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Isolation of Reducing Oligosaccharide Chains from the Chondroitin/Dermatan Sulfate–Protein Linkage Region and Preparation of Analytical Probes by Fluorescent Labeling with 2-Aminobenzamide[1]

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The glycosaminoglycan (GAG)-protein linkage regions of various proteoglycans share the common tetrasaccharide GlcA-Gal-Gal-Xyl–attached to Ser residues in the core proteins. In previous analysis we demonstrated unique modifications by epimerization, sulfation and phosphorylation of the component sugars. Here we developed a sensitive analytical method for the linkage region oligosaccharides to detect or monitor structural variations and changes. This will be useful for investigation of their biological roles, which are largely unknown, but they have been implicated in biosynthesis. A variety of linkage region–derived hexasaccharides was first prepared as reducing sugar chains from peptide chondroitin/dermatan sulfate of whale cartilage, shark cartilage, and bovine aorta by means of chondroitinase digestion in conjunction with β-elimination in the absence of reducing reagents, but involving a mild alkali, 0.5 M LiOH, at 4°C to prevent peeling reactions. The structures of these oligosaccharides were determined by the combination of HPLC, enzymatic digestion, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, and ^{1}H NMR spectroscopy, which revealed eleven different hexasaccharides including a novel structure, ΔHexAα1-3GalNAcβ1-4IdoAα1-3Gal(4-*O*-sulfate)β1-3Galβ1-4Xyl (ΔHexA and IdoA represent unsaturated hexuronic acid and L-iduronic acid, respectively). These oligosaccharides were labeled with a fluorophore, 2-aminobenzamide, to prepare analytical probes using the recently developed procedure [Kinoshita and Sugahara (1999) *Anal. Biochem.* 269, 367–378]. The fluorophore-tagged hexasacharides of low picomoles were well separated by HPLC and successfully analyzed by MALDI-TOF mass spectrometry. The principle of the method should be applicable to the analysis of the linkage region oligosaccharides derived from heparin and heparan sulfate as well.

Key words: 2-aminobenzamide, chondroitin sulfate, MALDI-TOF mass, ^{1}H NMR, glycosaminoglycan.

Proteoglycans (PGs) play indispensable roles in cell proliferation, differentiation and migration, and tissue morphogenesis, and constitute a specific protein core and sulfated glycosaminoglycan (GAG) side chains. Non-branched sulfated GAGs are classified into chondroitin sulfate (CS)/dermatan sulfate (DS) and heparan sulfate (HS)/heparin (Hep), the former being galactosaminoglycans and the latter glucosaminoglycans. Both types of GAG chains are attached to the respective core proteins through the so-called common GAG-protein linkage region, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser [for reviews (*1*, *2*)].

During biosynthesis, the linkage region is formed through sequential stepwise addition of each monosaccharide residue from the corresponding sugar nucleotide to a specific serine residue in a core protein (*1*, *2*). Once the first GalNAc has been transferred to this tetrasaccharide linkage region by alleged GalNAc transferase I, the repeating disaccharide region [(-4GlcAβ1-3GalNAcβ1-)$_n$] of CS/DS is synthesized through alternative addition of β-GalNAc and β-GlcA. In contrast, the first GlcNAc transfer to the linkage region by α-GlcNAc transferase I leads to the synthesis of the repeating disaccharide region [(-4GlcAβ1-4GlcNAc-

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Abbreviations: 2AB, 2-aminobenzamide; 2D, two-dimensional; COSY, correlation spectroscopy; CS, chondroitin sulfate; DS, dermatan sulfate; GlcA, D-glucuronic acid; GAG, glycosaminoglycan; HOHAHA, homonuclear Hartmann-Hahn; ΔHexA, unsaturated hexuronic acid or 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid; HexA, hexuronic acid; HexNAc, *N*-acetylhexosamine; Hex, hexose; IdoA, L-iduronic acid; Pen, pentose; DE MALDI-TOF, delayed extraction matrix–assisted laser desorption ionization time-of-flight; 2P, 2-*O*-phosphate; 4S, 4-*O*-sulfate; 6S, 6-*O*-sulfate.

α1-)$_n$] of HS/Hep [for a review (*3*)] through alternative addition of α-GlcNAc and β-GlcA through the action of HS polymerase (*4*). Hence, β-GalNAc transferase I and α-GlcNAc transferase I are the key enzymes that determine the GAG species to be synthesized on the common tetrasaccharide linkage region. However, the biosynthetic sorting mechanism for CS/DS and HS/Hep has not yet been fully clarified, especially due to the lack of information on the acceptor specificity of these key enzymes. It is conceivable that these enzymes recognize the linkage region tetrasaccharide sequence and the specific peptide sequences near the GAG attachment sites. Although certain characteristic peptide sequences near the attachment sites have been described (*5*, *6*) [for a review (*7*)], the detailed acceptor specificity of these enzymes has not been fully established.

Our structural studies, which were started over a decade ago based on the working hypothesis that some structural differences may exist in the linkage region of the different GAG chains and determine the type or character of the GAG species to be synthesized (*2*, *8*), led to the identification of the unique structures containing novel sulfated or phosphorylated modifications such as GlcAβ/IdoAα1-3Gal(4S)β1-3Galβ1-4Xyl (*8*, *9*), GlcAβ1-3Galβ1-3Gal(6S)β1-4Xyl (*10*), GlcAβ1-3Gal(6S)β1-3Gal(6S)β1-4Xyl (*11*), GlcAβ1-3Gal(4S)β1-3Gal(6S)β1-4Xyl (*11*), and GlcAβ1-3Galβ1-3Galβ1-4Xyl(2P) (*10*, *12–15*), where 4S, 6S, and 2P denote 4-*O*-sulfate, 6-*O*-sulfate, and 2-*O*-phosphate, respectively. Notably, sulfated Gal residues have been demonstrated in the linkage region of CS and DS, but not in HS or Hep (*16*, *17*), although Xyl(2P) has been found in both HS/Hep (*13*, *18*) and CS (*10*, *14*), indicating that the sulfation(s) on the Gal residue(s) may represent biosynthetic sorting signals for CS/DS. In this respect, it is intriguing that the α-GalNAc transferase activity towards the linkage tetrasaccharide-serine of α-*N*-acetylglucosaminyltransferase for HS chain initiation is inhibited by 4-*O*-sulfation of the Gal residue (*19*, *20*). However, it is largely unknown whether or not these modifications of the linkage region tetrasaccharide sequence play any biological role as recognition signals for biosynthetic enzymes or functional protein ligands such as extracellular matrix components or signal transducing factors.

To clarify the biological significance of these modifications, a sensitive analytical method is required. So far, linkage region oligosaccharides have been analyzed by proton NMR, which requires substantial amounts of samples, or after ^{3}H-labeling, which requires a well equipped laboratory. Recently, we developed a sensitive analytical method for GAG oligosaccharides involving fluorophore-tagging with 2-aminobenzamide (2AB) (*21*). In this study, we applied this method to the analysis of the linkage region. GAG polysaccharides or linkage region oligosaccharide stubs, produced by GAG lyase digestion, were completely released as reducing sugar chains through β-elimination in the absence of reducing reagents, but under mild alkaline conditions with 0.5 M LiOH at 4°C (*22*) to prevent the possible peeling reaction, and then labeled with 2AB, which generated useful probes for a sensitive analytical method for the GAG-protein linkage region.

MATERIALS AND METHODS

Materials—GAG-peptide fractions of whale cartilage CS, shark cartilage CS, and bovine aorta DS were prepared as reported previously (*9*, *10*, *23*). Chondroitinases ABC [EC 4.2.2.4], AC-II [EC 4.2.2.5] and B [EC 4.2.2.] were obtained from Seikagaku, Tokyo. Calf intestine alkaline phosphatase of special quality for molecular biology was from Boehringer Mannheim, Tokyo. 2AB was purchased from Nacalai Tesque, Kyoto.

Chondroitinase ABC Digestion of the GAG-Peptide Fraction of Whale Cartilage CS—The GAG-peptide fraction prepared from whale cartilage CS (100 mg) was digested with 1.0 IU of chondroitinase ABC in a total volume of 1 ml of 0.05 M Tris-HCl buffer, pH 8.0, containing 100 μg/ml bovine serum albumin at 37°C for 18 h. To complete the digestion, 0.2 IU of the enzyme was added to the reaction mixture, followed by further incubation for 4 h. After incubation, 0.11 ml of 50% trichloroacetic acid was added and the reaction mixture was centrifuged. The resultant precipitate was washed with 1.2 ml of 5% trichloroactic acid and the combined supernatant fluid was extracted with ether. The aqueous phase was neutralized with 1 M Na_2CO_3 and then applied to a Bio-Gel P-2 column (1 × 115 cm) with 0.25 M NH_4HCO_3/7% 1-propanol as the eluent. Fractions (1 ml) were collected and monitored as to UV-absorption at 232 nm. The glycopeptide fraction, which was separated from the disaccharide fraction and presumably contained hexasaccharide-peptides (*8*, *23*), was recovered and concentrated to dryness.

LiOH Treatment of GAG-Peptides—The glycopeptide fraction (0.75 nmol as unsaturated uronic acid) prepared from whale cartilage CS was treated with various concentrations (0.1, 0.3, 0.5, 0.7, or 1.0 M) of LiOH at different temperatures (4, 7, 10, 12, 15, or 20°C) for various times (13, 15, 17, 19, 21, or 24 h) to determine the optimal conditions. To prepare linkage oligosaccharides, the glycopeptide fraction prepared from whale cartilage CS (4.6 μmol as unsaturated uronic acid) was treated with 0.5 M LiOH at 4°C for 15 h. To prepare free GAGs, the GAG-peptide fractions prepared from shark cartilage (1.0 g) and bovine aorta (140 mg) were treated with 0.5 M LiOH at 4°C for 15 h to liberate GAG chains from the core peptides. The reactions were terminated by neutralization with 2.0 M acetic acid, and the reaction mixtures were then subjected to gel filtration on a column of Sephadex G-25 (1.5 × 47 cm) with 0.25 M NH_4HCO_3/7% 1-propanol as the eluent.

Chondroitinase ABC Digestion of the Free GAG Fractions of Shark Cartilage CS and Bovine Aorta DS—The free GAG fractions prepared from shark cartilage and bovine aorta were individually digested with 750 mIU of chondroitinase ABC in a total volume of 1 ml of the buffer as described above at 37°C for 15.5 h. To complete the digestions, 0.2 IU of the enzyme was added to each reaction mixture, followed by further incubation. After 16 h, trichloroactic acid precipitation was performed as described above and the supernatant fluid was extracted with ether. The aqueous phase was neutralized with 1 M Na_2CO_3 and then applied to a Bio-Gel P-2 column. Fractions (1 ml) were collected and monitored as to absorption at 232 nm. The oligosaccharide fractions, which were separated from the disaccharide fractions and presumably contained linkage region hexasaccharides (*8*, *23*), were recovered.

HPLC—Fractionation and analysis of the linkage oligosaccharide fractions and their 2AB derivatives were carried out by HPLC on an amine-bound silica column, as

reported previously (*24*). Eluates containing 2AB-derivatized or underivatized oligosaccharides were monitored as to fluorescence intensity with excitation and emission wavelengths of 330 and 420 nm, or as to absorption at 232 nm, respectively. The separated subfractions were concentrated and desalted on a column of Sephadex G-25 with distilled water as the eluent.

Delayed Extraction Matrix-Assisted Laser Desorption Ionization Time-of-Flight (DE MALDI-TOF) Mass Spectrometry—DE MALDI-TOF mass spectra, in the negative mode, of linkage oligosaccharides and their 2AB derivatives were recorded with a Voyager Elite XL (PE Biosystems, Framingham, MA) in the reflector mode (*25*). α-Cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid was used as the matrix at a concentration of 10 mg/ml in a water–MeCN mixture (1:1, v/v) containing 0.1% trifluoroacetic acid or in a water–MeOH mixture (9:1, v/v), respectively. An aqueous solution of a linkage oligosaccharide (20 pmol/μl) or a 2AB derivative of a linkage oligosaccharide (10 pmol/μl) was mixed with an equal volume of the matrix solution. A 0.5–1.0 μl aliquot of this sample-matrix mixture was placed on the sample plate well and then dried under a stream of air.

Alkaline Phosphatase Digestion—The linkage oligosaccharide fractions (0.25 nmol as ΔHexA) were incubated with 1 IU of the enzyme in a total volume of 60 μl of 0.07 M glycine/NaOH buffer, pH 9.9, containing 0.5 mM $MgCl_2$ at 37°C for 10 min (*14*).

500-MHz ^1H NMR Analysis—Linkage oligosaccharides for NMR analysis were repeatedly exchanged in ^2H_2O with intermittent lyophilization. The 500-MHz ^1H NMR spectra of fractions A, B, C, and D derived from whale cartilage CS as well as fractions 7, 14, 17, 22, 23, 24, and 31 derived from shark cartilage CS were measured with a Varian VXR-500 at a probe temperature of 26°C, as reported previously (*9, 26, 27*). The 500-MHz ^1H NMR spectra of fractions 19 and 20 derived from shark cartilage CS as well as fractions I, II, III, and IV derived from bovine aorta DS were measured using a Nano-NMR probe containing 40 μl of the sample solution, as reported previously (*28*). Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone (δ 2.225) in ^2H_2O (*29*). ^1H NMR spectra of fractions 32 and 33 isolated from shark cartilage CS were not measured because of the limited amounts of the samples.

Other Analytical Methods—Unsaturated uronic acid was spectrophotometrically quantified based upon an average millimolar absorption coefficient of 5.5 at 232 nm (*30*). Derivatization of linkage oligosaccharides (2.0 nmol each as ΔHexA) with 2AB was carried out as described previously (*21*).

RESULTS

Determination of the Optimum Conditions for the LiOH Treatment—The optimal reaction conditions for liberating oligosaccharides from core peptides were determined by means of a series of experiments involving glycopeptide fractions prepared from whale cartilage CS as described under "MATERIALS AND METHODS." Firstly, the glycopeptide fraction prepared from whale cartilage CS was treated with 0.5 M LiOH at various temperatures (4, 7, 10, 12, 15, or 20°C) for 15 h, and the resultant oligosaccharides were labeled with 2AB. This treatment resulted in four major 2AB-labeled putative hexasaccharides (see below), as presumed from the previous observation indicating the existence of four major linkage hexasaccharides (*23*). As shown in Fig. 1A, the maximum yield of the four 2AB-labeled hexasaccharides was obtained on incubation at 4 and 7°C. The following experiments were performed at 4°C rather than 7°C, to avoid an unnecessary shift to a higher temperature, since a sharp decrease in the recovery was observed at 10°C (Fig. 1A). Secondly, the glycopeptide fraction was treated with 0.5 M LiOH for various times (13, 15, 17, 19, 21, or 24 h). The reaction time did not affect the yield of 2AB-labeled hexasaccharides (Fig. 1B), indicating that the reaction reached a plateau within 13 h and that the liber-

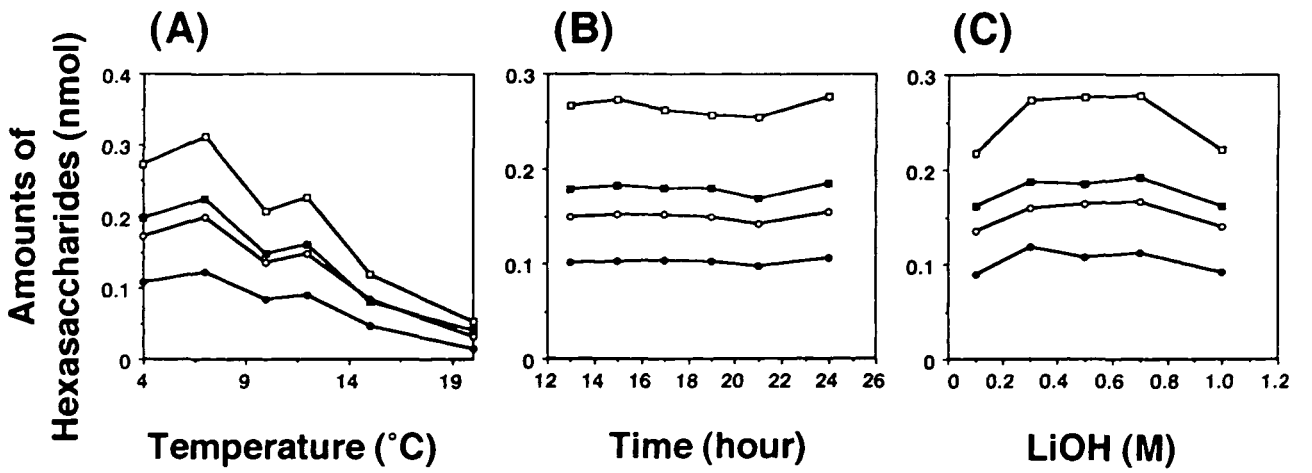

Fig. 1. **Liberation of hexasaccharides from whale cartilage CS on LiOH treatment.** The optimum temperature (A), reaction time (B), and LiOH concentration (C) for the liberation of hexasaccharides from the hexasaccharide-peptides prepared from whale cartilage CS were determined by means of a series of experiments, as described under "MATERIALS AND METHODS." Four major liberated hexasaccharides were labeled with 2AB and then analyzed by HPLC on an amine-bound silica column. The amounts of the four major 2AB-hexasaccharides, A–D, were calculated based on their fluorescent intensity. Open circles, fraction A; closed circles, fraction B; open squares, fraction C; closed squares, fraction D.

ated hexasaccharides were not degraded through side-reactions such as the peeling reaction. Thirdly, the glycopeptide fraction was incubated with various concentrations (0.1, 0.3, 0.5, 0.7, or 1.0 M) of LiOH at 4°C for 15 h, and the resultant oligosaccharides were labeled with 2AB. The 2AB-hexasaccharide products were separated and quantified based on their fluorescent intensity. The reaction was maximum when the concentration of LiOH was in the range of 0.3–0.7 M (Fig. 1C). Thus, 0.5 M LiOH was used for the following reactions. Under the established conditions, no shorter chains generated through possible peeling reactions were observed.

Isolation of the Linkage Oligosaccharides—The GAG-protein linkage region oligosaccharides of CS/DS proteoglycans of whale cartilage, shark cartilage and bovine aorta were isolated from the corresponding GAG-peptide fractions (see "MATERIALS AND METHODS") by the combination of chondroitinase digetion and a β-elimination reaction. The GAG-peptide fraction (100 mg) prepared from whale cartilage CS was depolymerized exhaustively by chondroitinase ABC digestion. The digest was subjected to gel permeation chromatography and the glycopeptide fraction obtained was treated with LiOH to liberate oligosaccharides from peptides. The resulting unsaturated oligosaccharides were fractionated by HPLC on an amine-bound silica column. As shown in Fig. 2A, four major peaks, designated as fractions A, B, C, and D, were detected, and the oligosaccharide gave a similar chromatographic profile to that of the linkage hexasaccharide alditol fraction prepared from whale cartilage CS reported previously (*23*). The structure of each oligosaccharide was determined from the elution position on the amine-bound silica column, and confirmed by structural analyses involving DE MALDI-TOF mass spectrometry and ^{1}H NMR spectroscopy, as described below.

In the cases of shark cartilage CS and bovine aorta DS, the CS and DS chains were first liberated from the core peptides, and then depolymerized into the linkage region oligosaccharides and disaccharides derived from the repeating disaccharide region. Large amounts (1.0 and 0.14 g) of the glycopeptide fractions prepared from shark cartilage and bovine aorta, respectively, were treated with LiOH. The liberated GAG fractions were digested with chondroitinase ABC, and then each digest was fractionated into disaccharides and oligosaccharides by gel filtration on Bio-Gel P-2. The amounts of the linkage-region-derived oligosaccharide fractions prepared from shark cartilage CS and bovine aorta DS were 19.7 and 2.1 μmol, respectively, based on the absorption of unsaturated uronic acid at 232 nm. The oligosaccharide fractions from shark cartilage CS and bovine aorta DS were subfractionated by HPLC on an amine-bound silica column into 38 (fractions 1 to 38) (Fig. 2B) and 4 fractions (fractions I to IV) (data not shown), respectively. For identification of hexasaccharides derived from the linkage region, the sensitivity of each isolated oligosaccharide to chondroitinases AC-II and B was examined. Chondroitinase ABC degrades all the β-galactosaminide bonds in CS/DS except for the innermost galactosaminidic linkage immediately adjacent to the GAG-protein linkage region tetrasaccharide (*23*). Chondroitinase ACII or B can cleave the innermost galactosaminidic linkage (*9*), although sulfation patterns influence the sensitivity of linkage hexasaccharides to the latter enzyme (*23*). Therefore, fractions that are resistant to chondroitinase ABC but sensitive to AC-II or B were assumed to contain the linkage region hexasaccharides. Fractions 7, 14, 17, 19, 20, 22, 23, 24, 31, 32, and 33, in addition to fractions I, II, III, and IV, were sensitive to

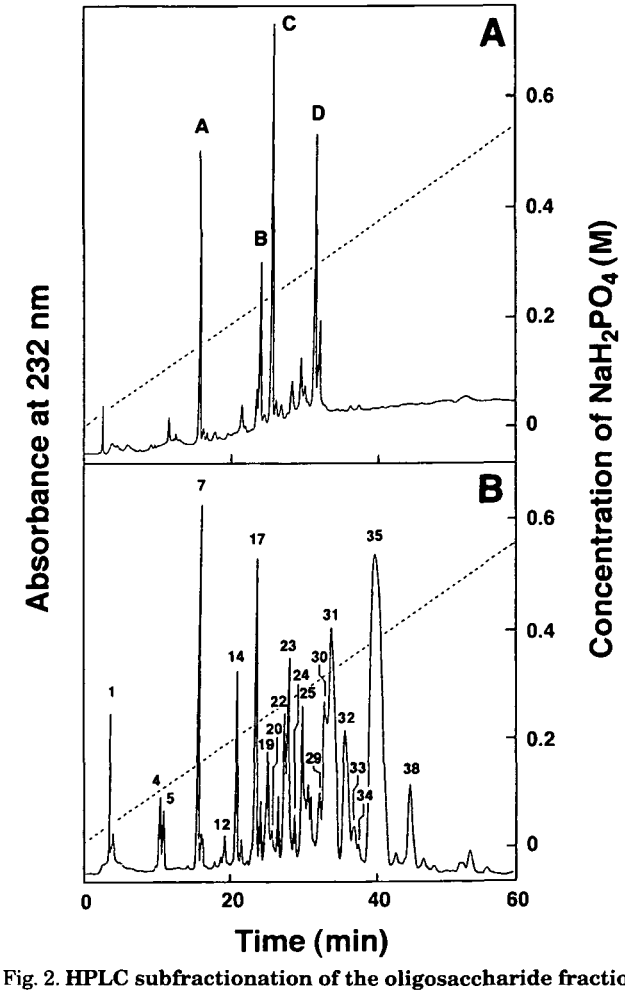

Fig. 2. **HPLC subfractionation of the oligosaccharide fraction prepared from the GAG-protein linkage region.** The oligosaccharide fractions prepared from whale cartilage CS (A) and shark cartilage CS (B), corresponding to 4.6 and 19.7 μmol of ΔHexA, respectively, were chromatographed on an amine-bound silica column using a linear gradient of NaH_2PO_4, as indicated by the dashed lines.

TABLE I. **Assignments of molecular ion signal afforded by the negative DE MALDI-TOF mass spectra of the fractions prepared from whale cartilage CS.**

Fraction	*m/z* for $[M - H]^-$	*m/z* for $[M + Na - 2H]^-$	*m/z* for $[M + 2Na - 3H]^-$	Molecular composition of M
A	1,010	1,032		$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1$
B	1,090	1,112		$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$
C	1,090	1,112	1,134	$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$
D		1,192		$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)_2$

either chondroitinase AC-II or B, indicating that they were most likely derived from the linkage region. These individual fractions were more than 80% pure, as judged on HPLC. Three (I, III, and IV) of the four fractions obtained from the linkage region oligosaccharide fraction of bovine aorta DS were eluted at the positions of fractions A, B, and C derived from whale cartilage CS, indicating that each corresponding fraction contained the same structure, respectively. This was confirmed by 500-MHz ^{1}H NMR analysis, as described below.

As described above, in the case of the whale cartilage sample, enzymatic digestion was applied first, then a β-elimination reaction was carried out, whereas the order of these treatments was reversed for the shark cartilage and bovine aorta samples. Although the linkage region oligosaccharides, attached to peptides, were better separated from the enzymatically produced disaccharides with the former method, both methods gave satisfactory results in terms of the separation and recovery of the linkage region oligosaccharides.

DE MALDI-TOF Mass Analysis—DE MALDI-TOF mass analysis of the 2AB-derivatized and underivatized hexasaccharide samples in the negative ion mode revealed their molecular weights, from which the composition and the maximum number of *O*-sulfate or *O*-phosphate groups present in each fraction were inferred. In the negative ion mode DE MALDI-TOF mass spectrum of sulfated oligosaccharides, alkali-metal-attached molecular ions of the $[M + xNa - (x+1)H]^-$ type (M represents the fully protonated acid form of an oligosaccharide) were preferentially observed. The assignments of the molecular ion signals afforded by each of the fractions are presented in Tables I, II, and III.

The molecular ion signal clusters at *m/z* 1,010 and 1,032 afforded by fraction A from whale cartilage CS (Table I) corresponded, respectively, to non- and mono-sodiated molecular ions of $\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1$ ($[M - H]^-$ and $[M + Na - 2H]^-$, respectively), where HexA, ΔHexA, HexNAc, Hex, and Pen represent hexuronic acid, unsaturated

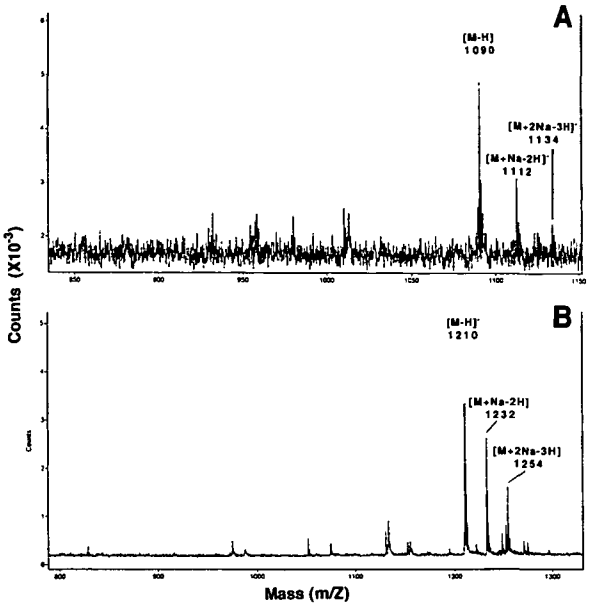

Fig. 3. **DE MALDI-TOF mass spectra of underivatized fraction II (A) and 2AB-derivatized fraction II (B).** DE MALDI-TOF mass spectra of fraction II and its 2AB derivative were recorded in the negative ion mode with 2,5-dihydroxybenzoic acid as the matrix. Major molecular ion signals were assigned as summarized in Table III.

TABLE II. **Assignment of molecular ion signals afforded by the negative DE MALDI-TOF mass spectra of the fractions prepared from shark cartilage CS.**

Fraction	*m/z* for					Molecular composition of M
	$[M-H]^-$	$[M+Na-2H]^-$	$[M+2Na-3H]^-$	$[M+3Na-4H]^-$	$[M+4Na-5H]^-$	
7	1,010	1,032				$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1$
14		1,112	1,134			$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$
17	1,090	1,112				$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$
19	1,090	1,112				$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$
20	1,090	1,112	1,134			$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OPO_3H)_2$
22		1,192				$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)_2$
23		1,192	1,214	1,236		$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)_2$
24		1,192				$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)_2$
31			1,294	1,316	1,338	$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)_3$
32			1,294	1,316	1,338	$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)_3$
33			1,294	1,316	1,338	$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)_3$

TABLE III. **Assignment of molecular ion signals afforded by the negative DE MALDI-TOF mass spectra of the fractions prepared from bovine aorta DS.** The molecular ion signals of the non-derivatized (A) and 2AB-derivatized (B) samples are shown.

Fraction	*m/z* for $[M - H]^-$	*m/z* for $[M + Na - 2H]^-$	*m/z* for $[M + 2Na - 3H]^-$	Molecular composition of M
A				
I	1,010			$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1$
II	1,090	1,112	1,134	$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$
III	1,090	1,112	1,134	$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$
IV	1,090	1,112	1,134	$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$
B				
I	1,130	1,152		$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1$-2AB
II	1,210	1,232	1,254	$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$-2AB
III	1,210	1,232	1,254	$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$-2AB
IV	1,210	1,232	1,254	$\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$-2AB

hexuronic acid, *N*-acetylhexosamine, hexose and pentose, respectively. In the negative ion mode DE MALDI-TOF mass spectra of fractions B and C (Table I), the molecular ion signal clusters at *m/z* 1,090 and 1,112 corresponded, respectively, to non- and mono-sodiated $\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1$ with an *O*-sulfate group. In the DE MALDI-TOF mass spectrum of fraction D, a molecular ion signal was observed as $[M + Na - 2H]^-$ at *m/z* 1,192, indicating that the component in this fraction was $\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1$ with two *O*-sulfate groups. Thus, the major components in fractions A, B, C, and D were estimated to be non-, mono-, mono-, and disulfated unsaturated hexasaccharides, respectively, derived from the GAG-protein linkage region.

In the DE MALDI-TOF mass spectra of fractions 7, 14, 17, 19, 20, 22, 23, 24, 31, 32, and 33 from shark cartilage CS, molecular ion signals characteristic of the linkage region hexasaccharides were observed, as shown in Table II, and the molecular compositions were estimated from the findings as in the case of the oligosaccharides derived from whale cartilage CS, and are listed in the right column. Although the detected molecular ion signals of the component in fraction 20 were the same as those in fractions 14, 17, and 19, the major component in this fraction was sensitive to calf intestine alkaline phosphatase (see below), indicating that it was phosphorylated but not sulfated.

The samples prepared from the GAG-protein linkage region of bovine aorta DS were analyzed by DE MALDI-TOF mass before and after derivatization with 2AB. The findings are summarized in Table III. In the negative ion mode DE MALDI-TOF mass spectra, the molecular ion signal for underivatized fraction I was detected at *m/z* 1,010, and molecular ion clusters for underivatized fractions II, III, and IV were observed at 1,090, 1,112, and 1,134 ($[M - H]^-$, $[M + Na - 2H]^-$, and $[M + 2Na - 3H]^-$, respectively). These findings indicated that the molecular compositions of fraction I and fractions II – IV were $\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1$ and $\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1(OSO_3H)$, respectively. Derivatization of oligosaccharides with 2AB increased the detection sensitivity and improved the quality of the spectra by lowering the background level (Fig. 3). In the negative ion mode DE MALDI-TOF mass spectra of the 2AB derivatives, the molecular ion signal clusters at *m/z* 1,130 and 1,152 as well as 1,210, 1,232, and 1,254 corresponded, respectively, to the molecular ions of non-sulfated as well as monosulfated $\Delta HexA_1HexA_1HexNAc_1Hex_2Pen_1$2AB, respectively (Table III), this being consistent with the composition findings on DE MALDI-TOF mass analysis of the underivatized samples.

Alkaline Phosphatase Digestion Analysis—Since the mass number of a sulfate group is the same as that of a phosphate group, it is not possible to distinguish them by DE MALDI-TOF mass analysis. Hence, each fraction was subjected to alkaline phosphatase digestion, and then the

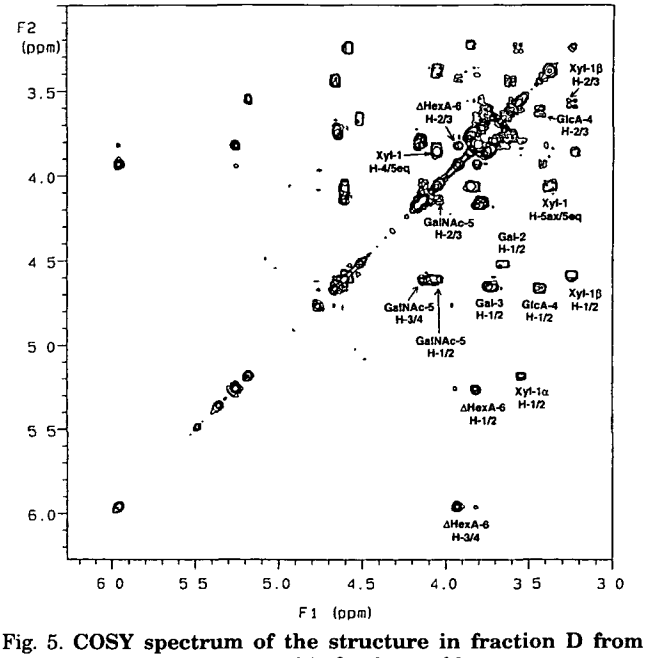

Fig. 5. **COSY spectrum of the structure in fraction D from whale cartilage CS recorded in ^2H_2O at 26°C.**

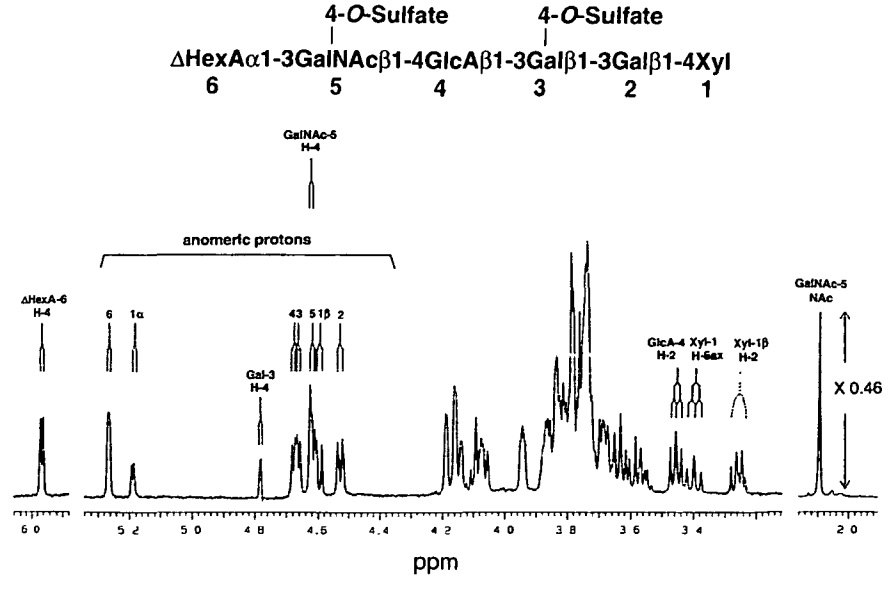

Fig. 4. **One-dimensional 500-MHz ^1H NMR spectrum of the structure in fraction D from whale cartilage CS recorded in ^2H_2O at 26°C.** The numbers and letters in the spectrum refer to the corresponding residues in the structure.

digests were analyzed by HPLC on an amine-bound silica column. All the fractions isolated in this study except for fraction 20 were resistant to this enzyme. Upon alkaline phosphatase digestion of fraction 20, the peak shifted by approximately 10 min to the position of authentic ΔHexAα1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl (data not shown), indicating that the major component in fraction 20 was phosphorylated.

500-MHz ^{1}H NMR Analysis—The structures of the major compounds in the linkage hexasaccharide fractions isolated from whale cartilage CS (fractions A, B, C, and D), shark cartilage CS (fractions 7, 14, 17, 19, 20, 22, 23, 24, and 31), and bovine aorta DS (fractions I, II, III, and IV) were individually analyzed by 500-MHz ^{1}H NMR spectroscopy. The proton chemical shifts were assigned by means of two-dimensional (2D) homonuclear Hartmann-Hahn (HOHAHA) and correlation spectroscopy (COSY). As representatives, the one-dimensional and 2D COSY spectra of fraction D are depicted in Figs. 4 and 5. The one-dimensional and 2D HOHAHA spectra of fraction II from bovine aorta are shown in Figs. 6 and 7, respectively. All the NMR findings are summarized in Table IV.

Compared with the NMR findings for the linkage hexasaccharide alditols isolated from CS/DS chains of whale cartilage, shark cartilage and bovine aorta, the chemical shifts of the protons of most of the linkage hexasaccharides prepared in the present study were identical with those of the corresponding hexasaccharide alditols (*9–11*, *23*) except for those of their reducing terminal xylose residues. Since the xylose residues of the linkage hexasaccharides prepared here were not reduced and were in equilibrium between the α and β anomers in solution, the anomerization effects resulted in the doubling of each proton signal. Anomeric proton signals of an αXyl and a βXyl in the linkage hexasaccharides prepared here were observed around δ 5.18 - 5.19 and 4.59 - 4.60, respectively. These values were consis-

TABLE IV. **^{1}H-Chemical shifts of structural-reporter-groups of the monosaccharide constituents of the isolated linkage hexasaccharides from whale cartilage, shark cartilage, and bovine aorta CS/DS.** The ^{1}H-chemical shifts of structural-reporter-groups of the monosaccharide constituents of the linkage hexasaccharides are shown. Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured indirectly relative to acetone (δ 2.225 ppm) in ^2H_2O at 26°C. The estimated error for the values to two decimal places was only ± 0.01 ppm because of partial overlapping of signals, that for the values to three decimal places being ± 0.002 ppm. Coupling constants, $J_{1,2}$ (in Hz), are given in parentheses.

Residue	Reporter group	Fraction A[a]	Fraction B[b]	Fraction C[c]	Fraction D	Fraction 14
Xyl-1	H1α	5.185 (4.0)	5.190 (3.5)	5.191 (3.5)	5.190 (3.5)	5.178 (3.0)
	H1β	4.596 (8.0)	4.596 (8.0)	4.597 (7.5)	4.596 (8.0)	4.590 (8.0)
	H2α	3.547	3.546	3.540	3.546	3.56
	H2β	3.263	3.261	3.263	3.263	3.279
	H3	3.567	3.569	3.57	3.567	3.56
	H4	3.84	3.84	3.85	3.85	3.87
	H5ax	3.397	3.397	3.397	3.397	3.402
	H5eq	4.06	4.06	4.06	4.06	4.08
Gal-2	H1α[d]	4.526 (7.5)	4.523 (8.0)	4.524 (8.0)	4.524 (8.0)	4.550 (8.5)
	H1β[d]	4.528 (7.5)	4.528 (7.5)	4.529 (8.0)	4.528 (8.0)	4.558 (7.5)
	H2	3.67	3.67	3.67	3.67	3.68
	H3	3.81	3.81	3.81	3.80	3.85
	H4	4.187	4.186	4.188	4.172	4.251
	H5	ND[e]	ND	ND	ND	3.98
	H6	ND	ND	ND	ND	4.219
	H6′	ND	ND	ND	ND	4.206
Gal-3	H1	4.663 (8.0)	4.663 (7.0)	4.666 (7.5)	4.665 (8.0)	4.668 (7.5)
	H2	3.73	3.75	3.74	3.75	3.74
	H3	3.78	3.78	3.78	ND	3.79
	H4	4.154	4.154	4.159	4.783	4.154
	H5	ND	ND	ND	ND	ND
	H6	ND	ND	ND	ND	ND
	H6′	ND	ND	ND	ND	ND
HexA-4	H1	4.667 (8.0)	4.677 (7.0)	4.675 (7.5)	4.675 (7.5)	4.668 (7.5)
	H2	3.455	3.469	3.455	3.456	3.455
	H3	3.622	3.632	3.633	3.634	3.622
	H4	3.76	3.74	3.77	3.76	3.79
	H5	ND	ND	ND	ND	ND
GalNAc-5	H1	4.543 (8.0)	4.576 (8.5)	4.619 (7.5)	4.619 (7.5)	4.542 (8.0)
	H2	4.004	4.023	4.07	4.072	3.98
	H3	3.903	3.942	4.149	4.147	3.902
	H4	4.098	4.173	4.623	4.624	4.098
	H5	ND	4.00	ND	ND	ND
	H6	ND	4.233	ND	ND	ND
	H6′	ND	4.221	ND	ND	ND
	NAc	2.057	2.052	2.095	2.095	2.057
ΔHexA-6	H1	5.185 (4.0)	5.181 (5.0)	5.265 (2.0)	5.266 (2.5)	5.185 (4.0)
	H2	3.78	3.77	3.82	3.82	3.78
	H3	4.094	4.106	3.945	3.944	4.094
	H4	5.896	5.882	5.968	5.967	5.895

[a]The spectral data were identical for fractions A, 7, and I. [b]The spectral data were identical for fractions B, 17, and III. [c]The spectral data were identical for fractions C, 19, and IV. [d]These values may be interchanged. [e]ND, not determined.

tent with the chemical shifts of protons belonging to Xyl-**1** of the reference compound, ΔHexAα1-4GlcNAcα1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl, isolated from the GAG-protein linkage region of bovine kidney heparan sulfate (*26*), confirming the presence of a non-reduced xylose residue at the reducing ends of the isolated hexasaccharides. Thus, the following structures have been elucidated, where 4S and 6S denote 4-*O*- and 6-*O*-sulfate, respectively.

Fractions A, 7, I: ΔHexAα1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl

Fractions B, 17, III: ΔHexAα1-3GalNAc(6S)β1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl

Fractions C, 19, IV: ΔHexAα1-3GalNAc(4S)β1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl

Fraction D: ΔHexAα1-3GalNAc(4S)β1-4GlcAβ1-3Gal-(4S)β1-3Galβ1-4Xyl

Fraction 14: ΔHexAα1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Gal(6S)β1-4Xyl

Fraction 22: ΔHexAα1-3GalNAc(6S)β1-4GlcAβ1-3Galβ1-3Gal(6S)β1-4Xyl

Fraction 23: ΔHexAα1-3GalNAcβ1-4GlcAβ1-3Gal (6S)β1-3Gal(6S)β1-4Xyl

Fraction 24: ΔHexAα1-3GalNAc(4S)β1-4GlcAβ1-3Galβ1-3Gal(6S)β1-4Xyl

Fraction 31: ΔHexAα1-3GalNAc(6S)β1-4GlcAβ1-3Gal-(6S)β1-3Gal(6S)β1-4Xyl

The proton signals in the spectrum of fraction 20, which contained a phosphorylated linkage hexasaccharide, were not well resolved due to the limited amount of the sample. The chemical shifts of H-2, H-3, and H-4 of Xyl-**1** of the compound in fraction 20 were in the bulk region and overlapped with other proton signals, and thus could not be assigned (Table IV). Compared with the chemical shifts of protons of the non-sulfated linkage hexasaccharide, ΔHexAα1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl (fractions A, 7, and I), downfield and upfield shifts of the anomeric protons of αXyl-**1** (δ 5.334) and βXyl-**1** (δ 4.544) of the compound in fraction 20 (by approximately Δ0.15 and 0.05 ppm), respectively, were found, whereas the chemical shifts of protons belonging to Gal-**2**, Gal-**3**, GlcA-**4**, GalNAc-**5**, and ΔHexA-**6** were very similar to those of the corresponding residues of the non-sulfated linkage hexasaccharide (data not shown), indicating that the Xyl residue was phosphorylated. Although it was not possible to distinguish between

TABLE IV. (Continued)

Residue	Reporter group	Fraction 20	Fraction 22	Fraction 23	Fraction 24	Fraction 31	Fraction II
Xyl-**1**	H1α	5.334 (3.5)	5.183 (2.5)	5.185 (3.0)	5.187 (3.5)	5.183 (4.0)	5.188 (4.0)
	H1β	4.544 (8.5)	4.591 (7.5)	4.590 (8.0)	4.591 (8.0)	4.592 (7.5)	4.600 (8.0)
	H2α	3.91	3.57	3.56	3.57	3.56	3.548
	H2β	ND	3.279	3.286	3.281	3.286	3.265
	H3	ND	3.579	3.582	3.58	3.584	3.572
	H4	ND	3.86	3.86	3.86	3.87	3.85
	H5ax	ND	3.402	3.407	3.404	3.407	3.401
	H5eq	ND	4.07	4.07	4.07	4.08	4.08
Gal-**2**	H1α	4.529 (6.0)	4.549 (8.0)	4.550 (8.0)	4.551 (8.0)	4.550 (8.0)	4.529 (7.5)
	H1β	ND	4.556 (8.0)	4.559 (7.5)	4.558 (7.5)	4.559 (8.0)	4.534 (8.0)
	H2	3.67	3.67	3.67	3.68	3.68	3.66
	H3	3.80	3.85	3.84	3.84	3.840	3.83
	H4	4.179	4.250	4.283	4.251	4.285	4.180
	H5	ND	3.97	3.97	3.97	3.97	ND
	H6	ND	4.220	4.239	4.220	4.241	ND
	H6′	ND	4.208	4.222	4.208	4.220	ND
Gal-**3**	H1	4.665 (7.5)	4.667 (7.5)	4.678 (7.5)	4.670 (8.0)	4.679 (8.0)	4.726 (7.5)
	H2	3.74	3.75	3.75	3.74	3.76	3.76
	H3	3.78	3.79	3.80	3.79	3.840	4.00
	H4	4.154	4.154	4.197	4.159	4.285	4.661
	H5	ND	ND	3.91	ND	3.97	ND
	H6	ND	ND	4.187	ND	4.241	ND
	H6′	ND	ND	4.175	ND	4.220	ND
HexA-**4**	H1	4.665 (7.5)	4.676 (8.0)	4.676 (7.5)	4.676 (7.5)	4.685 (8.0)	5.168 (3.0)
	H2	3.454	3.468	3.455	3.457	3.468	3.71
	H3	3.624	3.632	3.622	3.635	3.631	3.97
	H4	3.74	3.74	3.75	3.73	3.74	4.136
	H5	ND	ND	ND	ND	ND	4.725
GalNAc-**5**	H1	4.544 (8.5)	4.576 (7.5)	4.544 (8.0)	4.619 (7.5)	4.579 (8.5)	4.615 (8.0)
	H2	4.005	4.021	3.98	4.068	4.00	3.98
	H3	3.90	3.945	3.989	4.149	3.94	3.90
	H4	4.097	4.175	4.101	4.626	4.176	4.08
	H5	ND	4.00	ND	ND	4.00	ND
	H6	ND	4.232	ND	ND	4.231	ND
	H6′	ND	4.220	ND	ND	4.217	ND
	NAc	2.057	2.052	2.059	2.096	2.055	2.08
ΔHexA-**6**	H1	5.186 (4.0)	5.180 (5.0)	5.187 (4.5)	5.266 (3.0)	5.183 (4.0)	5.188 (4.0)
	H2	3.78	3.78	3.78	3.82	3.77	3.78
	H3	4.094	4.105	4.095	3.93	4.105	4.095
	H4	5.897	5.879	5.895	5.966	5.879	5.898

C2 and C3 with regard to the phosphorylation position of the Xyl residue, it was most probably the C2 position of the Xyl, judging from previous reports (*10*, *12–15*). Thus, the following structure is proposed for fraction 20, where 2P represents 2-*O*-phosphate.

ΔHexAα1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xyl(2P).

The NMR findings for the linkage hexasaccharide in fraction II did not correspond to any of those for the linkage hexasaccharide alditols reported so far, indicating that the hexasaccharide in fraction II was a novel structure. The resonances between 4.5 and 5.2 ppm are characteristic of anomeric protons, and those at δ 5.188, 4.600, 4.529, 4.534, 4.726, 4.615, and 5.188 ppm were identified as H-1 resonances of Xyl-**1**α, Xyl-**1**β, Gal-**2**α, Gal-**2**β, Gal-**3**, GalNAc-**5**, and ΔHexA-**6**, respectively. The signal at δ 5.168 had a coupling constant, $J_{1,2}$, of 3.0 Hz, indicating that it was the H-1 signal of IdoA-**4** but not GlcA (Figs. 6 and 7, and Table IV) (*9*, *27*). Compared with the NMR findings for the reference compound, ΔHexAα1-3GalNAc(4S)β1-4IdoAα1-3Galβ1-3Galβ1-4Xyl-ol, isolated from bovine aorta DS (*9*), a large downfield shift of H-4 of Gal-**3** (Δ 0.503 ppm) and a large upfield shift of H-4 of GalNAc-**5** (Δ 0.54 ppm) were observed on NMR of fraction II, indicating that the Gal-**3** residue was 4-*O*-sulfated and that the C4 position of the GalNAc-**5** residue was not sulfated. Based on these NMR findings as well as those of DE MALDI-TOF mass analysis, the following structure was elucidated for the major compound in fraction II:

ΔHexAα1-3GalNAcβ1-4IdoAα1-3Gal(4S)β1-3Galβ1-4Xyl.

HPLC Analysis of the 2AB-Derivatives of the Linkage Region–derived Hexasaccharides—The isolated linkage region hexasaccharides were labeled with a fluorophore, 2AB, to prepare analytical probes for HPLC. Chromatographic separation of the 2AB-derivatives was demonstrated by HPLC on an amine-bound silica column. As representative chromatograms, the HPLC profiles of two mixtures, each of which contained five different fractions isolated from shark cartilage CS (fractions 7, 14, 17, 22, and 24, or 19, 20, 23, 31, and 32), are shown in Fig. 8, A and B, respectively. Individual peaks were identified by means of co-injection experiments. The fractions in both mixtures were well separated from each other, and the

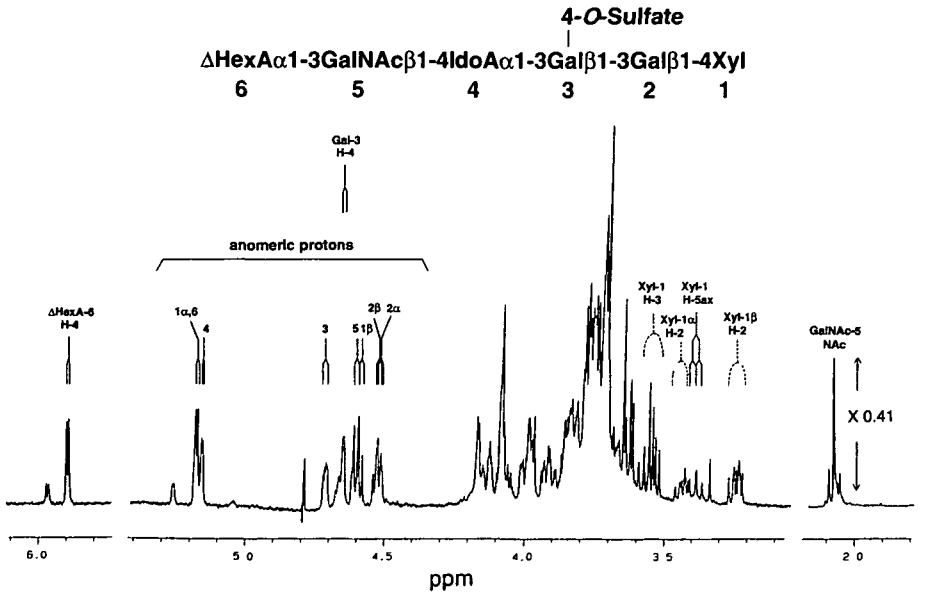

Fig. 6. **One-dimensional 500-MHz ^{1}H NMR spectrum of the structure in fraction II from bovine aorta DS recorded in ^2H_2O at 26°C.** The numbers and letters in the spectrum refer to the corresponding residues in the structure.

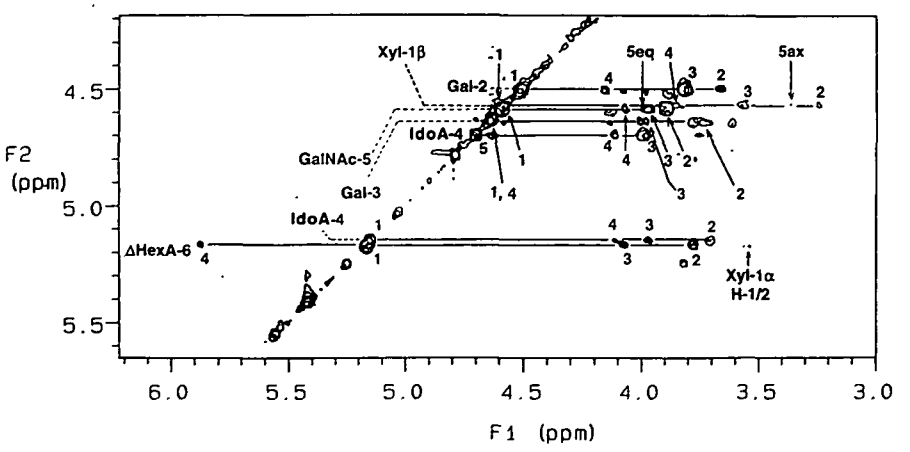

Fig. 7. **2D HOHAHA spectrum of the structure in fraction II from bovine aorta DS recorded in ^2H_2O at 26°C.** Anomeric protons are denoted by horizontal lines.

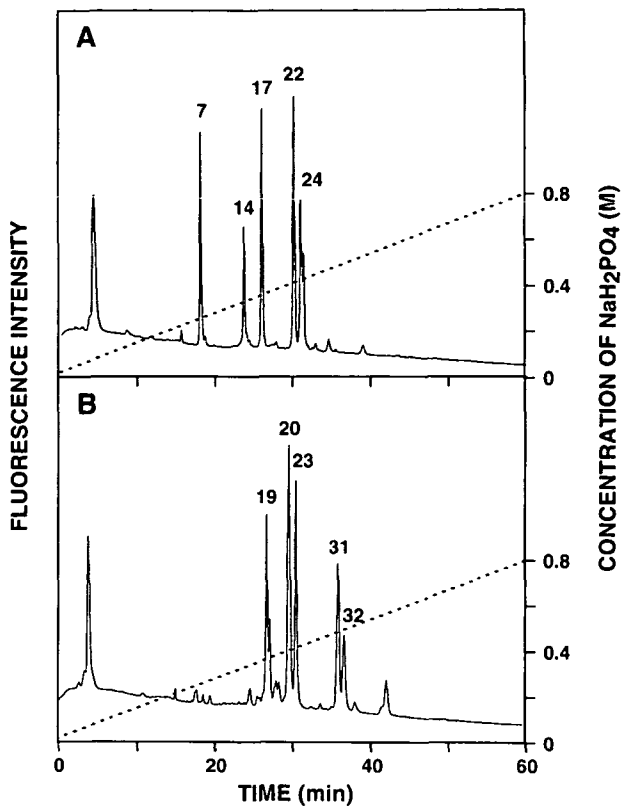

Fig. 8. **HPLC separation of the 2AB-derivatives of the linkage hexasaccharides from shark cartilage CS.** Separation of the 2AB-labeled linkage region hexasaccharides was performed on HPLC. Mixtures of 2AB-derivatives of the linkage hexasaccharide fractions prepared from shark cartilage CS were chromatographed on an amine-bound silica column with a linear gradient of NaH_2PO_4, as indicated by the dashed lines. A, mixture of fractions 7, 14, 17, 22, and 24 (15–30 pmol); B, mixture of fractions 19, 20, 23, 31, and 32 (15–45 pmol). The peaks at 3–5 min were attributable to 2AB carried over during the purification procedure. The right shoulders of fractions 24 (in panel A) and 19 (in panel B) were due to minor contaminants.

order of their elution positions was consistent with that of the unlabeled corresponding precursor hexasaccharides. These results indicate that LiOH treatment/2AB-derivatization can produce sensitive analytical probes, which facilitate analysis of the GAG-protein linkage region.

DISCUSSION

In the present study, we developed a sensitive analytical method for the GAG-protein linkage region. The conditions for the LiOH treatment to release sugar chains from the corresponding core proteins were optimized using the linkage region-derived glycopeptides, and a number of linkage region hexasaccharides were prepared. The conditions were also used to release GAG polysaccharides, which were then digested with chondroitinase ABC to yield linkage region hexasaccharides. The structures of these reducing hexasaccharides were determined by DE MALDI-TOF mass spectrometry and proton NMR spectroscopy, followed by tagging with a fluorophore, 2-AB. The structures of the 2AB-labeled hexasaccharides were confirmed by DE MALDI-TOF mass spectrometry. The 2AB-derivatization showed high sensitivity as to analysis of the linkage region in the low picomole range without radiolabeling.

Multiple linkage region hexasaccharides were obtained from whale cartilage CS, shark cartilage CS and bovine aorta DS, respectively. The structures of the major components in most of the isolated fractions were determined by 500 MHz ^{1}H NMR spectroscopy and are summarized in Table V, although the ^{1}H chemical shifts of the spectra of fractions 20, 32, and 33 isolated from shark cartilage CS could not be assigned due to insufficient resolution resulting from the limited sample amounts. Each major component in fractions A, B, and C from whale cartilage CS was identical to one in fractions 7, 17, and 19 from shark cartilage CS as well as to one in fractions I, III, and IV from bovine aorta DS, respectively (Table V). Thus, eleven kinds of linkage hexasaccharides were obtained altogether. Although the structures of most of the isolated linkage hexasaccharides corresponded to those of the known hexasaccharide alditols or hexasaccharide-serines, they were utilized as authentic reducing oligosaccharides to prepare 2AB-labeled standards, which will, in turn, facilitate investigation of the unknown linkage region structures. These linkage hexasaccharides prepared here will also be valuable enzyme substrates for specificity studies on GAG biosynthetic enzymes such as sulfotransferases and GAG-degrading enzymes.

The usefulness of the developed method is evident. The method has already been successfully applied to analysis of the linkage region of α-thrombomodulin, a recombinant part-time PG, which revealed that it contains the truncated linkage region tetrasaccharide, GlcA-Gal-Gal-Xyl (*31*). More recently, we also successfully analyzed a linkage hexasaccharide of appican, the CS-PG form of the Alzheimer amyloid precursor protein (*32*). In the present study, this method led to the discovery of a novel structure, ΔHexAα1-3GalNAcβ1-4IdoAα1-3Gal(4S)β1-3Galβ1-4Xyl, in fraction II from bovine aorta DS. This structure is related to, yet different from, those of the previously isolated IdoA-containing hexasaccharide alditols, ΔHexAα1-3GalNAc(4S)β1-4IdoAα1-3Galβ1-3Galβ1-4Xyl-ol and ΔHexAα1-3GalNAc-(4S)β1-4IdoAα1-3Gal(4S)β1-3Galβ1-4Xyl-ol, after alkaline $NaBH_4$ treatment of the same DS-peptide preparation (*9*). It will be interesting to determine the relationship between the 4-*O*-sulfation of the Gal residue and the C5-epimerization of the GlcA residue attached to the Gal residue.

The developed method should also be applicable to analysis of the linkage region of PGs isolated from cultured cells or precious tissue specimens, which provide only small amounts of samples. Such samples may reveal the linkage region structures of the biosynthetic intermediates of PGs, and investigation of the structural variations and possible dynamism of the modifications may help clarify the biological functions of these unique modifying groups. Notably, *O*-sulfation of the Gal residues and 2-*O*-phosphorylation of the Xyl residue have not yet been demonstrated concomitantly on the same hexasaccharide backbone. It is possible that these modifications exhibit dynamic metabolism, being coupled with regulated addition of the monosaccharide building units of the linkage region tetrasaccharide sequence. In addition, since the linkage region is first constructed during biosynthesis, differences in the structure of this region may influence the structure of the repeating disaccharide region to be synthesized later, for example

TABLE V. **Structures of the unsaturated hexasaccharides isolated from the GAG-protein linkage region.**

Fractions[a]	Structure			
A, 7, I	ΔHexAα1-3GalNAcβ1	-4GlcAβ1-3Galβ1	-3Galβ1	-4Xyl
B, 17, III	ΔHexAα1-3GalNAc(6S)β1	-4GlcAβ1-3Galβ1	-3Galβ1	-4Xyl
C, 19, IV	ΔHexAα1-3GalNAc(4S)β1	-4GlcAβ1-3Galβ1	-3Galβ1	-4Xyl
D	ΔHexAα1-3GalNAc(4S)β1	-4GlcAβ1-3Gal(4S)β1	-3Galβ1	-4Xyl
14	ΔHexAα1-3GalNAcβ1	-4GlcAβ1-3Galβ1	-3Gal(6S)β1	-4Xyl
20	ΔHexAα1-3GalNAcβ1	-4GlcAβ1-3Galβ1	-3Galβ1	-4Xyl(2P)
22	ΔHexAα1-3GalNAc(6S)β1	-4GlcAβ1-3Galβ1	-3Gal(6S)β1	-4Xyl
23	ΔHexAα1-3GalNAcβ1	-4GlcAβ1-3Gal(6S)β1	-3Gal(6S)β1	-4Xyl
24	ΔHexAα1-3GalNAc(4S)β1	-4GlcAβ1-3Galβ1	-3Gal(6S)β1	-4Xyl
31	ΔHexAα1-3GalNAc(6S)β1	-4GlcAβ1-3Gal(6S)β1	-3Gal(6S)β1	-4Xyl
II	ΔHexAα1-3GalNAcβ1	-4IdoAα1-3Gal(4S)β1	-3Galβ1	-4Xyl

[a]Fractions A–D were isolated from whale cartilage CS, fractions 7–31 from shark cartilage CS, and fractions I–IV from bovine aorta DS, respectively.

through possible regulation of the β-GalNAc transferase I and α-GlcNAc transferase I activities.

Another prominent example of the linkage region structure has been revealed for inter-α-trypsin inhibitor (*33*) and urinary trypsin inhibitor (*34*), which bear a single CS chain with a uniform linkage region structure, GlcAβ1-3Gal(4S)-β1-3Galβ1-4Xyl. This is in contrast to the structural heterogeneity demonstrated in the linkage region oligosaccharides isolated from cartilagenous CS-PGs bearing multiple GAG chains. These heterogeneous linkage regions may represent a number of different CS attachment sites of the cartilage PGs. However, structural variations have also been demonstrated in the linkage region of the recombinant decorin with a single CS/DS synthesized in cloned Chinese hamster ovary cells (*35*), suggesting that even a single cell or a single population of a certain cell type can synthesize a CS/DS chain with heterogeneous structures. Thus, the uniformity and the heterogeneity of the structural modifications of the linkage region pose interesting scientific questions in terms of the biosynthetic mechanisms of GAGs and the possible molecular interactions with other molecules. Although the specific biological significance of the modifications found in the linkage region is unknown, individual CS/DS chains with distinct structural modifications in the linkage region may reflect discrete subclasses with different biological roles. The analytical method developed in the present study will facilitate invesitigation of these unsolved problems concerning the linkage region.

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