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Polydispersity in sulfation profile of oligosaccharide alditols isolated from the protein-linkage region and the repeating disaccharide region of chondroitin 4-sulfate of bovine nasal septal cartilage

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Proteoglycans of bovine nasal septal cartilage bear predominantly chondroitin 4-sulfate. After exhaustive chondroitinase ABC digestion of a chondromucoprotein preparation rich in proteoglycans and subsequent reductive β-elimination, five hexasaccharide alditols were isolated from the glycosaminoglycan-protein linkage region. They were analyzed by enzymatic digestion in conjunction with HPLC and by one-dimensional and two-dimensional ^{1}H-NMR spectroscopy. They share the conventional core saccharide structure $\Delta^{4,5}$HexAα1−3GalNAcβ1−4GlcAβ1−3Galβ1−3Galβ1−4Xyl-ol (where $\Delta^{4,5}$HexA is 4,5-unsaturated hexuronic acid), but have different sulfation profiles. One compound (I) does not contain sulfate. Two of the three monosulfated compounds (II and III) have an *O*-sulfate group at either C6 or at C4 of the GalNAc residue. The other monosulfated compound (IV) is hitherto unreported and has a *O*-sulfate at C4 of the Gal residue preceding the GlcA residue, whereas the GalNAc is not sulfated. The disulfated compound (V) has sulfate groups at C4 of both the Gal residue preceding GlcA and the GalNAc residue. The molar ratio of compounds I−V is 38.3:5.9:43.0:1.6:11.2. The structural heterogeneity of these hexasaccharide alditols reflects the polydispersity in the linkage region of the chondroitin sulfate chains. In addition, two trisaccharide and two tetrasaccharide alditols derived from the repeating disaccharide region of the chondroitin sulfate chains were also isolated. Their structures were unambiguously determined by enzymatic analysis and by ^{1}H-NMR spectroscopy as $\Delta^{4,5}$HexAα1−3GalNAc(4-*O*- or 6-*O*-sulfate)β1−4GlcA-ol and $\Delta^{4,5}$HexAα1−3GalNAc(4-*O*- or 6-*O*-sulfate)β1−4GlcAβ1−3GalNAc(4-*O*-sulfate)-ol, respectively.

Keywords: chondroitin 4-sulfate; sulfated oligosaccharides; ^{1}H-NMR; cartilage.

Chondroitin sulfate is found in various tissues and exhibits a wide variety of biological functions. It is a ubiquitous component of the extracellular matrix of connective tissues and is also found on the surface of many cell types and in intracellular secretory granules (for reviews see [1−3]). Immunological studies using monoclonal antibodies have revealed the developmentally regulated expression of the chondroitin sulfate epitopes in the rodent fetus (for a review see [4]), and notably during rat central nervous system development [5]. Developmentally regulated expression and tissue-specific distribution of chondroitin sulfate isoforms suggest that chondroitin sulfate chains differing in sulfation profile perform distinct functions in development. For example, chondroitin/dermatan sulfate from mouse brain, which is recognized by a specific monoclonal antibody, promotes neurite outgrowth of neurons from the rat central nervous system [6]. However, the detailed structure and the biosynthetic mechanism that regulates the expression of chondroitin sulfate chains appear sophisticated and are not well understood.

The basic structure of a chondroitin sulfate chain is composed of variably sulfated repeating disaccharide units that are linearly polymerized and covalently attached to the protein core through the glycosaminoglycan(GAG)-protein linkage region, GlcAβ1−3Galβ1−3Galβ1−4Xylβ1-*O*-Ser. Thus, they usually occur as proteoglycans. It is noteworthy that this linkage region is shared with dermatan sulfate, heparan sulfate and heparin (for a review see [7]). Chinese hamster ovary cell mutants deficient in xylosyltransferase or galactosyltransferase do not produce chondroitin sulfate nor heparan sulfate [8], being consistent with the concept of the common linkage region. The basic biosynthetic principle of these GAG chains is an alternate stepwise transfer of monosaccharide residues onto a growing saccharide chain from the corresponding nucleotide sugars (for a review see [9]). However, the mechanism how different GAG chains

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Abbreviations. $\Delta^{4,5}$HexA, 4,5-unsaturated hexuronic acid; GalNAc, 2-deoxy-2-*N*-acetylamino-D-galactose; GlcA-ol, D-glucuronic acid alditol; ΔDi-0S, $\Delta^{4,5}$HexAα1−3GalNAc; ΔDi-4S, $\Delta^{4,5}$HexAα1−3GalNAc(4-*O*-sulfate); ΔDi-6S, $\Delta^{4,5}$HexAα1−3GalNAc(6-*O*-sulfate); ΔDi-diS$_D$, $\Delta^{4,5}$HexA(2-*O*-sulfate)α1−3GalNAc(6-*O*-sulfate); ΔDi-diS$_E$, $\Delta^{4,5}$HexAα1−3GalNAc(4,6-*O*-disulfate); ΔDi-triS, $\Delta^{4,5}$HexA(2-*O*-sulfate)α1−3GalNAc(4,6-*O*-disulfate); GAG, glycosaminoglycan; CS, chondroitin sulfate; C4S, chondroitin 4-sulfate; C6S, chondroitin 6-sulfate; CS-4- or CS-6-sulfatase, chondro-4-*O*- or 6-*O*-sulfatase, respectively; ROE, rotating-frame Overhauser enhancement; 1D, one-dimensional; 2D, two-dimensional; IdoA, iduronic acid.

Enzymes. chondroitinase AC-II (EC 3.1.6.9); chondroitinase ABC (EC 4.2.2.4); chondro-4-*O*-sulfatase (EC 3.1.6.9); chondro-6-*O*-sulfatase (EC 3.1.6.10); $\Delta^{4,5}$Hexuronate-2-sulfatase (EC 3.1.6.-).

are polymerized on the same linkage tetrasaccharide, which is synthesized first, is unsolved. Our studies stem from the working hypothesis that differences in the structures of the linkage regions of the various GAG chains may exist, and that they influence the structure to be formed thereafter [10].

Systematic structural studies on the GAG-protein linkage region of various chondroitin sulfate isoforms such as chondroitin 4-sulfate and 6-sulfate (C4S and C6S) as well as dermatan sulfate, all of which are classified as galactosaminoglycans, led us to the discovery of modified structures such as Gal4*S*, Gal6*S* and Xyl2*P*, where 4*S*, 6*S* and 2*P* represent 4-*O*-sulfate, 6-*O*-sulfate and 2-*O*-phosphate [11], respectively [10, 12–15]. In contrast, no sulfation of the Gal residues has been detected so far for heparan sulfate and heparin [16–18], both of which are glucosaminoglycans. These lines of accumulating evidence have not yet solved the biosynthetic sorting mechanism, but indicate the existence of the structural heterogeneity of the GAG-protein linkage region among various GAG. In this study, oligosaccharides were isolated from the GAG-protein linkage region of bovine nasal cartilage C4S to make a survey of new modification profiles.

MATERIALS AND METHODS

Materials. Chondromucoprotein prepared from bovine nasal septal cartilage [19] was a gift from the late Dr Albert Dorfman, University of Chicago. Other materials were obtained from the following sources: chondroitinase AC-II, the conventional and highly purified preparations of chondroitinase ABC (the latter being available as a protease-free preparation), chondro-4-*O*-sulfatase (abbreviated as CS-4-sulfatase), and chondro-6-*O*-sulfatase (abbreviated as CS-6-sulfatase) were from Seikagaku Corp. $\Delta^{4,5}$hexuronate-2-sulfatase (abbreviated as 2-sulfatase), purified from *Flavobacterium heparinum* [20] was a generous gift from Keiichi Yoshida Seikagaku Corp. Authentic tetrasaccharide and hexasaccharide alditols derived from the chondroitin-sulfate-protein linkage region were prepared from whale cartilage and shark cartilage chondroitin-sulfate-proteoglycans as reported previously [12–14]. Authentic unsaturated chondroitin sulfate/dermatan sulfate trisaccharides were prepared as reported [21].

Preparation of oligosaccharide alditols. Chondromucoprotein (2.03 g) from bovine nasal cartilage was suspended in 44 ml 0.06 M Tris/HCl, pH 8.0 containing 6 mM sodium acetate. The solution was adjusted to pH 7.0 with 1 M HCl, treated in boiling water for 10 min, cooled in ice water, and readjusted to pH 8.0 with 1 M NaOH. The sample was then exhaustively digested with 1 IU of highly purified chondroitinase ABC in a total volume of 47 ml. Additional enzyme (1 IU) was added after 18 h and the reaction proceeded for another 26 h. The reaction was terminated by adjusting the pH to 6.5 with 1 M HCl followed by heating in boiling water for 12 min. The digest was dialyzed extensively against distilled water at room temperature. A 5.8% (by vol.) aliquot of the sample corresponding to 24.1 μmol of oligosaccharides [as 4,5-unsaturated hexauronic acid ($\Delta^{4,5}$HexA)] was treated with 1 M $NaBH_4$/0.05 M NaOH in a total volume of 2.34 ml at room temperature overnight to release the chondroitin-sulfate-derived oligosaccharides from the protein cores. The reaction mixture was acidified with 1 M acetic acid and a 30% trichloroacetic acid solution was added to give a final trichloroacetic acid concentration of 5%. Insoluble materials were removed by centrifugation, and the supernatant was subsequently extracted with ether. The water phase was concentrated to dryness and desalted by gel filtration on a Sephadex G-25 column (0.9 cm×60 cm) resulting in separation between oligosaccharide fractions, and disaccharide fractions. The ultraviolet-absorbing oligosaccharide fractions were pooled, concentrated and redigested with 2 IU of the conventional preparation of chondroitinase ABC for 50 min in 2.0 ml of the buffer described above. The digest was separated into disaccharide (1.19 μmol as $\Delta^{4,5}$HexA) and oligosaccharide fractions (10.2 μmol as $\Delta^{4,5}$HexA) by gel filtration on a Sephadex G-15 column (1.0 cm×89 cm) using 0.25 M NH_4HCO_3/7% 1-propanol as eluent.

Enzymatic analysis of the isolated oligosaccharides. Enzyme digestion proceeded using 0.5 nmol of each isolated oligosaccharide fraction and the indicated amount of the enzyme (see below) in a total volume of 40 μl of the appropriate buffer at 37°C for 10 min, unless otherwise indicated. Chondroitinase AC-II digestion was performed with 10 mIU enzyme in 0.05 M sodium acetate, pH 6.0 [22]. Complete digestion of fraction 8-2A into two disaccharide units was accomplished by incubating 0.5 nmol of fraction 8-2A with 25 mIU of chondroitinase AC-II for 60 min. Digestion with 2-sulfatase was carried out for 20 min using 2 mIU of the enzyme in 10 mM imidazole/HCl, pH 6.5 [20]. For digestion with CS-4-sulfatase or CS-6-sulfatase 20 mIU enzyme was used in 34 mM Tris/HCl, pH 7.5, containing 34 mM sodium acetate and 0.01% (mass/vol.) BSA [22, 23]. After the incubations the reaction mixtures were boiled at 100°C for 2 min, cooled to room temperature, mixed with 360 μl of 16 mM NaH_2PO_4 and analyzed by HPLC.

HPLC. Fractionation of the oligosaccharide alditols and analysis of the chondroitinase AC-II digests of the isolated oligosaccharides were carried out by HPLC as reported previously for the separation of the chondroitin sulfate disaccharides [24, 25]. HPLC was performed on a 4.6 mm×250 mm polyamine-bound silica PA03 column (YMC Co.) using a linear gradient from 16 mM to 530 mM NaH_2PO_4 over a 60-min period at a flow rate of 1.0 ml/min at room temperature. Eluates were monitored by absorbance at 232 nm. Separated fractions were concentrated in a vacuum concentrator (Savant Instruments, Inc.) and desalted through a column (0.8 cm×56 cm) of Sephadex G-25 (fine) with distilled water as eluent.

^{1}H-NMR spectroscopy. The isolated oligosaccharides were repeatedly exchanged in ^2H_2O (99.96% ^{2}H, Aldrich) with intermediate lyophilization. One-dimensional (1D) ^{1}H-NMR, two-dimensional (2D) ^{1}H-NOE, 2D ^{1}H-rotating-frame Overhauser enhancement (ROE) and 2D ^{1}H-TOCSY spectra were recorded at a probe temperature of 285 K or 300 K using Bruker AMX-500 or AMX-600 spectrometers (Bijvoet Center, Utrecht University) or at 288 K using a Varian VXR-500 (Kobe Pharmaceutical University) essentially as described [15, 26].

Other analytical methods. Chondroitinase-produced oligosaccharides were quantified based on the absorbance (E_{232} = 5500 $M^{-1}cm^{-1}$) [21] caused by the Δ4,5 sites of the uronic acid at the non-reducing ends. Uronic acid was determined by the carbazole method [27] using GlcA as a standard.

RESULTS

Isolation of the linkage oligosaccharides. Chondroitin sulfate chains of bovine nasal septal cartilage proteoglycans contain GlcAβ1–3GalNAc(4S) as a predominant disaccharide unit [28] and is classified as C4S. To investigate the structure of the protein-carbohydrate linkage region of the proteoglycans, linkage oligosaccharides were isolated from the chondromucoprotein preparation that is rich in C4S-proteoglycans. A proteoglycans preparation was exhaustively digested with highly purified chondroitinase ABC and the core protein fraction was recovered by dialyzing the digest against distilled water. The core protein frac-

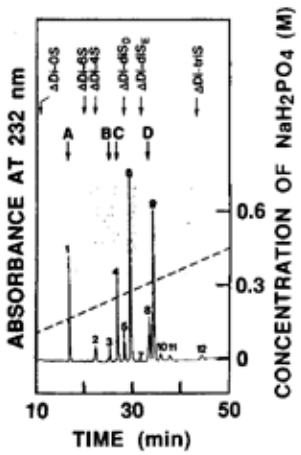

Fig. 1. HPLC separation of the oligosaccharide alditol fraction. The oligosaccharide alditol fraction was chromatographed on an amine-bound silica column as described in Materials and Methods. Elution was performed by using a linear gradient of NaH_2PO_4 as indicated by the dashed line. The elution positions of the authentic linkage hexasaccharide alditols (A–D) as well as the six authentic unsaturated chondroitin sulfate disaccharides are indicated by arrows: (A) ΔHexAα1–3GalNAcβ1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl-ol; (B) ΔHexAα1–3GalNAc6*S*β1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl-ol; (C) ΔHexAα1–3GalNAc4*S*β1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl-ol; (D) ΔHexAα1–3GalNAc4*S*β1–4GlcAβ1–3Gal4*S*β1–3Galβ1–4Xyl-ol. For abbreviations of the disaccharides see Abbreviations.

Table 1. Oligosaccharides isolated from C4S of bovine nasal cartilage.

Fraction	Amount	Recovery
	nmol	%
1	624	11.8
2	159	3.0
3	143	2.7
4	701	13.3
5	166	3.1
6	1401	26.5
7	>10	>1
8	416	7.9
9	1415	26.8
10	66	1.3
11	88	1.7
12	99	1.9
Total	5278	100.0

tion was subjected to reductive β-elimination using $NaBH_4$/NaOH to release the glycan stubs from the core proteins. The sample was acidified with acetic acid and oligosaccharide fractions were recovered by gel filtration on a Sephadex G-25 column. The oligosaccharide fraction was digested again with chondroitinase ABC (a conventional preparation) to achieve a complete digestion, and the digest was fractionated by gel filtration on a column of Sephadex G-15 into an oligosaccharide and a disaccharide fraction in a molar ratio of 9: 1.

The oligosaccharide fraction was then separated into 12 subfractions by HPLC on an amine-bound silica column (Fig. 1). Close inspection of fraction 3 indicated that the main peak had a shoulder ahead of it. They were designated as fractions 3-1 and 3-2 in the elution order. The yield of the isolated fractions are summarized in Table 1. As described below, enzymatic analysis in conjunction with HPLC and ^{1}H-NMR spectroscopy revealed that fractions 1, 3, 4 and 8 contain hexasaccharide alditols stemming from the proteoglycan linkage region. In addition to the linkage oligosaccharide alditol, fraction 8 contains a tetrasaccharide alditol derived from the repeating disaccharide region. Fraction 9 contains such an unsaturated tetrasaccharide alditol as well, whereas fractions 5 and 6 each contain an unsaturated trisaccharide alditol. Fraction 2 contains the disaccharide $\Delta^{4,5}$HexA(α1–3)GalNAc(4-*O*-sulfate) (ΔDi-4S), as evident from its elution position on HPLC (Fig. 1), and the conversion into $\Delta^{4,5}$HexA(α1–3)GalNAc (ΔDi-0S) upon CS-4-sulfatase digestion. The minor fractions 7, 10, 11 and 12 were not analyzed due to their small amounts.

Structural analysis of the oligosaccharides from the proteoglycan linkage region. Aliquots of fractions 1, 3-1, 3-2, 4 and 8 were subjected to chondroitinase AC-II digestion followed by HPLC analysis. The compounds in fractions 1, 3-2 or 4 were each degraded into equimolar amounts of a common unsaturated oligosaccharide and variable unsaturated disaccharides being ΔDi-0S, $\Delta^{4,5}$HexA(α1–3)GalNAc(6-*O*-sulfate) (ΔDi-6S), or ΔDi-4S, respectively (data not shown). The common oligosaccharide eluted on HPLC at the position of the authentic non-sulfated unsaturated tetrasaccharide alditol $\Delta^{4,5}$HexAα1–3Galβ1–3Galβ1–4Xyl-ol. As shown in Fig. 1, the intact hexasaccharides in fractions 1, 3-2 and 4 eluted at the positions of the authentic linkage hexasaccharide-alditols A [$\Delta^{4,5}$HexAα1–3GalNAcβ1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl-ol], B [$\Delta^{4,5}$HexAα1–3GalNAc6*S*β1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl-ol] and C [$\Delta^{4,5}$HexAα1–3GalNAc4*S*β1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl-ol], respectively, which were isolated from C4S-proteoglycan or from C6S-proteoglycan obtained from whale cartilage [12], or shark cartilage [13–14]. Subsequently, the oligosaccharides in fractions 1,3-2 and 4 were analyzed by ^{1}H-NMR spectroscopy. The chemical shifts of the structural-reporter-group protons (Table 2) are identical to those of the authentic hexasaccharide alditols A, B, C, respectively [12–14], showing that the structures of the compounds in these fractions are:

fraction 1, $\Delta^{4,5}$HexAα1–3GalNAcβ1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl-ol;

fraction 3-2, $\Delta^{4,5}$HexAα1–3GalNAc6*S*β1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl-ol;

fraction 4, D^{4,5}HexAα1–3GalNAc4*S*β1–4GlcAβ1–3Galβ1–3Galβ1–4Xyl-ol.

HPLC analysis of the chondroitinase AC-II digest of fraction 3-1 gave rise to equimolar amounts of ΔDi-0S and an oligosaccharide that eluted at the position of the authentic monosulfated tetrasaccharide alditol $\Delta^{4,5}$HexAα1–3Gal4*S*β1–3Galβ1–4Xyl-ol (standard 9; Fig. 2A). Upon subsequent treatment of the chondroitinase AC-II digest with CS-4-sulfatase, the latter component shifted to the position of the non-sulfated tetrasaccharide alditol $\Delta^{4,5}$HexAα1–3Galβ1–3Galβ1–4Xyl-ol (standard 7; Fig. 2B). The enzyme CS-4-sulfatase has been demonstrated to act not only on GalNAc4*S* but also on Gal4*S* in $\Delta^{4,5}$HexAα1–3Gal4*S*β1–3Galβ1–4Xyl-ol [12]. These results indicate that the compound in fraction 3-1 has the common core hexasaccharide structure with a sulfate group probably at the C4 position of Gal–3 (Fig. 3 for notation).

The 1D ^{1}H-NMR spectrum of the oligosaccharide in fraction 3-1 is depicted in Fig. 3 and includes its ^{1}H resonance assignments (see also Table 2). The resonances of ΔHexA-6 H4 (δ 5.899) and H1 (δ 5.185) were readily recognized by their downfield positions with respect to those of anomeric signals of the other monosaccharide constituents (Fig. 3). Subsequently, the connectivity pattern in the 2D ^{1}H-TOCSY spectrum (Fig. 4) in

Table 2. ^{1}H chemical shifts of monosaccharide constituents of the linkage oligosaccharides isolated from bovine nasal septa C4S, together with those of a reference compound (A; fraction 4 in [13]). Chemical shifts are given relative to acetone in ^2H_2O (δ 2.225) at 285 K (fraction 3-1), at 288 K (compound A) or at 299 K (fractions 1, 3-2 and 4). Compound A is identical to the compound in fraction 1; NMR data for compound A are from [13]. n.d., not determined; −, not occurring.

Residue	Proton	Chemical shift in					
		A	1	3−1	3−2	4	8−2A
Xyl-ol-1	H4	3.988	3.985	3.986	3.985	3.987	3.994
Gal-2	H1	4.617	4.615	4.615	4.618	4.618	4.625
	H2	3.720	n.d.	3.722	n.d.	n.d.	3.74
	H3	3.842	n.d.	3.83	n.d.	n.d.	3.86
	H4	4.200	4.195	4.188	4.203	4.196	4.195
Gal-3	H1	4.668	4.670	4.697	4.666	4.670	4.743
	H2	3.745	n.d.	3.792	n.d.	n.d.	n.d.
	H3	3.800	n.d.	4.010	n.d.	n.d.	4.143
	H4	4.160	4.156	4.747	4.165	4.161	4.586
GlcA-4	H1	4.668	4.670	4.737	4.683	4.674	−
	H2	3.455	3.457	3.434	3.470	3.457	−
	H3	3.624	3.625	3.606	n.d.	3.634	−
	H4	3.778	n.d.	3.692	n.d.	n.d.	−
GalNAc-5	H1	4.531	4.543	4.542	4.569	4.616	−
	H2	4.003	4.005	4.001	n.d.	4.072	−
	H3	3.901	n.d.	3.90	3.949	4.150	−
	H4	4.098	4.098	4.107	4.181	4.615	−
	H5	n.d.	n.d.	n.d.	4.013	n.d.	−
	H6	n.d.	n.d.	n.d.	4.234	n.d.	−
	H6′	n.d.	n.d.	n.d.	4.221	n.d.	−
	NAc	2.057	2.056	2.060	2.054	2.094	−
ΔHexA-6	H1	5.184	5.184	5.185	5.182	5.265	5.573
	H2	3.793	n.d.	3.793	n.d.	n.d.	4.072
	H3	4.094	4.094	4.094	4.112	3.943	3.947
	H4	5.899	5.895	5.899	5.886	5.965	6.018

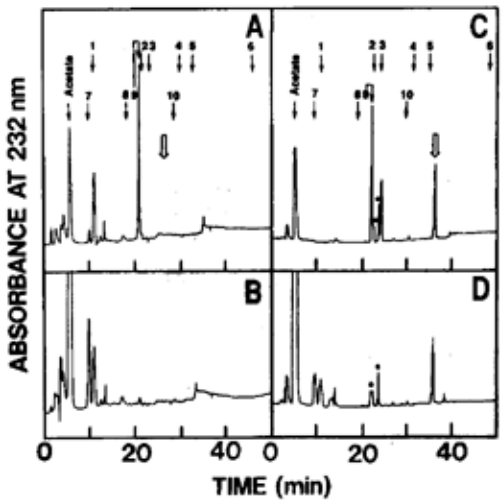

Fig. 2. Analysis of the isolated linkage sugar alditol fractions using chondroitinase AC-II and CS-4-sulfatase. The isolated sugar alditol fractions 3-1 or 8 were sequentially digested with chondroitinase AC-II and CS-4-sulfatase. Each chondroitinase digest and each double digest were then analyzed on an amine-bound silica column under the conditions described for Fig. 1. (A) Chondroitinase AC-II digest of fraction 3-1; (B) chondroitinase AC-II digest of fraction 8; (C) double digest of fraction 3-1; (D) double digest of fraction 8. The authentic linkage tetrasaccharide alditols (standard 1−6) as well as the elution positions of the standard unsaturated disaccharides (standard 7−10) are indicated by arrows at the top of both panels: 1, ΔDi-0S; 2, ΔDi-6S; 3, ΔDi-4S; 4, ΔDi-diS$_D$; 5, ΔDi-diS$_E$; 6, ΔDi-triS; 7, ΔHexAα1−3Galβ1−3Galβ1−4Xyl-ol; 8, ΔHexAα1−3Galβ1−3Gal6Sβ1−4Xyl-ol; 9, ΔHexAα1−3Gal4Sβ1−3Galβ1−4Xyl-ol; 10, ΔHexAα1−3Gal4Sβ1−3Gal6Sβ1−4Xyl-ol. The open arrows indicate the elution positions of the fractions 3-1 (A) and 8 (C) before digestions, respectively.

combination with values of the vicinal ^{1}H-^{1}H coupling constants led to the resonance positions of ΔHexA-6 H2 (δ 3.793) and H3 (δ 4.094). The set of chemical shifts of ΔHexA H1-H4 closely resembles that of ΔHexA protons in the non-sulfated ΔHexAβ1−3GalNAc disaccharide unit in the linkage region hexasaccharide A (Table 2), suggesting that neither of these monosaccharides is sulfated. The GalNAc spin system was located by the presence of a 6H1-5H3 correlation in the 2D ^{1}H-NOE spectrum (data not shown), and was further assigned through the connectivity pattern in the 2D ^{1}H-TOCSY spectrum (Fig. 4), aided by the spin-coupling topology in the 1D ^{1}H-NMR spectrum (Fig. 3). Comparison of the ^{1}H chemical shifts of this GalNAc residue with those of the GalNAc moiety at the corresponding position in reference compound A (Table 2) confirms that this GalNAc is not sulfated in structure 3-1. Inspection of the other anomeric tracks in the 2D ^{1}H-TOCSY spectrum (Fig. 4) shows that one of the Gal residues is sulfated at the C4 position, as is evident by the downfield shift of its H4 atom to 4.747 ppm, whereas the other monosaccharide constituents appear to be not sulfated. The GlcA spin system was identified by the characteristic upfield resonance position of its H2 atom (δ 3.434). Despite partial overlap of GlcA-4 H1 (δ 4.737) and the H4 signal of the sulfated Gal residue (δ 4.747), a 4H1-3H3 NOE could unmistakably be identified, showing that the Gal residue at position 3 is sulfated. We have previously reported a 4-sulfated Gal-3 residue in a linkage oligosaccharidyl-serine isolated from Swarm rat chondrosarcoma C4S-proteoglycan [10] as well as in a linkage hexasaccharide alditol isolated from whale carti-

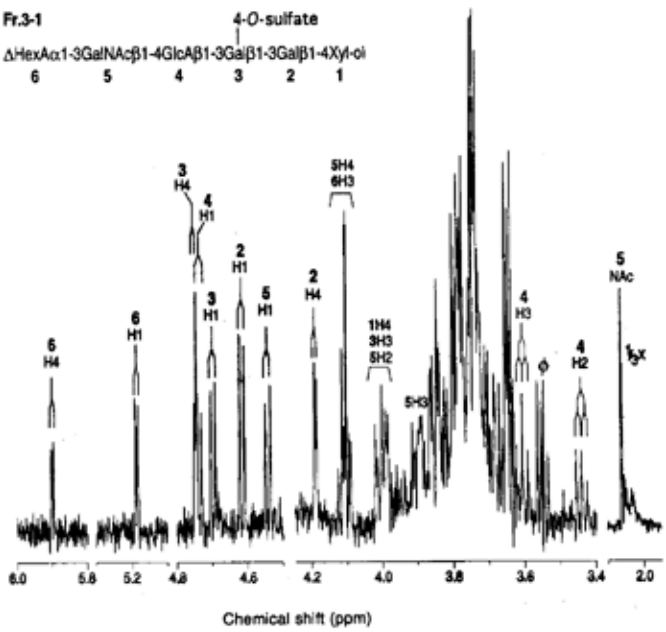

Fig. 3. 1D ^{1}H-NMR spectrum of the hexasaccharide alditol in fraction 3-1, recorded in ^2H_2O at 285 K. The letters in bold in the spectra refer to the position of the monosaccharide in the structure, and those in normal font stand for the protons.

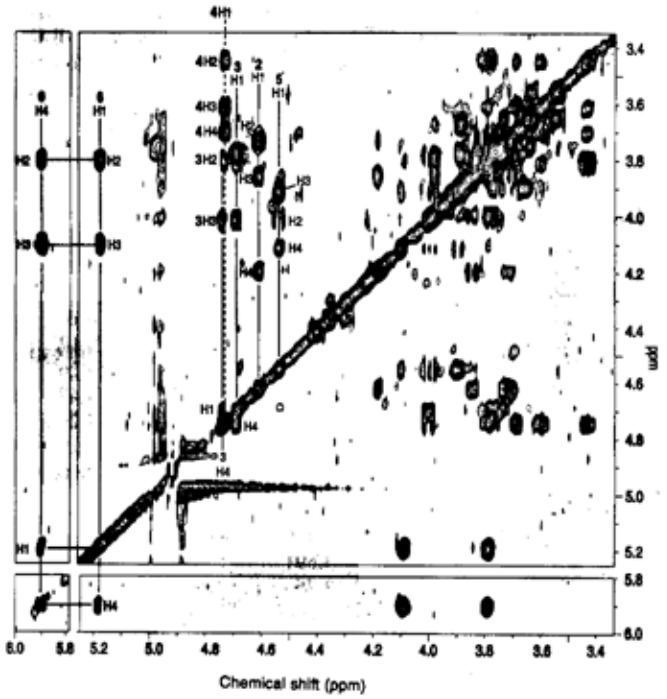

Fig. 4. 2D ^{1}H-TOCSY spectrum of the hexasaccharide alditol in fraction 3-1, recorded in ^2H_2O at 285 K. The letters in bold in the spectra refer to the position of the monosaccharide in the structure, and those in normal font designate the protons.

lage C4S-proteoglycan [12]. The close resemblance of the ^{1}H chemical shifts of the Gal-3 residues in these three compounds, the set of ^{1}H chemical shifts of Gal-2, NOEs between Gal-3 H1 and Gal-2 H3, and between Gal-2 H1 and Xyl-1 H4, unequivocally establish the exclusive 4-sulfation of Gal-3. In summary, the oligosaccharide in fraction 3-1 is

fraction 3-1, $\Delta^{4,5}$HexAα1−3GalNAcβ1−4GlcAβ1−3Gal4*S*β1−3Galβ1−4Xyl-ol.

Two major and two minor peaks were observed on HPLC after chondroitinase AC-II digestion of the oligosaccharide alditols in fraction 8. In addition to these peaks, a peak was present at the original elution position of the undigested oligosaccharide alditols (marked by an open arrow in Fig. 2C), indicating that one of the compounds is not digested under the employed digestion conditions. The structure of the compound resulting in this peak and in the two minor peaks will be discussed below. The peak areas of the major peaks are similar, suggesting that the respective compounds originate from a common parent oligosaccharide (designated as fraction 8-2). Their respective elution positions correspond to those of ΔDi-4S (standard 3) and $\Delta^{4,5}$HexAα1−3Gal4*S*β1−3Galβ1−4Xyl-ol (standard 9). Upon subsequent digestion by CS-4-sulfatase each of the two peaks shifted on HPLC to elution positions of ΔDi-0S (standard 1) and $\Delta^{4,5}$HexAα1−3Galβ1−3Galβ1−4Xyl-ol (standard 7), respectively (Fig. 2D). The presumably tetrasaccharide compound eluting with standard 9 (labelled as fraction 8-2A) was isolated and analyzed by ^{1}H-NMR spectroscopy. Comparison of the NMR spectral data with those of the hexasaccharide alditol in fraction 3-1 (Table 2) show that the compound in fraction 8-2A is indeed sulfated at the C4-position of Gal-3, and that the structure is $\Delta^{4,5}$HexAα1−3Gal4*S*β1−3Galβ1−4Xyl-ol. Therefore, the following structure is proposed for the compound in fraction 8-2.

fraction 8-2, $\Delta^{4,5}$HexAα1−3GalNAc4*S*β1−4GlcAβ1−3Gal4*S*β1−3Galβ1−4Xyl-ol.

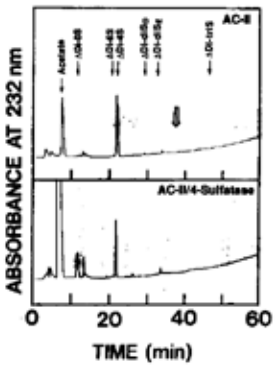

Fig. 5. Analysis of the isolated sugar alditol fraction 9 using chondroitinase AC-II and CS-4-sulfatase. The isolated sugar alditol fraction 9 was sequentially digested with chondroitinase AC-II and CS-4-sulfatase. The chondroitinase digest (upper) and the double digest (lower) were then analyzed on an amine-bound silica column under the conditions as described for Fig. 1. The elution positions of the authentic unsaturated chondroitin sulfate disaccharides are indicated by arrows. The open arrow indicates the elution position of the fraction 9 before the digestions.

Structural analysis of the oligosaccharides derived from the repeating disaccharide region. The oligosaccharide alditol in fraction 8 which was resistant to chondroitinase AC-II digestion (designated fraction 8-1, and indicated by the open arrow in Fig. 2C) was isolated, and was found to be completely digestable by chondroitinase AC-II under stronger incubation conditions (see Materials and Methods). It was converted into two compounds eluting at the same positions as the two minor compounds (marked by the asteriks in Fig. 2C). This shows that under the initially employed digestion conditions the oligosaccharide in fraction 8-1 was only partially cleaved. The intensities of these peaks are quantitatively equivalent indicating that the two compounds stem from a common ancestor. Thus, fraction 8 contains two compounds (8-1 and 8-2) in a molar ratio of 56:44. One of the two digested products of the oligosaccharide in fraction 8-1 eluted at the position of ΔDi-6S (standard 2; Fig. 2D) and the peak displays a characteristic doublet representing α and β anomers [13]. The other compound eluted slightly ahead of ΔDi-4S at the position of $\Delta^{4,5}$HexAα1−3GalNAc(4S)-ol (see below). The further structural characterization of the compound in fraction 8-1 had to await ^{1}H-NMR analysis described below.

HPLC analysis of the chondroitinase AC-II digest of fraction 9 showed two peaks; one compound eluted at the position of ΔDi-4S, the other eluted slightly ahead of it (Fig. 5). Upon subsequent treatment with CS-4-sulfatase the latter peak shifted to the position of ΔDi-0S (Fig. 5). The structure determination of the compound in fraction 9 was also accomplished by ^{1}H-NMR analysis as described below.

^{1}H-NMR spectroscopy demonstrated that fractions 8-1 and 9 each contain a tetrasaccharide alditol originating from the repeating disaccharide region (Fig. 6), and the ^{1}H-NMR data of these compounds have been collected in Table 3. Analysis of the spin-coupling topology, 2D ^{1}H-TOCSY and 2D ^{1}H-ROE connectivity patterns (data not shown), together with comparison of NMR data of appropriate reference compounds allowed the identification of a ΔHexAα1−3GalNAc6*S*β1−3GlcAβ1−3 fragment in fraction 8-1. The only difference between the oligosaccharide structures in fraction 8-1 and 9 lies in the differential sulfation of GalNAc-3; the structure in fraction 9 is sulfated at GalNAc C4 instead of at its C6 atom. The spin systems of the reduced monosaccharide in the ^{1}H-NMR spectra of both com-

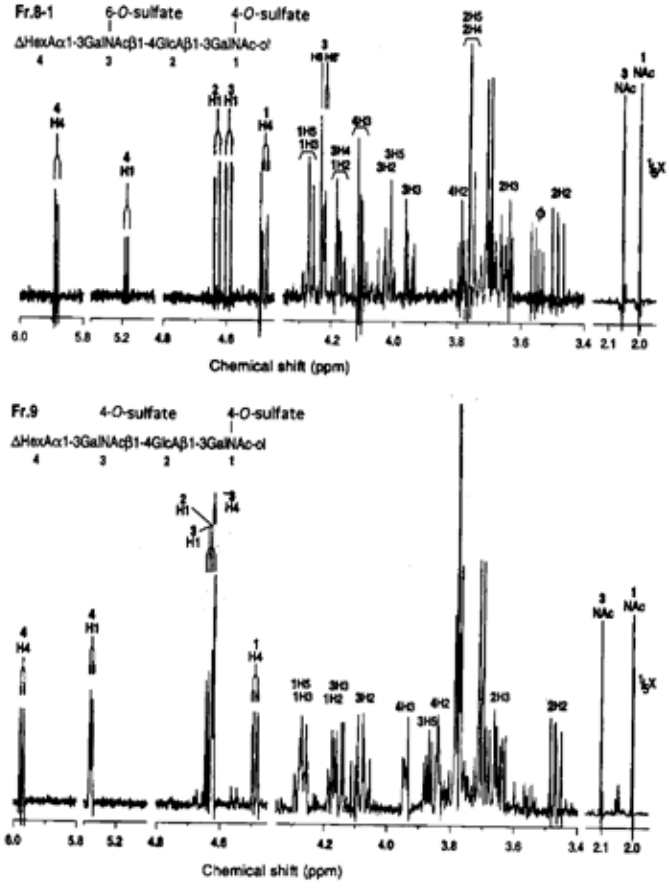

Fig. 6. 1D ^{1}H-NMR spectra of the tetrasaccharide alditols in fractions 8-1 and 9, recorded in ^2H_2O at 285 K. The letters in bold in the spectra refer to the position of the monosaccharide in the structure, and those in normal font stand for the protons.

Table 3. ^{1}H chemical shifts of monosaccharide constituents of tetrasaccharide fragments from the chondroitin sulfate repeating disaccharide region, together with those of a tetrasaccharide reference compound (D; CSC fraction 6 in [31]). Chemical shifts are given relative to acetone in ^2H_2O (δ 2.225) at 285 K. Compound D is ΔHexAα1−3GalNAc6*S*β1−4GlcAβ1−3GalNAc4*S*b1; NMR data were taken from [31]. n.d., not determined; −, not occurring.

Residue	Proton	Chemical shift in		
		D	8-1	9
GalNAc-ol-1	H1/H1′	−	3.695	n.d.
	H2	−	4.171	4.172
	H3	−	4.264	4.267
	H4	−	4.481	4.488
	H5	−	4.271	4.269
	H6/H6′	−	3.68	n.d.
	NAc	−	2.000	2.005
GlcA-2	H1	4.483	4.632	4.629
	H2	3.416	3.484	3.468
	H3	3.591	3.64	3.662
	H4	3.76	3.75	n.d.
	H5	3.680	3.75	n.d.
GalNAc-3	H1	4.593	4.596	4.636
	H2	4.03	4.030	4.073
	H3	3.941	3.949	4.151
	H4	4.180	4.179	4.623
	H5	n.d.	4.011	3.870
	H6	4.22	4.226	n.d.
	H6′	4.22	4.227	n.d.
	NAc	2.056	2.054	2.097
ΔHexA-4	H1	5.184	5.187	5.297
	H2	3.77	3.787	3.838
	H3	4.105	4.106	3.939
	H4	5.877	5.886	5.974

pounds were very similar and were in both cases assigned to a GalNAc-ol residue, sulfated at its C4 atom. The sulfation at GalNAc C4 was concluded from the resonance position of the H4 signal at about 4.48 ppm and the nature of the spin-topology pattern of this spin system. In summary, the structures in these fractions are listed below and are consistent with the results obtained by enzymatic analysis described above

fraction 8-1, $\Delta^{4,5}$HexAα1−3GalNAc6*S*β1−4GlcAβ1−3GalNAc4*S*-ol;

fraction 9, $\Delta^{4,5}$HexAα1−3GalNAc4*S*β1−4GlcAβ1−3GalNAc4*S*-ol.

The elution position of the compound in fraction 5 on HPLC was the same as that of the authentic trisaccharide $\Delta^{4,5}$HexAα1−3GalNAc6*S*β1−4GlcA [21]. However, unlike the authentic trisaccharide, the oligosaccharide in fraction 5 was resistant to both CS-4-sulfatases and CS-6-sulfatases, and also to chondroitinase AC-II (data not shown). The 1D ^{1}H-NMR spectrum and the resonance assignments of the oligosaccharide in fraction 5 are shown in Fig. 7. The two most downfield signals are characteristic of ΔHexA H4 (δ 5.882) and H1 (δ 5.187) resonances. The respective resonance positions of ΔHexA H2 (δ 3.790) and H3 (δ 4.122) were deduced through scalar correlations in a 2D ^{1}H-TOCSY spectrum and from values of vicinal ^{1}H-^{1}H coupling constants. Comparison of the set of ^{1}H chemical shifts of this ΔHexA moiety with those of ΔHexA in suitable reference compounds (i.e. B and C in Table 4) indicates that this residue is not sulfated. Furthermore, the values of the ΔHexA ^{1}H chemical shifts values strongly suggest that the preceding GalNAc residue is sulfated at the C6 position (i.e. B in Table 4). Only one additional anomeric signal was observed (δ 4.727), the respective scalar connectivity pattern and the spin-coupling topology being typical of a GalNAc residue. The presence of two signals stemming from GalNAc H6 and H6′ at 4.227 ppm and 4.18 ppm, respectively, affirms that this GalNAc residue is sulfated at C6 [21]. The lack of other anomeric signals indicate that the structure is a trisaccharide fragment, wherein the terminal residue is reduced. The doublet at 4.202 ppm was assigned as stemming from HexA-ol H5 and served as a starting point for the assignment of the remaining part of the spin system. Resonance positions of the other protons were determined using scalar connectivities in the 2D ^{1}H-TOCSY spectrum (data not shown) and values of the vicinal ^{1}H-^{1}H coupling constants. It is noteworthy that when structure B (Table 4) was reduced, it yielded an identical 1D ^{1}H-NMR spectrum as that of fraction 5, indicating that the HexA-ol residue is GlcA-ol. In summary, the structure of the trisaccharide in fraction 5 emanates from the repeating disaccharide region and is

fraction 5, $\Delta^{4,5}$HexAα1−3GalNAc6*S*β1−4GlcA-ol.

The elution position of fraction 6−2 on HPLC was the same as that of the authentic trisaccharide $\Delta^{4,5}$HexAα1−3GalNAc4*S*β1−4GlcA [21]. Fraction 6−2 was resistant to CS-6-sulfatase and chondroitinase AC-II, unlike the authentic trisaccharide, but sensitive to CS-4-sulfatase resulting in a compound that eluted at the position of the authentic non-sulfated trisaccharide

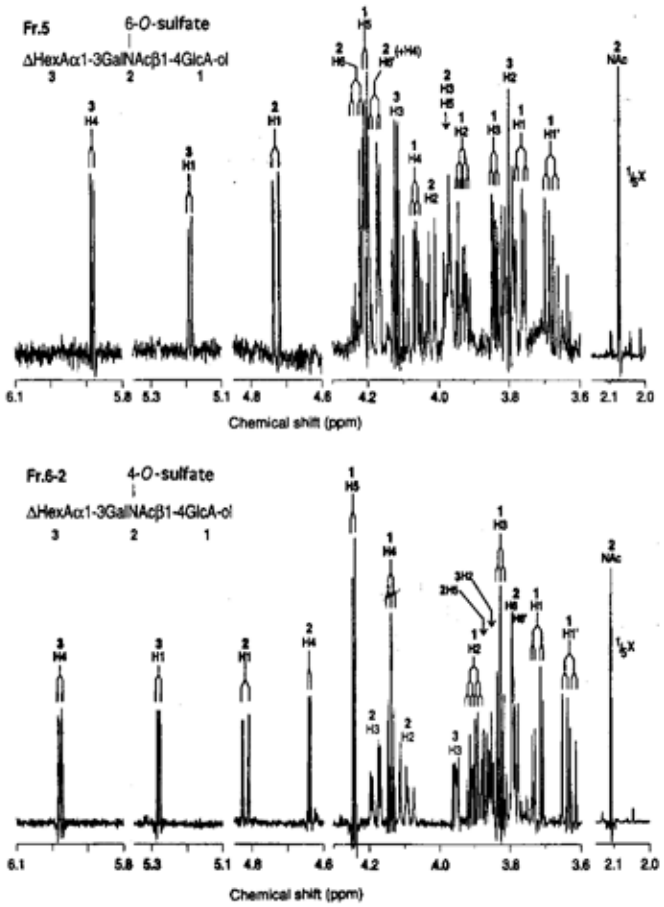

Fig. 7. 1D ^{1}H-NMR spectra of the trisaccharide alditols in fractions 5 and 6-2, recorded in ^2H_2O at 285 K. The letters in bold in the spectra refer to the position of the monosaccharide in the structure, and those in normal font stand for the protons. The spin-coupling topology has been included for the GlcA-ol moieties.

$\Delta^{4,5}$HexAα1−3GalNAcβ1−4GlcA [21]. Fraction 6−2 was then analyzed by ^{1}H-NMR spectroscopy. The 1D spectrum of fraction 6−2 is depicted together with its ^{1}H resonance assignments in Fig. 7. Also this fraction contains a trisaccharide derived from the chondroitin sulfate repeating unit. The structure of the compound in fraction 6−2 only differs from that in fraction 5 in that the GalNAc-2 residue is sulfated at C4 instead of at C6, which was concluded based on the characteristic chemical shifts of the ΔHexA protons (i.e. compare with the structure of C in Table 4), the chemical shift of GalNAc H4 (δ 4.639), and those of GalNAc H5, H6 and H6′ (Table 4). Thus, the compound in fraction 6−2 contains the following structure:

fraction 6−2, $\Delta^{4,5}$HexAα1−3GalNAc4*S*β1−4GlcA-ol.

DISCUSSION

For preparation of the oligosaccharides from the GAG-protein linkage region of C4S of bovine nasal cartilage, highly purified chondroitinase ABC was used in this study. It is an endo-type eliminase that cleaves *N*-acetylgalactosaminide linkages and the predominant digestion products are unsaturated disaccharides, derived from the repeating disaccharide region, and unsaturated hexasaccharides with the core structure $\Delta^{4,5}$HexAα1−3GalNAcβ1−4GlcAβ1−3Galβ1−3Galβ1−4Xyl-(*O*-Ser) from the linkage region [12, 29, 30]. Recently, this highly purified commercial enzyme preparation chondroitinase ABC was demonstrated not to act on chondroitin sulfate tetrasaccharides from the repeating region: unsaturated disaccharides and tetrasaccharides were generated in a molar ratio of approximately 1:3 upon digestion of various chondroitin sulfate isoforms [31]. The strategy used for isolating the linkage region oligosaccharides in this study was a digestion of a chondromucoprotein preparation with highly purified chondroitinase ABC, extensive dialysis of the digest to remove the resulting disaccharides and oligosaccharides from the non-dialyzable protein cores, reductive β-elimination of the dialysate to release the linkage oligsaccharide stubs from the protein cores, isolation of oligosaccharides by gel chromatography, and finally HPLC fractionation of the oligosaccharides. Thus, five linkage hexasaccharide alditols were isolated along with unsaturated trisaccharide and tetrasaccharide alditols derived from the repeating region. The trisaccharide and tetrasaccharide alditols were assumed to be produced by borohydride reduction of the corresponding reducing trisaccharides and tetrasaccharides that were generated by chondroitinase ABC digestion and remained present despite the extensive dialysis. They might, alternatively, be an artefact from the β-elimination reaction. The trisaccharides may be derived from the cleavage sites that were generated by the action of an endogenous endo-β-D-glucuronidase in the cartilage tissue as discussed for the chondroitin sulfate trisaccharides with the backbone structure of GlcAβ1−3GalNAcβ1−4GlcA [21].

The isolated linkage hexasaccharide alditols include one non-sulfated, three monosulfated and one disulfated component in fractions 1, 3-1, 3-2, 4 and 8-2, respectively, in a molar ratio of 38.3:1.6:5.9:43.0:11.2. Two of them (the compounds in fractions 3-1 and 8-2) contain a sulfate group on the C4 position of Gal-3 and accounted for 12.8 mol/100 mol of the isolated linkage hexasaccharides. Incubation of the disulfated hexasaccharide

Table 4. ^{1}H Chemical shifts of monosaccharide constituents of trisaccharide fragments from the CS repeating disaccharide region together with those of reducing trisaccharide reference compounds (B and C; DS fraction I and CSC fraction 9 in [21], respectively). Chemical shifts are given relative to acetone in ^2H_2O (δ 2.225) at 285 K. Compound B, ΔHexAα1−3GalNAc6*S*β1−4GlcAb1; compound C, ΔHexAα1−3GalNAc4*S*β1−4GlcAb1. NMR data of these structures were taken from [21]. ^{1}H chemical shifts are given for βGlcA. n.d., not determined; −, not occurring.

Residue	Proton	Chemical shift in			
		B	C	5	6-2
GlcA-ol-1	H1	−	−	3.769	3.722
	H1′	−	−	3.680	3.633
	H2	−	−	3.926	3.906
	H3	−	−	3.840	3.836
	H4	−	−	4.062	4.139
	H5	−	−	4.202	4.242
GalNAc-2	H1	4.575	4.622	4.727	4.818
	H2	4.02	4.090	4.029	4.093
	H3	3.946	4.151	3.956	4.183
	H4	4.174	4.628	4.17	4.639
	H5	4.02	n.d.	3.96	3.878
	H6	4.234	n.d.	4.227	3.794
	H6′	4.223	n.d.	4.18	3.782
	NAc	2.055	2.099	2.072	2.108
ΔHexA-3	H1	5.181	5.265	5.187	5.280
	H2	3.784	3.834	3.790	3.852
	H3	4.105	3.940	4.122	3.954
	H4	5.879	5.966	5.882	5.976

found in fraction 8 under the alkaline β-elimination conditions used in this study did not cause desulfation of galactose 4-sulfate (data not shown), confirming that the monosulfated hexasaccharide structure found in fraction 3-1 is not an artefact. The C4 sulfation of Gal-3 was first demonstrated in C4S from rat chondrosarcoma [10], later in that from whale cartilage [12] and recently in that of inter-α-trypsin inhibitor [32] and urinary trypsin inhibitor [33]. Thus, the monosulfated tetrasaccharide structure GlcAβ1−3Gal4*S*β1−3Galβ1−4Xyl seems to be characteristic of C4S. Recently, the following novel linkage structures containing iduronic acid (IdoA) in combination with or without Gal4*S* were isolated from bovine aorta dermatan sulfata: IdoA-Gal4*S*-Gal-Xyl and IdoA-Gal-Gal-Xyl [15]. They appear to be characteristic of dermatan sulfate. It should be noted that C4 sulfation of Gal-3 was also found in combination with C6 sulfation of Gal-2 in the linkage hexasaccharides isolated from shark cartilage C6S as in GlcAβ1−3Gal4*S*β1−3Gal6*S*β1−4Xyl [14]. Moreover, studies on shark cartilage C6S revealed other novel modified structures including GlcA-Gal-Gal6*S*-Xyl and GlcA-Gal6*S*-Gal6*S*-Xyl [13, 14]. However sulfation of C6 of Gal-2 and Gal-3 appears to be specific to C6S. In contrast to the linkage region of dermatan sulfate/chondroitin sulfate (galactosaminoglycans), no sulfation of the Gal residues has been detected so far for heparan sulfate and heparin (glucosaminoglycans) despite careful inspection [16−18]. Although the biological significance of these structures is as yet unknown, they may play essential roles in the biosynthesis of glycosaminoglycans, such as a contribution to the segregation of galactosaminoglycans and glucosaminoglycans, or to the chain elongation of chondroitin sulfate. Evaluation of this hypothesis requires characterization of the substrate specificities and of the subcellular localization of the hexosaminyltransferases and the sulfotransferases involved. It is noteworthy that an enzymatic transfer of a β-GalNAc residue to the linkage hexasaccharide-serine synthesized chemically using serum β-GalNAc transferase was markedly influenced by C4 sulfation of the Gal-3 residue (Kitagawa, H., Tsutsumi, K., Ujikawa, M., Goto, F., Tamura, J., Neumann, K., Ogawa, T. and Sugahara, K., unpublished results).

No phosphorylated Xyl was detected in the hexasaccharides isolated in this study in contrast to the linkage region of shark cartilage C6S [13], rat chondrosarcoma C4S [34] and bovine lung heparan sulfate [35]. Remarkably, about 50 mol/100 mol of the linkage hexasaccharides prepared from Engelbreth-Holm-Swarm mouse tumor [36] contain the Xyl2*P* structure when Glc6*P* is present during the isolation procedure to prevent dephosphorylation. It remains to be determined whether the multiple structures in the linkage region of CS reflect variable synthetic modifications by phosphorylation and sulfation or result in part from dephosphorylation and/or desulfation.

In any event, accumulating evidence indicates the existence of the structural heterogeneity of the GAG-protein linkage region among various proteoglycans, which in turn reflects the polydispersity of each GAG species. Isolation of multiple oligosaccharide structures has revealed the existence of a series of GAG polysaccharide subpopulations represented by these individual oligosaccharide structures [10, 12−18, 36]. Thus, the five hexasaccharide structures isolated in this study indicate that there are at least five species of structurally distinct C4S subpopulations in bovine nasal cartilage. It remains to be investigated if they all are derived from aggrecans since the chondromucoprotein preparation used in this study may possibly contain additional chondroitin sulfate proteoglycans. In C4S from rat chondrosarcoma and whale cartilage, three and four hexasaccharide structures were demonstrated, which corresponded to those in fractions 1, 3-2 and 4, and fractions 1, 3-2, 4 and 8-2, respectively. The present investigation added to the list of the C4S linkage hexasaccharides another hexasaccharide structure found in fraction 3-1, $\Delta^{4,5}$HexAα1−3GalNAcβ1−4GlcAβ1−3Gal4*S*β1−3Galβ1−4Xyl-ol. An individual chondroitin sulfate chain may have its intrinsic polymer sequence and function. Structural analysis of longer sequences from the linkage region would provide a more detailed picture of the subpopulations.

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