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Localization and characterization of acharan sulfate in the body of the giant African snail *Achatina fulica*

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Abstract

Acharan sulfate is a glycosaminoglycan (GAG), having the structure →4)-2-acetamido-2-deoxy-α-D-glucopyranose(1→4)-2-sulfo-α-L-idopyranosyluronic acid (1→, isolated from the body of the giant African snail *Achatina fulica*. This GAG represents 3–5% of the dry weight of this snail's soft body tissues. Frozen sections and polyester wax sections of the snail's body were stained by Alcian blue-periodic acid-Schiff's reagent (PAS) to localize acharan sulfate. Alcian blue staining indicated that GAG was mainly secreted into the outer surface of the body from internal granules. A highly mucous material was collected and treated and the acharan sulfate was recovered by ethanol and cetyl pyridinium chloride precipitation. Crude acharan sulfate was purified by DEAE-Sephacel ion-exchange chromatography. Depolymerization of intact mucus and purified acharan sulfate fractions by heparin lyase II (heparitinase I) from *Flavobacterium heparinum* produced an unsaturated disaccharide as a major product, establishing the repeating unit of acharan sulfate. These results demonstrate that mucus in the granule and secreted to the outside of the body is composed entirely of acharan sulfate.

Keywords: *Achatina fulica*; Snail; Acharan sulfate; Localization; Section analysis; Mucus; Granules; Heparinase

1. Introduction

Proteoglycans are complex macromolecules consisting of a core protein and one or more covalently attached glycosaminoglycan chains (Hardingham and Fosang, 1992). The biological functions of proteoglycans primarily result from the structurally dominant glycosaminoglycan (GAG) chains emanating from the protein core of the molecule. A large number of animal species contain GAGs and mollusks are a particularly rich source of these polysaccharides. GAGs are usually found in the extracellular matrix of vertebrate and invertebrate tissues. A structural investigation revealed that GAGs in invertebrate species often contain unusual variations of sulfate distribution and uronic acids (Chatziioannidis et al., 1999; Chavante et al., 2000; Pejler et al.,

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1987). While pursuing our long-term studies on heparin, we isolated a pure glycosaminoglycan, acharan sulfate, in large amounts from the giant African snail *Achatina fulica* with a unique structure. This GAG represents 3–5% of the dry weight of this snail's soft body tissues. This GAG is neither heparin nor heparan sulfate, but instead represents an unusual repeating sequence, → 4) -2-acetamido-2-deoxy-α-D-glucopyranose(1 → 4)-2-sulfo-α-L-idopyranosyluronic acid (1 → (→ GlcNpAc → IdoAp2S →) (Fig. 1). Acharan sulfate and chemically modified *N*-sulfoacharan sulfate (→ GlcNpS → IdoAp2S →) markedly decreased the mitogenic activity of basic fibroblast growth factor in a concentration dependent manner, showing an inhibition of angiogenesis (Wang et al., 1997).

Since this polysaccharide is of biological significance, we were interested in understanding the distribution of acharan sulfate in the tissues of snail and the confirmation of its unusual structure. The current study focuses on the collection and processing of tissue samples and the histological evaluation of stained sections.

2. Materials and methods

2.1. Materials

African giant snails (*Achatina fulica* Bowdich) were purchased from a greenhouse near Seoul, South Korea and live snails were captured on Okinawa Island, Japan. Heparinase (EC 4.2.2.7) and heparin lyase II (heparitinase II), and heparin lyase III (EC 4.2.2.8 heparitinase I) from *Flavobacterium heparinum* were provided by Dr Keiichi Yoshida in Seikagaku Co. (Tokyo, Japan). Acharan sulfate lyase was purified from *Bacteroides stercoris* as described previously (Ahn et al., 1998; Kim et al., 2001). Chondroitin lyase ABC (EC 4.2.2.4), chondroitinase AC II (EC 4.2.2.5) from *Proteus vulgaris*, proteinase K, and DEAE-Sephacel were purchased from Sigma Chemical Co. (St Louis, USA). Acharan sulfate standard was purified from *Achatina fulica* as described previously (Kim et al., 1996). High-performance liquid chromatography (HPLC) was performed to analyze the reaction products treated with heparin lyase II (heparitinase II). The system was equipped with AKTA^{TM} Purifier 10 controlled by Unicorn software 3.1 (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was a 5 μm Phenosphere strong-anion exchange (SAX) column from Phenomenex (Torrells, CA) of dimension 0.46×25 cm. UV spectrometry was performed on a JASCO model V550 (Tokyo, Japan). For ^{1}H-NMR spectroscopy, each sample was exchanged three times with 0.5-ml portions of $^{2}H_2O$ (99.90%, Sigma), followed by in vacuo desiccation over P_2O_5. The thoroughly dried sample was re-dissolved in 0.7 ml of $^{2}H_2O$ (99.9%), and spectra were obtained using a Bruker AMX5 spectrometer at the operating frequency of 500 MHz equipped with VAX 32 computer located at the Korea Research Basic Science Center (Seoul, Korea). The operation conditions for one-dimensional spectra were as follows: frequency, 500 MHz; sweep width, 5 kHz; flip angle, 45° (6.0 μs); sampling point, 32 K; accumulation, 256 pulses; temperature, 303 K. The water resonance was suppressed by selective irradiation during the relaxation delay.

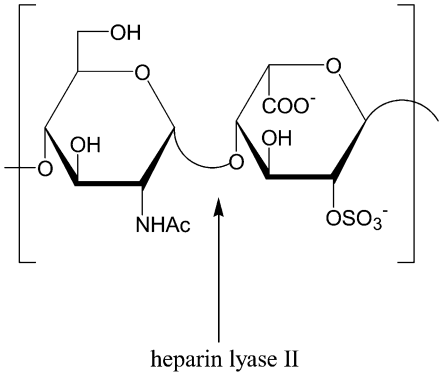

Fig. 1. Structure of acharan sulfate.

2.2. Preparation of tissue sections embedded in polyester wax

The tissues were fixed overnight in 3.7% formaldehyde solution, and then embedded in 90% polyester wax containing 10% ethanol as previously described (Koshiishi et al., 1999; Kusakabe et al., 1984). Sections with thickness of 6 μm were cut on a cryostat Coldtome (Sakura Seiki Co., Tokyo, Japan) at 0°C, layered on a water bath, and then taken up on glass slides stubbed with egg albumin. The slides were dried overnight at 4°C and stored at this temperature (Koshiishi et al., 1999).

2.3. Staining of sections

Staining was performed by submersing the gel in 5% alcian blue solution in water for 0.5 h and then put into periodic acid-Schiff's (PAS) reagent. The slides were destained and observed under the light microscope.

2.4. Collection and treatment of mucus from snails

Mucus (400 ml) was collected by stimulating the surface of live snails by poking with a small rod and freeze-dried. After decantation, the precipitate was dried and dissolved in two volumes of water. Then, five volumes of 1% potassium acetate in ethyl alcohol was added and the suspension was stored overnight at 4°C and then centrifuged for 30 min at $8000 \times g$. The precipitate was dissolved in 1 l water and 250 ml of cetylpyridinium chloride (5%) was added, stored at room temperature for 1 h. After centrifugation at $8000 \times g$ for 30 min, the pellet was dissolved by adding 100 ml of 2.5 M NaCl at 45°C for 30 min. Again, three volumes of ice-cold ethanol were added and the precipitate was collected. It was dissolved in water, dialyzed and freeze-dried to yield 215 mg of dry mucus containing crude acharan sulfate.

2.5. Purification of a mucous sample by anion-exchange chromatography

One hundred milligrams of the mucous sample was dissolved in 10 ml of water and applied to a column (1.5×35 cm) of DEAE-Sephacel prepared in 50 mM sodium phosphate buffer (pH 7.0). The column was eluted in a stepwise gradient with 50 mM sodium phosphate buffer containing 0.0 M, 0.5 M and 1.0 M NaCl. The elution was monitored at 210 nm and the flow rate was set at 30 ml/h. Each fraction was collected, dialyzed and freeze-dried to give a white powder. All samples were subjected to ^{1}H-NMR spectroscopy and agarose gel-electrophoresis. Simultaneously, the fractions were depolymerized by heparin lyase II and the reaction products were analyzed by strong anion-exchange (SAX)-HPLC as described below.

2.6. Chemical analysis of the mucous material

Protein was determined according to a Bradford assay using Bio-Rad kit (Bradford, 1976) and uronic acid was analyzed by a modified carbazole assay (Taylor and Buchanan-Smith, 1992).

2.7. Analysis of mucus by agarose gel-electrophoresis

Agarose gel electrophoresis was performed in a 1.5% gel poured in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA). One hundred micrograms of mucus containing crude acharan sulfate, acharan sulfate standard, or porcine mucosal heparin standard was loaded on the gel and constant voltage (80 V) was applied for 1 h at room temperature. The gel was visualized with 0.5% azure A in 1% acetic acid (Wu et al., 1998).

2.8. Depolymerization of mucus and acharan sulfate with polysaccharide lyases

Both mucus (100 μg) and acharan sulfate standard (10 μg) dissolved in 50 mM sodium phosphate buffer containing 50 mM KCl (pH 7.1) were treated with 100 munits of heparin lyase II (heparitinase II) at 37°C until the absorbance at 232 nm was constant. The digestion mixture was analyzed by SAX-HPLC. Other polysaccharide lyases (1 munits), including heparin lyase I, heparin lyase III and chondroitinase ABC (Linhardt, 1994; Kim et al., 1996), and an acharan sulfate lyase having similar specificity to heparin lyase II (Kim et al., 2001), were used to treat mucus and acharan sulfate standard.

2.9. SAX-HPLC analysis of glycan chain

The heparin lyase II (heparitinase II) digested samples were injected on an analytical SAX-HPLC column to monitor the reaction. A linear NaCl gradient of 0.1–1.6 M (pH 3.5), at a flow rate of 1.0 ml/min was used and the detection was at 232 nm. Each peak was pooled, lyophilized and desalted on a Bio-Gel P-2 column (1.8 cm × 60 cm). Elution from the Bio-Gel P-2 column was monitored by absorbance at 232 nm. The pooled fractions were lyophilized and each oligosaccharide was analyzed by ^{1}H-NMR (Kim et al., 1998).

2.10. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of mucus proteins

SDS-PAGE was performed for the analysis of proteins bound to mucus after treatment with

heparin lyase II (heparitinase II) using 12% polyacrylamide gel (Laemmli, 1970). Proteins were stained with Coomassie Brilliant Blue R-250 and silver stain kit from Bio-Rad.

3. Results and discussion

The polyester wax sections (width, 10 mm; length, 10 mm; thickness, 6 μm) of the tissue samples were prepared using standard histochemical techniques. Four sections were prepared and stained (Fig. 2-I). The light micrographs of these stained tissue samples are shown in Fig. 2. Fig. 2-IIA,C,D are the exterior sites of snail. Fig. 2-IIB represents the interior space of the body. The mucus containing GAGs could be visualized as a blue color by alcian blue staining and collagen fibers were stained as a red color by PAS reagent. Proteoglycans and GAGs can be stained by a number of dyes, such as alcian blue, azure A and toluidine blue (Cowman et al., 1984; Rice et al., 1987). These results indicate that the GAGs in the snail are primarily located inside granules (Fig. 2-II A,C,D) and are secreted onto the surface as a mucous material (Fig. 2-IIC). Cells in mammals usually secret proteoglycans into the extracellular environment on exposure to outer stress. Cells with storage granules concentrate proteoglycans along with other secretory products. These proteolgycans typically contain highly sulfated forms of GAGs including chondroitin sulfate, heparan sulfate and heparin.

The amount of protein in the mucus was determined to be ~26% based on a protein assay using a standard curve prepared with bovine serum albumin. The uronic acid in mucus corresponded to ~80% as determined by uronic acid assay using a standard curve prepared with purified acharan sulfate. These results demonstrate that the mucus is composed primarily of proteins and glycosaminoglycans. After recovery and fractionation of mucus on a DEAE-Sephacel anion-exchange chromatography (Fig. 3), mucus and fractionated mucus were analyzed, on the basis of charge and molecular weight, by agarose gel-electrophoresis (Fig. 4). Lanes 1–3 show acharan sulfate standard, DEAE-Sephacel non-interacting acharan sulfate and interacting acharan sulfate fractions, respectively. Lanes 4–6 represent snail mucus, DEAE-Sephacel non-interacting mucus and interacting mucus fractions, respectively. While agarose electrophoresis does not give a precise value of molecular weight, the average molecular weight of mucus was considerably larger

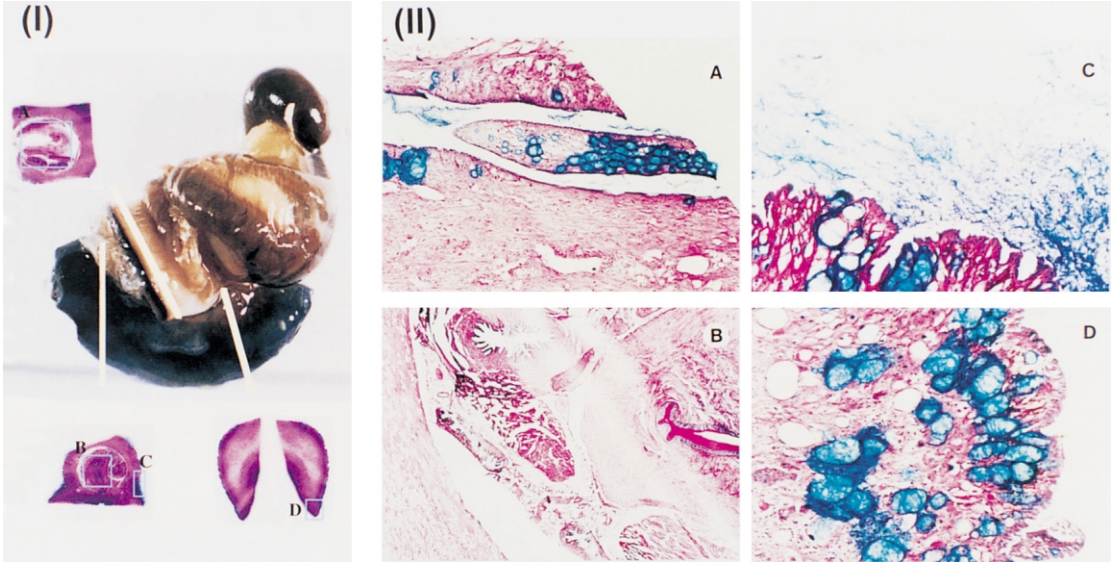

Fig. 2. Sections of the African giant snail prepared in polyester wax. (I) The whole body of the giant African snail. (II) Light micrographs of boxed areas (I) stained with alcian blue and PAS are shown at high magnifications. (A) 100×; (B) 40×; (C) 200×; (D) 200×. The surface is covered with mucus and it is visualized as a blue color by alcian blue staining. Collagen fibers are stained as a red color by Schiff's reagent.

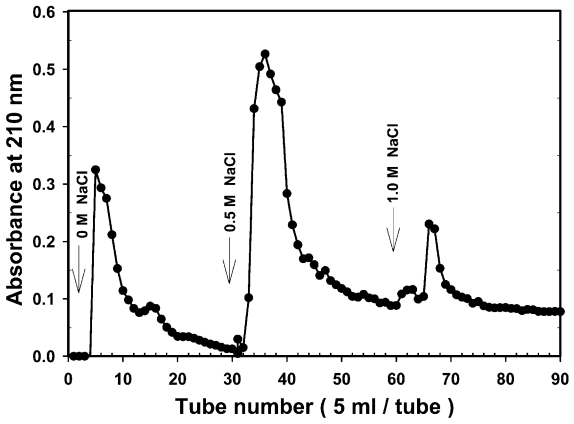

Fig. 3. The purification of acharan sulfate from mucus by DEAE-Sephacel ion-exchange chromatography. Mucus (100 μg) was dissolved in 20 ml of 50 mM sodium phosphate buffer and applied onto the column. The column was eluted with a stepwise salt gradient.

than that of acharan sulfate standard, having an average molecular weight of 30 000 (Kim et al., 1996) (Lanes 1 and 4, Fig. 4). No staining was observed for non-interacting mucus and the fraction eluting at 0.5 M NaCl. (Lanes 5 and 6, Fig. 4). The average molecular weight of the mucus fraction eluting at 1.0 M NaCl (Lane 7, Fig. 4) was similar to that of a acharan sulfate fraction eluting at 1.0 M NaCl (Lanes 3, Fig. 4). These results suggest that mucus is synthesized as a higher molecular weight species or it forms non-covalent complexes with other components, such as proteins. Mucus was next depolymerized using heparin lyase II (heparitinase I) and the resulting products were analyzed by SAX-HPLC (Fig. 5). The major peak, observed at 325 s corresponds to the repeating disaccharide unit of acharan sulfate (ΔUAp2S-GlcNpAcα,β, where ΔUAp is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid). Isolation of this major peak and NMR analysis confirmed its structure to be ΔUA2S-GlcNAc (Kim et al., 1996). The mucus and acharan sulfate standard were insensitive to chondroitin lyase ABC and heparin lyases I and III but both could be degraded with acharan sulfate lyase, resulting in the same disaccharide product obtained using heparin lyase II (not shown).

Evidence that acharan sulfate binds protein comes from the observation of minor bands (< 45 kDa) on SDS-PAGE after digestion of the mucus sample, heparin lyase II (heparitinase I) or acharan sulfate lyase (data not shown). It is unclear whether acharan sulfate exists as a proteoglycan, similar to those typically found in vertebrates, or is simply non-covalently associated with mucus protein. However, mucus has some similarity to heparin-proteoglycan exclusively produced by connective tissue mast cells, where it is stored in cytoplasmic granules (Lindahl et al., 1989). The linkage regions of core protein to GAG chains constituted of Ser-Gly-repeats named serglycin. Compositional analysis of amino acids in the mucus indicated that Gly, Asx and Glx each represent more than 10% the protein (Table 1). It is noteworthy that Ser, commonly associated with the attachment site of O-linked GAG chains in vertebrates, also represents over 8% of the protein component. Mucus is made up of many components, including water and electrolytes, mucous glycoproteins (Mitra et al., 1987), glycosaminoglycans, lectins (Yuasa et al., 1998) and hemocyanin (Deyrup-Olsen et al., 1983). The molecular mechanism that controls mucin storage inside the se-

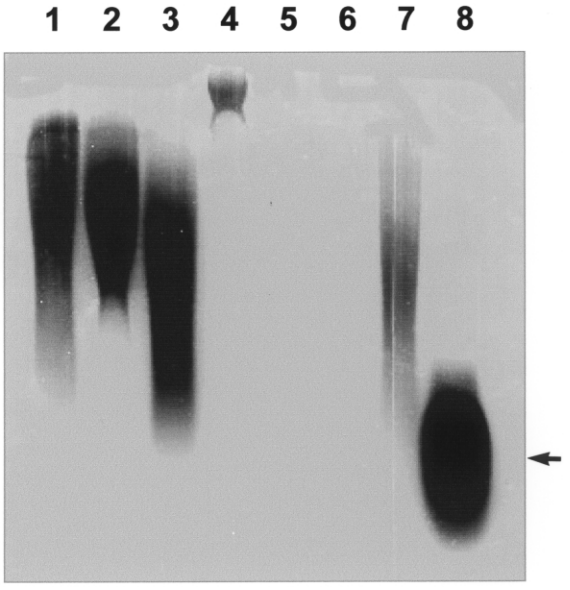

Fig. 4. Agarose gel-electrophoresis of the purified acharan sulfate. Lane 1, acharan sulfate standard; lane 2, the non-interacting acharan sulfate fraction with DEAE-Sephacel; lane 3, the acharan sulfate fraction eluting from DEAE-Sephacel at 1.0 M NaCl; lane 4, the mucus from snail; lane 5, the non-interacting mucus fraction with DEAE-Sephacel; lane 6, the mucus fraction from DEAE-Sephacel at 0.5 M NaCl; lane 7, the mucus fraction from DEAE-Sephacel at 1.0 M NaCl; lane 8, porcine mucosa heparin ($M_r = 12\,000$). The samples are loaded at the top of the gel (cathode) and migrate towards the anode at the bottom of the gel.

cretory cell is believed to be involved in maintaining the balance of polyanions with a shielding cations (Verdugo et al., 1987). The mucus secreted on the body surfaces and mucous membranes of mollusks is known to play crucial roles in locomotion, feeding, osmoregulation, reproduction and protection of epithelial and other surfaces (Deyrup-Olsen et al., 1983). The biological roles of acharan sulfate glycosaminoglycan in mucus secreted to the outer surface might have additional roles including: (1) the binding, uptake, and transport of divalent cations; (2) as an antidesiccant; (3) as a molecule linked to snail mobility; or (4) as an antibiotic or antipredator molecule. Another likely role of acidic glycoconjugates in snail is the neutralization of cations found in secretory granules (Verdugo et al., 1987).

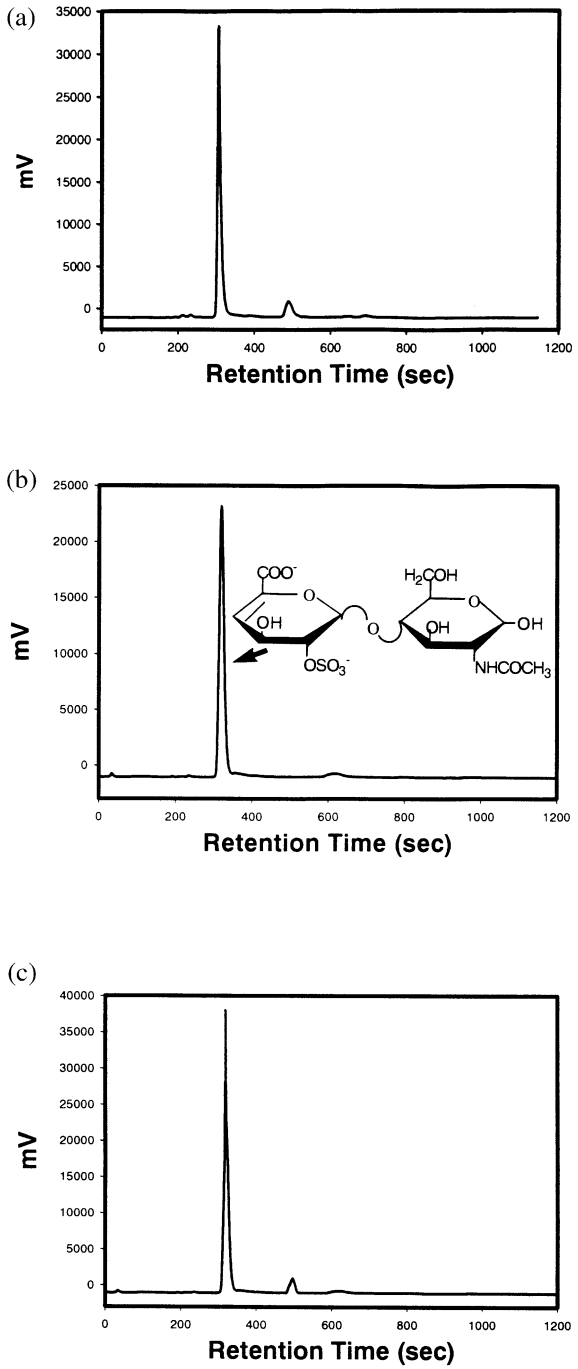

Fig. 5. HPLC analysis of the sample (Lane 7 in Fig. 4) after treatment of heparin lyase II. (a) The reaction mixture of the sample; (b) An authentic sample of unsaturated disaccharide (ΔUA2S-GlcNAc); (c) Coinjection of (a) and (b). The reaction conditions and analytical methods are described in Section 2.

Table 1
Compositional analysis of amino acids in mucus

Amino acid	Content (pmol)	Mol.%
Cys[a]	Nd	Nd
Asx[b]	535	10.8
Glx[b]	509	10.3
Ser	401	8.1
Gly	614	12.4
His	204	4.1
Arg	52	1.01
Thr	373	7.5
Ala	345	7.0
Pro	410	8.3
Tyr	64	1.3
Val	360	7.3
Met	17	0.3
Ile	228	4.6
Leu	420	8.5
Phe	221	4.4
Trp	Nd	Nd
Lys	208	4.2
Total	4958	100

[a] Nd, not determined.
[b] Asx and Glx are the sums of asparagine + aspartic acid, and glutamine + glutamic acid, respectively.

In conclusion, this study demonstrates that the major glycoconjugate of snail mucus is a glycosaminoglycan with a novel repeating unit composed of the disaccharide sequence, → 4) *N*-acetyl-α-D-glucosamine (1 → 4) 2-*O*-sulfo-α-L-iduronic acid (1 → . This GAG comes from granules within the snails body and is localized on the outer surface, possibly as a result of exposure of the snail to stress.

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