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Primary structure and chain conformation of fucoidan extracted from sea cucumber *Holothuria tubulosa*

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\section*{Highlights}

Fucoidan from sea cucumber *Holothuria tubulosa* (Ht-FUC) was extracted. > Structural characteristics of Ht-FUC were clarified at several hierarchical levels. > Ht-FUC was composed of an $\alpha$1,3-linked tetrafucose repeating unit. > It adapted random coil conformation in 0.15 M NaCl solution. > The chain conformation of sea cucumber fucoidan was elucidated for the first time.

\section*{Abstract}

Knowledge of the structure of polysaccharides is essential for understanding and controlling their functional properties. In this study, fucoidan was extracted from *Holothuria tubulosa*, a species of sea cucumber that has recently become commercially important. The primary structure of the *H. tubulosa* fucoidan was
clarified using a combination of enzymatic degradation, methylation analysis, and NMR, and its chain conformation was further elucidated by utilizing high performance size exclusion chromatography combined with multiple angle laser light scattering and viscometry. The fucoidan was composed of a tetrafucose repeating unit \([\rightarrow 3-\alpha-L-Fucp2(OSO_3^-)-1\rightarrow 3-\alpha-L-Fucp2,4(OSO_3^-)-1\rightarrow 3-\alpha-L-Fucp-1\rightarrow 3-\alpha-L-Fucp2(OSO_3^-)-1\rightarrow] \). Its sulfate content was determined to be 31.2±1.6% and the weight-average molecular mass was 1567.6±34.1 kDa. The molecule adapted a random coil conformation in 0.15 M NaCl solution (pH 7.4) at 25 °C, with a root-mean-square radius of 63.9±1.8 nm and a hydrodynamic radius of 44.5±4.5 nm. This is the first report on the chain conformation of sea cucumber fucoidan.

Keywords
Sea cucumber; Fucoidan; Primary structure; Conformation; Holothuria tubulosa

1. Introduction
Fucoidan (also referred to as sulfated fucan) is a polysaccharide mainly composed of fucose and sulfate groups that is found in brown algae and sea cucumber (Vo & Kim, 2013). Algal fucoidans have been shown to exhibit a broad range of biological activities (Senthilkumar, Manivasagan, Venkatesan & Kim, 2013; Wijesinghe & Jeon, 2012), and have already been used as nutraceutical ingredients in many food products. Sea cucumber fucoidans have also attracted increasing interest in the past two decades for their potential as nutraceutical ingredients. Various bioactivities of sea cucumber fucoidans have been demonstrated, including anticoagulant (Ribeiro, Vieira, Mourão & Mulloy, 1994), osteoclastogenesis inhibiting (Kariya et al., 2004), neural stem/progenitor cells proliferating (Zhang et al., 2010), protection against ethanol-induced gastric ulcer (Wang et al., 2012), anti-oxidation (Guo et al., 2014), insulin resistance modifying (Hu et al., 2014; Xu et al., 2014b), anti-adipogenic (Xu et al., 2014a) and intestinal mucositis preventing effects (Zuo et al., 2015). Meanwhile it has been suggested that sea cucumber fucoidans could also be utilized as functional food ingredients, e.g., coating agent of nano-emulsions (Chang & McClements, 2015).

The primary structures of several sea cucumber fucoidans have already been clarified, which were summarized in Fig. 1A. Compared to algal fucoidans whose structure are usually complex and heterogeneous (Morya, Kim & Kim, 2012), sea cucumber fucoidans may have relatively well-defined repeating structures, which would facilitate the interpretation of their performance at a molecular level (Pomin, 2009). Fucoidans from sea cucumber Ludwigothurea grisea (Ribeiro et al., 1994), Isostichopus badionotus (Chen et al., 2012), Acaudina molpadioides (Yu et al., 2014a; Yu et al., 2013) and Thelenota ananas (Yu et al., 2014b) are all linear polysaccharide consisting of \(\alpha 1\rightarrow 3\) linked tetrafucose repeating units, but with different sulfation patterns. Fucoidans from Stichopus japonicas (Kariya et al., 2004) and Apostichopus japonicas (Yu et al., 2015) are branched polysaccharides, and a pentasaccharide repeating unit was found as the major structural component of A. japonicas fucoidan.
Up to date, all reported primary structures of fucoids extracted from different species of sea cucumber are different from each other.

Recent studies have revealed that the functionality of sea cucumber fucoids is closely related to their structural characteristics. For instance, fucoidan from *T. ananas* shows a significantly higher inhibitory effect on superoxide radicals than *I. badionotus* fucoidan, which indicates that the sulfation pattern plays an important role in the bioactivity of sea cucumber fucoidan (Yu et al., 2014b). The viscosity and rheological behavior of *A. japonicas* fucoidan are distinctly different from those of *A. molpadioides* fucoidan, which demonstrates that the rheological characteristics of sea cucumber fucoidan are significantly influenced by branch structure (Yu et al., 2015). More precise structure-functionality relationships are expected to be established in future if more sea cucumber fucoids have their structure defined, and those relationships will further facilitate the rational design of functional foods. The structural characterization of sea cucumber fucoidan is therefore of both theoretical and practical interest.

It should be stressed that besides primary structure, structural characteristics at higher hierarchical levels, such as chain conformation, also have a profound influence on the functionality of polysaccharides. For example, (1→3)-β-D-glucan adopting triple helix conformation manifests significantly higher anticancer activity compared to that in single flexible chain fashion (Surenjav, Zhang, Xu, Zhang & Zeng, 2006). At present, the chain conformation of sea cucumber fucoidan has not been reported.

*Holothuria tubulosa* is a species of sea cucumber that is widely distributed in the Mediterranean Sea and eastern areas of the Atlantic Ocean, and has recently become commercially important (Aydin, 2008). In this study, we aimed to extract fucoidan from *H. tubulosa* (*Ht-FUC*) and investigate its structural characteristics at different hierarchical levels, i.e., primary structure and chain conformation.

### 2. Materials and methods

#### 2.1. Preparation of fucoidan from *H. tubulosa*

Dry sea cucumber *H. tubulosa* was purchased from a local market in Qingdao, China. Besides conventional morphological methods, a phylogenetic method employing 16S mitochondrial ribosomal DNA (16S rDNA) gene was also utilized to identify the species of purchased sea cucumber (Kerr et al., 2005). The similarity between 16S rDNA sequence of purchased sea cucumber (Supplementary Data 1) and the known 16S rDNA sequence of *H. tubulosa* (Genbank No. EU191976.1) was up to 99%.

*Ht-FUC* was prepared according to a method described previously (Ribeiro et al., 1994) with a few modification. Briefly, the milled body wall of sea cucumber *H. tubulosa* was hydrolyzed by papain (Nanning Pangbo Biological Engineering Co., China), and cetylpyridinium chloride was subsequently added to precipitate crude sulfated polysaccharide. The obtained crude sulfated polysaccharide was applied on an Express-Ion D (Whatman, USA) column using ÄKTAprime plus system (GE Healthcare, USA) and eluted with 0 to 2.0 M linear gradient of NaCl. Fraction containing fucoidan confirmed by the monosaccharide composition determination was collected, dialyzed, lyophilized, and further purified on a gel filtration column.
(HiPrep 26/60 Sephacryl S-500 HR column, GE Healthcare, USA) which was eluted with 0.2 M \( \text{NH}_4 \text{HCO}_3 \). The carbohydrate content in the eluate was measured by the \( \text{H}_2\text{SO}_4 \)-phenol method (Dubois, Gilles, Hamilton, Rebers & Smith, 1951). The finally purified fucoidan was collected, dialyzed, lyophilized, and then used in following analysis as Ht-FUC.

2.2. Compositional analysis

The monosaccharide composition of Ht-FUC was determined by high performance liquid chromatography (Strydom, 1994). Two microgram Ht-FUC was hydrolyzed by 1 mL 2 M trifluoroacetic acid at 110 °C for 8 h; After neutralization, the hydrolysate was labeled by 1-phenyl-3-methyl-5-pyrazolone (Sigma, USA) and subsequently analyzed employing a HPLC system (Agilent 1200, USA) equipped with a reversed-phase column (Zorbax Eclipse XDB-C18, 4.6mm × 150mm, 5µm, Agilent, USA); The composition and content of monosaccharides were determined referring to the retention time and ultraviolet absorbance response of monosaccharide standards. The content of sulfate groups in Ht-FUC was determined by the \( \text{BaCl}_2 \)-gelatin method (Silvestri, Hurst, Simpson & Settine, 1982).

2.3. Methylation analysis

The Ht-FUC was dissolved in deionized water, loaded on an AG50W-XS (H\(^+\) form, 100µL; Bio-Rad, USA) column and eluted with 10 mL water. The eluent was adjusted to pH 9.0 with pyridine to obtain fucoidan-pyridine salt. After rotary evaporation and lyophilisation, 10 mg fucoidan-pyridine salt was dissolved in 2 mL dimethyl sulfoxide/methanol solvent (v/v=9:1) and incubated 10 h at 80 °C for desulfation. The desulfated sample was subsequently methylated according to the method of Hakomori (Hakomori, 1964). Thereafter, the methylated polysaccharide was hydrolyzed in 2 M trifluoroacetic acid at 100 °C for 8 h and dried under a steam of nitrogen. The hydrolysate was then reduced with sodium borohydride at room temperature for 2 h and acetylated with acetic anhydride at 100 °C for 1 h. The obtained product was finally analyzed by gas chromatography-mass spectroscopy (GC-MS) (Agilent 6890, Agilent, USA) equipped with a DB-225MS fused-silica capillary column (30 mx0.32 mmx0.25 m, Agilent, USA). The analysis of GC-MS spectra referred to the Complex Carbohydrate Structural Database of University of Georgia.

2.4. Enzymatic preparation of low molecular weight polysaccharide

The fucoidanase employed to degrade Ht-FUC was prepared as a previously described method (Yu et al., 2014b). Briefly, the bacterial strain Flavobacteriaceae CZ1127 was cultivated and its intracellular supernatant was extracted. The fucoidanase was thereafter precipitated by \( \text{(NH}_4 \text{)}_2\text{SO}_4 \) from the intracellular supernatant and further purified by a cellulase sulfate column (Chisso, Japan). Active fractions were collected, pooled, and dialyzed against 20 mM Tris-HCl (pH 7.2). The resulted enzyme was named as CZ1127 fucoidanase.

Fifty milliliters of CZ1127 fucoidanase was mixed with 50 mL 0.4% (w/v) Ht-FUC solution containing 20 mM Tris-HCl (pH 7.2) and 0.3 M NaCl. This mixture was incubated at 35 °C for 24 h, and heated at 100°C for 10min. After centrifugation and
lyophilisation, the crude low molecular weight polysaccharide was obtained. It was subsequently applied on a HiPrep 26/60 Sephacryl S-300 HR column (GE healthcare, USA) and eluted with 0.2 M NH₄HCO₃ at 1.2 mL/min. The purified fractions was collected, dialyzed against water, lyophilized and nominated as Ht-LMW.

To investigate whether desulfation occurred during the degradation, sulfate content of Ht-LMW was determined and compared with that of Ht-FUC. The molecular mass of Ht-LMW was analyzed according to the method described in the section 2.5.

2.4. NMR Experiment

Fifty milligrams Ht-FUC or 25 mg Ht-LMW was co-evaporated twice with deuterium oxide (D₂O, 99.9%; Sigma, USA) by lyophilisation and finally dissolved in 500 µL D₂O containing 0.1 µL 4, 4-dimethyl-4-silapentane-1-sulfonic acid (DSS). NMR spectra were recorded by the AVANCE III 600 NMR equipped with a cryoprobe (Bruker, Germany). The 1-dimensional ¹H NMR spectra and 2-dimensional correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and nuclear overhauser effect spectroscopy (NOESY) spectra were all obtained at 600 MHz and 25 °C with sufficient acquisition time. The observed ¹H chemical shifts were calibrated according to DSS (δ 0.00 ppm).

2.5. HPSEC-MALLS-Vis-RI analysis

The molecular mass, root-mean-square radius (⟨s²⟩₁/₂), hydrodynamic radius (R₉ₐ), and chain conformation of Ht-FUC were determined by using high performance size exclusion chromatography combined with multiple angle laser light scattering, viscosity, and refractive index detectors (HPSEC-MALLS-Vis-RI). This system consisted of an Agilent 1200 HPLC (Agilent, USA), a size exclusion column (Shodex OHPAK SB-806 HQ, 8.0 mm × 300 mm, Shodex, Japan), a Dawn Heleos-II multi-angle laser light scattering instrument (Wyatt, USA), a Viscostar-II viscometer (Wyatt, USA) and an Opti-lab T-rEX refractive index detector (Wyatt, USA). A 0.15 M NaCl solution containing 5 mM phosphate buffer (pH 7.4) and 0.02 % (w/v) NaN₃ was employed as eluent. The temperature of column, viscometer and refractive index detector was set at 25 °C, and the flow rate was set to 0.4 mL/min. The injection volume was 50 µL with a concentration of 2 mg/mL for the sample. Data were collected and processed using ASTRA software (Version 6.1.2.84, Wyatt, USA). The curve fitting for calculating Mₐ was based on a first-order Zimm plot method. The specific refractive index increment (dn/dc) of Ht-FUC in the eluent was 0.1114 mL/g, which was measured by utilizing the off-line refractive index detector and Ht-FUC solutions with gradient concentrations (0.2-1 mg/mL). The HPSEC-MALLS-Vis-RI measurement was performed in triplicate.

2.6. Statistical analysis

The Tukey’s post-hoc test was performed to determine individual differences by using PASW Statistics 18.0 (IBM SPSS, USA). Statistical significance was considered when p value was less than 0.05.

3. Results and discussion

3.1. General composition
The yield of Ht-FUC from the dried body wall of *H. tubulosa* was 2.5%. Fucose was the only monosaccharide found in Ht-FUC. It was reported that besides fucose algal fucoids usually contain other monosaccharides, e.g., mannose, galactose, glucose, xylose, and uronic acids (Vo & Kim, 2013), while most reported sea cucumber fucoids including Ht-FUC solely contain fucose, and therefore in some studies sea cucumber fucoids are also referred to as “sulfated fucan” (Berteau & Mulloy, 2003; Chen et al., 2012; Ribeiro et al., 1994). The sulfate content of Ht-FUC was determined as 31.2±1.6%, which was similar to that of the fucoidan from sea cucumber *I. badionotus* (32.9%) (Chen et al., 2012), and higher than those of *T. ananas* fucoidan (28.2%) (Yu et al., 2014b) and *A. molpadioides* fucoidan (26.3%) (Yu et al., 2014a). It was suggested that the Ht-FUC possessed a similar ratio of sulfate group / fucose residue to *I. badionotus* fucoidan (1:1 as shown in Fig. 1A).

The Ht-FUC demonstrated a single peak with a polydispersity index of 1.15 in its HPSEC chromatogram (Fig. 2), which indicated that the Ht-FUC was well purified. The molar mass of Ht-FUC ranged from about 3091.1 kDa to 793.9kDa. The weight-average molar mass (*M*_w) was calculated to be 1567.6±34.1 kDa. Additionally, the number-average molar mass, z-average molar mass and peak molar mass were 1362.9±12.3 kDa, 1800.7±34.2 kDa and 1371.1±11.0 kDa respectively.

### 3.2. Primary structure

The result of methylation analysis showed that the major methylated alditol acetate derived from Ht-FUC is 1,3,5-tri-O-acetyl-2,4-di-O-methylfucitol. Its peak area in mass spectrometry was 80% of the total. Besides, 1,5-tri-O-acetyl-2,3,4-di-O-methylfucitol (16%) and trace amounts of 1,3,4,5-tetra-O-acetyl-2-O-methylfucitol (3%) were observed. These results indicated that Ht-FUC mainly consisted of 3-linked and terminal fucose residues, i.e., the major type of glycosidic linkage in Ht-FUC was 1→3.

The ^1^H NMR spectrum of Ht-FUC was ambiguous and overlapped (Fig. 3A), which might be attributed to the high *M*_w of Ht-FUC. Therefore, controlled degradation of Ht-FUC was performed to enhance the resolution of NMR spectra. After enzymatic degradation and purification, Ht-LMW with a *M*_w of 128.7 kDa was obtained (the purification chromatogram could be found in supplementary data Fig. S1). The yield of Ht-LMW was up to 82%, indicating that the majority of Ht-FUC was converted into Ht-LMW. There was no significant difference between the sulfate contents of Ht-LMW (30.5±2.0%) and Ht-FUC (31.2 ± 1.6%), which confirmed that the sulfate group was not cleaved during the degradation. Furthermore, the ^1^H NMR spectra of Ht-LWM (Fig. 3B) and Ht-FUC had similar patterns. In conclusion, the primary structure of Ht-FUC could be represented by that of Ht-LMW.

Ht-LWM gave much clearer NMR spectra than Ht-FUC. Strong signals around δ 1.2 ppm were observed in the ^1^H NMR spectrum of Ht-LWM (Fig. 3B), which are the characteristic signals of methyl protons of the L-fucose residue (Pereira, Mulloy & Mourão, 1999). Unambiguous signals at δ 5.36 ppm, δ 5.33 ppm, δ 5.29 ppm, δ 5.02 ppm were also observed. Correspondingly, four spin systems starting from them were found in the COSY spectrum (Fig. 4A). These signals could be attributed to the anomeric resonances of four α-configuration fucose residues A, B, C and D (Mourão
et al., 1996). Their intensity integrations were approximately equal (Fig. 3C), which suggested that a tetrasaccharide repeating unit was present in Ht-LMW. According to the correlation peaks in COSY and TOCSY (Fig. 4B), $^1$H chemical shifts of Ht-LWM could be well assigned and listed in Table 1 ($^1$H NMR spectrum with detailed annotation could be found in Fig. S2).

The positions of sulfate groups were further elucidated by comparing each proton chemical shift with that of nonsulfated fucose residue. The O-sulfation would render the chemical shift of oxymethine protons shifted to downfield by 0.4-0.8 ppm (Yamada, Yoshida, Sugiura & Sugahara, 1992). As H-2 and H-4 signals of residue A apparently shifted to downfield, residue A was deduced as 2,4-$O$-sulfated fucose residue. In the same way, residues B and C were clarified as 2-$O$-sulfated fucose residues, while residue A was nonsulfated fucose residue.

The glycosidic linkage and the sequence of residues were clarified according to the correlation peaks in NOESY (Fig. 4C). In detail, correlation from H-1 of residue B to H-3 of residue A, correlation from H-1 of residue A to H-3 of residue D, correlation from H-1 of residue D to H-3 of residue C, and correlation from H-1 of residue C to H-3 of residue B were observed. As the result, the sequence of residues in Ht-LWM could be confirmed as $\rightarrow$3B1$\rightarrow$3A1$\rightarrow$3D1$\rightarrow$3C1. This linkage type deduced from NMR was in a good accordance with the result of methylation analysis.

Combining all information of above analysis, the primary structure of Ht-LMW was elucidated as $\rightarrow$3-$\alpha$-L-Fucp2(OSO$_3$)$_{-1}$$\rightarrow$3-$\alpha$-L-Fucp2,4(OSO$_3$)$_{-1}$$\rightarrow$3-$\alpha$-L-Fucp1$\rightarrow$3-$\alpha$-L-Fucp2(OSO$_3$)$_{-1}$$\rightarrow$3A1$\rightarrow$3D1$\rightarrow$3C1$\rightarrow$. Since Ht-FUC possessed an identical primary structure with Ht-LWM, Ht-FUC was clarified to be composed of the tetrafucose repeating unit $\rightarrow$3-$\alpha$-L-Fucp2(OSO$_3$)$_{-1}$$\rightarrow$3-$\alpha$-L-Fucp2,4(OSO$_3$)$_{-1}$$\rightarrow$3-$\alpha$-L-Fucp1$\rightarrow$3-$\alpha$-L-Fucp2(OSO$_3$)$_{-1}$$\rightarrow$, which was schematically illustrated in Fig. 1B.

Ht-FUC and the fucoidan from sea cucumber L. grisea consisted of an identical repeating unit. It is worth mentioning that all primary structures of sea cucumber fucoidans reported before this study are different from each other (Fig. 1A). The identity between Ht-FUC and L. grisea fucoidan is the first exception. Interestingly, the sea cucumber L. grisea, whose synonymized name is Holothuria grisea, belongs to the same genus (the genus Holothuria) with H. tubulosa (Tommasi, 1969) (the taxonomic information of sea cucumbers mentioned in this paper is listed in Table S1). The species-specificity existing in the primary structure of sea cucumber fucoidan is certainly intriguing and requires further investigation. Nevertheless, this study showed that a one-to-one correspondence between the primary structure of fucoidan and the sea cucumber species should not be expected.

Meanwhile, it should be noticed that distinction between Ht-FUC and L. grisea fucoidan is still obvious. Especially, the $M_w$ of Ht-FUC is remarkably higher than that of L. grisea fucoidan (30 kDa) (Ribeiro et al., 1994), although these two fucoidans were extracted and purified by similar methods. Numerous studies have indicated that molecular mass has a significant influence on the bioactivities of fucoidan. For example, the ability of the degradation products of A. molpadioides fucoidan to protect intestinal mucosal immunity has been reported to be better for fractions containing larger molecules (50-500 kDa) than those containing smaller ones (10 kDa).
In addition, native *Lessonia vadosa* fucoidan (320 kDa) has been reported to manifest better anticoagulant activity than its depolymerized fraction (32 kDa) (Chandia & Matsuhiro, 2008). Therefore, it was expected that Ht-FUC and *L. grisea* fucoidan may perform diverse functionalities, and thus could be utilized in different applications.

3.3. Chain conformation

As an absolute method, HPSEC-MALLS-Vis-RI can determine conformational parameters and the true molecular mass without standard samples. The refractive index detector was utilized to provide information about the concentration of components in the eluate. The $<s_z^2>^{1/2}$ and $M_w$ of components could be calculated from the angular dependency of light scattering intensity which was measured by the MALLS detector. The tandem viscometer could simultaneously determine the intrinsic viscosity ([η]). And by combining the $M_w$ and [η] data, the $R_h$ could be calculated. (Fekete, Beck, Veuthey & Guillarme, 2014; Oberlerchner, Rosenau & Potthast, 2015).

The $<s_z^2>^{1/2}$ (weight-average) of Ht-FUC in 0.15 M NaCl solution (pH 7.4) at 25 °C was 63.9±1.8 nm, and the $R_h$ (weight-average) was 44.5±4.5nm. A 0.15 M NaCl solution was chosen for these experiments because it is widely used in many bioactivity assays. The chain shape of Ht-FUC could be further estimated from $<s_z^2>^{1/2}$, $M_w$ and [η] at each slice of HPSEC-MALLS-Vis-RI chromatogram (Morris, Adams & Harding, 2014). The relationship of $<s_z^2>^{1/2}$-$M_w$ and [η]-$M_w$ were shown in Fig. 5.

The Mark-Houwink-Kuhn-Sakurada equation for Ht-FUC was represented by

$$[\eta] = 3.48 \times 10^{-2} M_w^{0.65 \pm 0.01} \text{ (mL/g)}$$

The exponent in the equation is related to the macromolecular conformation. In general, a value of ~0 corresponds to spheres, 0.5-0.8 corresponds to random coils, and up to 1.8 for rigid rods (Morris et al., 2014). Our results therefore suggest that the sea cucumber fucoidan has a random coil shape in aqueous solution. Meanwhile, the relationship of $<s_z^2>^{1/2}$-$M_w$ was presented by the following equation:

$$\langle s_z^2 \rangle^{1/2} = 1.54 \times 10^{-2} M_w^{0.58 \pm 0.02} \text{ (nm)}$$

For this equation, the exponents of 0.33, 0.5-0.6, and 0.85 respectively reflect the chain shape of spheres, random coils and rigid rods (Morris et al., 2014), again suggesting that the sea cucumber fucoidan was a random coil. An estimate of chain conformation can also be deduced from the $\rho$ parameter, which has theoretical limits of 0.78, 1.7 and 2 for spheres, random coils and rigid rods (Burchard, 1988).
The exponent of the Mark-Houwink-Kuhn-Sakurada equation (0.65), the $<s^2>_z^{1/2}-M_w$ equation (0.58) and the $\rho$ value (1.44) consistently indicated that the Ht-FUC chain adapted a random coil chain conformation in 0.15 M NaCl solution.

The relative flexible conformation of Ht-FUC may be attributed to the abundant and evenly distributed sulfate groups in the molecule. It has been widely reported that the introduction of sulfate groups will result in chain expansion and increase stiffness of the polysaccharide (e.g. lentinan, locust bean gum, and $\alpha$-glucan) (Chen et al., 2014; Wang et al., 2014; Wang & Zhang, 2009). The negatively charged sulfate groups were expected to enhance the intramolecular repulsion and the steric hindrance between segments in the molecular chain, which prevented the chain from adapting a compact conformation.

4. Conclusion

Both the primary structure and chain conformation of Ht-FUC were clarified. Ht-FUC was composed of a tetrafucose repeating unit $[\rightarrow 3-\alpha-L-Fucp2(OSO_3-)1\rightarrow 3-\alpha-L-Fucp2,4(OSO_3-)1\rightarrow 3-\alpha-L-Fucp-1\rightarrow 3-\alpha-L-Fucp2(OSO_3-)1\rightarrow]$. Its sulfate content was determined as 31.2±1.6% and $M_w$ was 1567.6±34.1 kDa. It adapted a random coil conformation in 0.15 M NaCl solution (pH 7.4) at 25 °C, with a $<s^2>_z^{1/2}$ of 63.9±1.8 nm and a $R_h$ of 44.5±4.5nm. For sea cucumber fucoidan, this is the first report on its chain conformation, and also the first study that depicted its structure at several hierarchical levels.

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References


Fig. 1. (A) Reported primary structures of fucoidans from sea cucumber *L. grisea* (*Lg*), *A. molpadioides* (*Am*), *I. badionotus* (*Ib*), *T. ananas* (*Ta*), *A. japonicas* (*Aj*) and *S. japonicas* (*Sj*). (B) primary structure of Ht-FUC clarified in this study. To facilitate
the comparison, structures were aligned by 2-O-sulphated fucose residue in reducing-
terminal direction.

Fig. 2. The refractive index chromatogram of HPSEC analysis of Ht-FUC. The
variation of molar mass with elution volume was shown as the inserted dash line.

Fig. 3. The $^1$H NMR spectra of Ht-FUC (A) and Ht-LMW (B), and a close-up of
anomeric signals of Ht-LMW (C).

Fig. 4. The COSY (A), TOCSY (B) and ROESY (C) spectra of Ht-LMW. A1-A2
indicated the cross-peak between H-1 and H-2 of residue A, etc.

Fig. 5. Plots of $[\eta]$ versus $M_w$ (A) and $<s^2>^{1/2}$ versus $M_w$ (B) in double logarithmic
coordinate. Data were obtained from slices of HPSEC-MALLS-Vis-RI chromatogram
of Ht-FUC. Results of linear regression were shown as dash lines.

**Table 1** $^1$H chemical shifts of Ht-LMW

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Residue</th>
<th>$\delta_{H1}$</th>
<th>$\delta_{H2}$</th>
<th>$\delta_{H3}$</th>
<th>$\delta_{H4}$</th>
<th>$\delta_{H5}$</th>
<th>$\delta_{H6}$</th>
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<tbody>
<tr>
<td>Ht-LMW</td>
<td>A</td>
<td>5.36</td>
<td>4.54$^a$</td>
<td>4.35</td>
<td>4.87</td>
<td>4.37</td>
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<td></td>
<td>B</td>
<td>5.33</td>
<td>4.49</td>
<td>4.28</td>
<td>4.02</td>
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<td>1.18</td>
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<td>4.48</td>
<td>4.08</td>
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<tr>
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<tr>
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<td>$\rightarrow 3-\alpha-L-Fucp-1\rightarrow^b$</td>
<td>5.07</td>
<td>3.83</td>
<td>3.93</td>
<td>4.02</td>
<td>4.27</td>
<td>1.25</td>
</tr>
</tbody>
</table>

$a$: The chemical shifts highlighted in bold were down-field shifted compared with the corresponding $^1$H
chemical shifts of nonsulphated $\alpha-L-Fucp$. The chemical shifts were expressed in ppm. $^1$H chemical
shifts were referred to 4, 4-dimethyl-4-silapentane-1-sulfonic acid ($\delta$ 0.00 ppm).

$b$: A $\rightarrow 3-\alpha-L-Fucp-1\rightarrow$ residue in the low molecular weight polysaccharide derived from *Thelenota
ananas* fucoidan was employed as a reference, and its chemical shifts were determined under the
identical condition to Ht-LMW (Yu et al., 2014b).

Graphical Abstract
Structure of fucoidan from *Holothuria tubulosa* was clarified at different structural levels.