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Comprehensive analysis of glycosaminoglycans from the edible shellfish

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ABSTRACT

We have previously reported that the keratan sulfate (KS) disaccharide was branched to the C-3 position of glucuronate in chondroitin sulfate (CS)-E derived from the *Mactra chinensis*. We carried out the comprehensive disaccharide analysis of GAGs from 10 shellfish, *Ruditapes philippinarum*, *Scapharca broughtonii*, *Mizuhopecten yessoensis*, *Turbo cornutus*, *Crassostrea nippona*, *Corbicula japonica*, *Mytilus galloprovincialis*, *Neptunea intersculpta*, *Pseudocardium sachalinense* and *Crassostrea gigas*, to better understand the glycan structures in marine organisms. The contents of CS, heparan sulfate and hyaluronic acid and their compositions depend on the species of shellfish. Interestingly, a peak corresponding to a pentasaccharide containing KS disaccharide was observed when GAGs from *T. cornutus* was treated with chondroitinase (Chase) ACII but not Chase ABC. In addition, unidentified peaks were also observed when CS derived from *R. philippinarum*, *S. broughtonii* were treated with Chase ACII. These results suggest the presence of additional unidentified structure of CS in these shellfish.

1. Introduction

Glycosaminoglycans (GAGs) including chondroitin sulfate (CS), dermatan sulfate (DS), heparin (HP), heparan sulfate (HS) and keratan sulfate (KS) are a linear and sulfated polysaccharide chains composed of uronic acid (or galactose (Gal) in the case of keratan sulfate) and (*N*-acetyl or *N*-sulfo) hexosamine (Roden, 1980). CS consists of a distinctive repeating disaccharide unit [→4)-β-D-GlcA-(1 → 3)-β-D-GalNAc-(1 →]$_n$, where GlcA is glucuronic acid and GalNAc is *N*-acetylgalactosamine, and certain hydroxyl groups in disaccharide units are replaced with sulfo groups (S) responsible for a diversity of disaccharide units, GlcA-GalNAc (O-unit), GlcA-GalNAc4S (A-unit), GlcA-GalNAc6S (C-unit), GlcA (2S)-GalNAc (6S) (D-unit), GlcA-GalNAc4S,6S (E-unit) (Higashi, Okamoto, Mano, Wada, & Toida, 2014). A disaccharide unit containing iduronic acid (IdoA) in place of GlcA, a stereoisoform of CS that differs in the C-5 configuration of the hexuronic acid moieties, is commonly found in DS (Malmström, Bartolini, Thelin, Pacheco, & Maccarana, 2012). Thus, IdoA-GalNAc (iO-unit), IdoA-GalNAc4S (iA-unit) and IdoA2S-GalNAc4S are also constituents of dermatan sulfate (DS) and CS/DS hybrid chains.

There are several reports that highly sulfated and rare structures of CS are observed in marine organisms. For example, DS from adult and embryonic sea urchin contains 59% of iC-units (IdoA-GalNAc6S) and 74% of iE-unit (IdoA-GalNAc4S,6S), and B-unit (66%) and iD-unit (IdoA-GalNAc2S,6S) (> 90%) and these are predominant disaccharides in DS are from *Ascidian S. plicata* and *A. nigra* (Pavão et al., 1998; Pavão, Mourão, Mulloy, & Tollefsen, 1995; Vilela-Silva, Werneck, Valente, Vacquier, & Mourão, 2001). Oligosaccharides containing GlcA[Fuc4S-Fuc3S,4S]-GalNAc4S,6S are found in CS from sea cucumbers (Vieira, Mulloy, & Mourão, 1991), and GlcA3S-GalNAc4S (K-unit) are found in CS from king crab (*Tachypleus tridentataus*) cartilage, sea cucumber (*Ludwigothurea grisea*), squid cartilage and octopus cartilage (Higashi et al., 2015; Kinoshita et al., 1997; Seno, Yamashiro, & Anno, 1974; Vieira et al., 1991). Recently, we identified a particularly unusual KS disaccharide-branched CS from *Mactra chinensis* (Higashi et al., 2016).

It is of interest to note that CS, having a fucosylated GlcA, a sulfo group at the 3-position of GlcA residue IdoA residues and KS disaccharide attached to GlcA, show different susceptibility to chondroitinases (Chases) compared to the other types of CS. It is well known that almost all types of CS from vertebrates are depolymerized by Chase ABC to unsaturated disaccharides including ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-diS$_E$, ΔDi-diS$_B$ and ΔDi-diS$_D$ (Higashi et al., 2014). Both the (1–3)-linkage of K-unit and also (1–4)-linkage of disaccharides are digested by Chase ABC resulting in GalNAc4S residues being produced from the K-unit (Fongmoon et al., 2007). In contrast, the (1–4)-linkage between GalNAc and 3-*O*-sulfo GlcA in CS-K show resistance to Chase ACII digestion (Higashi et al., 2015; Sugahara et al., 1996). In addition, the (1–4)-linkage between GalNAc and IdoA in DS is also resistant to the action of Chase ACII (Williams et al., 2017). Recently, we solved structure of KS disaccharide-branched CS-E which exhibited resistance to the degradation by Chase ABC or ACII (at low concentrations) when compared to typical CS structures (Higashi et al., 2016). Glucose

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branched CS-E was also identified (Habuchi, Sugiura, & Kawai, 1977). It is well known that fucosylated (fucose-branched) CS, derived from sea cucumber, shows resistance to both Chase ABC and ACII, and partial acid hydrolysis is required to prepare the unsaturated disaccharide units (Vieira et al., 1991). While Vieira et al. suggested that these branches serve to prevent digestion of polysaccharide by microorganisms (Vieira et al., 1991), the actual physiological function of these branches in marine organisms are not fully understood.

The composition of GAGs including CS, DS, HP and HA in several kinds of shellfish has been well investigated (Cao et al., 2015; Cesaretti, Luppi, Maccari, & Volpi, 2004; Jordan & Marcum, 1986; Liu et al., 2017; Luppi, Cesaretti, & Volpi, 2005; Volpi & Maccari, 2009). However, disaccharide analysis of GAGs, especially CS has not been carried out after Chase ACII treatment. In this paper, a more comprehensive analysis of the GAG composition in 10 shellfish was performed to better understand glycan structures present in these shellfish.

2. Experimental

2.1. Materials and methods

The shellfishes used in the experiments were purchased from the Japanese common market. Actinase E was purchased from Kaken pharmaceutical Co., Ltd. (Tokyo, Japan). For the CS unsaturated disaccharide standards, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-β-D-*xylo*-hexenepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-*O*-(4-deoxy-β-D-*xylo*-hexenepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-*O*-(4-deoxy-β-D-*xylo*-hexenepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S), 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-β-D-*xylo*-hexenepyranosyluronic acid)-D-galactose (ΔDi-UA2S), 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*- sulfo-β-D-*xylo*-hexenepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-diS_B), 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-β-D-*xylo*-hexenepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-diS_D), 2-acetamido-2-deoxy-3-*O*-(4-deoxy-β-D-*xylo*-hexenepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose (ΔDi-diS_E) and 2-acetamido-2-deoxy-3-*O*-(4-deoxy-2-*O*-sulfo-β-D-*xylo*-hexenepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose (ΔDi-TriS) were obtained from Seikagaku Kougyo (Tokyo, Japan). Chondroitinase ABC (Chase ABC) and Chondroitinase ACII (Chase AC II) were purchased from Sigma-Aldrich (St. Louis, MO) and ChromaDex (CA, USA), respectively. For the HS unsaturated disaccharide standards, 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hexenepyranosyluronic acid)-D-glucose (ΔDi-0S_{HS}), 2-deoxy-2-sulfamido-4-*O*-(4-deoxy-α-L-*threo*-hexenepyranosyluronic acid)-D-glucose (ΔDi-NS), 2-acetamido-2-deoxy-4-*O*-(4-deoxy-α-L-*threo*-hexenepyranosyluronic acid)-6-*O*-sulfo-D-glucose (ΔDi-6S), 2-deoxy-2-sulfamido-4-*O*-(4-deoxy-α-L-*threo*-hexenepyranosyluronic acid)-6-*O*-sulfo-D-glucose (ΔDi-NS6S), 2-deoxy-2-sulfamido-4-*O*-(4-deoxy-2-*O*-sulfo −α-L-*threo*-hexenepyranosyluronic acid)-D-glucose (ΔDi-2SNS), 2-deoxy-2-sulfamido-4-*O*-(4-deoxy-2-*O*-sulfo-α-L-*threo*-hexenepyranosyluronic acid)-6-*O*-sulfo-D-glucose (ΔDi-TriS_{HS}) were obtained from Seikagaku Kougyo (Tokyo, Japan). Heparin lyases I and II, III were purchased from Iduron Ltd. (UK, Manchester), and the other reagents were of the analytical grade available.

2.2. Molecular phylogenetic analysis of shellfish

Phylogenetic tree of shellfish was constructed by using 16S rRNA gene sequences. Each genetic sequence data of shellfish, *Pseudocardium sachalinense* (Acession number: KP090052.1, GenBank), *Mactra chinensis* (DQ356386.1), *Ruditapes philippinarum* (HQ634142.1), *Corbicula japonica* (AB304508.1), *Crassostrea nippona* (AY007426.1), *Crassostrea gigas* (DQ839414.1), *Turbo cornutus* (AM403777.1), *Neptunea intersculpta* (AB044265.1), *Scapharca broughtonii* (AF305058.1), *Mizuhopecten yessoensis* (AB103394.1), *Mytilus galloprovincialis* (AB205194.1), was obtained from NCBI's database. The multiple sequence alignment was performed by Clustal X (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa, Kishino, & Yano, 1985). The phylogenetic analyses were conducted in MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

2.3. Isolation of crude GAG from shellfish

Each raw shellfish was separated from its shell and lyophilized and homogenized by using Blender (Vita-Mix, Osaka Chemical Co., Ltd). The dry powder of each shellfish was delipidated overnight with acetone (4 mL/g dry powder). After centrifugation and aspiration of supernatant, resulting precipitate was dried and proteolyzed at 45 °C with Actinase E (10 mg/g dry powder) in 50 mM Tris-acetate (pH 8.0) for 48 h. After the proteolysis, the sample solution was treated with 0.5 M NaOH containing 0.3 M sodium borohydride (20 mL/g dry powder) at 4 °C for overnight to release the GAG chains from the core proteins by chemical β-elimination reaction. The reaction mixture adjusted pH to 7 with 2.0 M acetic acid was added 60% perchloric acid (final concentration 5%) to remove the proteins and then centrifuged at 1000*g* for 30 min. The impurities in the supernatant were removed from residual GAG chains by dialysis (molecular weight cut-off (MWCO) membrane 3000) at room temperature for overnight. GAG was precipitated with cetyl pyridinium chloride. The GAG-CPC complex was collected by centrifugation at 2300*g* for 15 min. The precipitate was washed twice with 0.1% CPC. Crude GAGs were recovered from the GAG-CPC complex by addition of 2.5 M NaCl, and the mixture was centrifuged at 2300*g* for 15 min. Crude GAGs were precipitated from the supernatant through the addition of 4-volumes of cold ethanol for 16 h at 4 °C and were collected by centrifugation at 2300*g* for 15 min. Finally, the collected crude GAGs were dissolved in water, dialyzed against water, and freeze-dried. The GAG recovery for each sample is shown in Table 1.

2.4. High performance liquid chromatography

The crude GAG samples (10 μg) were incubated in the reaction mixture (17.5 μL) containing 28.6 mM Tris-acetate (pH 8.0) and 50 mIU of Chase ABC and/or Chase ACII. After 16 h at 37 °C, depolymerized samples were heated to 100 °C to inactivate the enzyme and the aqueous solvent was evaporated, the resulting unsaturated saccharide products derived from CS/DS were dissolved into 10 μL of H_2O for the sample injection to HPLC. In the case of the disaccharide analysis of HS, 10 μg of crude GAG samples were also incubated in 16 μL of reaction mixture (pH 7.0) containing 1 mIU of each heparin lyases I, II, and III,

Table 1
Amounts of the crude glycosaminoglycans derived from shellfish.

		GAGs		
Shellfish	Crude GAG[a] (mg)	CS[b] (μg)	HS[b] (μg)	HA[b] (μg)
M. chinensis	11.8	12.8	3.7	N.D.
R. philippinarum	12.3	125.7	10.3	0.4
P. sachalinense	0.9	2.8	102.6	N.D.
C. japonica	9.2	13.3	13.2	0.2
C. nippona	2.4	20.1	0.5	N.D.
C. gigas	4.9	N.D.	2.4	N.D.
N. intersculpta	2.8	2.1	0.4	N.D.
T. cornutus	3.5	86.6	65.1	N.D.
S. broughtonii	25.9	14.4	22.8	0.3
M. yessoensis	8.0	43.2	17.0	N.D.
M. galloprovincialis	3.2	11.2	1.0	N.D.

N.D.: Not detected.
[a] Per 1 g of dry sample.
[b] Per 1 mg of crude GAG.

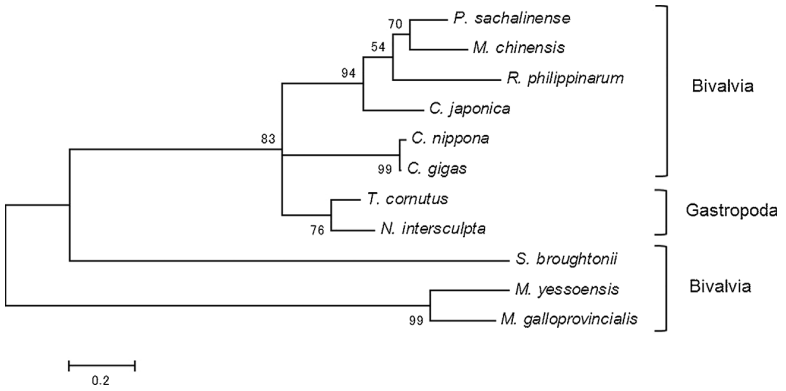

Fig. 1. Tree diagram based on 16S rRNA gene sequences (231 bp) of shellfish. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa et al., 1985). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The numbers in branches indicate the bootstrap values from 2000 bootstrap extractions.

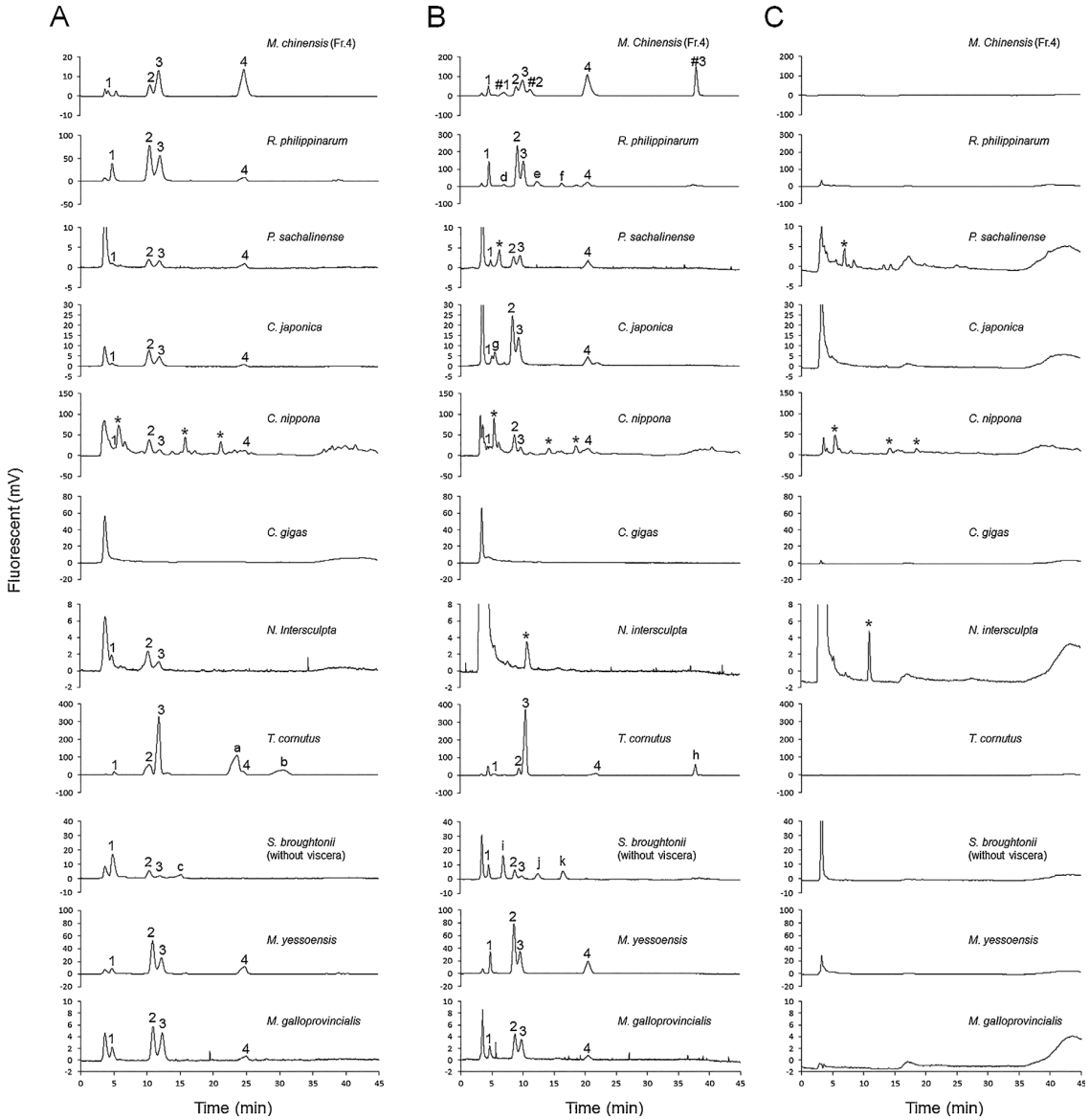

Fig. 2. Chromatogram of CS derived from shellfish after chondroitinase ABC and ACII treatment. Unsaturated disaccharide analysis was performed as follows. Crude GAGs (10 μg) were incubated in the reaction mixture (35 μL), which contained 28.6 mM Tris acetate (pH 8.0) and 25 mU of Chase ABC or ACII. Chromatogram were obtained with Chase ABC (A) or ACII (B) treatment and without treatment (C). Peaks: 1, ΔDi-0S; 2, ΔDi-4S; 3, ΔDi-6S; 4, ΔDi-diS_E; #1-3, KS-branched CS (mono-, di- and tri-sulfated CS); a-k, unknown peaks; *, impurity peaks.

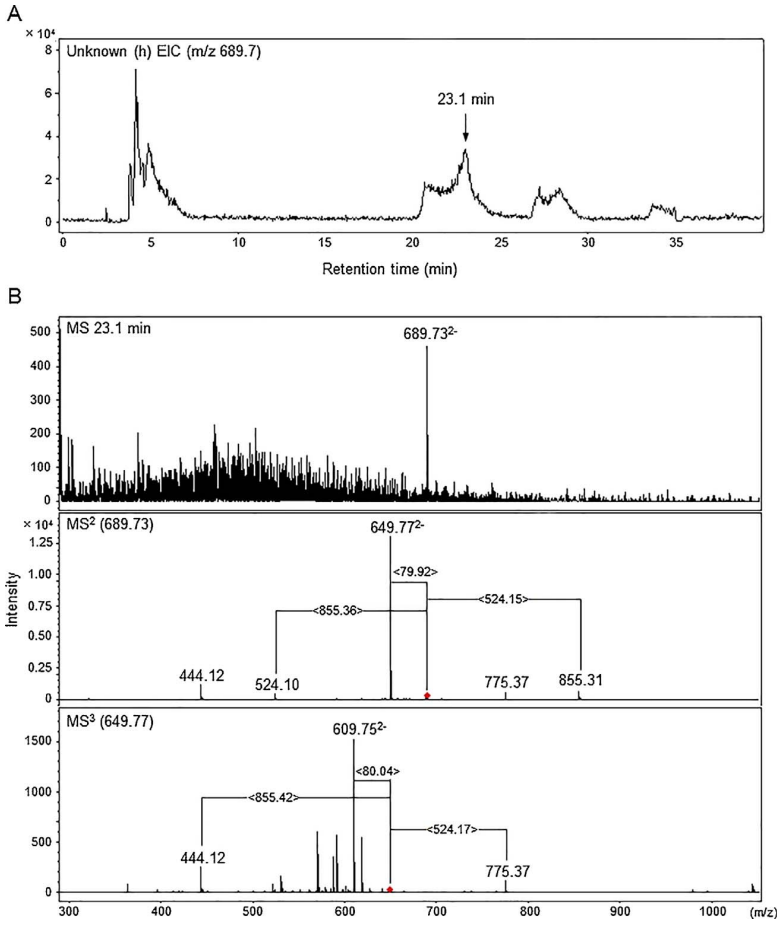

Fig. 3. LC–MS/MS analysis of unknown peak (h) in *T. cornutus*. AMAC labeling and LC–MS analysis were performed according to the method of Yang et al. (2012). Extracted ion chromatogram (EIC) of unknown peak (h) was obtained by Chase ACII treatment (A). Mass spectra of unknown peak (h) (B). MS^2 or MS^3 was performed using m/z 689.7^{2-} or 649.76^{2-} as a precursor ion.

Table 2
The comparison of theoretical and calculated ions with observed ions from *T. cornutus*.

	Theoretical mass	Calculated ions (charge)	Observed ions (charge)
Hex + HexNAc + SO_3 $-H_2O$	445.1	444.1 (−1)	
Hex + HexNAc + $2SO_3$ $-H_2O$	525.1	524.1 (−1)	
ΔUA + 2HexNAc + AMAC	776.3	775.3 (−1)	775.4 (−1)
ΔUA + 2HexNAc + SO_3 + AMAC	856.3	855.3 (−1)	855.3 (−1)
ΔUA + Hex + 3HexNAc + SO_3 + AMAC	1221.4	1220.4 (−1)	
		609.7 (−2)	609.8 (−2)
ΔUA + Hex + 3HexNAc + $2SO_3$ + AMAC	1301.4	1300.4 (−1)	
		649.7 (−2)	649.8 (−2)
ΔUA + Hex + 3HexNAc + $3SO_3$ + AMAC	1381.4	1380.4 (−1)	
		689.7 (−2)	689.7 (−2)

31.3 mM sodium acetate and 3.13 mM calcium acetate for 16 h at 37 °C. The determination of disaccharide composition was performed by monitoring the fluorescence detection with a post-column labeling reagent (Imamura et al., 2016). The post-column HPLC system was constructed with two LC-10Ai intelligent HPLC pumps (Shimadzu Co., Tokyo, Japan), a sample injector with a 20 μL loop (Model 7725i; Reodyne, CA, U.S.A.), a double-plunger pump for the fluorogenic reagents (NP-FX (II)-1U; Nihon Seimitsu Kagaku Co., Ltd, Tokyo, Japan), a column oven (L-7300; Hitachi, Tokyo, Japan), a chromato-integrator (D-2500; Hitachi, Tokyo, Japan), a dry reaction bath (DB-5; Shimamura Instrument), and FP–920 s intelligent Fluorescence Detector (Jasco, Tokyo, Japan). A DOCOSIL column (150 mm × 4.6 mm i.d.; Senshu Kagaku, Tokyo, Japan) was used at 60 °C. The HPLC conditions were as follows: eluent A, 1.2 mM tetrabutylammonium hydrogen sulfate in 12% methanol; eluent B, 1.2 mM tetrabutylammonium hydrogen sulfate and 0.2 M NaCl in 12% methanol; gradient for CS, 0–10 min (1% eluent B), 10–11 min (1–10% eluent B), 11–30 min (10% eluent B), 30–35 min (10–60% eluent B), 35–40 min (60% eluent B), 40–41 min (60-1% eluent B), 41–50 min (1% eluent B), gradient for HS, 0–10 min (1–4% eluent B), 10–11 min (4–15% eluent B), 11–20 min (15–25% eluent B), 20–22 min (25–53% eluent B), 22–29 min (53% eluent B), 29–30 min (53-1% eluent B), 30–40 min (1% eluent B); flow rate, 1.0 mL/min. The reaction reagents, aqueous 0.5% (w/v) 2-cyanoacetamide and 0.25 M NaOH, were delivered at the same flow rate of 0.25 mL/min by a double-plunger pump. The mixture reaction was performed at 110 °C in dry reaction bath and detected fluorometrically (Ex. 346 nm, Em. 410 nm). Measurement of HA contents in crude GAGs was performed according to the method of Toyoda, Muraki, Imanari,

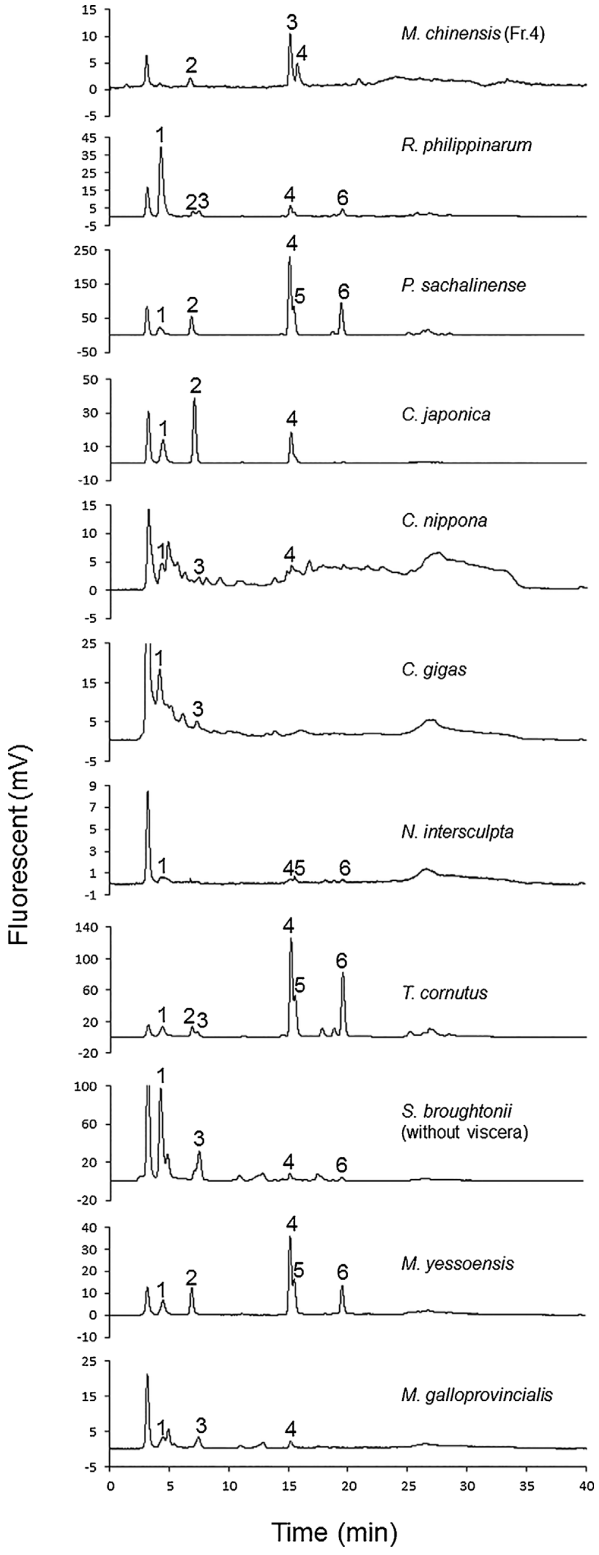

Fig. 4. Chromatogram of HS derived from shellfish after heparin lyases I and II, III treatment. Unsaturated disaccharide analysis was performed as follows. Crude GAGs (10 μg) were incubated in the reaction mixture (32 μL), which contained 31.3 mM sodium acetate with 3.1 mM calcium acetate (pH 7.0) and 2 mU of Heparin lyases I, II, and III. After incubation. Peaks: 1, ΔDi-$0S_{HS}$; 2, ΔDi-NS; 3, ΔDi-6S; 4, ΔDi-NS6S; 5, ΔDi-2SNS; 6, ΔDi-$TriS_{HS}$.

and Kinoshita-Toyoda, 2011). The HPLC conditions were as follows: column, HYPER CARB column (100 mm × 4.6 mm i.d.; Thermo Scientific, Tokyo, Japan); eluent, 60 mM phosphoric acid in 4.0% acetonitrile (without gradient); flow rate, 0.75 mL/min; column temperature, 40 °C.

2.5. Liquid chromatography-mass spectrometry

Freeze dried oligosaccharides obtained by Chase *Aci*I treatment were labeled with 2-aminoacridone (AMAC) according to the method of Yang, Chang, Weyers, Sterner, and Linhardt, 2012). with minor modifications. Oligosaccharides (100 μg) were added to 25 μL of 0.1 M AMAC solution in acetic acid/dimethyl sulfoxide (DMSO) (15/85, v/v) and mixed by vortexing for 5 min. Next, 25 μL of 1 M $NaBH_3CN$ was added in the reaction mixture and incubated at 45 °C for 4 h. Finally, the AMAC-tagged oligosaccharide mixture was diluted using 50% (v/v) aqueous DMSO. LC–MS analysis was performed on an AmaZon series SL ion-trap mass spectrometer (Bruker Daltonics, Bremen, Germany). MS data were collected by following conditions: ESI interface, negative mode; Capillary voltage, 3500 V; End plate offset 500 V; Gas flow, 6.0 L/min. The column used was an Acquity UPLC BEH C18 column (2.1 mm i.d. × 150 mm, 1.7 μm) at 50 °C in a column oven CO 631A (GL Science Corp., Tokyo, Japan) and flow rate was 0.1 mL/min. The eluent buffers were as follows: Eluent A, 80 mM ammonium acetate/methanol (88/12, v/v) and B, 80 mM ammonium acetate/methanol (12/88, v/v). The gradient program was as follows: 0–4 min (0% eluent B), 4–12.5 min (0–4% eluent B), 12.5–25 min (4–15% eluent B), 25–50 min (15–100% eluent B).

3. Results and discussion

3.1. Molecular phylogenetic classification of shellfish

At first, phylogenetic tree based on nucleotide sequences of 16S rRNA gene of mitochondrial DNA was constructed to examine the relationship between the GAGs composition and phylogenetic classification (Fig. 1). *T. cornutus* and *N. intersculpta* belong a class of Gastropoda. The others are classified into Bivalvia. *P. sachalinense*, *M. chinensis*, *R. philippinarum* and *C. japonica* are classified as the same order (veneroida), and are observed sequence similarity. On the other hands, *S. broughtonii*, *M. yessoensis* and *M. galloprovincialis* are separated from other bivalvia classifications.

3.2. Chondrotinase ACII afforded unknown peaks from CS of 4 shellfishes

Crude GAGs were extracted from 10 shellfishes by Actinase E digestion, and recovered by ethanol precipitation. The dried pellets (crude GAGs) were weighed after dialysis and freeze-drying. As a result, from 0.9 to 25.9 mg of GAG was recovered from 1 g of dry tissue (Table 1). Next, we investigated the contents and compositions of crude GAG components of the 10 shellfish, including CS, HS and HA. Unsaturated disaccharides from GAGs were obtained by the depolymerization with Chase ABC or Chase ACII, because Chase ABC can depolymerize the most types of CS, whereas Chase ACII cannot cleave the (1–4)-linkage between GalNAc and IdoA in DS (Hiyama & Okada, 1975).

When 10 μg of crude GAG was treated Chase ABC, peaks corresponding to ΔDi-0S, ΔDi-4S, ΔDi-6S and ΔDi-diS_E were found in *R. philippinarum*, *P. sachalinense*, *C. japonica*, *C. nippona*, *T. cornutus*, *M. yessoensis* and *M. galloprovincialis*, respectively (Fig. 2A). In *T. cornutus*, unknown peaks a and b were also observed. Many additional peaks (indicated with asterisks) surrounding the known disaccharides were also observed in *C. nippona*, however, we judged most of these peaks were impurities because similar control chromatogram was observed when a sample without Chase treatment was submitted to HPLC (Fig. 2C). Peaks corresponding to ΔDi-0S, ΔDi-4S and ΔDi-6S were observed for *N. intersculpta* and *S. broughtonii*, respectively, and no

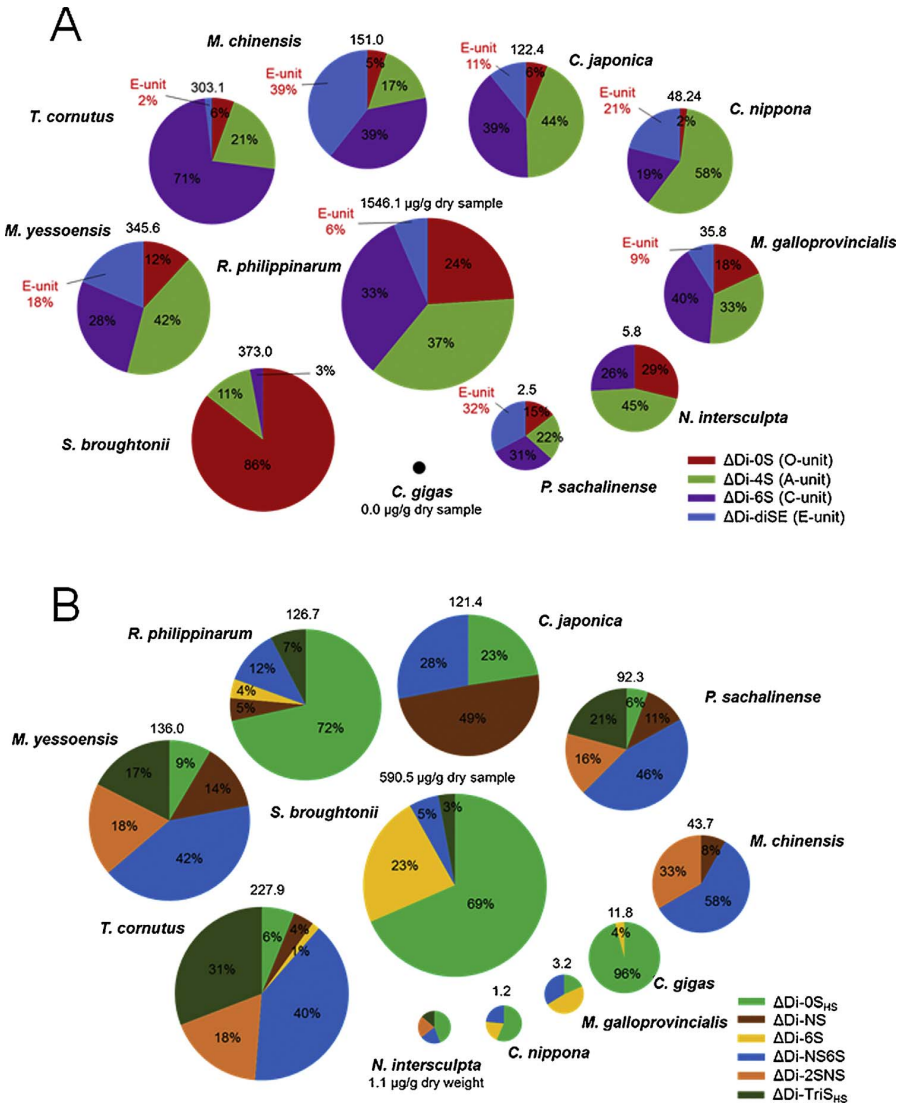

Fig. 5. Unsaturated disaccharide composition of CS (A) and HS (B) from 11 types of shellfish. For unsaturated disaccharide analysis, crude GAGs were treated with Chase ABC and AC II (A), or Heparin lyases I, II and III (B). Depolymerized samples were submitted to a reversed-phase ionpair chromatography and specific post-column with fluorescence detection. A comparison was performed with disaccharide standards.

peaks were observed for *C. gigas*.

We have previously reported that KS disaccharide-branched CS structures in *M. chinensis* showed the different susceptibility to Chase ACII compared with typical CS structures (Higashi et al., 2016). The production of KS disaccharide-branched unsaturated disaccharides (tetrasaccharides) depended on the Chase ACII treatment (Peaks #1, #2 and #3), and increased peak intensities of typical unsaturated disaccharides (ΔDi-0S, ΔDi-4S, ΔDi-6S and ΔDi-diS_E) were observed (Fig. 2B top chromatogram). Thus, we looked for the KS disaccharide-branched CS structures or other unknown structures in CS among 10 shellfish (Fig. 2B). As a result, increased peak intensities of the typical CS disaccharides and some unknown peaks were observed on Chase ACII treatment of GAG from *R. philippinarum* (peaks d, e and f) and *C. japonica* (peak g), respectively (Fig. 2B). Unexpectedly, unknown peaks were also observed in *T. cornutus* (peak h) and *S. broughtonii* (peaks i, j and k) despite an unchanged level of the typical unsaturated CS disaccharides. The glycan structures of unknown peaks a and b in *T. cornutus* may contain IdoA residues, because they are not detected by Chase ACII treatment.

3.3. UPLC–MS/MS analysis of CS oligosaccharides having unknown structures from T. cornutus

Since the elution time of unknown peak h was nearly identical with Peak #3 of *M. chinensis*, we speculated that *T. cornutus* also contained KS-branched structures. Thus, LC–MS/MS analysis of CS from *T. cornutus* was carried out to obtain structural information unknown peak h after pre-labeling with AMAC (Fig. 3).

Because the elution time of Peak #3, corresponding to tetrasaccharide (ΔUA + GalNAc + Gal + GlcNAc + $3SO_3$ + AMAC; *m/z* 588.17 $[M-2H]^{2-}$), was 23.4 min (Higashi et al., 2016), we looked for the candidate peak in the LC–MS at ~23 min after the injection of Chase ACII degraded CS from *T. cornutus*. Surprisingly, *m/z* 689.73 $[M-2H]^{2-}$ peak but not *m/z* 588.17 $[M-2H]^{2-}$ peak were observed at 23.1 min (Fig. 3). The product ions, including *m/z* 855.31 $[M-H]^{-}$, *m/z* 775.37 $[M-H]^{-}$, *m/z* 649.77 $[M-2H]^{2-}$, *m/z* 524.10 $[M-H]^{-}$ and *m/z* 444.12 $[M-H]^{-}$, were observed by fragmentation (MS^2) of *m/z* 689.70 $[M-2H]^{2-}$, whereas product ion *m/z* 775.37 $[M-H]^{-}$, 609.75 $[M-2H]^{2-}$ and 444.12 $[M-H]^{-}$ were also detected by further fragmentation (MS^3) of *m/z* 649.77 $[M-2H]^{2-}$. The structures, theoretical molecular masses and observed ions, shown in Fig. 3, are

summarized in Table 2. The product ions at *m/z* 649.77 $[M-2H]^{2-}$ and 609.75 $[M-2H]^{2-}$ were obtained by neutral loss of sulfo group from *m/z* 689.73 $[M-2H]^{2-}$ and *m/z* 649.77 $[M-2H]^{2-}$, respectively. The product ions *m/z* 855.31 $[M-H]^{-}$ and *m/z* 524.10 $[M-H]^{-}$ were probably obtained by the cleavage from *m/z* 689.73 $[M-2H]^{2-}$. We speculate that the product ion at *m/z* 444.12 $[M-H]^{-}$ in Table 2 was a disaccharide, Gal + GlcNAc6S because this product ion was previously observed by the fragmentation of *m/z* 588.17 $[M-2H]^{2-}$ (ΔUA + GalNAc 4S,6S + Gal + GlcNAc6S + AMAC) (Higashi et al., 2016). Considering that product ion at *m/z* 444.12 $[M-H]^{-}$ was obtained by neutral loss of sulfo group from *m/z* 524.10 $[M-H]^{-}$, disaccharides of Hex and HexNAc with two sulfo groups were present in peak (h). In addition, the product ion at *m/z* 775.37 $[M-H]^{-}$ was probably obtained by the deletion of 524 Da (Hex + HexNAc + $2SO_3$) from *m/z* 649.76 $[M\text{-}2H]^{2-}$. The remaining *m/z* 775.37 $[M-H]^{-}$ peak is suggested to correspond to ΔUA + 2HexNAc + AMAC. In addition, *m/z* 855.31 $[M-H]^{-}$ peak corresponds to ΔUA + 2HexNAc + SO_3 + AMAC. These results suggest that not only Hex but also HexNAc are branched in the CS backbone. On this basis, we can speculate that peak (h) is a pentasaccharide containing unsaturated disaccharide, a neutral sugar, two HexNAc and three sulfo groups (Table 2). Experiments are in progress to clarify the detailed structure of peak (h) as well as peaks (a) and (b) in Fig. 2B.

3.4. Composition of glycosaminoglycans in 10 shellfishes

Disaccharide analysis using reversed phase ion-pair chromatography was performed after treatment with chondroitinases or heparin lyases to better understand the composition and content of GAGs present in 11 shellfish, including *M. chinensis*. Although Chase ACII can depolymerize the CS and HA (Hiyama & Okada, 1975), the resulting ΔDi-0S (coming from CS and DS) and ΔDi-HA (coming from HA) cannot be separated under our reversed phase ion-pair chromatography conditions. For this reason, a graphitized carbon column was utilized to separate ΔDi-0S and ΔDi-HA according to the method of Toyoda et al. (2011). Chromatograms of unsaturated HS disaccharides from 11 shellfish, and their CS and HS disaccharide composition are shown in Figs. 4 and 5. We found that degree of sulfation in HS and CS depends on species and that ΔDi-diS_E was major disulfated disaccharide in CS (Fig. 5A). Among 11 shellfish, highly sulfated CS and HS are found in *M. chinensis, T. cornutus, P. sachalinense* and *M. yessoensis*, respectively. The contents of CS (or DS), HA and HS depend on species (Table 1). For example, CS (or DS) in major GAG in *M. galloprovincialis*, *C. nippona*, *M. chinensis*, *R. philippinarum*, *N. intersculpta* whereas HS is the major GAG in *P. sachalinense*. Comparable levels of CS and HS were found in *S. broughtonii*, *M. yessoensis*, *C. japonica* and *T. cornutus*. We examined the correlation between GAGs and phylogenetic classification, however, sulfation composition and contents of GAGs are not related (Fig. 1 and Table 1). These results are consistent with the analytical results of several kinds of fishes obtained by Arima et al. (2013). In addition, HA was not found in most of the shellfish. As mentioned above, peak intensities of several unknown structures were low in with typical CS disaccharides *R. philippinarum*, *C. japonica*, *T. cornutus* and *S. broughtonii*, however, total GAG contents are very low (less than 13%). Based on our prior observations, we thought that uronic acid-containing polysaccharides having unknown structures might be major components in crude GAGs (Cao et al., 2015; Liu et al., 2017). Further experiments are needed to understand the structures and physiological functions of acidic polysaccharides in marine organisms, particularly shellfish.

4. Conclusion

We analyzed composition and contents of CS (or DS), HA and HS in 11 shellfish including *M. chinensis*. We found that degree of sulfation in HS and CS depends on species and that ΔDi-diS_E was major disulfated disaccharide in CS in most shellfish. Particularly highly sulfated CS and HS were found in *M. chinensis, T. cornutus, P. sachalinense* and *M. yessoensis*. In addition, several unknown peaks were observed in *R. philippinarum*, *P. sachalinense*, *C. japonica* and *T. cornutus*, respectively. These results suggest that marine organisms are rich source of structurally unique sulfated CS GAGs.

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