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Structural elucidation of the exopolysaccharide produced by the mangrove fungus *Penicillium solitum*

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ABSTRACT

A water soluble extracellular polysaccharide, designated GW-12, was obtained from the liquid culture broth of the mangrove fungus *Penicillium solitum* by ethanol precipitation, anion-exchange and size-exclusion chromatography. Reversed-phase high performance liquid chromatography analysis showed that GW-12 mainly consisted of D-mannose, and its molecular weight was estimated to be about 11.3 kDa determined by high performance gel permeation chromatography. On the basis of chemical and spectroscopic analyses, including methylation analysis and nuclear magnetic resonance (NMR) spectroscopy, the structure of GW-12 may be represented as a mannan with branches. The main chain of GW-12 was composed of (1→2)-linked α-D-mannopyranose and (1→6)-linked α-D-mannopyranose residues, branched by single α-D-mannopyranose units attached to the main chain at C-6 positions of (1→2)-linked α-D-mannopyranose residues. There was three branch points for every seven sugar residues in the backbone.

1. Introduction

Marine microorganisms are recognized as an abundant source of bioactive substances (Kobayashi & Tsuda, 2004; Peng et al., 2012). In recent years, the interest in extracellular polysaccharides produced by marine fungi is increasing (Sun et al., 2009; Wang et al., 2013). The diversity of marine fungi determines the diversity of extracellular polysaccharides (Raghukumar, 2008). The extracellular polysaccharides produced by marine fungi hold a great potential application in biology and pharmacology (Selbmann, Onofri, Fenice, Federici, & Petruccioli, 2002).

Mangrove forest is considered a dynamic ecotone or transition zone between terrestrial and marine habitats, and is biodiversity spots for marine fungi (Gopal & Chauhan, 2006; Shearer et al., 2007). Mangrove fungi constitute the second largest ecological group of the marine fungi due to its complex and special environment (Sridhar, 2004). So far, few reports on the extracellular polysaccharides from mangrove fungi could be found (Chen et al., 2011). With today's interest in new renewable sources of chemicals and polymers, the extracellular polysaccharides isolated from mangrove fungi are recognized as a potential source to be explored. In this study, a homogenous extracellular polysaccharide from the liquid culture broth of the mangrove fungus *Penicillium solitum* was isolated, and its structural characterizations were elucidated by a combination of chemical and spectroscopic methods, including gas chromatography–mass spectrometry (GC–MS), one- and two-dimensional nuclear magnetic resonance (1D and 2D NMR) spectroscopy.

2. Materials and methods

2.1. Materials

Monosaccharides (D-glucose, D-mannose, D-galactose, L-rhamnose, D-xylose, L-arabinose, L-fucose, D-glucuronic acid, D-galacturonic acid and D-glucosamine), 1,1-diphenyl-2-picrylhydrazyl, trifluoroacetic acid, thiobarbituric acid, 1-phenyl-3-methyl-5-pyrazolone, L-cysteine methyl ester and *o*-tolyl isothiocyanate were from Sigma–Aldrich (St. Louis, MO, USA). Pullulan standards (M_w: 47.1, 21.1, 9.6, 5.9 and 1.0 kDa) were from Showa Denko K.K. (Tokyo, Japan). Dialysis membranes (flat width 44 mm, molecular weight cut-off 3500) were from Lvniao (Yantai, China). Q Sepharose Fast Flow and Sephacryl S-100 were from GE Healthcare Life Sciences (Piscataway, NJ, USA).

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2.2. Microbial strain and culture conditions

The fungus *P. solitum* was separated from the soil of *Rhizophora stylosa* collected from the mangrove of the gulf of Shankouyingluo, Guangxi, China (N: 21° 29′ 798″ E: 109° 45′ 470″). It was identified according to its morphological characteristics and internal transcribed spacer (ITS) rDNA sequences, and the accession number of Genbank was KJ577599. The extracellular polysaccharide producing strain was activated on potato dextrose agar slants and stored at 25 °C for 3 days. *P. solitum* was then grown in the liquid medium containing potato steep liquor (20 g/L), maltose (2 g/L), mannitol (2 g/L), glucose (1 g/L), monosodium glutamate (0.5 g/L), peptone (0.5 g/L) at 25 °C for 25 days on a reciprocal shaker. The cultivation experiment was performed in 1000 mL of Erlenmeyer flasks containing 300 mL of the medium in a vessel. Finally, 70 L of liquid culture broth were obtained.

2.3. Isolation and purification of the extracellular polysaccharide

The liquid culture broth was filtered through cheesecloth to separate the mycelium and supernatant. The supernatant was concentrated under reduced pressure at 40 °C, and precipitated by adding fourfold of the volume of 95% ethanol (v/v) and was kept at 4 °C for 24 h. The precipitate was collected by centrifugation at 3600 × *g* for 10 min, and dialyzed in a cellulose membrane (flat width 44 mm, molecular weight cut off 3500) against distilled water for 48 h. The retained fraction was vacuum-dried, and the protein in the fraction was removed as described by Sevag, David, and Smolens (1938). The crude polysaccharide was fractionated with a Q Sepharose Fast Flow column (300 mm × 30 mm) by eluting with a step gradient of 0–2 mol/L NaCl. The fractions were assayed for carbohydrate content by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The fractions eluted with distilled water and 0.05 mol/L NaCl were pooled, dialyzed and further purified on a Sephacryl S-100 column (100 cm × 1.6 cm) with 0.2 mol/L NH_4Cl as eluent. The major polysaccharide fraction was pooled and evaporated to dryness.

2.4. Analytical techniques

Total sugar content was measured by the phenol–sulfuric acid method using mannose as the standard (Dubois et al., 1956). Protein content was assayed according to the method of Bradford (1976) using bovine serum albumin as the standard. Sulfate content was measured according to Therho and Hartiala (1971). Amino sugar content was assayed according to the method of Wagner (1979). Uronic acid content was determined by the carbazole–sulfuric acid method using glucuronic acid as standard (Blumenkrantz & Asboe-Hansen, 1973). Purity and molecular weight was assessed by high performance gel permeation chromatography (HPGPC) on a Shodex OHpak SB-803 HQ column (8.0 mm × 300 mm), and the column calibration was performed with pullulan standards (Li et al., 2011).

2.5. Analysis of monosaccharide composition

Monosaccharide composition was determined by reversed-phase high performance liquid chromatography (HPLC) after pre-column derivatization (Qi et al., 2012). Briefly, 2 mg of polysaccharide was hydrolyzed with 2 mol/L trifluoroacetic acid at 105 °C for 6 h. Excess acid was removed by co-distillation with methanol after the hydrolysis. The dry hydrolysate was dissolved in 100 μL of 0.3 mol/L NaOH, and added to 120 μL of 0.5 mol/L methanol solution of PMP at 70 °C for 1 h. Finally, the mixture was added 100 μL of 0.3 mol/L HC1 solution and vigorously shaken and centrifuged for 5 min. The supernatant was filtered through 0.22 μm nylon membranes, and 10 μL of the resulting solution was injected into the Eclipse XDB-C_{18} column (4.6 mm × 250 mm). The chromatograms were performed on an Agilent 1260 Infinity HPLC instrument fitted with Agilent XDB-UV detector (254 nm). The mobile phase was a mixture of 0.1 mol/L KH_2PO_4 (pH 6.7) – acetonitrile (83:17). Sugar identification was done by comparison with reference sugars (L-rhamnose, L-arabinose, L-fucose, D-xylose, D-mannose, D-galactose, D-glucose, D-glucuronic acid, D-galacturonic acid and *N*-acetyl-β-D-glucosamine). Calculation of the molar ratio of the monosaccharide was carried out on the basis of the peak area of the monosaccharide.

2.6. Determination of sugar configuration

Sugar configuration was determined as described by Tanaka, Nakashima, Ueda, Tomii, and Kouno (2007). 5 mg of polysaccharide was hydrolyzed with 2 mol/L trifluoroacetic acid at 105 °C for 6 h. Excess acid was removed with methanol in a rotary evaporator. The hydrolysate was heated with L-cysteine 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 directly 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.

2.7. Methylation analysis

Methylation analysis was performed by the method of Hakomori (1964). In brief, polysaccharide in dimethyl sulfoxide was methylated using NaH and iodomethane, and the completeness of methylation was confirmed by infrared spectroscopy. After hydrolysis with 2 mol/L trifluoroacetic acid at 105 °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, and dissolved again in 100 μL of dichloromethane. The products were analyzed by gas chromatography–mass spectrometry (GC–MS) on a HP6890II/5973 instrument using a DB 225 fused silica capillary column (0.25 mm × 30 m) (Agilent Technologies Co. Ltd., USA). The temperature was increased from 100 to 220 °C at a rate of 5 °C/min then maintained at 220 °C for 15 min. Identification of partially methylated alditol acetates was carried out on the basis of the retention time and its mass fragmentation patterns.

2.8. IR and UV spectroscopy analysis

The Fourier-transform infrared (FTIR) spectrum of the polysaccharide was measured on a Nicolet Nexus 470 spectrometer. The polysaccharide was mixed with KBr powder, ground and pressed into a 1 mm pellets for FTIR measurements in the frequency range of 4000–400 cm^{-1}. Ultraviolet (UV) spectrum was recorded on a UV-2102 PCS spectrophotometer between 190 and 400 nm.

2.9. NMR spectroscopy analysis

50 mg of polysaccharide was deuterium exchanged by two successive freeze-drying steps in 99% D_2O and then dissolved in 0.5 mL of 99.98% D_2O. ^1H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were measured at 23 °C using a JEOL JNM-ECP 600 MHz spectrometer. ^1H–^1H correlated spectroscopy (COSY), ^1H–^{13}C heteronuclear multiple quantum coherence spectroscopy (HMQC)