Eur. J. Biochem. 202, 805–811 (1991)

Structural studies on sulfated oligosaccharides derived from the carbohydrate-protein linkage region of chondroitin sulfate proteoglycans of whale cartilage

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(Received April 15/June 24, 1991) – EJB 91 0488

From the carbohydrate-protein linkage region of whale cartilage proteoglycans, which bear predominantly chondroitin 4-sulfate, one nonsulfated, two monosulfated and one disulfated hexasaccharide alditols were isolated after exhaustive digestions with Actinase E and chondroitinase ABC, and subsequent β-elimination. Their structures were analyzed by chondroitinase ACII digestion in conjunction with HPLC and by 500-MHz ^{1}H-NMR spectroscopy. The nonsulfated compound (A) had the following conventional structure: ΔGlcA(β1–3)-GalNAc(β1–4)GlcA(β1–3)Gal(β1–3)Gal(β1–4)Xylol, where GlcA, ΔGlcA and GalNAc are glucuronic acid; 4,5-unsaturated glucuronic acid and 2-deoxy-2-*N*-acetylamino-D-galactose, respectively. The other compounds were sulfated derivatives of compound A. Two monosulfated compounds (B and C) had an ester sulfate on C4 or C6 of the GalNAc residue, respectively and the disulfated compound (D) had two ester sulfate groups, namely, one on C4 of the GalNAc and the other on C4 of the Gal residue substituted by GlcA. The molar ratio of A/B/C/D was 0.21:0.16:0.36:0.27. The compound containing Gal-4-*O*-sulfate was previously isolated by us in the form of a sulfated glycoserine [ΔGlcA(β1–3)GalNAc(4-*O*-sulfate)(β1–4)GlcA(β1–3)Gal(4-*O*-sulfate)(β1–3)-Gal(β1–4)Xylβ1-*O*-Ser] from the carbohydrate-protein linkage region of rat chondrosarcoma chondroitin-4-sulfate proteoglycans [Sugahara K., Yamashina, I., DeWaard, P., Van Halbeek, H. & Vliegenthart, J. F. G. (1988) *J. Biol. Chem. 263*, 10168–10174]. The discovery of this structure in the carbohydrate-protein linkage region of chondroitin 4-sulfate proteoglycans from nontumorous cartilage indicates that it is not a tumor-associated product but rather a physiological biosynthetic product since it represents a significant proportion. The biological significance of this structure is discussed in relation to glycosaminoglycan biosynthesis.

Proteoglycans are macromolecular glycoproteins that contain a protein core to which side chains of characteristic sulfated glycosaminoglycans including heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate, are covalently attached [1]. The structural studies by Lindahl, Rodén and co-workers revealed the occurrence of a common carbohydrate-protein linkage structure, GlcA(β1–3)Gal(β1–3)Gal(β1–4)Xylβ1-*O*-Ser, in proteoglycans bearing various glycosaminoglycans except keratan sulfate [2].

The question arises how different glycosaminoglycans are synthesized on the common structure. Sorting mechanisms in their biosynthesis are still unsolved. Recently we identified the compound ΔGlcA(β1–3)GalNAc(4-*O*-sulfate)(β1–4)-GlcA(β1–3)Gal(4-*O*-sulfate)(β1–3)Gal(β1–4)Xyl1β-*O*-Ser derived from the linkage region of chondroitin-4-sulfate proteoglycans from Swarm rat chondrosarcoma [3]. This sulfated structure has not been found in the linkage region of heparin [4, 5], and may play an important role in glycosaminoglycan biosynthesis, especially in the sorting mechanisms leading to galactosaminoglycans (chondroitin sulfate/dermatan sulfate) or to glucosaminoglycans (heparin/heparan sulfate). In the present study we carried out structural studies of the linkage region of proteoglycans from an apparently nontumourous whale cartilage which contains predominantly chondroitin 4-sulfate [6] in order to investigate the ubiquity of the novel sulfated structure. The preliminary results on the enzymatic characterization of the linkage oligosaccharides have been reported [5].

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Abbreviations. UA, uronic acid; ΔUA, 4,5-unsaturated uronic acid; GlcA, glucuronic acid; ΔGlcA, 4,5-unsaturated glucuronic acid; GalN, galactosamine; GalNAc, 2-deoxy-2-*N*-acetylamino-D-galactose; ΔDi-OS, $\Delta_{4,5}$GlcAβ(1–3)GalNAc; ΔDi-4S, $\Delta_{4,5}$GlcAβ(1–3)GalNAc 4-*O*-sulfate; ΔDi-6S, $\Delta_{4,5}$GlcAβ(1–3)-GalNAc 6-*O*-sulfate; ΔDi-diS_D, $\Delta_{4,5}$GlcA(2-*O*-sulfate) β(1–3)-GalNAc 6-*O*-sulfate; ΔDi-diS_E, $\Delta_{4,5}$GlcAβ(1–3)GalNAc 4,6-*O*-disulfate; Δ-Di-triS, $\Delta_{4,5}$GlcA(2-*O*-sulfate)β(1–3)GalNAc 4,6-*O*-disulfate.

Enzymes. Chondroitinase ABC (EC 4.2.2.4), chondroitinase ACII (EC 4.2.2.5), chondro-4-sulfatase (EC 3.1.6.9).

MATERIALS AND METHODS

Materials

Materials were obtained from the following sources: Actinase E, previously supplied as Pronase P, from Kaken

Pharmaceutical Co., Tokyo; chondroitinase ACII, chondro-4-sulfatase, and conventional and protease-free preparations of chondroitinase ABC from Seikagaku Kogyo Co., Tokyo; $NaBH_4$ from Wako Pure Chemical Industry, Osaka.

Preparation of peptidoglycans

Whale cartilage (400 g) was treated in boiling water for 30 min, minced and digested with 2 g of Actinase E in the presence of a small amount of toluene in 600 ml 0.1 M sodium borate, pH 8.0, containing 10 mM calcium acetate. After 24 h incubation at 54° C, an additional 2 g of the enzyme was added and the incubation was continued for 24 h. During the incubation the mixture was adjusted to pH 8.0 intermittently with 1 M NaOH. The digest was cooled to room temperature and filtered through Celite. Sodium acetate and then ethanol were added to the filtrate to give final concentrations of 3% (mass/vol.) and 54% (by vol.), respectively. The precipitate was collected by centrifugation, dissolved in 3% (mass/vol.) sodium acetate solution, pH 6.0 and precipitated with ethanol (final concentration 45%). The sample was redigested with Actinase E and the precipitate was recovered by ethanol precipitation, dissolved in 1% sodium acetate solution, pH 6.0 and mixed with 6 g activated charcoal at 50°C for 1 h. The mixture was filtered through Celite. Peptidoglycans were precipitated with ethanol (final concentration 85%) and dried. The yield was 29.4 g. The contents of protein and GlcA were 14.5% and 25.0% (mass/mass), respectively. A portion (15 g) of this peptidoglycan preparation was redigested with Actinase E (30 mg) in 80 ml of the buffer under the above-mentioned conditions and the peptidoglycans (9.79 g) were obtained. The contents of protein and GlcA were 2.6% and 41% (mass/mass), respectively. Based on the amino acid analysis, the molar ratios of the major amino acids were as follows: Ser/Asp/Thr/Glu/Pro/Gly/Ala/Val/Lys/Arg = 1.00: 1.74:0.42:1.99:1.53:4.22:1.00:0.52:0.63:0.65. The amounts of other amino acids were negligible.

DEAE-cellulose column chromatography

The peptidoglycan preparation (6.0 g) was dissolved in 120 ml 0.2 M LiCl/0.05 M sodium acetate, pH 4.0 and applied to a DEAE-cellulose column (9.3 × 8.8 cm) equilibrated with the same buffer. The column was washed stepwise with 500 ml of the buffer and 3 l each of the buffer containing 0.3, 1.0 and 2.0 M LiCl. Peptidoglycans were recovered by ethanol precipitation and dried. The yields of peptidoglycans in the flow-through and the separated fractions were 0.1, 1.24, 3.29 and 0.51 g. The major fraction (1.0 M LiCl fraction) was used for isolation of the linkage oligosaccharides.

Chondroitinase ABC treatment

The peptidoglycan preparation obtained after DEAE-cellulose chromatography (1.6 g corresponding to 67.1 μmol Ser) was digested with 1.9 U chondroitinase ABC (protease-free) in a total volume of 20 ml 0.05 M Tris/HCl buffer, pH 8.0, containing 50 mM sodium acetate and 100 μg/ml bovine serum albumin. The digestion was carried out for 27 h at 37°C; an additional 0.5 U enzyme was added after 18 h to complete the digestion. Following the incubation, the mixture was adjusted to pH 6.5 with 1 M AcOH and treated at 100°C for 3 min. The digest was fractionated by gel filtration using Sephadex G-25. Unexpectedly, the isolated oligosaccharide fraction I was not degraded any further by a protease-free preparation of chondroitinase ABC. Therefore this fraction (32.2 μmol as Ser) was redigested with a conventional preparation of chondroitinase ABC (2.5 U) for 3 h in a total volume of 6 ml, otherwise as described above. Analysis of the disaccharides produced, however, did not reveal any appreciable abnormality. The above observations are reproducible and the investigation of the differences in enzymatic action between the two enzyme preparations is currently in progress.

Reduction of the peptidoglycans

The glycopeptide preparation obtained after chondroitinase ABC digestions (10.4 μmol as unsaturated uronic acid) was treated with 2 ml 0.1 M $NaBH_4$/0.05 M NaOH overnight at 40°C. Acetic acid was added to adjust the incubation mixture to pH 5.5 and the mixture was applied to a column (2.8 × 4.5 cm) of Dowex 50 W × 8 (H^+ form). The column was washed with 120 ml of water. Following repeated evaporations of the flow-through fraction with methanol, the residue was chromatographed on a column (1.2 × 70 cm) of Sephadex G-25 (fine) with water as effluent. The fractions containing unsaturated uronic acid were pooled, concentrated to dryness by evaporation and reconstituted in water. The yield of unsaturated uronic acid in the oligosaccharide alditol fraction was 5.45 μmol.

Chondroitinase ACII treatments

The isolated linkage oligosaccharides (4 nmol) were incubated with 14 mU enzyme in a total volume of 40 μl 0.03 M sodium actate buffer, pH 6.0, at 37°C for 10 min. The incubation mixtures were treated at 100°C for 1 min, cooled to room temperature, mixed with 960 μl 16 mM NaH_2PO_4, filtered through a 0.45-μm membrane filter C3HV (Millipore) and 200-μl aliquots were analyzed by HPLC.

Successive treatment with chondroitinase ACII and chondro-4-sulfatase

The isolated linkage oligosaccharide, fraction D (2.4 nmol), was first incubated with 14 mU enzyme in a total volume of 30 μl 25 mM sodium acetate buffer, pH 6.0, at 37°C for 10 min. 70 μl water was added to the incubation sample and the mixture was treated at 100°C for 1 min. The sample (50%) was analyzed by HPLC as described above while the remainder was mixed with 40 μl water, 10 μl each of 0.4 M sodium acetate/0.4 M Tris/HCl buffer, pH 7.5, 0.1% bovine serum albumin, chondro-4-sulfatase (100 mU) and then incubated at 37°C for 12 min. The reaction was terminated by heat treatment at 100°C for 1 min. The sample was mixed with 135 μl water and 45 μl 100 mM NaH_2PO_4. A 100-μl aliquot of the sample was analyzed by HPLC as in the case of the chondroitinase ACII digests.

HPLC

Fractionation of the linkage oligosaccharides and analysis of the chondroitinase ACII or chondro-4-sulfatase digests of the isolated oligosaccharides were carried out by HPLC as previously reported for the separation of the chondro-disaccharides [7, 8]. Briefly, HPLC was performed on a 4.6 × 250 mm polyamine-bound silica PA03 column (YMC Co., Kyoto) using a linear gradient from 16 to 530 mM NaH_2PO_4 over a 60-min period at a flow rate of 1.0 ml/min at room temperature. For preparative purposes separated fractions were con-

centrated in a vacuum concentrator (Savant Instruments, Inc., Farmingdale, New York), desalted through a column of Sephadex G-25 and re-chromatographed as above. This process was repeated several times until the preparations became homogeneous as judged by HPLC.

Analysis of sulfate and phosphate

To samples (2 nmol) dissolved in water in glass tubes (pyrex No. 9820TST) 20 µl 0.02 M NaOH was added and concentrated to dryness. The samples were pyrolyzed for 12 s using a dental burner (Phenix Dent, Tokyo), reconstituted with 190 µl chromatography buffer, mixed with 10 µl 0.2 mM $NaNO_3$ as an internal standard and centrifuged at 10000 rpm for 5 min. The supernatant was analyzed by ion chromatography, which was performed using an ion chromatography HIC-6A system (Shimadzu, Kyoto) equipped with an anion-exchange Shim-pack IC-A1 column (4.6 × 100 mm). The elution was performed at 40 °C at a flow rate of 1.5 ml/min using 7.5 mM Tris/6 mM boric acid buffer, pH 8.0, containing 18 mM mannitol and 5 mM tri-*n*-butylphosphine oxide (Nacalai Tesqui Inc., Kyoto). Ions were detected conductometrically at 1.6 $mscm^{-1}$ full scale deflection and peak areas were quantified by an Intelligent Integrator Model 7000A (System Instruments Corp., Tokyo). Under the conditions used, negative ions including phosphate and sulfate were well separated.

500-MHz ^{1}H-NMR spectroscopy

Linkage oligosaccharides were repeatedly exchanged in ^2H_2O (99.96% ^{2}H, Aldrich) with intermediate lyophilization. 500-MHz ^{1}H-NMR spectra were recorded on a Bruker AM-500 spectrometer (Department of NMR spectroscopy, Utrecht University, The Netherlands) at a probe temperature of 15 °C to prevent disturbance by the HO^2H resonance. Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly to acetone in ^2H_2O (δ 2.225) [9]. Resolution enhancement of the one-dimensional spectra was achieved by Lorentzian-to-Gaussian transformation [10].

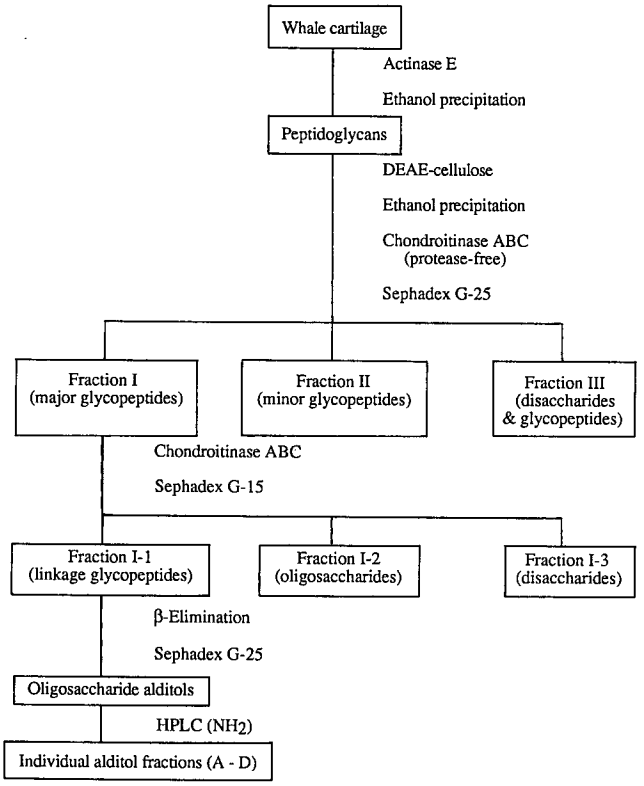
Other analytical methods

Chondroitinase-produced oligosaccharides were quantified based on the absorbance at 232 nm (E_{232} = 5500 $M^{-1} \cdot cm^{-1}$) [11] produced by the unsaturated Δ4,5 sites of the uronic acids at the non-reducing ends. Uronic acid was determined by the carbazole method [12] using GlcA as standard. Amino sugars and amino acids were analyzed with an amino acid analyzer after hydrolysis in 6 M HCl at 100 °C for 3 h and 110 °C for 20 h, respectively [13]. Protein was determined according to Lowry et al. [14].

RESULTS

Isolation of oligosaccharides from the carbohydrate-protein linkage region

To isolate the carbohydrate-protein linkage region of chondroitin sulfate proteoglycans of whale cartilage, a peptidoglycan fraction was prepared from the tissue after exhaustive Actinase E digestions, and subsequent purification by DEAE-cellulose column chromatography. The linkage region was then prepared by chondroitinase ABC digestion. The



Scheme 1. *Flow chart of the isolation of the hexasaccharide alditols from the carbohydrate-protein linkage region of whale cartilage chondroitin sulfate proteoglycans*

peptidoglycan preparation was digested with a protease-free preparation of chondroitinase ABC and fractionated by gel filtration on Sephadex G-25 (Fig. 1 A) into two faster-eluting fractions (fractions I and II) and a major disaccharide peak (fraction III). Disaccharide analysis by HPLC of fraction III showed that the molar ratio of ΔDi-OS/ΔDi-4S/ΔDi-6S was 3:70:27 (ΔDi-OS, ΔDi-4S and ΔDi-6S are $\Delta_{4,5}$GlcAβ-(1 − 3)GalNAc; $\Delta_{4,5}$GlcAβ(1 − 3)GalNAc 4-*O*-sulfate and $\Delta_{4,5}$GlcAβ(1 − 3)GalNAc 6-*O*-sulfate, respectively). Thus, the preparation contained the 4-sulfated disaccharide as a major repeating unit. Recoveries of Ser in fractions I, II and III were 48, 17 and 35%, respectively, suggesting that all three fractions contained glycopeptides. The molar ratios of the other amino acids to Ser in fractions I, II and III were 6.5, 8.1 and 2.6, respectively, indicating the size of the peptide moieties. In the present study the major glycopeptide fraction (fraction I) was employed for isolation of the linkage region. The isolated fraction I was resistant to a protease-free preparation of chondroitinase ABC and was redigested with a conventional preparation of the enzyme. The absorbance at 232 nm increased by 1.68-fold upon digestion. When chromatographed on Sephadex G-15, the digested resulted in two major peaks, the presumed glycopeptide fraction and the disaccharide fraction. The fractions were devided into three fractions I-1, I-2 and I-3 as shown in Fig. 1 B. Disaccharide analysis of fraction I-3 showed that the molar ratio of ΔDi-OS/ΔDi-6S/ΔDi-4S/ΔDi-S_D was 8.0:20.6:67.4:4.0 (ΔDi-S_D, $\Delta_{4,5}$GlcA(2-*O*-sulfate)β(1 − 3)GalNAc 6-*O*-sulfate). The recoveries of Ser in fractions I-1, I-2 and I-3 were 84, 1 and 15%, respectively. The carbohydrate and amino acid composition of fraction I-1 (Table 1) showed that it contains typical linkage-region glycopeptides with the innermost disaccharide unit. Xyl and

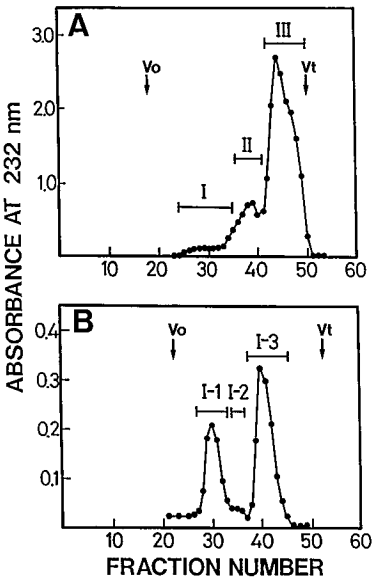


Fig. 1. *Gel filtration of the chondroitinase ABC digest.* The chondroitinase ABC digestions were carried out as described under Materials and Methods. (A) Chondroitinase ABC (protease-free) digest of the peptidoglycan fraction was chromatographed on a column (2.2 × 84 cm) of Sephadex G-25 (fine) with 0.25 M NH_4HCO_3/7% 1-propanol as eluent. Fractions (6 ml) were collected and an aliquot was measured for absorbance at 232 nm after 100-fold dilution. Fractions I, II and III were pooled as indicated. (B) Chondroitinase ABC (conventional) digest of Fraction I shown in (A) was chromatographed on a column (2.15 × 147 cm) of Sephadex G-15 with 0.25 M NH_4HCO_3/7% 1-propanol as eluent. Fractions (8 ml) were collected and an aliquot was measured for absorbance at 232 nm after 100-fold dilution

Table 1. *Chemical composition of the glycopeptide fraction prepared from whale cartilage*
The values are expressed by molar ratio to Ser. Amino acids and hexosamines were determined using an amino acid analyzer and the hexosamine values have been corrected for degradation (22.7%) during acid hydrolysis. Uronic acid (UA) and 4,5-unsaturated uronic acid (⊿UA) were estimated by the carbazole reaction and absorption at 232 nm respectively. UA includes value for ⊿UA also

Component	Content
	mol/mol
Ser	1.00
Asp	0.39
Glu	0.55
Gly	1.29
UA	2.18
⊿UA	1.33
GalN	0.93
GlcN	1.08

Gal were not analyzed at this stage. One mol GlcN may reflect keratan sulfate and/or Asn-linked oligosaccharide chains. The occurrence of *O*-glycosidic chains is unlikely since the GalNAc content is approximately 1.

Since the glycopeptide fraction strongly adsorbed to and could not be eluted from the polyamine column on HPLC, it was treated with alkaline $NaBH_4$ to release oligosaccharides from the peptides. The resulting oligosaccharide alditols were then subjected to separation on a polyamine column HPLC

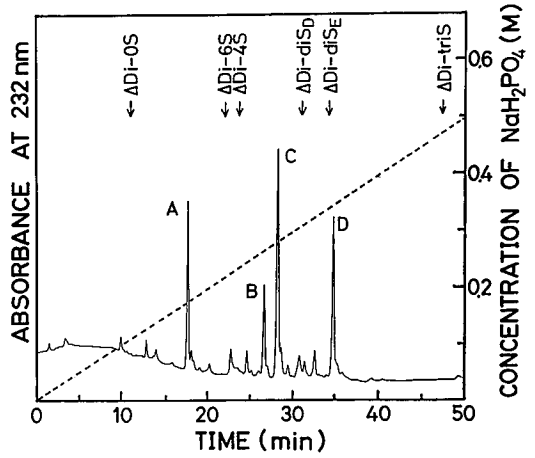


Fig. 2. *HPLC of the oligosaccharide alditol fraction prepared from the carbohydrate-protein linkage region.* The oligosaccharide alditol fraction corresponding to 12 nmol ⊿UA was chromatographed on a polyamine column. The elution positions of authentic unsaturated chondro-disaccharides are indicated by arrows. For the experimental details see Materials and Methods

Table 2. *Chemical composition of linkage oligosaccharides isolated from whale cartilage chondroitin sulfate*
Sulfate was quantified by ion chromatography. No glucosamine was detected in fractions A–D in an amino acid analyzer. For the other analytical methods see Table 1. The experimental details are described in text. UA includes value for ⊿UA also

Fraction	Amount of			
	GalN	⊿UA	UA	Sulfate
	mol/mol			
A	0.82	0.79	2.00	0.13
B	0.66	0.83	2.00	0.96
C	0.83	0.97	2.00	0.78
D	0.82	0.87	2.00	1.59

(Fig. 2). The recovery of the ultraviolet-absorbing materials on HPLC was 55%. The low recovery may be due to removal of the peptides. Four major fractions (A, B, C and D) were obtained, representing 14, 11, 24 and 18% of the recovered ultraviolet-absorbing material, respectively.

Characterization of the oligosaccharides

The carbohydrate analysis indicated that all four fractions contained 1 mol each of saturated uronic acid, unsaturated uronic acid and GalN (Table 2). Sulfate analysis indicated that fractions A, B, C and D contained 0, 1, 1 and 2 mol sulfate/mol oligosaccharide, respectively, which is consistent with the elution order upon HPLC. GlcN, which had been present in the glycopeptide fraction (Table 1), was not detected in the isolated fractions A–D. It remains to be determined what type of glycan GlcN represented in the glycopeptide fraction.

To determine the innermost disaccharide structure and to characterize the presumed tetrasaccharide cores, fractions A–D were subjected to chondroitinase ACII digestion followed by HPLC analysis. They were each degraded into

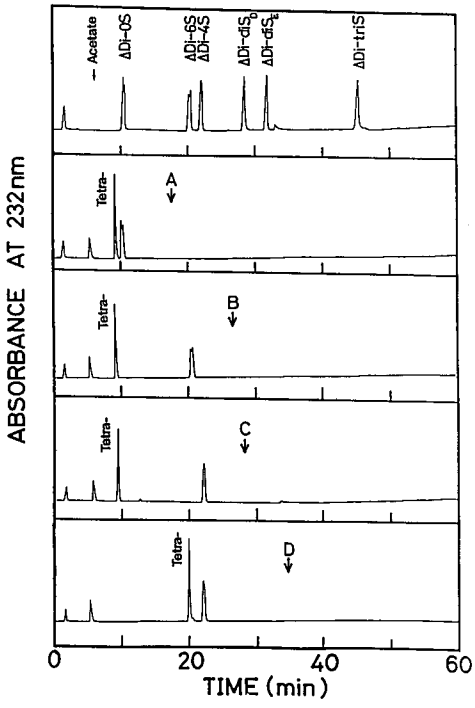


Fig. 3. *HPLC of the chondroitinase ACII digests of the isolated hexasaccharide alditols.* Top column, the authentic chondro-disaccharides; the lower columns, the chondroitinase ACII digests of fractions A, B, C and D. The arrows indicate the elution positions of the corresponding hexasaccharide alditols before digestion

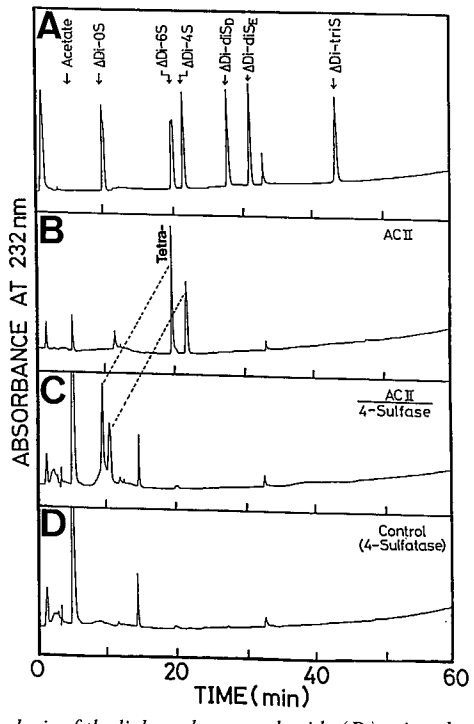


Fig. 4. *Analysis of the linkage hexasaccharide (D) using chondroitinase ACII and chondro-4-sulfatase.* The isolated linkage hexasaccharide (fraction D) was sequentially digested with chondroitinase ACII and chondro-4-sulfatase and then chromatographed on a polyamine column. (A) Standard chondro-disaccharides; (B) chondroitinase ACII digest of fraction D; (C) chondro-4-sulfatase digest of the above chondroitinase ACII digest; (D) chondro-4-sulfatase and the incubation buffer

equimolar amounts of an unsaturated disaccharide (α and β anomers) and a presumed unsaturated tetrasaccharide alditol as shown in Fig. 3. Notably, α- and β-anomers gave rise to double peaks of ΔDi-OS and ΔDi-6S. Fractions A–C were each degraded into the presumed unmodified tetrasaccharide core plus ΔDi-OS, ΔDi-6S or ΔDi-4S, respectively.

Fraction D yielded ΔDi-4S and the presumed monosulfated unsaturated tetrasaccharide with a longer retention time than that derived from the other fractions. In order to identify this tetrasaccharide alditol, chondro-4-sulfatase was utilized. This enzyme is known to hydrolyze the ester sulfate bond of ΔDi-4S [11] and that on the C4 of the GalNAc residue at the reducing end of the various tetrasaccharides derived from chondroitin sulfate [15]. In the present study the specificity of this enzyme was further evaluated using the authentic compound, ΔGlcA(β1–3)GalNAc(4-*O*sulfate)(β1–4)GlcA-(β1–3)Gal(4-*O*-sulfate)(β1–3)Gal(β1–4)Xylβ1-*O*-Ser, previously isolated from chondroitin-4-sulfate proteoglycans of Swarm rat chondrosarcoma [3]. Sequential treatment of this compound with chondroitinase ACII and then chondro-4-sulfatase revealed that the latter enzyme acted on both ΔDi-4S and the monosulfated unsaturated tetrasaccharide Ser as examined by a polyamine column HPLC (data not shown). The results indicate that the enzyme can hydrolyze the ester sulfate bond of ΔGlcA(β1–3)Gal(4-*O*-sulfate)(β1–3)-Gal(β1–4)Xylβ-1-*O*-Ser. The chondroitinase ACII digest of fraction D was then incubated with chondro-4-sulfatase followed by HPLC. As shown in Fig. 4, ΔDi-4S and the tetrasaccharide alditol produced by chondroitinase ACII were converted into their nonsulfated counterparts by chondro-4-sulfatase. Thus, the compound in fraction D is disulfated on C4 of both GalNAc and Gal substituted by GlcA, having most likely the structure ΔGlcA(β1–3)GalNAc(4-*O*-sulfate)(β1–

Table 3. *^{1}H-Chemical shifts of structural-reporter groups of the constituent monosaccharides of linkage oligosaccharides*
Chemical shifts are given in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly to acetone in ^2H_2O (δ 2.225) at 15°C. n.d., not determined

Residue	Reporter group	Chemical shift in			
		A	B	C	D
		δ			
Xylol	H4	3.988	3.987	3.988	3.986
Gal-2	H1	4.617	4.616	4.618	4.616
	H4	4.201	4.200	4.202	4.189
Gal-3	H1	4.668	4.669	4.671	4.700
	H3	n.d.	n.d.	n.d.	4.014
	H4	4.161	4.161	4.166	4.748
GlcA-4	H1	4.668	4.677	4.674	4.748
	H2	3.455	3.470	3.456	3.440
	H3	3.625	n.d.	3.635	3.618
GalNAc-5	H1	4.532	4.568	4.611	4.628
	H2	4.003	4.031	4.073	4.072
	H3	3.901	3.947	4.153	4.150
	H4	4.099	4.178	4.623	4.627
	H5	n.d.	4.010	n.d.	n.d.
	H6	n.d.	4.233	n.d.	n.d.
	H6′	n.d.	4.220	n.d.	n.d.
	NAc	2.058	2.052	2.097	2.099
ΔGlcA-6	H1	5.184	5.179	5.269	5.270
	H3	4.096	4.109	3.944	3.943
	H4	5.899	5.882	5.971	5.969

4)GlcA(β1 – 3)Gal(4-*O*-sulfate)(β1 – 3)Gal(β1 – 4)Xylol. The results in turn suggest that chondro-4-sulfatase is applicable to the structural study of the sulfation of the Gal-3 residue in the linkage region.

500-MHz ^{1}H-NMR spectroscopy

The ^{1}H-NMR data of fractions A, B, C and D are summarized in Table 3. Comparison of the spectral data of fraction A with those of the core structure $\Delta_{4,5}$GlcA-GalNAc-GlcA-Gal-Gal-Xyl-Ser (fraction D-1 in [3]) reveals that this fraction contains a similar structure, except for the reduced Xyl residue. Compared to the non-reduced reference compound, the Gal-2 H1 has a different chemical shift (δ 4.617), the Xyl H1 is absent in the anomeric region and the Xylol H4 is found at δ 3.988. The differences between the reduced compound in fraction A and the non-reduced reference compound are in accordance with earlier ^{1}H-NMR studies on fragments of the proteoglycan linkage region [16]. The structure of the compound present in fraction A is:

$\Delta_{4,5}$GlcA(β1 – 3)GalNAc(β1 – 4)GlcA(β1 – 3)Gal(β1 – 3)Gal(β1 – 4)Xylol.
6 5 4 3 2 1

In the spectrum of fraction B, signals are observed at δ 4.233 which have shifted out of the bulk region under the influence of a sulfate group [17]. Comparison with the data of fraction A indicates that the H1 signals of GlcA-4, GalNAc-5 and $\Delta_{4,5}$GlcA-6 are shifted. Therefore, GalNAc-5 is most likely the sulfated residue. In an elaborate NMR study on a fraction derived from the linkage region of chondroitin 6-sulfate of shark cartilage it was proven that these signals can be assigned to the H6/H6′ of a 6-*O*-sulfated GalNAc residue (unpublished results). An assignment pathway from H1 to H6/H6′ was deduced by a combination of two-dimensional homonuclear Hartmann-Hahn and rotating frame Overhauser enhancement spectroscopy. The structure of the compound in fraction B is:

6-*O*-sulfate
|
$\Delta_{4,5}$GlcA(β1 – 3)GalNAc(β1 – 4)GlcA(β1 – 3)Gal(β1 – 3)Gal(β1 – 4)Xylol.
6 5 4 3 2 1

In the ^{1}H-NMR spectrum of fraction C the GalNAc H4 has shifted downfield to a position in the anomeric region in the same way as in the spectrum of ΔGlcA-GalNAc(4-*O*-sulfate)-GlcA-Gal-Gal-Xyl-Ser (fraction D-3 in [3]). Furthermore, the chemical shifts of GalNAc H1, H2, H3, and $\Delta_{4,5}$GlcA H1, H3, and H4 are similar to those of this reference compound. Therefore, fraction C contains a compound with a 4-*O*-sulfated GalNAc residue:

4-*O*-sulfate
|
$\Delta_{4,5}$GlcA(β1 – 3)GalNAc(β1 – 4)GlcA(β1 – 3)Gal(β1 – 3)Gal(β1 – 4)Xylol.
6 5 4 3 2 1

In the ^{1}H-NMR spectrum of fraction D not only GalNAc H4 but also the Gal-3 H4 has shifted downfield to a position in the anomeric region. The chemical shift data of this compound are similar to those of ΔGlcA-GalNAc(4-*O*-sulfate)-GlcA-Gal(4-*O*-sulfate)-Gal-Xyl-Ser (fraction D-4F in [3]). The assignment of the 4-*O*-sulfated Gal-3 residue was based on two-dimensional homonuclear Hartmann-Hahn spectroscopy [3]. The influences on the chemical shifts of GlcA-4 and GalNAc-5 can be ascribed to the presence of sulfate on Gal-3. The structure of the compound in fraction D is:

4-*O*-sulfate 4-*O*-sulfate
| |
$\Delta_{4,5}$GlcA(β1 – 3)GalNAc(β1 – 4)GlcA(β1 – 3)Gal(β1 – 3)Gal(β1 – 4)Xylol.
6 5 4 3 2 1

DISCUSSION

In the present study we isolated and characterized four hexasaccharide alditols from the carbohydrate-protein linkage region of whale cartilage chondroitin sulfate proteoglycans in order to investigate the ubiquity of the Gal-4-*O*-sulfate structure previously demonstrated to occur in the linkage region of rat chondrosarcoma chondroitin 4-sulfate proteoglycans [3]. One of the alditols, fraction D, was an alditol counterpart of a disulfated hexasaccharide Ser, ΔGlcA(β1 – 3)GalNAc(4-*O*-sulfate)(β1 – 4)GlcA(β1 – 3)-Gal(4-*O*-sulfate)(β1 – 3)Gal(β1 – 4)Xylβ1-*O*-Ser isolated from rat chondrosarcoma, indicating that 4-*O*-sulfation of the Gal residue is not tumor associated, but a physiological modification.

Recently, we also isolated several hexasaccharide alditols from the linkage region of shark cartilage chondroitin 6-sulfate. Some of them bear a sulfate group on the C6 position of both Gal residues [18]. However, the Gal residues of the linkage region from porcine intestinal heparin [4, 5] are not sulfated. Thus, there seems to be distinct differences in the structure of the linkage region between chondroitin sulfate and heparin.

The structural differences in the linkage region may determine the character of the glycosaminoglycan species in biosynthesis. We have addressed a question how different glycosaminoglycans can be synthesized on the so called common carbohydrate-protein linkage structure, GlcA(β1 – 3)-Gal(β1 – 3)Gal(β1 – 4)Xylβ1-*O*-Ser [3]. Recent evidence obtained by analysis of the gene and the mRNA for the mast cell proteoglycan named serglycin, which can bear either one or both of chondroitin sulfate and heparin chains, clearly indicates that the attachment of these chains is not encoded by the amino acid sequence of the core protein [19, 20]. The concept of a multienzyme complex has been introduced for the synthesis of the linkage region of chondroitin sulfate chains [21, 22] and for the modification reactions of heparin glycosaminoglycan chains [23]. It is unknown, however, if this concept can be applicable to the sorting of different glycosaminoglycan chains. It is tempting to speculate that

sulfation of Gal residue(s) may be sorting signals favoring the synthesis of chondroitin sulfate. The committing biosynthetic step in sorting is the transfer of the first *N*-acetylhexosamine unit to the linkage tetrasaccharide core. It remains to be investigated whether the sulfated Gal structures are specific recognition signals for the *N*-acetylgalactosaminyltransferase while they may inhibit *N*-acetylglucosaminyltransferase. It should be noted, however, that recently Fransson et al. [24] isolated from pig skin dermatan sulfate, which is a galactosaminoglycan, a hexasaccharide serine in which neither one of the two Gal residues was sulfated.

A phosphorylated Xyl unit has been demonstrated in the carbohydrate-protein linkage region of chondroitin-4-sulfate proteoglycans from rat chondrosarcoma [25] and that of heparan sulfate proteoglycans from bovine lung [26]. The linkage region from porcine intestinal heparin has also been claimed to be phosphorylated [27]. The lack of this phosphate group from the alditols isolated in the present study is most likely due to the dephosphorylation during the biosynthetic processing or to the experimental manipulation.

We thank D. Masayuki Nishimura (Analytical Application Laboratory, Shimadzu Corp., Kyoto) for his assistance in establishing the analytical conditions for ion chromatography.

This investigation was supported by Grants-in-Aid for Scientific Research (C) from the Ministry of Education and Culture of Japan, the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO) and by the Netherlands Foundation for Cancer Research (KWF, grant UUKC 88-14).

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