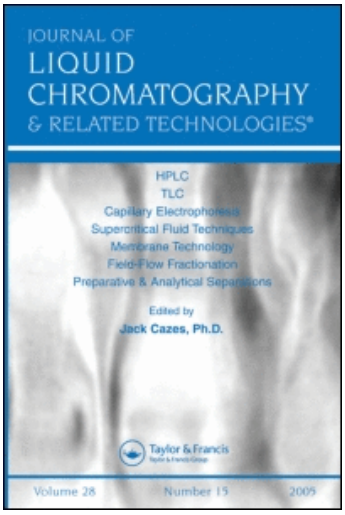

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DETERMINATION OF PHOSPHORYLATED AND SULFATED LINKAGE-REGION OLIGOSACCHARIDES IN CHONDROITIN/DERMATAN AND HEPARAN SULFATE PROTEOGLYCANS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The linkage-region oligosaccharides of chondroitin sulfate and dermatan sulfate, produced by the action of various chondroitinases, as well as of heparan sulfate, produced by the combined action of heparin-lyases I, II, and III, have been separated and characterized according to their size, number of sulfate residues, and the presence of phosphorylated xylose by HPLC. Glycosaminoglycans and/or proteoglycans were treated by tritiated borohydride and the [^{3}H]-labeled xylitole-containing glycan chains were degraded by the various chondro/dermato-lyases (chondroitinases ABC, AC and B) and/or heparin-lyases. The produced linkage-region Δ-oligosaccharides have been completely separated by ion-pair reversed phase HPLC, using tetrabutylammonium as ion-pairing reagent, and detected by a radiochemical detector.

Application of the method to chondroitin sulfate and dermatan sulfate revealed that the first uronic acid after the -Gal-Gal-Xyl linkage trisaccharide is always glucuronic acid and the next galactosamine residue is sulfated. In porcine skin dermatan sulfate studied, a three glucuronic acid-containing cluster following the linkage-region oligosaccharide was found. None of chondroitin sulfate and dermatan sulfate studied contains phosphorylated xylose. This phosphorylation was, however, a dominating feature in xylose linkage-region of the glycans derived from rat chondrosarcoma proteoglycans.

The linkage region oligosaccharide fragment of heparan sulfate had the same structure and identical retention time with that obtained from dermatan sulfate with chondroitinase AC.

INTRODUCTION

Uronic acid-containing glycosaminoglycans (GAGs) are highly charged linear biopolymers composed of repeating disaccharide units which contain one hexosamine and one hexuronic acid.[1] Except hyaluronic acid (HA), all GAGs are covalently bound to protein cores, forming proteoglycans (PGs).[1] The galactosaminoglycans (GalAGs) - chondroitin sulfate (CS) and dermatan sulfate (DS) - contain galactosamine as hexosamine, the uronic acid being glucuronic acid (GlcA) in CS and both iduronic acid (IdoA) and GlcA in DS. Heparan sulfate (HS) contains glucosamine as hexosamine and GlcA/IdoA as uronic acid. IdoA is formed by C-5 epimerization of GlcA in the chondroitin biosynthetic precursor.[2] Sulfation of the hexosamine and hexuronic acid is considered as an event after epimerization which results in variously sulfated glycan chains with a wide range of specificities in biological functions.[3-6] CS, DS, and HS chains are linked to serine or threonine of the protein core via the common linkage-region trisaccharide Gal-Gal-Xyl-Ser/Thr by an O-glycosidic linkage.[1-3, 5, 7-9]

This attachment region provides important information on the size of the glycan chain.[4] The xylose residue has been identified in some PGs to be phosphorylated at C-2 [5-11] and this phosphorylation may reflect a biosynthetic event required for specific functional properties.[1, 9]

Previous studies on DS and CSA revealed the GlcA being the next monosaccharide to Gal in the linkage trisaccharide.[4, 9-14] Electrophoretic and chromatographic studies have shown that the first GlcA in a DS fraction (DS-18) [average molecular size (M_r) 18 kDa] is followed by a disaccharide containing 6-sulfated galactosamine.[9, 11-13]

Using ion-pair HPLC[9, 13] it has also been shown that the linkage trisaccharide in DS-18 is followed by a cluster of three GlcA containing disaccharides which have only the first galactosamine sulfated at C-6.[4] Structural characterization of the linkage-region oligosaccharides from CSA however, suggested that the galactosamine after the first GlcA is sulfated at C-4.[10, 12]

Cleavage of CS/DS with the various chondroitinases (ABC, AC, and B), which selectively degrade the hexosaminidic bonds of galactosaminoglycans (GalAGs) at positions depending on the hexuronic acid type,[14] could provide useful information on the sequence of the linkage-region oligosaccharides.[12,13,15,16] Although the same trisaccharide is used for the attachment of HS to protein core, little is known about the structure of linkage-region oligosaccharides. The use of the three heparin lyases I, II, and III, which have different specificity concerning the uronic acid type and sulfation,[17] has been found to be of great importance in analyzing HS-disaccharide composition by HPLC and/or capillary electrophoresis.[17-21] The degradation of HS using a combination of the three heparin lyases may also provide information on the linkage-region of HS as well as of its molecular size.

Using an ion-pair HPLC method, the variously-sized and sulfated Δ-saccharides produced by the action of the various chondroitinases on CS and DS have been successfully separated according to the number of tetra-butylammonium cations (TBA^+) that bind to Δ-oligosaccharides.[14] It was, therefore, of great interest to study the sequence of the linkage-region fragments of various GalAGs by using this powerful ion-pair technique.

The aim of this study was, therefore, to develop an efficient HPLC method by which the different linkage-region Δ-oligosaccharides, produced by the various chondro/dermato-lyases on CS/DS and the combination of heparin-lyases I, II, and III on HS, could be completely separated and determined according to their size, sulfation pattern, and phosphorylation of xylose.

EXPERIMENTAL

Materials

CSA from whale cartilage, DS from porcine skin, HS (Na^+ salt) from bovine kidney, and heparin-lyase II (heparinase II, no EC number) derived from *Flavobacterium heparinium* were all supplied by the Sigma Chemical Co. (St. Louis, MO, USA). A_1D_1 fraction of rat chondrosarcoma PGs was a gift from

Prof. Bruce Caterson. Chondroitinases ABC, AC, and B, heparin-lyase I (heparinase EC 4.2.2.7.) and heparin-lyase III (heparitinase, EC 4.2.2.8.) were purchased from the Seikagaku Kogyo Co. (Tokyo, Japan). Standard preparations of non- and variously sulfated Δ-disaccharides: Δdi-nonS_{HA}, Δdi-nonS_{CS}, Δdi-mono4S, Δdi-mono6S, Δdi-mono2S, [Δdi-di(2,4)S or Δdi-diS_B], [Δdi-di(2,6)S or Δdi-diS_D], [Δdi-di(4,6)S or Δdi-diS_E] and [Δdi-tri(2,4,6)S or Δdi-triS] were also obtained from Seikagaku. Tritiated sodium borohydride was purchased from Dupont (Wilmington, DE, USA). Tetrabutylammonium hydrogen sulfate (0.5 M solution, pH 7.5) was from Alltech (Deerfield, IL, USA).

Enzymic Digestions

Digestions with chondroitinases ABC and AC were performed in 50 μL solution of 50 mM Tris/HCl, pH 7.5, at 37°C for 90 min,[15, 16, 22] using 0.01 units per 10 μg of uronic acid that was determined by the borate-carbazole method of Bitter and Muir.[23] Digestion with chondroitinase B was carried out in 50 μL solution 50 mM Tris/HCl, pH 8.0, at 37°C for 60 min, using 0.01 units per 40 μg uronic acid.[24, 25] Digestion of HS was performed in 100 μL of 20 mM acetate buffer, pH 7.0, containing 1 μmol calcium acetate and 0.05 units each of heparin lyases I, II, and III per 25 μg of uronic acid at 37°C, overnight. The reactions were terminated by heating the solutions at 100°C for 1 min. Digests were centrifuged in a Beckman (San Ramon, CA, USA) microfuge at 11,000 x *g* for 10 min and aliquots were taken for further treatment.

Preparation of Standard Δ-Oligosaccharides with Known Size

Non- and mono-sulfated HA- and CSA- derived Δ-oligosaccharides of various sizes were prepared by limited digestion with chondroitinase AC and subsequent gel permeation chromatography according to a protocol described by Karamanos et al.[14] Following 1 min heating in a boiling water bath, the various sized Δ-oligosaccharides were isolated by two sequential chromatographies of the digest on a Bio-Gel P-10 (100 x 0.6 cm) column eluted with 0.5M ammonium formate, pH 7.0. Eluted fractions were measured at 232 nm.[14,15,21]

Sample Treatment and Analysis of Linkage-Region Oligosaccharides

The linkage-region oligosaccharides of CS, DS, and HS were prepared using 5 μg of GAGs and 20 μg of A_1D_1 fraction of rat chondrosarcoma PGs.

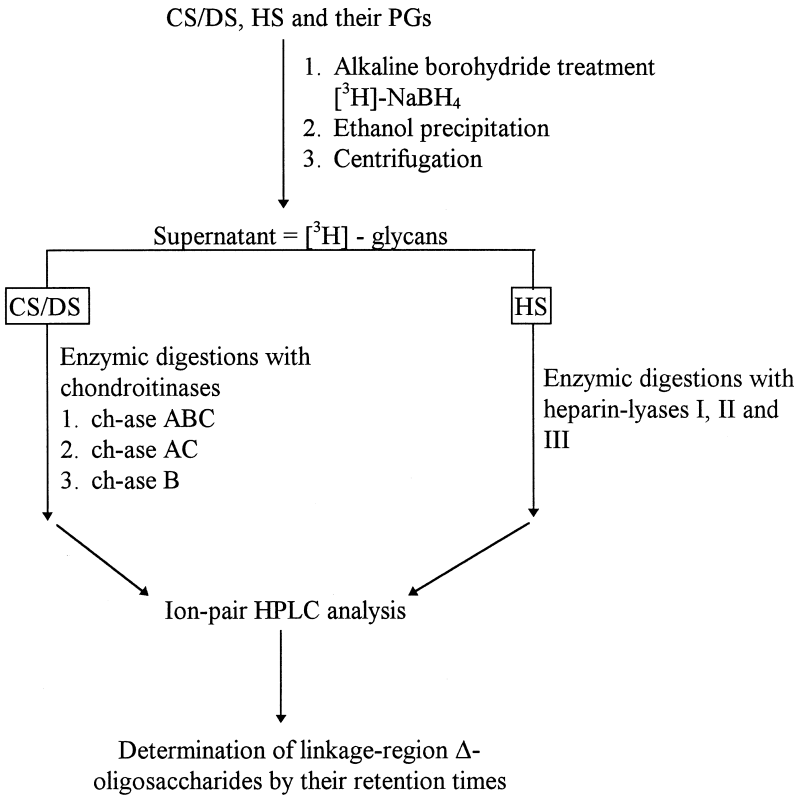

Figure 1. Flow chart of the strategy followed for sample treatment, separation and determination of the linkage-region Δ-oligosaccharides derived from CS/DS and HS.

They were first treated with alkaline-borohydride using 0.25 M [^{3}H]-$NaBH_4$ and 0.75 M $NaBH_4$ in 50 mM NaOH for 48 h at 45°C.[14,26] The obtained solutions were neutralized with 4 M acetic acid and [^{3}H]-labeled glycans were recovered by ethanol precipitation [4 vol. of 95% ethanol containing 2.5% (w/v) sodium acetate]. The CS/DS-labeled chains were then extensively split by separate digestions with chondroitinases ABC, AC, and B. The HS-labeled chain was extensively degraded (>90%) by digestion with the combination of the three heparin-lyases,[17,18,21] as mentioned above. Finally aliquots from 10 to 50 μL were taken for ion-pair HPLC analysis. The [^{3}H]-radioactivity positive peaks that contained the [^{3}H]-labeled linkage-region oligosaccharides were detected by using an HPLC radiochemical detector (EG&G Berthold, Germany). The entire procedure followed for sample treatment is summarized in Figure 1.

Separation of the linkage-region Δ-oligosaccharides, derived from the degraded polymers by enzymic treatments, was performed using ion-pair reversed phase chromatography on a Pharmacia LKB HPLC system using a 250 x 4.6 mm-i.d. Supelcosil LC-18 (Supelco) column that was connected with a 30 x 4.6 mm-i.d. RP-18 precolumn (Brownlee Labs.).[14]

A gradient elution was performed using a binary solvent system composed of 20% (v/v) aqueous acetonitrile (eluent A) and 75% (v/v) aqueous acetonitrile (eluent B). Both solvents contain 0.01 M of the ion-pairing reagent TBA phosphate at a final pH of 6.7. The flow rate was 1.2 mL/min. The elution program used was the following: isocratic elution with 100% A for 5 min, gradient elution to 50% B for 20 min, and finally one more gradient elution to 100% B for another 20 min. After each sample was totally eluted, the column was washed and re-equilibrated by further elution with 100% B for 2 min, returning to 100% A during 1 min, and continuing with 100% A for 10 min.

RESULTS AND DISCUSSION

Separation of Non- and Variously Sulfated Oligosaccharides

As it has been previously described,[14] HA-derived non-sulfated Δ-oligosaccharides and CSA-derived mono-sulfated Δ-oligosaccharides, bearing one sulfate residue per disaccharide unit, can be completely separated by ion-pair HPLC. The retention times for HA-derived Δ-di- to Δ-decahexasaccharides as well as of CSA-derived Δ-di- to Δ-octasaccharides are shown by arrows in Figure 2A. As shown in this figure, the increased number of carboxyl and sulfate groups resulted in higher retention times. This is well explained by the higher number of TBA cations which interact with the anionic charges of the saccharides - the carboxyl and the sulfate groups - and the hydrophobic sites of the C-18 column.

Resolution of Sulfated and Phosphorylated Linkage-Region Oligosaccharides

Analysis of [^{3}H]-labeled linkage-region fragments obtained from CSA, DS, and the glycan chains of A_1D_1 PGs fraction, after separate digestions with chondroitinase ABC, AC, or B and these obtained from HS after the combined action of heparin-lyases I, II, III was performed by ion-pair HPLC, as described above.

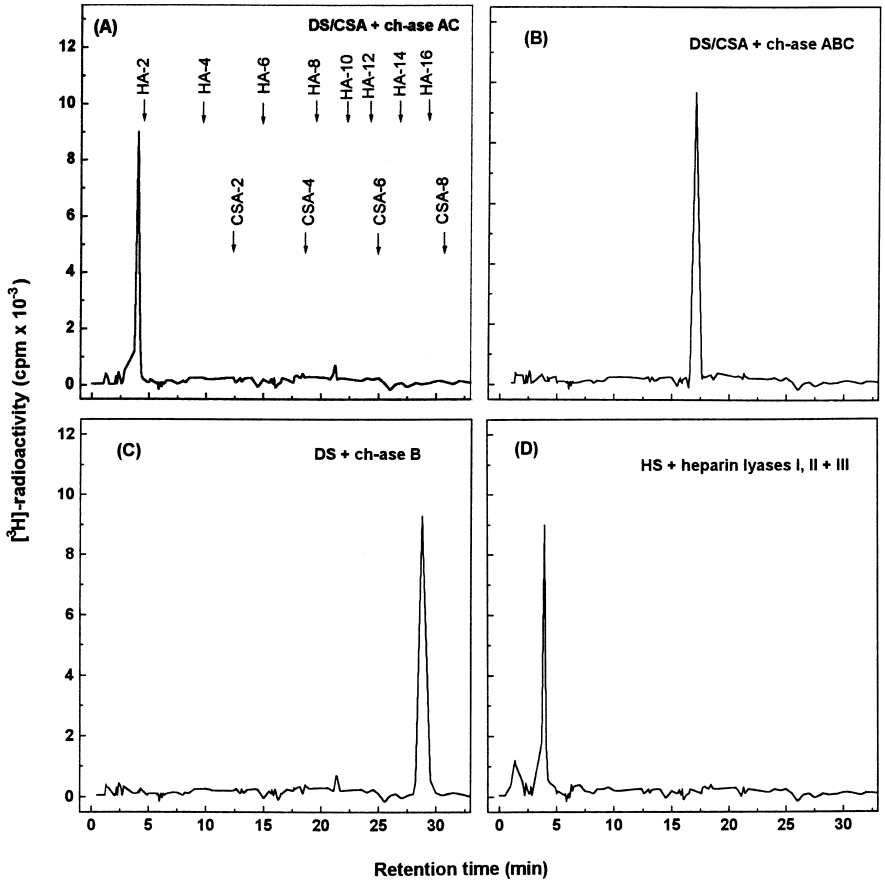

Figure 2. Resolution of [^{3}H]-labeled linkage-region Δ-oligosaccharides derived from CSA and DS following digestions with chondroitinase ABC (A and B), and from HS with combined digestion with heparin-lyases I, II and III (D). Arrows indicate the elution positions of HA-derived non-sulfated Δ-di (HA-2) to Δ-decahexasaccharides (HA-16) and of CSA derived mono-sulfated Δ-di- (CSA-2) to Δ-octasaccharides (CSA-8).

It has been explained[14] that action of chondroitinase ABC on CSA and DS produces the linkage-region ΔUA-GalNAc(SO_3H)-GlcA-Gal-Gal-Xyl-^{3}H, whereas chondroitinase AC the linkage-region fragment ΔUA-Gal-Gal-Xyl-^{3}H. The DS linkage-region fragment obtained with chondroitinase B digestion contains GlcA-containing repeats near the linkage-region and has the structure ΔUA-GalNAc(SO_3H)-GlcA-GalNAc(SO_3H)-GlcA-GalNAc(SO_3H)-GlcA-Gal-Gal-Xyl-^{3}H.

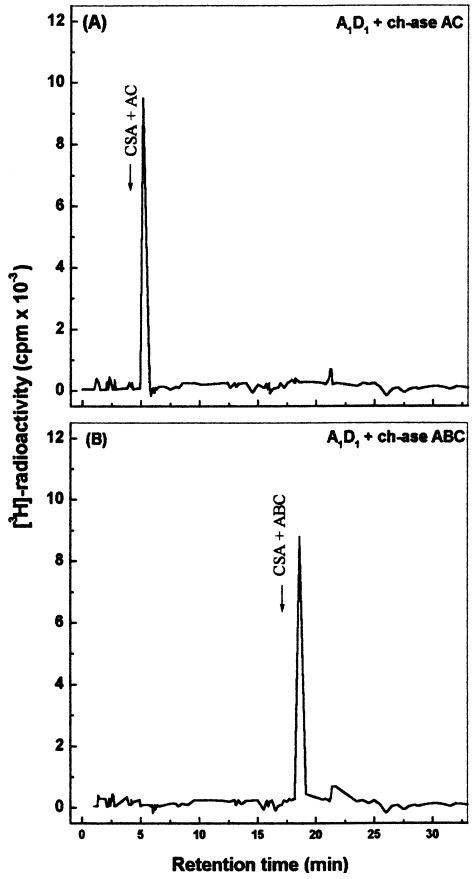

Figure 3. Determination of phosphorylated linkage-region Δ-oligosaccharides derived from A_1D_1 glycans from rat chondrosarcoma following digestion with chondroitinase AC (A) and ABC (B). Arrows indicate the elution positions of non-phosphorylated linkage-region Δ-oligosaccharides obtained from CSA following digestion with AC (arrow in A) and with ABC (arrow in B).

No phosphatase activity was detected in any of GAGs tested, concluding that the DS and CSA linkage-region contains no esterified phosphate groups. The linkage-region fragments obtained for CSA and DS, when different chondroitinases were used, were completely separated as it was expected since they contain different number of carboxyl and sulfate groups (Figures 2A, B and C). The HS linkage-region fragment obtained with a combined digestion with all three lyases has the same structure and identical retention time with that obtained from DS following digestion with chondroitinase AC (Figure 2D).

The linkage-region fragments obtained with chondroitinases ABC and AC from the A_1D_1 glycans have the same saccharide composition as the respective fragment obtained from CSA. However, both fragments contain phosphates, indicating the presence of phosphorylated xylose.[8-11,14] These linkage-region fragments for A_1D_1 glycans were also completely separated. These fragments showed different retention times from those obtained for CSA and DS and this may well be explained by the additional binding of TBA cations to phosphate groups (Figures 3A and B).

CONCLUSIONS

By the proposed ion-pair HPLC method the linkage-region oligosaccharide structure of GAGs can be easily and accurately determined. Presence of phosphorylated xylose can also easily be seen by the same set-up. The method could be helpful in determining the sequence of the linkage-region of GAGs isolated from various tissues as well as in identification of phosphorylated xylose which is related to specific biological functions and/or pathophysiological conditions. The amount of linkage-region oligosaccharide can be also used to determine the molecular size of the GAG chain.

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