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Analysis of structural heterogeneity of fucoidan from *Hizikia fusiforme* by ES-CID-MS/MS

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ABSTRACT

A fucoidan from the brown alga *Hizikia fusiforme* was extracted with hot water and fractionated by anion-exchange chromatography. The main fraction YF5 was depolymerized by partial acid hydrolysis and further purified by gel filtration chromatograph. The sequences of oligosaccharides generated were then determined by ES-CID-MS/MS, which gave directly information on the structural heterogeneity of fucoidan from *H. fusiforme*. It was shown that YF5 had a backbone of a repeating disaccharide unit of [→4GlcAβ1,2Manα1→], in good agreement with previous report by NMR and methylation analysis. In the product-ion spectra, the unique ^{0,2}A type fragmentation was important to establish the presence of a 2-linked Man in the backbone structure. In addition, abundant novel heterogeneous branched structural fragments were also detected and characterized: Fuc1,3Fuc, Fuc1,3Gal, Fuc1,4GlcA, Gal1,4Gal, Gal1,4GlcA and GlcA1,4GlcA. The sulfation mainly occurred at C2 or C4 of the fucose residue and C2, C4 or C6 of the galactose residue.

1. Introduction

Fucoidans, a family of complex sulfated polysaccharides extracted from brown algae and some marine invertebrates, have been extensively investigated because of their various biological activities, *e.g.* anticoagulant (Athukorala, Lee, Kim, & Jeon, 2007), antiviral (Lee, Takeshita, Hayashi, & Hayashi, 2011) and antitumor activities (Synytsya et al., 2010) and these activities differ according to the sulfate content, monosaccharide composition and molecular weight (Berteau & Mulloy, 2003). It is important to elucidate the structure of fucoidans to understand the relationship between their structure and biological activities. Unfortunately, the structures of native fucoidan from brown algae are usually heterogeneous and branched. Besides fucose (Fuc), they also contain additional monosaccharides, *e.g.* mannose (Man), galactose (Gal), glucose (Glc), xylose (Xyl), and glucuronic acid (GlcA) and many different sulfation patterns occurred in these molecules. As a result, chemical methods for structural analysis as well as NMR spectroscopy of native fucoidans can give only partial information on their structures.

Along with NMR spectroscopy, mass spectrometry has become a primary technique in carbohydrate structural analysis in the last two decades, due to its high sensitivity and selectivity. Recently, the negative-ion electrospray tandem mass spectrometry with collision-induced dissociation (ES-CID-MS/MS) has been successfully applied as an independent tool to investigation of fucooligosaccharides (Anastyuk, Shevchenko, Nazarenko, Dmitrenok, & Zvyagintseva, 2009; Anastyuk et al., 2010; Daniel et al., 2007; Tissot, Salpin, Martinez, Gaigeot, & Daniel, 2006). It is possible to determine the monosaccharide composition, the linkage type and the sulfation pattern of heterogeneous fucoidans by this technique. Since there are no widely available enzymes to depolymerize fucoidans, partial acid hydrolysis is often performed and the sequence of oligosaccharides is then determined by ES-CID-MS/MS, which can give directly structural information of fucoidans.

Hizikia fusiforme is one of the most widely consumed seaweeds in China, Japan and Korea. Recently, fucoidans from *H. fusiforme* have widely been investigated because of their biological activities including anticoagulant (Dobashi, Nishino, Fujihara, & Nagumo, 1989) and antioxidant activities (Choi, Hwang, & Nam, 2010; Choi, Kim, Kim, et al., 2010). Although a previous study by Li, Wei, Sun, and Xu (2006) has provided partial structural information of fucoidan from *H. fusiforme* by several chemical methods combined with NMR spectroscopy, little detailed information is available about fucoidans from this seaweed. In the present work, a fucoidan was isolated and purified from *H. fusiforme* and its heterogeneous structure was confirmed directly by ES-CID-MS/MS analysis of

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oligosaccharides prepared by partial acid hydrolysis of the polysaccharide.

2. Experimental

2.1. Materials

The brown seaweed *H. fusiforme* was collected in Dongtou (Zhejiang Province, China, May 2007). Monosaccharide standards (mannose, glucose, galactose, xylose, fucose, glucuronic acid), 1-phenyl-3-methyl-5-pyrazolone (PMP), $NaBD_4$ and 1-ethyl-3-(3-dimethyaminopropyl) carbodiimide (EDC) were purchased from Sigma (Shanghai, China). Dextran standards (788, 404, 212, 112, 47.3, 11.8, and 5.9 kDa) were purchased from Shodex (Tokyo, Japan). Superdex Peptide HR column (1.0 cm × 30 cm) and Q-Sepharose Fast Flow ion-exchange column (2.6 cm × 10 cm) were purchased from GE Healthcare (Uppsala, Sweden) and PL aquagel-OH column (300 mm × 8 mm, 8 μm) was from Perkin Elmer (Massachusetts, USA). Agilent ZORBAX Eclipse XDB-C_{18} column (4.6 mm × 150 mm, 5 μm) was from Agilent (Beijing, China). Fused-silica capillary columns DB-225 and DB-225MS (30 m × 0.32 mm, 0.25 μm) were all purchased from J&W Scientific (Folsom, USA). All other reagents were of analytical grade.

2.2. Extraction, isolation and purification of fucoidans

The extraction of fucoidan from *H. fusiforme* was carried out as previously described by Li et al. (2006). Briefly, 80 g of *H. fusiforme* powder was extracted with distilled water three times (80 °C, 3 h). The supernatants were combined, concentrated and dialyzed. The retained solution was treated with 3 mol/L $CaCl_2$ at 4 °C overnight. After centrifugation, the precipitate was removed and the supernatant was dialyzed, concentrated and lyophilized. The crude fucoidan (yield 2.4%) obtained was fractionated on a Q-Sepharose Fast Flow column, eluted with a step gradient of 0–3 mol/L NaCl at a flow rate of 3 mL/min. Seven fractions YF0–YF6 were collected respectively.

2.3. General analysis

Total sugar content was determined by the phenol–sulfuric acid method (Cuesta, Suarez, Bessio, Ferreira & Massaldi, 2003). The crude protein content was measured by the Lowry method (Ledoux & Lamy, 1986). The sulfate content was estimated by the method of Dodgson and Price (1962). The uronic acid content was analyzed by the carbazole–sulfuric acid method (Bitter & Muir, 1962).

The purity and relative molecular weight of polysaccharides were determined by gel filtration chromatography on a PL aquagel-OH column eluted with 0.2 mol/L Na_2SO_4 at a flow rate of 0.5 mL/min at 35 °C. The column was calibrated with dextran standards.

Monosaccharide compositions were determined by a PMP-HPLC method according to Chen et al. (2008). In brief, poly- or oligosaccharide was hydrolyzed with 2 mol/L trifluoroacetic acid (TFA) at 100 °C for 6 h. The monosaccharide released were derivatized with PMP and then analyzed by high performance liquid chromatography on a XDB-C_{18} column eluted with acetonitrile/phosphate buffer solution (18:82, pH 6.7) at a flow rate of 1.0 mL/min at 30 °C. The detection wavelength was set at 254 nm. The composition and content of monosaccharides were determined by retention times and peak areas, in comparison with monosaccharide standards (mannose, fucose, xylose, galactose, glucose and glucuronic acid; Sigma Company, Shanghai, China).

2.4. Preparation and fractionation of oligosaccharides from YF5

Polysaccharide YF5 was hydrolyzed with 0.2 mol/L TFA (10 mg/mL) at 100 °C for 5 h. Excess TFA were removed by repeated co-evaporation with MeOH. The hydrolysate was dissolved in distilled water and a same volume of ethanol was added to remove the residual fraction. The supernatant was concentrated and lyophilized. The oligosaccharide mixtures were fractionated on a Superdex Peptide HR column eluted with 0.1 mol/L NH_4HCO_3 at a flow rate of 0.1 mL/min. Nine fractions were collected and freeze-dried.

2.5. Carboxyl-group reductions and methylation analysis

Oligosaccharides with even degree of polymerization (DP) were subjected to reduction (two cycles) first to convert the carboxyl into hydroxyl groups, based on the method described by Taylor and Conrad (1972). Methylation of the reduced oligosaccharides was performed according to previous method by Hakomori (1964) and derivatized to PMAAs. The partially methylated alditol acetates were analyzed by GC–MS equipped with a DB-225MS fused-silica capillary column. Mass spectra of the derivatives were analyzed using Complex Carbohydrate Structural Database (CCSD) of Complex Carbohydrate Research Centre (http://www.ccrc.uga.edu/).

2.6. MS analysis of fucoidan oligosaccharides

ESI-MS was performed on a Micromass Q-TOF Ultima instrument (Waters, Manchester, UK) for the oligosaccharides as described by Yu et al. (2006). Source temperature was 80 °C and the desolvation temperature was 150 °C. Samples were dissolved in CH_3CN/H_2O (1:1, v/v), typically at a concentration of 10 pmol/μL, of which 10 μL was loop-injected. Mobile phase (CH_3CN/H_2O, 1:1, v/v) was delivered by a syringe pump at a flow rate of 5 μL/min. Capillary voltage was maintained at 3 kV while cone voltage was 50 V. For CID-MS/MS product-ion scanning, the collision energy was adjusted between 10 and 50 eV for optimal sequence information.

3. Results and discussion

3.1. Preparation of fucoidan oligosaccharides from H. fusiforme

A crude fucoidan was extracted from the powder of brown alga *H. fusiforme* with hot water. Alginate was precipitated as calcium alginate and the soluble fucoidan was fractionated by anion exchange chromatography to yield seven fractions named as YF0–YF6. The chemical compositions of fractions YF0–YF6 are given in Table 1. The results showed that all the seven fractions were heterogeneous fucoidans, differing in the molar ratio of monosaccharides, the degree of sulfation and molecular weight. The major fraction, YF5, gave a symmetrical peak on a PL aquagel-OH column, with average molecular weight of 177 kDa and had a composition similar to the other fractions. It was thus selected for further structural analysis.

In order to study its fine structure, Fraction YF5 was depolymerized by partial acid hydrolysis and oligosaccharide mixture generated was fractionated by gel filtration chromatography to yield nine oligosaccharide fractions, p1–p9 (Fig. 1). Their molecular masses and DP were determined by negative-ion ES-MS. According to monosaccharide composition, all the possible structural compositions of oligosaccharides are shown in Table 2. Different DP oligosaccharides with similar molecular mass and equal charge coexisted in one peak, due to the heterogeneous structure of YF5. The oligosaccharides in fractions p1–p3 were mainly composed of

Table 1
Composition analysis of fucoidans from *H. fusiforme*.

Sample	Yield (%)	Total sugar (%)	Protein (%)	Sulfate (%)	Relative molecular mass (kDa)	Monosaccharide composition (%, w/w)					
						Man	GlcA	Glc	Gal	Xyl	Fuc
YF0	11.2	67.3	2.3	12.1	442.0	10.8	10.2	4.1	8.1	6.8	40.9
YF1	10.1	66.8	1.2	13.9	314.4	10.3	11.9	5.7	22.5	6.7	21.7
YF2	12.0	60.9	1.3	16.5	263.2	14.8	14.7	2.9	16.1	2.4	28.4
YF3	12.9	59.0	1.1	17.3	441.3	15.1	13.8	Tr	19.8	Nd	28.0
YF4	13.5	58.3	1.2	18.5	217.3	13.9	16.1	Nd	17.5	Nd	26.6
YF5	28.5	54.3	1.0	19.8	177.3	14.8	14.0	Nd	16.7	Nd	28.6
YF6	11.8	65.1	0.8	23.2	220.2	7.4	5.6	Nd	23.5	Nd	35.7

Nd, not detected; Tr, trace.

Table 2
Structural compositions of fucoidan oligosaccharides from YF5.

Peak	DP	Ions found (charge)	*m*/*z*	Monosaccharide composition	Structural compositions
p1	1	243(−1)	243	Fuc:Gal	FucS
		259(−1)	259	(55:45)	GalS
p2	2	339(−1)	339	Fuc:Gal:GlcA (41:35:24)	FucGlcA
		355(−1)	355		GalGlcA
		369(−1)	369		$GlcA_2$
		389(−1)	389		Fuc_2S
		405(−1)	405		FucGalS
		421(−1)	421		Gal_2S
		435(−1)	435		GalGlcAS
p3	3	531(−1)	531	GlcA:Gal (62: 38)	$GlcA_2$
		545(−1)	545		$GlcA_3$
		583(−1)	583		Gal_3S
		597(−1)	597		Gal_2GlcAS
p4	4	346.1(−2)	693.2	GlcA:Man (50:50)	$GlcA_2Man_2$
p5	6	515.2(−2)	1031.4		$GlcA_3Man_3$
p6	8	684.4(−2)	1370.8		$GlcA_4Man_4$
p7	10	568.6(−3)	1708.8		$GlcA_5Man_5$
p8	12	681.4(−3)	2046.2		$GlcA_6Man_6$
p9	14	595.4(−4)	2365.6		$GlcA_7Man_7$

Fuc, fucose; Gal, galactose; GlcA, glucuronic acid; Man, mannose; S, sulfate.

Fuc, Gal and GlcA along with low degree of sulfation, while oligosaccharides in fractions p4–p9 were homogeneous with GlcA and Man in almost equal ratio (1:1), indicating the presence of a repeating disaccharide unit of —[GlcAMan]—.

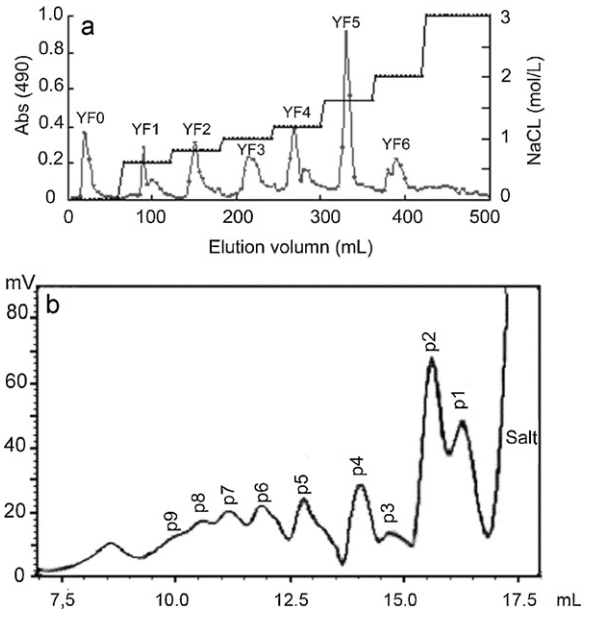

Fig. 1. Anion-exchange chromatography of fucoidans from *H. fusiforme* (a) and gel filtration chromatography of YF5-derived oligosaccharides on a Superdex Peptide column (b).

Two main ions at *m*/*z* 243 and 259 were detected in the mass spectrum of fraction p1, indicating the presence of monosulfated Fuc and Gal, respectively. The main component, p2, had the most complex composition with the ions detected indicating the presence of seven individual disaccharides differing in their monosaccharide composition and degree of sulfation. Four different trisaccharide ions were detected in the mass spectrum of the minor peak, p3. Notably, the DP of oligosaccharides composted of Fuc and Gal was no larger than DP3, suggesting that the linkages of Fuc and Gal residue in the fucoidan were unstable and easily degraded into oligosaccharides with low DP in acid conditions. In contrast, the mannosyl and hexuronosyl bonds were more stable to acid hydrolysis and the resulting regular disaccharide repeating oligosaccharides suggested that YF5 had a backbone structure composed of —[GlcA-Man]$_n$—. In order to derive the fine structure of the heterogeneous fucoidan YF5, the sequence of homogeneous and heterogeneous oligosaccharides was determined by ES-CID-MS/MS.

3.2. Analysis of backbone structure by negative-ion ES-CID-MS/MS

In order to determine the nonreducing end of the —[GlcA-Man]$_n$— disaccharide repeats, the fragmentation rules for hyaluronic acid (HA) oligosaccharides composed of —[GlcA-GlcNAc]$_n$— was used (Takagaki et al., 1998) due to their similar disaccharide composition. The CID-MS/MS spectra of tetra, hexa- and octa-saccharides are shown in Fig. 2. The characteristic fragment C_1 ion at *m*/*z* 193 only present when GlcA is at the

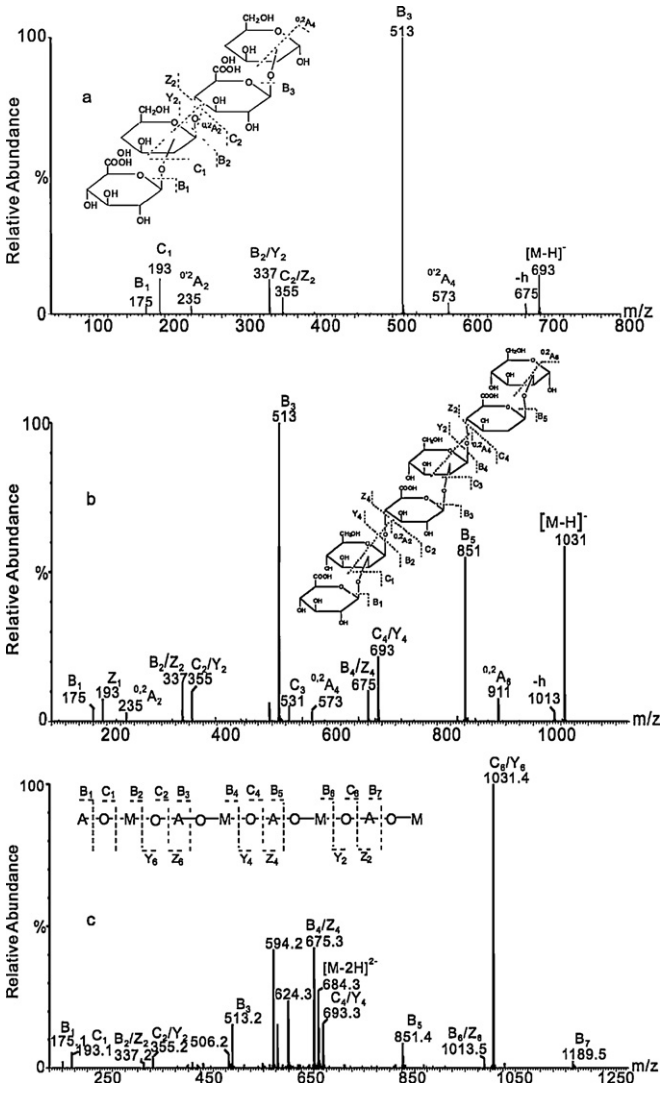

Fig. 2. ES-CID-MS/MS product-ion spectrum of tetrasaccharide ions. (a) Spectrum of ion at *m*/*z* 693; (b) spectrum of ion at *m*/*z* 1031; (c) spectrum of double charge ion at *m*/*z* 684.3. The ion marked with "-h" is fragment produced by dehydration of the major ion.

nonreducing end, demonstrated that GlcA was at the nonreducing end of the oligosaccharides of —[GlcA-Man]$_n$—. The CID-MS/MS spectrum of the tetrasaccharide (M−H *m*/*z* 693) showed a series of intensive B ions at *m*/*z* 175 (B_1), 333 (B_2) and 513 (B_3) from the nonreducing terminal, that together with C ion at *m*/*z* 193 (C_1) and 355 (C_2) from the reducing terminal indicating a linear sequence of GlcA-Man-GlcA-Man (Fig. 2a). Notably, the $^{0.2}A_4/^{0.2}A_2$ (*m*/*z* 573 and 235) were obtained from reducing Man and the internal Man respectively, by cross-ring cleavage of the C1—O (ring) and C2—C3 bonds of Man, leading to the loss of $C_4H_8O_4$ at *m*/*z* 120. This unique cross-ring cleavage revealed the presence of a 2-linked Man.

In order to further confirm the rules of the fragmentations of the disaccharide repeats, the sequence of hexasaccharide and octasaccharide were also determined (Fig. 2b and c) and similar fragment ions were observed. The intense B/C-ions from nonreducing end and Y/Z-ions from the reducing end further indicated a linear sequence of alternating GlcA and Man. The unique cross-ring cleavage $^{0.2}$A ion in each Man residue in these spectra provided further evidence for the presence of 2-Man in these oligosaccharides.

The linkage of GlcA residue was confirmed by carboxyl-reduction and methylation analysis, which showed that the disaccharide repeats consisted of 1,4,5-Ac$_3$-2,3,6-Me$_3$-Glc and 1,2,5-Ac$_3$-3,4,6-Me$_3$-Man, with the approximate molar proportion of 1:1, suggesting a linear sequence of a repeating disaccharide unit of alternating 4-linked GlcA and 2-linked Man. While there may be differences in hydrolysis rates among different linkages it is more likely that the different hydrolysis here is due to the stability of the uronic acid linkages. Therefore, the backbone of polysaccharide YF5 can be concluded to have the following structure: —[4GlcAβ1,2Manα1]$_n$—, which was in good agreement with the previous report by Li et al. (2006).

3.3. Analysis of heterogeneous structure by negative-ion ES-CID-MS/MS

According to the published papers (Saad & Leary, 2004; Tissot et al., 2006), the formation of $^{0.2}$X and $^{0.2}A_2$ ions need an hydrogen on the C3—OH, but it will not formed if the hydrogen is substituted by sulfated group or other sugar residues. These rules are very helpful for determination the linkage of oligosaccharides by mass spectrometry.

3.3.1. Hybrid-monosaccharides

The ES-CID-MS/MS spectra of the [FucSO$_3$]$^-$ ion at *m*/*z* 243 and the [GalSO$_3$]$^-$ ion at *m*/*z* 259 (figure not shown) were similar to previous report by Anastyuk et al. (2009). The cross-ring cleavage ion of $^{0.2}$X and $^{0.2}$A ion (*m*/*z* 139 and 183) indicated the sulfate group located at C2 or C4 position of the fucose. Similarly, a series cross-ring cleavage of $^{0.2}$A ion (*m*/*z* 199), $^{2.5}$A ion (*m*/*z* 181) and $^{0.3}$A ion (*m*/*z* 169) suggested the sulfate group locate at C4 or C6 position of the galactose.

3.3.2. Hybrid-disaccharides

In the ES-CID-MS/MS spectrum of ion at *m*/*z* 339 (Fig. 3a), the intense $^{0.2}X_1$ ion (*m*/*z* 235) suggested the fucose residue was at the nonreducing end. The presence of Z_1 and Y_1 ions (*m*/*z* 175 and 193), assigned to a dehydrated GlcA and GlcA, respectively, together with a unique $^{2.5}A_2$ ion (*m*/*z* 261), indicated that GlcA was at the reducing end (Zhang et al., 2006). In addition, the intensive $^{0.2}A_2$ ion at *m*/*z* 279 indicated that this residue was 4-linked and therefore this disaccharide was deduced to be Fuc1,4GlcA. The evidence of a fucose directly attached to a GlcA residue was not observed in the previous study of fucoidan from *H. fusiforme* (Li et al., 2006). The sequence of the disaccharide ion at *m*/*z* 355 can be readily elucidated as Gal1,4GlcA (data not shown), since the ions patterns were similar to those of the disaccharides ions of [FucGlcA]$^-$ observed above.

The product-ion spectrum of the ion at *m*/*z* 389 showed intense B_1 and C_1 ion (*m*/*z* 225 and 243) suggesting that the nonreducing end was a monosulfated Fuc (Fig. 3b). The $^{0.2}X_1$ and $^{0.3}X_1$ ion (*m*/*z* 285 and 315) indicated the sulfation occurred at C2 of the nonreducing Fuc. In addition, the low intensity of $^{0.2}A_2$ ion at *m*/*z* 329 suggested that 3Fuc was predominant. Therefore the disaccharide of Fuc2S1,3Fuc was main component in the disaccharide and was in agreement with the previous NMR analysis by Li et al. (2006).

In the ES-CID-MS/MS spectrum of the ion at *m*/*z* 405, an intense B_1 ion at *m*/*z* 241 indicated a monosulfated Gal was at nonreducing end (Fig. 3c). The $^{0.2}X_1$ and $^{0.3}X_1$ ions (*m*/*z* 285 and 315) suggested sulfation at the C2 of the nonreducing Gal. However, two less intensive B'_1 and C'_1 ions (*m*/*z* 225 and 243) were the cleavage products of the dehydrated monosulfated Fuc and monosulfated Fuc, respectively. In addition, the presence of $^{0.2}X'_1$ and $^{0.3}X'_1$ ions (*m*/*z* 301 and 331) indicated sulfation at the C2 of the nonreducing fucose residue. The $^{0.2}A_2$ ion at *m*/*z* 345 suggested the presence of a 1,3-type linkage, and according to the intensity, it predominantly contained Gal2S1,3Fuc, with a trace of Fuc2S1,3Gal (structure not shown).

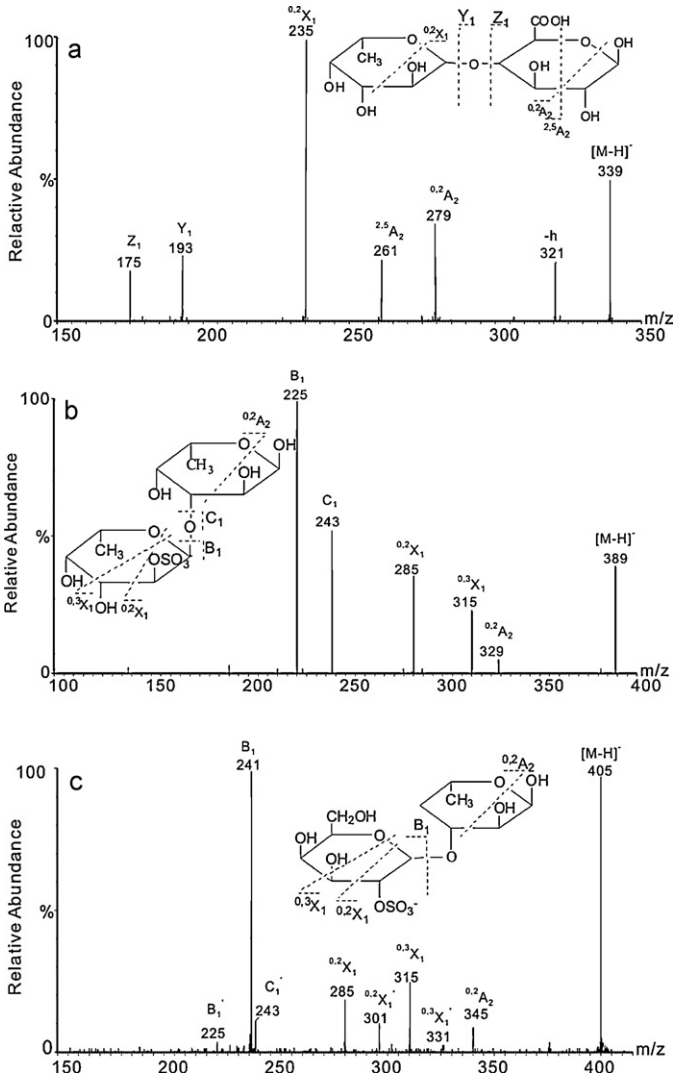

Fig. 3. ES-CID-MS/MS product-ion spectra of disaccharide ions. (a) Spectrum of ion at m/z 339; (b) spectrum of ion at m/z 389; (c) spectrum of ion at m/z 405. The ions marked with -h are fragments produced by dehydration of the major ions.

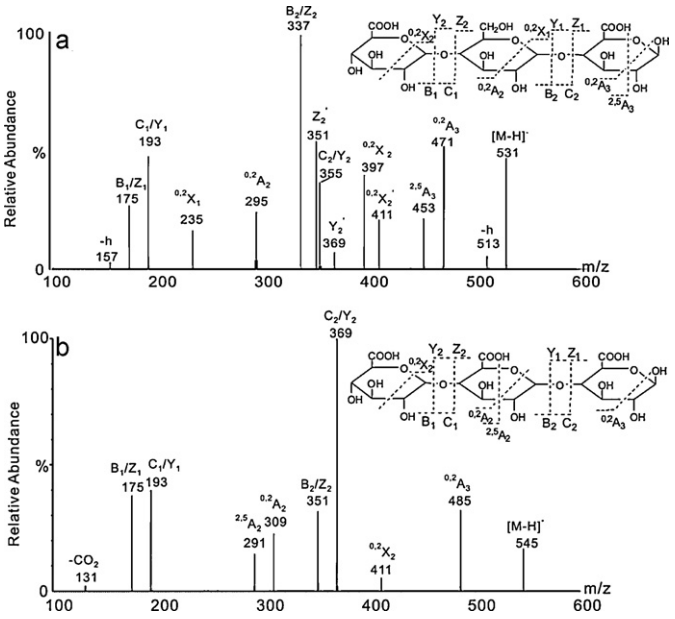

Fig. 4. ES-CID-MS/MS product-ion spectra of trisaccharide ions. (a) Spectrum of ion at m/z 531; (b) spectrum of ion at m/z 545. The ion marked with "-h" is fragment produced by dehydration of the major ion.

3.3.3. Hybrid-trisaccharides

The CID-MS/MS spectrum of the ion at m/z 531 (Fig. 4a) exhibited a complex fragmentation pattern. The presence of $^{0.2}X_2$ (m/z 397) and $^{0.2}X_2'$ ion (m/z 411) with similar intensity indicated Gal and GlcA located at the nonreducing terminal of the trisaccharides. Furthermore, the major daughter ions of B/Z- and C/Y-ions indicated successive cleavages of glycosidic bonds of the oligosaccharide chain: B_1/Z_1 and C_1/Y_1 ions (m/z 175 and 193) derived from the cleavages of the dehydrated GlcA residue and GlcA residue, respectively. The B_2/Z_2 and C_2/Y_2 ions (m/z 337 and m/z 355) suggested cleavages of dehydrated GlcAGal and GlcAGal, respectively. In addition, the Y_2' and Z_2' ion (m/z 369 and 351) indicated the glycosidic cleavage of a $GlcA_2$ residues and its dehydrated form the reducing terminal. Based on these data, we conclude that this trisaccharide had two major components with the sequences of GlcA-Gal-GlcA and Gal-GlcA-GlcA. The intense $^{0.2}A_3$ and $^{2.5}A_3$ ions (m/z 471 and 453) suggested a 1,4-type linkage of the reducing GlcA, while the $^{0.2}A_2$ at m/z 295 along with the $^{0.2}X_1$ at m/z 235 indicated the structural variant also had a predominate 1,4-type linkage of internal residue. Taken together, the mixture of isobaric trisaccharide variants contained GlcA1,4Gal1,4GlcA and Gal1,4GlcA1,4GlcA (figure not shown) as the main components.

The fragmentation of the selected ion at m/z 545 is shown in Fig. 4b. The regular ion pattern of B/C- and Y/Z-ions derived from the nonreducing and reducing termini respectively indicated a linear sequence of the trisaccharide with GlcA-GlcA-GlcA. The relatively high intensity of $^{0.2}A_2/^{0.2}A_3$ ion (m/z 309 and 485) suggested a prevalence of 1,4-type linkage in the trisaccharide, and thus the structure was deduced to be GlcA1,4GlcA1,4GlcA. The

Table 3
Fragment ions observed in the product-ion spectra from heterogeneous oligosaccharides.

Sequence	m/z	B_1	C_1	$^{2.5}A_2$	$^{0.2}A_2$	B_2	C_2	$^{2.5}A_3$	$^{0.2}A_3$	Z_1	Y_1	$^{0.2}X_1$	$^{0.3}X_1$	Z_2	Y_2	$^{0.2}X_2$	$^{0.3}X_2$
Fuc1,4GlcA	339			261	279					175	193	235					
Gal1,4GlcA	355			277	295						193	235					
GlcA1,4GlcA	369	175	193	291	309					175	193	235					
Fuc2S1,3Fuc	389	225	243		329							285	315				
Gal2S1,4Fuc	405	241			345							285	315				
Fuc2S1,4Gal	405	225	243		345							301	331				
Gal2S1,4Gal	421	241	259		361							301	331				
Gal2S1,4GlcA	435	241	259	357	375						193	295	325				
Gal6S1,4GlcA	435	241	259	357	375							235					
GlcA1,4Gal1,4GlcA	531	175	193		295	337	355	453	471	175	193	235		337	355	397	
Gal1,4GlcA1,4GlcA	531				295	337	355	453	471	175	193	235		351	369	411	
GlcA1,4GlcA1,4GlcA	545	175	193	291	309	351	369		485	175	193			351	369	411	
Gal2S1,4Gal1,4Gal	583	241	259		381	403	421		523							463	493

Gal, galactose; Gal2S, 2-O-sulfated galactose; Gal6S, 6-O-sulfated galactose; Fuc, fucose; Fuc2S, 2-O-sulfated fucose; GlcA, glucuronic acid.

fragmentation of this homoglucuronate oligosaccharide was similar to that of alginate oligosaccharides (homo- and hetero-oligomeric fragments composed of mannuronic acid and guluronic acid), which was previously determined by ES-CID-MS/MS (Zhang et al., 2006).

All the information of daughter ions from heterogeneous oligosaccharide ions is shown in Table 3, apart from the ions of $[Gal_2GlcASO_3]^-$ at m/z 597, since it was not even possible to distinguish the isomers due to the identical m/z values of the key fragments and the irregular patterns of sulfation.

4. Conclusions

The mass-spectrometric investigation of the homo- and hetero-oligosaccharides generated by partial acid hydrolysis of fucoidan YF5 from *H. fusiforme* has confirmed their heterogeneous structure. It was shown that the prevalence of novel glucuronomannan-derived disaccharide repeats indicating the presence of a backbone structure of YF5 with alternating repeating disaccharides unit of [→4GlcAβ1,2Manα→], in accordance with a previous report by NMR and methylation analysis (Li et al., 2006). The unique ^{0,2}A ion by cross-ring cleavage of each Man residue was important to establish a 2-linked Man. Polysaccharides with highly ordered disaccharide repeats are ubiquitous in marine algae (*e.g.* alginate and carrageenans), and this paper is among the first reports of such a disaccharide repeating backbone for fucoidans from brown algae.

In addition, the sequence analysis of fragments in heterogeneous structure fragments was also determined. The linkages between Fuc and Gal residues were predominantly 1,3-type whereas other heterogeneous fragments were 1,4-type linkage. The Fuc and Gal residues were mainly at nonreducing termini. Sulfation mainly occurred at C2 or C4 of Fuc and C2, C4 or C6 of Gal. All the data above reconfirmed the highly heterogeneity of the fucoidan from *H. fusiforme*.

It is interesting to note that the repeating disaccharide of —[GlcA-Man]— of backbone structure of YF5 is similar to the repeating disaccharide of —[GlcA-GlcNAc]— of hyaluronic acid. It is speculated that the novel glucuronomannan oligosaccharides may have some potential HA-liked biological activities. Further studies on the interactions of these novel oligosaccharides with proteins will be carried out using glycan arrays.

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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.carbpol.2012.05.084.

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