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Structure and rheological characteristics of fucoidan from sea cucumber *Apostichopus japonicus*

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ABSTRACT

Sea cucumber is a traditional health food consumed in East Asia. In this study, fucoidan from sea cucumber *Apostichopus japonicus* (Aj-FUC) was isolated, and its structure and rheological characteristics were elucidated for the first time. Aj-FUC was a branched polysaccharide mainly composed of a novel repeating unit [α-L-Fuc*p*2(OSO_3^-)-1 → 3,(α-L-Fuc*p*-1 → 4-α-L-Fuc*p*-1 →)4-α-L-Fuc*p*2(OSO_3^-)-1 → 3-α-L-Fuc*p*2(OSO_3^-)], clarified by using a combination of infrared spectroscopy, methylation analysis, enzymatic degradation and nuclear magnetic resonance. In steady shear measurement, Aj-FUC manifested a non-Newtonian shear-thinning behaviour at low shear rate (1–100 S^{-1}) while exhibiting a non-Newtonian shear-thickening behaviour at high shear rate (100–1000 S^{-1}); salts had limited impact on its flow curve. Comparative study on viscosity and rheological behaviour of Aj-FUC and a linear fucoidan extracted from sea cucumber *Acaudina molpadioides* suggested that the presence of branch structure might significantly influence the rheological characteristics of fucoidan.

1. Introduction

Sea cucumber is a health food traditionally consumed in East Asian countries (Bai, Qu, Luan, Li, & Yang, 2013; Kiew & Don, 2012). It is reported that at least 66 species of sea cucumber are harvested from more than 40 countries and exported to Asian markets (Gonzalez-Wanguemert, Aydin, & Conand, 2014). Among those editable sea cucumbers, *Apostichopus japonicus*, is one of the most important species with great economic value, and has obtained large-scale aquaculture. The culture area of *A. japonicus* in China is estimated at more than one million acres, and its production value exceeded 150 million dollars in 2009 (Li et al., 2012). In 2011, the yield of *A. japonicus* was 20,000 ton (dried weight) in China, Korea and Japan (Purcell et al., 2013).

Fucoidan, a bioactive polysaccharide with substantial percentages of L-fucose and sulphate group, is one of the major functional components in the body wall of sea cucumber (Berteau & Mulloy, 2003; Li, Lu, Wei, & Zhao, 2008; Morya, Kim, & Kim, 2012). Various biological activities of sea cucumber fucoidan have been established including anticoagulant, osteoclastogenesis inhibiting, protective effect against hyperglycaemia, proliferative effects on neural stem/progenitor cells and protective effect against ethanol-induced gastric ulcer (Hu et al., 2014; Kariya et al., 2004; Pereira, Mulloy, & Mourao, 1999; Wang et al., 2012; Zhang et al., 2010). Biological activity of polysaccharide has a profound relationship with its structure, and several studies have focused on the structural analysis of sea cucumber fucoidan. Mulloy, Ribeiro, Alves, Vieira, and Mourao (1994), Chen et al. (2012) clarified the structure of fucoidan from *Ludwigothurea grisea* and *Isostichopus badionotus* respectively, and the delicate structures of fucoidan from *Acaudina molpadioides* and *Thelenota ananas* were recently elucidated by us (Yu, Ge, et al., 2014; Yu, Xue, et al., 2014). All these fucoidans are linear polysaccharides, consisting of tetrasaccharide repeating units with diverse sulphated patterns (Supplementary Fig. 1). To date, the structural characterisation of fucoidan from sea cucumber *A. japonicus* (Aj-FUC) has not yet been reported.

Meanwhile, polysaccharides are widely employed in the food industry as thickening, stabilising or gelling agents (Stokes, Macakova, Chojnicka-Paszun, de Kruif, & de Jongh, 2011), depending on their unique rheological properties. Characterisation of rheological properties of this polysaccharide would facilitate its application (Ptaszek, Lukasiewicz, & Bednarz, 2013).

This research was aimed to clarify the structure and rheological characteristics of Aj-FUC. The structure was elucidated from the structural information of original Aj-FUC polysaccharide and its enzymatic degradation product, by using Fourier transform-infrared spectroscopy (FT-IR), methylation analysis and nuclear

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magnetic resonance (NMR). The apparent viscosity and rheological behaviour of Aj-FUC were analysed by steady shear measurement. Moreover, to investigate the influence of structural features on rheological characteristics of sea cucumber fucoidan, rheological properties of fucoidan from sea cucumber *A. molpadioides* (Am-FUC) were also comparatively analysed.

2. Material and method

2.1. Preparation of fucoidan from A. japonicus

Cultured sea cucumber *A. japonicus* was harvested from the Yellow Sea (Qingdao, China) in April 2013. The Aj-FUC was prepared according to the method of Chang et al. (2010) with little modification. Briefly, the body wall of *A. japonicus* was smashed and hydrolysed with papain; thereafter, cetylpyridinium chloride was added to precipitate the crude sulphated polysaccharide. The crude sulphated polysaccharide was then applied to an Express-Ion D (Whatman, USA) column using the AKTA™ UPC 100 (GE, USA) system and was eluted with 0–2.0 M linear gradient of NaCl. Fractions containing fucoidan (NaCl concentration 1.1–1.3 M) were collected, lyophilised and further purified by HiPrep 26/60 Sephacryl S-500 HR column (GE Healthcare, USA) which was eluted with 0.2 M NH_4HCO_3. The eluate was monitored by a refractive index detector (Aglient 1260, Aglient Technologies, USA), and the finally purified fucoidan was collected, dialysed, lyophilised and used in further analysis.

2.2. Compositional analysis

The monosaccharide composition of Aj-FUC was determined by high performance liquid chromatography (Strydom, 1994). The sulphate content was determined by the $BaCl_2$–gelatin method (Silvestri, Hurst, Simpson, & Settine, 1982). The mass average molecular mass (Mw) of Aj-FUC was determined by high performance size exclusion chromatography–multiangle laser light scattering (HPSEC–MALLS) according to the method of Yu, Xue, et al. (2014).

2.3. FT-IR assay

Two milligrams of Aj-FUC was mixed with 100 mg dried KBr and then pressed to transparent film. The film was scanned by using the Nicolet Nexus 470 FTIR spectrometer (Thermo Electron, USA) from 100 to 4000 cm^{-1}.

2.4. Methylation analysis

The Aj-FUC was dissolved in deionised water and passed through an AG50 W-XS (H^+ form, 100 μL) column eluted with 10 mL water. The eluent was adjusted to pH 9.0 with pyridine to obtain fucoidan-pyridine salt. Subsequently, the fucoidan-pyridine salt was rotatory evaporated and lyophilised. For desulphation, 10 mg fucoidan–pyridine salt were dissolved in 2 mL dimethyl sulphoxide/methanol solvent (v/v = 9:1) in a sealed tube and incubated at 80 °C for 10 h. After dialysis and lyophilisation, the desulphated sample was methylated by methyl iodide according to the method of Hakomori (1964). The methylated polysaccharide was then hydrolysed in 2 M trifluoroacetic acid at 100 °C for 8 h and dried under a steam of nitrogen. The hydrolysate was 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 analysed by gas chromatography–mass spectroscopy (GC–MS) (Agilent 1100, Agilent) with DB-225MS fused-silica capillary column (30 m × 0.32 mm × 0.25 m). The analysis of GC–MS spectra was referred to the Complex Carbohydrate Structural Database of University of Georgia.

2.5. Enzymatic preparation and purification of low molecular weight polysaccharide

The fucoidanase was prepared according to the method of Yu, Xue, et al. (2014). In brief, marine bacterium *Flavobacteriaceae* CZ1127 was cultured, and its intracellular supernatant was extracted and purified by ammonium sulphate precipitation and cellufine sulphate (Chisso, Kyoto, Japan) column successively. Active fractions were pooled and dialysed against 20 mM Tris–HCl (pH 7.2). The final product was nominated as CZ1127 fucoidanase.

Fifty milliliters of the CZ1127 fucoidanase was mixed with 50 mL 0.4% (w/v) fucoidan solution containing 20 mM Tris–HCl (pH 7.2) and 0.3 M NaCl. This mixture was incubated at 35 °C for 10 h and heated at 100 °C for 10 min. After centrifugation, desalting and lyophilisation, the crude low molecular weight polysaccharide was prepared. It was subsequently applied to HiPrep 26/60 Sephacryl S-300 HR column (GE, USA) and eluted with 0.2 M NH_4HCO_3. The finally purified product was lyophilised and nominated as Aj-LMW. To investigate whether desulphation occurred during enzymatic reaction, sulphate content of Aj-LMW was determined by the $BaCl_2$–gelatin method (Silvestri et al., 1982) and compared with that of Aj-FUC.

2.6. NMR analysis

Twenty-five milligrams of Aj-LMW and 50 mg of Aj-FUC were respectively co-evaporated with D_2O (99.9%) twice by lyophilisation, and dissolved in 500 μL D_2O (99.9%) containing 0.1 μL 4,4-dimethyl-4-silapentane-1-sulphonic acid (DSS). The NMR spectra were recorded by Bruker AVANCE III 600 (Bruker, German). Spectra of 1-dimensional ^1H NMR and 2-dimensional correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY) and rotating-frame overhauser effect spectroscopy (ROESY) were all obtained at 600 MHz under 25 °C with sufficient acquisition time. The observed ^1H chemical shifts were calibrated according to eternal DSS (0.00 ppm).

2.7. Steady shear measurement

Aj-FUC was dissolved in deionised water at concentrations of 0.10%, 0.25% and 0.50% (w/v) under mechanical stirring for 12 h at 25 °C. Solutions of Aj-FUC (concentration 0.25%) with 0.15 M NaCl, KCl and $CaCl_2$ were also prepared. Apparent viscosity of above samples under various shear rates (1–1000 s^{-1}) was measured utilising the Physica MCR301 rheogoniometer (Anton Paar Co., Ltd., Austria) equipped with PP50 flat plate (diameter 50 mm).

As a reference, Am-FUC was extracted from sea cucumber *A. molpadioides* according to the method described in Section 2.1, and its rheological properties were analysed in parallel with Aj-FUC.

3. Results and discussion

3.1. Composition and FT-IR spectrum of Aj-FUC

The yield of Aj-FUC was 3.8% against the dry weight of *A. japonicus* body wall. The compositional analysis showed that Aj-FUC only contained fucose with 23.3 ± 3.2% sulphate, and its Mw was 1970 kDa.

The composition of Aj-FUC was further confirmed by FT-IR. The FT-IR spectrum of Aj-FUC was shown in Fig. 1. The band at

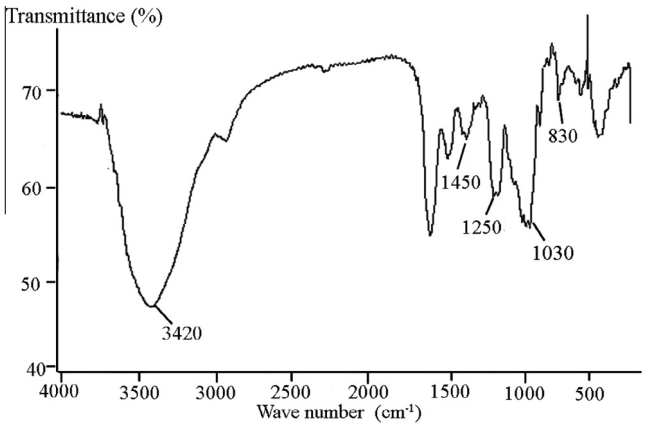

Fig. 1. FT-IR spectrum of Aj-FUC.

3420 cm^{-1} could be assigned to O—H stretching vibrations of hydroxyls and water. The envelope of bands and shoulders at 980–1160 cm^{-1} was mainly original from C—O and C—C stretching in pyranoid ring and C—O—C stretching of glycosidic bonds (Kacuráková, Capek, Sasinková, Wellner, & Ebringerová, 2000). The band at 1450 cm^{-1} was attributed to asymmetric bending of CH_3, confirming the presence of —CH_3 and fucose residue (Synytsya et al., 2010). Intense band at 1251 cm^{-1} arose from asymmetric O=S=O stretching vibration of sulphate groups (Pereira, Amado, Critchley, van de Velde, & Ribeiro-Claro, 2009), which is typical for sulphated polysaccharides. Moreover, the band at 830 cm^{-1}, which was assigned to equatorial C2—O—S or C3—O—S bending vibrations (Pereira et al., 2009; Sekkal & Legrand, 1993), suggested that *O*-sulphation might take place at C-2 and/or C-3 position.

3.2. Methylation analysis

The types of glycosidic linkage in Aj-FUC were investigated by methylation analysis. After desulphation, methylation, reduction and acetylation, methylated alditol acetates derived from Aj-FUC were detected as 1,3,5-tri-O-acetyl-2,4-di-O-methylfucitol, 1,4,5-tri-O-acetyl-2,3-di-O-methylfucitol and 1,3,4,5-tetra-O-acetyl-2-O-methylfucitol with an approximate molar ratio of 4:2:1. It indicated that Aj-FUC consisted of 3-linked, 4-linked and 3,4-linked fucose residues. The discovery of 3,4-linkage revealed the presence of branch structure in Aj-FUC.

Table 1
^{1}H chemical shift data for low molecular weight polysaccharide of fucoidan from sea cucumber *Apostichopus japonicus*.

Residue	δ_{H1}	δ_{H2}	δ_{H3}	δ_{H4}	δ_{H5}	δ_{H6}
→3-α-L-Fuc*p*-1→[a]	5.14	3.73	3.80	3.75	4.14	1.15
A	5.48	**4.50**[b]	4.30	4.08	4.42	1.23
B	5.40	**4.55**	4.26	3.95	4.08	1.30
C	5.35	**4.53**	4.11	3.93	4.27	1.27
D	5.09	3.97	3.94	4.07	4.42	1.23
E	5.07	3.93	4.10	4.05	4.42	1.23

[a] The chemical shifts of →3-α-L-Fuc*p*-1→ was referred to previous report (Yu et al., 2013).

[b] The chemical shifts highlighted in bold were significantly down-field shifted compared with the corresponding ^{1}H chemical shifts of →3-α-L-Fuc*p*-1→. The chemical shifts were expressed in ppm. ^{1}H chemical shifts were referenced to external 4,4-dimethyl-4-silapentane-1-sulphonic acid (δ 0.00 ppm).

3.3. Preparation and structural analysis of Aj-LMW

As the NMR spectra of Aj-FUC were ambiguous and overlapped (Supplementary Fig. 2), degradation product with lower molecular mass was prepared to facilitate the structural clarification. After enzymatic degradation, the product was purified into two fractions by using gel filtration chromatography (Supplementary Fig. 3). The minor and early eluted fraction (yield 17.4%) still possessed a high molecular mass (820–1160 kDa). It was suggested that this fraction might be a heterogeneous component existed in the molecular chain of Aj-FUC, which could not be recognised and degraded by the CZ1127 fucoidanase. Meanwhile, the major and late eluted fraction (yield 77.5%) was considerably degraded to 105 kDa. The major fraction was nominated as Aj-LMW and used in the further structural analysis.

The COSY spectrum of Aj-LWM was shown in Fig. 2A. Five spin systems could be found. The start signals of each spin system were δ 5.48, δ 5.40, δ 5.36, δ 5.09 and δ 5.07 ppm, which was, respectively, attributed to the anomeric resonance of α-configuration fucopyranoside residue A–E. The other proton chemical shifts were assigned by correlation peaks in COSY and TOCSY and listed in Table 1.

The positions of sulphated groups were determined by comparing each proton chemical shift with that of nonsulphated fucose residue. The *O*-sulphation would cause the chemical shift of oxymethine protons shifted to downfield by 0.4–0.8 ppm (Yamada, Yoshida, Sugiura, & Sugahara, 1992). As H-2 signals of residue A–C shifted to downfield compared with that of nonsulphated fucose residue (Yu et al., 2013), these residues were deduced as 2-*O*-sulphated fucose residues. Meanwhile residue D and E were deduced as nonsulphated fucose residues.

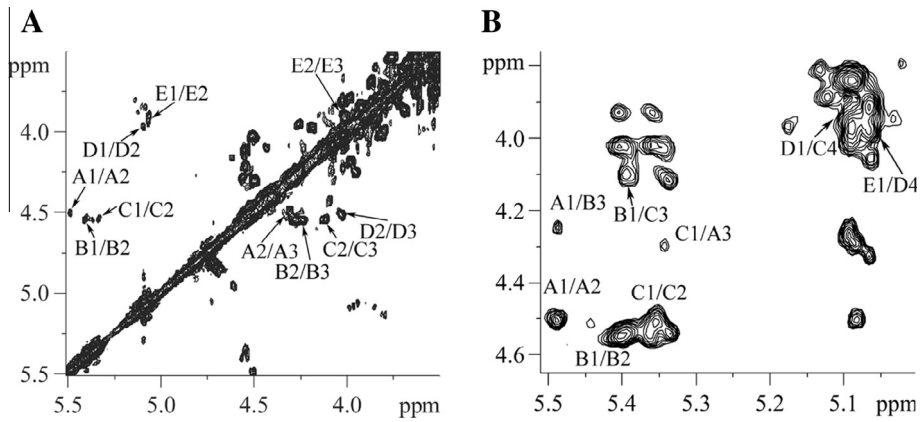

Fig. 2. 2D ^{1}H–^{1}H COSY (A) and ^{1}H–^{1}H ROESY (B) spectra of Aj-LMW. A1/A2 indicated the cross-peak between H-1 and H-2 of residue A, etc.

The glycosidic linkage and the sequence of residues were determined by the correlation peaks in ROESY (Fig. 2B). In detail, correlation from H-1 of residue A to H-3 of residue B, correlation from H-1 of residue B to H-3 of residue C, and correlation from H-1 of residue C to H-3 of residue A were observed. As the result, the sequence of residues in backbone structure could be determined as →3B1 → 3C1 → 3A1→. Additionally, correlation from H-1 of residue D to H-4 of residue C and correlation from H-1 of residue E to H-4 of residue D were found. It was indicated that a branch chain of E1 → 4D1→ presented at the O-4 position of residue C. In consequence, the sequence of residues in Aj-LWM could be deduced as →3B1 → 3, (E1 → 4D1 →) 4C1 → 3A1→.

To sum up, Aj-LWM was composed of a branched pentasaccharide repeating unit [α-L-Fuc*p*2(OSO_3^-)-1 → 3,(α-L-Fuc*p*-1 → 4-α-L-Fuc*p*-1 →)4-α-L-Fuc*p*2(OSO_3^-)-1 → 3-α-L-Fuc*p*2(OSO_3^-)]. This repeating unit possessed a novel structure that has not been described so far.

3.4. Structural elucidation of Aj-FUC

The similar sulphate contents (26.4 ± 1.9% versus 23.3 ± 3.2%) and similar ^{1}H NMR spectrum patterns (Supplementary Fig. 2) of Aj-LMW and Aj-FUC indicated that structural features of Aj-FUC were maintained during the enzymatic degradation. Moreover, as Aj-LMW was the major degradation product, its structure could reflect the major structure of original polysaccharide. Therefore, it could be deduced that Aj-FUC mainly consisted of the pentasaccharide repeating unit in Aj-LWM, although few heterogeneous component existed. The elucidated structure of Aj-FUC was shown in Fig. 3.

Aj-FUC was a branched polysaccharide mainly composed of repeating units. While fucoidan from sea cucumber *L. grisea*, *I. badionotus*, *A. molpadioides* and *T. ananas* are all linear polysaccharide; branch structure and 1,4-linkage are absent in those fucoidan (Chen et al., 2012; Mulloy et al., 1994; Yu, Ge, et al., 2014; Yu, Xue, et al., 2014). Although two fucoidans with branch structure have been found in *Stichopus japonicus* by Kariya et al. (2004), it should be noticed that they are extracted by using organic solvent (chloroform–methanol) while other reported sea cucumber fucoidans including Aj-FUC are all extracted in aqueous phase. Compared with Aj-FUC, branched fucoidans from *S. japonicus* possesses relatively low Mw (32 and 9 kDa), and do not have a regular repeating unit. In conclusion, the structure of Aj-FUC was distinct from those of reported sea cucumber fucoidans. This was also the first report of the branched repeating unit presented in sea cucumber fucoidan.

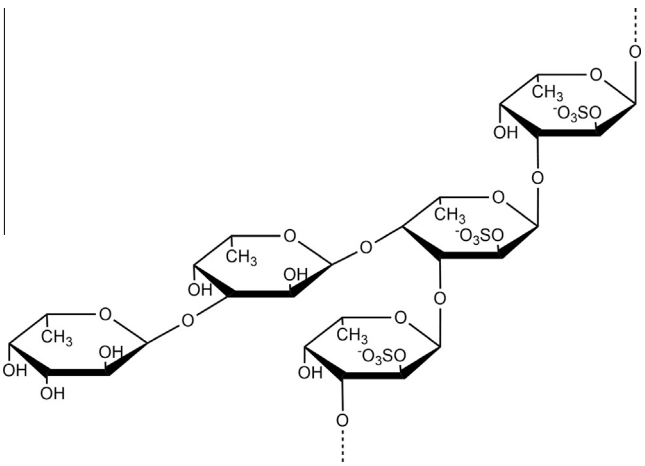

Fig. 3. The major structure of Aj-FUC.

However, it should be mentioned that structure of the pentasaccharide repeating unit could not perfectly explain the result of methylation analysis. The molar ratio of 3-linked, 4-linked and 3,4-linked fucose residues in the pentasaccharide repeating unit was 2:2:1 (versus 4:2:1 in methylation analysis). The inconformity might be attributed to the existence of the minor component with heterogeneous structure, which was observed in the enzymatic degradation of Aj-FUC. The tolerance to degradation and the complex NMR spectra (data not shown) of this component hampered its structural clarification.

3.5. Rheological properties of Aj-FUC

Apparent viscosity of Aj-FUC at different shear rates and concentrations was shown in Fig. 4. Viscosity of Aj-FUC increased gradually following the increasing of concentration. Aj-FUC demonstrated a non-Newtonian shear-thinning behaviour at low shear rate (1–100 S^{-1}), while a non-Newtonian shear-thickening behaviour at high shear rate (100–1000 S^{-1}).

A few reports have focused on the rheological characterisation of algal fucoidans. Tako (2003) studied the flow curve of fucoidan isolated from *Cladosiphon okamuranus*. Rioux, Turgeon, and Beaulieu (2007) investigated viscosity of fucoidans from brown seaweed *Saccharina longicruris*, *Ascophyllum nodosum* and *Fucus vesiculosus*, and found that viscosity of fucoidans was dependent on the molecular weight and the proportion of sulphates and uronic acids. Katayama, Nishio, Iseya, Kishimura, and Saeki (2009) studied on a highly viscous polysaccharide solution mainly containing fucoidan extracted from *Kjellmaniella crassifolia*, and found viscosity of the solution was affected by heat treatment, salt, and divalent cations. Possibly due to the complexity and irregularity in structure of algal fucoidans (Bilan et al., 2002), the relationship between structure and rheological properties of fucoidan could not be established in those studies. Sea cucumber fucoidans with regular structure might be employed as favourable tools to investigate how delicate structural features influence rheological characteristics of fucoidan.

Am-FUC was chosen as a representative of linear sea cucumber fucoidan, and its rheological properties were comparatively

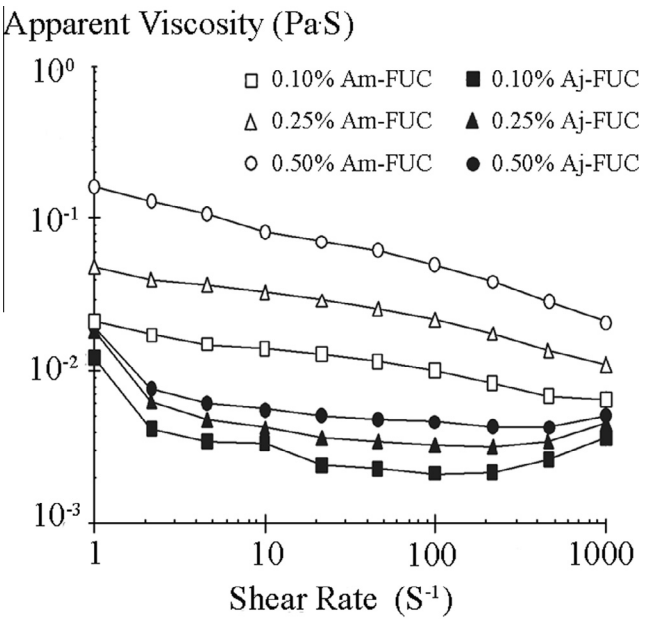

Fig. 4. Apparent viscosity as function of shear rate for Aj-FUC and Am-FUC solutions at concentrations of 0.10%, 0.25% and 0.50% (w/v) at 25 °C.

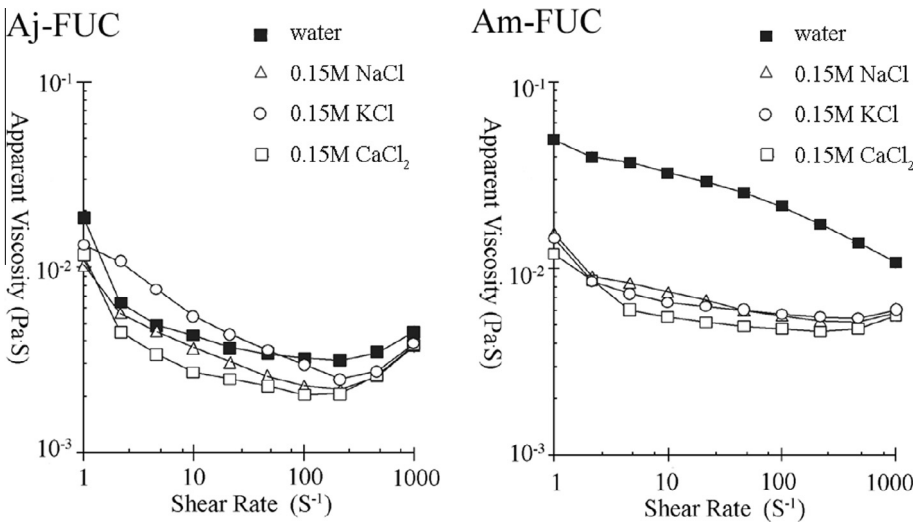

Fig. 5. Influence of salts (0.15 M NaCl, 0.15 M KCl, and 0.15 M $CaCl_2$) on the apparent viscosity of Aj-FUC and Am-FUC (0.25% w/v) solutions at 25 °C.

investigated. Interestingly, although Am-FUC and Aj-FUC had the identical compositional monosaccharide (fucose) and no significantly different sulphate contents (26.3 ± 2.7% for Am-FUC versus 23.3 ± 3.2% for Aj-FUC), and Mw of Am-FUC (1614 kDa) was even numerically lower than that of Aj-FUC (1970 kDa), Am-FUC manifested an obviously higher viscosity than Aj-FUC at the same concentration and shear rate (Fig. 4). Furthermore, Am-FUC consistently showed a non-Newtonian shear-thinning behaviour during the whole shear rate range, which also differentiated Am-FUC with Aj-FUC (Fig. 4).

Meanwhile, the influences of salt on rheological properties of Aj-FUC and Am-FUC were also distinct. As shown in Fig. 5, limited difference was found in flow curves of Aj-FUC solutions with and without salts, while the presence of salt remarkably decreased the apparent viscosity of Am-FUC and even changed its rheological behaviour. Due to the electrostatic repulsion, molecular chain of polyanionic polysaccharide in aqueous solution tends to expand, which causes a high viscosity (Simas-Tosin et al., 2010); Salt ions may alleviate this kind of repulsion by shielding charges, resulting in the decrease of viscosity, which was observed in the case of Am-FUC. However, the alleviation effect of salt for Aj-FUC was trifling.

The diversity between Aj-FUC and Am-FUC revealed in this study implied that rheological properties of fucoidan profoundly depended on its delicate structure. As the most prominent structural feature distinguishing Aj-FUC from Am-FUC, the branch structure might significantly contribute to the rheological characteristics of Aj-FUC. The presence of branch structure possibly rendered molecular chain of polysaccharide to adopt a more compact conformation (Wang et al., 2011), which leads to a weaker intermolecular force and lower apparent viscosity and restricts the charge shielding effect of salt. It was further suggested that the presence of branch structure might significantly influence the rheological characteristics of fucoidan.

4. Conclusion

The structure and rheological characteristics of Aj-FUC were elucidated for the first time. Aj-FUC was a branched polysaccharide mainly composed of a novel repeating unit [α-L-Fuc*p*2(OSO_3^-)-1 → 3,(α-L-Fuc*p*-1 → 4-α-L-Fuc*p*-1 →) 4-α-L-Fuc*p*2(OSO_3^-)-1 → 3-α-L-Fuc*p*2(OSO_3^-)]. The sulphate content of Aj-FUC was 23.2 ± 3.7%, and its Mw was 1970 kDa. Aj-FUC manifested a non-Newtonian shear-thinning behaviour at low shear rate while a non-Newtonian shear-thickening behaviour at high shear rate; salts had limited impact on flow curve of Aj-FUC. Apparent viscosity and rheological behaviour of Aj-FUC were distinct from those of linear fucoidan Am-FUC, which suggested that the presence of branch structure might significantly influence the rheological characteristics of fucoidan.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.02.034.

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