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Structural characterization and immuno-enhancing activity of a highly branched water-soluble β-glucan from the spores of *Ganoderma lucidum*

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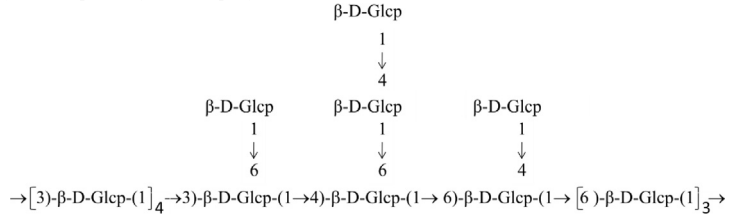
ABSTRACT

A water soluble β-glucan (GLSWA-I) with a weight average molecular weight ~1.57×10^5 g/mol, isolated from the aqueous extract of the broken cellular wall *Ganoderma lucidum* spores, was purified by anion-exchange and gel-permeation chromatography. The immunological activity test *in vivo* showed that GLSWA-I could significantly promote dinitrochlorobenzene (DNCB) induced delayed-type ear swelling in mice. Based on the monosaccharides composition analysis, methylation analysis, IR, 1D and 2D NMR spectroscopy, the repeating unit of polysaccharide GLSWA-I was elucidated as follows:



1. Introduction

Mushrooms have been valued as edible and medicinal resources, in which many biological active substances have been identified (Largeteau, Llarena-Hernández, Regnault-Roger, & Savoie, 2011; Meng, Liang, & Luo, 2016). In recent years, mushroom polysaccharides have drawn the attention of chemists and immunobiologists because of their immunomodulatory and antitumor properties (Meng et al., 2016). Fungal glucans, which constitute an obligatory part of cell walls in mycelia, fruiting bodies or other parts of different micro- and macromycetes, are structurally variable polymers of D-glucopyranose (Synytsya & Novak, 2013). β-D-glucans with (1 → 3), (1 → 4) and (1 → 6) linkages are the main glucans produced by fungi (Synytsya & Novak, 2013). It is generally known that linear (1 → 3)-β-D-glucans, especially those having β-D-glucopyranosyl units attached by (1 → 6) linkages as single unit branches, are biological response modifiers (BRMs) as they could enhance the immune system systemically (Bohn & Bemiller, 1995). Therefore, study on fungal β-glucans is a hot pot in the research field of natural medicine and functional food.

The mushroom *Ganoderma lucidum*, known as 'Lingzhi' in China, belongs to the family Polyporaceae and is well-known as a famous traditional Chinese medicine. It has a long history of use in the treatment of various diseases in China. *G. lucidum* contains many kinds of bioactive components, including triterpenoids, polysaccharides, nucleotides, sterols and other bioactive ingredients (Boh, Berovic, Zhang, & Zhibin, 2007). Recent studies demonstrated that polysaccharides extracted from the fruit bodies, mycelia, and spores of *G. lucidum* had diverse pharmacological activities such as antioxidant (Heleno et al., 2012; Kan, Chen, Wu, Wu, & Wu, 2015), antitu-

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mor (Liu, Yuan, Chung, & Chen, 2002; Wang, Zhu, & Lin, 2012), immunomodulating (Bao, Wang, Dong, Fang, & Li, 2002; Zhang et al., 2010; Zhu, Chen, & Lin, 2007) and others. Although more than 200 polysaccharides have been isolated from *Ganoderma* spp., in many cases the *in vitro/in vivo* effects of the polysaccharides still remain unclear due in large part to a lack of structural data. *Ganoderma* spores are extremely tiny mist-like brown oval-shaped spores which are released at the pileus of mature *G. lucidum*. These spores contain the entire genetic materials and biological substances of *Ganoderma*. They were thought to be of greater pharmaceutical values than the fruit bodies of *Ganoderma*. However, the polysaccharides in *G. lucidum* spores have been less studied due to difficulties associated with the collection.

The structural study of fungal polysaccharides is important for understanding the mechanism of action and structure-function relationship of the polysaccharides with therapeutic effects and to select new targets for drugs (Ferreira, Passos, Madureira, Vilanova, & Coimbra, 2015). Some scientific investigations have been performed on the structural analysis and biological properties of polysaccharides from water extracts of *G. lucidum* spores. Most polysaccharides are glucans with different combinations of glucose, including linear (1 → 3)-α-D-glucan (Bao, Duan, Fang, & Fang, 2001), branched α-D-glucan with (1 → 4)-α-D-glucan backbone (Bao & Fang, 2001), β-D-glucan with (1 → 3)-β-D-glucan backbone (Bao, Liu, Fang, & Li, 2001; Bao, Dong, & Fang, 2000), β-D-glucan with (1 → 6)-β-D-glucan backbone (Bao, Fang, & Li, 2001; Dong et al., 2012) and mixed-linkage α, β-D-glucans (Guo et al., 2009; Zhang, Zhang, & Zhang, 2011). Recent studies demonstrated that polysaccharides extracted from *G. lucidum* spores had antitumor (Wang et al., 2012), antioxidant (Klaus, Kozarski, & Niksic, 2011; Zhu, Chen, Xie, Wang, & Su, 2012) and immunomodulating activities (Bao, Fang et al., 2001; Bao, Liu et al., 2001; Bao et al., 2002; Guo et al., 2009; Zhang et al., 2011; Wang et al., 2012; Yue, Fung, Leung, & Lau, 2008). Compared with the intensive investigation of water-soluble polysaccharides from the spores of *G. lucidum*, researches on cell wall polysaccharides of *Ganoderma* spores were seldom reported. In the present study, a water-soluble cell wall β-D-glucan was purified and characterized by a combination of chemical and instrumental analysis, such as monosaccharide analysis, methylation analysis, 1D and 2D NMR spectroscopy. This work will offer useful information for understanding the structural characteristics of *G. lucidum* spore polysaccharides, and will be helpful for further investigating the structure-activity relationships.

2. Materials and methods

2.1. Materials

The spores of *G. lucidum* were provided by the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences. DEAE-Sepharose Fast Flow and Sephacryl S-300 High Resolution were purchased from GE Healthcare. Dextrans and the monosaccharide standards were from Sigma-Aldrich. Lentinan tablets were purchased from Zhejiang Apeloa Pharmaceutical Co. Ltd. Prednisone Acetate Tablets were bought from Xianju Zhejiang pharmaceutical Limited by Share Ltd. All other reagents were from Chinese sources and were analytical grade unless otherwise specified.

2.2. Animals

Male and female BALB/c mice (18–21 g) were purchased from the Center of Comparative Medicine of Yangzhou University (Yangzhou, China). Animal quality certificate number: SCXK (Su) 2012-0004. The animals were maintained with free access to laboratory chow and tap water in plastic cages (five mice per cage) at a controlled temperature (22 ± 2 °C), relative humidity (50–60%) and under a diurnal 12 h light/12 h dark cycle. All experimental procedures involving animals were performed in accordance with the Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation.

2.3. Isolation and purification of polysaccharide GLSWA-I

The dried spores of *G. lucidum* were extracted twice with 15 vols of distilled water for 2 h at 100 °C to remove intracellular polysaccharides. The combined aqueous extracts were filtrated and centrifuged at 8000 g for 20 min. The residues were collected and oven-dried at 50 °C and then processed with superfine grinding vibration mill at 10 °C for 30 min to get cell wall broken spores. The sporoderm-broken spores were refluxing extracted with 15 vols 95% (v/v) ethanol twice for 2 h to eliminate the lipids. After filtration, the residues were dried in sunshine and then extracted two times with 15 vols of distilled water for 2 h at 100 °C to obtain cell wall polysaccharides. After being centrifuged, the supernatant was concentrated to 500 mL using vacuum evaporator, followed by precipitation in 55% (v/v) ethanol at 4 °C. The precipitate was collected by centrifuging at 8000 g for 20 min and freeze-dried to yield fraction GLSW.

For further separation, a portion of GLSW was dissolved in distilled water and then applied to a DEAE-Sepharose Fast-Flow column (XK26 × 100 cm) eluting with 500 mL filtered (0.22 μm membrane) distilled water and then with a 0–2 M gradient of NaCl. Carbohydrate was determined using the phenol-sulfuric acid assay. The fraction eluted with distilled water was collected and named as GLSWA. Fraction GLSWA was further purified by gel-permeation chromatography (GPC) on a Sephacryl S-300 HR column (XK16 × 100 cm) using distilled water as eluent, and a portion (GLSWA-I) was collected according to the chromatography profile detected by a refractive index detector. GLSWA-I was further purified by the previously described GPC method to get a homogeneous fraction. Content of total sugar was determined using the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.4. Determination of homogeneity and molecular weight

The homogeneity and weight average molecular weight (M_w) of polysaccharide GLSWA-I was determined using high-performance size exclusion chromatography (HPSEC) equipped with multiple detectors: a UV detector and a refractive index detector (RI) for concentration determination, a multiple angle laser light scattering detector (MALLS) for direct molecular weight determination and a differential pressure viscometer (DP) for viscosity determination. The chromatographic system was Waters HPLC 2695. The columns were composed of a guard column (TSK PWxl, Japan) and a Super Multipore PW-H gel filtration column (TSK PWxl, 6.0 mm × 15 cm, Japan) in series eluting with 0.15 M $NaNO_3$ and 0.05 M NaH_2PO_4 containing 0.02% (w/w) NaN_3 (pH = 7) at the flow rate of 0.5 mL/min. The column and RI detector temperature were maintained at 30.0 ± 0.1 °C. The normalization of MALLS detector and the determination of volume delay between MALLS and RI detectors were carried out using bovine serum albumin (BSA). The *dn/dc* value was set to be 0.146 mL/g for polysaccharide polymers. Data acquisition and analysis were carried out using ASTRA software (Version 6.1.1).

2.5. Monosaccharide composition analysis

GLSWA-I (2.0 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 110 °C for 3 h, and the monosaccharide composition analysis was conducted by high performance anion exchange chro-