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Antioxidant activity of polysaccharides produced by *Hirsutella* sp. and relation with their chemical characteristics

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ABSTRACT

Extracellular polysaccharide (EPS) and intracellular polysaccharide (IPS) were produced in a mycelial liquid culture of the *Hirsutella* sp. liquid fermentation. The polysaccharides were precipitated with 50% ethanol (EPS-1, IPS-1), 65% ethanol (EPS-2, IPS-2) and 80% ethanol (EPS-3, IPS-3). The polysaccharide fragments precipitated in lower ethanol percentages had a lower neutral sugar content and a larger molecular weight. EPS-1, EPS-2, IPS-1 and IPS-2 were composed of glucose (Glu), galactose (Gal) and mannose (Man). Galactose was not detected in EPS-3 and IPS-3. Evaluated by the $1/IC_{50}$ values of hydroxyl radical scavenging activity, the polysaccharides with higher protein content, lower neutral sugar content and molecular weight about 10–20 kDa were found to have better radical scavenging activity. Significant correlations demonstrated that the antioxidant effect of the polysaccharides was influenced by monosaccharide composition (mannose, $r = 0.942$; glucose, $r = -0.905$).

1. Introduction

Cordyceps sinensis, one of the most valuable medicinal fungi in traditional Chinese medicine, is initially recorded in Ben-Cao-Cong-Xin (New Compilation of Material Medical) and used for the treatment of various diseases (Li et al., 2003; Zhu, Halpern, & Jones, 1998). Recent studies have demonstrated its multiple pharmacological actions such as anti-aging, anti-tumor, immuno-stimulation and antioxidant activities (Chen, Wang, & Nie, 2013). *C. sinensis* is proven to have broad medicinal effects, which is beneficial to several systems in human body, including the circulatory, immune, cardiovascular, respiratory and glandular systems (Ma et al., 2008; Wang & Fang, 2004).

Polysaccharide has been reported to be the major bioactive substance of *C. sinensis* and most of other medicinal fungi (Leung, Zhao, Ho, & Wu, 2009; Li, Yang, & Tsim, 2006; Yang, Gao, Han, & Tan, 2005). Pharmacological research has declared polysaccharide to have great medical merits, such as anti-tumor, anti-oxidant, hypoglycemic effect, anti-fibrosis, anti-fatigue, kidney protection, increasing plasma testosterone levels and radiation protection (Wang, Jin, & Zhang, 2013; Yan, Wang, & Wu, 2013). The excellent bioactivities of polysaccharide subsequently make it the focus of looking for new drugs (Kiho, Yamane, Hui, Usui, & Ukai, 1996). The intracellular polysaccharide (IPS) from fungi documented and applied in commercial products is mostly extracted from the fruit bodies or mycelia, while the extracellular polysaccharide (EPS) is isolated from the liquid media used for mycelial cultivation (Wasser, 2002; Zhang, Cui, Cheung, & Wang, 2007). Since natural *C. sinensis* is very rare and not sufficient to meet the increasing demand, mycelial fermentation has become a major and more economical source for IPS isolation (Leung et al., 2009).

The chemical composition of polysaccharide, such as degree of polymerization, glycosidic bonds and molecular weight, has great influence on its antitumor activity, antiviral activity and hypoglycemic activity (Lou, Wang, Hong, Tang, & Liu, 2013). Studies on polysaccharide structure and activities are the premise for its application in the field of medicine and health care. Although, the preliminary study shed light on the molecular weight, monosaccharide compositions and activities of polysaccharides from *C. sinensis*, studies on the relationship between structure and activities, especially how the different molecular weight fractions and chemical constituents contribute to the bioactivities, are lacking (Huang, Siu, Wang, Cheung, & Wu, 2013).

To investigate the antioxidant activity of polysaccharide produced by *Hirsutella* sp. mycelia culture and relation with their Chemical characteristics, gradient precipitation was applied to separate and purify the EPS and IPS (from mycelium). The characteristics of EPS and IPS, including molecular weight, chemical compositions, neutral sugar and protein content, were analyzed. Hydroxyl radical scavenging ability was used to evaluate the

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antioxidant activity of polysaccharides. The correlation between antioxidant activity and characteristics of polysaccharide was studied by statistical analysis.

2. Material and method

2.1. Strain and culture condition

Hirsutella beakdumountain X.L.Jiang, sp.nov., sharing the same morphology and molecular biologic characteristics with *C. sinensis*, was identified as a new species of *Hirsutella* and deposited in China Center for Type Culture Collection (Li, 2009).

The fungus was maintained on Potato Dextrose Agar (PDA) slant, which was incubated at 25 °C for 15 days, and then stored at 4 °C. The fungus was initially grown on PDA medium in a Petri dish, and then transferred to the seed culture medium. Cultivation in liquid media was carried out in 250-mL Erlenmeyer flasks containing 100 mL of medium (200 g/L potato, 20 g/L sucrose, 4 g/L yeast, 1 g/L $NaNO_3$, 1.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, with an initial pH 6.8, at 25 °C in a rotary shaker incubator at 160 rpm for 48 h.

2.2. Polysaccharides isolation and purification

After fermentation, the supernatant liquid medium and mycelial biomass were harvested from the fermentation broth by centrifugation. The biomass was rinsed repeatedly with distilled water.

The supernatant liquid was concentrated by evaporation under reduced pressure at 50 °C. Ethanol was added slowly at 1 volume ratio to the concentrated extract liquid, left at 4 °C overnight for precipitation, followed by centrifugation. The precipitate was collected and the supernatant was subjected to the next step of precipitation with a higher ethanol volume ratio. In this way, the precipitated fractions were collected successively at 50%, 65% and 80% ethanol concentration, designated EPS-1, EPS-2 and EPS-3, respectively. All EPS precipitates were washed twice with ethanol.

IPS extraction from the mycelial biomass was conducted by the method of hot water extraction, referring to the method of Yan, Wang, Li, & Wu (2011) with little modification. Wet mycelia were frozen at −20 °C, and then thawed overnight at 4 °C. After centrifugation, the solid content was mixed with distilled water and heated at 100 °C in a water bath for 40 min followed by centrifugation. The extraction was repeated once and the extract liquid collected was concentrated by evaporation under reduced pressure. With the method of EPS-1, EPS-2 and EPS-3 isolation, IPS-1, IPS-2 and IPS-3 were isolated.

The crude EPS and IPS were dissolved in distilled water, extracted by the Sevag method to remove the dissociative protein (Staub, 1965) and purified by Q-Sepharose FF and Sephacryl S-200 (Amersham, Pharmacia Biotech, Uppsala, Sweden). The active peaks were collected, concentrated and lyophilized for following assays.

2.3. Molecular weight estimation

The molecular weight (MW) of polysaccharides was determined by gel permeation chromatography (GPC) according to the method of Liu (2012). Agilent 1100 HPLC system equipped with a TSKgel GMPWXL GPC column (300 × 7.8 mm^2, Tosoh Corporation, Tokyo, Japan), a Wyatt Optilab rEX and Agilent ChemStation were used. Polysaccharide samples were dissolved in distilled water and filtered through membrane prior to sample injection. The column was operated at 40 °C and eluted by sodium sulfate at a flow rate of 0.5 mL/min with an injection volume of 20 μL. The molecular weight was derived from the calibration curve measured with blue dextrin, molar masses of which was 180, 2500, 4600, 7100, 21400, 41100 and 84400 Da (Sigma, USA).

2.4. Component analysis

The total sugar content of the polysaccharide fractions was determined by the phenol–sulfuric acid method, using glucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The protein content in the samples was measured according to the Kjeldahl nitrogen method (Lloret, Andrés, & Legua, 2005).

The monosaccharide constituents of EPS and IPS fractions were analyzed by gas chromatography (GC), following the method of Chen, Du, & Zeng (2003). GC was performed on Agilent 6890 GC instrument (USA), with flame ionization detection (FID) and a J&W Scientific column (DB-225, 0.25 mm ID, 30 m). Arabinose, xylose, mannose, galactose and glucose were chosen as standards.

2.5. Hydroxyl radical scavenging ability

Hydroxyl radical (•OH) scavenging activity was measured according to the method of Ma et al. (2008) with some modifications. The reaction mixture contained 1 mL of ferrous sulfate (9 mM), 1 mL of salicylic acid–ethanol (9 mM), and 1 mL of various concentrations of polysaccharide (0–12 mg/mL). The reaction was started by 1 mL of hydrogen peroxide (8 mM). After incubating at 37 °C for 0.5 h, the absorbance of samples (A_i) was measured at 510 nm, using Vitamin C (Vc) as a positive control. The hydroxyl radical scavenging activity was calculated as the following formula:

$$\text{Inhibition rate}(\%) = (A_0 - (A_i - A_{i0}))/A_0 \times 100$$

where A_0 was the absorbance of the control group, A_i was the absorbance of samples/Vc, and A_{i0} was the absorbance of polysaccharide.

2.6. Statistical analysis

All analyses were performed in triplicate and the data was analysed by SPSS (version 19, SPSS Inc). Difference in antioxidant activities was tested by analysis of variance (ANOVA). Student–Newman–Keuls test was used to determine significant differences. Correlations among data obtained were calculated using Pearson's correlation coefficient. Linear regression analysis was used to calculate the linear regression equation.

3. Results

3.1. Molecular weight determination

The percentage content and molecular weight of these polysaccharides were different (Table 1). The polysaccharide with larger molecular weight was obtained with lower ethanol concentration. Compared with EPS, the molecular weight distribution of IPS was narrow, ranging from 23.1 kDa to 10.4 kDa, while the molecular mass distribution of EPS changed from 43.0 kDa to 4.7 kDa. The main composition of EPS and IPS were EPS-3 and IPS-3, the contents of which were 70.02% and 49.27%, respectively.

3.2. Chemical compositions

Protein, neutral sugar, and monosaccharide composition of the polysaccharides were summarized in Table 2. The result showed that all the polysaccharide fractions contained neutral sugar and protein, which indicated the polysaccharides were protein-bound polysaccharides. For both EPS and IPS, fractions obtained at lower