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Structural comparison, antioxidant and anti-inflammatory properties of fucosylated chondroitin sulfate of three edible sea cucumbers

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ABSTRACT

Three fucosylated chondroitin sulfate (fCS) were obtained from edible sea cucumbers *Apostichopus japonicus, Stichopus chloronotus* and *Acaudina molpadioidea* collected from China. fCS from *Stichopus chloronotus* was firstly reported. The detailed structures of fCSs, particularly the fucose branches, were investigated and compared. ^{1}H and ^{13}C NMR of the polysaccharide identified three sulfation patterns of fucose branches: 4-O-, 2,4-di-O, and 3,4-di-*O*-sulfation variously existed in different fCSs. The backbone structure was confirmed by the monosaccharide composition and two-dimensional NMR. Antioxidant properties of fCSs were evaluated by the scavenging abilities on 1,1-diphenyl-2-picrylhydrazyl, nitric oxide radicals and inhibition of lipid peroxidation. The results showed that their activities could be affected by the sulfation patterns of the fucose branches, and O-4 sulfation is particularly important for its activities. The anti-inflammatory assays of fCS-*Am* showed significant reduction of the carrageenan induced edema in a dose depended manner, which could be used as a potential antiallergic agent.

1. Introduction

Sea cucumbers, belonging to the class Holothuroidea, have been used for tonic food and folk medicine in Asian countries for centuries, especially for China. There were many species of edible sea cucumbers distributed along the coast of China. *Apostichopus japonicus* is mostly commercially cultivated species in shallow ponds and by sea ranching in Dalian, Qingdao and Yantai of China (Yamana, Hamano, & Goshima, 2009). Besides, many sea cucumbers, for instance *Stichopus chloronotus* and *Acaudina molpadioidea*, were derived from the Huanghai Sea and Nanhai Sea areas of China (Bordbar, Anwar, & Saari, 2011). In spite of its extensive usage as food commodity and medicinal cure, the composition and identities of the bioactive chemical components of those sea cucumbers have not been fully investigated.

The main components of sea cucumber body wall were collagen and acidic polysaccharide, together with some triterpene glycosides and gangliosides (Chen et al., 2011). There were two kinds of sulfated polysaccharide isolated from sea cucumbers: fucosylated chondroitin sulfate (fCS) and sulfated fucan (Kariya, Kyogashima, Ishii, & Watabe, 1997; Kariya, Watabe, Hashimoto, & Yoshida, 1990; Ustyuzhanina et al., 2017). Due to its unique structures, fCSs have attracted considerable interest because of its various biological activities, such as antitumor, antiviral, immunomodulatory, anticoagulant and antithrombotic properties (Chen et al., 2011; Kale et al., 2013). However, the antioxidant activities of fCSs were rarely reported (Liu et al., 2012).

The structures of fCSs significantly effected its activities. There were many reports on the structures of fCSs isolated from different sea cucumbers, *Stichopus japonicus* (Kariya et al., 1997), *Ludwigothurea grisea* (Mourão et al., 1996), *Thelenata ananas* (Wu et al., 2012) and *Pearsonothuria graeffei* (Chen et al., 2011). The fCSs contained almost equal amount of β-D-glucuronic acid (GlcA) and N-acetyl-β-D-galactosamine (GalNAc), assembling the chondroitin sulfate like backbone structure. Besides, they had a unique sulfated fucose branches stretching from the O-3 positon of GlcA residues, while the sulfation patterns of the fucose branches of fCSs were the main differences in their structures (Chen et al., 2011). With the development of one and two dimensional (1/2D) NMR spectroscopy, the detailed structure of fCSs could be defined, which had been achieved for different holothurian glycosaminoglycans.

In the present research, we isolated fCSs from three sea cucumbers, which were edible seafood with great commercial value from sea waters of different geographical areas of China: *A. japonicus* from Bohai Sea, *S. Chloronotus* from the Xisha Islands and Nansha Islands of Nanhai Sea,

Abbreviations: fCS, fucosylated chondroitin sulfate; *Aj*, *Apostichopus japonicus*; *Sc*, *Stichopus chloronotus*; *Am*, *Acaudina molpadioidea*; PMP, 1-phenyl-3-methyl-5-pyrazolone; DPPH, 1,1-diphenyl-2-picrylhydrazyl; TFA, trifluoroacetic acid; GlcA, glucuronic acid; GalNAc, N-acetylgalactosamine; Fuc, fucose; Man, mannose; GlcN, glucosamine; Rha, rhamnose; GalA, galacturonic acid; GalN, galactosamine; Gal, galactose; Xyl, xylose; Arb, Arabinose; Fuc4S, O-4 sulfated fucose; Fuc2, 4S,2,4-*O*-disulfated fucose; Fuc3, 4S,3,4-*O*-disulfated fucose

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and *A. molpadioidea* from Fujian province of China. The structure of fCS from *S. Chloronotus* has not been reported previously. The structures, antioxidant and anti-inflammatory activities of three fCSs were investigated and compared. The detailed structures of three fCSs were defined by chemical composition analysis and 1/2D NMR spectroscopy as the primary analytical methods. The effects of sulfation patterns of fucose branches on the antioxidant activities were fully investigated.

2. Materials and methods

2.1. Materials and animals

Dry sea cucumbers *A. japonicus*, *S. chloronotus* and *A. molpadioidea* were purchased from a local market in Qingdao (China). Monosaccharide standards and 1-phenyl-3-methyl-5-pyrazolone (PMP) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Diastase Vera (EC 3.3.21.4) was purchased from Xuemei Zymin Technology Ltd. (Wuxi, China). Dextran T-series standards were from National Institutes for Drugs and Biological Products (Beijing, China). Ascorbic acid (Vitamin C, Vc), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and thiobarbituric acid were from Sigma-Aldrich (USA). Q Sepharose Fast Flow, Sephadex G25 and Sephadex G-10 were from Baoman Bio-Sciences Company Ltd., Shanghai, China. All other chemical reagents were of analytic grade.

Male Balb/c mice (30–35 g) were recruited and housed in temperature-controlled rooms and received food and water ad libitum. All experiments were carried out according to the currently established principles for the care and use of research animals and approved by the Ethics Committee of the Weifang Medical University, China.

2.2. Isolation and purification of fCSs from different sea cucumbers

Isolation and purification of fCSs from different sea cucumbers was carried out according to our previously reported method. Briefly, the sea cucumber body wall (ca. 100 g) was minced, homogenized and diluted to 1 L with distilled waters. 56 g KOH was added under continuous stirring at 60 °C for 1 h. The pH was adjusted to 8.5 with cold HCl. 5 g of Diastase vera (EC 3.3.21.4) was added and stirred at 50 °C for 3 h. The resulting solution was cooled down and centrifuged to remove the precipitate. One fold 95% (v/v) ethanol was added to the supernatant to precipitate the polysaccharide. The formed precipitate was collected and dissolved in distilled water at the ratio of 1:20 (g/mL). 1.5 M KAc was added into the supernatant and kept at 4 °C overnight. The crude polysaccharide was collected by centrifugation, dissolved in distilled water, dialyzed against distilled water for 36 h and lyophilized.

The crude polysaccharide was further fractionated by anion exchange chromatography on a Q Sepharose Fast Flow column (2.6 cm × 50 cm) eluted with 1.5 M NaCl and detected by the phenol-sulfuric acid method. The major polysaccharide fractions were further desalted on a Sephadex G25 column (1.6 cm × 100 cm).

2.3. Analysis of purification and average molecular weight

The purity and molecular weight distribution of obtained fCSs were 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 Na_2SO_4 at a flow rate of 0.5 mL/min. The molecular weight was calculated by a reference to a calibration curve made by a series of dextran T-series standards.

2.4. Determination of chemical compositions

Monosaccharide composition was determined by high performance liquid chromatography (HPLC). Briefly, 5 mg of polysaccharide was hydrolyzed by 2 M trifluoroacetic acid (TFA) at 110 °C for 8 h. The excess acid was removed by co-evaporating with methanol. The hydrolysate was derived with 100 μL 0.1 M NaOH and 100 μL of 0.5 M PMP (dissolved in methanol) at 70 °C for 30 min. The solution was neutralized with 0.1 M HCl and extracted by chloroform. The supernatant was subject to HPLC analysis on a Agilent ZORBAX Eclipse C18 column (5 μm, 4.6 mm × 150 mm) at 30 °C with detection at 245 nm.

Moreover, Sulfate ester content was tested according to the method previously described (Terho and Hartiala, 1971). Briefly, 1 mg of polysaccharide was hydrolyzed by 2 M TFA at 110 °C for 8 h. The hydrolysate was lyophilized, dissolved in 0.5 mL distilled water and barium determined by barium chloride-gelatin colorimetric method.

Table 1
Chemical composition of fucosylated chondroitin sulfates isolated from three different sea cucumbers.

Sea cucumbers	Mw (kDa)	Mole ratio			
		GlcA	GalNAc	Fuc	Sulfate
A. japonicus	98.1	0.98	1.00	1.15	3.65
S. chloronotus	111.0	0.90	1.00	1.08	3.18
A. molpadioidea	93.3	0.82	1.00	0.88	3.04

2.5. IR and NMR spectroscopy

The fCSs were 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 NMR, ^{13}C NMR, ^1H-^1H COSY, HSQC and NOESY spectra of polysaccharide (ca. 40 mg) were recorded at 25 °C using a JEOL-ECP 600 MHz spectrometer after co-evaporation with 500 μL D_2O (99.9%, Sigma-Aldrich) twice followed by dissolution in 500 μL D_2O. The tetramethylsilane (TMS) was used as the external standard.

2.6. In vitro free radical scavenging activities of fCSs

2.6.1. 1.1-Diphenyl-2-picryl hydrazyl (DPPH) scavenging assay

Scavenging ability of DPPH radicals was tested based on previous method (Shibata, Suzuki, Kobayashi, & Okawa, 2007). Briefly, 1 mL of polysaccharide solution at different concentrations (0.25–4.0 mg/mL) was mixed with 3 mL of 0.1 mM solution of DPPH in ethanol. The mixture was measured at 517 nm after 30 min. Vc was used as positive control in all assays.

2.6.2. Nitric oxide scavenging assay

The nitric oxide scavenging assay was carried out by the previous method (Green et al., 1982). 5 mM of sodium nitroprusside in phosphate buffered saline was mixed with 3 mL of fCSs of different concentrations and reacted at 25 °C for 150 min. The above mixture were applied to react with Greiss reagent. The nitric oxide scavenging abilities of different samples were determined by measuring the absorbance of chromophoric substances at 546 nm. Vc was used as positive control in all assays.

2.6.3. Lipid peroxidation inhibition assay

0.8 mL of lecithovitellin, which was made by mixing the yolk with PBS (pH 7.4) in the ratio of 1:25, was mixed with polysaccharide solution with different concentration (2, 4, 8, 10 mg/mL) and 0.4 mL of 25 mmol/L $FeSO_4$. The mixture was kept at 37 °C for 60 min. Afterwards, 1.0 mL of 20% trichloroacetic acid (w/v) and 1 mL of 0.8% thiobarbituric acid were added to the mixture and kept in boiling water bath for 15 min. The mixture was centrifuged at 3500 rpm/min for 10 min. The extent of lipid peroxidation was determined by estimation of thiobarbituric acid reactive substances (TBARS) level by measuring