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Purification, characterization and biological activity on hepatocytes of a polysaccharide from *Flammulina velutipes* mycelium

Xiubing Pang, Wenbing Yao, Xiaobing Yang, Chen Xie, Dong Liu, Jian Zhang, Xiangdong Gao *

School of Life Science and Technology, China Pharmaceutical University, Nanjing 210009, PR China

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Abstract

A water-soluble polysaccharide named as FVP2 has been isolated from *Flammulina velutipes* mycelium by hot water extraction, anion-exchange and gel-permeation chromatography. Structure study shows FVP2 is an α-(1→4)-D-glucan, with a single α-D-glucan at the C-6 position approximately every seven residues, along the main chain. The weight-average molecular weight is 1.89×10^4 Da. Its biological activity was tested on hepatocytes. *In vitro* study indicates FVP2 can enhance the livability of primary culture of mouse hepatocytes and decrease the release of ALT as well as apoptosis of hepatocytes after CCl_4 intoxication.

Keywords: *Flammulina velutipes*; Polysaccharide; Mycelium; Structure; D-Glucan; Hepatocyte

1. Introduction

Malignant neoplasm is presently one of the diseases threatening people's health. Recently, several Chinese herbal medicines, which possess anti-tumor and/or immune-stimulating properties, have been obtained increasing attention and used clinically in prevention and treatment of cancer as adjuvants such as lingzhi (*Ganoderma lucidum*), shiitake (*Lentinus edodes*), yiner (*Tremella fuciformis*), *Coriolus versicolor*, and krestin (*Cordyceps sinensis*) (Cui & Chisti, 2003; Wu, Sun, & Pan, 2006; Zhang, Cui, Cheung, & Wang, 2007). Many species in polysaccharide, a big class of natural macromolecules, have been found to carry significant biological activities. It has been reported that polysaccharides derived from medicinal mushrooms have activities of anti-tumor, anti-infection, anti-aging, anti-radiation etc. All these activities are due to its contribution to enhance immune function of the human body (Cui & Chisti, 2003; Leung, Liu, Koon, & Fung, 2006; Zhang et al., 2007).

Flammulina velutipes, ascribed to Eumyeota, Basidiomycotina, Hgmenomycetes, Holobaeidiomycetidae, Agaricel, Tricholomataceae, *Flammulina*, is one of the most popular edible fugus in China and Japan. Its production and consumption ranks fourth in the edible mushrooms (Leifa, Pandey, & Soccol, 2001). The first study of *F. velutipes* polysaccharide (FVP) was reported by Kamasuka et al. (Kamasuka, Momoki, & Sakai, 1968). Since then several other polysaccharides have been isolated from *F. velutipes* fruit bodies and mycelium (Ikekawa et al., 1982; Leung, Fung, & Chay, 1997; Ohkuma, Tanaka, & Ikekawa, 1983). All these studies reveal FVPs stimulate concanavalin A-induced mitogenic activity of T lymphocytes, promotes T-cell to produce antibodies and also induces expression of interferon, indicating that FVPs possess a great potential. However, there is no report what the exact active compound in FVP is and its function on liver. In the present study, we report a new polysaccharide isolated from *F. velutipes* mycelium and its structure characteristic as well as its biological effects on hepatocytes.

* Corresponding author. Tel.: +86 25 83271298; fax: +86 25 83271249.
E-mail address: xiangdong_gao@hotmail.com (X. Gao).

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2. Materials and methods

2.1. Materials

Flammulina velutipes mycelium (Jiangsu Shenhua Pharmaceutical Co. Ltd., China), was air dried and ground. Dextran standards of T-500, T-70, T-40, T-10 were purchased from Pharmacia Co. Ltd. Hepatocyte growth factor (HGF) was purchased from Nanjing, Nanjing University Pharmaceutical Co. Ltd. (China).

2.2. General methods

The specific rotation was determined at 20 ± 1 °C with an automatic polarimeter (Model WZZ-2, China). The FTIR spectra was recorded on a Nicolet Impact 410 spectrophotometer with KBr pellets. Total carbohydrate content was determined by the anthrone–sulphuric acid method as D-glucose equivalents (Scott & Melvin, 1953). Protein was analyzed by the Coomassie Brilliant G-250 method. GC was analyzed on a Hewlett-Packard model 6890 instrument equipped with a capillary column of HP-5.5% phenyl methyl siloxane (30 m × 0.25 mm × 0.25 μm) and a flame-ionization detector, and programmed from 150 to 220 °C at 2 °C/min and from 220 to 280 °C at 30 °C/min. GC–MS was conducted with a UAR1AN Saturn 2000 GC/MS/MS instrument, using a DB5 capillary column (30 m × 0.25 mm × 0.25 μm). The column temperature was held at 140 °C for 3 min, increased to 250 °C at 3 °C/min, and kept for 10 min. The ionization potential was 70 eV and the temperature of the ion source was 220 °C. All NMR (^{1}H, ^{13}C, and HSQC) experiments were accomplished at 30 °C in D_2O on a Bruker AV-500 NMR spectrometer (Liu et al., 2007).

2.3. Isolation and purification of Flammulina velutipes polysaccharides

Flammulina velutipes mycelium (50 g) was extracted with 1000 mL water for 8 h at 70–80 °C. The extraction was collected by centrifugation and concentrated till 200 mL under reduced pressure, then protein was removed with Sevag method (Staub, 1965), followed by dialyzing against distilled water. The nondialyzable phase was diluted with three times of volume with 95% EtOH and kept at 4 °C for 4 h. After centrifugation, the resulting precipitate was washed sequentially with ethanol, acetone, ether, and vacuum-dried. The yield of crude polysaccharide was 9.5% of the dried material.

The crude polysaccharide was applied to a DEAE–Sephadex A-50 column (2.0 × 44 cm), eluted with H_2O. Fractions were collected and measured for carbohydrate by the anthrone–sulphuric acid method (Scott & Melvin, 1953). Further purified on a Sephacryl S-400 column (1.5 × 65 cm) eluted with H_2O at a flow rate of 9 mL/h. Two polysaccharide fractions named as FVP1 and FVP2 were separated. The yield of FVP1 and FVP2 were about 16.6% and 43.7% from the crude polysaccharide, respectively, of which the FVP2 was used in the subsequent studies.

2.4. Homogeneity and molecular weight determination

The molecular weight of FVP2 was determined by gel chromatographic technique (Rodriguez & Vanderwieles, 1979; Wu et al., 2006). Measurements were carried out on a Sephacryl S-400 column (1.0 × 100 cm), eluted with H_2O at a flow rate of 9 mL/h. A series of different weight-average molecular weights of standard dextrans T-500, T-70, T-40, and T-10 were prepared as 0.1% (w/v) solutions and 1 mL of solution was subjected in each run, and then the elution volumes were plotted against the logarithms of their respective molecular weights. A solution of FVP2 (3 mg) in distilled water (0.5 mL) was passed through the column. The elution volume of FVP2 was then plotted in the same graph, and the weight-average molecular weight of FVP2 was obtained. The homogeneity of FVP2 was determined on an Agilent 1100 HPLC system equipped with a Shodex SUGAR KS-805 column (8 × 300 mm) according to the protocol of gel-permeation chromatography (GPC) previously described by Yang et al. (Yang et al., 2005), and was also measured by polyacyrlamide gel electrophoresis.

2.5. Monosaccharide composition

The polysaccharide (10 mg) was hydrolyzed in 2 M trifluoroacetic acid (2 mL) at 100 °C for 8 h. The hydrolysate was co-analyzed by TLC and GC methods. TLC was performed on thin layer plate (silica gel G) with a solvent system of *o*-butanol, acetoacetate, iso-propanol and H_2O in a ratio of 7:4:7:2 (v/v). D-mannose, D-glucose, D-fucose, D-arabinose and D-rhamnose were used as standard sugars. Sugar spots were visualized by spraying *o*-phthalic acid reagent. For GC analysis, the hydrolysate of FVP2 was converted into its respective alditol acetates and analyzed by gas chromatography with myo-inositol as the internal standard (Honda, Suzuki, Kakehi, Honda, & Takai, 1981).

2.6. Methylation analysis

The polysaccharide was methylated three times by the Needs and Selvendran method (Needs & Selvendran, 1993). The methylated product as depolymerized with 90% HCOOH at 100 °C for 6 h and further hydrolyzed with 2 M TFA at 100 °C for 2 h. The partially methylated residues were reduced and acetylated (Blakeney, Harris, Henry, & Stone, 1983). The resulting products were analyzed by GC–MS. The GC temperature program was carried out as described previously (Bao, Liu, Fang, & Li, 2001). Partially methylated alditol acetates were identified by their fragment ions in EI-MS and by relative retention times in GC, and the molar ratios were estimated from