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Conformational and physicochemical properties of fucosylated chondroitin sulfate from sea cucumber *Apostichopus japonicus*

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ABSTRACT

This study aimed at investigating the chain conformation and physicochemical properties of fucosylated chondroitin sulfate extracted from sea cucumber *Apostichopus japonicas* (*Aj*-fCS). By using HPSEC-MALLS-Visc-RI, M_w, $\langle s^2 \rangle_z^{1/2}$, R_h and [η] for *Aj*-fCS were determined as 58.0 ± 4.4 kDa, 21.8 ± 1.3 nm, 12.5 ± 1.3 nm and 27.8 ± 0.5 mL/g respectively. Conformation parameter α_s derived from the relationship of M_w-$\langle s^2 \rangle_z^{1/2}$ (0.39) and structure-sensitive parameter ρ (1.74) consistently indicated that *Aj*-fCS adopted a random coil conformation in solution, which was also supported by atomic force microscopy. Stiffness parameters of *Aj*-fCS chains including *q* (2.72 nm), *d* (1.03 nm) and C_∞ (5.28) were furthermore deduced from the worm-like cylinder model. *Aj*-fCS demonstrated a shear-thinning rheological behavior, relatively low apparent viscosity, negative charge in wide pH and ionic strength ranges, and favorable thermostability. These results have important implications for designing and fabricating functional foods or drugs based on *Aj*-fCS.

1. Introduction

Fucosylated chondroitin sulfate, a structurally distinct glycosaminoglycan derived from the body wall of the sea cucumber, consists of a chondroitin sulfate backbone with sulfated or non-sulfated fucose side chains (Myron, Siddiquee & Al Azad, 2014). Various bioactivities of fucosylated chondroitin sulfate have been established, such as anti-coagulatory (Chen et al., 2012; Mourão et al., 2001), anti-thrombotic (Chen et al., 2012, 2013), anti-hyperglycemic (Hu et al., 2014) and anti-tumor (Borsig et al., 2007) effects. Fucosylated chondroitin sulfate has been therefore considered as a potential ingredient for the development of new functional foods and drugs (Hu, Tao, Wang, Xiao & Wang, 2016; Myron et al., 2014; Suleria, Gobe, Masci & Osborne, 2016).

Studies have shown that the chain conformation of polysaccharides significantly affects their bioactivity (Blaschek, Käsbauer, Kraus & Franz, 1992; Falch, Espevik, Ryan & Stokke, 2000; Kojima, Tabata, Itoh & Yanaki, 1986). For example, (1 → 3)-β-D-glucan, in the triple helical state, is capable of suppressing the growth of S-180 tumor; however, this compound was found to be ineffective in the random coil state (Yanaki et al., 1983). The anti-tumoral activity of triple helical lentinan decreases significantly with transition into a single chain (Maeda, Watanabe, Chihara & Rokutanda, 1988; Surenjav, Zhang, Xu, Zhang & Zeng, 2006; Zhang, Li, Xu & Zeng, 2005). The specific physicochemical properties of polysaccharides are considered useful for application in the health care and food industries. For example, the favorable gelling properties of κ-carrageenan and ι-carrageenan (Funami et al., 2007), ability of alginate to form edible wet coatings (Comaposada, Gou, Marcos & Arnau, 2015), and negative charged properties of fucoidan to form multilayer nano-emulsions (Chang and McClements, 2015) make these compounds suitable for use as functional food ingredients. Therefore, an understanding of chain conformation and physicochemical propeties of fucosylated chondroitin sulfate is crucial for elucidation of the relationship between its structure and function as well as for its application in the food and pharmaceutical industries.

Apostichopus japonicas is one of the most important species of sea cucumber with high commercial value. The primary structure of fucosylated chondroitin sulfate from *A. japonicus* (*Aj*-fCS) comprises of an alternating 4-linked glucuronic acid and 3-linked N-acetyl galactosamine backbone; the most abundant branched residues in this compound are 2, 4-di-*O*-sulfated and 3,4-di-*O*-sulfated fucose (Yang, Wang, Jiang & Lv, 2015). To date, the chain conformation and physicochemical properties of *Aj*-fCS remains unknown. There are only few reports involving the chain information, weight average molecular weight (M_w) and intrinsic viscosity ([η]) of fucosylated chondroitin sulfate from other sea cucumbers. Fucosylated chondroitin sulfate of *Thelenata ananas* has been found to possess an

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intermediate conformation between the open coil and stiff rod conformations, in 0.1 M NaCl (Wu, Xu, Zhao, Kang & Ding, 2010b), and M_w and [η] of this compound were estimated to be 65 kDa and 48.7 mL/g, respectively. The morphology of fucosylated chondroitin sulfate from *Acaudina molpadioidea* (Zou, Pan, Dong, He & Wang, 2016) comprises a fibrous net with a height of nearly 3 nm as visualized by atomic force microscopy (AFM). Although fucosylated chondroitin sulfate extracted from different species of sea cucumber may have similar monosaccharide composition, difference could be obviously found on their delicate primary structure characteristics including branched structure and sulfated patterns (Dong et al., 2014; Wu, Xu, Zhao, Kang & Ding, 2010a), which would result in variance of their chain conformation and physicochemical properties. Therefore, research is needed to identify the specific properties for a particular polysaccharide.

This study was aimed at elucidating the chain conformation and physicochemical properties of *Aj*-fCS. The molecular characteristics and chain conformation were calculated by high-performance size-exclusion chromatography combined with multi-angle laser scattering, viscometry, and differential refractive index detection (HPSEC-MALLS-Visc-RI) and AFM. The physicochemical properties of *Aj*-fCS are analyzed in terms of rheological characteristics, ζ-potential, and thermal stability.

2. Experimental methods

2.1. Materials

Dried sea cucumber *A. japonicas* was purchased from Nanshan market (Qingdao, China) in April 2015. The homology between the CO1 sequence of the sea cucumber specimen and the known CO1 sequence of *A. japonicas* was up to 99%, which confirmed that the specimen studied was *A. japonicas*. All chemicals and reagents used were of analytical grade.

2.2. Extraction and purification of Aj-fCS

Fucosylated chondroitin sulfate was extracted and purified from the body wall of the present *A. japonicas* specimen, according to the method of Yang et al. (2015) with some modification. Briefly, the milled dried body wall of the sea cucumber was hydrolyzed using papain and precipitated using cetylpridinium chloride to obtain crude sulfated polysaccharides. Then, the crude sulfated polysaccharides were applied to an Express-Ion D (Whatman, USA) column using the AKTA UPC 100 (GE, USA) system and eluted with 0 M to 2.0 M linear gradient of NaCl. Collected fractions containing *Aj*-fCS (NaCl concentration: 0.6–1.0 M) were then purified using a Sephacryl S-500 (GE Healthcare, USA) column with 0.2 M NH_4HCO_3 as elution buffer. A refractive index detector (Agilent 1260, Agilent Technologies, USA) was employed for detection and the final purified *Aj*-fCS samples were collected, dialyzed, and lyophilized.

2.3. HPSEC-MALLS-Visc-RI analysis

An HPSEC-MALLS-Visc-RI system consisting of a pump (Agilent 1200, Agilent Technologies, USA, Santa Clara, CA), a SEC column (OHpak SB-806 HQ, 8.0 mm × 300 mm, Shodex, Japan), an Optilab T-rEX differential refractive index detector (Wyatt Technology Co., Santa Barbara, CA), a Viscostar-II viscometer (Wyatt Technology Co., Santa Barbara, CA), and a multi-angle laser light scattering (MALLS) instrument equipped with a He-Ne laser (λ = 633 nm) (DAWN DSP, Wyatt Technology Co., Santa Barbara, CA) was used in this study. A 0.15 M NaCl solution (pH 7.4, buffered by 10 mM Na_2HPO_4—NaH_2PO_4) was used in these experiments owing to its high utility in numerous bioactivity assays. The flow rate and the column temperature were set at 0.4 mL/min and 25 °C respectively. *Aj*-fCS solution was prepared in 0.15 M NaCl solvent (pH 7.4) at a concentration of 2 mg/mL and stirred for 12 h. Then, the 50 μL sample solution was injected into the system. The M_w and mean square radius of gyration ($\langle s^2 \rangle_z^{1/2}$) were calculated using the Zimm method. The instrumental software ASTRA 6.1.2 (Wyatt Technology Corp.) was utilized for data acquisition and analysis. The specific refractive index increments (dn/dc) of *Aj*-fCS in 0.15 M NaCl solvent (pH 7.4) were determined using the refractive index detector (Optilab T-rEX, Wyatt Technology Co., Santa Barbara, CA) at 25 °C. Samples were dissolved in 0.15 M NaCl solvent (pH 7.4) with increasing concentrations, from 0.5 mg/mL to 2.5 mg/mL, in order to determine the slope of the increment. All polysaccharide solutions were purified using a 0.2 μm filter (PTFE, Puradisc 13-mm Syringe Filters, Whatman, England) before use.

2.4. Molecular morphology observation

The molecular morphology was visualized using an atomic force microscope (Agilent Technologies, USA). The polysaccharide solution (10 μL), at a concentration of 10 μg/mL in water, was placed drop-wise onto freshly cleaved muscovite mica substrate and allowed to dry for more than 1.5 h. Freshly prepared samples were mounted on the AFM stage and imaged under tapping mode in air (25 °C, ambient pressure and humidity).

2.5. Steady shear measurement

Aj-fCS was dissolved in 0.15 M NaCl (pH 7.4) solution at concentrations of 1 mg/mL–15 mg/mL, with mild stirring, for 12 h at 25 °C. The apparent viscosity of the above samples was determined using a Physica MCR301 rheogoniometer (Anton Paar Co., Ltd., Austria) equipped with a PP50 flat plate (of diameter 50 mm). Viscosity data were collected at shear rates from 1 s^{-1} to 300 s^{-1} at a constant temperature of 25 °C, and fitted against the Cross model using OriginPro 9.0 software (OriginLab., USA).

2.6. ζ-Potential analysis

The ζ-potential of fucosylated chondroitin sulfate samples was determined using a microelectrophoresis device (Nano-ZS90, Malvern Instruments, Worcestershire, UK). For analyzing the influence of pH, *Aj*-fCS was dispeased in a series of 0.15 M NaCl solutions with different pH from 2.0 to 12.0 (adjusted by HCl or NaOH solution) at 2 mg/mL. For analyzing the influence of ionic strength, *Aj*-fCS (2 mg/mL) solutions were prepared by the 0.05–0.80 M NaCl solution with a constant pH value of 7.4 (buffered by Na_2HPO_4—NaH_2PO_4). All experiments were performed at 25 °C, with the laser beam operated at 659.0 nm.

2.7. Thermal properties of Aj-fCS

Thermal gravimetric analysis (TGA) data were obtained using a NETZSCH TG 209F1 analyzer (NETZSCH, Selb, Germany) in a nitrogen atmosphere. Approximate 30 mg of *Aj*-fCS sample was heated from 30 °C to 800 °C at a ramp rate of 10 °C/min. Differential scanning calorimetry (DSC) analysis was conducted using a NETZSCH DSC 200PC analyzer (NETZSCH, Selb, Germany). Thirteen milligrams of sample were run from 30 °C to 600 °C, at a rate of 10 °C/min.

2.8. Statistical analysis

All experimental data were obtained out in triplicate (n = 3). Data are presented as mean values with standard deviation. Multiple mean values were tested for significance using analysis of variance