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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.