Novel Sulfated Oligosaccharides Containing 3-O-Sulfated Glucuronic Acid from King Crab Cartilage Chondroitin Sulfate K

UNEXPECTED DEGRADATION BY CHONDROITINASE ABC

(Received for publication, March 20, 1996, and in revised form, June 10, 1996)

Kazuyuki Sugahara, Yukako Tanaka, Shuhei Yamada, Nobuko Seno, and Hiroshi Kitagawa
From the Department of Biochemistry, Kobe Pharmaceutical University, Higashinada-ku, Kobe 658 and the Department of Chemistry, Ochanomizu University, Tokyo 112, Japan

Stuart M. Haslam, Howard R. Morris, and Anne Dell
From the Department of Biochemistry, Imperial College, London SW7 2AZ, United Kingdom

We prepared a series of oligosaccharides from king crab cartilage chondroitin sulfate K after exhaustive digestion with testicular hyaluronidase, and determined the structures of four tetrasaccharides and a pentasaccharide by fast atom bombardment mass spectrometry and high performance liquid chromatography analyses. The tetrasaccharides shared the common core structure GlcAβ1–3GalNAcβ1–4GlcAα1–3GalNAc with various sulfation profiles. One structure was GlcAβ1–3GalNAcβ1–4GlcAβ1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S), whereas three of them have the following hitherto unreported structures including a novel glucuronate 3-O-sulfate: GlcAβ1–3GalNAcβ1–4GlcAβ1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S), GlcAβ1–3GalNAcβ1–4GlcAβ1–3GalNAc(4S), and GlcAβ1–3GalNAcβ1–4GlcAβ1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S), whereas three of them have the following hitherto unreported structures including a novel glucuronate 3-O-sulfate: GlcAβ1–3GalNAcβ1–4GlcAβ1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S), GlcAβ1–3GalNAcβ1–4GlcAβ1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S), where 3S or 4S represents 3-O- or 4-O-sulfate, respectively. The structure of the pentasaccharide was determined as GlcAβ1–3GalNAcβ1–4GlcAβ1–3GalNAcβ1–4GlcAβ1–3GalNAcβ1–4GlcA. Chondroitinase ABC digestion of the tetrasaccharides with GlcA(3S) at the internal position destroyed the disaccharide unit containing GlcA(3S) derived from the reducing side and resulted in only the disaccharide unit from the non-reducing side. In contrast, these tetrasaccharides remained totally resistant to chondroitinase AC-II. The results indicated that it is necessary to reevaluate the disaccharide composition of chondroitin sulfate poly- or oligosaccharides purified from various biological sources, since they were usually determined after chondroitinase ABC digestion. It is probable that the structures containing GlcA(3S) would not have been detected.

Chondroitin sulfates (CS) are complex polysaccharides synthesized by many mammalian cells, and they occur as proteoglycans that can be expressed on the cell surface. It has become increasingly evident that the CS proteoglycans of cartilage are dynamic components that play several key roles in the normal physiology of cartilage tissues and regulate the biological processes such as cell migration and recognition, extracellular matrix deposition, and morphogenesis (for reviews, see Refs. 1–3). Because many of their functions are associated with the glycosaminoglycan moieties (1), the structure of the CS chains is of considerable interest. However, the complexity and diversity of the structures have prevented detailed structural analysis.

The structural complexity of CS chains arises from an extensive series of modifications of a non-sulfated precursor with the structure (4GlcAβ1–3GalNAcβ1–). These modifications are achieved by specific modifying enzymes such as sulfotransferases and epimerases that mature the CS chains. Thus, the controlled expression of these biosynthetic enzymes is thought to be regulating factors in the elaboration of various CS isoforms. The CS chains from the cartilages of various animal species also have highly variable sulfation profiles, incorporating both oversulfated and non-sulfated structures, and they have been roughly classified into types A, B, C, D, E, and K (4–7).

Preliminary chemical analyses have indicated that unlike the CS from many other cartilages, CSK isolated from that of the king crab seems to contain 3-O-sulfated GlcA residues (7). The 3-O-sulfated GlcA was later found in sea cucumber CS as well (8). This novel structure forms a unique carbohydrate epitope on glycoproteins and glycolipids in nervous tissues, which is recognized by the HNK-1 monoclonal antibody (9).

Immunobots of the CS proteoglycans of the brain as well as a number of nervous tissue glycoproteins are intensely stained by the HNK-1 antibody (10). We have been conducting system-

CSE, or CS, chondroitin sulfate A, D, E, or K, 2-AB, 2-amino benzamide; DS, dermatan sulfate; FAB-MS, fast atom bombardment-mass spectrometry; GalNAc, N-acetylated-galactosamine; GlcA, α-glucuronic acid; GlcA(3S), 4,5-unsubstituted hexuronic acid or 4-deoxy-α-L-threo-4-ene-pyranosyluronic acid; HexA, hexuronic acid; HexNAc, N-acetylated-hexosamine; HPLC, high performance liquid chromatography; NFU, national formulary unit; α, β, γ, δ, θ, υ, U, G, U, 2S, 3S, 4S, and 6S represent α, β, γ, δ, θ, υ, U, G, U, 2S, 3S, 4S, and 6S monosaccharides, respectively. α, β, γ, δ, θ, υ, U, G, U, 2S, 3S, 4S, and 6S represent α, β, γ, δ, θ, υ, U, G, U, 2S, 3S, 4S, and 6S monosaccharides, respectively.
Novel Oligosaccharides Containing Glucuronate 3-O-Sulfate

EXPERIMENTAL PROCEDURES

Materials—CSK peptide preparation was purified from king crab cartilage as reported (15). Six unsaturated standard CS disaccharides, chondroitinases ABC (EC 4.2.2.4) and AC-II (EC 4.2.2.5), chondro-4-O-sulfate (EC 3.1.6.9, abbreviated as CS-4-sulfate), and chondro-6-O-sulfate (EC 3.1.6.10, abbreviated as CS-6-sulfate) were purchased from Seikagaku Corp., Tokyo, Japan. Sheep testicular hyaluronidase (EC 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.

Preparation of Oligosaccharide Fractions—The CSK peptide preparation (20 mg) was digested with 2 mg (approximately 3000 NFU) 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 NFU corresponds to the amount of the enzyme that hydrolyzes 74 mg of hyaluronate/min) (16, 17). Digestion proceeded at 37 °C for 18 h, after which an additional 1 mg (1500 NFU) of the enzyme was added and the incubation was continued for 7 h to complete the digestion. Thereafter, the digest was mixed with 0.42 ml of 30% trichloroacetic acid and centrifuged. The precipitate was washed with 0.5 ml of 5% trichloroacetic acid. The combined supernatant obtained from trichloroacetic acid precipitation was extracted with ether, the aqueous phase was neutralized with 1 ml Na2CO3, and the sample was then applied to a Bio-Gel P-10 column (1.6 × 95 cm) using 1 M NaCl, 10% ethanol as the eluent. Eluates were monitored by the absorbance at 210 nm, which is attributable mainly to the N-acetyl group of GalNAc. Tetra-, hexa-, and octasaccharide fractions were pooled (Fig. 2), concentrated, desalted through a column (1.5 × 46 cm) of Sephadex G-25 (fine), and lyophilized. The smallest size fraction, which was presumed to contain tetrasaccharides, was subfractionated by HPLC on an amine-bound silica PA03 column (4.6 × 250 mm; YMC Co., Kyoto, Japan). The HPLC was performed in an LC-10AS system (Shimadzu Co., Kyoto, Japan) using a linear gradient from 16 to 798 mM NaH2PO4 over a 60-min period at a flow rate of 1.0 ml/min at room temperature. Samples prepared as described above were diluted to contain 50 pmol of disaccharides in 200 μl of 16 mM NaH2PO4. Eluates were monitored using an RF-535 fluorometric detector (Shimadzu Co.) with excitation and emission wavelengths of 330 and 420 nm, respectively.

Disaccharide Composition Analysis of CSK Peptides—The CSK peptide preparation purified from king crab cartilage was digested with sheep testicular hyaluronidase, as CSK was largely resistant to the enzymatic action of chondroitinases ABC and AC. As shown in Fig. 1, it was readily digested by hyaluronidase and was more susceptible than the other CS isoforms. The digest was fractionated by gel filtration on a column of Bio-Gel P-10. Three peaks were observed when monitored by absorbance at 210 nm caused primarily by N-acetyl groups and they were assigned as tetra-, hexa-, and octasaccharide fractions as indicated in Fig. 2. The amounts of these fractions obtained from 20 mg of CSK were approximately 4.9, 13, and 1.7 mg, respectively. The elution profile was significantly different from that reported for a commercial CSD preparation (15), in that there were no fragments larger than octasaccharides among the CSK degradation products. The large peak at around fraction number 80 was attributable to the buffer salts. The tetrasaccharide fraction was subfractionated into Fractions 1–5 by HPLC on an amine-bound silica column. As shown in Fig. 3, Fractions 1 and 2 were eluted at the positions of authentic saturated tetrasaccharides with two and three sulfate groups, respectively, whereas Fractions 3–5 were eluted at higher salt concentrations. They were
Novel Oligosaccharides Containing Glucuronate 3-O-Sulfate

Further purified by rechromatography to apparent homogeneity, as judged by HPLC and capillary electrophoresis (data not shown), after which their structures were analyzed as described below. The amounts of the purified oligosaccharides in Fractions 1–5 from 20 mg of CSK are summarized in Table I. FAB-MS Analysis—FAB-MS analyses of the undervariated oligosaccharide samples in the negative-ion mode defined their molecular weights, from which the composition and the maximum number of O-sulfate groups present in each fraction were inferred, as in the case of heparin and heparan sulfate oligosaccharides (19, 21). In the negative ion mode FAB spectrum, alkali-metal-attached molecular ions of the type \([M - (x + 1)H + xNa]^+\) (M represents the fully protonated acid forms of oligosaccharides) were preferentially observed. The assignments of the molecular ion signals afforded by each of the analyzed fractions are listed in Table I.

The molecular ion signal clusters at \(m/z\) 935, 957, 979, and 1001 afforded by Fraction 1 corresponded respectively to \([M - H]^+, [M + Na - 2H]^+, [M + 2Na - 3H]^+, and [M + 3Na - 4H]^+\) of a disulfated saturated tetrasaccharide HexA\(_2\)HexNAc\(_4\)(OSO\(_3\)H)\(_2\) (data not shown).

Fractions 2 and 3 afforded similar FAB-MS data, suggesting that they are isomers of identical composition. The samples yielded molecular ion signals at \(m/z\) 1015, 1037, 1059, and 1081, corresponding to \([M - H]^+, [M + Na - 2H]^+, [M + 2Na - 3H]^+, and [M + 3Na - 4H]^+\) respectively, of a trisulfated saturated tetrasaccharide HexA\(_2\)HexNAc\(_2\)(OSO\(_3\)H)\(_3\) (Fig. 4A).

As shown in Fig. 4B, Fraction 4 afforded molecular ion signals at \(m/z\) 1095, 1117, 1139, and 1161, corresponding to \([M - H]^+, [M + Na - 2H]^+, [M + 2Na - 3H]^+, and [M + 3Na - 4H]^+\) respectively, of a tetrasulfated saturated pentasaccharide HexA\(_2\)HexNAc\(_3\)(OSO\(_3\)H)\(_4\).

Enzymatic Characterization of the Oligosaccharide Fractions—As shown in Fig. 3, Fraction 1 was eluted upon HPLC at the position of the authentic disulfated tetrasaccharide GlcA\(_1\)-3GalNAc\(_4\)(Di-4S)\(_1\)-4GlcA\(_1\)-3GalNAc\(_4\)(OSO\(_3\)H)\(_2\) (4S), where 4S denotes 4-O-sulfate (Fraction 2 derived from shark cartilage CSD in Ref. 13). The disaccharide analysis of this fraction, which was carried out by exhaustive chondroitinase AC-II digestion followed by HPLC on an amine-bound silica column, gave rise to a major peak at the elution position of \(\Delta\)HexA\(_1\)-3GalNAc\(_4\)(Di-4S) and a minor one at the position of the saturated disaccharide GlcA\(_1\)-3GalNAc\(_4\)(Di-4S) slightly ahead of \(\Delta\)Di-4S when monitored by the absorbance at 210 nm (data not shown). Only the major peak absorbed at 232 nm. The data are summarized in Table II with those obtained from the other fractions. Thus, the major and the minor peaks detected by absorbance at 210 nm were judged to be due to an unsaturated and a saturated disaccharide derived from the reducing and non-reducing side of the parent tetrasaccharide, respectively, as discussed (13). Therefore, the compound in Fraction 1 was concluded to be a tetrasaccharide with the structure GlcA\(_1\)-3GalNAc\(_4\)(Di-4S)\(_1\)-4GlcA\(_1\)-3GalNAc\(_4\)(OSO\(_3\)H)\(_2\).

When digested with chondroitinase AC-II or ABC, Fraction 2 yielded major and minor peaks at the elution positions of \(\Delta\)Di-4S and between \(\Delta\)HexA\(_1\)-3GalNAc\(_4\)(Di-4S) (\(\Delta\)Di-4S) and \(\Delta\)HexA\(_1\)-3GalNAc\(_4\)(Di-4S) (\(\Delta\)Di-6S) (data not shown). These succeeeding digestion of Fraction 2 with chondroitinase AC-II and CS-4-sulfatase yielded a major peak at the elution position of \(\Delta\)HexA\(_1\)-3GalNAc\(_4\)(Di-4S) (\(\Delta\)Di-4S) and a minor one shortly before the position of \(\Delta\)HexA\(_1\)-3GalNAc\(_4\)(Di-6S) (\(\Delta\)Di-6S) (data not shown). The successive digestion of Fraction 2 with chondroitinase AC-II and CS-4-sulfatase yielded a major peak at the elution position of \(\Delta\)Di-4S and a minor one shortly before the position of \(\Delta\)HexA\(_1\)-3GalNAc\(_4\)(Di-6S) (\(\Delta\)Di-6S) (data not shown). The results summarized in Table II indicated that the compound in Fraction 2 is a trisulfated tetrasaccharide composed of a Di-4S unit on the reducing side and a unidentified disulfated disaccharide unit with GalNAc(4S) on the non-reducing side, namely GlcA(S)\(_1\)-3GalNAc\(_4\)(Di-4S)\(_1\)-4GlcA\(_1\)-3GalNAc\(_4\)(OSO\(_3\)H)\(_2\), where S represents a sulfate group at an unidentified position.

Since Fraction 3 was resistant to chondroitinase AC-II, it was digested with chondroitinase ABC and the products were analyzed in the above HPLC system. HPLC analysis of the chondroitinase ABC digest gave rise to a single peak at the position of authentic Di-4S, slightly ahead of \(\Delta\)Di-4S when monitored by absorbance at 210 nm (Table II). No peak was detected when monitored by the absorbance at 232 nm. The peak of Fraction 3 shifted on HPLC by 10 min upon CS-4-sulfatase digestion under the standard and harsh incubation conditions (see “Experimental Procedures”) (data not shown), indicating that it contained at least one sulfate group on C-4 of...
summarized in Table I.

a GalNAc residue. Fraction 3 was resistant to CS-6-sulfatase. This enzyme specifically attacks GalNAc(6S) at the reducing end of unsaturated CS oligosaccharides (11, 12). We also showed that the enzyme digested saturated CS tetra- and hexasaccharides as well. In contrast, CS-4-sulfatase preferentially removes a sulfate group from GalNAc(6S) under the standard incubation conditions but also releases sulfate groups from internal GalNAc(6S) residues under harsh conditions (11) (see “Experimental Procedures”). The susceptibility to CS-4-sulfatase and the insusceptibility to CS-6-sulfatase suggested that the reducing GalNAc is monosulfated only at its C-4 position, and that the third sulfate group is probably on the internal GlcA residue, since the tetrasaccharide was resistant to chondroitinase AC-II (7, 8). Thus, the compound in Fraction 3 was assumed to be a trisulfated tetrasaccharide composed of a Di-4S unit on the non-reducing side and an unidentified disulfated disaccharide unit including the structure GlcAβ1–3GalNAc(4S) on the reducing side, possibly GlcAβ1–3GalNAc(4S)β1–4GlcA(S)β1–3GalNAc(4S).

Since Fraction 4 was also resistant to chondroitinase AC-II, we digested it with chondroitinase ABC followed by HPLC analysis as described above. The digest gave rise to a single HPLC peak at a position between ΔDi-diSD and ΔDi-diSE when monitored by the absorbance at 210 nm (Fig. 5B). There was no peak when monitored by the absorbance at 232 nm (Fig. 5C and Table II). One sulfate group was lost upon CS-4-sulfatase digestion of Fraction 4 under the standard and harsh incubation conditions (see “Experimental Procedures”) as judged by HPLC (data not shown). These results and those from FAB-MS (Table I) indicated that the compound in Fraction 4 is a tetrasulfated tetrasaccharide composed of an unidentified disulfated disaccharide unit on the non-reducing side and an unidentified disulfated disaccharide unit including the GlcAβ1–3GalNAc(4S) structure on the reducing side; i.e. [disulfated (GlcAβ1–3GalNAc)]β1–4GlcA(S)β1–3GalNAc(4S).

Since Fraction 5 was also resistant to chondroitinase AC-II, we digested it with chondroitinase ABC and analyzed the products by HPLC as described above. Chondroitinase ABC digestion resulted in two discrete peaks, one at the position of authentic Di-4S and the other between ΔDi-diSD and ΔDi-diSE when monitored by the absorbance at 210 nm (Table II). No peak was detected unexpectedly at 232 nm (data not shown). One sulfate group of the parent compound in Fraction 5 was lost upon CS-4-sulfatase digestion under the harsh incubation conditions (see “Experimental Procedures”) as judged by HPLC (data not shown). These results together with those from FAB-MS indicate that the compound in Fraction 5 is a tetrasulfated pentasaccharide containing a GalNAc(4S) residue. However, the structure determination of the compound had to await 1H NMR analysis as described below.

1H NMR Analysis—Each isolated tetrasaccharide fraction was characterized by 500-MHz 1H NMR spectroscopy. As presumed from the enzymatic analysis described above, the one-dimensional 1H NMR spectrum of Fraction 1 (data not shown) was confirmed to be identical with that of the reference tetrasaccharide R1, GlcAβ1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S) obtained from shark cartilage CSD (13). The NMR data are summarized in Table III with those of the other fractions. The one-dimensional 1H NMR spectra of Fractions 2, 4, and 5 are shown as representatives in Fig. 6. Signals found in the anomeric proton region between δ 4.4 and 5.3 ppm were readily identified as H-1 resonances of the constituent saccharide residues by comparison with the NMR spectra of the tetrasaccharides obtained from shark cartilage CSD (13). Other proton signals in the one-dimensional spectra were assigned using the two-dimensional HOHAHA (homonuclear Hartmann-Hahn) and COSY (correlation spectroscopy) spectra (data not shown), as were oligosaccharides isolated from various CS isoforms (11, 13, 22), dermatan sulfate (DS) (23), and hyaluronic acid (17). Isomer types of the two uronic acid residues in each isolated tetrasaccharide were identified as GlcA based upon the chemical shifts (δ 4.467–4.635) of the anomeric proton signals. Anomeric proton signals of an idoA and a

![FAB-MS analysis of the five oligosaccharides isolated from CSK of king crab cartilage](image_url)

**FIG. 4. Negative FAB/MS spectra of underivatized Fractions 2 (A), 4 (B), and 5 (C).** Major molecular ion signals were assigned as summarized in Table I.

**TABLE I**

| Fraction | Yield* | m/z for [M + H]+ | m/z for [M + Na+ - 2H]- | m/z for [M + 2Na+ - 3H]- | m/z for [M + 3Na+ - 4H]- | m/z for [M + 4Na+ - 5H]- | Assignment |
|----------|--------|-----------------|-----------------|----------------|----------------|----------------|----------------|
| 1        | 65     | 935             | 957             | 979           | 1001          | HexAβHexNAc(S)(OSO3H)2 |
| 2        | 649    | 1015            | 1037            | 1059          | 1081          | HexAβHexNAc(S)(OSO3H)3 |
| 3        | 93     | 1015            | 1037            | 1059          | 1081          | HexAβHexNAc(S)(OSO3H)3 |
| 4        | 367    | 1095            | 1117            | 1139          | 1161          | HexAβHexNAc(S)(OSO3H)4 |
| 5        | 146    | 1271            | 1293            | 1315          | 1337          | HexAβHexNAc(S)(OSO3H)4 |

* Yield is given in nanomoles obtained from 20 mg of CSK.

---

2 Nadanaka, S., and Sugahara, K., *Glycobiology*, in press.
Each oligosaccharide was digested with chondroitinase AC-II or ABC or CS-4-sulfatase, and the digest was analyzed by HPLC as described under “Experimental Procedures.” Saturated and unsaturated disaccharides were monitored by absorbance at 210 and 232 nm, respectively.

### Table II

| Fraction | Chondroitinases | Chondroitinase digestion products | Sensitivity to CS-4-sulfatase | Proposed structures
|----------|----------------|---------------------------------|-----------------------------|------------------|
| 1        | AC-II          | Disulfated disaccharide         | U-G(4S)-U-G(4S)             |                  |
|          | ABC            | Di-4S                           |                            |                  |
| 2        | AC-II          | Di-4S                           | U-G(4S)-U-G(4S)             |                  |
|          | ABC            | Disulfated disaccharide         |                            |                  |
| 3        | AC-II          | Di-4S                           | U-G(4S)-U-G(4S)             |                  |
|          | ABC            | Disulfated disaccharide         |                            |                  |
| 4        | AC-II          | Mono- and disulfated disaccharide |                            |                  |
| 5        | ABC            | Disulfated disaccharide         |                            |                  |

* Under the standard incubation conditions (see “Experimental Procedures”) a sulfate group on the reducing GalNAc(4S) is preferentially removed.

The proposed structures were consistent with the m/z values determined by FAB/MS (see Table I).

The chemical shifts of protons belonging to GlcA in CS/DS oligosaccharides are observed at around δ 5.0–5.2 and 4.5–4.8 ppm, respectively (11, 12, 23, 24).

O-Sulfation causes downfield shifts of protons bound to the O-sulfated carbon atoms by about 0.4–0.8 ppm (11, 22, 25). Thus, the sulfation positions of the saccharide residues were estimated by comparison with the proton signals of non-sulfated saccharide residues. The one-dimensional spectrum of Fraction 2 is shown in Fig. 6A, and the NMR data are summarized in Table III with those of Fractions 3 and 4. The chemical shifts of protons belonging to GalNAc at the reducing terminal trisaccharide region were very similar to those of the other fractions containing tetrasaccharides. Characteristic H-1 resonances at δ 5.204 and 4.635 identified an αGlcA or a βGlcA residue at the reducing end, respectively (12). Since the resonances of the α anomer of GlcA, but not of GalNAc, were observed, the reducing terminal sugar residue was confirmed not to be GalNAc but rather GlcA.

The one-dimensional spectrum of Fraction 5 is shown in Fig. 6C, and the NMR data are summarized in Table IV. The spectrum contained additional H-1 signals in the anemic region when compared with those of the other fractions containing tetrasaccharides. Characteristic H-1 resonances at δ 5.204 and 4.635 identified an αGlcA or a βGlcA residue at the reducing end, respectively (12). Since the resonances of the α anomer of GlcA, but not of GalNAc, were observed, the reducing terminal sugar residue was confirmed not to be GalNAc but rather GlcA.

The signals of the non-reducing terminal trisaccharide region were very similar to those of the other fractions containing tetrasaccharides. Characteristic H-1 resonances at δ 5.204 and 4.635 identified an αGlcA or a βGlcA residue at the reducing end, respectively (12). Since the resonances of the α anomer of GlcA, but not of GalNAc, were observed, the reducing terminal sugar residue was confirmed not to be GalNAc but rather GlcA.

The chemical shifts of the H-3 proton signal of Glc-A was 0.808 ppm lower than that (δ 3.466) of the corresponding GlcA residue of the reference compound, indicating the 3-O-sulfation of this GlcA residue. Therefore, the following structure is proposed for the compound in Fraction 2.

Glc(A3S)β1–3GalNAc(4S)β1–4GlcAβ1–3GalNAc(4S)

**Fraction 2**

The one-dimensional spectrum of Fraction 4 is shown in Fig. 6B, and the NMR data are summarized in Table III. Downfield shifts of H-3 of GlcA-2 and H-3 of GlcA-4 by ∆ 0.812 and 0.803 ppm were found, respectively, when compared with the reference compound R1, indicating the 3-O-sulfation of the corresponding carbon atoms. The structure proposed for the compound in Fraction 4 is listed below. Likewise, the proton chemical shifts of the compound in Fraction 3 were also assigned (Table III) and the structure was determined as listed below.

GlcAβ1–3GalNAc(4S)β1–4GlcA(3S)β1–3GalNAc(4S)

**Fraction 3**

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

**Fraction 4**

The one-dimensional spectrum of Fraction 5 is shown in Fig. 6C, and the NMR data are summarized in Table IV. The spectrum contained additional H-1 signals in the anemic region when compared with those of the other fractions containing tetrasaccharides. Characteristic H-1 resonances at δ 5.204 and 4.635 identified an αGlcA or a βGlcA residue at the reducing end, respectively (12). Since the resonances of the α anomer of GlcA, but not of GalNAc, were observed, the reducing terminal sugar residue was confirmed not to be GalNAc but rather GlcA.

The one-dimensional spectrum of Fraction 5 is shown in Fig. 6C, and the NMR data are summarized in Table IV. The spectrum contained additional H-1 signals in the anemic region when compared with those of the other fractions containing tetrasaccharides. Characteristic H-1 resonances at δ 5.204 and 4.635 identified an αGlcA or a βGlcA residue at the reducing end, respectively (12). Since the resonances of the α anomer of GlcA, but not of GalNAc, were observed, the reducing terminal sugar residue was confirmed not to be GalNAc but rather GlcA.

The signals of the non-reducing terminal trisaccharide region were very similar to those of the other fractions containing tetrasaccharides. Characteristic H-1 resonances at δ 5.204 and 4.635 identified an αGlcA or a βGlcA residue at the reducing end, respectively (12). Since the resonances of the α anomer of GlcA, but not of GalNAc, were observed, the reducing terminal sugar residue was confirmed not to be GalNAc but rather GlcA.
GlcA residues are given in parentheses.

with those of the reported CS oligosaccharides (11, 13, 22). In a

indirectly in reference to acetone (δ 2.225 ppm) in 2H2O at 26 °C. The estimated error for the values to two decimal places was only ±0.01 ppm because of partial overlap of signals. That for the values to three decimal places was ±0.002 ppm. Coupling constants J1,2 (in Hz) of GalNAc and GlcA residues are given in parentheses.

pentasaccharide structure, which contains a GlcA residue extension on the reducing side. Thus, we propose the following tetradsulfated pentasaccharide structure for the compound in Fraction 5, and its molecular composition was in good agreement with that afforded by the results of FAB/MS analysis.

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

Enzymatic Action of Chondroitinase ABC for Glucuronate 3-O-Sulfate-containing Oligosaccharides—As described above, chondroitinase ABC digestion of the tetrasaccharides with GlcA(3S) at the internal position caused the disappearance of the disaccharide unit containing GlcA(3S) to be formed from the reducing side and resulted only in the disaccharide unit from the non-reducing side. To investigate whether or not the disaccharide that disappeared was destroyed, the chondroitinase ABC digests of the oligosaccharides, Fractions 3, 4, and 5, were labeled with the fluorophore 2-AB for high sensitivity, then the derivatives were analyzed by HPLC with a fluorescence detector. Only a single major peak was detected at the position of the 2-AB derivative of authentic Di-4S, slightly ahead of that of Di-diSE for the digest of Fraction 3, or between the 2-AB derivatives of ΔDi-diSE and ΔDi-diSP for the digest of Fraction 4 (Fig. 7, A or B). The results indicated that the disaccharide unit containing GlcA(3S) derived from the reducing side was indeed destroyed upon chondroitinase ABC digestion. In contrast, two discrete peaks, one at the position of the

The sulfation sites determined by 1H NMR of each oligosaccharide in Fractions 1–5 were in good agreement with those of the pentasaccharide in Fraction 5, and its molecular composition was in good agreement with that afforded by the results of FAB/MS analysis.

Enzymatic Action of Chondroitinase ABC for Glucuronate 3-O-Sulfate-containing Oligosaccharides—As described above, chondroitinase ABC digestion of the tetrasaccharides with GlcA(3S) at the internal position caused the disappearance of the disaccharide unit containing GlcA(3S) to be formed from the reducing side and resulted only in the disaccharide unit from the non-reducing side. To investigate whether or not the disaccharide that disappeared was destroyed, the chondroitinase ABC digests of the oligosaccharides, Fractions 3, 4, and 5, were labeled with the fluorophore 2-AB for high sensitivity, then the derivatives were analyzed by HPLC with a fluorescence detector. Only a single major peak was detected at the position of the 2-AB derivative of authentic Di-4S, slightly ahead of that of Di-diSE for the digest of Fraction 3, or between the 2-AB derivatives of ΔDi-diSE and ΔDi-diSP for the digest of Fraction 4 (Fig. 7, A or B). The results indicated that the disaccharide unit containing GlcA(3S) derived from the reducing side was indeed destroyed upon chondroitinase ABC digestion. In contrast, two discrete peaks, one at the position of the
2-AB derivative of authentic Di-4S and the other between those of \( \Delta \text{Di-diS}_\alpha \) and \( \Delta \text{Di-diS}_\beta \), were observed for the digest of Fraction 5 that contains a pentasaccharide (Fig. 7C), implying that only the internal GlcA(3S) residue, but not the remaining disaccharide structure of the trisaccharide sequence on the reducing side, was destroyed upon chondroitinase ABC digestion.

**Chondroitinase ABC Digestion of the CSK Peptide Preparation Isolated from King Crab Cartilage**—In view of the unexpected action of chondroitinase ABC toward GlcA(3S)-containing oligosaccharides, it was of particular interest to reevaluate the disaccharide composition of intact CSK-polysaccharides. The CSK peptide preparation was digested with chondroitinase ABC.
ABC, and the disaccharides in the products were analyzed by HPLC. As shown in Fig. 8, only one major peak at the position of Di-4S and a few minor peaks were detected. No peak was observed in the region of authentic disaccharide units with two or more sulfate groups, despite the fact that the CSK peptide preparation contained such disaccharide units in considerable amounts as demonstrated in this study. In addition, the total GlcA recovery calculated on the basis of the disaccharide yield was only 46% upon chondroitinase ABC digestion, being consistent with the analytical data obtained in this study, showing that about half of the GlcA residues in CSK oligosaccharides were sulfated at the C-3 position. These results suggested that chondroitinase ABC digestion of CS with GlcA(3S) specifically destroys the disaccharide units containing GlcA(3S).

### DISCUSSION

In this study, four sulfated tetrasaccharides and one sulfated pentasaccharide were isolated after testicular hyaluronidase digestion of CSK from king crab cartilage. The tetrasaccharide in Fraction 1 has been described elsewhere (13), whereas those in Fractions 2–4 and the pentasaccharide in Fraction 5 were novel. The pentasaccharide may be derived from the cleavage site that was generated by an endogenous endo-β-d-glucuronidase in the cartilage tissue as has been discussed for the CS trisaccharides with the backbone structure of GlcAβ1–3GalNAcβ1–4GlcA (12). Seno and Murakami (7) originally proposed the GlcA(3S) structure in the saturated disulfated disaccharide unit GlcA(S)β1–3GalNAc(4S) isolated from king crab cartilage CSK after digestion with testicular hyaluronidase followed by chondroitinase ABC. The unique 3-O-sulfated GlcA structure was identified based upon the results that a uronic acid residue of a saturated disulfated disaccharide unit GlcA(2S)β1–3GalNAc(6S) from shark cartilage CSD was rapidly and completely oxidized, whereas that of the CSK disaccharide was quite stable upon oxidation using sodium periodate. This study demonstrated by means of solid chemical analysis by FAB-MS and 500-MHz 1H NMR, that the oligosaccharides derived from CSK contained GlcA(3S) residues.

GlcA(3S) has been detected in a glycolipid isolated from human peripheral nerves using the mouse monoclonal antibody HNK-1, raised against human natural killer cells (9, 27). The terminal GlcA(3S) residue is essential for the immunoreactivity with the HNK-1 antibody and the carbohydrate epitope...
Novel Oligosaccharides Containing Glucuronate 3-O-Sulfate

is expressed not only on glycolipids but also on glycoproteins (28). The HNK-1-reactive carbohydrates are temporally and spatially regulated during the development of the nervous system (29), and implicated in cell-cell adhesion, as well as the recognition of neurons and astrocytes (30). In addition, the HNK-1-reactive carbohydrate is a ligand for selectins, which are leukocyte-endothelial cell adhesion molecules (31). Although the structures of the HNK-1-reactive sugar chains on glycoproteins have not yet been determined, similar epitopic structures reportedly occur on CS proteoglycans of mammalian tissues (32). Karamanos et al. (33) have reported that the HNK-1 antibody reacts with an intact CS polymer from squid skin and that unsaturated oversulfated disaccharides with GlcA(3S) residues. The results would suggest that the oligosaccharides isolated in this study occur on proteoglycans in the nervous system and are potentially useful tools for elucidating the function of putative polysaccharide structures.

Considering the well defined mechanism of action of testicular hyaluronidase (34, 35), the elution profile of the oligosaccharides produced by hyaluronidase from CSK (Fig. 2) was unique, in that no peak corresponding to deca- or larger oligosaccharides was observed. When shark cartilage CSD was digested with testicular hyaluronidase under the same incubation conditions, a series of tetra- to dodeca- and larger oligosaccharide fractions were obtained (13). Thus, CSK is more sensitive to the hyaluronidase than other CS isoforms. Among the four isoforms (CSA, CSD, CSE, and CSK), CSK was the best substrate for the testicular hyaluronidase, followed by CSA, as determined by the turbidity assay (Fig. 1), suggesting that the sulfation profile affects the hyaluronidase action and the enzyme probably cleaves more preferentially the N-acetylgalactosamidic linkage in the GalNAc(4S)β1–4GlcA than that in the GalNAc(6S)β1–4GlcA. However, mammalian hyaluronidases exhibit transglycosylation activity through the reverse reaction of hydrolysis (36, 37). Therefore, the unique elution profile observed in Fig. 1 may be due in part to the inhibition of the transglycosylation activity by the GlcA(3S) residue at the non-reducing terminus of the hyaluronidase digest from CSK.

Characterization of CS-4-sulfatase using the isolated tetrasaccharides revealed unexpected substrate specificity. This enzyme can digest disaccharide units containing GalNAc(4S) at both the reducing and non-reducing sides of tetrasaccharides, unless the GalNAc(4S) bears another sulfate group on the C-6 position (11, 38). However, the enzyme removed a sulfate group only from the reducing GalNAc(4S) of tetrasaccharides with an internal GlcA(3S) residue like GlcAβ1–3GalNAc(4S)β1–4GlcA(3S)β1–3GalNAc(4S). The 3-O-sulfation of a GlcA residue seems to render the adjacent GalNAc(4S) residue resistant to CS-4-sulfatase.

This study also revealed an unexpected enzymatic action of chondroitinase ABC using Fractions 3–5 that contained oligosaccharides with internal GlcA(3S) residues as substrates. A study has indicated that chondroitinase ABC, as well as chondroitinase AC-II, cannot cleave the N-acetylgalactosamidic linkage in the GalNAc(4S)β1–4GlcA(3S) structure, while testicular hyaluronidase can (7). In this study, however, chondroitinase ABC digestion of Fractions 3 and 4 tetrasaccharides with GlcA(3S) at the internal position unexpectedly destroyed the disaccharide unit containing GlcA(3S) derived from the reducing side, resulting in only the disaccharide unit from the non-reducing side (Figs. 5 and 7, A and B), whereas these tetrasaccharides were resistant to chondroitinase AC-II. Moreover, about half of the GlcA residues were destroyed upon chondroitinase ABC digestion of the intact CSK peptide preparation. Although the degradation mechanism of the disaccharide containing GlcA(3S) upon chondroitinase ABC digestion remains to be investigated, our data show unequivocally that degradation occurs. Therefore, it will be essential to reevaluate the disaccharide composition of oversulfated CS chains, which was estimated previously by means of chondroitinase ABC digestion. Indeed, an analysis of the disaccharide composition of CSK polysaccharides using chondroitinase ABC first led us to temporarily speculate that the CSK preparation consisted primarily of the GlcAβ1–3GalNAc(4S) units and that it did not contain disulfated disaccharide units such as GlcA(3S)β1–3GalNAc(4S) (see Fig. 8). Thus, it is possible that the GlcA(3S) structure might have been missed in other CS chains analyzed after chondroitinase ABC digestion. In fact, we found that squid cartilage CSE also contains GlcA(3S) residues.3

Acknowledgments—We thank Satomi Kaji and Yukiko Iwamoto for technical assistance.

REFERENCES
1. Poole, A. R. (1986) Biochem. J. 236, 1–14
2. Fransson, L. Å. (1987) Trends Biochem. Sci. 12, 406–411

3. A. Kinoshita, S. Yamada, S. M. Haslam, H. R. Morris, A. Dell, and K. Sugahara, manuscript in preparation.
Novel Sulfated Oligosaccharides Containing 3-O-Sulfated Glucuronic Acid from King Crab Cartilage Chondroitin Sulfate K: UNEXPECTED DEGRADATION BY CHONDROITINASE ABC
Kazuyuki Sugahara, Yukako Tanaka, Shuhei Yamada, Nobuko Seno, Hiroshi Kitagawa, Stuart M. Haslam, Howard R. Morris and Anne Dell

J. Biol. Chem. 1996, 271:26745-26754.
doi: 10.1074/jbc.271.43.26745

Access the most updated version of this article at http://www.jbc.org/content/271/43/26745

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 15 of which can be accessed free at http://www.jbc.org/content/271/43/26745.full.html#ref-list-1