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Purification, characterization, and complement fixation activity of acidic polysaccharides from *Tuber sinoaestivum*

Xiaolin Li [a], Bo Zhang [a, b], Juan Li [c], Jie Zhou [a], Xiaolan He [a], Lei Ye [a], Jie Zou [a, b], Chenguang Wu [a, b], Xiaoping Zhang [b], Weihong Peng [a, *]

[a] Soil and Fertilizer Institute, Sichuan Academy of Agricultural Sciences, Chengdu 610066, China
[b] Department of Microbiology, College of Resource and Environment, Sichuan Agricultural University, Chengdu 611130, China
[c] Institute of Animal Science, Chengdu Academy of Agriculture and Forestry Sciences, Chengdu 611130, China

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Chemical compounds studied in this article:
D-Mannose (PubChem CID: 18950)
L-Rhamnose (PubChem CID: 25310)
D-glucuronic acid (PubChem CID: 94715)
D-galactose (PubChem CID: 6036)
D-glucose (PubChem CID: 5793)
D-mannuronic acid (PubChem CID: 439630)
D-xylose (PubChem CID: 135191)
L-Arabinose (PubChem CID: 439195)
D-fructose (PubChem CID: 5984)
Trifluoroacetic acid (PubChem CID: 6422)

ABSTRACT

Tuber sinoaestivum, Chinese black truffle, which is very commercially important in China, is not only well known for its delicious taste but also for its high medical value. Polysaccharides from its European sister species *Tuber aestivum* have been shown to have some interesting bioactivities, but very few studies have been performed on it. In this study, 2 acidic polysaccharides, *T. sinoaestivum* polysaccharide-I (TSP-I) and *T. sinoaestivum* polysaccharide-II (TSP-II), were obtained from 100 °C water extracts of *T. sinoaestivum* fruiting bodies by diethylaminoethyl (DEAE) anion exchange chromatography and gel filtration. The polysaccharides presented similar monosaccharide composition, molecular size, and types of linkage. The results from methylation, Fourier transform infrared spectroscopy (FT-IR), and nuclear magnetic resonance (NMR) indicated the polysaccharides as the rhamno-mannan-like types that also contain mannuronic acid and acetyl groups. Both of TSP-I and TSP-II exhibited potential complement fixation activity. The higher molecular weight and lower amount of glucose in TSP-II may explain why it had higher complement fixation activity. Accordingly, the Chinese truffle *T. sinoaestivum* might serve as a potential immunomodulator.

1. Introduction

Truffles, of the genus *Tuber*, are recognized as delicious edible mushrooms and are of great economic value (Wang & Marcone, 2011). Surprisingly, recent investigations have shown that truffles contain quite a number of bioactive compounds that have potential biological activities such as antioxidant, antiviral, anti-microbial, hepatoprotective, anti-mutagenic, anti-inflammatory, anti-carcinogenic, and anti-tuberculoid (Wang & Marcone, 2011). Dozens of low molecular weight compounds such as ethanol, carbon dioxide, 2-butanone, and ethyl acetate have been reported in the genus *Tuber*, such as *T. aestivum*, *T. rufum*, *T. simonea*, *T. brumale*, *T. melanosporum*, and *T. miesentericum* (Wang & Marcone, 2011; Zhang, Liu, & Chen, 2012). It has been confirmed that polysaccharides isolated from mushrooms have antioxidant, anti-diabetic, antitumor, and immunomodulating biological activities (Meng, Liang, & Luo, 2016; Wang, Zhao, Yang, Wang, & Kuang, 2016). To date, only a few polysaccharides have been isolated from the *Tuber* genus. Heteroglycans from *T. indicum* (Luo et al., 2010; 2011), which mainly consist of manose (Man), glucose (Glc), galactose (Gal), and rhamnose (Rha). A heteroglycan

* Corresponding author.
E-mail address: pwh424@163.com (W. Peng).

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containing Glc, Man, and Gal and a glucan were isolated from *T. huidongense* (Chen, Jiang, Zhang, Kan, & Liang, 2016; Chen, Zhang, Ran, Wang, & Kan, 2016). Pattanayak et al. (2017) reported another heteroglycan from *T. rufum* which was mainly comprised of Glc, Gal, and fucose. However, all of these reported polysaccharides are neutral polysaccharides, and few of them have been well characterized. Chinese black truffles such as *T. indicum*, *T. formosanum*, and *T. pseudohimalayense* have attracted much scientific and commercial interest since their export to Europe in the beginning of the 1990s (Chen, Liu, & Wang, 2005; Wang et al., 2006). As a very important commercial Chinese black truffle in China, *Tuber sinoaestivum* is not only well known for its delicious taste but also has high medicinal value. However, prior to 2012, this type of mushroom had been mistaken for the species *T. aestivum* for a long time (Zhang et al., 2012). There are few reports about polysaccharides from the Chinese black truffle *T. sinoaestivum*, and thus it was of interest to investigate the structural properties and biological activities of its polysaccharides.

The complement system is an important part of the innate immune system which also cooperates with the adaptive immune system in many ways (Dunkelberger & Song, 2010). The complement system, among other things, plays a direct part in defense, as it is the primary defense against bacterial invasions and viral infections. Complement fixation activity of polysaccharides has previously been used as an indicator for effect on the immune system (Inngjerdingen et al., 2013; Michaelsen, Gilje, Samuelsen, Høgåsen, & Paulsen, 2000). Screening of polysaccharides with complement fixing activity could promote the discovery of potential immunomodulators.

In the present study, we described the isolation, structure elucidation, and complement fixation activity of polysaccharides from fruiting bodies of *T. sinoaestivum*. The cruder water extracts were purified by diethylaminoethyl (DEAE) anion exchange chromatography and gel filtration, and 2 acidic polysaccharides were thereby obtained. Gas chromatography, gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance were employed for structural elucidation. The complement fixation activities of polysaccharides obtained were also investigated.

2. Materials and methods

2.1. Materials

The fruiting bodies of *T. sinoaestivum* were collected from Huidong County, Sichuan Province, China, and deposited at the Sichuan Academy of Agricultural Sciences (No.20151105). They were then further confirmed by sequencing internal transcribed spacer sequences. The fruiting bodies were cleaned, dried, cut into small pieces, and pulverized to a fine powder. All other chemicals used were of analytical grade and were obtained from Chengdu Kelong Chemical factory (Chengdu, China) and Sigma-Aldrich (St. Louis, MO, USA).

2.2. Purification of polysaccharides

One hundred grams of the powdered fruiting bodies were pre-extracted with 96% ethanol (EtOH) to remove lipophilic and low molecular compounds. After filtration, the residue was further extracted 2 more times with 100 °C distilled water, followed by concentration, dialysis (cut-off 3500 Da), and lyophilization. The crude polysaccharides were thereby obtained.

The crude polysaccharides were purified first by ion exchange chromatography and then by gel filtration. The crude polysaccharide was filtered through 0.45 μm filters and applied to a DEAE Sepharose (Fast Flow, FF) column (Beijing Rui Da Heng Hui Sci-Tech Development Co. Ltd., Beijing, China). The neutral fraction was firstly eluted with distilled water at 2 mL/min, and then the acidic fractions were eluted with linear NaCl gradients (0–1.5 M) at 2 mL/min. The carbohydrate elution profiles were monitored using the phenol-sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The acidic fractions of interest were pooled, dialyzed at cut-off 3500 Da against distilled water for removal of NaCl, and lyophilized.

The acidic fractions dissolved in 10 mM NaCl were filtered through a Millipore 0.45 μm filter (Merck, Kenilworth, NJ, USA) followed by gel filtration with a Sepharose 6FF column (Beijing Rui Da Heng Hui Sci-Tech Development Co. Ltd., Beijing, China) and elution with 10 mM NaCl at 1.0 mL/min. Fractions were pooled based on the elution profile, as determined by the phenol-sulfuric acid assay, dialyzed, and lyophilized.

2.3. Chemical compositions and linkage determination

Monosaccharide compositions of the purified fractions were determined by gas chromatography (GC) of the trimethylsilylated (TMS) derivatives of the methyl-glycosides obtained after methanolysis with 3 M hydrochloric acid in anhydrous methanol for 24 h at 80 °C (Austarheim et al., 2012b; Barsett, Paulsen, & Habte, 1992; Chambers & Clamp, 1971). Mannitol was used as an internal standard. The TMS derivatives were analyzed by capillary gas chromatography on a Focus GC (Thermo Scientific, Milan, Italy). The injector temperature was 250 °C, the detector temperature was 300 °C, and the column temperature was 140 °C at the time of injection, which was then increased by 1 °C/min to 170 °C, followed by 6 °C/min to 250 °C and then 30 °C/min to 300 °C.

Glycosidic linkage was analyzed by methylation. Prior to the methylation, uronic acids at the polymeric level were reduced with $NaBD_4$ to their corresponding neutral sugars. After reduction, methylation, hydrolysis, reduction, and acetylation (Kim & Carpita, 1992) were carried out sequentially. The derivatives were analyzed by GC-MS using a GCMS-QP2010 (Shimadzu, Kyoto, Japan) attached to a Restek Rxi-5MS column (30 m; 0.25 mm i.d.; 0.25 μm film, Shimadzu, Kyoto, Japan). The injector temperature was 280 °C, the ion source was temperature 200 °C, and the interface temperature was 300 °C. The column temperature was 80 °C when the sample was injected, and was then increased at 10 °C/min to 140 °C, followed by 4 °C/min to 210 °C and then 20 °C/min to 300 °C. Helium served as the carrier gas (pressure control: 80 kPa). The compound in each peak was characterized by an interpretation of the retention times and the characteristic mass spectrum. The estimation of the relative amounts of each linkage type was related to the total amount of each monosaccharide type as determined by methanolysis (Sweet, Shapiro, & Albersheim, 1975).

2.4. Molecular weight determination

The homogeneity and molecular weight of the native purified polysaccharide fractions were determined by size exclusion chromatography on a Hiload™ 16/60 Superdex™ 200 prep grade column combined with an Äkta system (FPLC, GE Healthcare, Uppsala, Sweden). Dextran polymers (Pharmacia, Uppsala, Sweden) of 10, 40, 70, 500, and 2000 kDa were used as calibration standards (Zhu et al., 2011).

2.5. Fourier transform infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) spectroscopy

Approximately 1 mg of polysaccharide samples were mixed with 150 mg of dried KBr powder and pressed into a 1 mm thick disk for analysis by a PerkinElmer FT-IR spectrophotometer. The IR