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Purification, characterization and hepatoprotective activities of mycelia zinc polysaccharides by *Pleurotus djamor*

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ABSTRACT

This study was designed to investigate the physicochemical properties (molecular weights, bond types and monosaccharide compositions), antioxidant activities, and hepatoprotective effects on carbon tetrachloride (CCl_4)-induced acute liver damage of mycelia zinc polysaccharides (MZPSs) and its major fractions (MZPS-1, -2 and -3) separated from *Pleurotus djamor*. *In vitro* assays, the MZPS-3 demonstrated relatively strong antioxidant activities in dose-dependent manners. For *in vivo* hepatoprotective activities, administration of MZPS-3 at 800 mg/kg significantly decreased the levels of AST, ALT, MDA and LPO, remarkably increased the levels of TC, TG and ALB, and prominently restored the activities of SOD, GSH-Px, CAT and T-AOC in serum/liver homogenate against CCl_4-induced injures. Findings presented in this study clearly demonstrated that MZPSs, especially MZPS-3, might be suitable for functional foods and natural drugs in preventing the CCl_4-induced acute liver damage.

1. Introduction

In recent years, "oxidative stress" and its adverse effects on human health have been received more and more academic attentions due to its role involved in the human pathological diseases. It is well known that the reactive oxygen species (ROS), including superoxide anion radicals, hydroxyl radicals and DPPH radicals, were considered to be fatal in tissue damage and the loss of body functions. The liver is the major site of xenobiotic metabolism (Lu, Zhao, Sun, Yang, & Yang, 2013). The metabolism of these xenobiotics will induce the generation of ROS in hepatocytes and result in hepatic damage, gross cellular change and cell death causing hepatotoxicity or liver damage (Sabir et al., 2012). Notoriously, the hepatotoxicity induced by carbon tetrachloride (CCl_4) *via* trichloromethyl-free radicals ($\bullet CCl_3$ or $CCl_3OO\bullet$) is the well-characterized animal model for evaluating the therapeutic potential of drugs with treating liver diseases. Recently, many evidences have indicated that natural substances from edible and medicinal mushrooms show potential antioxidant capacities which may antagonize the hepatic toxicity caused by CCl_4 (Liu et al., 2014; Nitha, Fijesh, & Janardhanan, 2013). For this reason, there is a considerable desiderating in complementary and alternative medicines for the treatment of liver injuries.

Pleurotus djamor, which is classified in the genus *Pleurotus* of the family *Pleurotaceae*, has been appreciated as an edible and medicinal mushroom. Documented pharmacology researches have indicated that polysaccharides from *Pleurotus* spp. usually showed various biological activities, such as antioxidant, immunomodulatory and antitumor (Jayakumar, Ramesh, & Geraldine, 2006; Ahmed, Yossef, & Ibrahim, 2010; Jayakumar, Sakthivel, Thomas, & Geraldine, 2008). Meanwhile, zinc is an essential trace element in the human body and it participates in various pathways of metabolism (Jia et al., 2006). Interestingly, it has been proved by our previous report that, after submerged fermentation with zinc-compound (zinc acetate), zinc-enriched polysaccharides exhibit significantly higher antioxidant activities than the regular polysaccharides (Zhang et al., 2014). Taken together, it is quite necessary and significative to explore the antioxidant activity of the polysaccharides extracted from the zinc-enriched mycelia of *P. djamor* and evaluate their hepatoprotective activities in preventing CCl_4-induced hepatotoxicity.

The objective of the present study was aimed to describe the structural characteristics of the mycelia zinc polysaccharides (MZPS) and its major fractions (MZPS-1, -2 and -3) purified by

Abbreviations: ALB, albumin; ALT, alamine aminotransferase; AST, aspertate aminotransferase; CAT, catalase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FT-IR, Fourier-transform infrared; GSH-Px, GSH peroxide; HPLC, high performance liquid chromatography; LPO, lipid peroxidation; MC, model control; MDA, malondialdehyde; *Mn*, number-average molecular weight; *Mw*, weight-average molecular weight; MZPS, Mycelium zinc polysaccharides; MZPSs, MZPS and it fractions; NC, normal control; PC, positive control; prot, protein; ROS, reactive oxygen species; SOD, superoxide dismutase; T-AOC, total antioxidant capacity; TC, total cholesterol; TG, triglyceride.

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DEAE-52 cellulose column chromatography. Furthermore, Antioxidant activities *in vitro* and hepatoprotective effects by means of CCl_4-induced hepatic injury in mice were also investigated.

2. Materials and methods

2.1. Organism and chemicals

The strain of *P. djamor* was provided by our laboratory and used in this experiment. The diagnostic kits for assaying superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), total antioxidant activity (T-AOC), malondialdehyde (MDA) and lipid peroxidation (LPO) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), dextrans and DEAE-52 cellulose were purchased from Sigma Chemicals Company (St. Louis, USA). All other chemicals used in this work were of analytical grade and purchased from local chemical suppliers.

2.2. Preparation and purification of MZPS

The liquid fermentation technology was used to produce *P. djamor* zinc mycelia. Each 250-mL flask, containing 100 mL basal medium of (g/L) potato 200, glucose 20, KH_2PO_4 1.5, $MgSO_4{\cdot}7H_2O$ 1 and with zinc acetate (3 g/L, 3 mL) for supplying the zinc, was kept cultivation at 25 °C with a shaking of 140 × *g* for 10 days.

The polysaccharides of *P. djamor* were isolated by employing our previous procedure (Zhang et al., 2014). After filtration, concentration, sterilization and lyophilization, the powered of mycelia were extracted twice with proper water for 4 h (60 °C). The supernatant liquid was obtained by centrifugation (3600 × *g*, 15 min), and precipitated with ethanol (1:3, v/v) at 4 °C overnight. After centrifugation, the precipitate was washed 3 times by 85% ethanol. The precipitate was collected by centrifugation and deproteinated by employing the Sevage method (Miao et al., 2013). Finally, the deproteinated supernatant was extensively dialyzed against distilled water and lyophilized to give *P. djamor* mycelia zinc polysaccharides (MZPS).

The lyophilized powered of MZPS (0.5 g) was dissolved in distilled water (5 mL), then applied to a DEAE-cellulose column (1.6 × 20 cm), and eluted at a flow rate of 3.0 mL/min with distilled water and gradient NaCl solutions of 0.1, 0.3, 0.5, and 1 M. The eluent was collected automatically, and monitored by the phenol–sulfuric acid colorimetric method (Dubois, Gillers, & Hamilton, 1956). The major fractions was pooled and lyophilized to yield MZPS fractions (MZPSs), which were used for further studies.

2.3. Preliminary characterization of MZPSs

The molecular weights and homogeneities were determined by high performance liquid chromatography (HPLC) that was operated with a HPLC system (Shimadzu LC-2010AT, Japan) equipped with an Atlantis C18 column (250 mm × 4.6 mm × 5 μm) and a refractive index detector. The injection volume was 20 μL. The deionized water was used as mobile phase at a flow rate of 1 mL/min, and the column temperature was maintained at 30 °C. A series of standard dextrans were used to make the calibration curve. Molecular weight was analyzed by Agilent GPC software.

Spectroscopy analysis of Fourier-transform infrared (FT-IR) was recorded on a 6700 Nicolet Fourier transform-infrared spectrophotometer (Thermo Co., Madison, WI, USA) within the range from 4000 to 400 cm^{-1}, using the KBr disc method to prepare the specimen.

The monosaccharide analyses were determined by gas chromatography (GC, CP3800, Varian, USA) according to the previously described methods (Luo et al., 2010). The samples were hydrolyzed with trifluoroacetic acid (TFA, 2 M, 110 °C) for 4 h, and the excess TFA was completely removed by co-distillation with ethanol. The hydrolyzed products were acetylated with hydroxylamine hydrochloride and pyridine. The supernate (1 μL) was injected into an HP-5 fused silica capillary column (3000 × 0.32 × 0.25 mm) and equipped with flame ionization detector. Monosaccharide components were investigated using rhamnose, D-ribose, arabinose, xylose, inositol, allose, mannose, glucose and galactose as reference sugars. The relative molar ratios of monosaccharides were calculated by the area normalization method according to the chromatogram.

2.4. Analysis of antioxidant effects in vitro

The scavenging abilities on super anion were determined according to our previous report (Zhang et al., 2014). The mixture, contained 1 mL samples (0–1500 mg/L), 4.5 mL Tris–HCl buffer (pH 8.2, 50 mM), and 3.2 mL deionized water, were kept warm at 25 °C for 20 min. Another 20 min warming (25 °C) was processed after the addition of 0.3 mL pyrogallol. The mixtures were reacted in the bath for 20 min (25 °C), and the reaction was ended by adding 1 mL vitamin C (5%, w/v). The absorbance was measured at 420 nm against deionized water as a blank. The scavenging ability was calculated as follows.

$$\text{Scavenging ability}\,(\%) = \frac{A_0 - A}{A} \times 100$$

where A_0 was the absorbance of isometric water, and A was the absorbance of samples.

The hydroxyl radical scavenging assay was measured according to the method of Koksal, Bursal, Dikici, Tozoglu, and Gülçin (2011) with slight modifications. The reaction mixture contained 1 mL of phenanthroline (7.5 mM), 1 mL of ferrous sulfate (0.75 mM), 5 ml of phosphate buffer (pH 7.4), 1 mL of 3% (v/v) hydrogen peroxide and 1 mL of the sample (0–1500 mg/L). After incubating at 37 °C for 60 min, the absorbance was measured at 560 nm and calculated as follows.

$$\text{Scavenging ability}\,(\%) = \frac{A_0 - A}{A} \times 100$$

where A_0 was the absorbance of isometric water, and A was the absorbance of samples.

The DPPH radical scavenging effects were processed using the method reported by Cheng et al. (2013) with slight modifications. Briefly, 0.2 mL of the sample (0–1500 mg/L) was added to 0.6 mL of DPPH solution (0.004% in methanol, m/v). The mixture was shaken thoroughly and kept in the dark for 30 min at the room temperature. The absorbance was measured at 517 nm. The scavenging percentage was calculated using the following equation:

$$\text{Sacvenging activity}\,(\%) = \frac{A_0 - A}{A} \times 100$$

where A_0 was the absorbance of control (water used instead of sample) and A was the absorbance in the presence of the sample.

The reducing power was assayed according to the reported method with some modifications (Liu, Luo, Ye, & Zeng, 2012). One milliliter of phosphate buffer (0.2 M, pH 6.6), 1.0 mL of 1% potassium ferricyanide solution and 0.5 mL of samples at different concentrations (0–1500 mg/L) were incubated at 50 °C for 20 min. After addition of trichloroacetic acid (1 mL, 10%, w/v), the mixture was centrifuged for 10 min at 3600 × *g*. The upper layer of solution (1.5 mL) was mixed with isovolumetric water and trichloroacetic acid (0.1%, w/v), and the absorbance was measured at 700 nm.