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A New Glycosaminoglycan from the Giant African Snail *Achatina fulica**

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A new glycosaminoglycan has been isolated from the giant African snail *Achatina fulica*. This polysaccharide had a molecular weight of 29,000, calculated based on the viscometry, and a uniform repeating disaccharide structure of →4)-2-acetyl,2-deoxy-α-D-glucopyranose (1→4)-2-sulfo-α-L-idopyranosyluronic acid (1→. This polysaccharide represents a new, previously undescribed glycosaminoglycan. It is related to the heparin and heparan sulfate families of glycosaminoglycans but is distinctly different from all known members of these classes of glycosaminoglycans. The structure of this polysaccharide, with adjacent *N*-acetylglucosamine and 2-sulfo-iduronic acid residues, also poses interesting questions about how it is made in light of our current understanding of the biosynthesis of heparin and heparan sulfate. This glycosaminoglycan represents 3–5% of the dry weight of this snail's soft body tissues, suggesting important biological roles for the survival of this organism, and may offer new means to control this pest. Snail glycosaminoglycan tightly binds divalent cations, such as copper(II), suggesting a primary role in metal uptake in the snail. Finally, this new polysaccharide might be applied, like the *Escherichia coli* K5 capsular polysaccharide, to the study of glycosaminoglycan biosynthesis and to the semisynthesis of new glycosaminoglycan analogs having important biological activities.

Glycosaminoglycans (GAGs)[1] are a family of linear anionic polysaccharides that are typically isolated as proteoglycans linked to a protein core. The biological functions of proteoglycans, including the regulation of cell growth, result, in large part, through the interaction of the GAG chains in proteoglycans with proteins, such as growth factors and their receptors (1). There are two major classes of GAGs: 1) glucosaminoglycans, including heparin, heparan sulfate, hyaluronic acid, and keratan sulfate; and 2) galactosaminoglycans, including chondroitin and dermatan sulfates (1).

Heparin and heparan sulfate have been the subject of intensive study because of their well recognized ability to bind many different proteins that regulate a variety of important biological processes (2). Heparin and heparan sulfate GAGs are comprised of alternating 1→4 linked glucosamine and uronic acid residues. Heparan sulfate is composed primarily of monosulfated disaccharides of *N*-acetyl-D-glucosamine and D-glucuronic acid, while heparin is composed mainly of trisulfated disaccharides of *N*-sulfoyl-D-glucosamine and L-iduronic acid (2).

GAGs have been isolated from various tissues obtained from a large number of animal species including both vertebrates and invertebrates (3, 4). An exhaustive assessment showed that while a large number of invertebrate species contain GAGs, mollusks are a particularly rich source of these sulfated polysaccharides (4). Invertebrates were first shown to contain a heparin or heparan sulfate type GAG by Burson and co-workers in 1956 (5). Heparin has only been found in one invertebrate phylum, the Mollusca, and it often corresponds to up to 90% of the total GAG content of these organisms. While the heparins isolated from various mollusks are structurally different from human heparin (6) and pharmaceutical heparins (7, 8), mollusk heparins contain antithrombin-dependent anticoagulant activity associated with the presence of the unique 3-*O*-sulfated glucosamine residue found in the antithrombin pentasaccharide binding site common to all anticoagulant heparins (5, 8, 9–11).

While pursuing our long term study of heparin's structure, we isolated a pure GAG in large amounts from the giant African snail *Achatina fulica* having a unique structure. This GAG is neither heparin nor heparan sulfate, but instead represents a new type of 1→4 linked GAG. A number of biological roles are likely for this molecule in the snail.

EXPERIMENTAL PROCEDURES

Materials

Giant snails (*A. fulica* Bowdich) originated in East Africa and were purchased in Seoul, Korea, where they are a local food source. Porcine mucosal heparin and heparan sulfate (12) (sodium salt) were from Celsus (Cincinnati, OH). Heparin lyase II used in the large-scale depolymerization was from IBEX (Montreal, Canada). Heparin lyase I (EC 4.2.2.7), heparin lyase II (no EC number), and heparin lyase III (EC 4.2.2.8) used in structural studies were purified in our laboratory to homogeneity from *Flavobacterium heparinum* (13). Heparin disaccharide standards were from Grampian Enzymes (Aberdeen, UK). All other reagents used were analytical grade. Alkaline protease mixture, Alcalase, was from Novo (New York, NY). Gel filtration chromatography was performed on Sephadex G-50 (superfine) from Pharmacia Biotech Inc. and on Bio-Gel P-2 (fine) from Bio-Rad. Spectrapore dialysis membranes with a molecular weight cut-off of 1000 were from Spectrum Medical (Los Angeles, CA). Strong anion exchange HPLC was performed on a 5-μm Spherisorb 2.5 × 25-cm column from Phase Separation (Norwalk, CT) using dual face programmable LC-7A titanium-based pumps (Shimadzu, Kyoto, Japan) equipped with a Pharmacia variable wavelength detector.

Gradient polyacrylamide gel electrophoresis (PAGE) was performed on a 20-cm vertical slab gel (Protean® II, equipped with a model 1420B

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[1] The abbreviations used are: GAG, glycosaminoglycan; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; CE, capillary electrophoresis; M_v, viscometric molecular weight; GC, gas chromatography; COSY, correlation spectroscopy; ΔUAp, 4-deoxy-α-L-*threo*-hex-4-enopyranosyl uronic acid; IdoAp, idopyranosyluronic acid; GlcAp, glucopyranosyluronic acid; GlcNp, 2-amino-2-deoxyglucopyranose; S, sulfate; Ac, acetate.

power source from Bio-Rad. Capillary electrophoresis (CE) was performed using a Dionex Capillary Electrophoresis system with advanced computer interface, model I, equipped with high voltage power supply capable of constant or gradient voltage control using a fused silica capillary from Dionex Corporation (Sunnyvale, CA). The Cannon-Ubbelohde semimicro capillary viscometer was from Cannon Instruments (State College, PA). A Perkin-Elmer (Ueberlingen, Germany) model 141 polarimeter was used to determine optical rotations.

Methods

Preparation of GAG—The shell of a giant snail (*A. fulica*) was removed, and the whole soft body was defatted using three 24-h extractions with acetone. The fat-free dried snail was cut into a fine powder using a razor blade. Approximately 4 g of dried, defatted, pulverized powder was suspended in 40 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 2 ml of Alcalase (2.4 Anson units/g). The digestion mixture was cooled to 4 °C, and trichloroacetic acid was added to a final concentration of 5%. The sample was mixed, allowed to stand for 10 min, and then centrifuged for 20 min at 8000 × *g*. The supernatant was recovered by decanting. Three volumes of 5% potassium acetate in ethanol was added to one volume of supernatant. After mixing, the suspension was stored overnight at 4 °C and then centrifuged for 30 min at 8,000 × *g*. The supernatant was discarded, and the precipitate was washed with absolute alcohol. The precipitate (1 g) was dissolved in 40 ml of 0.2 M NaCl and centrifuged for 30 min at 8,000 × *g*, and 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 precipitate 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 0.18 g of GAG as a white powder.

Analysis of the Physical Properties of the Intact Polysaccharide—A stock solution of polysaccharide (snail GAG, heparin, or heparan sulfate) was prepared in 0.5 M sodium chloride at a concentration of 5 mg/ml. The viscosity of each polysaccharide was determined using a capillary viscometer (14). The intrinsic viscosity was calculated from the specific viscosity (η_{SP}) of each polysaccharide. The viscometric molecular weight (M_v) was determined from the Mark-Houwink equation using constants ($K = 3.55$, $a = 0.09$) obtained from heparin by Liberti and Stivala (15). The optical rotation $[\alpha]_D$ was obtained for each polysaccharide in water at concentrations of 5 and 10 mg/ml.

Analysis of Monosaccharide Composition by Gas Chromatography (GC)—Sample (200 μg) was thoroughly dried under P_2O_5 *in vacuo* and dissolved in 0.5 ml of methanolic 1 M HCl using a screw-capped tube with a Teflon-lined cap (16). Nitrogen gas was bubbled through the solution for 15 s, and then the tube was sealed. After methanolysis for 24 h at 80 °C, the acid solution was neutralized by the addition of 0.15 ml of pyridine. Re-*N*-acetylation was carried out by the addition of 0.1 ml of acetic anhydride. This mixture was kept at room temperature for 1 h. The sample solution was evaporated with nitrogen gas flow at 35 °C. The residue was dried for 16 h *in vacuo* over P_2O_5. Finally, the sample was trimethylsilylated with 50 μl of silylating reagent (pyridine/*N*,*O*-Bis-(trimethylsilyl) trifluoroacetamide, 1:2 (v/v)) for 30 min at room temperature.

Compositional analysis by GC (16) was performed using a capillary column AT-1, 0.53 mm × 30-m (1.5-μm thickness), from Alltech Associates (Deerfield, IL) on a Shimadzu (Kyoto, Japan) gas chromatograph, model GC-14A, with a flame ionization detector, equipped with Shimadzu Chromatopac CR501 integrating recorder. The injection port temperature and the detector temperature were 270 and 280 °C, respectively. For the analysis of mixtures of monosaccharides, the oven temperature was programmed 120–260 °C at 10 °C/min.

Analysis of Oligosaccharides Formed from Lyase Treatment of GAGs—The composition of disaccharides and oligosaccharides produced from intact polysaccharide by each heparin lyase was analyzed by CE (12, 17). The CE system was operated in the reverse polarity mode by applying the sample at the cathode and run with 20 mM phosphoric acid adjusted to pH 3.5 with 1 M dibasic sodium phosphate as described previously (18). The capillary (75-μm inner diameter, 375-μm outer diameter, 68 cm long) was manually washed before use with 0.5 ml of 0.5 M sodium hydroxide followed by 0.5 ml of distilled water and then 0.5 ml running buffer. Samples were applied using gravity injection (20 s) by hydrostatic pressure (45 mm), resulting in a sample volume of 9.2 nl. Each experiment was conducted at a constant 18,000 V. Data collection was at 232 nm. Peaks were identified by co-injection with disaccharide and oligosaccharide standards prepared and characterized in our laboratory (18, 20) The size of products formed on treating snail GAG with heparin lyase II was determined by gel permeation chromatography on a Sephadex G-50 (superfine) column (1.5 × 25 cm) eluted with 0.2 M sodium chloride and monitored using a carbazole assay (19). The disaccharide product of heparin lyase II treatment was prepared for analysis by semipreparative strong anion exchange HPLC (21) and desalted by chromatography on a 5-cm × 0.5-m Bio-Gel P-2 column.

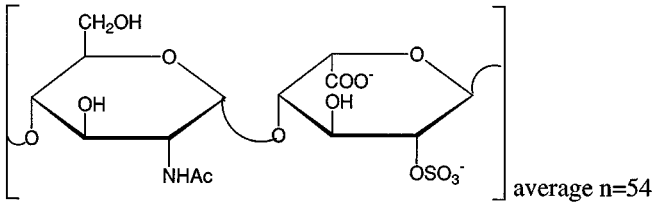

FIG. 1. **Structure of snail GAG.**

^1H NMR Spectroscopic Analysis—For ^1H NMR spectroscopy, approximately 1 mg of each sample was exchanged three times with 0.5-ml portions of ^2H_2O (99.96%; Sigma), followed by *in vacuo* desiccation over P_2O_5. The thoroughly dried sample was redissolved in 0.7 ml of ^2H_2O (99.96%), and spectra were obtained using a UNITY-500 spectrometer at the operating frequency of 500 MHz equipped with a VXR 5000 computer system from Varian Instruments. The operation conditions for one-dimensional spectra were as follows: frequency, 500 MHz; sweep width, 6 kHz; flip angle, 90° (11.1 μs); sampling point, 48 K; accumulation, 256 pulses; temperature, 298 K for oligosaccharides and 333 K for polysaccharides. Quantitative analysis was performed at each temperature with a pulse delay 5 times the longest t_1 (5.5 s), estimated from an inversion-recovery experiment. Chemical shifts were indicated by parts per million from the signal of 3-(trimethylsilyl)-d_4-propionic acid sodium salt as an internal reference. The water resonance was suppressed by selective irradiation during the relaxation delay.

Two-dimensional double quantum-filtered COSY and multiple relayed COSY spectra were recorded using the phase-sensitive mode. All two-dimensional spectra were recorded with 512 × 2048 data points and a spectral width of 3200 Hz. The water resonance was suppressed by selective irradiation during the relaxation delay. A total of 128–256 scans were accumulated for each t_1, with a relaxation delay of 2 s. The digital resolution was 1.6 Hz/point in both dimensions with zero-filling in the t_1 dimension. A phase-shifted sine function was applied for both t_1 and t_2 dimensions in the case of double quantum-filtered COSY, and a Lorentz-Gauss function was applied in all other cases.

Gradient PAGE Analysis—Gradient PAGE was performed on a polyacrylamide linear gradient resolving gel (14 × 16 cm, 12–22% total acrylamide) prepared and run as described previously (22). The molecular sizes of these oligosaccharide samples were determined by comparing them with a banding ladder of heparin oligosaccharide standards prepared from bovine lung heparin (23). Oligosaccharides were visualized by Alcian blue staining (22).

RESULTS

The GAG component of the soft body tissue of the giant African snail was isolated by proteolysis of defatted tissue and purified by fractional precipitation. Carbazole assay (19) of the polysaccharide component showed that it contained uronic acid, and Azure A dye binding assay (24) demonstrated the presence of sulfate groups, consistent with it being a GAG. The M_v of snail GAG showed it to have a molecular weight of 29,000, significantly higher than the values of 12,000 and 15,500 measured for porcine mucosal heparin and heparan sulfate, respectively (12, 23). The optical rotation $[\alpha]_D$ of the snail GAG was +44°. Monosaccharide compositional analysis of the polysaccharide isolated from giant African snail using GC showed it to be composed of 47% IdoAp, 3% GlcAp and 50% GlcNpAc (16). The uronic acid component of the original GAG might have been sulfated, since *O*-sulfoyl groups are labile and lost on acidic methanolysis (25). While GlcNp and GlcNp2S residues might have been present in the original polysaccharide and subsequently converted to GlcNpAc following methanolysis and re-*N*-acetylation, it is well known that the glycosidic linkage to these residues is resistant to acidic

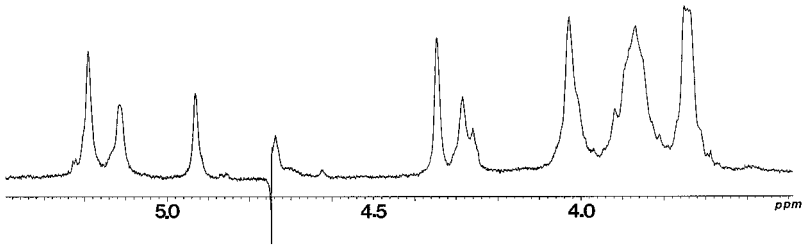

FIG. 2. **^{1}H NMR spectrum of snail GAG.** Assignment of each signal is shown in Table I.

TABLE I
Assignment of ^{1}H NMR of intact snail GAG

	L-IdoAp2S	D-GlcNpAc
H-1	5.189	5.114
H-2	4.345	4.020
H-3	4.284	3.74
H-4	4.027	3.74
H-5	4.930	3.867
H-6		3.87, 3.90
NAc		2.083

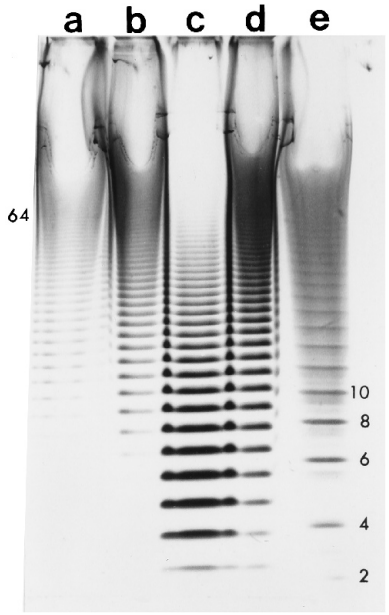

FIG. 3. **Gradient PAGE analysis of snail GAG.** Analysis was on a 12–22% linear gradient gel with visualization using Alcian blue staining. *Lane a*, snail GAG untreated (the degree of polymerization of 64 corresponds to a molecular weight of ~29,000); *lane b*, snail GAG treated with heparin lyase I; *lane c*, snail GAG treated with heparin lyase II; *lane d*, snail GAG treated with heparin lyase III; *lane e*, heparin oligosaccharide standards (degree of polymerization is as marked).

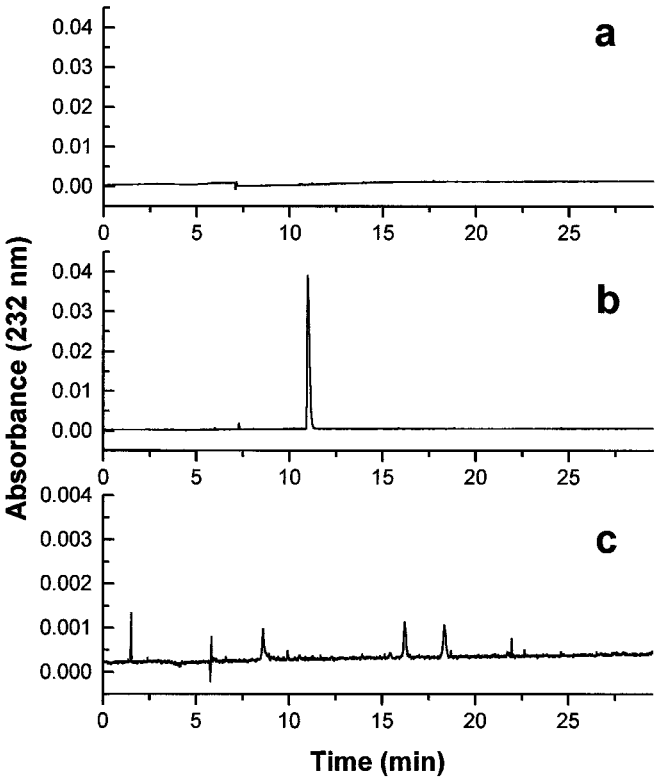

FIG. 4. **CE analysis of snail GAG on treatment with heparin lyases.** *Electropherogram a*, heparin lyase I; *electropherogram b*, heparin lyase II; *electropherogram c*, heparin lyase III. The peak observed at 11 min in *electropherogram b* co-migrates with ΔUA2S(1→4)-D-GlcNAc α,β. Intact polysaccharide (control, no heparin lyase treatment (not shown) and *electropherogram a*) has no chromophore absorbing at 232 nm and shows no peak. The peaks in *electropherogram c* migrating at <11 min correspond to oligosaccharide products that have >1 sulfate per disaccharide residue, and those migrating at >11 min correspond to oligosaccharides that have <1 sulfate per disaccharide residue.

methanolysis. Since no disaccharide peaks were observed in the GC chromatogram obtained from the snail GAG (several such peaks are detected in the acidic methanolysis of heparin) these data are consistent with a GAG made up of IdoAp:GlcNpAc at approximately a 1:1 composition.

^{1}H NMR analysis of the intact polysaccharide demonstrated the presence of two anomeric protons having chemical shifts corresponding to the H-1 of GlcNpAcα1→ at δ5.1 and H-1 of IdoAp2Sα1→ at δ5.2, respectively. The H-1 of GlcNpAc is detected at δ5.4 in the spectrum of heparan sulfate (12). The observation of this upfield shift caused on only the anomeric proton of GlcNpAc appeared to be attributable to the unusual sequence GlcNpAcα→IdoAp2S (Fig. 1). Complete assignment of the ^{1}H NMR spectrum of snail GAG were obtained using two-dimensional NMR spectroscopy (Fig. 2 and Table I).

Snail GAG was examined by gradient PAGE with Alcian blue staining (Fig. 3). Intact GAG showed a pattern of discrete banding consistent with a polysaccharide of a relatively homogenous structure with the greatest staining intensity at a degree of polymerization of ~64 (Fig. 3, *lane a*) corresponding to a molecular weight of ~29,000 (23). Next, the sensitivity of the snail GAG toward heparin lyases I, II, and III was examined. No degradation when the polysaccharide was treated with heparin lyase I (Fig. 3, *lane b*), little degradation on treatment with heparin lyase III (Fig. 3, *lane d*) and substantial degradation on treatment with heparin lyase II (Fig. 3, *lane c*) was found. Products having a low level of negative charge (<−3) do not stain, so that a disaccharide having a single sulfate group would not be visualized. Analysis of snail GAG treated with heparin lyase II, on Sephadex G-50 (21), demonstrated that >90% of the weight of product mixture corresponded to disaccharide. CE analysis confirmed that the polysaccharide was converted by heparin lyase II into a single product (Fig. 4*b*). This product was prepared in milligram amounts using semi-preparative strong anion exchange HPLC (21), and on CE

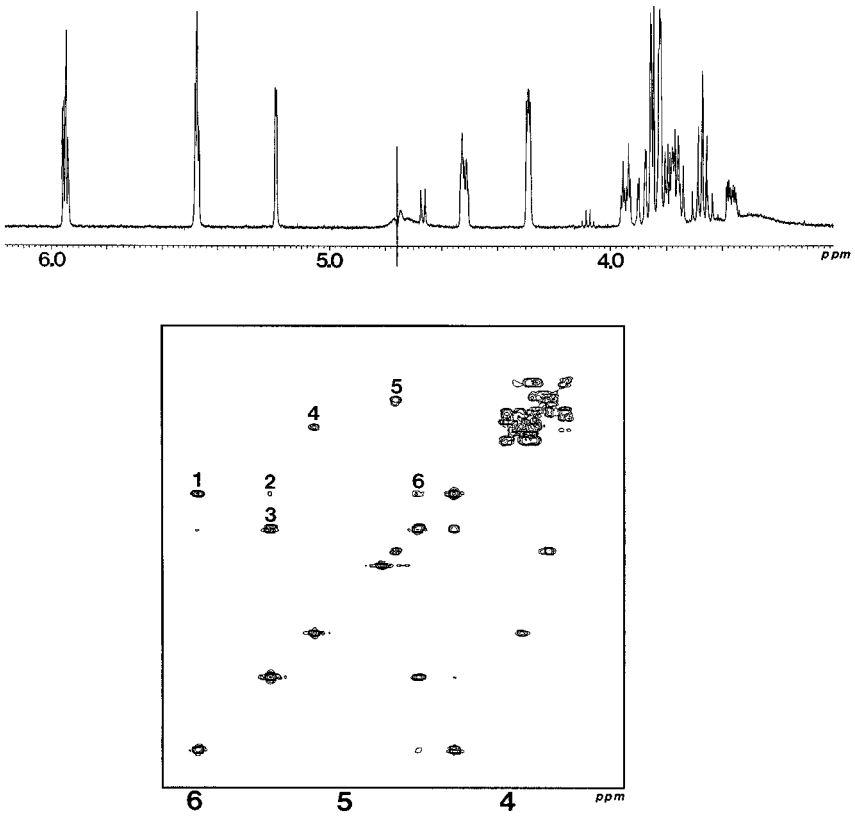

FIG. 5. **Two-dimensional COSY spectrum of disaccharide obtained from snail GAG on treatment with heparin lyase II.** *Cross-peak 1*, H-1/H-3 of ΔUAp2S; *Cross-peak 2*, H-1/H-3 long range coupling of ΔUAp2S; *Cross-peak 3*, H-1/H-2 of ΔUAp2S; *Cross-peak 4*, H-1/H-2 of GlcNpAcα; *Cross-peak 5*, H-1/H-2 of GlcNpAcβ; *Cross-peak 6*, H-2/H-3 of ΔUAp2S.

analysis it co-migrated with a disaccharide standard having the structure, ΔUAp2S(1→4)-D-GlcNpAcα,β. CE analyses also demonstrated that the snail GAG was only very slightly degraded by heparin lyase III and gave no products on treatment with heparin lyase I (Fig. 4). The spectrum of the disaccharide obtained by heparin lyase II treatment of snail GAG confirmed its structure to be ΔUAp2S(1→4)-D-GlcNpAcα,β (Fig. 5 and Table II), consistent with that reported by Yamada and co-workers (26).

The absorbance spectrum of the copper(II) complex polysaccharide shows a λ_{max} at 240 nm, confirming the presence of sulfate and carboxylate groups in the polysaccharide (27). CE analysis of snail GAG in copper(II) sulfate solution gave a response identical to low molecular heparin (27). The addition of copper(II) sulfate (5 mM) to snail GAG (5 mg/ml) afforded an insoluble blue precipitate. Neither heparin nor heparan sulfate precipitate under similar conditions.

TABLE II
Chemical shifts and coupling constants of disaccharide

	UAp2S-αGlcNpAc	UAp2S-βGlcNpAc	αGlcNpAc	βGlcNpAc
H-1	5.477	5.471	5.192	4.665
$^3J_{1,2}$	3.0	3.0	2.6	8.1
H-2	4.526	4.511	3.851	3.674
$^3J_{2,3}$	3.2	2.5	6.5	6.9
H-3	4.291	4.291	3.851	3.668
$^3J_{3,4}$	4.7	4.8	8.6	8.4
H-4	5.952	5.942	3.771	3.758
$^3J_{4,5}$	1.2 ($J_{2,4}$)	1.2 ($J_{2,4}$)	6.7	7.9
H-5			3.945	3.567
$^3J_{5,6a}$			5.2	5.5
H-6a			3.828	3.887
$^2J_{6a,6b}$			9.9	10.1
H-6b			3.824	3.792
$^3J_{5,6b}$			3.4	2.6

DISCUSSION

Heparin has been prepared from a number of different species ranging from humans (6) to clams (5, 8–11). While structural differences have been observed between heparins obtained from different species and tissues (8) all heparins characteristically exhibit a molecular weight of 10,000–25,000 and contain a high content of IdoAp2S and GlcNpS (6S or 6OH) residues (8). Also common to heparin is the presence of a unique pentasaccharide sequence containing a central GlcNpS3S6S residue that interacts specifically with antithrombin and is primarily responsible for heparin's anticoagulant activity (28). The heparin isolated from clams, a member of the Mollusca phylum, is similar to heparins obtained from higher organisms (8, 11). Disaccharide and oligosaccharide analyses show that clam heparin and porcine mucosal heparin contain →4)α-D-GlcNp2S6S(1→4)α-L-IdoAp2S(1→ as their major sequence, corresponding to 70 and 87%, respectively (Table III) and have saccharide compositions falling in the ranges shown in Table III. Indeed, the oligosaccharide map of clam heparin shows it to have a somewhat more complex structure than heparins obtained from vertebrates (8). No peptidoglycan was detected in the snail polysaccharide preparation using an amine-specific fluorescent reagent (29). Thus, it is unclear whether it is biosynthesized as a proteoglycan. Additional studies aimed at the extraction of intact proteoglycan from the defatted snail body will be required to clarify this question.

Heparan sulfate has a distinctly different structure from that of heparin (Tables III and IV). Heparan sulfate GAGs have molecular weights of 8,000–35,000 (30) and are comprised primarily of sequences of →4)α-D-GlcNpAc(1→4)β-D-GlcAp(1→ (45–73%) and to a lesser extent →4)-α-D-GlcNp2S(1→4)-β-D-GlcAp(1→ and →4)-α-D-GlcNpAc6S(1→4)-β-D-GlcAp (1→. Analyses of heparan sulfate isolated from various species and tissues have been compared with those obtained on heparin (31, 32).

A plot of *N*-sulfate as a function of total *O*-sulfate or 2-*O*-sulfate for various heparin and heparan sulfate samples demonstrated that these GAGs were structurally distinct (31) (Fig. 6). Heparin, having 2.4–2.7 sulfates/disaccharide unit, is also

TABLE III
Sequence composition of heparin, heparan sulfate, and snail GAG

Sequences	Heparin[a,b]	Heparan sulfate[a,b]	Snail[a,c]
→4)D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→	70–90	2–21	<1
→4)D-GlcNpAc(1→4)-β-D-GlcAp(1→	0–1	45–73	<1
→4)D-GlcNp2S(1→4)-β-D-GlcAp(1→	0–2	7–12	<1
→4)D-GlcNpAc(1→4)-α-L-IdoAp2S(1→	0–1	0–1	95–100

[a] Frequency of disaccharide sequence in mol %.
[b] Calculated (6) from published disaccharide analyses of heparins and heparan sulfates prepared from a variety of tissues and species (6–8, 32).
[c] Calculated from disaccharide analysis by CE (17).

TABLE IV
Saccharide composition of heparin and heparan sulfate observed in various species and tissues and snail GAG

Residue	Heparin[a,b]		Heparan Sulfate[a,b]		Snail GAG[b]
	Average	Range	Average	Range	
	%	%	%	%	%
L-IdoAp	5	4–15	8	6–10	<1
L-IdoAp2S	86	70–94	10	8–15	95
D-GlcAp	6	5–12	72	55–89	<5
D-GlcNp2S	2	0–2	15	4–27	<1
D-GlcNp2S6S	84	72–92	7	2–21	<1
D-GlcNp2S3S6S	1	1–3	<1	0–2	<1
D-GlcNpAc6S	2	1–5	17	6–29	<1
D-GlcNpAc	5	1–6	66	47–85	95

[a] The range percentage of residues are obtained from previously published oligosaccharide analyses (6–8, 32). The average percentage of residues are obtained by averaging published values. Thus, the average percentage of the uronic acid residues or the glucosamine residues may not total to 100%.
[b] The percentage of each monosaccharide residue was calculated from GC, CE, and ^{1}H NMR analysis.

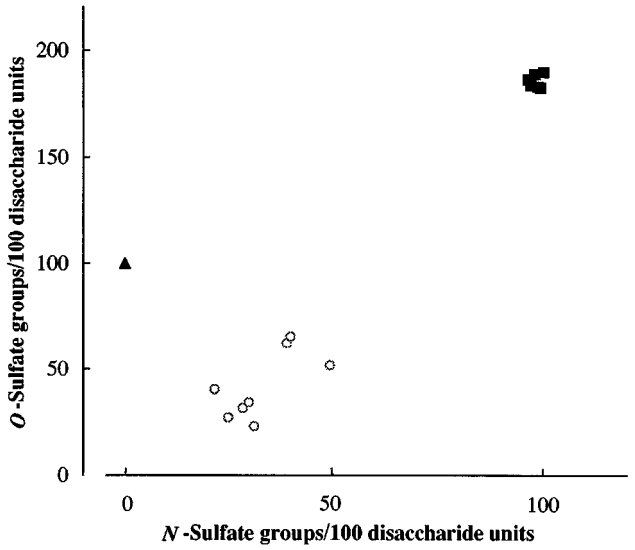

FIG. 6. **Comparison of structural features of snail GAG, heparin, and heparan sulfate.** A plot of *N*-sulfate groups/100 disaccharide units *versus* *O*-sulfate groups/100 disaccharide units is shown for heparins (■) from a variety of tissues and species (8), heparan sulfates (○) (32), and snail GAG (▲).

considerably more highly sulfated than heparan sulfate, having 0.6–1.0 sulfates/disaccharide unit.

Extraction of the soft body tissue of the giant African snail and subsequent purification of its GAGs showed that this tissue contained a large amount of GAG, corresponding to nearly 30–50 mg of GAG/g of dry defatted tissue. This GAG was sulfated, had a higher molecular weight (M_v 29,000) than either heparin or heparan sulfate, and exhibited an optical rotation $[\alpha]_D$ of +44 °, similar to porcine mucosal heparin's value of +53° but significantly below the $[\alpha]_D$ of +75° observed for heparan sulfate (12). Monosaccharide analysis showed snail GAG to have a saccharide backbone comprised of an equal amount of IdoAp and GlcNpAc. The resistance of this polysaccharide to heparin lyase I and III suggested that this polysaccharide was neither heparin nor heparan sulfate (33). No heparin has been observed that is completely resistant to heparin lyase I, since this enzyme acts at the major repeating disaccharide sequence →4)α-D-GlcNpS6S (or 6OH) (1→4) -α-L-IdoAp2S (1→ found in all heparins (Table III). No heparan sulfate has been found to be completely resistant to heparin lyase III, which primarily acts at →4)-α-D-GlcNpAc (or 2S) 6S (or 6OH) (1→4)α-D-GlcAp (1→ sequence found in all heparan sulfates (Table III). Snail GAG is broken down by heparin lyase II into ΔUA2S(1→4)-D-GlcNpAcα,β as confirmed by co-migration on CE (not shown) and ^{1}H NMR spectroscopy. ^{1}H NMR of the intact polymer unequivocally establishes the structure of the snail GAG as →4)-α-D-GlcNpAc (1→4)-α-L-IdoAp2S(1→. This sequence represents at least 95% of the polymer structure. The small amounts of GlcAp observed in the monosaccharide analysis (and resulting in its slight sensitivity to heparin lyase III) and unsulfated IdoAp observed in the ^{1}H NMR may either be associated with minor structural heterogeneity in this GAG or be due to a small amount of a contaminating GAG of different structure.

The high molecular weight, sequence, and structural homogeneity of this GAG are inconsistent with its classification as either heparin or heparan sulfate. Additionally, the current pathway proposed for heparin/heparan sulfate biosynthesis requires *N*-deacetylation and *N*-sulfation of glucosamine residue prior to the C-5 epimerization and 2-*O*-sulfation of adjacent uronic acid residues (28, 34, 35). Thus, the snail polysaccharide represents a new GAG that displays different and unique structural properties than either the heparin or heparan sulfate GAGs.

The presence of such a polysaccharide with a high molecular weight and a simple but unique sequence raises questions about its biosynthesis, including whether or not it is synthesized as a proteoglycan. While snails are known to synthesize neutral polysaccharides and glycoproteins, very little is known about their biosynthesis of acidic polysaccharides, such as GAGs (36). Uridine diphosphate precursors, such as UDP-*N*-acetylglucosamine have been identified in the tissues of snails. Thus, the biosynthesis of the GAG isolated from snail probably proceeds through the polymerization of nucleotides of uronic acid and *N*-acetylglucosamine. Snails contain basophilic cells in their digestive glands, that bind divalent metals (37). These cells are similar to mast cells that synthesize heparin in vertebrates and may play some role in snail GAG biosynthesis. Snails also biosynthesize glycoproteins that contain *N*-linked and *O*-linked glycans composed of mannose, fucose, xylose, and *N*-acetylglucosamine (38, 39). The *O*-linked glycans from snails are believed to be mucins containing both sialic acids and sulfate groups (40) and are linked to threonine (41). Thus, all the biosynthetic machinery appears to be in place to permit the biosynthesis of proteoglycans and GAGs in the snail.

The large amounts of this GAG found in snail also raise some interesting questions about its biological function(s). Many

roles can be proposed for this GAG including 1) binding, uptake, and transport of divalent cations (42, 43); 2) an antidesiccant (40, 44); 3) a molecule linked to snail mobility (45, 46); and 4) an antibiotic or antipredator molecule. The most likely role of snail GAG is its involvement in cation binding. *A. fulica* is a very large gastropod that requires substantial quantities of calcium for its shell (37, 47). In addition, other divalent ions are critical components of their diets (37). The blood of snails is blue, as hemocyanin is the copper-based carrier of oxygen in these animals (47). This study shows that snail GAG binds copper(II) much more tightly than heparan sulfate and with about the same avidity of heparin, which has a 3-fold higher level of sulfation. GAGs are known to organize and hold water (48). Since snails are particularly prone to dehydration, this suggests a second role for this polysaccharide. Snails move on a mucus slime through wave-like undulations of their foot muscle (46, 47). This high molecular polysaccharide is extremely viscous and may represent a component of this slime. Antibiotic properties have been reported for heparin (49), and *A. fulica* is known to make a bactericidal glycoprotein that is found in its mucus (50), suggesting that snail GAG may have a protective role. Further studies are required to define the precise role(s) of the high concentration of this GAG in *A. fulica*.

The discovery of the new GAG poses interesting new questions about its biosynthesis and that of the related GAGs, heparin and heparan sulfate. Are the biosynthetic enzymes for all three GAGs similar, and if so, how is glucuronic acid epimerized to iduronic acid in the presence of adjacent *N*-acetylglucosamine residues? This GAG is easily prepared and purified and affords a valuable source of a potentially useful polysaccharide for the study of heparin/heparan sulfate biosynthesis and biological activities. A similar bacterial polysaccharide, K5, has proved very useful in such studies (51). Chemical modification of snail polysaccharide using relatively simple methods, *i.e.* de-*N*-acetylation and re-*N*-sulfation, should lead to a structurally homogenous polysaccharide with the minimum structural features for binding fibroblast growth factors (52). Finally, the giant African snail is considered a major pest in many parts of the world (47). The use of heparin lyase II or *F. heparinum* (a soil isolate) capable of degrading its major polysaccharide might provide a biological means for controlling *A. fulica*. (53).

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