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Galactomannan with novel structure produced by the coral endophytic fungus *Aspergillus ochraceus*

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ABSTRACT

The homogeneous extracellular polysaccharide, AW1, was obtained from the fermented broth of the fungus *Aspergillus ochraceus* derived from coral *Dichotella gemmacea*. AW1 was a galactomannan with a molar ratio of mannose and galactose of 2.16:1.00 and a molecular weight of about 29.0 kDa. The structure of AW1 was investigated by chemical and spectroscopic methods, including methylation analysis, one- and two-dimensional nuclear magnetic resonance (1D, 2D NMR) and electrospray mass spectrometry with collision-induced dissociation (ES-CID MS/MS) spectroscopic analyses. The results showed that the backbone of AW1 consisted of (1 ⟶ 2)-linked α-D-mannopyranose residues. The mannopyranose residues in the backbone were substituted at C-6 by the (1 ⟶)-linked α-D-mannopyranose units and (1 ⟶ 5)-linked β-D-galactofuranose oligosaccharides with different degrees of polymerization. The investigation demonstrated that AW1 was a novel galactomannan with different structural characteristics from other fungal galactomannans, and could be a potential resource of the (1 ⟶ 5)-linked β-D-galactofuranose oligosaccharides.

1. Introduction

Polysaccharides produced by marine fungi present unique structures and diverse properties due to their specific marine environment (Selbmann et al., 2002). Many marine fungi are new or inadequately described species, especially the endophytic fungi from marine organisms (Olson & Kellogg, 2010; Raghukumar, 2008). With today's interest in new renewable sources of polymers, the polysaccharides produced by endophytic fungi from marine organisms are recognized as a potential source to be explored (Chen et al., 2012).

Some galactomannans from mycelia of *Aspergillus* species have been reported (Gómez-Miranda & Leal, 1981; Nakajima & Ichishima, 1994). The main chain of the galactomannans generally consists of (1 ⟶ 2) linked α- mannopyranose units. Differences are mainly based on the side chain composition and linkages. The galactofuranose residues are attached to the mannan backbone by the (1 ⟶ 2), (1 ⟶ 3) and (1 ⟶ 6) glycosidic linkages (Gómez-Miranda et al., 2003; Tisher, Gorin, de Souza, & Barreto-Bergter, 2002). Recently, the interest in galactomannans with galactofuranose units is increasing because of their novel structures and properties (Peltier et al., 2008). However, the structures of galactomannan from the culture medium of *Aspergillus* species have not yet been fully characterized. In the paper, a galactofuranose-containing galactomannan was isolated from the fermented broth of the fungus *Aspergillus ochraceus* derived from coral *Dichotella gemmacea*, and its structural characteristics were investigated by a combination of chemical and spectroscopic methods, including one- and two-dimensional nuclear magnetic resonance (1D, 2D NMR) and electrospray mass spectrometry with collision-induced dissociation (ES-CID MS/MS) spectroscopy techniques.

2. Materials and methods

2.1. Materials

Pullulan standards (M_w: 344, 200, 107, 49.1, 21.1, 9.6 and 5.9 kDa) were from Showa Denko K.K. (Tokyo, Japan). D-Glucose, D-mannose, D-galactose, L-rhamnose, D-xylose and L-fucose were from Sigma–Aldrich (St. Louis, MO, USA). Dialysis membranes (flat width 44 mm, molecular weight cut off 3500) were from Lvniao (Yantai, China). Q Sepharose Fast Flow and Sephadex G 150 were

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from GE healthcare (Piscataway, NJ, USA). Bio-Gel P-4 was from BioRad (Richmond, CA). Silica Gel 60 high performance thin layer chromatography (HPTLC) plates (with aluminium backing) were from Merck (Darmstadt, Germany).

2.2. Microorganism and culture conditions

A. ochraceus was isolated from the coral *Dichotella gemmacea,* which was collected from South Sea, China. It was identified according to its morphological characteristics and 18 S rRNA sequences. The producing strain was activated on potato dextrose agar (PDA) slants at 3.33% salt concentration and stored at 20 °C for 7 days. *A. ochraceus* was grown under static conditions at 20 °C for 30 days in the liquid medium containing mannitol (20 g/L), maltose (20 g/L), glucose (10 g/L), yeast extract paste (3 g/L), maize paste (1 g/L) monosodium glutamate (10 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4$ (0.3 g/L), and sea salt (33.3 g/L), pH 6.5. The fermented broth of 60 L was obtained.

2.3. Isolation and purification of the extracellular polysaccharides

The procedures used for the isolation and purification of the extracellular polysaccharides are similar to the method described by Sun et al. (2009). The crude polysaccharide was fractionated by a Q Sepharose Fast Flow column (2.6 cm × 50 cm) coupled to an AKTA FPLC system, eluted with a step-wise gradient of 0 and 0.25 M NaCl. The fractions were assayed for carbohydrate content by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The fractions eluted with water were pooled, dialyzed, and further purified on a Sephadex G 150 column (2.6 cm × 90 cm) with 0.2 M NH_4HCO_3 as eluent. The major polysaccharide fraction was pooled, freeze–dried and designated as AW1.

2.4. Mild acid hydrolysis of AW1 and preparation of oligosaccharide fractions

10.0 mL of AW1 (5 mg/mL) was taken, and then 1.0 M hydrochloric acid was added to adjust pH to 1.5 under magnetic stirring. The mixture was kept at 70 °C for 4 h. The hydrolysis product was neutralized with ammonium bicarbonate, concentrated under reduced pressure at 40 °C, and a two-fold volume of 95% (v/v) ethanol was added. The resulting supernatant and precipitate were recovered by centrifugation (3600 g, 10 min), and designated as AW1-S and AW1-E, respectively. The precipitate AW1-E was washed with ethanol and vacuum-dried. The supernatant AW1-S was concentrated, and further fractionated with a Bio-Gel P-4 column (1.3 cm × 90 cm) by elution with 0.2 M NH_4HCO_3 and detection by refractive index. The oligosaccharide fractions were collected, freeze–dried and designated as 1–9, respectively.

2.5. Chemical analysis

Total sugar content was determined by the phenol–sulfuric acid method using mannose and galactose as the standard (Dubois et al., 1956). Protein content was measured by the method of Bradford (1976) using bovine serum albumin as the standard. Sulfate ester content was detected according to the method reported by Therho and Hartiala (1971). Uronic acid content was measured by the carbazole–sulfuric acid method using glucuronic acid as standard (Bitter & Muir, 1962).

2.6. Analysis of monosaccharide composition

The monosaccharide compositions were measured by gas chromatography (GC) (Mao et al., 2008). Briefly, polysaccharide (5 mg) was hydrolyzed with 2 M TFA (1.0 mL) at 110 °C for 6 h. Excess acid was removed by co-distillation with methanol after the hydrolysis was completed. The dry hydrolysate together with hydroxylamine hydrochloride (5.0 mg) and inositol (1.0 mg) were dissolved in pyridine (0.5 mL), and heated at 90 °C for 30 min. The mixture was cooled before acetic anhydride (0.5 mL) was added to the mixture, and then incubated at 90 °C for another 30 min. GC was performed on a HP6890 instrument with a SE-54 fused silica capillary column (320 μm × 50 m) (Agilent Technologies Co., Ltd., USA) equipped with flame-ionization detector. The operation was performed using the following conditions: H_2: 1.5 mL/min; air: 200 mL/min; N_2: 1.5 mL/min; injection temperature: 250 °C; detector temperature: 250 °C; column temperature: 212 °C. Sugar identification was done by comparison with reference sugars (D-glucose, D-mannose, D-galactose, L-rhamnose, D-xylose and L-fucose). Calculation of the molar ratio of the monosaccharide was carried out on the basis of the peak area of the monosaccharide.

2.7. Determination of sugar configuration

Sugar configuration was determined as described by Tanaka (Tanaka et al., 2007). Briefly, 5.0 mg of polysaccharide was hydrolyzed with 2 M trifluoroacetic acid at 105 °C for 6 h. Excess acid was removed with methanol in a rotary evaporator. The hydrolysate was heated with l-cycteine methyl ester in pyridine at 60 °C for 60 min. A solution of the *o*-tolyl isothiocyanate was added to the mixture, and was further heated at 60 °C for 60 min. The reaction mixture was analyzed on an Agilent 1260 Infinity HPLC instrument using an Eclipse XDB-C_{18} column (4.6 mm × 250 mm) and detected by an Agilent XDB-UV detector at 250 nm. Sugar configuration was identified by comparison with reference sugars.

2.8. Determination of purity and molecular weight

Purity and molecular weight were determined by high performance gel permeation chromatography (HPGPC) with a Shodex Ohpak SB-804 (7.8 cm × 30 cm, Tokyo, Japan) column eluting with 0.1 M Na_2SO_4 (Mao et al., 2008). The molecular weight was estimated by reference to a calibration curve made by pullulan standards (M_w: 344, 200, 107, 49.1, 21.1, 9.6 and 5.9 kDa).

2.9. High performance thin layer chromatography

The purity of oligosaccharide was checked on a silica gel HP-TLC plate (2 cm × 4.5 cm) developed with a solvent system of triethylamine/*n*-butanol/water (0.7:60:30, v/v/v). The developed plates were stained by dipping in diphenylamine/aniline/phosphoric acid reagent for 2 s, and heated at 105 °C for 10 min for color formation.

2.10. Methylation analysis

Methylation analysis was performed by the method of Hakomori (1964) with some modifications. Briefly, polysaccharide in dimethyl sulfoxide was methylated using anhydrous sodium hydride and iodomethane, and the completeness of methylation was confirmed by infrared spectroscopy. After hydrolysis with 2 M trifluoroacetic acid at 110 °C for 6 h, the methylated sugar residues were converted to partially methylated alditol acetates by reduction with $NaBH_4$, followed by acetylation with acetic anhydride. The derivatised sugar residues were extracted into dichloromethane and evaporated to dryness, dissolved again in 100 μL dichloromethane. The products were analyzed by gas chromatography–mass spectrometry (GC–MS) on a DB 225 fused silica capillary column (0.25 mm × 30 m) (Agilent Technologies Co.

Ltd., USA), using a temperature gradient: first 100–220 °C with a rate of 5 °C /min, then keeping at 220 °C for 15 min. Identification of partially methylated alditol acetates was carried out on the basis of retention time and mass fragmentation patterns.

2.11. NMR spectroscopy analysis

^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were recorded at 23 °C using a JEOL JNM-ECP 600 MHz spectrometer. Polysaccharides (20 mg) or oligosaccharides (5 mg) were deuterium exchanged by two successive freeze–drying steps in 99% D_2O and then dissolved in 0.5 mL of 99.98% D_2O. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C. Distortionless enhancement by polarization transfer spectroscopy (DEPT), ^{1}H—^{1}H correlated spectroscopy (COSY), ^{1}H—^{13}C heteronuclear multiple quantum coherence spectroscopy (HMQC), ^{1}H—^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC) and nuclear Overhauser effect spectroscopy (NOESY) experiments were also carried out.

2.12. Mass spectrometry

Electrospray mass spectrometry (ES-MS) analysis was performed on a Micromass Q-Tof Ultima instrument (Waters, Manchester, UK). Oligosaccharides were dissolved in acetonitrile/1.0 mM NH_4HCO_3, (1:1, v/v), with a final concentration of 5.0 μg/μL, of which 10 μL was loop-injected. Nitrogen was used as the desolvation and nebulizer gas, at a flow rate of 250 L/h and 15 L/h, respectively. The source temperature and the desolvation temperature were 80 °C and 150 °C, respectively. The cone voltage and capillary voltage were maintained at 50 V and 3 kV, respectively. For negative-ion ES-CID MS/MS product-ion scanning, argon was used as the collision gas at the pressure of 1.7 bar, and the collision energy was adjusted between 15∼100 eV for an optimal sequence information.

3. Results and discussion

3.1. Isolation and composition analysis of the extracellular polysaccharide AW1

The crude extracellular polysaccharide was obtained from the fermented broth of the coral endophytic fungus *A. ochraceus,* and fractionated using a Q Sepharose Fast Flow column into two fractions, the polysaccharide fraction AW eluted with distilled water and the polysaccharide fraction AS eluted with 0.05 M NaCl. Here, the fraction AW was further fractionated using a Sephadex G 150 column, and two extracellular polysaccharides, AW1 and AW2, were obtained. The two fractions were composed of mannose and galactose with similar molar ratios. In this paper, we mainly focus on the structure of AW1. The yield of AW1 was about 0.03 g/L. AW1 appeared as a single and symmetrical peak in the HPGPC chromatogram (Supplemental Fig. 1), indicating its homogeneity and its molecular weight was estimated to be about 29.0 kDa. GC analysis showed that AW1 consisted of mannose and galactose in a molar ratio of 2.16:1.0. Reversed-phase HPLC analysis of the polysaccharide exhibited peaks at 13.30 and 9.41 min, which were coincided with derivatives of D-galactose and D-mannose, identifying the absolute configuration of the sugar components. The monosaccharide composition of AW1 is different from that of the polysaccharides from the yeast extract (Komura et al., 2010), and the polysaccharides containing only mannose are typical for yeast extracts. No any sulfate ester, uronic acid and protein were detected in AW1.

3.2. Mild acid hydrolysis of AW1 and preparation of oligosaccharide fractions

To determine the structural characteristics of AW1, the oligosaccharides were prepared by mild acid hydrolysis of AW1. As shown in Supplemental Fig. 2a, more and more oligosaccharide fragments were released from AW1 with the increasing of hydrolysis time. Further incubation (>4 h) under the same condition did not result in the increase of oligosaccharide fragments. Therefore, the extracellular polysaccharide AW1 was hydrolyzed under mild acid condition at 70 °C for 4 h.

Two types of hydrolysis products, AW1-S and AW1-E, were obtained. AW1-S was fractionated by gel filtration chromatography, the oligosaccharide fractions were obtained (Supplemental Fig. 2b) and analyzed by HP-TLC (Supplemental Fig. 2c). The result showed that each oligosaccharide migrated as a single band, indicating its homogeneity. Fraction 1 was identified to be a galactose. AW1-E showed a sharp and symmetrical peak on Shodex OHpak SB-804 HQ column, and its molecular weight was about 26.1 kDa. Compared with the GC result of AW1, the galactose content of AW1-E significantly decreased. The molar ratio of mannose and galactose in AW1-E was 4.35:1.0, while the oligosaccharide fractions were only composed of galactose. The result suggested that galactose was easier to be released from the chain of AW1 than mannose by mild acid hydrolysis, and the galacto-oligosaccharides could be located at the side chains of AW1.

The molecular weights of the oligosaccharide fractions were determined by ES-MS. From the ES-MS spectrum of the oligosaccharide fraction 2, the oligosaccharide fraction 2 was identified to be a disaccharide. The oligosaccharide fraction 3 was deduced to be a trisaccharide, and the oligosaccharide fraction 4 was proposed to be a tetrasaccharide. A similar regular increment of 162 Da was found for oligosaccharide fractions 5–9 (Supplemental Table 1).

3.3. Structural characteristics of the oligosaccharide fractions

3.3.1. Methylation analysis

Methylation analysis could provide important information for the linkage position assignments of each monosaccharide. The results indicated that all of the galacto-oligosaccharides showed two ion peaks. 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl-galactitol was originated from Gal*f* (1 ⟶ .1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol could be assigned to ⟶ 4) Gal*p*(1 ⟶ if the galactose was in the pyranose form, or ⟶ 5) Gal*f*(1 ⟶ if the galactose was in the furanose form. In order to elucidate the linkage pattern of the galacto-oligosaccharides, the oligosaccharide fraction 5 was randomly chosen for NMR spectroscopy analysis.

3.3.2. NMR spectroscopy analysis of the oligosaccharide fraction 5

From the ^{1}H NMR spectrum of the oligosaccharide fraction 5 (Fig. 1a), the anomeric proton signals at 5.01, 4.99 and 4.88 ppm were observed, and had relative integrals of 1:3:1. The chemical shifts from 3.56 to 3.98 were assigned to protons of H2–H6. In the ^{13}C NMR spectrum of the oligosaccharide fraction 5 (Fig. 1b), two anomeric carbon signals at 107.02 and 105.69 ppm were assigned to signals of β-galactofuranose because of extremely low field shift. The anomeric carbon signals of α- and β-galactopyranose and α-galactofuranose do not exceed 105 ppm (Nagaoka et al., 1996). Other signals were located at the region of 60.94–82.58 ppm. The signal appearing at 60.94 ppm was correlated to C6 non-substituted sugar units (Giménez-Abián et al., 2007). Theoretically, both

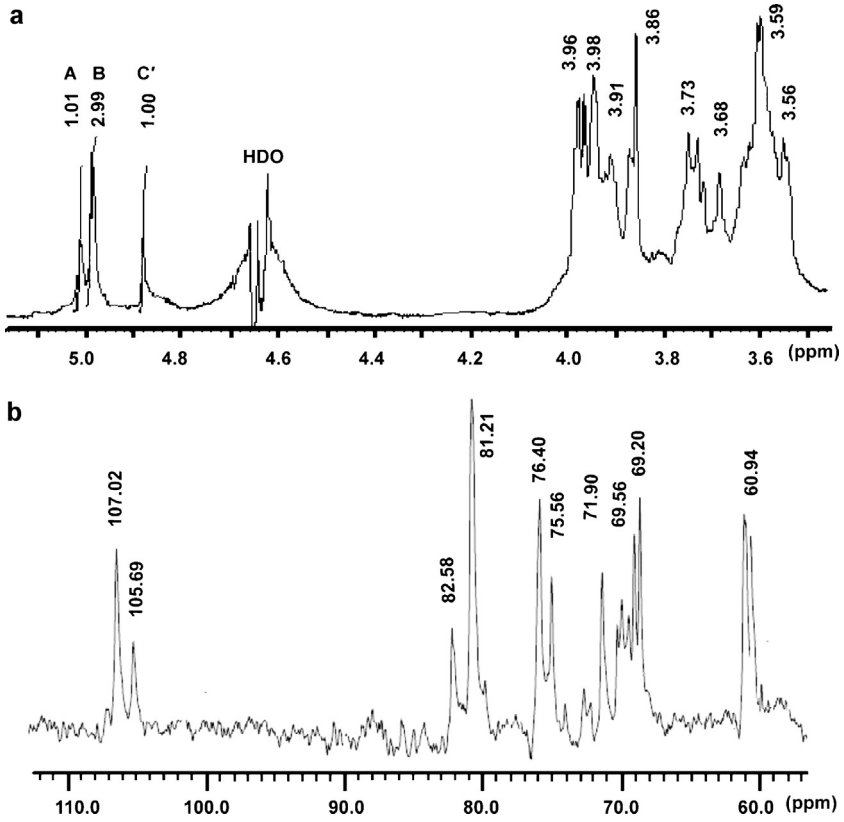

Fig. 1. 1D NMR spectra of the oligosaccharide fraction 5. (a) ^{1}H NMR spectrum; (b) ^{13}C NMR spectrum. Spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C. A–C\ correspond to β-D-Gal*f*(1 ⟶, ⟶ 5)-β-D-Gal*f*(1 ⟶, and ⟶ 5)-β-D-Gal*f*, respectively. Gal*f*: galactofuranose.

α- and β-anomers in the NMR spectrum maybe observed. However, only β-anomer could be distinguished in the NMR spectrum of the oligosaccharide fraction 5. It may be due to the dominant configuration of the oligosaccharide fraction 5 is β-configuration in the solution and the concentration of the oligosaccharide used, and it may be also related to the resolution of the NMR spectrometer.

The ^{1}H—^{1}H COSY spectrum (Fig. 2a) gave various proton correlations of the sugar residues. The direct C–H coupling was determined by the ^{1}H—^{13}C HMQC spectrum (Fig. 2b). The anomeric proton signal at 5.01 ppm correlated to the anomeric carbon signal A at 107.02 ppm. The anomeric proton signal at 4.99 ppm was related to the anomeric carbon signal B at 107.02 ppm, while the anomeric proton signal at 4.88 ppm correlated with the anomeric carbon signal C\ at 105.69 ppm. By combining the data from the ^{1}H—^{13}H COSY and ^{1}H—^{13}C HMQC spectra, the assignment of almost all the proton and carbon signals of A–C\ monosaccharide units were completed. Comparison of the experiment values with those of polysaccharides with similar structure, suggested that B was a (1 ⟶ 5)-linked β-galactofuranose residue while A was a (1 ⟶)-linked β-galactofuranose unit, because the chemical shift of C5 of B changed to low displacement at 75.56 ppm relatively to that of A at about 70.01 ppm. Furthermore, the NOESY experiment (Fig. 2c) gave important correlation signals. The presences of the cross signal of H1 in A and H5 in B, and the cross signal of H1 in B and H5 in B confirmed that B lied at the middle positions of the oligosaccharide with the linkage pattern of ⟶ 5)-β-D-Gal*f*(1 ⟶ unambiguously, whereas A and C\ were assigned to the non-reducing end and reducing end, respectively.

The results suggested that the oligosaccharide fraction 5 contained consecutive ⟶ 5)-β-D-Gal*f*(1 ⟶ residues. The proposed structure of the oligosaccharide fraction 5 was shown in Fig. 2d.

3.3.3. *Fragmentation pattern of the oligosaccharide fractions by ES-CID MS/MS analysis*

The detailed sequences of the oligosaccharide fractions were also investigated by ES collision-induced dissociation (CID) MS/MS. The nomenclature used to define the fragmentations of oligosaccharide is based on that introduced by Domon and Costello (1988). Some uncommon fragment ions were designated as following: from the non-reducing end, the fragment ion produced by the disintegration between C4 and C5 of the first glycosyl was named as D_1, and the same kind fragment ion produced by the second glycosyl was named as D_2, and so forth. From the reducing end, the fragment ion produced by the disintegration between C4 and C5 of the first glycosyl was named as W_1, and the same kind fragment ion produced by the second glycosyl was named as W_2, and so forth.

For the negative-ion ES-CID MS/MS, $[M-H]^-$ was used as precursors for optimal sequence information. The results demonstrated that all the galacto-oligosaccharides had the same kind of fragment ions. As an example, the product-ion spectrum of the oligosaccharide fraction 5, $[M-H]^-$ at *m/z* 827.5, was shown in Fig. 3a. The predominant ions observed were attributed to C/Y-type glycosidic bond cleavage yielding the ion at *m/z* 655.5, *m/z* 503.3, *m/z* 341.2 and *m/z* 179.1 corresponding to the loss of one to four glycosyl (162 Da), respectively. B/Z-type fragment ions were also present. Cross-ring fragment ions at m/z 233.1, *m/z* 395.2, *m/z* 557.4 and *m/z* 719.5 were attributed to ^{3,4}A2, ^{3,4}A3, ^{3,4}A4, and ^{3,4}A5, respectively (Fig. 3b). ^{0,3}A-type fragment ions could also be found. Furthermore, the unusual D/W-type fragment ions were present. For example, the fragment ions at *m/z* 281.1, *m/z* 443.2, *m/z* 605.4 and *m/z* 767.6 were attributed to W1, W2, W3 and W4, respectively. For the positive-ion ESI-MS/MS of the oligosaccharide fraction 5, $[M+H]^+$ at *m/z* 829.5 was used as precursors for optimal sequence

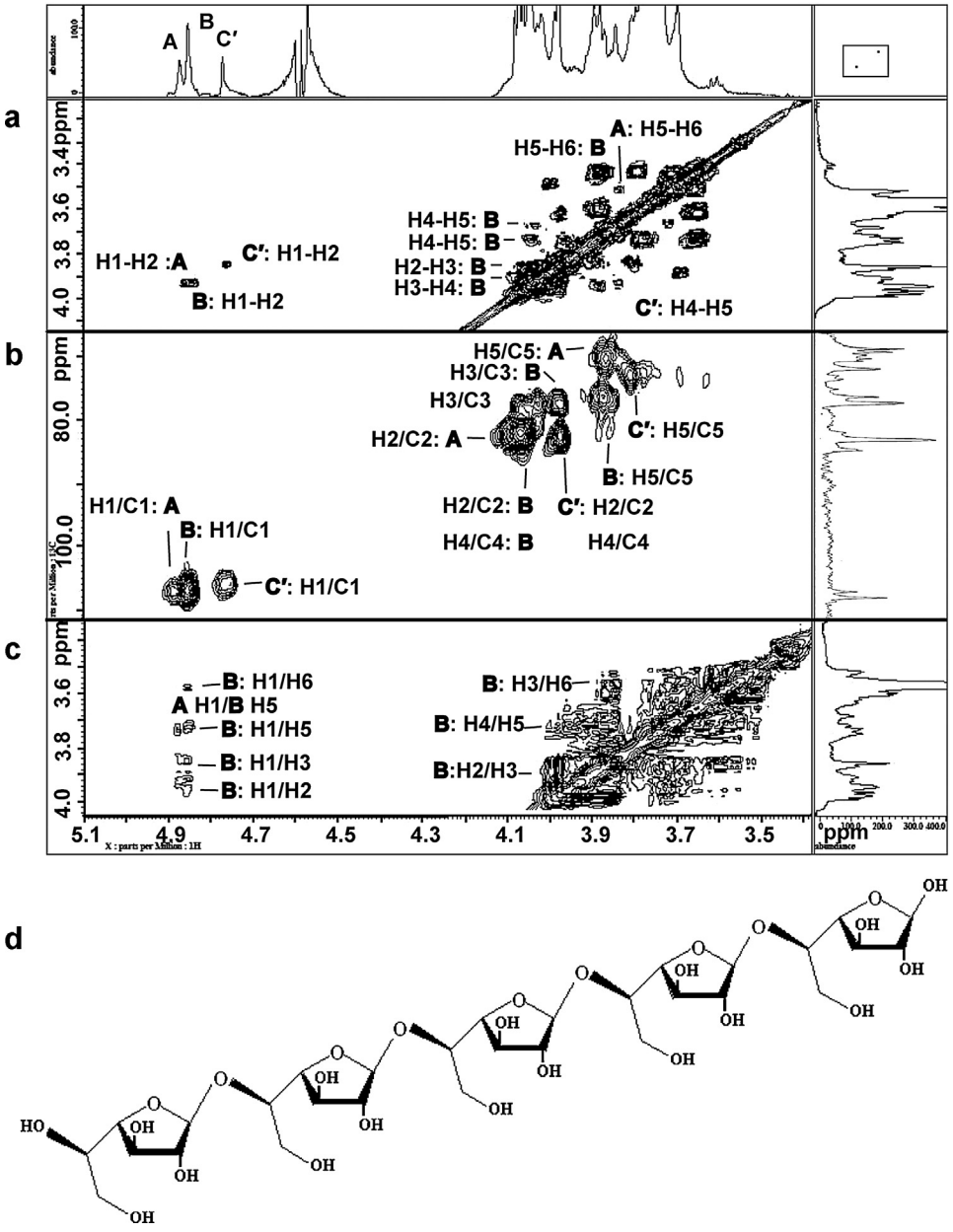

Fig. 2. 2D NMR spectra of the oligosaccharide fraction 5 and proposed structure of the oligosaccharide fraction 5. (a) $^{1}H—^{1}H$ COSY spectrum; (b) $^{1}H—^{13}C$ HMQC spectrum; (c) NOESY spectrum; (d) proposed structure of the oligosaccharide fraction 5. Spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C. A–C\ correspond to β-D-Gal*f*(1 ⟶, ⟶ 5)-β-D-Gal*f*(1 ⟶, and ⟶ 5)-β-D-Gal*f*, respectively. Gal*f*: galactofuranose.

information. As shown in Fig. 3c, both of B/Z- and C/Y-type fragment ions were present. The results suggested that all of the galacto-oligosaccharides had the same kind of disintegration pattern. These oligosaccharide fractions were deduced to be linear β-D-(1 ⟶ 5)-linked Gal*f* oligosaccharides

3.4. Structural characteristics of AW1

3.4.1. Methylation analysis

In order to determine the linkage pattern of the sugar residues, AW1 and AW1-E were subjected to methylation analysis. A large amount of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol, which originated from ⟶ 5) Gal*f*(1 ⟶ was found in AW1 (Table 1). 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl-galactitol was also detected, indicating the presence of Gal*f*(1 ⟶. Compared with the results of AW1, the molar ratio of the 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol in AW1-E decreased observably, indicating that the galactose was easier to be released from the chain of AW1 by mild acid hydrolysis. 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-mannitol and 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl- mannitol were also detected, indicating the presence of ⟶ 2) Man*p*(1 ⟶ and Man*p*(1 ⟶ residues in AW1 and AW1-E. The molar ratio of ⟶ 2) Man*p*(1 ⟶ in AW1-E was higher than that in AW1, suggesting that the ⟶ 2) Man*p*(1 ⟶ residues were in the main chain of AW1. In addition, AW1 should have a highly branched structure because of the presence of a large amount of 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-mannitol, which originated from the ⟶ 2,6) Man*p*(1 ⟶. The result suggested that AW1 was a galactomannan with highly branch. The main chain of the polysaccharide may be composed of (1 ⟶ 2)-linked mannopyranose residues, and

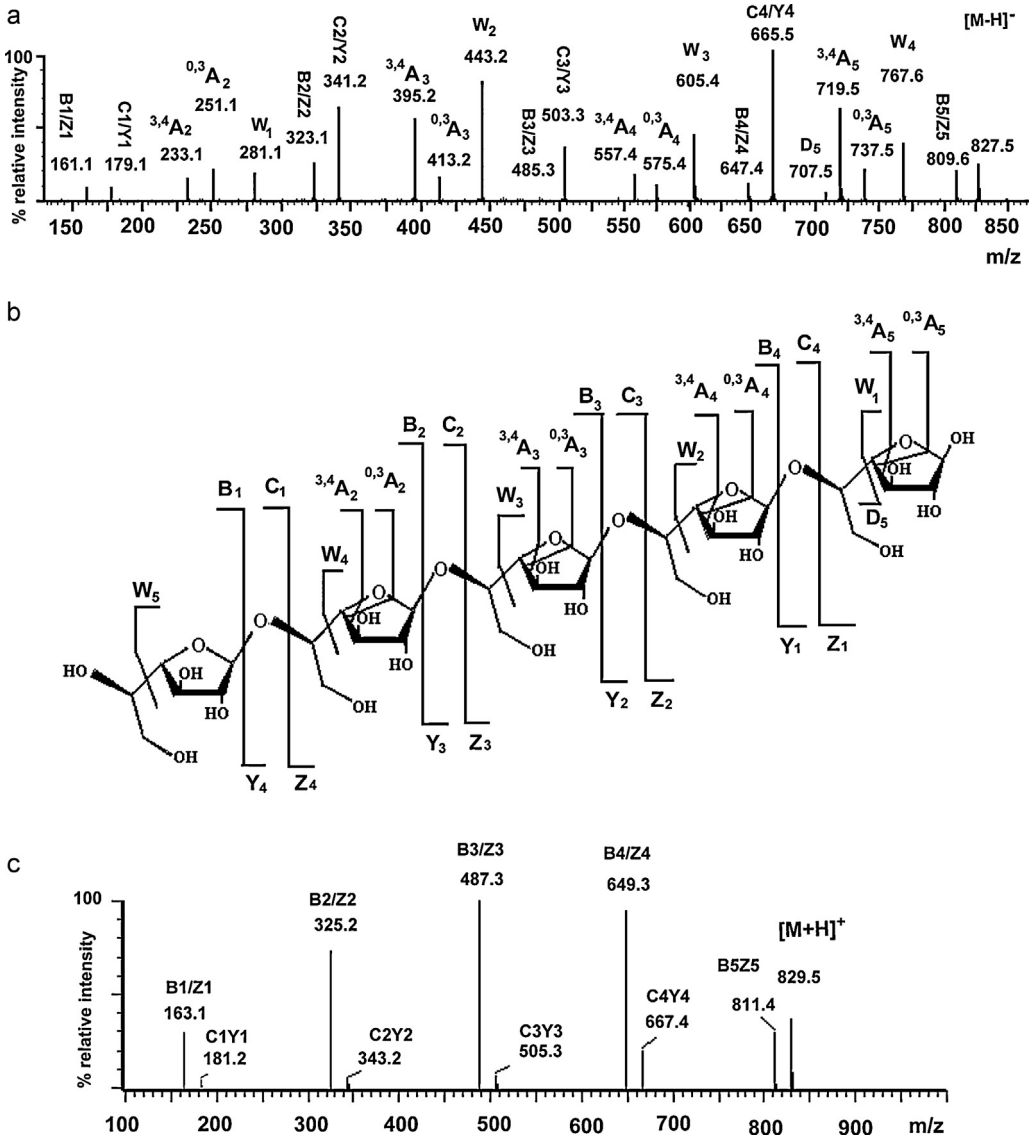

Fig. 3. ES-CID MS/MS product ion spectrum and assignments of the ions of the oligosaccharide fraction 5. (a) Negative-ion ES-CID MS/MS product ion spectrum; (b) assignments of the ions of the oligosaccharide fraction 5; (c) position-ion ES-CID MS/MS product ion spectrum. The sequence is shown to indicate the proposed fragmentations.

the galactose residues should be linked to the C-6 of the ⟶ 2) Man*p*(1 ⟶ units.

It is found that the ratio of 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methyl-galactitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactitol does not coincide with the dimensions of oligosaccharides obtained by partial acid hydrolysis. The difference may be due to side substituents of the (1 ⟶ 5)-linked β-D-galactofuranose oligosaccharides with different degrees of polymerization. The amount of each oligosaccharide in the side chain is different, and the oligosaccharides with lower DP could be major compositions. In addition, the result was less quantitative probably because of β-elimination of AW1 during methylation and loss of the degradation products during dialysis of the derivatized samples.

3.4.2. NMR spectroscopy analysis

The ^{13}C NMR spectrum (Fig. 4a) of AW1 showed anomeric carbon signals at 107.70, 107.08, 102.21, 100.65 and 98.20 ppm, which were labeled A–E from low to high field. A and B were assigned to the C1 of β-galactofuranose because of extremely low field shifts. The anomeric carbon signals of α- and β-galactopyranose and α-galactofuranose do not exceed 105 ppm (Nagaoka et al., 1996). C–E

Table 1
Results from methylation analysis of AW1 and AW1-E.

Retention time	Permethylated alditol acetate	Molar ratio		Deduced linkage type
		AW1	AW1-E	
27.639	2,3,5,6-Me$_4$-Gal*f*	1.00	1.00	Gal*f* (1 ⟶
30.860	2,3,6-Me$_3$-Gal*f*	1.61	1.29	⟶ 5) Gal*f* (1 ⟶
27.247	2,3,4,6-Me$_4$-Man*p*	1.49	2.70	Man*p*(1 ⟶
29.971	3,4,6-Me$_3$- Man*p*	1.63	3.65	⟶ 2) Man*p*(1 ⟶
35.664	3,4-Me$_2$-Man*p*	2.51	3.62	⟶ 2,6) Man*p*(1 ⟶

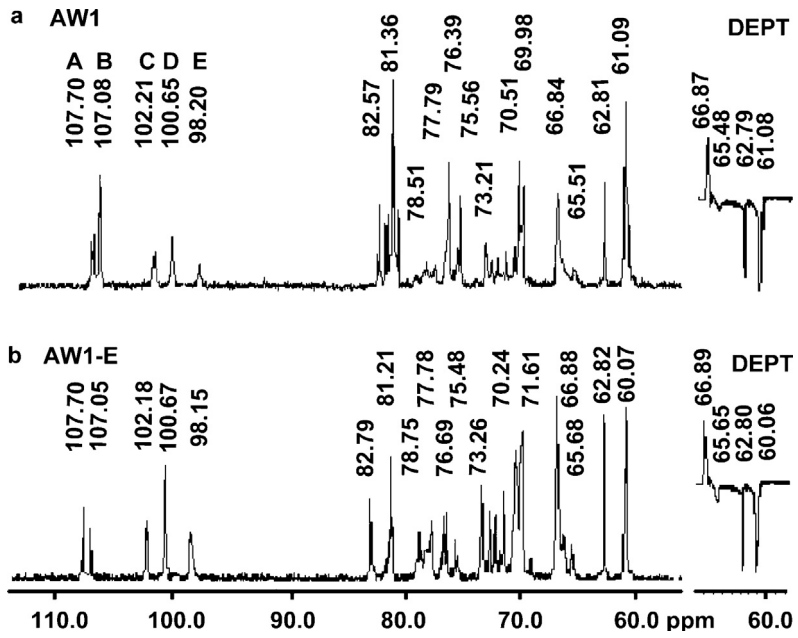

Fig. 4. ^{13}C NMR and DETP spectra of AW1 and AW1-E. (a) ^{13}C NMR and DETP spectra of AW1; (b) ^{13}C NMR and DETP spectra of AW1-E. Spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C. A–E correspond to β-D-Gal*f* (1 ⟶, ⟶ 5)-β-D-Gal*f* (1 ⟶, α-D-Man*p*(1 ⟶, ⟶ 2)-α-D-Man*p*(1 ⟶, and ⟶ 2,6)-α-D-Man*p*(1 ⟶, respectively. Gal*f*: galactofuranose, Man*p*: mannopyranose.

might be the signals of the mannopyranose linkages (Sassaki et al., 2002; Tischer et al., 2002). Compared with the ^{13}C NMR spectrum of AW1, the remarkably decreased carbon signals at 107.08, 81.36, 76.39, 81.36, 75.56 and 61.09 ppm in the ^{13}C NMR spectrum (Fig. 4b) of AW1-E, could be assigned to the (1 ⟶ 5)-linked β-galactofuranose residue. As shown in the DEPT spectrum of AW1, the signal at 65.51 ppm was due to the C-6 substituted residues, while the signals at 62.81 and 61.09 ppm were due to the C-6 non-substituted residues.

The ^{1}H—^{1}H COSY spectrum (data not shown) of AW1 allowed the assignment of most of the signals of the proton spin systems. The ^{1}H—^{13}C HMQC spectrum of AW1 also permitted assignment of the relevant carbon signals (Fig. 5a). The anomeric carbon signal of A at 107.70 ppm correlated to anomeric proton signal at 4.86 ppm. The anomeric carbon signal of B at 107.08 ppm was related to the anomeric proton signal at 5.03 ppm. The carbon signals at 107.70 and 107.08 ppm were the anomeric carbon signals of β-galactofuranose residues. Comparison of the experiment values with those of polysaccharides with similar structure, suggested that A was a (1 ⟶)-linked β-galactofuranose unit while B was a (1 ⟶ 5)-linked β-galactofuranose residue, because the chemical shift of C5 of B changed to low displacement at 75.56 ppm relatively to that of A at about 71.14 ppm. The anomeric carbon signal of C at 102.21 ppm correlated with the anomeric proton signal at 5.02 ppm, thus C was attributed to the (1 ⟶)-linked α-mannopyranose residue (Kobayashi et al., 1995). The anomeric carbon signal of D at 100.65 ppm was related with the anomeric proton signal at 5.06 ppm, and D was assigned to the (1 ⟶ 2)-linked α-mannopyranose, which was also proved by the downfield shifting of the chemical shift of C2 (77.79 ppm). The cross signal of the H2/C2 in D further indicated that D was the (1 ⟶ 2)-linked α-mannopyranose residue. The anomeric carbon signal of E at 98.20 ppm correlated with the anomeric proton signal at 4.90 ppm, and E was assigned to the (1 ⟶ 2,6)-linked α-mannopyranose (Omarsdottir et al., 2006; Tischer et al., 2002), which was also proved by the downfield shifting of the chemical shift of C2 (78.51 ppm) and C6 (65.51 ppm). The cross signals of the H2/C2 and H2/C6 in E further indicated that E was the (1 ⟶ 2,6)-linked α-mannopyranose residue. Combining with the data from the ^{1}H—^{1}H COSY and ^{1}H—^{13}C HMQC spectra, and comparison with the chemical shift data of similarly substituted sugar residues (Carbonero et al., 2005; Corsaro et al., 2001; Omarsdottir et al., 2006; Vinogradov et al., 1998), the main signals of the sugar residues of AW1 were assigned (Table 2).

The ^{1}H—^{13}C HMBC spectrum (Fig. 5b) of AW1 exhibited the linkage sequence of different glycosidic residues. The cross peak H1A/C1B suggested that the non-reducing end Gal*f* was linked to the (1 ⟶ 5)-linked β-galactofuranose residue. The cross peak H1/C5B confirmed the presence of the successive linkage of the (1 ⟶ 5)-linked β-galactofuranose residues. The results suggested the presence of the galacto-oligosaccharides as side chains in AW1. The cross-peaks between Gal*f* and Man*p* residues were hard to be distinguished. Compared with the ^{13}C NMR spectrum of AW1, the intensity of residue D increased remarkably in the ^{13}C NMR spectrum of AW1-E, which indicated the galacto-oligosachharides should be linked to the C-6 position of (1 ⟶ 2)-linked α-mannopyranose residue. Further efforts to investigate the linkage of α-mannopyranose units in AW1 were ineffective for lack of interpretable correlations in the ^{1}H—^{13}C HMBC spectrum. Combined with the data on the methylation analysis, the high proportion of (1 ⟶ 2,6)-linked α-mannopyranose residue was present in AW1. Thus, the non-reducing terminal α-mannopyranose residues in AW1 could be linked to the C-6 position of (1 ⟶ 2)-linked α-mannopyranose residue.

Based on the results above, the hypothetical structure of AW1 was established as shown in Fig. 6. The structure of AW1 was different from those of the extracellular polysaccharides from *Aspergillus* species (Chen et al., 2012; Gómez-Miranda & Leal, 1981; Leal & Rupérez, 1978). AW1 had similar structural characteristics to the cell wall galactomannan isolated from *A. versicolor* and *A. fumigatus* (Gómez-Miranda et al., 2003; Tischer et al., 2002), and they had a mannan core with side chains of (1 ⟶ 5)-linked Gal*f* oligosaccharides. Galactomannans from some fungi mainly had two types of mannan core, including α-(1 ⟶ 6) mannan backbone and α-(1 ⟶ 2)/α-(1 ⟶ 6) mannan backbone (Leal et al., 2010). The mannan backbone of the galactomannans isolated from *A. niger*,

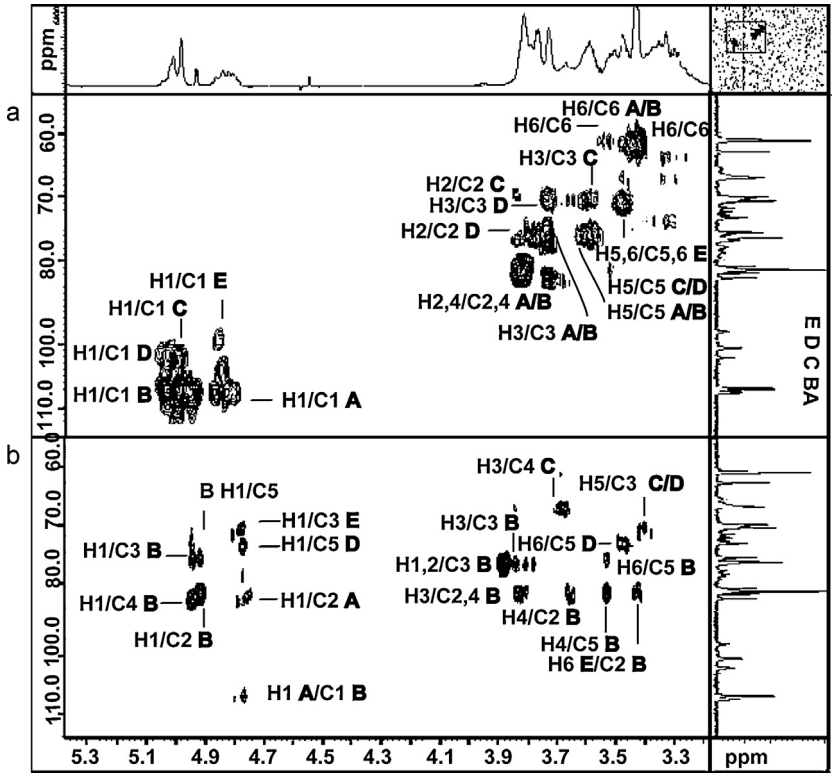

Fig. 5. 2D NMR spectra of AW1. (a) $^{1}H—^{13}C$ HMQC spectrum; (b) $^{1}H—^{13}C$ HMBC spectrum. Spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C. A–E correspond to β-D-Gal*f* (1⟶, ⟶5)-β-D-Gal*f* (1⟶, α-D-Man*p*(1⟶, ⟶2)-α-D-Man*p*(1⟶, and ⟶2,6)-α-D-Man*p*(1⟶, respectively. Gal*f*: *galactofuranose*, Man*p*: mannopyranose.

Table 2
^{1}H and ^{13}C chemical shifts for AW1.

Sugar residues [b]	$^{1}H/^{13}C$ chemical shifts (ppm)[a]					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
β-D-Gal*f* (1⟶ A	4.86/4.92107.70	3.9781.36	3.95 76.39	3.9782.57	3.8471.14	3.64/3.6461.09
⟶5)-β-D-Gal*f* (1⟶ B	5.03/5.05107.08	3.9881.36	3.9576.39	3.9881.36	3.7975.56	3.64/3.6461.09
α-D-Man*p*(1⟶ C	5.02102.21	3.9970.51	3.7869.98	3.6766.84	3.5773.21	–
⟶2)-α-D-Man*p*(1⟶ D	5.06100.65	3.9977.79	3.9169.98	3.6766.84	3.5773.21	–
⟶2,6)-α-D-Man*p*(1⟶E	4.9098.20	3.9978.51	3.9170.51	3.6766.84	3.6870.51	3.57/–65.51

[a] The spectra were performed at 23 °C on a JEOL ECP 600 MHz spectrometer. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^{1}H and 31.07 ppm for ^{13}C.
[b] Gal*f*: galactofuranose, Man*p*: mannopyranose.

Trichophyton species and *Penicillium charlesii*, mainly consisted of a α-(1⟶2)-linked mannotetraose repeat units, and the mannopyranose residues in the backbone were substituted mainly at C-6 (Ikuta et al., 1997; Latgé et al., 1994; Unkefer & Gander, 1990). AW1 had a similar mannan core to the galactomannans from fungi, but the side chain of AW1 was different from previously described fungal galactomannans. The branching of AW1 was mainly composed of the homogeneous (1⟶5)-linked Gal*f* oligosaccharide with DP 2–9. This is the first report of such kind of galactomannan isolated from the culture medium of microorganism. Komura et al. (2010) found that polysaccharides could have arisen from yeast extract used in the cultures media, and the possibility that polymers originated from the culture medium cannot be ruled out. Compared with the data of the literatures (Kath & Kulicke, 1999; Komura et al., 2010), AW1 was different from the polysaccharides from yeast extracts including monosaccharide component, main-chain and side-chain. The present result suggested that endophytic fungi from marine organisms could be a potential source of exopolysaccharides with unique structures to be worth being further studied.

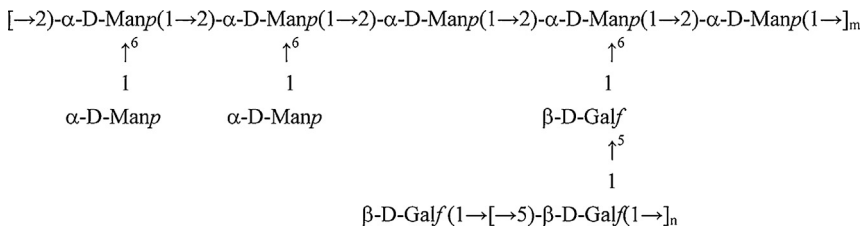

Fig. 6. One of the possible structures of AW1 (Gal*f*: galactofuranose, Man*p*: mannopyranose, m≈16, *n* = 0∼7).

4. Conclusion

The extracellular polysaccharide AW1 produced by the coral endophytic fungus *A. ochraceus* is a novel galactomannan with a mannan core. The side chains were linked to the C-6 of (1 ⟶ 2)-linked α-D-mannopyranose of backbone by the (1 ⟶)-linked α-D-mannopyranose units and (1 ⟶ 5)-linked β-D-galactofuranose oligosaccharides with different DP. AW1 may be a potential source of galactofuranose oligosaccharides. Further investigation on the preparation of (1 ⟶ 5)-linked galactofuranose oligosaccharides by controlled acid hydrolysis will play an important role in the applications of the extracellular polysaccharide AW1 in the food and pharmacology.

Acknowledgments

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Appendix 1

Table 1 Molecular weights of the oligosaccharide fractions obtained from mild acid hydrolysis of AW1.

Fractions	2	3	4	5	6	7	8	9
$[M-H]^-$	341.2	503.3	665.4	827.5	989.7	1151.8	1314.1	1476.3
DP	2	3	4	5	6	7	8	9

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.2014.01.079.

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