Carbohydrate Polymers 145 (2016) 71–77

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Purification, characterization and antioxidant activity of polysaccharides from *Flammulina velutipes* residue

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ARTICLE INFO

Article history:
Received 30 November 2015
Received in revised form 6 March 2016
Accepted 9 March 2016
Available online 11 March 2016

Keywords:
Flammulina velutipes residue
Polysaccharide
Purification
Characterization
Antioxidant activity

ABSTRACT

In this study, we isolated polysaccharides from *Flammulina velutipes* residue (FVRP) using microwave-assisted extraction and then purified the polysaccharides by column chromatography to yield FVRP-1, FVRP-2 and FVRP-3. The structural characteristics of FVRP-1, FVRP-2 and FVRP-3 were investigated, and their antioxidant activities against ABTS$^+$, DPPH and hydroxyl radicals were also analyzed *in vitro*. FVRP-1 was found to be neutral and rich in galactose. However, FVRP-2 and FVRP-3 were acidic polysaccharides and were rich in glucose. The average molecular weight of FVRP-1, FVRP-2 and FVRP-3 were 29,930, 62,290, and 36,310 Da, respectively. The glycosyl residue of FVRP-1 was an α-type glycosidic linkage, whereas FVRP-2 and FVRP-3 were β-type glycosidic linkages. We found FVRP-1, FVRP-2 and FVRP-3 had strong potential antioxidant activities in the order of FVRP-1 < FVRP-3 < FVRP-2. Our results thus suggested that FVRPs might be suitable for use as functional foods and as potential therapeutic agents.

1. Introduction

Flammulina velutipes (*F. velutipes*), also known as golden needle mushroom or enokitake, is the fourth most popular edible mushrooms in the world (Jing et al., 2014). Consumption of *F. velutipes* has increased dramatically due to its delicious taste and high nutritional properties (Yang et al., 2012). In recent studies, *F. velutipes* has been shown to be a low calorie mushroom with high levels of essential amino acids, vitamins, fiber, and polysaccharides (Leifa, Pandey, & Soccol, 2001; Zhang et al., 2013). It has been reported that both the fruiting bodies and the fungal mycelia of *F. velutipes* contain bioactive polysaccharides with beneficial immunomodulatory, anti-tumor, antioxidant, anti-inflammatory activities and biological activity on hepatocytes (Leung, Fung, & Choy, 1997; Pang et al., 2007; Sheng et al., 2007; Wu, Duan, Liu, & Cen, 2010; Shi, Yang, Guan, Zhang, & Zhang, 2012; Zhang et al., 2013). The antioxidant activity of polysaccharides mainly depends on several structural parameters including sugar composition, molecular weight, type of glycosidic bond in the main chain, and the degree of sulfuric acid esterification (Wang, Li, & Chen, 2009).

In recent years, a number of production facilities have been set up for the large-scale cultivation of *F. velutipes* in Asian countries, especially in China and Japan (Yan, Liu, Mao, Li, & Li, 2014). Along with increasing golden needle mushroom cultivation, the amount of solid waste from the spent mushroom culture medium (usually called mushroom residue) has been increasingly discarded as industrial waste (Bao, Ochiai, & Ohshima, 2010). This high accumulation of local mushroom residue has become a serious environment pollution concern (Song et al., 2014). Although many studies have focused on increasing the production of the golden needle mushroom, only a few studies have targeted *F. velutipes* residue. Thus far, *F. velutipes* residue has been utilized primarily as compost (Bao, Shinomiya, Ikeda, & Ohshima, 2009). However, *F. velutipes* residue can be used as a bioresource of natural antioxidants for controlling the oxidation of not only lipids but also myoglobin (Bao et al., 2010).

Thus, the objective of this study was to purify and characterize polysaccharides from *F. velutipes* residue and to determine the antioxidant activity of FVRP, including ABTS$^+$, DPPH and hydroxyl radicals scavenging activity.

2. Materials and methods

2.1. Materials

F. velutipes residue was obtained from Wuhan Edible Mushroom Biological Technology Co., Ltd. (Hubei Province, China), and then dried and further ground into powder. DEAE cellulose-52

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http://dx.doi.org/10.1016/j.carbpol.2016.03.020
0144-8617/

was purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. (Beijing, China). Standard reagents including D-glucose, D-galactose, D-galactose, D-xylose, D-rhamnose, D-mannose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents used were of analytical grade.

2.2. Preparation and purification of polysaccharides from F. velutipes residue

F. velutipes residue powders were extracted with petroleum ether at 80 °C for 2 h to remove lipids and pigments. This process was repeated three times. The residue was then extracted with distilled water using microwave assisted extraction under an optimized condition (ratio of water to material of 20 ml/g, microwave power of 650 W, microwave time of 2 min). The cooled solution was centrifuged at 5000 × *g* using an Avanti® J-E refrigerated centrifuge (Beckman Coulter, USA) for 10 min. The supernatant was then concentrated to one tenth of its volume using a rotary evaporator (RE-2000A, YaRong, China) at 50 °C and precipitated with anhydrous ethanol at 4 °C for 12 h. This process was repeated 4 times. The precipitate thus obtained was dissolved again in water and deproteinated using the Sevag method (Staub, 1965). This procedure was repeated 5 times. Next, the solution was dialyzed against deionized water over a period of 2 days. Finally, the retentate was freeze-dried using a freeze drier (Betr 2-8 LD plus, Christ, Germany) for 48 h to obtain crude polysaccharide from the *F. velutipes* residue (FVRP).

The crude FVRP was dissolved in deionized water, filtered through 0.45 μm of membrane and loaded onto a DEAE-52 cellulose column (2.6 × 50 cm). The column was then stepwise eluted with 0, 0.1 and 0.3 M NaCl solution at a flow rate of 1.0 ml/min. Three completely separated fractions, FVRP-1, FVRP-2 and FVRP-3, were collected by checking the absorbance at 490 nm using the phenol-sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The three fractions were dialyzed against deionized water for 2 days and freeze-dried for further study.

2.3. Determination of contents of total sugar, protein, uronic acid and sulfate

The content of total sugar in FVRP, FVRP-1, FVRP-2 and FVRP-3 were determined by the phenol-sulphuric acid method (Dubois et al., 1956), with glucose as the standard. The content of protein was determined by the Bio-Rad Protein Assay (Bradford, 1976), using bovine serum albumin (BSA) as the standard. The content of uronic acid was determined by the sulfuric acid-carbazole method (Blumenkrantz & Asboe-Hansen, 1973) and with D-glucuronic acid as the standard. The content of sulfate was determined by the barium chloride-gelatin method (Dodgson & Price, 1962), with potassium sulfate as the standard.

2.4. Determination of molecular weight

Molecular weight determination of FVRP samples was determined by the method described by Yi et al. (2012). A gel chromatography was coupled with multi-angle laser light photometer (GPC-MALLS) system, which consisted of a MALLS detector (DAWN HELEOS-II, Wyatt Technology, Santa Barbara, CA, USA), a RI detector (Optilab Rex, Wyatt Technology, Santa Barbara, CA, USA), a Waters 515HPLC pump (Waters Corporation, USA) and a Shodex Ohpak SB-805HQ column (Shoko, Japan) was used. The concentration of polysaccharide solution was 0.1 mg/ml, and 200 μl of sample was injected after passing through a 0.2 μm syringe filter (Waters, Milford, MA, USA). The isocratic mobile phase was 0.1 M sodium nitrate at a flow rate of 0.5 ml/min, and the column temperature was kept at 25 °C. Astra software was used for data collection and analysis.

2.5. Analysis of monosaccharide compositions

The monosaccharide compositions of FVRP, FVRP-1, FVRP-2 and FVRP-3 were analyzed by the reported method (Dai et al., 2010) with some modifications. The dried polysaccharide sample (10 mg) was hydrolyzed with 10 ml of 2.0 M trifluoroacetic acid (TFA) at 120 °C for 4 h in a sealed glass tube. After hydrolysis, excess TFA was removed by the addition of methanol and then evaporated at reduced pressure. The hydrolysate was acetylized with the mixture of 10 mg hydroxylamine hydrochloride, 2 mg inositol (as internal reference) and 1.0 ml pyridine at 90 °C for 30 min. The tube was cooled to room temperature, and then 1.0 ml of acetic anhydride was added and incubated at 90 °C for 30 min again. After cooling, 1.0 ml of water was added and stirred, and the solution was extracted with chloroform. This process was repeated 3 times after which, the combined chloroform extracts layer was evaporated to dryness for GC analysis.

The derivatives were analyzed by an Agilent 6890N Network GC System (Agilent, USA) equipped with an HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 μm) and a flame-ionization detector (FID). The following program was adopted for gas chromatography analysis: injection temperature: 270 °C, detector temperature: 250 °C. The oven temperature was maintained at 150 °C for 5 min, and then programmed from 150 °C to 190 °C at 10 °C/min. Subsequently, the temperature was adjusted up from 190 °C to 210 °C at 2 °C/min and for 10 min at 210 °C. Several kinds of standard monosaccharides were used as a control using the same process (Khaskheli et al., 2015).

2.6. Fourier-transform infrared spectra (FT-IR) analysis

The dried samples of FVRP-1, FVRP-2and FVRP-3 were mixed with spectroscopic-grade potassium bromide powder and then ground and pressed into pellets for FT-IR measurement. FT-IR spectra were recorded with a Nexus 470 FT-IR spectrometer (Thermo Nicolet, USA) at the frequency range of 4000–400 cm^{-1}.

2.7. Triple helical structure analysis

The triple helical structures of FVRP-1, FVRP-2 and FVRP-3 were analyzed by their interaction with Congo red (Rout, Mondal, Chakraborty, & Islam, 2008). Two milliliters of polysaccharide solution (2 mg/ml) were mixed with 2.0 ml of 80 μmol/l of Congo red in a gradient of sodium hydroxide solutions (0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 M). The absorbance was measured in the range of 200–800 nm, and the maximum absorption wavelength at different concentrations of sodium hydroxide was plotted. Distilled water without added polysaccharide was measured as the control.

2.8. Evaluation of antioxidant activity

2.8.1. $ABTS^+$ radical scavenging activity

The $ABTS^+$ radical scavenging activities of FVRP-1, FVRP-2, and FVRP-3 were measured by the reported method (Re et al., 1999) with some modifications. The stock solution of $ABTS^{\bullet+}$ was diluted to an absorbance of 0.70 ± 0.02 at 734 nm and stocked before use. Two hundred microliters of sample solutions at various concentration were mixed with 4 ml of $ABTS^{\bullet+}$ diluent and shaken gently for 30 s. The reaction was kept at room temperature for 6 min, and then the absorbance was measured at 734 nm, and Vc was used as the control. $ABTS^{\bullet+}$ scavenging activity was calculated as a scavenging rate (%) using the equation: scavenging rate (%) = $[1 - (A_1 - A_2)/A_0] \times 100$, where A_0 was the absorbance of the