Novel Tetrasaccharides Isolated from Squid Cartilage Chondroitin Sulfate E Contain Unusual Sulfated Disaccharide Units GlcA(3-O-sulfate)β1–3GalNAc(6-O-sulfate) or GlcA(3-O-sulfate)β1–3GalNAc(4,6-O-disulfate)*

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We previously isolated novel tetrasaccharides containing 3-O-sulfated glucuronic acid from king crab cartilage chondroitin sulfate K and demonstrated that the disaccharide units containing 3-O-sulfated glucuronic acid were decomposed by chondroitinase ABC digestion (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). The findings indicated the necessity to re-evaluate the disaccharide compositions of chondroitin sulfate preparations purified from other biological sources and analyzed using the above enzyme. In this study, to evaluate squid cartilage chondroitin sulfate E a series of even-numbered oligosaccharides were isolated after exhaustive digestion with sheep testicular hyaluronidase and subsequent fractionation by gel chromatography. The tetrasaccharide fraction was subfractionated by high performance liquid chromatography on an amine-bound silica column. Systematic structural analysis of five major fractions, h, l, m, n, and q, by fast atom bombardment mass spectrometry, enzymatic digestions in conjunction with capillary electrophoresis, and 500-MHz 1H NMR spectroscopy revealed one disulfated, three trisulfated, and one tetrasulfated tetrasaccharide structure: fraction h, GlcAβ1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S); fraction l, GlcA(3S)β1–3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S); fraction m, GlcA(3S)β1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S); fraction n, GlcAβ1–3GalNAc(4S,6S)β1–4GlcAβ1–3GalNAc(4S); and fraction q, GlcA(3S)β1–3GalNAc(4S,6S)β1–4GlcAβ1–3GalNAc(4S), where 3S, 4S, and 6S represent 3-O- and 4-O- and 6-O-sulfate, respectively. The structures found in fractions h and m as well as the unsaturated counterpart of that found in fraction n have been reported, whereas those in fractions l and q are novel in that they contained unusual disulfated and trisulfated disaccharide units where GlcA(3S) is directly linked to GalNAc(6S) and GalNAc(4S,6S), respectively. These novel tetrasaccharide sequences are distinct from those found in other chondroitin sulfate isoforms and may play key roles in the biological functions and activities of chondroitin sulfate E not only from squid cartilage but also from mammalian cells and tissues.

Chondroitin sulfate (CS) proteoglycans are ubiquitous components of the extracellular matrix of connective tissues and are also found 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. 1–3). Immunological studies using CS-specific antibodies have revealed the developmentally regulated expression of the epitopes in the rodent fetus and in the rat central nervous system (for reviews, see Refs. 4 and 5). Some CS epitopes are distributed differentially in distinct tissues and in functionally distinct domains within these tissues (6). These observations suggest that CS chains differing in degree and profile of sulfation perform distinct functions in development.

Oversulfated CS isoforms contain rare structural building units; thus, they may form domain structures that interact specifically with other molecules. Oversulfated CSs were originally discovered in cartilages of shark (7) and invertebrates such as squid and horseshoe crab (king crab) (8; for a review, see Ref. 9). Oversulfated CS isoforms from the shark, squid, and horseshoe crab cartilage are characterized by the distinct disulfated disaccharide units, GlcA(2S)β1–3GalNAc(6S), GlcAβ1–3GalNAc(4S,6S), and GlcA(3S)β1–3GalNAc(4S), where 2S, 3S, 4S, and 6S represent 2-O-, 3-O-, 4-O-, and 6-O-sulfate and are designated as CS-D, CS-E, and CS-K, respectively (10–12). Oversulfated CSs are not limited to invertebrates or marine vertebrates and have been identified in various tissues and cells from higher land-dwelling vertebrates. CS-D and CS-E have been found in human rib cartilage in small proportions (13). Another disaccharide unit, IdecA-GalNAc(4S,6S), has also been identified in dermatan sulfate

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1 The abbreviations used are: CS, chondroitin sulfate; AMAC, 2-amino-2-methyl-1-propanol; DS, dermatan sulfate; FAB-MS, fast atom bombardment mass spectrometry; HOHAHA, homonuclear Hartmann-Hahn; HexA, 4,5-unsaturated hexuronic acid or 4-deoxy-o-α-threo-hex-4-ene-pyranosyluronic acid; HexA, hexuronic acid; HexNAc, N-acetylgalactosamine; HPLC, high performance liquid chromatography; ΔDi-4S, Δ4HexA-1–3GlcNAc(4-O-sulfate), Δ-Di-Dis, Δ4HexA(2-O-sulfate)1–3GlcNAc(6-O-sulfate); Δ-Di-Dis, Δ4HexA-1–3GlcNAc(4-O-sulfate), Δ-Di-Dis, Δ4HexA-1–3GlcNAc(4, 6-O-sulfate); 2S, 3S, 4S, and 6S, 2-O-, 3-O-, 4-O-, and 6-O-sulfate, respectively.
(DS) from hagfish notochord (14), mammalian liver (15), and bovine kidney (16). This type of DS is designated as DS-E in this article.

Although the specific physiological functions of oversulfated CS isoforms have not been clarified, they show differentiation-associated expression. Elevated CS-E synthesis has been observed in the terminal differentiation of embryonic chick chondrocytes (17). Oversulfated CS isoforms are differentiation markers for different mast cell (MC) subsets (Refs. 18 and 19; for a review, see Ref. 20). While heparin is found in the secretory granules of “connective tissue” type MC, as typified by the “serosal” MC (21), CS-E or DS-E is usually found in those of “mucosal” MC, “bone marrow-derived” MC, and related cells (22–26). A proposed role for secretory granule proteoglycans involves their concentration and the stabilization of secretory granule enzymes (27). Close association between histamine and CS-E proteoglycan release has also been observed in human colonic mucosa, which is assumed to contain mast cells (24). DS-E has been demonstrated in guinea pig peritoneal macrophages (28). Kolset et al. (29, 30) showed CS-E and DS-E in human monocyte-derived macrophages as well as CS-E or CS-D in mature peritoneal macrophages and suggested that CS-E could be a marker for differentiation of monocytes into macrophages. Recently, Edwards et al. (31) demonstrated that human macrophages exhibited the differentiation-associated expression of CS-E or DS-E, which bound to lipoprotein lipase, and hypothesized that the marked increase in the synthesis of the surface proteoglycans containing CS-E/DS-E increased the macrophage uptake of plasma low density lipoprotein, i.e., the atherogenic potential of these cells.

CS-E exhibits biological activities in vitro. An in vitro anti-coagulant property has been reported for squid cartilage CS-E (32). This property is mediated primarily by acceleration of heparin cofactor II interaction with thrombin (33). Thrombomodulin, which is an integral membrane protein with anticoagulant activity, bears a single CS or DS chain with a unique GalNAc(4S,6S)–1–4GlcA–1–3GalNAc(4S,6S) sequence of a CS-E type at the nonreducing terminus (34). The CS or DS chain is involved in its anticoagulant activity through direct binding to thrombin and through activating protein C (for a review, see Ref. 35). More recently, monocyte proteoglycan activities of purified coagulation factors, VIIIa and IXa, were reported to be specifically inhibited on monocyte surfaces by size-defined CS-E oligosaccharides, indicating that the CS-E expressed on monocyte membranes inhibits factor X-activating reactions in the intrinsic pathway (36). Squid cartilage CS-E and murine bone marrow-derived MC CS-E (or DS-E) were noted to specifically inhibit the function of activated protein C in the alternative complement pathway (37). The former activates the contact (Hageman factor) system of plasma in vitro as does heparin (38).

In view of the importance of CS chains in various biological systems, we have been conducting systematic structural studies of various CS isoforms including CS-D, CS-E, and CS-K. Recent studies have revealed that the structural complexity and diversity of CS chains resulted from an extensive series of modifications by various sulfation reactions (39–43). Novel oligosaccharides that contained an unusual GlcA(3S) residue were isolated from CS-K of king crab cartilage (44, 45). To our surprise, when these oligosaccharides were digested with chondroitinase ABC, the resultant disaccharide units containing a GlcA(3S) residue were undetectable when monitored by UV-absorbance and were assumed to have been decomposed during the enzyme treatment. Thus, we reinvestigated squid cartilage CS-E that had been characterized by chondroitinase ABC digestion (11, 46) to see if it contained GlcA(3S).

In this study, five tetrasaccharides were isolated after digestion of a commercial squid cartilage CS-E preparation with hyaluronidase instead of chondroitinase ABC to prevent degradation of possible structures that may contain GlcA(3S) residues. The analysis by 500-MHz 1H NMR spectroscopy in conjunction with fast atom bombardment-mass spectrometry (FAB-MS) unambiguously demonstrated GlcA(3S) residues in some of the major tetrasaccharide components. Preliminary findings were reported in abstract form (47).

**EXPERIMENTAL PROCEDURES**

**Materials**—CS isoform preparations (super special grade), including squid cartilage CS-E, whale cartilage CS-A, shark cartilage CS-C, and shark cartilage CS-D, were purchased from Seikagaku Corp., Tokyo, Japan. A king crab cartilage CS-K peptide preparation (48) was a gift from the late Dr. X. Seno (Ochanomizu University, Tokyo). Sheep testicular hyaluronidase (EX 3.2.1.35) was obtained from Sigma, Bio-Gel P-10 and Sephadex G-25 (fine) were obtained from Bio-Rad and Pharmacia Biotech Inc., respectively. 2-Aminoacridone (AMAC) and NaCNBH3 were obtained from LAMBDA Corp. (Graz, Austria) and Aldrich, respectively. The following tetrasaccharides were prepared from shark cartilage CS-D as described (41): GlcAβ1–3GalNAc–(6S)β1–4GlcAβ1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S), GlcAβ1–3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S), GlcAβ1–3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S), GlcA(4S)β1–3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S), GlcAβ1–3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S), GlcAβ1–3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S), GlcAβ1–3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S), GlcAβ1–3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S), and GlcAβ1–3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S). Tetrasaccharides from shark cartilage CS-E were prepared from king crab cartilage as reported (44).

**Chondroitinase ABC Digestion of Various CS Isoforms**—Various CS isoform preparations (CS-A, CS-C, CS-D, CS-E, and CS-K) of 0.4 mg each were digested with 50 μIU of chondroitinase ABC in a total volume of 400 μl of Tris-HCl, pH 8.0, containing 0.06 mM sodium acetate at 37 °C. At appropriate time intervals, a 10-μl sample of the digest of CS-E was carried out by HPLC on an amine-bound silica PA03 column to determine the disaccharide composition as reported (50).

**Preparation of Oligosaccharide Fractions**—A commercial squid cartilage CS-E (100 mg) was digested with 10 mg (approximately 15,000 National formulary units) of sheep testicular hyaluronidase in a total volume of 2.0 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 which hydrolyzes 74 μg of hyaluronate/min) (51, 52). Digestion proceeded at 37 °C for 18 h, and then an additional 8 mg (6000 National formulary units) of the enzyme was added and the incubation was continued for 7 h to complete the digestion. Thereafter, the digest was mixed with 0.44 ml of 30% trichloroacetic acid and centrifuged at 2500 rpm for 10 min. The precipitate was washed with 0.5 ml of 5% trichloroacetic acid. The combined supernatant obtained from the trichloroacetic acid precipitation was extracted with ether, and the aqueous phase was neutralized with 1 N Na2CO3. Then the sample was applied to a Bio-Gel P-10 column (1.6 × 95 cm) using 1 N NaCl, 10% ethanol as the eluent. Eluates were monitored by absorbance at 210 nm, which is attributable mainly to carbohydrate chains. Fractions 1–IX were collected (Fig. 1). Analysis of the chondroitinase ABC digest of CS-E was carried out by HPLC on an amine-bound silica PA03 column to determine the disaccharide composition as reported (50).

**Preparation of Tetrasaccharides**—Excised fractions (FAB-MS unambiguously demonstrated GlcA(3S) residues in some of the major tetrasaccharide components. Preliminary findings were reported in abstract form (47).
as described (39). Reactions were terminated by boiling for 1 min, and
the reaction mixture was analyzed by capillary electrophoresis after
derivatization with AMAC as described below.

**CE Analysis of Underivatized Oligosaccharides and the AMAC Derivatives—**The homogeneity of each purified oligosaccharide fraction was
judged by CE (53) as well as by HPLC. For derivatization of
oligosaccharide fractions, samples (1 nmol each) were first digested
with chondroitinase AC-II as described above and concentrated to dry-
ess in a vacuum concentrator. Then the digests were derivatized with
AMAC according to Jackson (54). The labeled disaccharides were ana-
yzed by CE according to the method of Kitagawa et al. (55). The
electrophoresed fractions of the underivatized oligosaccharides and the
AMAC derivatives were detected by absorption at 185 or 254 nm,
respectively.

**FAB-MS and 500-MHz-1H NMR Spectroscopy—**The sugar and sul-
fate 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. Data were acquired and processed using the
VG Analytical Opus software. Monothioglycerol was used as the
matrix.1H NMR spectra of the oligosaccharides were measured on a
Varian-500 at a probe temperature of 26 or 60 °C as reported (39, 41).
Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapenta-
tane-1-sulfonate in 2H2O. Nevertheless, the actual assignment of the
chemical shifts and their correlation to the particular monosaccharide
units cannot be made due to the large number of possible structu-
ral possibilities for the oligosaccharide structures. Therefore, the 1H
NMR spectra of the oligosaccharides were measured on a Varian-500 at
20 °C and 60 °C, and the aldonic acid protons were observed as
singlets at 8 2.225 in 2H2O (56). Tetrasaccharides for NMR analysis were
repeatedly exchanged in 2H2O with intermediate lyophilization.

**RESULTS**

The previous study on the GlcA(3S)-containing oligosaccha-
rides isolated from king crab cartilage CS-K indicated that the
disaccharide units containing GlcA(3S) were decomposed by
the action of chondroitinase ABC (44) and that re-evaluation of
disaccharide compositions was required for CS preparations that
were purified from other biological sources and analyzed
using the above enzyme. Therefore, in this study, various CS
isoforms were first digested using chondroitinase ABC to com-
pare the degree of unsaturated oligosaccharide formation from
various CS isoforms.

**Differential Susceptibility of Various CS Isoforms to Chon-
droitinase ABC—**Various individual CS isoforms were digested
with chondroitinase ABC, and the reactions were monitored by
absorbance at 232 nm caused by HexA produced by the elimi-
nae action of the enzyme. The digestion degree was calculated
based on the comparison of the amounts of the resultant unsaturated
oligosaccharides estimated by the absorption at 232 nm and the uronic acid values determined for each parent CS
isoform by the carbazole reaction. CS-A, -C, and -D yielded unsaturated products corresponding to 121, 118, and 121% of
each parent isoform, respectively (Fig. 1). The apparent over-
production of the products was most likely due to the use of the
average millimolar absorption coefficient (5.5) to calculate the
unsaturated products. In contrast, the digestion of king crab
cartilage CS-K produced unsaturated oligosaccharides corre-
sponding to only 46% of the uronic acid contained in the parent
carrier, consistent with the recent finding that GlcA(3S)-con-
taining disaccharide units produced were destroyed by the
enzymatic action and undetectable when monitored by absorbance
at 232 nm (44). The production of UV-absorbing materials
from squid cartilage CS-E reached 90% of the level calculated
based on the uronic acid content but was significantly lower when compared with that of CS-A, -C, or CS-D. The
disaccharide composition analysis by HPLC of the digest showed
HexA1–3GalNAc, HexA1–4GlcA (4S,6S), and HexA1–3GalNAc (6S,8S), and HexA1–3GalNAc (4S,6S) in a molar ratio
of 8.4:21.1:8.6:61.5. No other components were detected by absorbance at 232 nm. The findings may indicate that CS-E
also contained the GlcA(3S) structure that was destroyed by the
chondroitinase ABC treatment as in the case of CS-K. Therefore, squid cartilage CS-E was digested by hyaluronidase
to obtain oligosaccharide fragments for structural analysis as below.

**Preparation and Isolation of Tetrasaccharides—**A commer-
cial preparation of squid cartilage CS-E was exhaustively di-
gested with sheep testicular hyaluronidase. Although the previous
study demonstrated that CS-E as well as CS-D were less
susceptible to testicular hyaluronidase when compared with
CS-K and CS-A, it was digested to a significant degree (44). 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 that were caused primarily by carbohydrate
groups, and they were divided into nine fractions, I–IX, as
indicated in Fig. 2. The large peak around fraction number 85
was attributable to the buffer salts. The elution profile was
markedly different from that reported for king crab CS-K (44) and
that for a commercial CS-D preparation (41), reflecting the
differential susceptibility of the CS isoforms to the enzyme
probably due to the linkage specificity of the enzyme. There
were no fragments larger than octasaccharides among the
CS-K degradation products, and there were small proportions
of fragments larger than hexasaccharides among the CS-D
degradation products. In contrast, larger oligosaccharides were
predominant among the CS-E degradation products as shown
in Fig. 2, being consistent with the relative unsusceptibility of
CS-E to the hyaluronidase action (see Fig. 1 in Ref. 44). In this
study, the smallest size fraction, fraction IX, was investigated.
It represented approximately 6.4% of the resultant oligosaccha-
rides and was judged to contain tetrasaccharides based on the
well defined mechanism of action of hyaluronidase (57, 58). It
was subfractionated into fractions a–v as indicated in Fig. 3 by
HPLC on an amine-bound silica column. Five major fractions, k,
l, m, n, and q, marked by asterisks, were further purified by
rechromatography to apparent homogeneity as judged by
HPLC and CE (data not shown). Then their structures were
analyzed as described below. As shown in Fig. 3, fraction k was
eluted at the position of an authentic disulfated saturated
tetrasaccharide GlcAβ1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc (4S).
Fractions l, m, and n were eluted at the positions of as a
natural tetrasaccharides with three sulfate
fractions q was eluted at the position of those
with three or four sulfate groups. The elution position of
fraction l was shortly after that of authentic GlcAβ1–3GalNAc(4S)β1–4GlcA(2S)β1–3GalNAc(6S), and that of frac-

![Fig. 1. Differential susceptibility of various CS isoforms to chondroitinase ABC.](image)
FAB-MS analyses of the underivatized oligosaccharides (44, 53, 59). In the negative ion mode FAB spectrum, alkali-metal-attached molecular ions of the type \([M + nNa − (κ + 1)H]^−\) (M represents the fully protonated acid forms of oligosaccharides) were preferentially observed. Representative FAB spectra are shown in Fig. 4, and assignments of the molecular ion signals afforded by each of the analyzed fractions are summarized in Table I.

The molecular ion signal clusters at \(m/z\) 1037, 1059, and 1081 afforded by fraction \(n\) corresponded, respectively, to \([M + Na − 2H]^−\), \([M + 2Na − 3H]^−\), and \([M + 3Na − 4H]^−\) of a trisulfated saturated tetrasaccharide \(\text{HexA}_2\text{HexNAc}_2\text{OSO}_3\text{H}_2\) (Fig. 4A). The molecular ion signal clusters at \(m/z\) 1161, 1183, and 1205 afforded by fraction \(q\) corresponded, respectively, to \([M + 2Na − 4H]^−\), \([M + 4Na − 5H]^−\), and \([M + 5Na − 6H]^−\) of a tetrasulfated saturated tetrasaccharide, \(\text{HexA}_2\text{HexNAc}_3\text{OSO}_3\text{H}_3\) (Fig. 4B). The molecular ion signal clusters at \(m/z\) 957, 979, and 1001 afforded by fraction \(h\) corresponded, respectively, to \([M + Na − 2H]^−\), \([M + 2Na − 3H]^−\), and \([M + 3Na − 4H]^−\) of a disulfated saturated tetrasaccharide, \(\text{HexA}_2\text{HexNAc}_2\text{OSO}_3\text{H}_2\) (Table I). The molecular ion signal clusters at \(m/z\) 1059, 1081, and 1103 afforded by fraction \(l\) corresponded, respectively, to \([M + 2Na − 3H]^−\), \([M + 3Na − 4H]^−\), and \([M + 4Na − 5H]^−\) of a disulfated saturated tetrasaccharide, \(\text{HexA}_2\text{HexNAc}_2\text{OSO}_3\text{H}_2\) (Table I).

Enzymatic Analysis of the Oligosaccharide Fractions in Conjunction with CE—The disaccharide composition of the isolated tetrasaccharides was determined by our microanalytical procedure (55). The chondroitinase AC-II digest of each tetrasaccharide was labeled with the fluorophore, AMAC. Then disaccharides were analyzed by CE. Chondroitinase AC-II, which is a bacterial eliminase, should degrade a CS-tetrasaccharide to yield 1 mol each of an unsaturated disaccharide unit and a saturated disaccharide unit derived from the reducing and the nonreducing terminus, respectively. The high resolution CE quantitatively resolves each authentic unsaturated disaccharide and the corresponding saturated disaccharide with a sulfation profile identical to that of the former. Therefore, this procedure not only gives the disaccharide composition but also gives information about the sequential arrangement of disaccharide units in a given oligosaccharide sequence (43, 55).

CE analysis of the chondroitinase AC-II digest of fraction \(h\) after the AMAC derivatization demonstrated AMAC-derivatives of \(\text{GlcA}_1\text{GlcNAc}_4\text{OSO}_3\text{H}_4\) (Di-4S) and \(\Delta\text{HexA}1\text{GlcNAc}_4\text{OSO}_3\text{H}_4\) (4S) in a molar ratio of 0.90:1.00 (Fig. 5A). The molar ratio was determined using the integrated peak areas reported for each standard CS-disaccharide (55). The disaccharides, Di-4S and \(\Delta\text{Di-4S}\), which share the same sulfation profile and differ only in the nonreducing terminal uronic acid residues in terms of saturation or unsaturation, were distinctly separated as described. The saturated disaccharide unit Di-4S was derived from the nonreducing terminus, whereas the unsaturated disaccharide unit \(\Delta\text{Di-4S}\) was derived from the reducing terminus. The internal uronic acid is GlcA but not IdcA, since the tetrasaccharide was digested by chondroitinase AC-II. Hence, the compound in fraction \(h\) has the disulfated tetrasaccharide structure \(\text{GlcA}_1\text{GlcNAc}_4\text{OSO}_3\text{H}_4\) (4S) or \(\Delta\text{GlcA}1\text{GlcNAc}_4\text{OSO}_3\text{H}_4\) (4S).

CE analysis of the chondroitinase AC-II digest of fraction \(n\) after the AMAC derivatization showed AMAC derivatives of \(\text{GlcA}_1\text{GlcNAc}_4\text{OSO}_3\text{H}_4\) (Di-4S) and \(\Delta\text{Di-4S}\) in a molar ratio of 1.20:1.00 (Fig. 5D), the former being derived from the non-
reducing terminus and the latter from the reducing terminus. Hence, the major compound in fraction $l$ has the hexasaccharide structure $\text{GlcA} \beta_1 \text{-} 3\text{GalNAc}(4\text{S}) \beta_1 \text{-} 4\text{GlcA} \beta_1 \text{-} 3\text{GalNAc}$.

CE analysis of the chondroitinase AC-II digest of fraction $l$ after AMAC derivatization showed AMAC derivatives of $\Delta\text{Di-4S}$ and an unidentified component X in a peak area ratio of 1.00:0.63, respectively (Fig. 5B). Likewise, fraction $m$ resulted in AMAC derivatives of $\Delta\text{Di-4S}$ and an unidentified component Y in a peak area ratio of 1.00:0.64, and fraction $q$ yielded AMAC derivatives of $\Delta\text{Di-4S}$ and an unidentified component Z in a peak area ratio of 1.00:0.68. The unidentified components X and Y migrated to the positions of AMAC derivatives of disulfated disaccharide units, whereas the component Z migrated to the position of an AMAC derivative of a trisulfated disaccharide unit. The results indicated that the compounds in fractions $l$, $m$, and $q$ share the disaccharide unit $\text{GlcA} \beta_1 \text{-} 3\text{GalNAc}(4\text{S})$ on the reducing sides but differ in the disaccharide structures on the nonreducing sides. The smaller peak areas of the unidentified peaks compared with that of $\Delta\text{Di-4S}$ are probably due to the structural differences between $\Delta\text{Di-4S}$ and the unidentified compounds. However, the structure determination of the compound was not possible due to the lack of authentic compounds and had to await $^1\text{H}$ NMR analysis as described below.

500-MHz $^1\text{H}$ NMR Analysis—Each isolated tetrasaccharide fraction was characterized by 500-MHz $^1\text{H}$ NMR spectroscopy. The sites of sulfation and the types of the internal uronic acid residues were determined by $^1\text{H}$ NMR analysis. The one-dimensional $^1\text{H}$ NMR spectra of fractions $l$, $m$, and $q$ and two-dimensional HOHAHA spectra of fractions $l$ and $q$ are depicted as representatives in Figs. 6 and 7, respectively. The chemical shifts were assigned by two-dimensional HOHAHA and correlation spectroscopy analyses (41, 60), and the NMR data are summarized in Table II. The resonances were well resolved in the structural-reporter group regions between 4.4 and 5.3 ppm and at around 2.0 ppm, being separated from other signals in the bulk region (3.6–4.3 ppm). The resonances between 4.4 and

| Fraction | Yield$^a$ (nmol) | $m/z$ for $[M + \text{Na} - 2\text{H}]^-$ | $m/z$ for $[M + 2\text{Na} - 3\text{H}]^-$ | $m/z$ for $[M + 3\text{Na} - 4\text{H}]^-$ | $m/z$ for $[M + 4\text{Na} - 5\text{H}]^-$ | $m/z$ for $[M + 5\text{Na} - 6\text{H}]^-$ | Assignment |
|----------|-----------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|----------------------------------------|-------------|
| h        | 720             | 957                                    | 979                                    | 1001                                   | 1103                                   | 1205                                   | $\text{HexA}_1\text{HexNAc}_2(\text{OSO}_3\text{H})_3$ |
| l        | 280             | 1059                                   | 1081                                   | 1103                                   | 1183                                   | 1205                                   | $\text{HexA}_1\text{HexNAc}_2(\text{OSO}_3\text{H})_3$ |
| m        | 710             | 1037                                   | 1059                                   | 1081                                   | 1103                                   | 1205                                   | $\text{HexA}_1\text{HexNAc}_2(\text{OSO}_3\text{H})_3$ |
| n        | 530             | 1037                                   | 1059                                   | 1081                                   | 1103                                   | 1205                                   | $\text{HexA}_1\text{HexNAc}_2(\text{OSO}_3\text{H})_3$ |
| q        | 930             | 1161                                   | 1183                                   | 1205                                   | 1205                                   | 1205                                   | $\text{HexA}_1\text{HexNAc}_2(\text{OSO}_3\text{H})_3$ |

$^a$ Yield is given in nmol obtained from 100 mg of CS-E.

FIG. 4. Negative ion mode FAB mass spectra of underivatized fractions $n$ (A) and $q$ (B). Major molecular ion signals were assigned as summarized in Table I.

FIG. 5. CE analysis of the AMAC derivatives of the chondroitinase AC-II digests of the isolated tetrasaccharide fractions. The purified tetrasaccharide fractions $h$, $l$, $m$, $n$, or $q$, corresponding to 1 nmol, were each digested with chondroitinase AC-II, derivatized with AMAC, and analyzed by CE. (A, fraction $h$; B, fraction $l$; C, fraction $m$; D, fraction $n$; E, fraction $q$). Fractions were monitored by absorbance at 254 nm with AMAC. The elution positions of the authentic unsaturated CS disaccharides are indicated in the top panel. The peaks marked by asterisks are attributable to a reagent.
FIG. 6. Structural-reporter group regions of the 500-MHz one-dimensional $^1$H NMR spectra of fractions $l$ (A), $n$ (B), and $q$ (C) recorded in $^2$H$_2$O at 20 °C. The numbers and letters in the spectra refer to the corresponding sugar residues in the structures. The insets are the spectra recorded at 60 °C to suppress disturbance by the HOD line. $U$ and $G$ represent GlcA and GalNAc, respectively.
recorded at 26 °C and was identical with those reported previously for the disulfated tetrasaccharide GlcAβ1–3GalNAc (4S)β1–4GlcAβ1–3GalNAc(4S) isolated from shark cartilage CS-D (fraction 2 in Ref. 41). The NMR data are summarized in Table II together with those of the other fractions. Thus, the structure of the compound in fraction h is as follows, consistent with the results from the enzymatic analysis.

\[
\text{GlcAβ1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S)}
\]

**Figure 7.** Two-dimensional HOHAHA spectra of fractions l (A) and q (B) recorded in \( ^2\text{H}_2\text{O} \). The numbers and letters in the spectra refer to the corresponding sugar residues in the structures. The spectrum of fraction l was recorded at 26 °C, whereas that of fraction q was recorded at 60 °C to suppress the disturbance by the HOD line. U and G represent GlcA and GalNAc, respectively.

5.3 ppm are characteristic of anomeric protons, whereas those at around 2.0 ppm are characteristic of the NAc protons of GalNAc. When recorded at 60 °C to suppress the disturbance by the HOD line, the weak resonances at around 4.8 ppm were recognizable in the one-dimensional spectra (Fig. 6), which are shown in the **insets**. The types of the two uronic acid residues in each isolated tetrasaccharide were identified as GlcA based on the chemical shifts (δ 4.46–4.59) of the anomeric proton signals. Anomeric proton signals of an αLacEa and a βGlC in oligosaccharides derived from DS and CS are observed at around δ 5.0–5.2 and 4.5–4.8, respectively (39, 40, 61, 62). Two NAc proton signals were observed for each tetrasaccharide component. They were assigned by comparison with those of CS oligosaccharides reported previously (39, 41, 60).

The one-dimensional spectrum (not shown) of fraction h was

The one-dimensional \(^1\text{H}\) NMR spectrum (not shown) of fraction m was indistinguishable from that of the trisulfated tetrasaccharide GlcA(3S)β1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S) isolated from squid cartilage CS-E after chondroitinase ABC digestion (fraction 9 in Ref. 39). The chemical shifts of protons belonging to the nonreducing uronic acid residue were very similar to those of the saturated tetrasaccharides isolated from shark cartilage CS-D (fraction 3 in Ref. 41) and king crab cartilage CS-K (44), indicating the presence of a saturated GlcA residue (GlcA-4) at the nonreducing end. Large downfield shifts of H-1, H-2, and H-3 of GlcA-4 by 0.09, 0.17, and 0.82 ppm, respectively. This indicates that the compound in fraction h is a tetrasaccharide composed of the above trisulfated tetrasaccharide sequence (-3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S)) on the reducing side and a 3-O-sulfated GlcA at the nonreducing terminus (44). These results are consistent with the m/z value obtained by FAB-MS and those from the enzymatic analysis described above.

\[
\text{GlcAβ1–3GalNAc(4S,6S)β1–4GlcAβ1–3GalNAc(4S)}
\]

**Figure 8.** Two-dimensional HOHAHA spectra of fractions l (A) and q (B) recorded in \( ^2\text{H}_2\text{O} \). The numbers and letters in the spectra refer to the corresponding sugar residues in the structures. The spectrum of fraction l was recorded at 26 °C, whereas that of fraction q was recorded at 60 °C to suppress the disturbance by the HOD line. U and G represent GlcA and GalNAc, respectively.

The results from FAB-MS and the enzymatic analysis indicated that the compound in fraction l is a trisulfated tetrasaccharide composed of a GlcAβ1–3GalNAc(4S) unit on the reducing side and a GlcAβ1–3GalNAc unit with two sulfate groups on the nonreducing side, namely (GlcAβ1–3GalNAc(disulfate)β1–4GlcAβ1–3GalNAc(4S)). The one- and two-dimensional \(^1\text{H}\) NMR spectra of fraction l are presented in Figs. 6A and 7A, respectively. Compared with the spectral data of the disulfated tetrasaccharide GlcAβ1–3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S) isolated from shark cartilage CS-D (fraction 3 in Ref. 41), no significant differences were observed except for the downfield shifts of H-1, H-2, and H-3 of GlcA-4 by 0.09, 0.17, and 0.82 ppm, respectively. This indicates that the compound in this fraction is a tetrasaccharide composed of the above trisaccharide sequence -3GalNAc(6S)β1–4GlcAβ1–3GalNAc(4S) on the reducing side and a 3-O-sulfated GlcA at the nonreducing terminus (44). These results are consistent with the m/z value obtained by FAB-MS and those from the enzymatic analysis described above. Thus, the compound in fraction l has the following structure.

\[
\text{GlcAβ1–3GalNAc(4S,6S)β1–4GlcAβ1–3GalNAc(4S)}
\]
are shown in Figs. 6C and 7B, respectively. In the one-dimensional spectrum, the chemical shifts of protons belonging to GalNAc\(^{-1}\) and GlcA\(^{-2}\) were very similar to those of the compound in fraction \(l\), indicating the presence of the monosulfated disaccharide sequence of -4GalNAc -3GlcA. Compared with the NMR data for fraction \(l\) (Table II), no significant differences were observed except for the downfield shifts of H-3, H-4, and H-5 of GalNAc-3 by \(0.002\) ppm because of partial overlap of signals. That for the values to three decimal places was only \(\pm 0.001\) ppm because of partial overlap of signals. That for the values to three decimal places was only \(\pm 0.001\) ppm

**Table II**

| Fraction | Chemical Shifts of the NAc Proton Signals of the Tetrasaccharides Isolated from CS-E |
|----------|----------------------------------------------------------------------------------|
|          | H-4, H-5 of GalNAc-3 by reported CS oligosaccharides (39, 41, 42, 60). In a previous 1H NMR study of saturated CS tetrasaccharides (41, 44), the chemical shifts of the NAc proton signals of GalNAc(4S)-1 and GalNAc(4S)-2 were observed around \(\delta 2.018-2.023\) and \(\delta 2.041-2.044\), respectively, and were influenced by \(O\)-sulfation of the neighboring uronic acid residue (39, 41, 60). Based on these observations, the NAc proton signals of the tetrasaccharides in the isolated fractions were assigned as shown in Table II. The NAc proton signals of GalNAc-1 were observed at a higher magnetic field than those of GalNAc-3.

**DISCUSSION**

In this study, CS-E tetrasaccharides were prepared by testicular hyaluronidase digestion, which exhibits transglycosylation activity through the reverse reaction of hydrolysis (64, 65). However, since disaccharides do not serve as acceptors, the isolated tetro saccharides most likely originated from the natural sequences as has been noted (43). The possibility that they were released from higher oligo- or polysaccharide chains newly formed by transglycosylation reactions is unlikely, due to the low concentrations of such chains. Indeed, the structures of the tetra- and hexasaccharides prepared by hyaluronidase digestion of CS-D (41, 43) were in agreement with those of the oligosaccharides prepared by chondroitinase digestion (39, 42).

The structures of the isolated tetro saccharides strongly indicate that the enzyme preferentially cleaves the N-acetylgalactosaminidic linkage in sequences containing GalNAc(4S) and GalNAc(3S) has been observed for king crab CS-K that contains a higher magnetic field than those of GalNAc-3.

**Structure 5. Fraction q**

Two NAc proton signals were observed for each oligosaccharide component except for fraction \(l\), which gave a single signal at \(\delta 2.020\). They were assigned by comparison with those of the reported CS oligosaccharides (39, 41, 42, 60). In a previous 1H NMR study of saturated CS tetrasaccharides (41, 44), the chemical shifts of the NAc proton signals of GalNAc(4S)-1 and GalNAc(4S)-2 were observed around \(\delta 2.018-2.023\) and \(\delta 2.041-2.044\), respectively, and were influenced by \(O\)-sulfation of the neighboring uronic acid residue (39, 41, 60). Based on these observations, the NAc proton signals of the tetrasaccharides in the isolated fractions were assigned as shown in Table II. The NAc proton signals of GalNAc-1 were observed at a higher magnetic field than those of GalNAc-3.
ably reflects the CS-E structure that contains a high content (61%) of GalNAc(4S,6S)β1–4GlcA units (46) resistant to the hyaluronidase action.

The isolated tetrasaccharides in fractions h and m as well as an unsaturated counterpart of that in fraction n have been reported (39, 41, 44), whereas those in fractions l and q are novel in that they contain GlcA(3S) directly linked to GalNAc(6S) and GalNAc(4S,6S) forming unusual disaccharide units GlcA(3S)β1–3GalNAc(6S) and GlcA(3S)β1–3GalNAc(4S,6S), respectively. These disaccharide structures have been suggested for oversulfated CS isolated from squid skin (66–68) as will be discussed below. The present study demonstrated their structures in the novel tetrasaccharide sequences. Thus, the GlcA(3S)-containing structure in squid cartilage CS-E seems to have been missed in previous studies where a disaccharide composition analysis was performed by chondroitinase ABC digestion (11, 39, 46). The lower recovery of unsaturated oligosaccharide products from the chondroitinase ABC digest (Fig. 1) seems to indicate the probable decomposition of the GlcA(3S)-containing disaccharide units. The GlcA(3S) content in the CS-E preparation may be roughly estimated to be up to 10% based on the recovery of unsaturated oligosaccharides. The structures of oversulfated CSs isolated from other biological sources, especially those containing GalNAc(4S,6S), should be reinvestigated to see if GlcA(3S) is contained.

Unique fucose-branched CS isolated from sea cucumber also contains GlcA(3S) (69) as well as a disaccharide unit GlcA-GalNAc(4S,6S) (70), although the sequential arrangement of these structural elements in the polysaccharide chain has not been reported. A proteoglycan bearing oversulfated CS chains has also been isolated from squid skin, and unsaturated counterparts of the disaccharide units (GlcA(2S)-GalNAc(6S), GlcA(3S)-GalNAc(4S), and GlcA-GalNAc(4S,6S)) as well as the GlcA(3S)-containing trisulfated disaccharide unit have been noted (66, 67). The proposal of the GlcA(3S)-containing structure for the disulfated and the trisulfated units was based on the elution positions on HPLC, the reactivity to the HNK-1 antibody, and the resistance to periodate oxidation. However, there is an apparent discrepancy between our findings and the isolation procedures used in the above studies. Karamanos et al. (67, 68) have reported that the GlcA(3S)-containing disaccharide units were obtained by digestion of squid skin CS with chondroitinase AC (presumably AC-II as in Karamanos et al. (71) although not specified), which strikingly contrasts to the findings by us and others that such structures were resistant to this particular enzyme (44, 45, 69). We could obtain GlcA(3S)-containing oligosaccharides only by hyaluronidase digestion (12, 44, 45). This discrepancy remains to be clarified.

The structures found in fractions l and q are hybrids. The compound in fraction l contained structural elements for CS-A (GalNAc(4S)), CS-C (GalNAc(6S)), and CS-K (GlcA(3S)) in a single sequence, whereas that in fraction q contained elements for CS-A, CS-K, and CS-E (GalNAc(4S,6S)). A tetrasaccharide, ΔHexA-GalNAc(4S,6S)-GlcA(2S)-GalNAc(6S), with a hybrid structure of the CS-D and CS-E structural elements had been isolated from shark fin cartilage after chondroitinase AC-I digestion, taking advantage of the resistant nature of the GalNAc-GlcA(2S) to the enzyme (46). This finding and ours may indicate that various combinations of the structural units representative of different CS isoforms result in more domain structures than anticipated, which would be embedded in oversulfated CS chains. It should be emphasized that immunological studies using CS-specific monoclonal antibodies including MO-225 that interact specifically with this hybrid structure (46) revealed developmentally regulated expression of distinct CS epitopes during the odontogenesis in the mouse fetus (4).

Thus, rare hybrid structural domains would be extremely unique so that they would specifically be recognized by other molecules and be involved in various biological processes. The structural bases for the in vitro activities of squid cartilage CS-E and for the physiological functions of mammalian CS-E/DS-E have to be reinvestigated in regard to the new structural feature demonstrated in this study. It remains to be clarified whether or not the novel sequences exist in chondroitin sulfate chains of mammalian origin and whether or not they have specific functions.

It is well known that GlcA(3S) is detected in a glycolipid isolated from human peripheral nerves using the mouse monoclonal antibody HNK-1, raised against human natural killer cells (72, 73). The terminal GlcA(3S) residue is essential for the immunoreactivity, and the carbohydrate epitope is expressed also on glycoproteins (74). The trisaccharide sequence GlcA(3S)β1–3Galβ1–4GlcNAc has been demonstrated for the epitope on bovine peripheral myelin glycoprotein P0 (75). The HNK-1-reactive carbohydrates are temporally and spatially regulated during the development of the nervous system (76) and implicated in cell-cell adhesion as well as in the recognition of neurons and astrocytes (77). In addition, the HNK-1-reactive carbohydrate is a ligand for selectins, which are leukocyte-endothelial cell adhesion molecules (78). It has been reported that the HNK-1 antibody reacted with an intact CS polymer from squid skin (67). It will be interesting to investigate whether GlcA(3S)-containing CS chains exist in the nervous system.

Biosynthetic mechanisms for the production of GlcA(3S) in oversulfated CSs or HNK-1 antigens are unknown. There may exist multiple 3-O-sulfotransferases, which synthesize CS-K, CS-E, and the GlcA(3S)-containing HNK-1 epitopes in glycolipids and glycoproteins, although none of them has been demonstrated. In contrast, two 6-O-sulfotransferases involved in the synthesis of GalNAc(4S,6S) have been reported. The one that synthesizes internal GalNAc(4S,6S) units was demonstrated in squid cartilage (79). The other 6-O-sulfotransferase that specifically transfers a sulfate group to the GalNAc(4S) residue at the nonreducing terminus to form GalNAc(4S,6S) was discovered in human serum (79, 80). GalNAc(4S,6S) has been found at the nonreducing terminus of the newly synthesized CS in the in vitro culture of chick and rat embryo cartilage (81, 82) and in the cell culture of chick chondrocytes and rat chondrosarcoma (17, 83) and is suggested as the possible chain termination signal in the CS biosynthesis. In the recent enzymological studies, sulfated CS tetra- or hexasaccharides with GlcAβ1–3GalNAc(4S,6S) or GlcA(3S)β1–3GalNAc(4S) at the nonreducing ends did not serve as acceptors for chondroitin GalNAc transferase (84), supporting the notion of the termination signal. Interestingly, CS-E but not CS-C synthesis in fetal calf articular cartilage in culture was stimulated by dibutyryl cyclic AMP, indicating specific control mechanisms for the CS-E biosynthesis (85). Regulatory mechanisms for the CS-E synthesizing system including the putative 3-O-sulfotransferase will be interesting to investigate.

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