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Structural elucidation of an extracellular polysaccharide produced by the marine fungus *Aspergillus versicolor*

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ABSTRACT

A homogenous extracellular polysaccharide, designated AWP, was isolated from the fermented liquid of the marine fungus *Aspergillus versicolor* from the coral *Cladiella* sp. and purified by anion-exchange and size-exclusion chromatography (SEC). Chemical and spectroscopic analyses, including one- and two-dimensional nuclear magnetic resonance (1D and 2D NMR) spectroscopy showed that AWP consisted of glucose and mannose in a molar ratio of 8.6:1.0, and its average molecular weight was estimated to be 500 kDa. AWP is a slightly branched extracellular polysaccharide. The backbone of AWP is mainly composed of (1→6)-linked α-D-glucopyranose residues, slightly branched by single α-D-mannopyranose units attached to the main chain at C-3 positions of the glucan backbone. The investigation demonstrated that AWP is a novel extracellular polysaccharide different from those of other marine microorganisms.

1. Introduction

Marine organisms, such as sponges, sea squirts, corals, worms and algae, host diverse and abundant symbiotic microorganisms (Olson & Kellogg, 2010). The microorganisms may be saprophytic, pathogenic, or may serve important positive functions in the ecosystem, and protect their hosts from pathogens (Armstrong, Yan, Boyd, Wright, & Burgess, 2001; Harder, Lau, Dobretsov, Fang, & Qian, 2003; Harel et al., 2008; Kramarsky-Winter et al., 2006; Rohwer, Breitbart, Jara, Azam, & Knowlton, 2002; Siboni et al., 2010). Recently, the interest in extracellular polysaccharides produced by symbiotic microorganisms from marine organisms is increasing because of the microorganisms are mostly new or inadequately described species (Miranda et al., 2008). The marine bacterium *Shewanella colwelliana* from *Crassostrea virginic* excreted a kind of acidic polysaccharide, which contained mannose, glucose, galactose and pyruvic acid (Sledjeski & Weiner, 1993). *Aeromonas* sp. HYD154 from a deep-sea hydrothermal vent polychaete annelid produced an acidic polysaccharide, with glucose, galactose, glucuronic acid, galacturonic acid and 4,6-pyruvic acidic galactose (Cambon-Bonavita, Raguenes, Jean, Vincent, & Guezennec, 2002).

The coral-associated fungus *Aspergillus versicolor* LCJ-5-4 could produce a mannoglucan, with the backbone of (1→6)-linked α-D-glucopyranose and (1→2)-linked α-D-mannopyranose units (Chen et al., 2012). The extracellular polysaccharides produced by symbiotic microorganisms from marine organisms represent a potential source to be explored (Dobretsov & Qian, 2004). In the present work, a novel extracellular polysaccharide was obtained from the culture liquids of the marine fungus *A. versicolor* from the coral *Cladiella* sp., and its structure was investigated by a combination of chemical, chromatographic (SEC, HPGPC, GC–MS) and spectroscopic (FTIR, 1D and 2D NMR) methods.

2. Methods

2.1. Materials

Dextran standards (M_w: 788, 404, 112, 47.3, 22.8, 11.8 and 5.9 kDa) were from Showa Denko K.K. (Tokyo, Japan). D-Glucose, D-mannose, D-galactose, L-rhamnose, D-xylose, L-fucose and D-glucuronic acid 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 Sephacryl S-400/HR were from Amersham Biosciences (Uppsala, Sweden).

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2.2. Microbial strains and culture conditions

A. versicolor was isolated from the coral *Cladiella* sp. collected from Hainan, China. It was identified according to its morphological characteristics and 18S rRNA sequences. Briefly, the fungus was cultivated in a liquid medium containing sorbitol (20 g/L), maltose (20 g/L), monosodium glutamate (10 g/L), KH_2PO_4 (0.5 g/L), $MgSO_4{\cdot}7H_2O$ (0.3 g/L), tryptophane (0.5 g/L), yeast extract (3 g/L), sea salt (33.3 g/L), pH 6.5 at 20 °C for 30 days, and 90 L of fermented broth were collected (Zhuang et al., 2011).

2.3. Isolation and purification of the extracellular polysaccharide

The fermented broth was filtered through cheesecloth to separate into fermentation liquids and mycelium. The fermentation liquids from *A. versicolor* cultures were concentrated, and three volumes of 95% (v/v) ethanol were added. The resulting precipitate was recovered by using centrifugation at 8000 rpm 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 by the method of Sevag (Matthaei, Jone, Martin, & Nirenberg, 1962). The crude polysaccharide was fractionated on a Q Sepharose Fast Flow column (300 × 30 mm) coupled to an AKTA FPLC system, eluting with 0, 0.3 and 2.5 M NaCl (Chen et al., 2012). The total sugar content of the fractions was determined by the phenol–sulfuric acid method. The fractions eluted with distilled water were pooled, and further purified on a Sephacryl S-400 column (100 cm × 3 cm) with 0.2 M NH_4HCO_3 as eluent. The major polysaccharide fraction was gathered and freeze-dried.

2.4. Analytical techniques

Total sugar content was measured by the phenol–sulfuric acid method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein content was assayed according to the modified Lowry method (Bensadoun & Weinstein, 1976). Sulfate content was measured according to Silvestri, Hurst, Simpson, and Settine (1982). Uronic acid content was determined by the carbazole–sulfuric acid method using glucuronic acid as standard (Blumenkrantz & Asboe-Hansen, 1973). The homogeneity and molecular weight was determined by high performance gel permeation chromatography (HPGPC) on a Shodex OHpak SB-804 HQ column, and the column calibration was performed with dextran standards (Li et al., 2012). The monosaccharide compositions were measured by gas chromatography (GC) (Chen et al., 2011). Sugar identification was done by comparison with reference sugars (D-glucose, D-mannose, D-galactose, L-rhamnose, D-xylose and L-fucose), and inositol was added as the internal standard. Calculation of the molar ratio of the monosaccharide was carried out on the basis of the peak area of the monosaccharide.

2.5. Methylation analysis

Methylation analysis was performed according to the modified Hakomori method (Ciucanu & Kerek, 1984). Briefly, the polysaccharide in dimethyl sulfoxide was methylated using NaH and iodomethane. The completion of methylation was confirmed by FTIR spectroscopy as the disappearance of OH bands. After hydrolysis with 2 M 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 derivatized sugar residues were extracted into dichloromethane and evaporated to dryness, dissolved again in 100 μL dichloromethane, and then analyzed by gas chromatography–mass spectrometry (GC–MS) on a HP6890II/5973 instrument (Agilent Technologies Co. Ltd., USA) using a DB 225 fused silica capillary column (0.25 mm × 30 m). Identification of partially methylated alditol acetates was carried out on the basis of the retention time (t_R) and its mass fragmentation patterns (Chen et al., 2012; Hung et al., 2012).

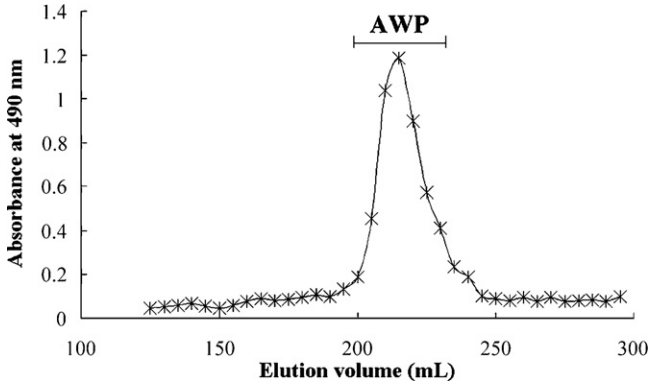

Fig. 1. Purification of the extracellular polysaccharide AWP from the marine fungus *A. versicolor* on a Sephacryl S-400 column.

2.6. IR spectroscopy analysis

For FTIR spectroscopy, the polysaccharide was mixed with KBr powder, ground and then pressed into a 1 mm pellets for FTIR measurement in the frequency range of 4000–500 cm^{-1} with the resolution of 4.0 cm^{-1} and 320 scans co-addition. FTIR spectrum of the polysaccharide was measured on a Nicolet Nexus 470 spectrometer by using the Nicolet Omnic software.

2.7. NMR spectroscopy analysis

Seventy mg of polysaccharide was deuterium exchanged by two successive freeze-drying steps in 99% D_2O (Sigma–Aldrich, Canada) 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 by using Delta NMR processing and control software. ^1H–^1H correlated spectroscopy (COSY), ^1H–^{13}C heteronuclear multiple quantum coherence spectroscopy (HMQC) and ^1H–^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC) experiments were also carried out. Chemical shifts are expressed in ppm using acetone as internal standard at 2.225 ppm for ^1H and 31.07 ppm for ^{13}C.

3. Results and discussion

3.1. Purification and chemical composition of the extracellular polysaccharide AWP

The two crude extracellular polysaccharides from the fermented broth of coral-associated fungus *A. versicolor* were isolated on a Q Sepharose Fast Flow column, and the polysaccharide fraction eluted with 0.3 M NaCl was reported (Chen et al., 2012). Here, the polysaccharide fraction eluted with distilled water was further purified by a Sephacryl S-400 column (Fig. 1), and an extracellular polysaccharide AWP was obtained. The yield of AWP from crude extracellular polysaccharide (0.24 g/L) was about 25%. As shown in Fig. 2, AWP appeared as a single peak in the HPGPC chromatogram, and its average molecular weight was about 500 kDa. AWP did not contain any sulfate esters and protein, and its uronic acid content was below the detection limit. GC analysis demonstrated that AWP was composed of glucose and mannose in a molar ratio of 8.6:1.0. It is noted that the chemical composition of AWP was different from that of