Glycosaminoglycans in *Hydra magnipapillata* (Hydrozoa, Cnidaria): demonstration of chondroitin in the developing nematocyst, the sting organelle, and structural characterization of glycosaminoglycans

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The hydrozoan is the simplest organism whose movements are governed by the neuromuscular system, and its *de novo* morphogenesis can be easily induced by the removal of body parts. These features make the hydrozoan an excellent model for studying the regeneration of tissues *in vivo*, especially in the nervous system. Although glycosaminoglycans (GAGs) and proteoglycans (PGs) have been implicated in the signaling functions of various growth factors and play critical roles in the development of the central nervous system, the isolation and characterization of GAGs from hydrozoans have never been reported. Here, we characterized GAGs of *Hydra magnipapillata*. Immunostaining using anti-GAG antibodies showed chondroitin or chondroitin sulfate (CS) in the developing nematocyst, which is a sting organelle specific to cnidarians. The CS—PGs might furnish an environment for assembling nematocyst components, and might themselves be components of nematocysts. Therefore, GAGs were isolated from *Hydra* and their structural features were examined. A considerable amount of CS, three orders of magnitude less heparan sulfate (HS), but no hyaluronic acid were found, as in *Caenorhabditis elegans*. Analysis of the disaccharide composition of HS revealed glucosamine 2-N-sulfation, glucosamine 6-O-sulfation, and uronate 2-O-sulfation. CS contains not only nonsulfated and 4-O-sulfated *N*-acetylgalactosamine (GalNAc) but also 6-O-sulfated GalNAc. The average molecular size of CS and HS was 110 and 10 kDa, respectively. It has also been established here that CS chains are synthesized on the core protein through the ubiquitous linkage region tetrasaccharide, suggesting that indispensable functions of the linkage region in the synthesis of GAGs have been conserved during evolution.

Key words: chondroitin sulfate/cnidarian/glycosaminoglycan/heparan sulfate/*Hydra magnipapillata*

Introduction

Glycosaminoglycans (GAGs) have been implicated in the regulation and maintenance of cell adhesion, motility, proliferation, differentiation, tissue morphogenesis, and embryogenesis (Kjellén and Lindahl 1991; Brickman and Gerhart 1994; Itoh and Sokol, 1994). GAGs include chondroitin sulfate (CS)/dermatan sulfate (DS) and heparan sulfate (HS)/heparin. The backbones of HS and CS consist of repeating disaccharide units: D-glucuronic acid (GlcA)β1-4GlcNAcα1-4 for HS and GlcAβ1-3N-acetylgalactosamine (GalNAc)β1-4 for CS. These simple structures acquire a considerable degree of variability through extensive modifications involving sulfation and uronate epimerization (Fransson 1985). This structural variability of GAGs is the basis for a wide variety of domain structures with different biological activities (Kjellén and Lindahl 1991), and is generated by the elaborate, concerted actions of biosynthetic enzymes.

Investigation of the occurrence of GAGs shows their wide distribution in tissues of vertebrates and invertebrates (Nader and Dietrich 1989), suggesting that GAGs have been phylogenetically conserved through evolution, and play fundamental, biological roles in animal development. The nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* are ideal experimental organisms for studying a wide range of fundamental biological disciplines in development using modern techniques of molecular and cell biology (Bellaiche et al. 1998; Sen et al. 1998; Seppo and Tiemeyer 2000). Biochemical studies of GAGs from *Drosophila* and *C. elegans* have been reported in Yamada et al. (1999) and Toyoda et al. (2000). Although HS chains are sulfated at various positions in both organisms, the structure of the CS of these organisms is unique in that the CS from *Drosophila* is composed of nonsulfated and 4-O-sulfated disaccharides, whereas only nonsulfated chondroitin is detected in *C. elegans*. These results might suggest that GalNAc 4-O-sulfation was acquired during evolution from nematodes to arthropods and plays an important role in some special function characteristic of higher animals. CS containing a GalNAc 6-O-sulfated structure has never been detected in *C. elegans* and in adult *Drosophila*. Although a small quantity of 6-O-sulfated GalNAc was detected in the third instar stage in *Drosophila larvae* (Pinto et al. 2004), the specific CS 6-O-sulfotransferase in *Drosophila* has not been identified. Mollusks such as squids are the lowest animals reported to contain...
GalNAc 6-O-sulfated or multi-sulfated structures in their CS chains (Matthews and Person 1962; Suzuki et al. 1968).

The hydrozoan has also been utilized as a model to study tissue regeneration in vivo (Fujisawa 2003; Holstein et al. 2003). It is one of the lowest multicellular animals and assumed to be a lower form of life than *C. elegans*. It has an extracellular matrix (ECM) boundary zone between two functionally distinct tissue layers, ectodermal and endodermal epithelial layers, and the ECM components of *Hydra vulgaris* have been characterized in an immunocytochemical study (Sarras et al. 1991). Although the presence of the core protein of HS–proteoglycan (PG) was indicated, it is not clear whether it is glycanated and the structural characterization of hydra GAG chains has not been attempted. On the basis of previous findings concerning the structure of CS/DS chains from *Drosophila* and *C. elegans* (Yamada et al. 1999; Toyoda et al. 2000), hydrozoans may be assumed to produce only nonsulfated chondroitin or no CS.

Accumulating evidence suggests that GAGs play important roles in neural development by regulating neuronal adhesion and migration, the formation of neurites, and axonal guidance (Sugahara et al. 2003). Immunological studies using monoclonal antibodies have revealed that CS isoforms, differing in the position and degree of sulfation, perform distinct functions during development in the mouse and rat fetus (Mark et al. 1990). Oversulfated disaccharides and l-iduronic acid (IdoUA) residues from CS/DS chains play critical roles in growth factor-binding and neuritogenic activities during development of the brain (Faisstner et al. 1994; Clement et al. 1998; Nadanaka et al. 1998; Deepa et al. 2002; Bao et al. 2004; Bao, Mikami et al. 2005; Bao, Muramatsu et al. 2005; Li et al. 2007). Since the hydrozoan is the simplest organism, in which movements are governed by the neuromuscular system, it provides the most appropriate model system to investigate the basic mechanism of neuritogenesis. GAGs may be present in hydrozoans and perform critical functions during neuroregeneration. To understand the development of nervous system in terms of the evolution of GAG chains and their functions in the regeneration of the nervous system of hydrozoans, structural information about GAGs will be required, which can be used together with genetic and molecular data. Hence, GAGs from *Hydra magnipapillata* were isolated and their structural features were characterized in this study.

**Results**

**Immunohistochemistry of GAGs in Hydra**

Immunostaining of *Hydra* using five different antibodies, including 473A12, 2H6, CS-56, HepSS-1, and F58-10E4, against chondroitin, CS, and HS was carried out. Only the anti-chondroitin antibody 473A12 detected the corresponding epitope (Figures 1 and 2). The anti-HS antibodies (HepSS-1 and F58-10E4) and anti-CS antibodies (2H6 and CS-56) showed no staining. In a whole-mount sample, clusters of cells in the body column were stained (Figure 1). They are nests of nematoblasts (Figure 2, lower panel). When stem cells enter the nematocyte differentiation pathway, they undergo a series of synchronous cell divisions up to five rounds (David and Gierer 1974). However, the cytokinesis is incomplete and the daughter cells are connected to each other by cytoplasmic bridges, thus forming clusters or nests of cells. Cells at this stage are called dividing nematoblasts. Upon the cessation of division, nests of usually 8 or 16 cells start producing nematocytes, the stinging organelle. First, a hole appears in the cytoplasm and becomes a larger hollow space. Finally, the nematocyte structures develop. Cells at this stage are called differentiating nematoblasts. In a single nest, nematocytes of the same type are synchronously produced. There are four types of nematocytes. To examine the precise stage of the stained cells, polyps were dissociated into single cells by maceration and then immunostained with 473A12 for chondroitin (Figure 2). Stained cells had a large hollow space in the cytoplasm (Figure 2B), where the staining occurred as can be seen in a merged image (Figure 2C). These cells are actively producing nematocytes, but the nematocytes have not yet fully developed.

**Characterization of GAGs in Hydra**

To characterize GAGs in *Hydra* chemically, a GAG–peptide fraction was prepared from the acetone powder of the crude homogenate of *Hydra* by digestion with actinase E, followed by trichloroacetic acid treatment and ethanol precipitation. The GAG–peptide fraction was further fractionated by anion-exchange chromatography on a Sep-Pak Accell Plus QMA anion-exchange cartridge (Waters Corp., Milford, MA) using stepwise elution with a 0.3 M phosphate buffer, pH 6.0, containing 0.15, 0.5, 1.0, or 1.5 M NaCl. Each fraction was analyzed by anion-exchange high-performance liquid chromatography (HPLC) to identify the disaccharides produced by digestion with specific GAG lyases.

Upon digestion with chondroitinase ABC (Table 1), disaccharides were detected mainly in the fraction eluted with 1.0 M NaCl on the Accell Plus QMA cartridge. The major disaccharide was the nonsulfated disaccharide 4-deoxy-α-L-threo-hex-4-enepyranosyluronic acid (ΔHexA–GalNAc (Figure 3 and Table 1). Small proportions of monosulfated disaccharides were also detected. Each peak was co-eluted with the corresponding standard disaccharide (results not shown), and upon digestion with chondro-4- or 6-sulfatase, the peak that eluted at the position of ΔHexA–GalNAc(4-O-sulfate) or ΔHexA–GalNAc(6-O-sulfate) was shifted to the position, respectively, of ΔHexA–GalNAc (data not shown), confirming the identity of these structures. To evaluate whether the *Hydra* GAG fraction contains DS, enzymatic digestions with chondroitinase AC-I and B were carried out. Chondroitinase AC-I and B split GalNAc–GlcA and GalNAc–IdoUA linkages, respectively (Yoshida et al. 1993). The *Hydra* GAG fraction was resistant to the action of chondroitinase B but sensitive to that of chondroitinase AC-I and B were carried out. Chondroitinase AC-I and B split GalNAc–GlcA and GalNAc–IdoUA linkages, respectively (Yoshida et al. 1993). The *Hydra* GAG fraction was resistant to the action of chondroitinase ABC, although the enzyme depolymerizes not only CS but also hyaluronate, indicating the absence of hyaluronate in *Hydra*. However, no disaccharides from hyaluronate were detected in the digest with chondroitinase ABC, although the enzyme depolymerizes not only CS but also hyaluronate, indicating the absence of hyaluronate in *Hydra*. Approximately 1 μmol of the unsaturated DS disaccharide was recovered by digestion with chondroitinase ABC from 120 g of the *Hydra*.

The *Hydra* GAG fraction was also subjected to a compositional analysis of HS disaccharides after digestion with a mixture of heparinase and heparitinase. The total amount of HS disaccharides produced by digestion with HS lyases was
only 0.35 nmol per 120 g of the *Hydra*, three orders of magnitude less than that of CS. The chromatogram obtained after labeling with 2-aminobenzamide (2AB) is shown in Figure 4. The yield of each disaccharide was calculated based on the fluorescence intensity, and the data are summarized in Table I. Peaks of unidentified contaminants derived from the 2AB reagent were often observed as a result of the sensitivity of the analysis just before the elution of monosulfated disaccharides, as indicated by asterisks in Figure 4. The identity of the disaccharide peaks was established based on co-chromatography with standard disaccharides.

When the digest of the 1.0 M NaCl-eluted fraction with chondroitinase ABC was analyzed by gel-filtration HPLC after 2AB labeling, only disaccharides but no oligosaccharides were detected (data not shown). The amount of uronic acid in the 1.0 M NaCl-eluted fraction was determined by the carbazole reaction and its concentration in the starting material was calculated to be 1.56 μg/g wet tissue. Taking this value as 100%, the recovery of the total disaccharides generated by chondroitinase ABC, heparinase, and heparitinase was 99%, suggesting that essentially all the GAG polysaccharides were degraded to disaccharides by the GAG lyases.

The molecular size of the *Hydra* CS and HS was analyzed by gel-filtration chromatography on a Superdex 200 column (Figure 5). To monitor small amounts of *Hydra* GAGs, aliquots of individual fractions collected at 4.0 min intervals were lyophilized and digested with chondroitinase ABC or a mixture of heparinase and heparitinase, then the products were derivatized using a fluorophore, 2AB, and analyzed by anion-exchange HPLC. The amounts of the derivatized disaccharide products were calculated based on their fluorescence intensity. From comparisons with the calibration plot generated using the data obtained with size-defined commercial polysaccharides (Figure 5, inset), the average molecular size was calculated to be 1.56 μg/g wet tissue. Taking this value as 100%, the recovery of the total disaccharides generated by chondroitinase ABC, heparinase, and heparitinase was 99%, suggesting that essentially all the GAG polysaccharides were degraded to disaccharides by the GAG lyases.

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Fig. 2. Immunostaining on macerated cells with anti-chondroitin antibody 473A12. (A) Immunostaining. (B) Part of a nematoblast nest under phase contrast optics. (C) A merged image of (A) and (B). The lower panel illustrates the differentiation of multipotent interstitial stem cells in Hydra. Cells in the red square correspond to those shown in the upper panels. They are at a later stage of nematocyst differentiation.

Table 1. Disaccharide composition of Hydra CS and HS

<table>
<thead>
<tr>
<th>CS disaccharides</th>
<th>Proportion (%)</th>
<th>Total amount in wet tissue (nmol/g)</th>
</tr>
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<tbody>
<tr>
<td>∆HexA–GalNAc</td>
<td>71</td>
<td>6.01</td>
</tr>
<tr>
<td>∆HexA–GalNAc(4-O-sulfate)</td>
<td>15</td>
<td>1.29</td>
</tr>
<tr>
<td>∆HexA–GalNAc(6-O-sulfate)</td>
<td>14</td>
<td>1.18</td>
</tr>
<tr>
<td>Total</td>
<td>100 (0.29)b</td>
<td>8.48</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HS disaccharides</th>
<th>Proportion (%)</th>
<th>Total amount in wet tissue (pmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>∆HexA–GlcNAc</td>
<td>24</td>
<td>0.70</td>
</tr>
<tr>
<td>∆HexA–GlcNAc(6-O-sulfate)</td>
<td>18</td>
<td>0.52</td>
</tr>
<tr>
<td>∆HexA–GlcN(2-N-sulfate)</td>
<td>12</td>
<td>0.36</td>
</tr>
<tr>
<td>∆HexA–GlcN(2-N,6-O-disulfate)</td>
<td>25</td>
<td>0.73</td>
</tr>
<tr>
<td>∆HexA(2-O-sulfate)–GlcN(2-N-sulfate)</td>
<td>10</td>
<td>0.30</td>
</tr>
<tr>
<td>∆HexA(2-O-sulfate)–GlcN(2-N,6-O-disulfate)</td>
<td>11</td>
<td>0.31</td>
</tr>
<tr>
<td>Total</td>
<td>100 (1.33)b</td>
<td>2.93</td>
</tr>
</tbody>
</table>

*aThe purified GAG fraction was digested with chondroitinase ABC or a mixture of heparinase and heparitinase, and the digests were analyzed by HPLC as described in the “Materials and methods” section.

*bTotal sulfate groups/100 disaccharides are given in parentheses.

Fig. 3. HPLC analysis of the chondroitinase ABC digest of the Hydra GAG fraction. The chondroitinase ABC digest of the Hydra GAG fraction was analyzed by reversed-phase ion-pair chromatography with post-column detection. The positions of authentic unsaturated disaccharides are indicated by arrows. 1, ∆HexAα1-3GalNAc; 2, ∆HexAα1-3GalNAc(4-O-sulfate); 3, ∆HexAα1-3GalNAc(6-O-sulfate); 4, ∆HexAα1-3GalNAc(4,6-O-disulfate); 5, ∆HexA(2-O-sulfate)α1-3GalNAc (6-O-sulfate); 6, ∆HexA (2-O-sulfate)α1-3GalNAc(4,6-O-disulfate).
of CS and HS was estimated to be 110 and 10 kDa, respectively.

The structure of the GAG–protein linkage region was also analyzed. The GAG–peptide fraction was treated with LiOH to liberate O-glycan chains including GAGs from the core proteins (Sakaguchi et al. 2001). The liberated saccharides were labeled with 2AB, and the excess 2AB-derivatizing reagents were removed by paper chromatography and gel-filtration chromatography as described in the Materials and methods section. The flow-through fraction of the sample was pooled and analyzed further. For the analysis of the linkage region of CS, the sample was digested with chondroitinase ABC and/or AC-II, and the digests were subjected to anion-exchange HPLC. Depolymerization of CS chains by chondroitinase ABC results in sulfated disaccharide units and core hexasaccharides derived from the linkage region (Sugahara et al. 1988). Chondroitinase AC-II degrades a linkage region hexasaccharide into a disaccharide unit and a core tetrasaccharide (Sugahara et al. 1991).

When a chondroitinase ABC digest of the 2AB derivative prepared after the LiOH treatment was analyzed by anion-exchange HPLC, two major peaks were observed at the position of the authentic 2AB-labeled nonsulfated hexasaccharide, ΔHexAα1-3GalNAcβ1-4GlcAβ1-3Galβ1-4Xyl-2AB, and monophosphorylated hexasaccharide, ΔHexAα1-3GalNAcβ1-4GlcAβ1-3Galβ1-4Xyl(2-O-phosphate)-2AB (Figure 6A). Upon digestion with chondroitinase ABC and AC-II, two major peaks were observed at the positions of the authentic 2AB-labeled tetrasaccharides, ΔHexAα1-3Galβ1-3Galβ1-4Xyl-2AB and ΔHexAα1-3Galβ1-3Galβ1-4Xyl(2-O-phosphate)-2AB, in a molar ratio of 55:45 (Figure 6B). These samples were co-eluted when co-chromatographed (data not shown) with the corresponding standard linkage tetrasaccharides (Sakaguchi et al. 2001), confirming
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Discussion

In the present study, we characterized GAGs isolated from Hydra and demonstrated the existence of CS as well as HS. This is the first study to demonstrate the existence of GAGs in Hydra, although the presence of HS–PG core protein in Hydra vulgaris has previously been suggested by an immunocytochemical analysis (Sarras et al. 1991). It has been reported that Drosophila and C. elegans synthesize GAGs (Yamada et al. 1999; Toyoda et al. 2000). Although various positions in HS chains of these model animals are sulfated, their CS chains of Drosophila are nonsulfated or sulfated predominantly at the C4 position of GalNAc residues. On the basis of these findings, we had expected Hydra to have no CS chains or only nonsulfated chondroitin chains, because Drosophila and C. elegans are assumed to be higher forms of life than H. magnipapillata. Interestingly, however, Hydra CS turned out to be more heterogeneous in terms of sulfation than C. elegans chondroitin and Drosophila CS. Since the CS chains from lower animals are more complex than those from higher animals, there seems to be no direct correlation between CS structure and evolution. It cannot be ruled out that Hydra acquired the genes of various CS sulfotransferases through horizontal transfer.

Hydra CS contains not only nonsulfated and 4-O-sulfated GalNAc residues but also 6-O-sulfated GalNAc residues. In contrast, CS chains from C. elegans and adult Drosophila are not 6-O-sulfated on GalNAc residues (Yamada et al. 1999; Toyoda et al. 2000), and a very small quantity of 6-O-sulfated GalNAc was detected only in the third instar stage in Drosophila larvae (Pinto et al. 2004). The complete genome sequences of C. elegans (1 × 10^8 bp) and Drosophila (1.4 × 10^8 bp) have been determined, but no ortholog of the human CS 6-O-sulfotransferase gene has been reported for these genomes. Although the complete genome sequence of Hydra has not been determined or reported, some genes, such as those for CS 4-O-sulfotransferase, CS 6-O-sulfotransferase, HS 2-O-sulfotransferase, and HS–CS synthase, have been identified in the GenBank database. The findings of this study suggest that the enzymes required for the 6-O-sulfation of GalNAc residues of CS chains exist in Hydra. Indeed, a gene (accession number CN554591) encoding a protein with significant homology to human CS 6-O-sulfotransferase is present in the expressed sequence tags of Hydra.

We also isolated and characterized the Hydra CS–core protein linkage region and demonstrated that Hydra CS chains are attached to core proteins through the common linkage region tetrasaccharide, −4GlcAβ1-3Galβ1-3Galβ1-4Xylβ1−. This indicates that Hydra CS exists as a PG, although the core proteins remain to be identified. Some bacterial strains synthesize GAG-like polysaccharides and are produced as extracellular polysaccharide capsules that serve as virulence factors. These polysaccharides are not attached to core proteins and are synthesized on the inner surface of the cytoplasmic membrane (DeAngelis 2002). Studies on the GAG–protein linkage region of invertebrate GAGs are limited, and the linkage region tetrasaccharide GlcA-Gal-Gal-Xyl was isolated as a discrete structure from chondroitin of C. elegans as well as HS and CS of Drosophila (Yamada et al. 2002). Although the hydrozoan is a lower form of life than the nematode, it has now been clarified that at least the conventional linkage region tetrasaccharide of PGs has been conserved from Hydra through evolution. The tetrasaccharide linkage structure was not detected in Hydra HS, because of the limited amount of HS.

A chain length analysis of Hydra CS and HS was also carried out in this study. The average molecular size of Hydra CS was estimated to be approximately 110 kDa, which is similar to that of mammalian CS, but relatively large in size. In contrast, the average molecular size of HS chains from Hydra was only approximately 10 kDa.
Although the chains of Drosophila HS have been demonstrated to be relatively short (20 kDa) compared with mammalian HS chains (Yamada et al. 2002), the average molecular size of the HS chains from Hydra was half that of the Drosophila HS chains. The reason why the chains of Hydra HS are much shorter than those of Hydra CS remains to be elucidated.

Immunostaining showed that the epitope of the anti-chondroitin antibody 473A12 was present in the developing nematocysts. Although their precise role in Hydra is unknown, chondroitin PGs might furnish an environment for assembling the components of nematocysts, or themselves be components. Since chondroitin in C. elegans is indispensable for cytokinesis early during embryonic development (Mizuguchi et al. 2000), Hydra chondroitin may also be essential in cell division and morphogenesis. The lack of staining with anti-HS antibodies early during embryonic development (Mizuguchi et al. 2000; Yamada et al. 1999) and the reason why the chains of Hydra CS are much shorter than those of the anti-CS antibodies in C. elegans is indispensable for cytokinesis early during embryonic development (Mizuguchi et al. 2000), Hydra chondroitin may also be essential in cell division and morphogenesis. The lack of staining with anti-HS antibodies early during embryonic development (Mizuguchi et al. 2000). Therefore, the role of chondroitin in cell division (Mizuguchi et al. 2000) and cell division (Mizuguchi et al. 2000) may have similar roles in Hydra. Thus, Hydra is a useful organism for studying functions of CS chains especially in cell division, cytokinesis, and regeneration in the absence of hyaluronan.

Materials and methods

Materials

Chondroitinases ABC (EC 4.2.2.4), AC-I (EC 4.2.2.5), AC-II (EC 4.2.2.5), and B (EC 4.2.2.), chondro-4-sulfatase (EC 3.1.6.9), chondro-6-sulfatase (EC 3.1.6.10), heparinase (EC 4.2.2.7), heparitinase (EC 4.2.2.8), and standard unsaturated disaccharides derived from CS were obtained from Seikagaku Corp. (Tokyo, Japan). Enzymatic digestion

The Hydra samples fractionated by chromatography with the Accell QMA Plus cartridge (corresponding to 0.1–6.0 g of Hydra) were digested with chondroitinase ABC, AC-I, AC-II or B, chondro-4- or 6-sulfatase, or a mixture of heparinase and heparitinase as described previously (Sugahara et al. 1994, 1995; Yamada et al. 1994; Yamada and Sugahara 2003). Unsaturated disaccharides produced enzymatically from HS and CS were derivatized with 2AB, and then analyzed by anion-exchange HPLC on an amino-bound silica PA-03 column (YMC Co., Kyoto, Japan) and gel-filtration HPLC on a Superdex Peptide column (Amersham Pharmacia Biotech) as reported previously (Kinoshita and Sugahara 1999; Bao, Mikami et al. 2005), or directly analyzed by reversed-phase ion-pair chromatography with post-column detection (Toyoda et al. 1999).

Preparation of the GAG–protein linkage region from Hydra

The major Hydra GAG–peptide fraction, which was eluted using 1.0 M NaCl on an Accell QMA Plus cartridge (corresponding to 30 g of Hydra), was digested with chondroitinase ABC, AC-I, AC-II or B, chondro-4- or 6-sulfatase, or a mixture of heparinase and heparitinase as described previously (Sugahara et al. 1994, 1995; Yamada et al. 1994; Yamada and Sugahara 2003). Unsaturated disaccharides produced enzymatically from HS and CS were derivatized with 2AB, and then analyzed by anion-exchange HPLC on an amino-bound silica PA-03 column (YMC Co., Kyoto, Japan) and gel-filtration HPLC on a Superdex Peptide column (Amersham Pharmacia Biotech) as reported previously (Kinoshita and Sugahara 1999; Bao, Mikami et al. 2005), or directly analyzed by reversed-phase ion-pair chromatography with post-column detection (Toyoda et al. 1999).

Immunohistochemistry

Fluorescence immunohistochemical staining for whole-mount and macerated samples was performed as described previously (Nishimiya-Fujisawa and Sugiyama 1993; Yum et al. 1998). Single cell samples were prepared by a maceration procedure (David 1973). The following monoclonal antibodies against GAGs used in this study were obtained from Seikagaku Corp.: the anti-HS antibodies HepSS-1 and F58-10E4, anti-CS antibodies 2H6 and CS-56, and anti-chondroitin antibody 473A12.

Preparation of a GAG-peptide fraction from Hydra

Frozen tissue of H. magnipapillata (120 g wet weight) was homogenized in ice-cold acetone and lyophilized as described previously (Yamada et al. 1999). The acetone powder was digested with actinase E, treated with 5% trichloroacetic acid, and precipitated with 80% ethanol as described previously (Yamada et al. 2002). The resultant precipitate, a GAG–peptide fraction, was subjected to anion-exchange chromatography on an Accell QMA Plus cartridge using step-wise elution with a 0.3 M phosphate buffer, pH 6.0, containing 0.15, 0.5, 1.0, or 1.5 M NaCl. Each fraction was desalted by gel filtration on a PD-10 column using 50 mM pyridine-acetate, pH 5.9, as the eluent, lyophilized, and dissolved in water. Uronic acid in each fraction was determined by the carbazole method (Bitter and Muir 1962).

Enzymatic digestion

The Hydra samples fractionated by chromatography with the Accell QMA Plus cartridge (corresponding to 0.1–6.0 g of Hydra) were digested with chondroitinase ABC, AC-I, AC-II or B, chondro-4- or 6-sulfatase, or a mixture of heparinase and heparitinase as described previously (Sugahara et al. 1994, 1995; Yamada et al. 1994; Yamada and Sugahara 2003). Unsaturated disaccharides produced enzymatically from HS and CS were derivatized with 2AB, and then analyzed by anion-exchange HPLC on an amino-bound silica PA-03 column (YMC Co., Kyoto, Japan) and gel-filtration HPLC on a Superdex Peptide column (Amersham Pharmacia Biotech) as reported previously (Kinoshita and Sugahara 1999; Bao, Mikami et al. 2005), or directly analyzed by reversed-phase ion-pair chromatography with post-column detection (Toyoda et al. 1999).
for 30 min. Alkaline phosphatase treatment of the chondroitinase digest was carried out using 4 IU of the enzyme in a total volume of 100 μL of 0.08 M glycine–NaOH buffer, pH 9.9, containing 0.5 mM MgCl₂ at 37 °C for 30 min (Sugahara, Mizuno et al. 1992). The enzymatic reactions were terminated by heating at 100 °C for 1 min, and each digest was analyzed by anion-exchange HPLC on an amine-bound silica PA-03 column as described previously (Kinosita and Sugahara 1999).

**Gel-filtration chromatography of the Hydra GAG preparation**

The major Hydra GAG fraction, which was eluted with 1.0 M NaCl on an Accell Plus QMA cartridge (corresponding to 30 g of hydra as the starting material), was analyzed by gel-filtration chromatography on a column (10 × 300 mm) of Superdex 200 eluted with 200 mM ammonium bicarbonate at a flow rate of 0.3 mL/min. Fractions were collected at 40 min intervals, lyophilized, and digested with chondroitinase ABC or a mixture of heparinase and heparitinase. The digests were derivatized with 2AB then analyzed by HPLC on an amine-bound silica column (Kinosita and Sugahara 1999).

**Conflict of interest statement**

None declared.

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**Abbreviations**

2AB, 2-aminobenzamide; CS, chondroitin sulfate; DS, dermatan sulfate; ECM, extracellular matrix; GAG, glycosaminoglycan; GalNAc, N-acetylgalactosamine; GlcA, D-glucuronic acid; ΔHexA, 4-deoxy-α-L-threo-hex-4-enepyranosyluronic acid; HPLC, high-performance liquid chromatography; HS, heparan sulfate; IdoUA, L-iduronic acid; 2P, 2-O-phosphate; PG, proteoglycan

**References**


