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Glycosaminoglycans from earthworms (*Eisenia andrei*)

A-Rang Im · Youmie Park · Joon-Soo Sim · Zhenqing Zhang · Zhenling Liu · Robert J. Linhardt · Yeong Shik Kim

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Abstract The whole tissue of the earthworm (*Eisenia andrei*) was lyophilized and extracted to purify glycosaminoglycans. Fractions, eluting from an anion-exchange column at 1.0 M and 2.0 M NaCl, showed the presence of acidic polysaccharides on agarose gel electrophoresis. Monosaccharide compositional analysis showed that galactose and glucose were most abundant monosaccharides in both fractions. Depolymerization of the polysaccharide mixture with glycosaminoglycan-degrading enzymes confirmed the presence of chondroitin sulfate/dermatan sulfate and heparan sulfate in the 2.0 M NaCl fraction. The content of GAGs (uronic acid containing polysaccharide) in the 2.0 M NaCl fraction determined by carbazole assay was 2%. Disaccharide compositional analysis using liquid chromatography–electrospray ionization mass spectrometry (LC–ESI–MS) analysis after chondroitinase digestion (ABC and ACII), showed that the chondroitin sulfate/dermatan sulfate contained a 4-*O*-sulfo (76%), 2,4-di-*O*-sulfo (15%), 6-*O*-sulfo (6%), and unsulfated (4%) uronic acid linked *N*-acetylgalactosamine residues. LC–ESI–MS analysis of heparin lyase I/II/III digests demonstrated the presence of *N*-sulfo (69%), *N*-sulfo-6-*O*-sulfo (25%) and 2-*O*-sulfo-*N*-sulfo-6-*O*-sulfo (5%) uronic acid linked *N*-acetylglucosamine residues.

Keywords Glycosaminoglycans · Earthworm · *Eisenia andrei* · Disaccharide compositional analysis · Monosaccharide compositional analysis · Heparan sulfate · Chondroitin sulfate/dermatan sulfate

A.-R. Im · Y. Park · J.-S. Sim · Y. S. Kim (✉)
Natural Products Research Institute, College of Pharmacy,
Seoul National University,
599 Gwanangno, Gwanak-gu,
Seoul 151-742, Republic of Korea
e-mail: kims@snu.ac.kr

J.-S. Sim
National Academy of Agricultural Science, RDA,
Suwon 441-707, Republic of Korea

Z. Zhang · Z. Liu · R. J. Linhardt (✉)
Departments of Chemistry and Chemical Biology and Chemical and Biological Engineering, Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute,
110 8th Street,
Troy, NY 12180, USA
e-mail: linhar@rpi.edu

Z. Liu
College of Chemistry and Chemical Engineering,
State Key Laboratory of Applied Organic Chemistry,
Lanzhou University,
Lanzhou 730000, People's Republic of China

Introduction

The presence of carbohydrates in worms has been reported from an examination of cuticle collagen of *Neveis virens* (clam worm) [1]. Alkaline borohydride treatment of cuticle collagen released 80% of its carbohydrates, indicating the presence of *O*-glycan units. The released glycans are found to be neutral mono-, di-, and trisaccharides of galactose and an uronic acid containing disaccharide, glucuronylmannitol. Comparative studies of two earthworms, *Neris* (a polychaete) and *Lumbricus* (an oligochaete) showed that *Lumbricus* earthworm did not contain the acidic disaccharides but had more galactose units [1, 2]. Interestingly, *Lumbricus terrestris* earthworm produces a collagen-like substance detected by the garter snake [3]. The carbohydrate content of this collagen-like substance, reveals a high content of galactose (11 wt%) with lesser amounts of fucose, mannose, glucose, *N*-acetylglucosamine (GlcNAc), and *N*-acetylgalactosamine (GalNAc) [3]. Glycosaminoglycans (GAGs) are composed

of repeating uronic acid (glucuronic acid (GlcA) or iduronic acid (IdoA)) residues and either GlcNAc or GalNAc residues. GAGs, such as heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS) are acidic, sulfated polysaccharides commonly found *O*-linked to core proteins through a tetrasaccharide linkage region, →4)-β-D-GlcA (1→3)-β-D-Gal (1→3)-β-D-Gal (1→4)-β-D-Xyl (1→ *O*-serine (where Xyl is xylose). These proteoglycans are commonly found on the cell surface as well as in the extracellular matrix of all animals ranging from *C. elegans* to man [4–6].

GAG chains are biosynthesized through the action of *N*-acetyl hexosamine transferase (GalNAc or GlcNAc transferases) acts in concert with GlcA transferase to assemble the disaccharide repeating unit structure, which can be further modified by *N*-deacetylation, *N*-sulfonation (in the case of HS), C5 epimerization (converting GlcA to IdoA), and *O*-sulfonation at different sites [7–10]. HS has a major repeating disaccharide backbone of →4) β-D-GlcA (1→4) β-D-GlcNAc with some α-L-IdoA residues and *N*-sulfo and *O*-sufo group substitution. The structural differences in HS are common based on tissue and species of origin [11, 12]. CS is composed of repeating disaccharide backbone of →4) β-D-GlcA (1→3) β-D-GalNAc with some α-L-IdoA residues and *O*-sufo group substitution. CS generally contains less sequence heterogeneity than HS. [13–15]. Biosynthetic modifications of GAGs in the Golgi generate variably sulfated disaccharide units, and it has been extensively reported that the sulfation pattern is closely related with their biological functions [16–20]. While the sugars present in GAGs are found in earthworms no studies have been reported on the characterization of GAGs from earthworms.

Carbohydrate-binding proteins have been reported in worms. Lectins from *Lumbricus terrestris* earthworm have also been isolated by affinity chromatography [21], and the C-terminal domain of this lectin was characterized by ^{1}H-, ^{13}C-, and ^{15}N-NMR [22, 23]. Earthworms are also known to have carbohydrate-hydrolyzing enzymes. Ueda *et al*. purified and characterized α-amylase from *Eisenia foetida,* which hydrolyzes raw starch into glucose, maltose, and maltotriose as end products [24]. The presence of ceramide-glycanase, which hydrolyzes the linkage between the ceramide and the glycan chain in various glycosphingolipids, was also reported in the earthworm (*Lumbricus terrestris*) [25]. Amphoteric glycosphingolipids that enhance interleukin-8 production in a tumor necrosis factor-α stimulated granulocytic HL-60 cells have also been purified from whole tissues of *Pheretima hilgendorfi* earthworm [26, 27]. To date, no GAG degrading enzymes in the earthworms have been reported.

Materials and methods

Materials

Earthworms *Eisenia andrei* were obtained from a local supplier (Hwasun, Cheollanam-Do, Republic of Korea, earthworm picture is shown in Fig. 1). HS unsaturated disaccharides standards of (0S, ΔUA-GlcNAc; (where ΔUA is Δ-deoxy-α-L-threo-hex-4-enopyranosyl uronic acid); NS, ΔUA-GlcNS; 6S, ΔUA-GlcNAc6S; 2S, ΔUA2S-GlcNAc; 2SNS, ΔUA2S-GlcNS; NS6S, ΔUA-GlcNS6S; 2S6S, ΔUA2S-GlcNAc6S; NS2S6S, ΔUA2S-GlcNS6S), CS unsaturated disaccharides standards (0S, ΔUA-GalNAc; 2S, ΔUA2S-GalNAc; 6S, ΔUA-GalNAc6S; 4S, ΔUA-GalNAc4S; 2S6S, ΔUA2S-GalNAc6S; 4S6S, ΔUA-GalNAc4S6S; 2S4S, ΔUA2S-GalNAc4S and 2S,4S6S, ΔUA2S-GalNAc4S6S), and chondroitinase ABC (from *Proteus vulgaris*) and chondroitinase ACII (*Arthrobacter aurescens*) were obtained from Seikagaku Co. (Tokyo, Japan). Recombinant heparin lyase I, II, and III were prepared in our laboratory as previously described [18]. Alcalase® (2.4 Anson Units per gram) from *Bacillus licheniformis* was purchased from Novozymes (Bagsvaerd, Denmark). Tributylamine (TrBA), hexylamine (HXA), 1,1,1,3,3,3-hexafluoro isopropanol (HFIP), DS, heparin, Dowex® macroporous resin (strong anion chloride, 16–50 mesh), cetylpyridinium chloride, tris(hydroxymethyl)amino-

Fig. 1 Picture of earthworm

methane (Trizma® base), 1,2-diaminopropane, toluidine blue, and Stains-All were purchased from Sigma (St. Louis, MO, USA). Diethylaminoethyl (DEAE) anion-exchange resin was purchased from Bio-Rad (Hercules, CA, USA). The dialysis membrane (Spectra/Por® 1, molecular weight cut-off (MWCO) 6~8 kDa) was purchased from Spectrum® Laboratories (Rancho Dominguez, CA, USA). Agarose was purchased from Cambrex Bio Science (Rockland, ME, USA). All other reagents were of analytical grade.

Extraction

Two kilograms of earthworms were cleaned, lyophilized, and ground. The dried powder was suspended in chloroform/methanol mixtures (2:1, 1:1, 1:2, v/v) to remove organic solvent soluble fats and dried under vacuum. The resulting dried powder was resuspended in 50 mM sodium carbonate buffer (pH 7.0) containing alcalase solution (5 wt%) and incubated for 12 h at 60°C with shaking (200 rpm). After boiling for 10 min, the sample solution was filtered and cooled to 4°C. The trichloroacetic acid solution (6.1 M) was mixed with the filtrate to a final concentration of 5% (v/v), and the precipitate was removed by centrifugation at 887*g* for 30 min at 4°C. Ethanol (80%, v/v) was added to the supernatant to precipitate polysaccharide, which was recovered by centrifugation at 887*g* for 30 min at 4°C. Polysaccharide precipitate was dissolved in water and mixed with cetylpyridinium chloride solution (final concentration 1%, w/v) to precipitate anionic polysaccharides. The resulting suspension was stored at room temperature for 1 h, and centrifuged at 887*g* for 30 min at 4°C. The recovered precipitate was dissolved in 2.5 M of NaCl solution, again precipitated with ethanol (80%, v/v), and centrifuged at 887*g* for 30 min at 4°C. The precipitate was recovered and dissolved in water, dialyzed (MWCO 6~8 kDa) against water for 2 days at 4°C and freeze-dried.

Macroporous strong anion-exchange (SAX) and DEAE-Sepharose ion-exchange chromatography

The crude anionic polysaccharide sample was loaded to a column packed with 500 mL of Dowex® macroporous resin in distilled water. The column was washed with 50 mL distilled water, followed by elution with 50 mL of 0.5, 1.0 and 2.0 M aqueous NaCl. Each fraction (monitored at 210 nm), was collected, dialyzed, and freeze-dried. The dried sample from the 2.0 M NaCl fraction was loaded on a column (5×50 cm) packed with 500 mL of DEAE-Sepharose in 50 mM sodium phosphate buffer (pH 7.0) [28]. The column was eluted with 50 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 0.0, 0.5, 1.0 and 2.0 M NaCl. Each fraction was again monitored at 210 nm, collected, dialyzed, and freeze-dried.

Agarose gel electrophoresis

Agarose gel-electrophoresis was performed on 1 wt% gels in TBE buffer (45 mM Tris-borate, 1 mM EDTA). CS, hyaluronan, DS, heparin and HS standards (50 μL at 5 mg/mL) and fractions obtained from earthworms (50 μL at 5 mg/mL, 1.0 M and 2.0 M NaCl fractions) were mixed with 50 μL of 60% sucrose solution and loaded on the gel. Electrophoresis was performed at constant voltage (100 V) for 1 h. The gel was stained with 0.5% Azure A (in 1% acetic acid) solution for 10 min, destained with water-methanol-acetic acid (60:30:10, v/v/v) and visualized.

Carbazole assay

Sodium tetraborahydrate (190.7 mg) dissolved in 20 mL of sulfuric acid 125 mg of carbazole dissolved in 1 mL of ethanol [29]. Sodium tetraborahydrate solution (1 mL) was placed in a test tube and cooled on ice. Multiple concentrations (1, 5, 10, 15, 20 μg, in 100 μL of water) of D-glucuronic acid and 100 μL of the samples were mixed with the cooled sodium tetraborahydrate solution. Tubes were heated for 10 min at 100°C, cooled to room temperature then 40 μL of 0.125% carbazole solution was added. The tubes were heated again at 100°C for 10 min, cooled at room temperature and absorbance at 530 min was measured on a UV/VIS Spectrophotometer (JASCO, Tokyo, Japan).

Monosaccharide compositional analysis by high performance anion exchange chromatography (HPAEC)-pulsed amperometric detection (PAD)

Monosaccharide peaks from the samples were confirmed by the injection of a mixture of authentic monosaccharide samples (250, 500, 1000, 2000, 3000, and 4000 pmol), including fucose, rhamnose, galactosamine, glucosamine, galactose, glucose, mannose, and xylose, were individually quantified based on their calibration curves. HPAEC-PAD analyses were performed on the Bio-LC system (Dionex, Sunnyvale, CA) equipped with gradient pumps (GS50, Dionex), and PAD (ED50A, Dionex) with a gold working electrode and an autosampler (AS50, Dionex). PeakNet 6.3 software (Dionex) was used to control apparatus and to collect data. Separation of monosaccharides was performed on a CarboPac™ PA-1 column (4×250 mm, Dionex) at 1 mL/min. 18 mM NaOH (eluent A) and 1 M NaOH containing 1 M sodium acetate (eluent B) were used as follows: 0–25 min 0% B; 25–40 min 30% B; 40–70 min 0% B. The pulse potential of detector 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 mobile

phases used for HPAEC-PAD analyses were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA).

Glycosaminoglycan disaccharide compositional analysis by LC-ESI-MS [30]

For LC-ESI-MS analysis, the DEAE fraction (2.0 M NaCl) was incubated with the chondroitinase ABC (10 mU) and chondroitinase ACII (5 mU) at 37°C for 10 h. The enzymatic products were recovered by the centrifugal filtration (YM-3, MWCO 3000 Da, Millipore, Bedford, MA). CS/DS disaccharides, passed through the filter, were freeze-dried and ready for LC-ESI-MS analysis. Next, the heparinase I, II, and III (5 mU each) were added to the remainder and incubated at 37°C for 10 h. The products were again recovered by centrifugal filtration and the HS disaccharides were similarly collected and freeze-dried and ready for LC-ESI-MS analysis.

The LC-ESI-MS analysis for HS disaccharides was performed on a LC-ESI-MS system (Agilent, LC/MSD trap MS). Solutions A and B for HPLC were 15% and 70% acetonitrile, respectively, containing the same 37.5 mM NH_4HCO_3 and 11.25 mM tributylamine. The pH values of the solutions were adjusted to 6.5 with acetic acid. The flow rate was 10 μL/min. Separation was performed on a C-18 column (Agilent), using solution A for 20 min, followed by a linear gradient of 0% to 50% solution B for 20 to 45 min. The column effluent entered the source of the ESI-MS for continuous detection by MS. The electrospray interface was set in the negative ionization mode with the skimmer potential of −40.0 V, capillary exit at −40.0 V, and a source of temperature at 325°C to obtain the maximum abundance of the ions in full scan spectra (150–1500 Da, 10 full scans/s). Nitrogen was used as a drying (5 L/min) and nebulizing gas (20 p.s.i.).

CS/DS disaccharide analysis was performed on an ACQUITY UPLC™ BEH C18 column (2.1 × 150 mm, 1.7 μm) (Waters Corporation, Milford, MA) using solution C for 10 min, followed by a linear gradient from 10 to 40 min of 0% to 50% solution D. The column temperature was maintained at 45°C. The flow rate was 100 μL/min. The solution C and D was 0% and 75% acetonitrile, respectively, containing the same concentration of 15 mM HXA as an ion-pairing reagent and 100 mM HFIP, as an organic modifier. Buffers were filtered by using 0.2 μm membrane filters (Millipore). The column effluent also entered the source of the ESI-MS for continuous detection by MS. The ESI interface was set in positive ionization mode with the skimmer potential 40.0 V, capillary exit 40.0 V and a source of temperature of 350°C to obtain maximum abundance of the ions in a full scan spectra (350–2000 Da, 10 full scans/s). Nitrogen was used as a drying (8 L/min) and nebulizing gas (40 p.s.i.).

Results

Purification procedures

The crude GAG extract was purified according to previously published procedures with minor modifications [31]. The yield at each step is shown in Table 1. Wet earthworms generated 770 g of powder after lyophilization, a yield of 39%. These results demonstrate that approximately 61% of earthworm weight consisted of water. Lyophilized earthworm (770 g) yielded 9.2 g of crude acidic polysaccharides after initial purification. Crude acidic polysaccharides were further purified by macroporous SAX chromatography affording 290 mg of acidic polysaccharide from the 2.0 M NaCl fraction. The elution profile of DEAE Sepharose ion-exchange chromatography is shown in Fig. 2. Final fractionation by DEAE Sepharose ion-exchange chromatography yielded 170 mg of purified acidic polysaccharide.

Analysis of the 0.0 M and 0.5 M NaCl fractions by agarose gel electrophoresis showed no acidic polysaccharides by Azure A staining. In addition, these fractions were not susceptible to the digestion with GAG lyases (chondroitinase ABC, heparin lyase I and III), demonstrating no CS/DS and HS. Both 1.0 M and 2.0 M NaCl fractions (Fig. 3 lane 7 and lane 8) were analyzed by agarose gel electrophoresis and compared with known GAGs. Azure A staining confirmed that 1.0 M and 2.0 M NaCl fractions contain acidic polysaccharides. The complete digestion of both fractions (1.0 M and 2.0 M) by chondroitinase ABC and heparin lyase I/III showed that only the 2.0 M NaCl fraction contains CS/DS and HS. The carbazole assay result showed that the acidic polysaccharide content in the 2.0 M NaCl was 2 wt%. In contrast, the 1.0 M NaCl fractions

Table 1 Amounts and yield of each purification process

Purification steps	Amount	Yield (%)[a]	Yield (%)[b]
Wet earthworms	2.0 kg	–	–
Freeze-dried earthworm powder	770 g	39	–
Crude GAG extracts	9.2 g	0.46	1.2
Macroporous SAX column (2.0 M fraction)	290 mg	0.015	0.038
DEAE column (2.0 M Fraction)	170 mg	0.0087	0.023

[a] Yield was calculated based on starting material (wet earthworms)

[b] Yield was calculated based on freeze-dried powder

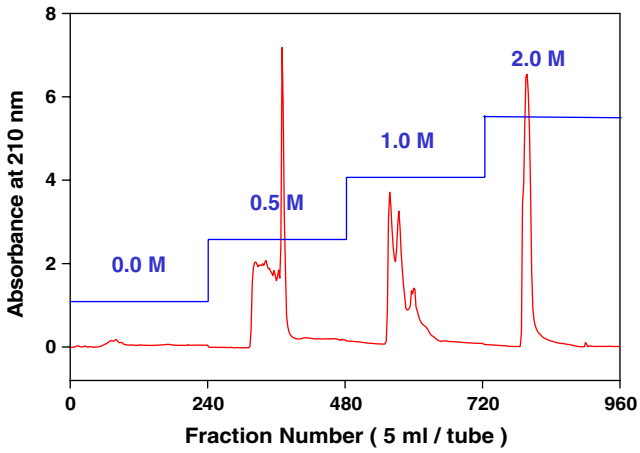

Fig. 2 Profile of DEAE-Sepharose ion-exchange chromatography. No. 0–240 is the 0.0 M NaCl fraction, No. 241–480 is the 0.5 M NaCl fraction, No. 481–720 is the 1.0 M NaCl fraction and No. 721–960 is the 2.0 M NaCl fraction

(lane 7) did not produce any GAG oligosaccharides or disaccharides on enzyme digestion (data not shown). These results suggest that this fraction contains other acidic polysaccharides. Therefore, we focused on the 2.0 M NaCl fraction for the disaccharide compositional analysis by LC-ESI-MS. The final amount of 2.0 M NaCl fraction was 170 mg, which amounts to a 0.023% yield from the lyophilized powder.

Monosaccharide compositional analysis

Monosaccharide compositional analyses of amino/neutral sugars in the 1.0 M and 2.0 M NaCl fractions was next performed (Table 2). Fucose (6.0 mol%), rhamnose (14.5 mol%), galactosamine (3.4 mol%), arabinose (2.2 mol%), glucosamine (7.0 mol%), galactose (28.9 mol %), glucose (17.6 mol %), mannose (14.5 mol %), and xylose (5.9 mol %) were detected in the 1.0 M NaCl fraction. Galactose mole % was the highest in the 1.0 M fraction followed by glucose.

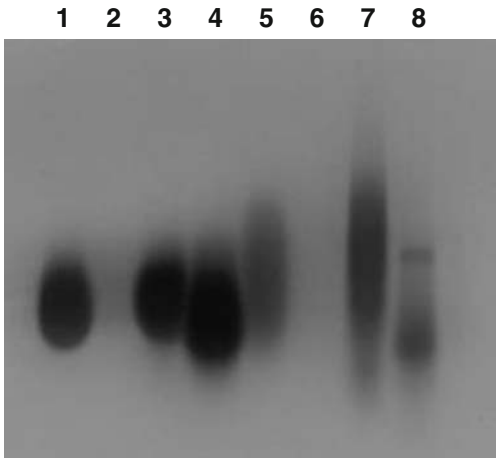

Fig. 3 Agarose gel electrophoresis. Lane 1, chondroitin sulfate; lane 2, hyaluronan; lane 3, DS; lane 4, heparin; lane 5, HS; lane 6, blank; lane 7, 1.0 M NaCl fraction from DEAE Sepharose chromatography; lane 8, 2.0 M NaCl fraction from DEAE Sepharose chromatography. 25 μg of each sample was loaded and stained with Azure A

Table 2 Mole (%) of neutral and amino monosaccharides from 1.0 and 2.0 M NaCl fractions of DEAE Sepharose ion-exchange chromatography

Monosaccharides	1.0M NaCl fraction mole (%)	2.0M NaCl fraction mole (%)
Fucose	6.0	1.4
Rhamnose	15	28
Galactosamine	3.4	n.d.[a]
Arabinose	2.2	2.6
Glucosamine	7.0	11
Galactose	29	27
Glucose	18	22
Mannose	15	5.5
Xylose	5.9	1.8

[a] n.d.: not detected

Glycosaminoglycan disaccharide compositional analysis

The 2.0 M NaCl fraction was further analyzed and its GAGs content was characterized. Following chondroitinase ABC and AC II digestion, the disaccharide composition was analyzed by LC-ESI-MS (Fig. 4. and Table 3). The major disaccharide (76 % and 64 % of disaccharide ratio in EIC and UV, respectively) was ΔUA-GalNAc4S. In addition, ΔUA-GalNAc, ΔUA-GalNAc6S and ΔUA2S-GalNAc4S were observed. All these three peaks are identified by retention time and through their mass spectra. All the peaks in EIC detection were conclusively identified from their corresponding MS spectrum.

Discussion

Earthworms belong to the class Oligochaeta and are commonly used for the decomposition of organic wastes in agroecology. There are 6,000 species of earthworms known throughout the world [32]. *E. fetida* and *E. andrei* are the most commonly used earthworms in waste treatment. These are rugged worms having a wide temperature tolerance range [32]. The acidic polysaccharide composition of this important earthworm has not previously been examined in any detail. Acidic polysaccharides, specifically GAGs serve important biological functions including: contributing to the structural elements of tissues as a major component of the extracellular matrix, in cell signaling particularly in devel-

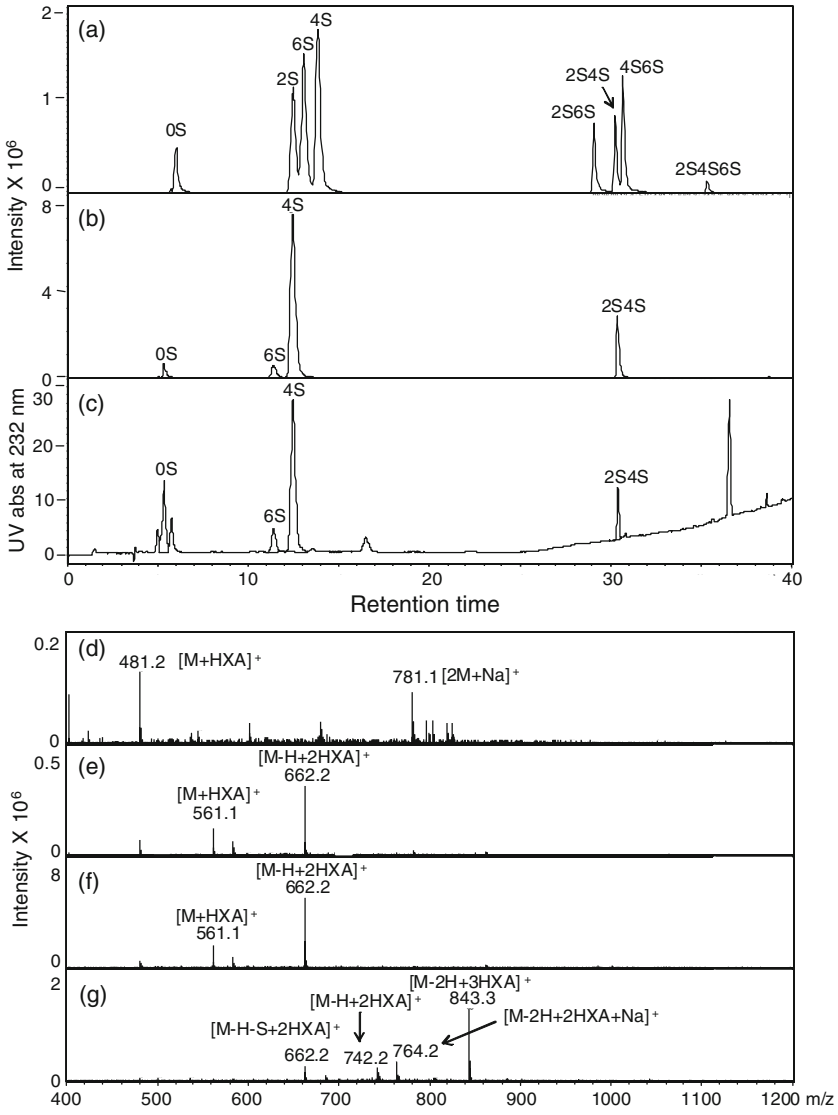

Fig. 4 Disaccharide compositional analysis of CS by LC-ESI-MS. **a** CS/DS disaccharide standards, **b** EIC of CS/DS disaccharide analysis, **c** UV (232 nm) detection of CS/DS disaccharide analysis, **d** MS spectrum of 0S, ΔUA-GalNAc, **e** MS spectrum of 4S, ΔUA-GalNAc4S, **f** MS spectrum of 6S, ΔUA-GalNAc6S, and **g** MS spectrum of 2S4S, ΔUA2S-GalNAc4S

opment, and in response to wounding and infection [6, 12]. The acidic polysaccharide extracts from earthworm (1.0 and 2.0 M fractions) represents less than 1.0 % of their dry weight (Table 1) and show a variety of monosaccharides present (Table 2). Galactose is a major component that likely originates from collagenous material from earthworm cuticles. Muir and Lee [2] reported that approximately 90% of the neutral sugars present in whole cuticles from earthworm (*Lumbricus terrestris*) was galactose [33]. Along with galactose, they also detected smaller amounts of other neutral sugars such as fucose and xylose, and hexosamines (glucosamine and galactosamine) from *L. terrestris* cuticles. Another abundant monosaccharide was glucose. It has been reported that freezing induced glucose accumulation in the enchytraeid worms [34], suggesting a role of glucose in enhancing tolerance towards freezing. In addition, the presence of insulin in earthworms and insects was reported, showing a stimulation of glucose oxidation or lipogenesis by isolated rat adipocytes [35]. Rhamnose, another major component, has also been found in surface-layer glycoproteins of prokaryotic (*Archaea* and bacteria) organisms [36]. However, it is more likely that rhamnose may come from the food source in the farm for growing earthworms. Food is mainly composed of fruits, vegetables, and wet paper. Mannose (14.5 mol %) is the third abundant monosaccharide followed by glucosamine (7.0 mol %), fucose (6.0 mol %), and xylose (5.9 mol %) in the 1.0 M NaCl fraction, suggesting a possible presence of truncated acidic *N*-glycans with fucosylation and/or xylosidation. Alternatively, the detection of mannose might suggest the presence of glucuronosylmannose disaccharide units, which was first reported by Spiro and Bhoyroo [1]. They characterized this acidic disaccharide unit after partial acid hydrolysis from cuticle collagen of clam worm (*Nereis virens*) [1] and also

Table 3 Disaccharide compositional analysis of CS/DS and HS

CS/DS disaccharide composition[a]								
	0S	2S	6S	4S	2S6S	2S4S	4S6S	2S4S6S
EIC	3.9[b] %	n.d.	5.6 %	76 %	n.d.	15 %	n.d.	n.d.
UV	20.2[c] %	n.d.	8.7 %	64 %	n.d.[d]	8.0 %	n.d.	n.d.
HS disaccharide composition[e]								
	0S	NS	6S	2S	NS6S	NS2S	2S6S	NS2S6S
EIC	n.d	67 %	n.d.	n.d.	29 %	n.d.	n.d.	3.7 %
UV	n.d	69 %	n.d.	n.d.	25 %	n.d.	n.d.	5.3 %

[a] CS/DS disaccharide composition by LC-ESI-MS is shown in Fig. 4

[b,c] % of each disaccharide=(peak area of each disaccharide from EIC or UV at 232 nm) / (total peak area from EIC or UV 232 nm) × 100

[d] n.d.: not detected

[e] HS disaccharide composition by LC-ESI-MS is shown in Fig. 5

reported that it was *O*-glycosidically linked to a threonine residue which was within or adjacent to a characteristic collagen tripeptide sequence. Muir and Lee [2] and Spiro and Bhoyroo [1] have shown that earthworm cuticle collagen has at least 23 attachment sites, on serine and threonine residues, per 1,000 residues of *O*-glycosidically bound carbohydrates.

GAGs are composed of uronic acid, observed by carbazole assay, and glucosamine or galactosamine residues observed in the acidic polysaccharide fraction (1.0 and 2.0 M fractions in Table 2). Treatment with specific GAGs digesting enzymes, chondroitinases and heparinases, and disaccharide analysis confirmed that both CS/DS and HS GAGs were present. Disaccharide compositional analysis relied on LC-ESI-MS with detection by UV or extracted ion chromatography (EIC). Differences observed in disaccharide composition, particularly for non-sulfated disaccharide, as measured using EIC and UV detection (Table 3), are likely caused by overlapping, UV absorbing peaks of unknown origin. Thus, the composition by EIC detection provides the most reliable values as well as offering confirmation of assignment based on ESI-MS analysis of each peak. The number of $HXAH^+$ moieties adducted in each peak varied based on the charge of each CS/DS disaccharide. The non-sulfated disaccharide showed a molecular ion of m/z 481.2, corresponding to the addition of one $HXAH^+$ (Fig. 4d). Monosulfated disaccharides (m/z 662.2) had two $HXAH^+$, and minor peaks at m/z 561.1 corresponding to $[M+HXA]^+$ were also observed in these spectra (Fig. 4e and f). Disulfated disaccharides with three $HXAH^+$ were observed at m/z 843.3 (Fig. 4g). This spectrum also showed minor molecular ions, corresponding to $[M-2H+2HXA+Na]^+$ and $[M-H+2HXA]^+$ at m/z 764.2 and 742.2, respectively. In addition, fragment

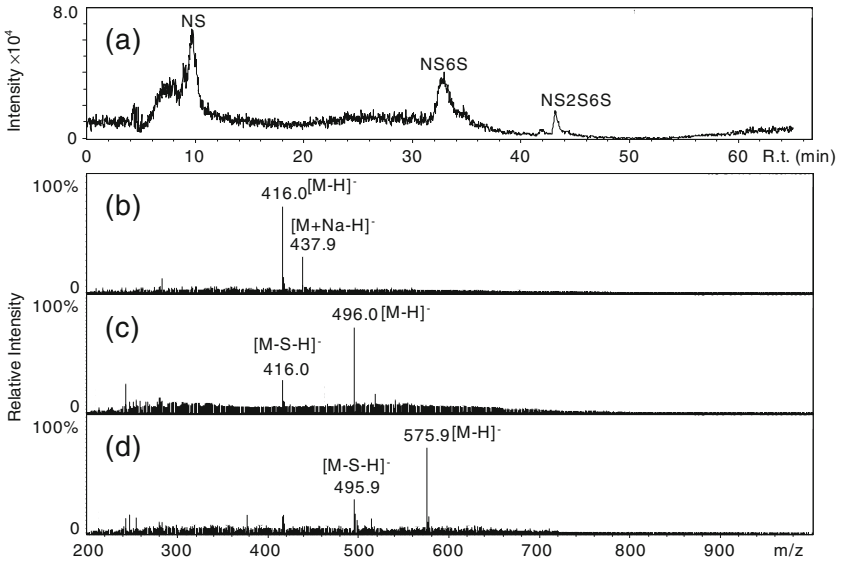

Fig. 5 Disaccharide compositional analysis of HS by LC-ESI-MS. **a** EIC of heparin/HS disaccharides, **b** MS spectrum of NS, ΔUA-GlcNS, **c** MS spectrum of NS6S, ΔUA-GlcNS6S, and **d** MS spectrum of NS2S6S, ΔUA2S-GlcNS6S

ion peaks, observed at 662.2, corresponding to desulfonation and these were assigned as $[M\text{-}H\text{-}S+2HXA]^+$. The disaccharide composition of earthworm CS/DS is typical of many animal-derived CS/DS consisting of a 4-*O*-sulfo (76 %), 2, 4-di-*O*-sulfo (15 %), 6-*O*-sulfo (6 %), and unsulfated (4 %) chondroitin.

The extracted ion chromatography (EIC) of HS disaccharides and the mass spectrum of each peak are shown in Fig. 5. Three peaks, ΔUA-GlcNS (NS), ΔUA-GlcNS6S (NS6S), and ΔUA2S-GlcNS6S (NS2S6S), were observed (Fig. 5a). These peaks were confirmed by their retention times and their masses observed in the corresponding mass spectra. The molecular ions $[M\text{-}H]^-$ and its sodium form $[M+Na\text{-}2H]^-$ of disaccharide ΔUA-GlcNS were observed at m/z 416.0 and 437.9, respectively (Fig. 5b). The molecular ion $[M\text{-}H]^-$ of disaccharide ΔUA-GlcNS6S was observed at m/z 496.0 in Fig. 5c. The disaccharide ΔUA2S-GlcNS6S was confirmed in Fig. 5d by m/z 575.9. The disaccharide composition was calculated by the ratio of these three peak areas in EIC (Fig. 5a) and UV chromatography at 232 nm (figure not shown) and listed in Table 3. The results from EIC and UV chromatography are consistent. It is interesting to note that the HS composition is extremely simple. The HS backbone is→4)GlcA/IdoA (1→4)GlcNS(1→with 30–33 % of the GlcNS residues containing a 6-*O*-sulfo group and 3–6 % containing both 2-*O*-sulfo uronic acid and a 6-*O*-sulfoglucosamine. Surprisingly, no *N*-acetyl group was observed in this polysaccharide. Because of the very small amount of this HS in earthworm insufficient sample was available for NMR analysis. This is a very unusual composition and warrants further investigation. Other invertebrates including snail also show unusual structures [31].

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References

1. Spiro, R.G., Bhoyroo, V.D.: Studies on the carbohydrate of collagens. Characterization of a glucuronic acid-mannose disaccharide unit from *Nereis cuticle* collagen. J. Biol. Chem. **255**, 5347–5354 (1980)
2. Muir, L., Lee, Y.C.: Structures of the D-galactose oligosaccharides from earthworm cuticle collagen. J. Biol. Chem. **244**, 2343–2349 (1969)
3. Kirschenbaum, D.M., Schulman, N., Halpern, M.: Earthworms produce a collagen-like substance detected by the garter snake vomeronasal system. Proc. Natl Acad. Sci. USA **83**, 1213–1216 (1986)
4. Nelson, R.M., Venot, A., Bevilacqua, M.P., Linhardt, R.J., Stamenkovic, I.: Carbohydrate-protein interactions in vascular biology. Annu. Rev. Cell. Dev. Biol. **11**, 601–631 (1995)
5. Hileman, R.E., Fromm, J.R., Weiler, J.M., Linhardt, R.J.: Glycosaminoglycan-protein interactions: definition of consensus sites in glycosaminoglycan binding proteins. Bioessays **20**, 156–167 (1998)
6. Capila, I., Linhardt, R.J.: Heparin-protein interactions. Angew. Chem. Int. Ed. Engl. **41**, 391–412 (2002)
7. Lindahl, U., Hook, M., Backstrom, G., Jacobsson, I., Riesenfeld, J., Malmstrom, A., Roden, L., Feingold, D.S.: Structure and biosynthesis of heparin-like polysaccharides. Fed. Proc. **36**, 19–24 (1977)
8. Bodevin-Authelet, S., Kusche-Gullberg, M., Pummill, P.E., DeAngelis, P.L., Lindahl, U.: Biosynthesis of hyaluronan: direction of chain elongation. J. Biol. Chem. **280**, 8813–8818 (2005)
9. Lindahl, U.: Biosynthesis of heparin. Biochem. Soc. Trans. **18**, 803–805 (1990)
10. Lindahl, U., Kusche, M., Lidholt, K., Oscarsson, L.G.: Biosynthesis of heparin and heparan sulfate. Ann. N. Y. Acad. Sci. **556**, 36–50 (1989)
11. Casu, B.: Structure and biological activity of heparin. Adv. Carbohydr. Chem. Biochem. **43**, 51–134 (1985)
12. Linhardt, R.J.: 2003 Claude S. Hudson Award address in carbohydrate chemistry. Heparin: structure and activity. J. Med. Chem. **46**, 2551–2564 (2003)
13. Deutsch, A.J., Midura, R.J., Plaas, A.H.: Structure of chondroitin sulfate on aggrecan isolated from bovine tibial and costochondral growth plates. J. Orthop. Res. **13**, 230–239 (1995)
14. Hoffman, P., Linker, A., Lippman, V., Meyer, K.: The structure of chondroitin sulfate B from studies with *Flavobacterium* enzymes. J. Biol. Chem. **235**, 3066–3069 (1960)
15. Winter, W.T., Arnott, S., Isaac, D.H., Atkins, E.D.: Chondroitin 4-sulfate: the structure of a sulfated glycosaminoglycan. J. Mol. Biol. **125**, 1–19 (1978)
16. Linhardt, R.J., Dordick, J.S., Deangelis, P.L., Liu, J.: Enzymatic synthesis of glycosaminoglycan heparin. Semin. Thromb. Hemost. **33**, 453–465 (2007)
17. Munoz, E., Xu, D., Avci, F., Kemp, M., Liu, J., Linhardt, R.J.: Enzymatic synthesis of heparin related polysaccharides on sensor chips: rapid screening of heparin-protein interactions. Biochem. Biophys. Res. Commun. **339**, 597–602 (2006)
18. Zhang, Z., McCallum, S.A., Xie, J., Nieto, L., Corzana, F., Jimenez-Barbero, J., Chen, M., Liu, J., Linhardt, R.J.: Solution structures of chemoenzymatically synthesized heparin and its precursors. J. Am. Chem. Soc. **130**, 12998–13007 (2008)
19. Jin, L., Abrahams, J.P., Skinner, R., Petitou, M., Pike, R.N., Carrell, R.W.: The anticoagulant activation of antithrombin by heparin. Proc. Natl. Acad. Sci. USA **94**, 14683–14688 (1997)
20. Richard, B., Swanson, R., Olson, S.T.: The signature 3-O-sulfo group of the anticoagulant heparin sequence is critical for heparin binding to antithrombin but is not required for allosteric activation. J. Biol. Chem. **284**, 1–23 (2009)
21. Hirabayashi, J., Dutta, S.K., Kasai, K.: Novel galactose-binding proteins in Annelida. Characterization of 29-kDa tandem repeat-type lectins from the earthworm *Lumbricus terrestris*. J Biol Chem **273**, 14450–14460 (1998)
22. Hemmi, H., Kuno, A., Ito, S., Suzuki, R., Hasegawa, T., Hirabayashi, J.: NMR studies on the interaction of sugars with the C-terminal domain of an R-type lectin from the earthworm *Lumbricus terrestris*. FEBS J **276**, 2095–2105 (2009)
23. Nielbo, S., Thomsen, J.K., Graversen, J.H., Jensen, P.H., Etzerodt, M., Poulsen, F.M., Thogersen, H.C.: Structure of the plasminogen kringle 4 binding calcium-free form of the C type lectin-like domain of tetranectin. Biochemistry **43**, 8636–8643 (2004)

24. Ueda, M., Asano, T., Nakazawa, M., Miyatake, K., Inouye, K.: Purification and characterization of novel raw-starch-digesting and cold-adapted alpha-amylases from *Eisenia foetida*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **150**, 125–130 (2008)
25. Li, Y.T., Ishikawa, Y., Li, S.C.: Occurrence of ceramide-glycanase in the earthworm, *Lumbricus terrestris*. Biochem. Biophys. Res. Commun. **149**, 167–172 (1987)
26. Sugita, M., Fujii, H., Dulaney, J.T., Inagaki, F., Suzuki, M., Suzuki, A., Ohta, S.: Structural elucidation of two novel amphoteric glycosphingolipids from the earthworm, *Pheretima hilgendorfi*. Biochim. Biophys. Acta **1259**, 220–226 (1995)
27. Hada, N., Shida, Y., Shimamura, H., Sonoda, Y., Kasahara, T., Sugita, M., Takeda, T.: Synthetic studies on glycosphingolipids from *Protostomia phyla*: syntheses and biological activities of amphoteric glycolipids containing a phosphocholine residue from the earthworm *Pheretima hilgendorfi*. Carbohydr. Res. **343**, 2221–2228 (2008)
28. Kim, H.S., Lee, Y.H., Lee, Y.R., Im, S.A., Lee, J.K., Kim, Y.S., Sim, J.S., Choi, H.S., Lee, C.K.: Activation of professional antigen presenting cells by acharan sulfate isolate from giant African snail, *Achatina fulica*. Arch. Pharm. Res. **30**, 866–870 (2007)
29. Kosakai, M., Yosizawa, Z.: A partial modification of the carbazole method of Bitter and Muir for quantitation of hexuronic acids. Anal. Biochem. **93**, 295–298 (1979)
30. Zhang, Z., Xie, J., Liu, H., Liu, J., Linhardt, R.J.: Quantification of heparan sulfate disaccharides using ion-pairing reversed-phase microflow high-performance liquid chromatography with electrospray ionization trap mass spectrometry. Anal. Chem. **81**, 4349–4355 (2009)
31. Kim, Y.S., Jo, Y.Y., Chang, I.M., Toida, T., Park, Y., Linhardt, R.J.: A new glycosaminoglycan from the giant African snail (*Achatina fulica*). J. Biol. Chem. **271**, 11750–11755 (1996)
32. Edwards, C.A., Bohen, P.J.: Biology and ecology of earthworms. Chapman & Hall, London (1996)
33. Muir, L., Lee, Y.C.: Structures of the D-galactose oligosaccharides from earthworm cuticle collagen. J. Biol. Chem. **244**, 2343–2349 (1969)
34. Slotsbo, S., Maraldo, K., Malmendal, A., Nielsen, N.C., Holmstrup, M.: Freeze tolerance and accumulation of cryoprotectants in the enchytraeid *Enchytraeus albidus* (Oligochaeta) from Greenland and Europe. Cryobiology **57**, 286–291 (2008)
35. LeRoith, D., Lesniak, M.A., Roth, J.: Insulin in insects and annelids. Diabetes **30**, 70–76 (1981)
36. Kahlig, H., Kolarich, D., Zayni, S., Scheberl, A., Kosma, P., Schaffer, C., Messner, P.: N-acetylmuramic acid as capping element of alpha-D-fucose-containing S-layer glycoprotein glycans from *Geobacillus tepidamans* GS5–97T. J. Biol. Chem. **280**, 20292–20299 (2005)