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Identification of pivotal components on the antioxidant activity of polysaccharide extract from *Ganoderma atrum*

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ABSTRACT

The pivotal components responsible for the antioxidant activities of polysaccharide from *Ganoderma atrum* (PSG) were identified by chemical composition and antioxidant activity analysis after separating PSG into different fractions. PSG was determined to be a mixture with neutral fraction and acidic fraction, as well as protein and phenolic compounds. The fractions with different ionic density were separated by anion exchange chromatography (AEC). The neutral fraction (Fw) was identified as a (1→6)-linked-heterogalactan, while the ionic fractions ($F_{0.2}$, $F_{0.5}$ and F_2) were acidic (1→3, 1→6)-linked-heteroglucans with non-sugar components. Phenolic compounds and proteins bonded/crosslinked with PSG were enriched in the very ionic fractions ($F_{0.5}$ and F_2). Antioxidant activities of PSG and its fractions via chemical assays showed good correlation to the total phenolic and protein contents, while cell culture assay indicated that acidic (1→3, 1→6)-linked-heteroglucan could significantly stimulate the macrophage cell RAW264.7 to release nitric oxide. These results clarified that the antioxidant activities of PSG ascribed to the phenolic and protein components, rather than the carbohydrates part which would be more responsible to the immunomodulatory activity of PSG.

1. Introduction

Ganoderma has been a popular edible mushroom in Asia and eastern Russia for more than 2000 years. Kinds of compounds from the fruiting bodies of *Ganoderma*, such as polysaccharides, proteins, peptides and a variety of small organic molecules (phenolics, flavonoids and sterols) have been reported to possess bioactivities (Paterson, 2006). *Ganoderma atrum* (*G. atrum*), which is widely cultivated in China, is one of the major species of *Ganoderma*. Recently, lots of work has been focused on a bioactive polysaccharide from the fruiting bodies of *G. atrum*. Chen et al. (Chen, Xie, Nie, Li, & Wang, 2008) firstly extracted this kind of polysaccharide using hot water and designated it as PSG. The monosaccharide composition of the purified PSG (PSG-1) was characterized to be mannose, galactose and glucose in a molar ratio of 1: 1.28: 4.91. Based on this study, the bioactivities of PSG have been investigated extensively. Results showed that PSG-1 possessed variety of bioactivities, such as immunomodulatory (Yu et al., 2013) and antitumor activities (Li et al., 2011). The mechanism and signaling pathway involved in the immunomodulatory effect of PSG have been elucidated, and the results demonstrated that PSG-1 could activate macrophages via TLR4-dependent signaling pathways, improve immunity and inhibit tumor growth (Zhang, Nie, Huang, Li, & Xie, 2013). In addition to the well-understood immunomodulatory and antitumor properties, significant antioxidant ability of PSG has also been reported (Chen et al., 2008). However, the complex chemical composition of PSG poses a major challenge in evaluating and understanding the free radical scavenging capacity.

Many polysaccharide extracts from plants and mushrooms have been reported to be excellent natural antioxidants. However, the antioxidant effect of pure polysaccharides was considered to be weak in comparison with classical free radical scavenging agents, such as pyrrolidine dithiocarbamate (PDTC) or Trolox. For naturally produced polysaccharides, only polyelectrolytes, e.g. sulfated or phosphorylated glycans and lipopolysaccharide, showed considerably higher scavenging activities (Tsiapali et al., 2001). Most reported antioxidant polysaccharides were crude polysaccharides or polysaccharide conjugates which contained protein, uronic acid and/or other undefined compounds. Nie et al. reported that the antioxidant abilities of TPS-protein conjugates depended on the protein content (Nie, Xie, Fu, Wan, & Yan, 2008). Chen et al. identified that uronic acids were the key factor for the antioxidant activities of tea polysaccharide (Chen, Zhang, & Xie, 2004). In this way, the antioxidant activities assigned to some polysaccharides need to be reassessed.

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The objectives of this study tried to establish the correlation between chemical composition and bioactivity to clarify the pivotal components responsible to the antioxidant activities of PSG. PSG was firstly separated into five fractions with different chemical composition by anion-exchange chromatography (AEC). The antioxidant activities of these fractions were evaluated via chemical assays, including 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity, ferric reducing ability of plasma (FRAP) and oxygen radical absorbance capacity (ORAC) assays. Additionally, cell culture assay was used to screen the immunomodulatory fractions in order to distinguish the different active factors exerting on the antioxidant and immunomodulatory activities.

2. Materials and methods

2.1. Materials and chemicals

The fruiting bodies of *G. atrum* were purchased from Ganzhou, Jiangxi province, China. The fruiting bodies were cut and smashed by edge-type pulverizer. Fluorescein, Trolox and DPPH were obtained from Sigma (St. Louis, MO). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). All other chemicals were of analytical grade unless otherwise specified.

2.2. Extraction and fractionation of Ganoderma atrum polysaccharide

The polysaccharide from *G. atrum* (PSG) was extracted by hot water extraction method, precipitated by ethanol with the final concentration of 80%, and deproteinized by Sevage reagent following our previous report (Chen et al., 2008). PSG was then fractionated by anion-exchange chromatography using Q-Sepharose Fast Flow resin (GE Healthcare, Uppsala, Sweden) as shown in Fig. 1. The resin was exchanged with pure water to obtain water-saturated resin, and then poured into a column ($1.5 \times 10\ cm^2$) which connected to a filtration funnel. The sample solution (5 mg/mL, 20 mL) was injected into the column to let it mix with the resin for about 10 min The un-adsorbed sample was rinsed off with pure water by suction, and the adsorbed sample was eluted sequentially with 0.1, 0.2, 0.5 and 2 M of NaCl solutions. The flow rate was adjusted at about 10 mL/min by vacuum. All fractions were desalted by dialysis against de-ionized water, concentrated and lyophilized, yielding five fractions designated as Fw, $F_{0.1}$, $F_{0.2}$, $F_{0.5}$ and F_2, respectively.

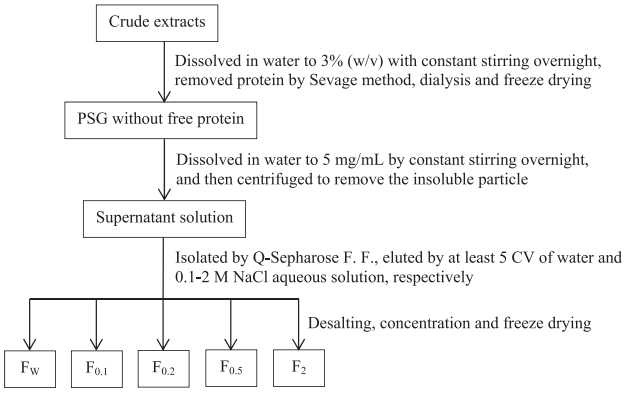

Fig. 1. The fractionation procedure for the polysaccharide from *Ganoderma atrum* (PSG).

2.3. Analysis of total sugar, uronic acid, protein and phenolic contents

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 (Wang, Meckling, Marcone, Kakuda, & Tsao, 2011). Briefly, 25 μL of gallic acid standards or polysaccharide samples were mixed with 125 μL of Folin-Ciocalteu reagent in 96-well microplate and allowed to react for 10 min at 25 °C. Then 125 μL of 7.5% sodium carbonate (Na_2CO_3) solution was added and allowed to stand for 30 min at 25 °C before the absorbance of the reaction mixture was read at 765 nm using a visible-UV micro plate kinetic reader (EL 340, Bio-Tek Instruments Inc., Winooski, VT, USA). The results were expressed as percentage of gallic acid equivalent (GAE) per gram of dry weight extract (%). All standards and samples were tested in triplicate.

2.4. High-performance size-exclusion chromatography (HPSEC) analysis

The HPSEC equipped with multiple detectors (Wyatt Technology Co., USA): a multi-angle laser light scattering detector for absolute molecular weight determination, a differential pressure viscometer for viscosity determination, a refractive index detector and a diode array detector for concentration determination, was used to determine the molecular weight and distribution. 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 × 300 mm, Showa Denko K.K., Tokyo, Japan), were used. The columns, viscometer and RI detector were maintained at 35 °C. 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.

2.5. Phenolic profile analysis by HPLC

The phenolic compounds were released from polysaccharides by treating with 1 M NaOH for 2 h at room temperature (Rao & Muralikrishna, 2001). The supernatants were collected and acidified to pH 1.5 using 4 M HCl after centrifugation. The phenolic compounds were extracted with ethyl acetate (5 × 20 mL), followed by drying with anhydrous sodium sulphate and evaporating to dryness. The extracts were dissolved in methanol and analyzed on a Kinetex XB-C18 column (100 mm × 4.6 mm, 2.6 μm) (Phenomenex Inc., Torrance, CA). Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) system was used with an auto sampler and a diode array detector (DAD). The mobile phase consisted of 5% formic acid in water (*v*/*v*) (solvent A) and 95% methanol mixed with 5% acetonitrile (*v*/*v*) (solvent B). The solvent gradient was as follows: 0–40 min, 0–80% B; 40–42 min, 80–100% B; 42–44 min, 100% B; 44–44.5 min, 100–0% B. Peaks were monitored at 260, 280 and 360 nm for the different phenolic compounds. Standards were used for identification of phenolic compounds present in the sample. The sample without the treatment of 1 M NaOH was used as the control group.

2.6. Monosaccharide composition analysis

Monosaccharide compositions were determined by treating sample (10 mg) with 0.5 mL of 12 M H_2SO_4 at room temperature