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Characterization of a bioactive polysaccharide from *Ganoderma atrum*: Re-elucidation of the fine structure

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ABSTRACT

The fine structure in terms of backbone and branch chain features of a bioactive polysaccharide from *Ganoderma atrum* (PSG-1) was re-elucidated systematically using high performance anion-exchange chromatography (HPAEC), methylation and GLC–MS analysis, and 1D & 2D NMR spectroscopy. Monosaccharide composition analysis revealed that PSG-1-$F_{0.2}$ fraction mainly consisted of glucose (73.8%) and glucuronic acid (15.3%), with small amount of mannose (5.7%) and galactose (5.2%). Based on methylation, multistep partial acid hydrolysis and NMR study, T-Glc*p*-(1→6)-Glc*p*-(1→, T-Glc*p*-(1→4)-GlcA*p*-(1→, and T-Glc*p*-(1→4)-GlcA*p*-(1→6)-Glc*p*-(1→ were proposed to substitute at the *O*-6 position of β-(1 → 3)-glucan. The small amount of mannose and galactose residues were considered to be from the other fraction in PSG which was very difficult to be separated from PSG-1-$F_{0.2}$. This revised structure as an acidic β-(1 → 3, 1 → 6)-glucan is considered to be more accurate than the previous proposal of PSG-1.

1. Introduction

Ganoderma is a well-known genus of polypore macrofungi medicine which has been identified to possess multifarious functions in terms of antibacterial, antioxidant, antitumor and adjusting immunity (Paterson, 2006). The water-soluble polysaccharides from *Ganoderma* are considered to play an important role in acting the healthy beneficial and have been extensively studied regarding their structure and functions. According to previous reports, *Ganoderma* polysaccharides were mainly consisted of glucan, galactan and/or other heteropolysaccharides composed of several monosaccharides such as glucose, galactose, mannose and fucose, and possessed excellent antitumor and immunomodulatory activities (Bao, Wang, Dong, Fang, & Li, 2002; Chen, Xie, Nie, Li, & Wang, 2008; Liu et al., 2014; Miyazaki & Nishijima, 1981; Wang et al., 2011; Zhu, Chen, & Lin, 2007). Several reviews have also summarized their structural and bioactive properties and tried to establish the structure-activity relationship (Ferreira et al., 2015; Nie, Zhang, Li, & Xie, 2013). However, due to the complexity and diversity of polysaccharide, the fine structure and conformational characterization of *Ganoderma* polysaccharides are still a challenge which made the structure-activity relationship unclear.

In recent years, a bioactive polysaccharide from *Ganoderma atrum* (PSG-1) has been found by Chen, Xie, Nie, Li, and Wang (2008) who firstly isolated PSG-1 from the fruiting bodies and identified its composition and antioxidant activities. The bioactivities in terms of antitumor, immunomodulatory, cardioprotective effect and antidiabetic activities have also been investigated via in *vitro* and in *vivo* assays (Li et al., 2010, 2011; Yu et al., 2013; Zhu, Nie, Li, Gong, & Xie, 2014). Based on these studies, the primary structure of PSG-1 was proposed to be a heteropolysaccharide by methylation and NMR analysis (Zhang et al., 2012). However, as we studied furtherly, more and more questions emerged from the reported structure, which needs to be further revised and re-elucidated.

The current study tried to re-purify PSG-1 by our reported method (Zhang et al., 2016). Afterwards, the fine structure of the purified PSG-1 was elucidated by monosaccharide composition, partial acid hydrolysis, methylation and GLC–MS analysis, and 1D & 2D NMR spectroscopy including homonuclear $^{1}H/^{1}H$ correlation spectroscopy (DQF-COSY, TOCSY), and heteronuclear $^{13}C/^{1}H$ multiple-quantum correlation experiments (HSQC, HMBC). This

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study will present a better understanding for the bioactive polysaccharide from *Ganoderma atrum*.

2. Materials and methods

2.1. Materials

The polysaccharide of PSG-1 was prepared in our lab using hot water extraction method. The monosaccharide standards (D-glucose, D-galactose, D-mannose, L-fucose, D-arabinose, D-fructose, D-ribose, D-galacturonic acid and D-glucuronic acid) and deuterium oxide (99.9% D) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were of analytical grade unless otherwise specified.

2.2. Preparation of the polysaccharide sample

The extraction and purification procedures of polysaccharides from *Ganoderma atrum* (PSG) followed our previous method (Chen et al., 2008) as demonstrated in Fig. 1a. Smashed fruiting bodies were firstly soaked by 95% ethanol for 24 h to remove the alcohol soluble components. Afterwards, the residues were extracted twice by hot water at 90 °C. Four volumes of ethanol was added to the extracted solution to precipitate the crude polysaccharide. The protein was removed by Sevage method (Staub, 1965) to get refined sample. The Superdex-G200 prep grade column (2.6 × 60 cm) was used to fractionate PSG into two fractions (PSG-1 and PSG-2). Due to the dark color appearance, anion exchange chromatography was applied to decolorize and purify PSG-1 as shown in Fig. 1a according to previous report (Zhang et al., 2016).

2.3. Homogeneity analysis of different fractions

The high performance size exclusion chromatography (HPSEC) equipped with a refractive index (RI) detector (Wyatt Technology Co., USA) was used to detect the homogeneity of different fractions. A Model 1500 HPLC Pump (Scientific Systems, Inc., Woburn, MA, USA) with two columns in series, a SB-806 HQ and a SB-804 HQ (Shodex OHpak, 8 mm x 300 mm, Showa Denko K.K., Tokyo, Japan), were used. The eluent was 0.9% NaCl aqueous solution (containing 0.02% NaN_3) at a flow rate of 0.6 mL/min. Samples were prepared at the concentration of 1.0 mg/mL.

2.4. Chemical components analysis

Total sugar contents of PSG and its fractions were determined following the phenol sulfuric acid assay using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Total uronic acid was measured by the *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973) as glucuronic acid equivalents. The protein content was determined following the spectrophotometric assay (Bradford, 1976) using bovine serum albumin as the standard. Total phenolic content (TPC) was determined according to Folin–Ciocalteu assay (Slinkard & Singleton, 1977) with some modifications (Zhang et al., 2016).

2.5. Determination of monosaccharide and uronic acid composition

Monosaccharide and uronic acid compositions were determined by treating sample (10 mg) with 0.5 mL of 12 M H_2SO_4 at room temperature for 30 min, then diluting to 2 M H_2SO_4 with water followed by hydrolysis at 100 °C for 2 h. Analysis was carried out using a high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD, Dionex ICS-5000, USA) equipped with a CarboPac™ PA20 guard column and a CarboPac™ PA20 analytical column (4 mm × 250 mm). The gradient elution program was 0.8% of 250 mmol/L NaOH for the first 20 min (to elute the neutral sugar), then added 1 M NaOAc from 5% to 20% in the next 10 min (to elute the acid sugar). The system was then equilibrated by 0.8% of 250 mmol/L NaOH for 25 min. Standards (glucose, galactose, mannose, fucose, arabinose, fructose, ribose, galacturonic acid and glucuronic acid) were used to determine the composition and corresponding molar percentages. All measurements were repeated three times.

2.6. Methylation and GLC–MS analysis of PSG

The high percentage of uronic acids in PSG-1-$F_{0.2}$ created difficulties for methylation analysis. In the current study, uronic acid was firstly reduced into neutral sugars prior to methylation analysis following a published procedure (Blumenkrantz & Asboe-Hansen, 1973; York, Darvill, McNeil, Stevenson, & Albersheim, 1986) with some modification as described by Guo et al. (2015). The reduced sample (RPSG-1-$F_{0.2}$) was hydrolyzed for HPAEC analysis to confirm that no uronic acid was detected.

Methylation analysis procedure was conducted according to the method of Ciucanu and Kerek (1984) with slight modification (Guo et al., 2015). The methylated sample was detected by FT-IR spectrum to make sure that the peak of —OH band (3200–3700 cm^{-1}) disappeared completely. Then the dried methylated sample was hydrolyzed and derived as partially methylated alditol acetates (PMAA) which were then analyzed by GLC–MS system (THERMO 1310 GC-ISQ LT MS, USA) equipped with a TG-200MS (Thermo Fisher, USA) capillary column (30 m × 0.25 mm × 0.25 μm) programmed from 160 to 210 °C at 2 °C/min, and then 210–240 °C at 5 °C/min.

2.7. Multistep partial acid hydrolysis analysis

Sample of PSG-1-$F_{0.2}$ (50 mg) was firstly hydrolyzed with 10 mL of 0.01 M TFA at 100 °C for 2 h. After cooling to room temperature, TFA was evaporated under a stream of nitrogen and the hydrolysate was dissolved and dialyzed against distilled water (Mw cut-off 3600 Da) for 24 h. The solution in the dialysis tube was lyophilized to obtain a high molecular mass fraction, which was designated as H-1. The H-1 fraction was further hydrolyzed in turn with 0.01 M and 0.05 M TFA at the same conditions to obtain fractions H-2 and H-3, respectively. The three fractions were then applied for HPSEC, monosaccharide composition and methylation analysis.

2.8. Nuclear magnetic resonance (NMR) spectroscopy analysis

Samples (50 mg) were exchanged with deuterium by lyophilizing against deuterium oxide (D_2O) for 3 times, and finally dissolved in D_2O at room temperature for 3 h before NMR analysis. High-resolution of ^1H and ^{13}C NMR spectra were recorded at 600.10 and 151.01 MHz, respectively, on a Bruker AVIII 600 NMR spectrometer (Brucker, Rheinstetten, Germany) at 295 K. The homonuclear $^1H/^1H$ correlation (DQF-COSY, TOCSY), heteronuclear single-quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) experiments were conducted using the standard Bruker pulse sequence. Chemical shifts are reported relative to trimethylsilyl propionate (TSP) in D_2O for ^1H (0.0 ppm, external standard).

2.9. Statistical analysis

The data of chemical components, monosaccharide composition and methylation analysis were calculated with triplicate and shown