Novel branch patterns and anticoagulant activity of glycosaminoglycan from sea cucumber Apostichopus japonicus

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Abstract

A novel glucosidic pattern of fucose branches was found in the glycosaminoglycan from the sea cucumber Apostichopus japonicus in China. The methylation of desulfated/carboxyl-reduced polysaccharides and analysis of unsaturated disaccharides generated from the enzymolysis of the defucosed polysaccharides demonstrated that the branch is formed by one fucopyranosyl residue, 46.5% of which is linked through the O-3 position of β-Gal-glucuronic acid, while 8.7% and 43.9% are linked through the O-6 and O-4 positions of the N-acetylgalactosamine moiety. The β-1-Gal-glucuronic acid, N-acetyl-β-D-galactosamine, α-L-fucose and sulfate ester with the molecular ratio of 0.97:1.00:1.13:3.85 composed the backbone →4)GalGALβ(1→3)GalNACβ(1→ and sulfated fucose branches. The sulfation patterns of fucose branches and the linkage pattern of the backbone structure were determined by 1D/2D dimension NMR. The most abundant branch species were 2,4-di-O-sulfated and 3,4-di-O-sulfated fucose, but 4-monosulfated residue was also present. The structure of previously obtained glycosaminoglycan is different from that previously obtained from Stichopus japonicus (Kariya et al., Carbohydr. Res. 297 (1997) 273–279), which suggests that the structures of glycosaminoglycans from the same species of different regions somehow differ. The anticoagulant assay indicated that the polysaccharide possessed a high anticoagulant activity and the sulfated fucose branches were essential to the activity.

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1. Introduction

Sea cucumber has been used as a fundamental source of food and drug in traditional Chinese medicine for centuries. The sea cucumber Apostichopus japonicus is a species with high commercial value and is found along the coast of Russia, China, Japan and Korea [1]. A. japonicus is commercially cultivated in shallow ponds and by sea ranching in China with production reaching 2.7 × 10^5 t in 2008 [2]. Qingdao is one of the main production places of A. japonicus, where the sea cucumber is regarded as a high value seafood. The usage of A. japonicus ranched from the Qingdao sea area, however, is simple, and the composition and identities of its bioactive chemical components have not been fully investigated.

Holothurian glycosaminoglycan is a novel polysaccharide isolated from the body wall of sea cucumbers, which have a chondroitin sulfate-like backbone structure and sulfated α-L-fucose (Fuc) branches. Mourao and Bastos [3] found a new fucose-rich sulfated polysaccharide from the body wall of the sea cucumber Ludwigiairesa, and studied its detailed structures in their papers. The results showed that the fucose branches, which were formed by two fucopyranosyl residues linked through the α-1,2 band, mainly stretched from the O-3 position of the β-Gal-glucuronic acid residues (GlcUA) on the non-reducing end of the polysaccharide chain. The fucose branches were mostly 4 mono-sulfated residues, meanwhile 2,4-di-sulfated and 3,4-di-sulfated residues were also present [4–6]. The glycosaminoglycan isolated from the sea cucumber Stichopus japonicas was attentively studied as well. It was found that the mono-fucopyranosyl residue with 2,4-di-sulfation, 3,4-di-sulfation and 4 mono-sulfation were linked to the O-3 position of the β-Gal-glucuronic acid residues or the 4/6 position of β-4-acetyl-galactosamine (GalNAc) [7–9]. Meanwhile, there have been several reports about the glycosaminoglycans from the sea cucumbers Thelenena ananas, Pearsonothuria graeffei, Stichopus tremulus, Holothuria vagaabunda, Holothuria edulis, A. japonicas and Holothuria nobilis mainly focusing on the sulfation patterns of the fucose branches [10,11]. Even though A. japonicas from the Qingdao sea area is of the same species as S. japonicas from Japan, the glycosaminoglycans structures from both sources have not been
compared and studied yet. In addition, the question whether the environment would influence the structure of the bioactive compounds from the same species is still unknown.

There have been many reports about the various biological activities of holothurian glycosaminoglycan, such as antitumor, antiviral, antithrombogenic and angiogenesis modulation properties [12–15]. Glycosaminoglycan from sea cucumbers have been found to possess a heparin-like anticoagulant activity [16], and the branches of sulfated fucose have been shown to be essential for the anticoagulant activities [6,10,17,18]. The mechanisms of their anticoagulant activities have been proven to be multiple, including acceleration of thrombin inhibition by heparin cofactor II (HCII), inhibition of factor VIII activation by thrombin, and inhibition of factor X activation by the intrinsic tenase complex [19,20]. Due to the lack of antithrombin-dependent activities, the glycosaminoglycan from the sea cucumber decreased the bleeding risk compared with heparin [20]. This advantage offers the promise of a potential anticoagulant drug.

The variety of biological activities of sea cucumber glycosaminoglycans are related to their physicochemical properties. Structural characterization of the complex carbohydrate has become possible with the development of modern analytic techniques, especially 1D/2D NMR spectroscopy and methylation analysis. The NMR spectra have been used to analyze the sulfated sugar contaminants in heparin materials associated with adverse clinical effects [21]. Methylation analysis proved to be an efficient tool to identify the glycosidic linkage patterns of the sulfated fucose branches [9].

In the present study, we extracted and analyzed fucosylated chondroitin sulfate from the body wall of a sea cucumber (holothurian glycosaminoglycan from A. japonicus, named AHG). The defined structure of AHG was clarified systematically by mild acid hydrolysis, enzymatic degradation, methylation analysis and 1D/2D of NMR spectroscopy, which was shown to differ from glycosaminoglycans from S. japonicas and L. grisea. Furthermore, the anticoagulant activity of the A. japonicus glycosaminoglycan was investigated and compared with unfractionated heparin.

2. Material and methods

2.1. Materials

Monosaccharide standards, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 1-phenyl-3-methyl-5-pyrazolone (PMP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Unsaturated disaccharides of the chondroitin sulfate family, including ΔUA-GalNAc (ΔDi-0S), ΔUA-GalNAc4S (ΔDi-4S), ΔUA-GalNAc6S (ΔDi-6S) and ΔUA-GalNAc4S6S (ΔDi-di6S), were from Iduron (Manchester, England). Dextran T-series standards, were from National Institutes for Drugs and Biological Products (Beijing, China). Heparin (150 IU/mg) was from Huixing Biochemistry Reagents Company Ltd., Shanghai, China. Q Sepharose Fast Flow and Sephadex 25 were from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). All other reagents were of analytical grade from Sinopharm Chemical Reagents Co., Ltd (Shanghai, China).

2.2. Isolation and purification of native AHG

AHG was extracted from the body wall of the sea cucumber A. japonicus, purchased from the Nanshan market of Qingdao, China. The body wall of fresh sea cucumber A. japonicus (1 kg) was grinded into homogenate and diluted to 1 L. Fifty-six grams of KOH was added under continuous stirring at 60 °C for 1 h. The pH was adjusted to 8.5 with cold HCl. Five grams of Diastase verA (EC 3.2.1.4) was added, and the mixture was maintained at 50 °C for 3 h. The mixture was adjusted to pH 7.0 with HCl and centrifuged to remove the precipitate. A threefold 95% (v/v) ethanol was added to precipitate the polysaccharides. The formed precipitate was collected and dissolved in distilled water at the ratio of 1:20 (g/mL). 1.5 M KOAc was added into the supernatant and kept at 4 °C overnight. The crude AHG was collected by centrifugation, dissolved in distilled water, dialyzed against distilled water for 36 h and lyophilized. The crude AHG was fractionated by a Q Sepharose Fast Flow column (300 mm × 30 mm) coupled with a peristaltic pump, eluted with a step-wise gradient of 0, 0.75 and 1.5 mol/L NaCl and detected by the phenol–sulfuric acid method [22]. The fractions eluted by 1.5 M aqueous NaCl were pooled, diazylzed and further purified on a Sephadex 25 column (100 cm × 2.6 cm) with deionized water. The major polysaccharide fractions were pooled and lyophilized.

2.3. General analysis

Total sugar content was determined by the phenol–sulfuric acid method using fucose as the standard [22]. The protein content was measured by the method as described [23]. Sulfate ester content was estimated according to the high performance capillary electrophoresis (HPCE) method reported [24]. The purity of AHG was determined by cellulose acetate membrane electrophoresis [25].

2.4. Determination of molecular weight

Molecular weight was determined by high performance gel permeation chromatography (HPGPC) on a Waters Ultrahydrogel™ Linear column (7.8 mm × 300 mm, Japan) with a Waters 2410 refractive index detector, eluted by 0.2 M Na2SO4 at a flow rate of 0.5 mL/min. Twenty microliter of a 10 mg/mL sample dissolved in 0.2 M Na2SO4 was injected. The molecular weight was calculated by a reference to a calibration curve made by a series of dextran T-series standards (Mr: 133.8, 84.4, 41.1, 21.4, 10.0, 7.1 kDa) [26].

2.5. Analysis of monosaccharide composition

Five mg of AHG was hydrolyzed by 2 mol/L trifluoroacetic acid at 110 °C for 8 h. The excess acid was removed by co-evaporating with methanol. The residue was dissolved in 0.5 mL distilled water and lyophilized. The samples were dissolved in 1.0 mL. One hundred microliter of sample was mixed with 100 μL of 0.1 M NaOH and 100 μL of 0.5 M PMP (dissolved in methanol) and incubated in 70°C for 30 min. The mixture was neutralized with 0.1 M HCl and the excess PMP was extracted by chloroform. The supernatant was subject to reversed phase high performance liquid chromatography (HPLC) and UV detection as described [27]. The analysis was performed on a Zonran Bondysil AQ-C18 column (5 μm, 4.6 mm × 150 mm, Zonran technologies company Ltd., Shanghai, China) at 30 °C with detection at a UV wavelength of 245 nm. The mobile phase was aqueous 50 mM KH2PO4 (pH 6.7) with 20% acetonitrile. The flow rate was 1.0 mL/min. The identification and quantitation of each monosaccharide were done by comparison with reference monosaccharides.

2.6. Analysis of unsaturated disaccharides composition

2.6.1. Partial acid hydrolysis

Partial removal of sulfated fucose branches from the glycosaminoglycan AHG was performed by the modified method as described [6]. Briefly, 50 mg of AHG was dissolved in 1.0 mL of 150 mM H2SO4, kept at 100 °C for 30 min, and adjusted to pH 7.0 with saturated aqueous Ba(OH)2. The mixture was centrifuged to remove the precipitate and the supernatant was dialyzed against
distilled water for 24 h. The defucosylated AHG (dsAHG) was obtained after lyophilization.

2.6.2. Analysis of the disaccharide products formed by digestion of dsAHG with chondroitin ABC lyase

The dsAHG (7 mg) was dissolved in 100 μL distilled water and filtered through 0.22 μm nylon membranes. The sample was incubated at 37 °C for 6 h by addition of 800 μL 50 mM Tris/HCl buffer (pH 8.0) and 0.1 unit of chondroitin ABC lyase (EC 4.2.2.4) from Proteus vulgaris (Seikagaku American Inc., Rockville, MD) dissolved in 100 μL Tris/HCl buffer. The mixture was boiled for 5 min, and centrifuged 10,000 × g for 20 min. The supernatant was then prepared HPLC analysis.

The identification and quantitation of each disaccharide were determined by an anion exchange HPLC method [28]. The analysis was performed on a Waters Spherisorb 55 NH2 column (2.0 mm × 150 mm) at 30 °C with UV detection at 232 nm. A linear gradient from 10 mM NaH2PO4 to 400 mM NaH2PO4 over 50 min was carried out. The identification and quantitation of each unsaturated disaccharide was done by comparison with reference disaccharides.

2.7. IR and NMR spectroscopic analysis

The AHG was mixed with dried KBr, ground and punched into 1 mm pellets for Fourier-transform infrared (FT-IR) spectral analysis in the frequency range of 4000–500 cm−1. FT-IR spectra were obtained on a Nicolet Nexus 470 spectrometer. 1H nuclear magnetic resonance (NMR) and 13C NMR spectra of AHG (30 mg) were performed at 25 °C using a JEOL-ECP 600 MHz spectrometer after co-evaporation with 500 μL D2O (99.9%, Sigma-Aldrich) twice followed by dissolution in 500 μL D2O containing tetramethylsilane (TMS) as the internal standard. Two-dimensional COSY and heteronuclear multiple quantum correlation (HMOC) were recorded at 20 °C.

2.8. Methylation analysis

2.8.1. Desulfation of intact AHG

Desulfation of AHG was performed as described [29]. The AHG (50 mg) was dissolved in 9 mL anhydrous dimethyl sulfoxide. Two milliliters of anhydrous pyridine, 50 mg of oxalic acid and 50 mg of antimony trioxide were added. The reaction was stopped by addition of 6% NaHCO3, and the mixture was dialyzed against distilled water for 24 h. The desulfated AHG (dsAHG) was obtained after lyophilization. The desulfation extent was determined by IR spectroscopy.

2.8.2. Reduction of carboxyl groups

Reduction of hexuronic acid groups in dsAHG was performed as described [4]. Briefly, the dsAHG (10 mg) was dissolved in 1 mL distilled water, and the pH of the solution was adjusted to 4.75. Solid EDC (10 mg) was added over a period of 30 min and the mixture was stirred for 1 h while maintaining the pH at 4.75 with 0.1 M HCl. The solution was kept at 50 °C by dropwise addition of 4 mL of 2 M aqueous NaBH4 over 2 h. The reaction was stopped by addition of acetic acid to destroy the excess borohydride. The reduction product of dsAHG (redAHG) was obtained by lyophilization after dialysis against distilled water over 24 h. The extent of reduction of the carboxyl groups was estimated by the HPLC method described in Section 2.5.

2.8.3. Methylation analysis of AHG derivatives

Methylation of dsAHG and redAHG were performed as described [30]. Briefly, 1.0 mg of polysaccharide was made completely dried in decomposition drier containing P2O5 for 5 h, and dissolved in 1.0 mL anhydrous dimethyl sulfoxide. By adding 100 mg anhydrous NaH powder, the mixture was stirred at room temperature for 1 h with N2 protection. 1.0 mL iodomethane was added to the mixture, and stirred with lightproof for a further 1 h. The reaction was stopped by addition of 1.0 mL water, and the residue was extracted with CHCl3. The extract was washed with distilled water and dried under vacuum. The extent of methylation was tested by the disappearance of OH groups in FT-IR spectrum. The former extract was hydrolyzed with 3 M trifluoroacetic acid at 105 °C for 6 h. The methylated monosaccharide residues were transformed into their partially methylated alditol acetates by reduction with NaBH₄ followed by acetylation with acetic anhydride and pyridine. The mixture was evaporated to dryness, dissolved in 100 μL dichloroethane. The analysis of partially methylated alditol acetate was performed on an Agilent DB 225 column (0.25 mm × 30 m) with an HP6890 instrument using a temperature gradient as follows: 100 °C to 220 °C with a rate of 5 °C/min, then held at 220 °C for 20 min. The identification and quantitation of each methylated sugar linkage were done by retention time and fragmentation pattern.

2.9. Anticoagulant assay

The anticoagulant activities of intact AHG were performed on lamb plasma according the specifications with a coagulometer. All assay kits were obtained from Nanjing Jiancheng Bioengineering Institute, China. The activated partial thromboplastin time (APTT) assay was carried out by mixing the lamb plasma (90 μL) with 10 μL of solutions with different amounts of polysaccharide in 0.9% NaCl and incubated at 37 °C for 5 min after addition of 100 μL prewarmed APTT assay reagent. Pre-warmed calcium chloride (100 μL, 0.25 M) was added and the clotting time was recorded. The results were compared with the heparin standard (150 unit/mL, Huixing Biochemistry Reagents Company Ltd., Shanghai, China).

The prothrombin time (PT) assay was conducted by mixing 90 μL of lamb plasma with 10 μL of sample and incubating at 37 °C for 3 min. Two hundred microliter of prewarmed PT assay reagent was added, and the PT was recorded.

The thrombin time (TT) assay was carried out by mixing 90 μL of lamb plasma with 10 μL of sample and incubating at 37 °C for 3 min. One hundred microliter of prewarmed TT assay reagent was added and the TT was recorded.

3. Results and discussion

3.1. Isolation and purification

The yield of AHG isolated from the fresh sea cucumber A. japonicus was 0.51% by weight. The extraction of AHG on a Sepharose Fast Flow column showed one single peak when eluted with 1.5 mol/L NaCl (Fig. 1a). The average molecular weight of AHG based on HPGPC was 58.07 kDa (dispersity: 1.48) and the chromatogram showed a single and symmetrical sample peak (Fig. 1b). The purity of AHG was confirmed by cellulose acetate membrane electrophoresis as it gave a single band in the electrophoretogram (Fig. 2). The migration rate of AHG was faster than the chondroitin sulfate standard (from Sigma-Aldrich) because of a higher ratio of charge/molecular weight.

3.2. Analysis of the chemical composition of AHG

According to the method described in Section 2.3, the AHG contained 58.54% total carbohydrate, 1.04% protein and 33.20% sulfate ester (Fig. 3a). Moreover, monosaccharide composition analysis based on pre-column derivatization reversed-phase HPLC showed that AHG consisted of GlcUA, GalNAc and Fuc (Fig. 3b and Table 1).
Fig. 1. Isolation of fucosylated chondroitin sulfates (AHG) from sea cucumber A. japonicus. (a) The crude polysaccharide was eluted with 1.5 mol/L NaCl on a Q Sepharose Fast Flow column; (b) HPGPC chromatogram of AHG on a Waters Ultrahydrogel™ Linear column (7.8 mm × 300 mm).

Fig. 2. Electrophoretogram of the cellulose acetate membrane electrophoresis of the chondroitin sulfate standard (1) and AHG (2).

Fig. 3. (a) HPCE chromatography for analysis of sulfate ester content; (b) HPLC chromatography for analysis of monosaccharide composition of AHG; (c) HPLC chromatography for analysis of core disaccharide units of AHG after partially acid hydrolysis. The disaccharides generated from exhaustive action of chondroitin ABC lyase on acid-resistant fragments of AHG were tested, and the numbered peaks corresponded to the elution positions of known disaccharide standards as follows: peak 1, ΔDi-0S; peak 2, ΔDi-6S; peak 3, ΔDi-4S; peak 4, ΔDi-diS. The peaks marked with x and y are from containments of the reaction system.

AHG was assigned to chondroitin sulfate as the GlcUA and GalNAc were approximately in a 1:1 ratio, which composed the backbone structure of →4)GlcUAβ(1 → 3)GalNAcβ(1 →. In addition, the sulfated fucose branch occurred in every disaccharide unit, which was similar to the acid polysaccharide obtained from S. japonicus [8].
Fig. 4. IR spectrum of the desulfated AHG (a) and the native AHG (b).

Fucose branches were more sensitive to the acid than the backbone formed by glucuronic acid and hexosamine, therefore they were removed by mild hydrolysis with acid [5]. The analysis of disaccharide composition showed that the core disaccharide units consisted of ΔDi-4S, ΔDi-6S and ΔDi-diS with a molar ratio of 0.20:1.00:1.08 (Fig. 3c). It was also demonstrated that 47.4% of the disaccharide units of the core AHG polymer were of the E-type structure of chondroitin sulfate.

3.3. Analysis of IR and NMR spectroscopy

As shown in Fig. 4a, the signal at 2932 cm⁻¹ (vC=H) indicated the presence of a fucose methyl group. The signal at 1632 cm⁻¹ (vN-H) was attributed to the acetamido group of the N-acetyl-D-galactosamine. The absorptions at 1239 cm⁻¹ (vC-O) and 820–860 cm⁻¹ (vC-O-S) confirmed the presence of sulfate in AHG. Furthermore, the signals at 820–860 cm⁻¹ possibly indicated the pattern of the sulfates: the absorption at 822 cm⁻¹ indicated the presence of 2,4-O-disulfated Fuc or 6-O-sulfated GalNAc; and the signal at 849 cm⁻¹ indicated the presence of 4-O-sulfated Fuc and/or GalNAc [31,32]. All the results showed that the AHG was a fucosylated chondroitin sulfate.

In the ¹H NMR spectra of intact AHG (Fig. 5a), the anomic proton signals at δ = 5.56 to 5.00 ppm were assigned to the α-configuration of pyranose units of various sulfated fucose residues. The regions from δ 3.4 to 4.2 ppm were attributed to protons of C-2 to C-6 of the hexosyl glycosidic ring. Finally, the signals at δ = 1.21 and 1.93 ppm were assigned to the methyl protons of Fuc (CH₃) and GalNAc (CH₃CO), respectively. In the ¹³C NMR spectra (Fig. 5b), three anomic carbon signals at δ = 105.3, 101.2 and 97.7 ppm were attributed to GlcUA, GalNAc and Fuc moieties respectively, according to the HMQC (Fig. 5d). The ¹H NMR spin system of the intact AHG was shown by the ¹H−¹H COSY spectra (Fig. 5c). The direct C−H coupling was determined by the ¹H−¹³C HMQC spectra. The major peak at 5.56 ppm in the ¹H NMR spectra was assigned to 2,4-disulfated α-fucose (Fuc2,4S), while the peaks at 5.22 and 5.27 ppm were assigned to 3,4-disulfated α-fucose (Fuc3,4S) and 4-sulfated α-fucose (Fuc4S), respectively, with a molar ratio of 1.00:0.49:0.32 based on peak area. The COSY spectra showed cross-peaks between adjacent protons of the hexosyl glycosidic ring. The data of direct C−H correlation obtained from HMQC and the ¹H and ¹³C chemical shifts of the glycosidic ring of intact AHG are summarized in Table 2.

Fig. 5. NMR spectra of the intact AHG. Spectra were determined at 23 °C on a JEOL ECP 600MHz spectrometer using acetone as internal standard. (a) ¹H NMR spectra; (b) ¹³C NMR spectra; (c) ¹H−¹H COSY spectra; (d) ¹H−¹³C HMQC spectra. Signals designated with A, A′, B and C refer to those produced by 2,4-di-SO₃-Fuc, 3,4-di-SO₃-Fuc, GalNAc and GlcUA, respectively.
### Table 1
Composition analysis of native AHG isolated from sea cucumber *A. japonicus*.

<table>
<thead>
<tr>
<th>Sea cucumber</th>
<th>M&lt;sub&gt;n&lt;/sub&gt; (kDa)</th>
<th>Mole ratio</th>
<th>GlcUA</th>
<th>GalNAc</th>
<th>Fuc</th>
<th>Sulfate</th>
</tr>
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<tr>
<td><em>A. japonicus</em></td>
<td>98.07</td>
<td>0.97</td>
<td>1.00</td>
<td>1.13</td>
<td>3.85</td>
<td></td>
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### Table 2

<table>
<thead>
<tr>
<th>Fucosylated chondroitin sulfate AHG</th>
<th>DHG&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>GlcUA</td>
<td>GalNAc&lt;sub&gt;4,6S&lt;/sub&gt;</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;/C&lt;sub&gt;1&lt;/sub&gt;</td>
<td>4.32/105.3</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;/C&lt;sub&gt;2&lt;/sub&gt;</td>
<td>3.47/73.9</td>
</tr>
<tr>
<td>H&lt;sub&gt;3&lt;/sub&gt;/C&lt;sub&gt;1&lt;/sub&gt;</td>
<td>3.56/78.3</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;/C&lt;sub&gt;4&lt;/sub&gt;</td>
<td>3.79/73.4</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;/C&lt;sub&gt;3&lt;/sub&gt;</td>
<td>3.45/74.3</td>
</tr>
<tr>
<td>H&lt;sub&gt;0&lt;/sub&gt;/C&lt;sub&gt;0&lt;/sub&gt;</td>
<td>4.18/4.08/67.6</td>
</tr>
<tr>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.93/23.9</td>
</tr>
<tr>
<td>C=O</td>
<td>--/176.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data from Yoshida et al. [8]. Chemical shifts were determined relatively to external TMS, 70 °C in D<sub>2</sub>O.

<sup>b</sup> Chemical shifts of the sulfation sites of intact AHG are highlight in bold.

### 3.4. Methylation analysis

The desulfation method used in the present work was efficient as identified by the absence of absorptions peaks at 1239 cm<sup>−1</sup> (ν<sub>SO</sub>) and 820–860 cm<sup>−1</sup> (ν<sub>C–O</sub>) in the IR spectrum (Fig. 4b). Reverse-phase HPLC indicated that the reduction of dsAHG was complete after two repetitions (data not shown). The extent of methylation of dsAHG and redsAHG was determined by the absence of the hydroxy signal in the IR spectrum (data not shown). The methylation analysis gave the linkage pattern of the sugar residues of AHG. The identification and the proportions of the methylated alditol acetates of dsAHG and redsAHG are listed in Table 3. The presence of 1,5-tri-O-acetyl-6-deoxy-2,3,4-tri-O-methyl-l-galactitol in both dsAHG and redsAHG showed that the fucose branch of intact AHG contained one single fucose residue. The presence of 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-d-glucitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-d-glucitol indicated that only a portion of the fucose branches (46.5%, based on molecular ratio) were linked to the O-3 position of a GlcUA moiety, and the remaining fucose branches were likely linked to the GalNAc moiety (53.5%). This was similar to the report previously described [10]. The sum of 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-d-glucitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-d-glucitol was in a mole ratio of 1:14:1.00 to 1,5-tri-O-acetyl-6-deoxy-2,3,4-tri-O-methyl-l-galactitol, which was well matched with the mole ratio of GlcUA and Fuc. According to the results from the unsaturated disaccharide composition analysis, the remaining fucosaccharides could link through the O-4/6 positions of the GalNAc moiety. To be precise, 8.8% of the remaining sulfated fucose branches were linked to the O-6 position of the GalNAc moiety and 43.8% through the O-4 position of GalNAc based on the molecular ratio of three unsaturated disaccharides present in AHG. The percentage distribution of core disaccharide units of AHG and possible branching positions through core polymers are summarized in Table 4. Fucosylation through either GlcUA or GalNAc possibly occurred at almost every disaccharide unit. If fucosylation through the O-3 position of a GlcUA moiety did not occur in the disaccharide units, a GalNAc moiety was fucosylated through the O-4/6 positions.

With all of the information combined, the hypothetical structures for fucosylated chondroitin sulfates in AHG were determined as shown in Fig. 6. The backbone in AHG was found to be a chondroitin sulfate-like structure of →4GlcUA[1 →3]GalNAc[1 →, and the sulfation occurred at the O-4 or O-6 positions of GalNAc.

The fucose branches (R) were depicted in two types depending on the sulfation patterns and were attached to the O-3 position of GlcUA moiety or the O-4/6 positions of GalNAc moiety in different proportions.

Previous reports demonstrated that the glycosaminoglycan obtained from sea cucumber *S. japonicus* contained branches composed of two fucopyranosyl moiety. 20% of which linked through O-3 position of GlcUA, 60% linked through the O-4 position of GalNAc, 10% through the O-6 position of GalNAc, and 10% through the O-4 and 6 positions of GalNAc. In the present study, similar linkage patterns were observed in glycosaminoglycan isolated from the sea cucumber *A. japonicus*, but the distribution of the fucose branches were quite different. The structures of the glycosaminoglycans in *S. japonicus* and *A. japonicus* therefore differed from each other, despite being of the same species. They were from different regions, however, which suggested that the environment could influence the structure of these bioactive compounds.
Table 3
Results of methylation analysis of the derivatized AHG.

<table>
<thead>
<tr>
<th>Methylation products</th>
<th>Mole ratio</th>
<th>Linkage pattern</th>
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<tbody>
<tr>
<td></td>
<td>dsAHG</td>
<td>redsAHG</td>
</tr>
<tr>
<td>1,5-tri-O-acetyl-6-deoxy-2,3,4-tri-O-methyl-L-Galactitol</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-α-glucitol</td>
<td>nd†</td>
<td>0.61</td>
</tr>
<tr>
<td>1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-β-glucitol</td>
<td>nd†</td>
<td>0.53</td>
</tr>
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</table>

† Not detected.

Table 4
Percentage distribution of core disaccharide units GlcUA(1 → 3)GalNAc derivatives and possible branching positions.

<table>
<thead>
<tr>
<th>Disaccharide unit</th>
<th>Zero-sulfated</th>
<th>4-O-sulfated</th>
<th>6-O-sulfated</th>
<th>4,6-di-sulfated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage</td>
<td>nd†</td>
<td>8.8</td>
<td>43.8</td>
<td>47.4</td>
<td>100</td>
</tr>
<tr>
<td>GalNAc</td>
<td>O-4,6b</td>
<td>0-6</td>
<td>0-4</td>
<td>–</td>
<td>53.5</td>
</tr>
<tr>
<td>GlcUA</td>
<td>O-310</td>
<td>0-3</td>
<td>0-3</td>
<td>0-3</td>
<td>46.5</td>
</tr>
</tbody>
</table>

† Not detected.
b Possible fucose-branching positions in GalNAc moiety or GlcUA moiety.
c Percentage of 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-α-glucitol compared with the sum of 1,3,4,5-tetra-O-acetyl-2,6-di-O-methyl-β-glucitol and 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-β-glucitol (see Table 3).

AHG was mostly attributed to the sulfated fucose branches according to a previous report [33]. The same results were observed in the present work where the APTT of intact AHG was 244.50 s, while that of defucosylated AHG was 44.24 s at the same concentration of 250 µg/mL.

For TT activity (Fig. 7b), the activities of AHG were slightly higher than heparin at the same concentration. Prolongation of APTT usually suggests inhibition of the intrinsic and/or common pathway, whereas prolongation of TT indicates inhibition of thrombin activity or fibrin polymerization. Thus, AHG inhibited both the intrinsic and/or common pathways of coagulation and thrombin activity or conversion of fibrinogen to fibrin.

4. Conclusions

We have isolated a novel fucosylated chondroitin sulfate from the sea cucumber A. japonicus collected from Qingdao, China. Detailed structure of the glycosaminoglycan, which differed from the glycosaminoglycan from sea cucumbers S. japonicus derived from the Japan sea area, was obtained and characterized by systematically using mild acid hydrolysis, enzymolysis, HPLC, 1/2D NMR and methylation analysis. The results indicated that the glycosaminoglycan had a chondroitin sulfate-like backbone structure of →4)GlcUAβ(1 → 3)GalNAcβ(1 → , with O-4 and/or O-6 positions of sulfation. Meanwhile, the sulfated fucose branches with different sulfation patterns may have occurred at the O-3 position of the n-GlcUA moiety or the O-4/6 position of n-GalNAc with distinguishable proportions. The anticoagulant assay indicated that the glycosaminoglycan possessed a high anticoagulant activity similar to heparin, which may be attributed to an interference with intrinsic and/or common pathways of coagulation and thrombin activity or conversion of fibrinogen to fibrin. The glycosaminoglycan isolated from A. japonicus could therefore be a potential anticoagulant drug. The structural studies on the glycosaminoglycan isolated from A. japonicus played an indispensable role in the understanding of the mechanism of anticoagulant activity. The detailed mechanism of anticoagulant action of the glycosaminoglycan is currently being investigated.

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Fig. 7. Analysis of the anticoagulant activities by APTT (a) and TT (b) on the intact AHG and heparin with different concentration.

3.5. Anticoagulant activities

The anticoagulant activities assay of intact AHG showed that the APTT and TT were effectively prolonged, and clotting inhibition was not observed in the PT assay (data not shown). The results indicated that the glycosaminoglycan isolated from A. japonicus had potential anticoagulant activity. For APTT activities, the potencies of AHG were similar to that of the standard unfractionated heparin (Fig. 7a). Below the concentration of 170 µg/mL, the anticoagulant activity of heparin was almost the same as the intact AHG at the same concentration, whilst the anticoagulant activity of AHG was lower at higher concentrations (>170 µg/mL). The anticoagulant activity of
References