed by ^{1}H NMR spectroscopy (Supporting Information, Table S3). The sequence of the other component is possibly C-E-E-A (35%), since a C-unit is released as the nonreducing terminal residue, a ΔE-unit is the major internal disaccharide, and ΔE-A is the reducing terminal tetrasaccharide (Table 3). The sequence of the other minor component with ΔC-A as the reducing terminal residue (8.4%) could be E-E-C-A (Table 3).

Sequencing of Fraction IXo. The major compound in fraction IXo is a heptasulfated octasaccharide as evidenced by the results of MALDI-TOF/MS (Table 2). A disaccharide analysis of the chondroitinase ABC digest after 2AB labeling identified 2AB derivatives of E, ΔC, and ΔE as major products in a molar ratio of 1.0:0.73:2.2 (Table 1), indicating the presence of three E-units and one C-unit. HPLC of the chondroitinase AC-II and ABC digests of the 2AB-labeled IXo revealed 2AB derivatives of ΔC and ΔE-C to be the major products, accounting for 87.4% and 89.4%, respectively, of all the di- and tetrasaccharides released, indicating that fraction IXo is 87−89% pure (Table 3).

Chondroitinase ABC released ΔE-A (6.9%) or ΔE-E (3.7%) from the reducing terminus of the minor compounds in IXo (Table 3), indicating the presence of at least three components in the fraction. The other minor di- and tetrasaccharides released by these digestions are listed in Table 3. 2AB labeling and HPLC of the chondroitinase AC-II digest identified E, ΔE, and ΔC in a molar ratio of 1.0:1.7:1.3 (Supporting Information, Table S1), suggesting the sequence E-E-E-C. Derivatization with 2AB and HPLC of the chondrotinase ABC digest of the 2AB-labeled IXo showed 2AB derivatives of E, ΔE, and ΔE-C to be present in a molar ratio of 1.0:1.1:1.5 (Supporting Information, Table S1), which is consistent with the proposed sequence. On the basis of these results, the sequence of the major compound in fraction IXo was concluded to be E-E-E-C [GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(6S)] (Table 4). This sequence was confirmed by ^{1}H NMR spectroscopy (Supporting Information, Table S3). One of the minor compounds in fraction IXo is E-E-E-A (6.9%), while the sequence of the other component having an ΔE-E tetrasaccharide at the reducing terminus is E-A-E-E (3.7%), since a small proportion of ΔA-unit, probably derived from the internal position, was detected by digestion with chondroitinase ABC (Supporting Information, Table S1).

Sequencing of Fraction IXq. The data from the MALDI-TOF/MS analysis suggested that the major compound in fraction IXq is a heptasulfated octasaccharide (Table 2). A disaccharide analysis of the chondroitinase ABC digest after 2AB labeling identified 2AB derivatives of E, ΔA, and ΔE as major products in a molar ratio of 1.0:0.82:2.2, indicating the presence of three E-units and one A-unit (Table 1). HPLC of the chondroitinase AC-II digest of the 2AB-labeled IXq showed ΔA-2AB (79.7%) (Table 3), while that of the chondroitinase ABC digest showed ΔE-A-2AB (98.5%), to be the major product (Table 3), suggesting an E-E-E-A sequence for the major octasaccharide. The purity of fraction IXq is about 98% as indicated by the digestion with chondroitinase ABC.

Digestion with chondroitinase AC-II revealed ΔA-2AB (79.7%) and a small proportion of undigested ΔE-A-2AB (18.4%), which is most likely derived from the major component due to incomplete digestion. In addition, a small proportion of ΔE-C (1.5%) was also released by the chondrotinase, probably derived from a minor component contaminating the preparation. Anion-exchange HPLC of the 2AB-labeled chondroitinase AC-II digest revealed a 2AB-labeled E-unit at the nonreducing terminus and a 2AB-labeled ΔE at the internal position, in addition to the 2AB-labeled ΔA at the reducing terminus with 2AB derivatives of E, ΔA, and ΔE in a ratio of 1.0:1.0:2.0, being consistent with the proposed structure. Derivatization with 2AB of the chondroitinase ABC digest followed by HPLC identified 2AB derivatives of E, ΔE, and ΔE-A in a ratio of 0.63:0.72:1.0 (Supporting Information, Table S1), confirming the proposed sequence. On the basis of these results, the sequence of the major compound in fraction IXq was concluded to be E-E-E-A [GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc-(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S)] (Table 4). This sequence was confirmed by proton NMR spectroscopy (Supporting Information, Figure S2 and Table S3). Besides the major compound, fraction IXq contains a minor component with the sequence E-E-E-C (1.5%), which was deduced on the basis of the digestions described above, and turned out to be the major compound in fraction IXo.

Sequencing of Fraction IXs. The results of the MALDI-TOF/MS analysis indicated a molecular mass of 2176.0 for the major compound, which corresponded to an octasulfated octasaccharide (Table 2). A disaccharide composition analysis of a chondroitinase ABC digest showed only E and ΔE as major components in a molar ratio of 1:2.8 (Table 1), suggesting that the major compound in fraction IXs is probably composed of four E-units. In addition to the E-units, a small proportion of the A-unit was also released from the nonreducing terminal, indicating the presence of a minor component with an A-unit at the nonreducing terminus. The analytical data obtained by the digestion of the 2AB-labeled IXs with chondroitinases AC-II and ABC showed 2AB derivatives of ΔE-E to be the major product, accounting for 87.4% and 94.1%, respectively, of all the di- or tetrasaccharides (Table 3). Since chondroitinase AC-II released ΔE-unit (6.8%) in addition to the ΔE-E tetrasaccharide (87.4%), the apparent purity of fraction IXs is 94% as shown by digestion with chondroitinase ABC. In addition, a small proportion of ΔE-A (5.8%) was also detected, which was probably derived from the reducing terminal of a minor contaminant in fraction IXs. Derivatization with 2AB of the chondroitinase AC-II and ABC digests followed by HPLC identified 2AB derivatives of E, ΔE, and ΔE-E in a ratio of 1.0:1.1:0.8 and 1.0:1.1:1.1, respectively (Supporting Information, Table S1), which is in good agreement with the proposed sequence. Hence, the sequence of the major compound in fraction IXs, which accounts for 94% of all the octasaccharides, was concluded to be E-E-E-E [GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc-(4S,6S)] (Table 4). This sequence was confirmed by ^{1}H NMR spectroscopy (Supporting Information, Table S3). The sequence of the minor compound was deduced as E-E-E-A (5.8%), based on the data from the enzymatic digestions described above. Small proportions of the ΔC-unit (1.3−2.1%) were released from the internal position, suggesting the existence of an E-C-E-E sequence. The presence of an A-unit (1.1−2.2%) at the nonreducing terminal suggests another minor contaminant with an A-E-E-E sequence.

Sequencing of Fraction VIIIm. The major compound in fraction VIIIm is an octasulfated decasaccharide, as indicated by the data obtained from MALDI-TOF/MS (Table 2). A disaccharide analysis of the chondroitinase ABC digest of fraction VIIIm after 2AB labeling identified 2AB derivatives of E, ΔA, and ΔE as major products in a molar ratio of 1.0:1.5:3.0 (Table 1). In addition, a considerable proportion (33%) of the C-unit was released from the nonreducing terminus, suggesting the presence of another compound in fraction VIIIm. Anion-exchange HPLC of the chondroitinase AC-II digest of the 2AB-labeled VIIIm showed a 2AB derivative of ΔA (82.6%) at the reducing terminus as the major compound, while the chondroitinase ABC digest released ΔA-A-2AB (58.4%) and ΔE-A-2AB (34.9%) as major products in addition to ΔC-A-2AB (6.7%) derived from the minor contaminant (Table 3), suggesting that fraction VIIIm is a mixture of at least three compounds. HPLC of the 2AB-labeled chondroitinase AC-II digest revealed a 2AB-labeled E-unit and C-unit at the nonreducing terminus, a 2AB-labeled ΔE and ΔA as major units at the internal position and/or reducing terminus, and 2AB-labeled C, E, ΔA, and ΔE in a ratio of 0.3:1.0:1.1:3.1 (Supporting Information, Table S1). The 2AB-labeled chondroitinase ABC digest contained C, E, ΔE, ΔA-A, and ΔE-A in a ratio of 0.3:1.0:2.9:0.6:0.4 (Supporting Information, Table S1). Since ΔA-A released from the reducing terminus was the major tetrasaccharide product, an E-unit was the major disaccharide at the nonreducing terminus, and ΔE was released from the internal position, the sequence of the major compound in fraction VIIIm is E-E-E-A-A [GlcUAβ1−3GalNAc(4S,6S)-β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc-(4S,6S)β1−4GlcUAβ1−3GalNAc(4S)β1−4GlcUAβ1−3GalNAc(4S)].

Since ΔE-A was the second major tetrasaccharide released from the reducing terminal (34.9%), a C-unit was released from the nonreducing terminal (33%), and ΔE was released from the internal position, the sequence of the other major compound in fraction VIIIm is C-E-E-E-A [GlcUAβ1−3GalNAc(6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S)] (Table 4). The sequence of the other component, which yielded ΔC-A (6.7%) from the reducing terminus, is E-E-E-C-A.

Sequencing of Fraction VIIIq. The major compound in fraction VIIIq is a nonasulfated decasaccharide, as evidenced by the data obtained from the MALDI-TOF/MS analysis (Table 2). A disaccharide composition analysis of the chondroitinase ABC digest identified E, ΔA, and ΔE in a molar ratio of 1.0:0.77:3.1 (Table 1), suggesting the presence of four E-units and one A-unit. For sequence determination, 2AB-labeled VIIIq was digested with chondroitinase AC-II, and HPLC of the digest showed 2AB-labeled ΔA derived from the reducing terminus to be the major product (75.7%) along with a minor proportion of undigested ΔE-A (19.2%), while chondroitinase ABC released ΔE-A-2AB as the major product (96.4%) from the reducing terminal, suggesting an apparently high purity (96%) for the major compound in this fraction (Table 3). In addition, a small proportion of ΔE-C (3.6%) was released probably from the reducing terminus of a minor component. Derivatization with 2AB of the chondroitinase AC-II digest followed by HPLC identified 2AB derivatives of E, ΔA, and ΔE in a ratio of 1.0:0.69:2.9, while E, ΔE, and ΔE-A were detected in a ratio of 1.0:2.0:0.92 from the analysis of the chondroitinse ABC digest (Supporting Information, Table S1). From these results, the sequence of the major compound in fraction VIIIq, which accounted for approximately 96% of all the decasaccharides, was concluded to be E-E-E-E-A [GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S)]. Digestion of 2AB-labeled VIIIq with chondroitinase AC-II or ABC revealed a ΔC-unit (5.1%) and ΔE-C (3.6%), respectively (Table 3), suggesting that the sequence of the minor component in VIIIq is E-E-E-E-C [GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1-3GalNAc(6S)], which is the major component in fraction VIIIo.

Sequencing of Fraction VIIIs. The data from the MALDI-TOF/MS analysis suggested that the major compound in fraction VIIIs contains a decasulfated decasaccharide and a nonasulfated decasaccharide (Table 2). A disaccharide composition analysis of a chondroitinase ABC digest showed only E and ΔE as major components in a molar ratio of 1.0:4.3 (Table 1), suggesting that the major compound in fraction VIIIs is probably composed of five E-units. The analytical data obtained by digestion of the 2AB-labeled VIIIs with chondroitinase AC-II revealed 2AB derivatives of ΔE-E and ΔE to be the major products, the sum of which accounted for 67.4% of all the di- or tetrasaccharides, while digestion with chondroitinase ABC identified a 2AB derivative of ΔE-E as the major product (79.5%) (Table 3), suggesting an apparent purity of 67.4−79.5% for the major product. In addition, a small proportion of ΔE-A (20.5%) was also detected in the chondroitinase ABC digest, which was probably derived from the reducing terminal of a minor component in fraction VIIIs. Derivatization with 2AB of the chondroitinase AC-II and ABC digests followed by HPLC identified 2AB derivatives of E, ΔE, and ΔE-E in a ratio of 1.0:3.3:0.46 and 1.0:2.7:0.65, respectively (Supporting Information, Table S1). The apparent inconsistency in the ratio with the proposed sequence could be due to the presence of undigested ΔE-E tetrasaccharides in both digests. From these results, the sequence of the major compound in VIIIs, which accounted for 79% of all the decasaccharides in the fraction, was concluded to be E-E-E-E-E [GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)β1−4GlcUAβ1−3GalNAc(4S,6S)]. The sequence of the minor component (21%) in fraction VIIIs was deduced to be E-E-E-E-A, based on the data from the enzymatic digestions described above, and is the major component in fraction VIIIq.

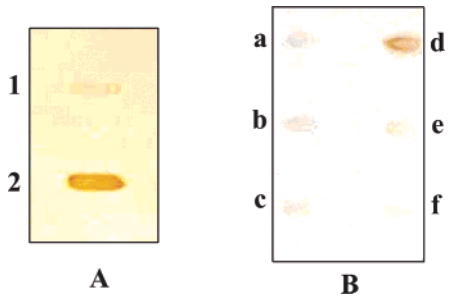


FIGURE 6: Reactivity of MO-225 with a mixture of CS-E octa- and decasaccharides and structurally defined decasaccharides. A mixture of octa- (A1) or decasaccharides (A2) or structurally defined decasaccharides (B) derived from squid cartilage CS-E was derivatized with DPPE, and 25 pmol (for A1 and A2) or 5 pmol (B) of each derivative was immobilized on a nitrocellulose membrane, and their reactivity with MO-225 was tested as described in Experimental Procedures. The structurally defined octasaccharides are as follows: a, fraction VIIIk, and b, fraction VIIIl, sequences unknown; c, fraction VIIIm with E-E-E-A-A as a major component; d, fraction VIIIo with E-E-E-E-C as a major component; e, fraction VIIIq with E-E-E-E-A as a major component; f, fraction VIIIs with E-E-E-E-E as a major component.

Reactivity of the mAb MO-225 with Structurally Defined Decasaccharides. MO-225 was developed against PG-M purified from the chick embryonic limb bud, and its determinant contains D-units (*34*). In contrast, squid cartilage CS-E, which does not contain D-units, also provides significant inhibition (*34*) though the reason for this unrelated action is unclear. Even though it has been reported that A-D and E-D tetrasaccharide sequences are capable of binding MO-225 (*34*), the determinant in CS-E which binds MO-225 is not known. Hence, the interaction of MO-225 with the structurally defined CS-E octa- and decasaccharides, isolated in this study, was investigated using an oligosaccharide microarray (*36*).

A mixture of octa- and decasaccharides (25 pmol each) was derivatized with DPPE as described in Experimental Procedures, and the resultant neoglycolipids were separately immobilized on a nitrocellulose membrane, and their reactivity with MO-225 was tested to examine the minimum size requirement of the oligosaccharides for the antibody to bind (Figure 6A). While at the tested concentration an octasaccharide mixture did not show any reactivity, a decasaccharide mixture showed strong reactivity with MO-225, suggesting that a CS-E decasaccharide could be the minimal size for the binding. Consistent with this observation, none of the structurally defined octasacchairdes, which were isolated in this study, showed a positive reaction with MO-225 (data not shown).

Since MO-225 exhibited strong reactivity with a CS-E decasaccharide mixture, its reactivity with structurally defined individual decasaccharides was tested. Six different decasaccharide fractions (VIIIk, VIIIl, VIIIm, VIIIo, VIIIq, and VIIIs), the major components of four of which were

structurally defined, were derivatized with DPPE and tested to identify the binding sequence. Among the tested fractions, only fraction VIIIo, which contained E-E-E-E-C as the major compound, was recognized by MO-225 (Figure 6B). It is interesting that MO-225 did not react with fractions VIIIm, VIIIq, and VIIIs, which contained E-E-E-A-A, E-E-E-E-A, and E-E-E-E-E sequences, respectively, as the major compounds. All of the four decasaccharides share a common E-unit at their nonreducing terminal with two other E-units adjacent to it, while they differ from each other in their reducing terminal tetrasaccharide sequences. Hence, it was concluded that the reactivity of MO-225 with fraction VIIIo could be due to an E-C sequence at the reducing terminal, since none of the other fractions gave a positive reaction. Interestingly, MO-225 did not show any reactivity with the octasaccharide fraction IXm (E-E-E-C), which also has an E-C tetrasaccharide at the reducing terminal, suggesting that a decasaccharide with an E-C unit at the reducing terminal is the minimal requirement for recognition by the antibody. Hence, in the absence of a D-unit, which is an essential requirement for MO-225's recognition, a CS-E oligosaccharide longer than an octasaccharide having an E-C tetrasaccharide unit at the reducing terminal can bind MO-225. It should be noted, however, that the reducing terminal GalNAc in each neoglycolipid has a linear form since it is conjugated with DPPE and may be only partially recognized by the antibody.

DISCUSSION

In the present study, we generated octa- and decasaccharides from squid cartilage CS-E by exhaustive digestion with testicular hyaluronidase. Even though testicular hyaluronidase exhibits transglycosylation activity, the oversulfated CS variants, CS-D and CS-E,[3] do not serve as substrates for transglycosylation reactions under the established optimum conditions (*46*). By anion-exchange HPLC, the mixture of octa- and decasaccharides was separated into 20 and 23 subfractions, respectively, and each of these fractions turned out to be a mixture of two or four different sequences, reflecting the complexity of the parent CS-E polysaccharides. CS-E derived from squid cartilage contains the E-unit as a major component (62.3%) along with A- (20.9%), C- (11.3%), and O-units (5.5%) (*41*). Hence, the presence of multiple E-units in all of the deduced sequences is not surprising. The major compounds in fractions IXs and VIIIs are composed solely of E-units, and the existence of such sequences with consecutive E-units is interesting, since they can form highly charged domains and hence may explain the Hep-like activity exhibited by CS-E, such as the anticoagulant activity (*47*). Even though the octasaccharide sequences E-E-E-C (IXo), E-E-E-A (IXq), and E-E-E-E (IXs) have been reported previously (*45*), all of the other sequences reported here are novel.

While an E-unit is present at the nonreducing end of the majority of octa- and decasaccharides, either an A-, C-, or E-unit was present at the reducing terminal end. The presence of two or three consecutive E-units in octasaccharides and three or four consecutive E-units in decasaccharides is a common feature. While consecutive A-units are found together with E-units forming an E-A-A sequence, such sequences are not formed with C-units. In addition, the presence of an E-E tetrasaccharide unit is a common feature in all of the deduced sequences and hence may reflect the specificity of the machinery producing this complex polysaccharide. Comparison of the sequences of the major components of octa- and decasaccharides revealed the reducing side octasaccharides of the decasaccharides to be similar to the isolated octasaccharides, except for the presence of an additional E-unit at the nonreducing terminus.

The sequencing was accomplished by the enzymatic digestion of 2AB-labeled oligosaccharides in conjunction with anion-exchange HPLC, a strategy that was successfully used to sequence octasaccharides from shark cartilage CS-C (Deepa et al., unpublished results).[2] In the present study, we have demonstrated that the strategy is applicable to the sequencing of even decasaccharides, and this is the first demonstration of the successful sequencing of a decasaccharide by enzymatic digestion in conjunction with HPLC. Previously, various tetra- and hexasaccharides were isolated from squid cartilage CS-E and sequenced by ^{1}H NMR spectroscopy in conjunction with HPLC (*29*, *30*). It is surprising that while both of these preparations contained a 3-*O*-sulfate GlcUA in most of the isolated fractions, such a unit is absent in the present octa- and decasaccharide preparations and hence may reflect the differences in the parental CS-E preparations used for their isolation. The enzymatic digestion of octa- and decasaccharides revealed the specificity of various chondroitinases toward 2AB-labeled CS-E oligosaccharides. Chondroitinase AC-I did not effectively digest 2AB-labeled octa- or decasaccharides (data not shown), which may indicate that clusters of the E-unit resist further action by the enzyme. In contrast, chondroitinase AC-II effectively cleaved the 2AB-labeled octa- and decasaccharides into four and five disaccharides, respectively, but ΔE-E-2AB and ΔE-A-2AB were partially resistant to the enzymatic action (Table 3). In contrast, digestion of octa- and decasaccharides with chondroitinase ABC released two and three disaccharides, respectively, along with 2AB-labeled tetrasaccharides from the reducing terminus, reflecting that 2AB-labeled tetrasaccharides are resistant to the action of chondroitinase ABC (*41*).

The reactivity of MO-225 toward these structurally defined octa- and decasaccharides was achieved using an oligosaccharide microarray, revealing the importance of chain length and sequence specificity for antibody recognition. Our study demonstrated that only the E-E-E-E-C sequence among the octa- and decasaccharides tested was recognized by MO-225. Our previous findings had shown that MO-225 specifically recognizes a C-A-D-C octasaccharide sequence derived from shark cartilage CS-C (Deepa et al., unpublished results,)[2] and all of the reported MO-225-binding sequences have A-D-C or A-D-A (*35*) at the reducing terminus. The present finding strongly supports our previous hypothesis (Deepa et al., unpublished results)[2] that a single antibody can recognize multiple sequences, suggesting that the common conformation shared by the multiple primary sequences in the intact CS chain is important as an epitope for the antibody. Recent studies using ^{1}H NMR spectroscopy and molecular modeling on the conformation of multiple structurally defined octasaccharides, which were isolated from

[3] K. Takagaki, Hirosaki University, Hirosaki, Japan, personal communication.

CS-D and recognized by MO-225 and CS-56, have revealed the importance of the 2-*O*-sulfate group of GlcUA and an exocyclic negative tail in C and ΔC disaccharides on the same core A-D tetrasaccharide sequence (*48*) and support the above hypothesis.

Even though marine organisms are the sources of highly sulfated CS chains, the presence of E-units has been demonstrated in bovine brain (*11*), chicken brain (*16*), mouse brain (*14*, *17*), the serglycine PG of mouse mast cells (*49*, *50*), and human knee cartilage, where the E-unit content of the nonreducing terminal region changes with age (*51*), indicating the existence of this unit in higher animals. The biological significance of the E-unit has been demonstrated by the inhibition of MK-mediated neuronal cell adhesion by CS-E (*15*), stimulation of the outgrowth of neurites in hippocampal neurons by CS-E (*26*), CS/DS hybrid chains containing E-units from shark skin (*52*) and shark liver (*53*), and a synthetic tetrasaccharide composed of two E-units (*54*). Other CS-E-related observations include the strong binding of MK to a GAG fraction with 32.3% E-unit from the brain of a 13-day-old mouse embryo (*55*), the binding of the appican CS chain from rat C6 glioma cells with 14.3% E-unit to several growth/differentiation factors (*56*), the binding of CS-E to type V collagen (*45*), the binding of photomedilins, novel olfactomedin domain-containing proteins, to CS-E (*57*), and the recent discovery of the formation of collagen fibrils of perlcan CS-PG containing E-units (*58*). Infections of herpes simplex virus also involve CS chains containing E-units at the cell surface (*24*). These findings suggest the importance of preparing different GAG oligosaccharides as investigational tools to analyze structural−functional relationships, which in turn will lead to new therapeutic strategies.

Even though oligosaccharides have been prepared by chemical synthesis (*54*, *59*−*61*), taking advantage of transglycosylation activities of glycosidases (*62*, *63*) and also using various bacterial chondroitinases (*5*, *64*), the current strategy of generating a library of oligosaccharides from a parent polysaccharide opens up the opportunity to generate more diverse structures that exist naturally. The sequencing of such CS/DS oligosaccharides by NMR (*5*, *65*, *66*) requires large amounts of materials or sequential digestions with various chondroitinases in conjunction with high-resolution HPLC (*5*) or capillary electrophoresis (*64*). In the future, with advances in technology, it should be possible to sequence oligosaccharides even from different cell types. The methodology utilizing a combination of enzymatic digestion and mass spectrometry (ESI-MS and ESI-MS^n) has recently been developed for sequencing of heparin hexa- (*67*) and octasaccharides (*68*) and has a potential for application to the sequencing of CS/DS oligosaccharides.

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SUPPORTING INFORMATION AVAILABLE

HPLC analysis of di- and tetrasaccharides released from 2AB-derivatized octa- or decasaccharides by chondroitinase AC-II and ABC digestions (Table S1) and 500 MHz ^{1}H NMR analysis of the isolated octa- and decasaccharides (Figures S1 and S2 and Tables S2 and S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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