



Characterization and antioxidant activities of extracellular and intracellular polysaccharides from *Fomitopsis pinicola*



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ABSTRACT

Fomitopsis pinicola (*F. pinicola*) is a kind of medicinal fungi, and few studies has been carried out on *F. pinicola* polysaccharides from liquid submerged cultivation. The characterization and antioxidant activities of extracellular polysaccharide (EPS) and intracellular polysaccharide (IPS) isolated from *F. pinicola* were investigated. The results showed that the molecular weight of EPS was 2.30×10^4 Da, and EPS was composed of mannose, rhamnose, xylose and galactose with the molar ratio of 0.1:1.0:0.3:0.5. The molecular weight of IPS was 4.07×10^5 Da, and the monosaccharide compositions included glucose, mannose, rhamnose, xylose and galactose with the molar ratio of 1.0:0.9:0.8:1.1. Antioxidant activities of both EPS and IPS including *in vitro* scavenging activities on 1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals, cellular protective effects on yeast cells from ultraviolet (UV) radiation and H_2O_2 oxidative damage were tested. Both EPS and IPS showed antioxidant activities in a dose dependent manner, and IPS had higher antioxidant activity than EPS. So EPS and IPS could be potential novel antioxidants for functional food.

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1. Introduction

Reactive oxygen species (ROS) and free radicals are produced with the normal metabolism of oxygen in human body (Serviddio, Bellanti, & Vendemiale, 2013), and their high level especially under environmental stress conditions often cause tissue injury and diseases (Sies, 1997). Exotic antioxidants are usually employed to reduce the damage of free radical species to human body.

In recent years, medicinal edible fungi have been a research hotspot for exploiting natural antioxidants. Polysaccharides from fungi are usually being involved in scavenging free radicals and ROS (Ge, Duan, Fang, Zhang, & Wang, 2009; Sun et al., 2009). Some investigations have proved that polysaccharides from medicinal fungi (*Cordyceps sinensis*, *Agaricus bisporus*, *Dictyophora indusiata*) had significant antioxidant activity on 1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion and hydroxyls radical et al. (Huang, Siu, Wang, Cheung, & Wu, 2013). *Fomitopsis pinicola* (*F. pinicola*) is a member of basidiomycetes fungus, which is widely used as a

medicinal mushroom in Asia (Cheng, Lin, Lur, Chen, & Lu, 2008). Choi, Park, Ding, and Cha (2007) has investigated the biological activities of *F. pinicola* extract by examining the antioxidant and antitumor activities *in vitro* and *in vivo*. Cheng et al. (2008) also conducted a thorough research on polysaccharide and ethanolic extract of cultured *F. pinicola* in regulating angiogenic process and inflammation. Although there have been several relevant reports about properties and biological activities of extract from *F. pinicola* fruiting body, few studies has been carried out on the comparison of characteristics and antioxidant activities of *F. pinicola* extracellular polysaccharide (EPS) and intracellular polysaccharide (IPS). Significant differences in structure or composition are expected in polysaccharides from different sources (Hsieh, Hsu, & Yang, 2005). In this study, the characterization and antioxidant activities of *F. pinicola* polysaccharides including IPS and EPS were investigated. Moreover, the protective effects of IPS and EPS on oxidative damage of yeast cells were also evaluated.

2. Materials and methods

2.1. Materials

F. pinicola used in this study was provided by the Quartermaster Equipment Institute of General Logistics Department of People's

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Liberation Army. The strain was inoculated at petri plate containing potato dextrose agar for 7 days at 30 °C. *F. pinicola* fruiting body was obtained from Changbaishan in Jilin province, China. Butylated hydroxytoluene (BHT) was purchased from Solarbio Co. Ltd (Beijing, China). 1-phenyl-3-methyl-4-benzene formyl pyrazolone (PMP) was from Ziyi Bio. Co. Ltd (Shanghai, China). 1-Diphenyl-2-picrylhydrazyl (DPPH) and reference monosaccharides (D-glucose, D-xylose, L-arabinose, D-mannose, L-rhamnose, D-galactose) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of crude polysaccharide extract

IPS was extracted twice from *F. pinicola* fruiting body with hot water at 70 °C in a 1:30 (w/v) ratio for 2 h, and then four volumes of ethanol were added and precipitated overnight at 4 °C. The precipitate was collected by centrifugation at $10,000 \times g$ for 15 min and lyophilized, thus yielded a crude polysaccharide extract. In the preparation of EPS, fermentation medium (sucrose 5%, yeast extract 1%, KH_2PO_4 0.2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 2%, w/v) was inoculated, and then incubated for 5 d. Similar to IPS, the fermentation broth was processed by concentration, precipitation, centrifugation and lyophilization to obtain crude polysaccharide extract.

2.3. Isolation and purification of the polysaccharides

Both the crude EPS and IPS (100 mg each) were dissolved in 10 mL of distilled water and centrifuged. The supernatant was added onto an anion exchange column (2.6 cm $\times$ 50 cm) with DEAE-cellulose. Eluting process was performed with a linear gradient from 0 to 0.5 mol/L NaCl at a flow rate of 1 mL/min. Total sugar content of the eluent was determined by phenol-sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The fractions eluted with linear gradient of NaCl solution were pooled, desalted and further purified on a Sephadex G-200 column eluting with distilled water at 0.5 mL/min. The major polysaccharide fractions were pooled and lyophilized. From this process, the purified polysaccharides were obtained for further identification of structure and monosaccharide compositions.

2.4. Analysis of monosaccharide compositions

Monosaccharide compositions were determined by high performance liquid chromatography (HPLC) after pre-column derivatization (Sun et al., 2009). In brief, 50 mg of purified polysaccharide powder was hydrolyzed with 2 mol/L of trifluoroacetic acid aqueous solution at 120 °C for 6 h in a sealed tube. After the hydrolysis was completed, the excess acid was removed by co-distillation with methanol to yield dry hydrolysate, which was dissolved in 0.3 mol/L of NaOH (100 μL), then 0.5 mol/L of methanol solution (200 μL) of PMP was added. The resulted solution was incubated at 70 °C for 1 h. After derivatization, the solution was cooled to room temperature followed by adjustment of pH to neutral with 0.3 mol/L HCl, then 1.5 mL of distilled water was added by vigorously shaking. Finally, the mixture was extracted with chloroform three times, and the aqueous phase was filtered through a 0.22 μm nylon membrane (Westborough, MA, USA). The resulted solution (10 μL) was injected onto a C18 column (4.6 mm $\times$ 250 mm) connected with a DAD-UV detector (Agilent Technologies, USA). The mobile phase was a mixture of 0.1 mol/L KH_2PO_4 (pH 10)–acetonitrile (83:17) at a flow rate of 1.0 mL/min. The column temperature was 30 °C. Sugar identification was done by comparison with reference monosaccharides (D-glucose, D-xylose, L-arabinose, D-mannose, L-rhamnose, D-galactose). Calculation of the molar ratio of monosaccharides was based on the peak area of the monosaccharides.

2.5. Determination of molecular weight

The molecular weights of EPS and IPS were determined on an Agilent 1200 HPLC system equipped with an evaporative light scattering detector (ELSD) and TSK-gel G5000 PWXL (7.8 mm $\times$ 30 cm, TOSOH Corp., Japan). The column was eluted with double-distilled water at a flow rate of 0.8 mL/min. Standard dextrans (T10, T40, T70, T100, T380, T500, Sigma, USA) were used for molecular weight measurement.

2.6. Infrared spectroscopy

The Fourier-transform infrared (FTIR) spectrum of the polysaccharides was detected on a Bruker-Vector 22 spectrometer (German). The polysaccharides were mixed with KBr powder, ground and pressed into 1 mm pellets in the frequency range of 4000–500 cm^{-1} .

2.7. Assay of antioxidant activities in vitro of EPS

2.7.1. DPPH radical scavenging activity

The methods of Shimada et al. was employed with some modifications (Shimada, Fujikawa, Yahara, & Nakamura, 1992). Different concentrations of EPS and IPS (2.0–10.0 mg/mL) were prepared with distilled water. A total of 0.5 mL each sample with different concentrations was mixed with 1.5 mL of 0.4 mmol/L methanol solution of DPPH, and the mixed solution was reacted for 30 min in darkness. Then absorbance was measured at 517 nm with BHT as control. The activity of scavenging the DPPH radical was calculated using the following equation: $Y\% = [1 - (A_1 - A_2)/A_0] \times 100$, where A_0 was the absorbance of the blank group (distilled water + DPPH), A_1 was the absorbance of the sample reaction (sample + DPPH), and A_2 was the background absorbance of the sample (distilled water replaced DPPH).

2.7.2. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured according to Fenton method described before (Zhong, Jin, Lai, Lin, & Jiang, 2010). Polysaccharides of different concentrations (2.0–10.0 mg/mL) were prepared. Then added 5 mmol/L of FeSO_4 (2 mL), 5 mmol/L of salicylic acid-ethanol solution (2 mL), 2 mL of polysaccharide sample solution sequentially. Then the reaction started with 5 mmol/L H_2O_2 (2 mL) in water bath at 37 °C. Absorbance was measured at 510 nm with distilled water as reference and BHT as positive control. The hydroxyl radical scavenging effect was calculated as follows: $Y\% = [1 - (A_1 - A_2)/A_0] \times 100$, where A_0 was the absorbance of blank, A_1 was the absorbance of the sample, and A_2 was the background absorbance of the sample.

2.7.3. Protective effect on yeast cells from UV

Firstly, the yeast cells were cultured to the early stage of the logarithm in yeast extract peptone dextrose medium. Then 20 mL of fermentation broth was centrifuged for 5 min at $4000 \times g$, and the supernatant was discarded for yeast paste collection. Then the cells were washed with phosphate buffer solution twice, and the yeast paste was mixed with 20 mL of saline. A total of 1 mL yeast suspension was mixed with 4 mL of different concentrations of EPS and IPS (5–25 mg/mL) in petri dish. Then the cells were disposed with UV radiation for lethal time (1 min treatment could kill all yeast cells in the control group) respectively, then the blank group was mixed with distilled water. Finally, the mixture after UV radiation were diluted to appropriate concentration for spread plate viable count method at 28 °C for 2 d with BHT as control. The survival rate was calculated as follows: $Y\% = [(A_0 - A_1)/A_0] \times 100$, where A_0 was the amount of cells which had not been disposed by UV radiation,

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