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Characterization of heparan sulfate from the unossified antler of *Cervus elaphus*

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Abstract—The antler is the most rapidly growing tissue in the animal kingdom. According to previous reports, antler glycosaminoglycans (GAGs) consist of all kinds GAGs except for heparan sulfate (HS). Chondroitin sulfate is the major antler GAG component comprising 88% of the total uronic acid content. In the current study, we have isolated HS from antler for the first time and characterized it based on both NMR spectroscopy and disaccharide composition analysis. Antler GAGs were isolated by protease treatment and followed by cetylpyridinium chloride precipitation. The sensitivity of antler GAGs to heparin lyase III showed that this sample contained heparan sulfate. After incubation of antler GAGs with chondroitin lyase ABC, the HS-containing fraction was recovered by ethanol precipitation. The composition of HS disaccharides in this fraction was determined by its complete depolymerization with a mixture of heparin lyase I, II, and III and analysis of the resulting disaccharides by the reversed-phase (RP) ion pairing–HPLC, monitored by the fluorescence detection using 2-cyanoacetamide as a post-column labeling reagent. Eight unsaturated disaccharides (ΔUA-GlcNAc, ΔUA-GlcNS, ΔUA-GlcNAc6S, ΔUA2S-GlcNAc, ΔUA-GlcNS6S, ΔUA2S-GlcNS, ΔUA2S-GlcNAc6S, ΔUA2S-GlcNS6S) were produced from antler HS by digestion with the mixture of heparin lyases. The total content of 2-*O*-sulfo disaccharide units in antler HS was higher than that of heparan sulfate from most other animal sources.

Keywords: Antler; Heparan sulfate; Disaccharide composition; Characterization; HPLC

1. Introduction

Velvet antlers are the fastest growing mammalian tissue, reaching growth rates of approximately two centimeters per day during the growing season.[1] They grow in length by a continuous process of the cellular differentiation from undifferentiated progenitors in the perichondrium into chondroblasts and chondrocytes.[2,3] However, unlike embryonic or growth plate cartilage, antler cartilage is highly vascularized.[4,5]

Cartilage and blood vessels generally contain heparan sulfate proteoglycan (HSPG) that consists of one or more heparan sulfate (HS) chains attached to core protein. It has been reported that cartilage HSPG regulates differentiation and proliferation of chondrocytes.[6,7] In vascular endothelial cells, HSPG regulates not only the anticoagulant activity but also the binding of basic fibroblast growth factor (bFGF, FGF-2) to its receptor.[8] Therefore, it is highly likely the antler contains HS in blood vessels and cartilages before calcification. According to a previous report, however, antler glycosaminoglycans (GAGs) are known to contain all kinds of GAGs except for HS.[9] The present study focuses on the separation and characterization of antler HS from the unossified antler tip.

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2. Results and discussion

The whole spectrum of cellular differentiation and bone development can be identified in the growing antler tip. Thus, the antler tip section was used to obtain HS. Homogenization of 50 g of a tip part of antler was defatted using extractions with acetone and 2:1 chloroform–methanol. The defatted tissue was next subject to proteolytic digestion, followed by trichloroacetic acid (TCA) precipitation. Crude polysaccharides (8 g, 16%) were obtained by addition of ethanol on supernatant from TCA precipitation. Antler GAGs (400 mg, 0.8%) were isolated by cetylpyridinium chloride precipitation, followed by desalting and lyophilization.

To confirm the presence of HS in antler, heparin lyase III, specific to low sulfated sequence in HS, was used to degrade the GAG isolated from antler. This enzyme cuts the *N*-acetylglucosamine–glucuronate linkage by an elimination reaction, leaving a C-4–C-5 unsaturated bond in the product, which can then be easily detected by ultraviolet (UV) absorbance at 232 nm.[10–12] However, in preliminary experiments heparin lyase III was found to be inactive against the antler GAG mixture. Chondroitin sulfate (CS) has been previously shown to inhibit heparin lyase activity.[13] Since CS is a major GAG component of antler comprising 88% total uronic acid,[9] its removal from antler GAGs was undertaken using chondroitin lyase ABC, followed by ethanol precipitation to recover the HS-containing fraction. After removal of CS, heparin lyase III acted on the antler GAG preparation, suggesting that it actually contained HS (Fig. 1).

The hexosamines and uronic acids in GAG standards including hyaluronic acid, CS, dermatan sulfate, and acharan sulfate (–Ido2S-GlcNAc–) were determined by hydrolysis of these GAGs with trifluoroacetic acid followed by Bio-LC analysis (not shown). When the crude antler GAGs were subjected to the same analysis, major peaks corresponding to galactosamine (GalN) and glucuronic acid (GlcA) and minor peaks corresponding to glucosamine (GlcN), and iduronic (IdoA) acid were observed (Fig. 2I). In contrast, when the chondroitinase-treated, purified fraction was analyzed, the major hexosamine peak observed corresponded to GlcN (Fig. 2II). While the total antler GAGs showed a significant amount of GalN, which is consistent with the major GAG in antler being CS, only a small amount of GalN was observed in the chondroitinase-treated, purified GAG fraction and GlcN and GlcA were the major monosaccharide components (Fig. 2II). This provides strong evidence that the antler contains HS as the minor GAG component. The identity of the peak labeled 'X' is unknown, but it is apparently related to carbohydrate or a decomposition product.[23]

A general approach for structural analysis of GAGs is to characterize the disaccharide composition following their complete enzymatic depolymerization.[14,15] The chondroitinase-treated, purified GAG fraction isolated from antler was incubated with a mixture of heparin lyase I, II, and III to ensure the complete breakdown of HS to disaccharides, and the composition of unsaturated disaccharides was determined. The composition of HS disaccharides was analyzed by a reversed-phase (RP) ion-pairing HPLC and was monitored by fluorescence

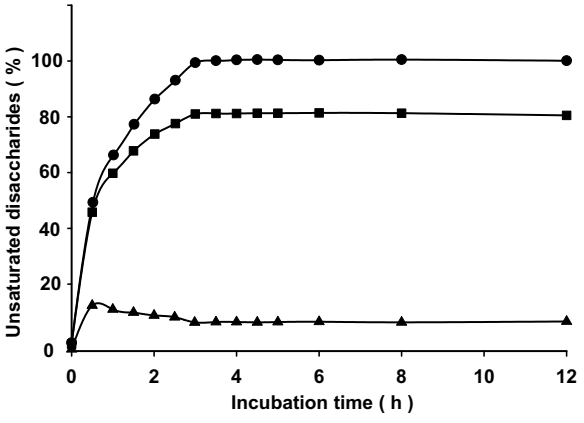
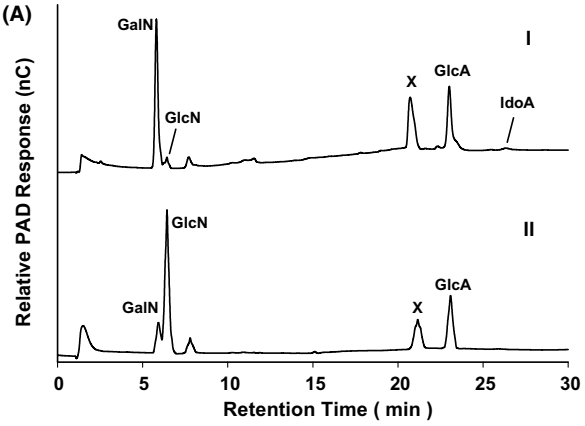


Figure 1. Time-course incubation of antler GAGs with heparin lyase III. Bovine kidney HS (●) (10 μg), antler GAGs after removal of CS (■) (800 μg) and antler GAGs (▲) (800 μg) were treated for 12 h at 37 °C with heparin lyase III.



(B)

	Hexosamine		Uronic acid	
Types	Galactosamine	Glucosamine	Glucuronic acid	Iduronic acid
Intact antler GAGs	95.2[a]	4.8[a]	93.2[b]	5.8[b]
Antler HS fraction	7.2[a]	92.8[a]	100[b]	n.d[b]

[a, b] See legend.

Figure 2. (A) HPLC chromatograms of a hydrolyzed crude antler GAGs and antler HS-containing fraction. (I) Crude antler GAGs; (II) antler HS-containing fraction. GalN, D-galactosamine; GlcN, D-glucosamine; GlcA, α-/β-D-glucuronic acid; IdoA, α-/β-D-iduronic acid; X, unknown peak. (B) Comparison of hexosamine and uronic acid from intact antler GAGs and antler HS fraction. [a]Ratio of hexosamines by relative peak area ($n = 3$). [b]Ratio of uronic acids by relative peak area ($n = 3$), nd: not detected.

detection using 2-cyanoacetamide as a post-column fluorogenic reagent (Fig. 3). The composition of unsaturated disaccharides produced from antler HS was compared with that of bovine kidney HS[16] as summarized in Figure 4. Antler HS was composed of eight unsaturated disaccharides (ΔUA-GlcNAc, ΔUA-GlcNS, ΔUA-GlcNAc6S, ΔUA2S-GlcNAc, ΔUA-GlcNS6S, ΔUA2S-GlcNS, ΔUA2S-GlcNAc6S, and ΔUA2S-GlcNS6S). As shown in Figure 4, the antler HS contains a higher proportion of ΔUA-GlcNS, ΔUA2S-GlcNAc, and ΔUA2S-GlcNS than bovine kidney HS.

The structure of antler HS was further characterized by 600 MHz NMR spectroscopy, and the spectra are shown in Figure 5. Because of the small amount of sample available, only the important structural reporter signals in HS could be identified. Anomeric protons of GlcNX (where X is acetyl or sulfo), IdoA or Ido2S are strongly indicative of HS. While the ratio of the disaccharides containing a ΔUA2S unit were relatively high compared with those of bovine kidney and most other animal-derived heparan sulfates, the intensity of the anomeric signal corresponding to IdoA2S in Figure 5

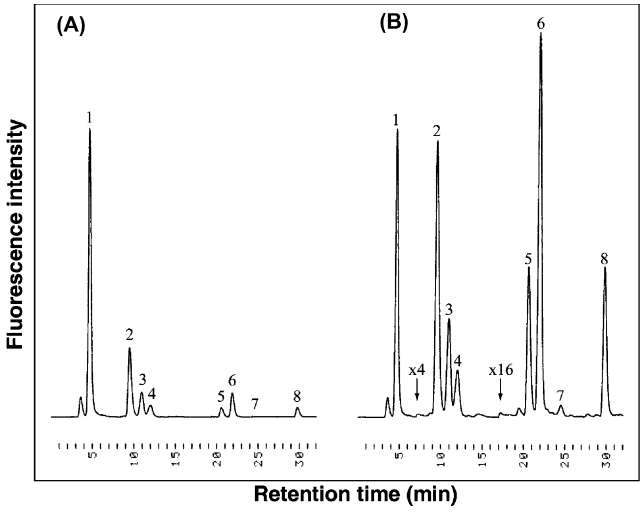


Figure 3. HPLC chromatogram of unsaturated disaccharides produced from antler GAGs after enzymatic digestion with heparin lyase III. (A) Unsaturated disaccharides from antler GAGs. Peaks represent as follows: (1) ΔUA-GlcNAc; (2) ΔUA-GlcNS; (3) ΔUA-GlcNAc6S; (4) ΔUA2S-GlcNAc; (5) ΔUA-GlcNS6S; (6) ΔUA2S-GlcNS; (7) ΔUA2S-Glc6S; (8) ΔUA2S-GlcNS6S. (B) The partially expanded chromatography of unsaturated disaccharides from antler GAGs. ×4 and ×16 indicate that the following peaks were expanded at a ratio of 1:4 and 1:16, respectively.

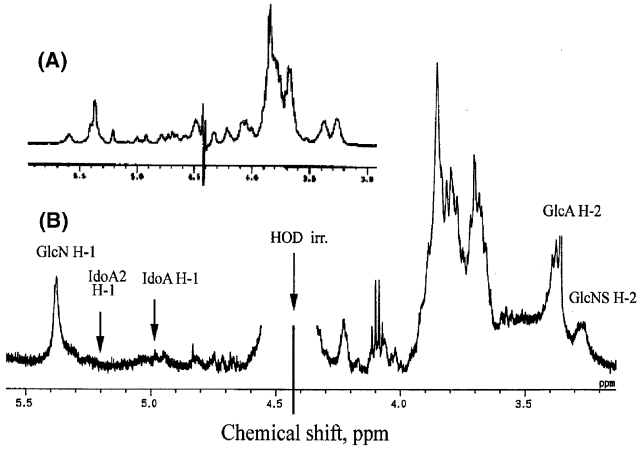
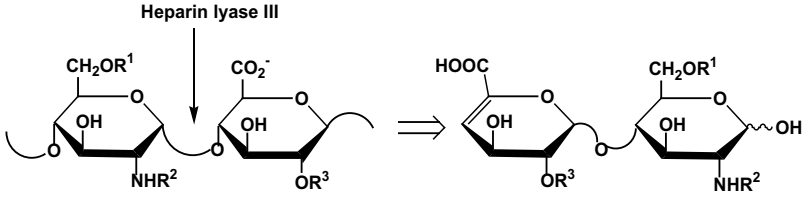


Figure 5. Part of ^{1}H NMR spectra of porcine mucosa HS (A) and antler HS (B).



Ratio of unsaturated disaccharide (%)									
	ΔUA-GlcNAc	ΔUA-GlcNS	ΔUA-GlcNAc6S	ΔUA2S-GlcNAc	ΔUA-GlcNS6S	ΔUA2S-GlcNS	ΔUA2S-GlcNAc6S	ΔUA2S-GlcNS6S	[a]2S/ sulfated disaccharide
R^1	H	H	SO^{3-}	H	SO^{3-}	H	SO^{3-}	SO^{3-}	
R^2	Ac	SO^3	Ac	Ac	SO^3	SO^3	Ac	SO^3	
R^3	H	H	H	SO^3	H	SO^3	SO^3	SO^3	
Bovine Kidney	51.7	18.7	10.7	0.6	7.2	7.1	0.0	4.0	24.2
Antler	51.6	20.9	6.3	3.4	3.2	10.3	0.3	4.1	37.4

[a] See legend.

Figure 4. Comparison of unsaturated disaccharides produced from bovine kidney and antler HS. [a]Percentage of 2-*O*-sulfo disaccharides in total sulfo disaccharides.

is very low. These results suggest that antler HS might contain the unusual saccharide, GlcA2S.

The proportion of disaccharide that contained 2-*O*-sulfo groups in antler HS was higher than those of bovine kidney and most other animal tissue sources.[17] The high content of 2-*O*-sulfo groups in uronate residues of HS is required for the activation of both FGF-1 and FGF-2.[18] Recently, several studies indicate that the specific positioning of sulfo groups along the HS chain is important in FGF signaling. There is an absolute requirement for *N*- and 2-*O*-sulfation for FGF-2 binding, and 6-*O*-sulfo groups are required for FGF receptor binding and mitogenicity.[19–22] Our results show that antler HS is composed of a high proportions of disaccharides that contain 2-*O*-sulfo groups, and thus, may be more active on FGF-2 binding. More detailed studies are being planned to examine the effect of antler HS on mitogenicity. The growing antler mainly consists of chondrocytes. HS in the cartilage regulates differentiation and proliferation of chondrocytes. Cell-surface HS is involved in the binding of FGF-2 to its receptors, and downregulation of this glycosaminoglycan is part of the pathway that leads to terminal differentiation of growth plate chondrocytes.[7] Syndecan-3, a cell-surface HSPG, is expressed by proliferating chondrocytes in vivo and is a direct and selective regulator on FGF-2 of the mitotic behavior of chondrocytes.[6] Antler HS may play a role in chondrocyte proliferation and antlerogenesis.

In conclusion, we demonstrated that the upper tip of antler tissue contains HS for the first time. Eight unsaturated disaccharides were produced after enzymatic depolymerization of antler HS, and their composition was analyzed by RP ion-pair HPLC after removal of chondroitin sulfate. The antler HS was further characterized by ^{1}H NMR spectroscopy. The proportion of disaccharides that contain 2-*O*-sulfo groups was higher than those of HS obtained from bovine kidney and most other animal sources. Further studies are needed to determine the localization of HS in antler tissues and to understand the relationship between the sequence of antler HS and the biological function of the growing antler.

3. Experimental

3.1. Materials

Antlers (*Cervus elaphus*) were obtained from male elks that were bred at Konkuk University Nokyong Research Center. Each antler was equally divided into four sections (tip, upper, middle, and bottom). Samples were freeze-dried, homogenized, and stored at −20 °C until they were analyzed. Heparin and HS disaccharides, chondroitin lyase ABC (*Proteus vulgaris*, EC 4.2.2.4) were obtained from Sigma Chemical Co. (St. Louis, MO). Heparin lyases III was obtained from Seikagaku (Tokyo, Japan). Alcalase from *Bacillus subtilis* was a product from NovoNordisk (Bagsvaerd, Denmark).

3.2. Preparation of antler HS

Unossified antler tissues were lyophilized and homogenized in a Waring blender. The antler tissues (50 g) were defatted by extraction with acetone and a 2:1 chloroform–methanol mixture. The fat-free dried antler was suspended in 200 mL of 0.05 M sodium carbonate buffer (pH 9.2). The suspension was shaken for 48 h at 200 rpm at 60 °C after adding 4 mL of alcalase (*B. subtilus*, 2.4 Anson units/g). The digestion mixture was cooled to 4 °C, and TCA was added to a final concentration of 5%. The sample was mixed, allowed to stand for 1 h, and then centrifuged for 20 min at 8000*g*. The supernatant was recovered by decantation. Three volumes of 5% potassium acetate in ethanol were added to one volume of supernatant. After mixing, the suspension was stored overnight at 4 °C and then centrifuged for 30 min at 8000*g*. The precipitate (8 g) was dissolved in 50 mL of 0.2 M NaCl and centrifuged for 30 min at 8000*g*, and the insoluble material was discarded. To the supernatant 0.5 mL of cetylpyridinium chloride (5%) was added, and the precipitate was collected by centrifugation. The precipitate was dissolved in 10 mL of 2.5 M NaCl, 5 volumes of ethanol was added, and the sample was centrifuged for 30 min at 10,000*g*. The precipitate was dissolved in water and dialyzed against 100 volumes of water, and the dialyzate was freeze-dried to obtain 400 mg of GAGs as a white powder. Antler GAGs (10 mg) were redissolved in 1 mL of 0.2 M Tris–HCl buffer (pH 8.0). To the solution, 100 μL of an aqueous solution containing 1 U of chondroitin lyase ABC were added, and it was incubated at 37 °C overnight after which 5 mL of ethanol and the HS-containing precipitate (400 μg) was collected by centrifugation at 8000*g* for 15 min at 4 °C.

3.3. Time-course assay of HS by heparin lyase III

For measuring heparin lyase III (*Flavobacterium heparinum*, Sigma) activity, 100 μL of aqueous solution containing heparin lyase was added to a cuvette containing 800 μL of 1 mg/mL HS fraction (50 mM sodium phosphate buffer, pH 7.6) at 37 °C and the increase in absorbance at 232 nm was measured as a function of time.

3.4. Enzymatic digestion of HS

A 10-μL aliquot of the sample solution containing up to 4 μg of HS, 10 μL of 0.1 M acetate buffer containing 10 mM calcium acetate (pH 7.0) and 10 μL of an

aqueous solution containing heparin lyases I, II, and III (0.4 mU each, one unit was defined as the quantity of the enzyme that catalyzes the formation of 1 μmol of unsaturated disaccharide per minute at 37 °C, pH 7.5.) were mixed and incubated at 37 °C for 12 h. A 5-μL volume of the reaction mixture was subjected to the post-column HPLC.

3.5. Analysis of hexosamines and uronic acids by Bio-LC

The types of hexosamine and uronic acid were determined from the antler HS-containing fraction according to the procedure previously reported.[23] Bio-LC (Sunnyvale, CA) was performed with Dionex gradient pumps, a pulsed amperometric detector with a gold working electrode and equipped with a 20 μL sample loop. A CarboPac PA-1 column (4 × 250 mm) was used with an adequate eluent system at a flow rate 1 mL/min. Conditions were as follows: eluent A, 50 mM NaOH; eluent B, 50 mM NaOH/150 mM NaOAc; gradient, 0–10 min (0–20% B), 10–20 min (20–100% B), 20–30 min (100% B), 30–40 min (100–0% B) and then equilibrated with 0% B for 10 min. The pulse potential was set at $E1 = 0.05$ V ($t_1 = 120$ ms), $E2 = 0.60$ V ($t_2 = 120$ ms), $E3 = -0.80$ V ($t_3 = 300$ ms) with an output range of 1–3 knA. The antler HS-containing fraction (200 μg) was treated with 500 mM TFA (50 μL) at 100 °C for 3 h, and neutralized with 2 M ammonia water. The resulting products were subjected to Bio-LC analysis.

3.6. Analysis of HS disaccharide composition by the post-column HPLC

HPLC analysis of unsaturated disaccharides from HS was essentially carried out as previously described.[24] In brief, a 5-μL portion of the sample solution was loaded onto the Hitach L-6000 instrument equipped with a fluorescence spectrophotometer (Hitach F-1050) and a TSKgel Super-Octyl column (4.6 mm i.d. × 100 mm, particle size 2 μm). HPLC conditions were as follows: eluent A, 1.2 mM TBA (tetrabutylammonium hydrogensulfate) in 4% acetonitrile; eluent B, 0.1 M cesium chloride in 4% acetonitrile; gradient, 0–3 min (1–15% B), 3–3.5 min (15–50% B), 3.5–8.5 min (50–75% B), 8.5–11.5 min (90% B), and then equilibrated with 1% B for 10 min. To the effluent were added the aqueous 1% 2-cyanoacetamide solution and 0.25 M sodium hydroxide at the same flow rate of 0.25 mL/min by using a double plunger pump (Shimamura Instrument SPU-2.5W). The mixture passed through a reaction coil (0.5 mm i.d. × 10 m) set in a dry reaction bath (Shimamura instrument DB-3) thermostated at 120 °C, followed by a cooling coil (0.25 mm i.d. × 3 m). The effluent was monitored at 346 nm of excitation and 410 nm of emission with a fluorescence detector.

3.7. ^{1}H NMR spectroscopy

^{1}H NMR spectroscopy was performed under the conditions as previously described.[25] Briefly, a sample (approximately 30–50 μg for purified HS from antler) was dissolved in 0.5 mL of D_2O (99.9%) and freeze-dried repeatedly to remove exchangeable protons. After two precipitations from 75% ethanol to further purify the HS from the GAG mixture treated with chondroitinase ABC, the sample was kept in a desiccator over phosphorus pentoxide in vacuo overnight at room temperature. The thoroughly dried sample was then dissolved in 0.5 mL of D_2O (99.96%) and passed through 0.45-μm syringe filter and transferred to a NMR tube (5.0 mm o.d. × 25 cm; Wilmad Glass Co. (Buena, NJ). 1D NMR experiments were performed on a JEOL ECP600 spectrometer (Tokyo, Japan) equipped with a 5-mm field gradient tunable probe with standard JEOL software at 60 °C on 500 μL samples. The HOD signal was suppressed by presaturation during 3 s for 1D spectra.

Acknowledgements

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