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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

Table 1
Molecular weight and percentage content of each polysaccharide fractions.

EPS/IPS	EPS			IPS		
	EPS-1	EPS-2	EPS-3	IPS-1	IPS-2	IPS-3
Content of each composition (%)	1.19	28.79	70.02	15.73	35.00	49.27
MW (kDa)	43.0	19.5	4.7	23.1	21.5	10.4

Table 2
Components of monosaccharide and properties of EPS and IPS.

EPS/IPS	EPS-1	EPS-2	EPS-3	IPS-1	IPS-2	IPS-3
Neutral sugar (%)	70.71	75.08	92.64	52.71	77.23	85.55
Protein (%)	19.86	20.30	2.00	33.97	20.38	1.90
Sugar component (%)						
Man (%)	10.6	32.7	15.2	59.0	42.2	27.2
Gal (%)	5.4	10.9	ND[a]	32.6	38.0	ND[a]
Glc (%)	84.0	57.3	84.8	8.5	19.8	72.8
Man:Gal:Glc(mol%)	1.97:1:15.63	3.24:1:5.68	1:0:5.56	1.81:1:0.26	1.11:1:0.52	1:0:2.67

[a] Not detected.

ethanol concentration (EPS-1 and IPS-1) had lower neutral sugar content (70.71% and 52.71%, respectively). Quite the opposite, fractions obtained at higher ethanol concentration (EPS-3 and IPS-3) had higher neutral sugar content (92.64% and 85.55%, respectively).

EPS-1, EPS-2, IPS-1 and IPS-2 were consisted of mannose, glucose and galactose, while galactose was not detected in EPS-3 and IPS-3. In EPS, glucose was the major component (84.0% in EPS-1, 32.7% in EPS-2 and 15.2% in EPS-3), while the galactose was the least components (10.6% in EPS-1, 57.3% in EPS-2 and 84.8% in EPS-3). EPS-2 had the highest content of mannose (32.7%) and the lowest content of glucose (57.3%) than EPS-1 and EPS-3. The mannose contents evaluated in IPS-1, IPS-2 and IPS-3 decreased gradually from 59.0% to 27.2%, while the glucose increased from 8.5% to 72.8%. The main component of IPS-1 and IPS-2 was mannose (59.0% and 42.2%, respectively), with the lowest glucose content (8.5% and 19.8%). Compared with IPS, the polysaccharide fractions EPS had higher glucose content than that of IPS, but lower mannose content than that of IPS.

3.3. Hydroxyl radical scavenging ability

The polysaccharide fractions were discovered to have the ability of scavenging hydroxyl radical (Fig. 1). The scavenging effects of EPS and IPS varied significantly with different concentrations of polysaccharides ($p < 0.01$). Higher scavenging activities were found when the content of polysaccharide increased, presenting dosage dependence relations. The difference between the scavenging activities of different polysaccharide fragments was also significant ($p < 0.01$). EPS-2 had higher hydroxyl radicals scavenging rate than EPS-1 and EPS-3. And among the tested three IPS, the scavenging activity of IPS-1 was the best, followed by IPS-2. Compared with EPS, IPS had better activity.

The IC_{50} values (95% of confidence limits) of the samples were calculated by probit analyses and listed in Table 3. With regard to IC_{50} values in antioxidant activity, the scavenging activities of IPS were obviously stronger than EPS. The results were in agreement with the above analysis.

4. Discussion

4.1. Gradient ethanol precipitation for isolation and fractionation of EPS and IPS

Ethanol precipitation was an effective method for fractionation and purification of polysaccharide in aqueous solutions. Polysaccharides from broth and mycelia of *Hirsutella* sp. were precipitated by gradient ethanol precipitation. The polysaccharides, although isolated from the same strain, were distinct from each other in the aspects of molecular weight, monosaccharide composition, neutral sugar and protein content. Other studies also found that the increase in ethanol concentration in certain range could lead to a decrease in molecular weight of the polysaccharide (Bai, 2009; Bian, Peng, Peng, Xu, & Sun, 2010; Zha et al., 2009). Ethanol could disrupt the hydration layer of polysaccharide and facilitate the interactions between the polymer molecules to form aggregates. The size of aggregates grows with increasing ethanol concentration until precipitation (Huang et al., 2013). Therefore, the biopolymers with a higher molecular weight tend to precipitate earlier at a lower ethanol concentration.

In addition to molecular weight, protein content and monosaccharide composition were also influenced by ethanol concentration

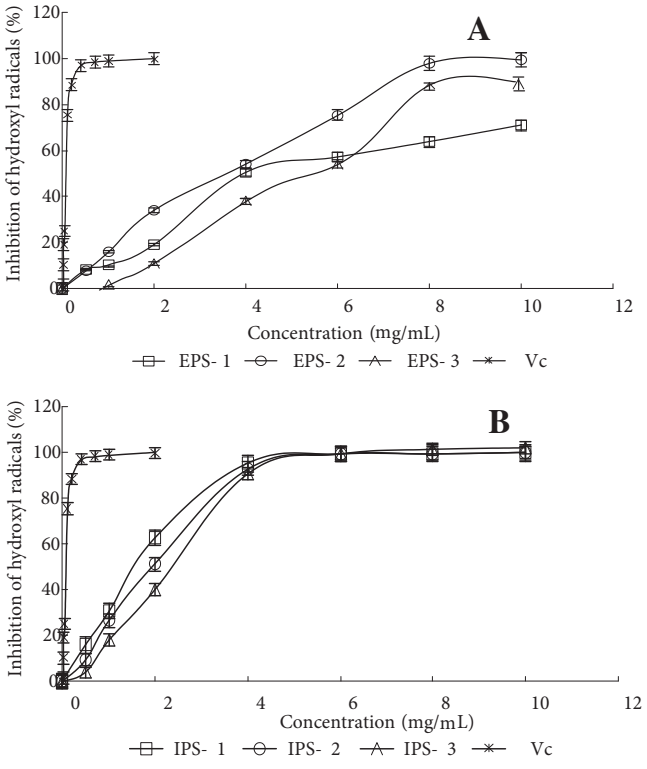

Fig. 1. Scavenging effects of EPS (A) and IPS (B) on hydroxyl radicals.

Table 3
The IC_{50} values of EPS and IPS on hydroxyl radicals.

IC_{50} value[b] (mg/mL)	EPS-1	EPS-2	EPS-3	IPS-1	IPS-2	IPS-3	Vc
Scavenging ability on •OH radicals	4.84	2.69	4.27	1.32	1.58	1.91	0.064

[b] IC_{50} value: the concentration at which the antioxidant activity was 50%.

(Yu et al., 2007). Polysaccharides precipitated by lower ethanol concentration had higher protein content. EPS-1, EPS-2, IPS-1 and IPS-2 were consisted of mannose, glucose and galactose. There was no galactose detected in EPS-3 and IPS-3. The main component of EPS was glucose, while mannose was the major constituents of IPS.

4.2. Correlation of antioxidant activities with molecular properties

The biological activities of polysaccharides were heavily depend on numerous factors including chemical components, molecular mass, structure, even the extraction and isolation methods (Chen, Zhang, Qu, & Xie, 2008). The polysaccharides from *Hirsutella* sp. had different hydroxyl radical scavenging activitiy, which was affected by MW and chemical composition.

The scatter diagram for OH $1/IC_{50}$ values and molecular properties were shown in Fig. 2. Polysaccharides with molecular weight about 10–20 kDa had better activities (Fig. 2-A), suggesting that the molecular weight of polysaccharides could play an important role in the antioxidant activity (Asker, Ahmed, & Ramadan, 2009). It is reported polysaccharide fractions with relatively lower molecular weight were reported to have better antioxidant activities (Chen et al., 2008; Ge et al., 2013), which was different with the present study. And some other polysaccharide fractions with best bioactivities have smaller molecular weight, less than 10 kDa (Zhou et al., 2004), indicating that IPS-3 and IPS-3 might have excellent activities in other aspects, such as antitumor and

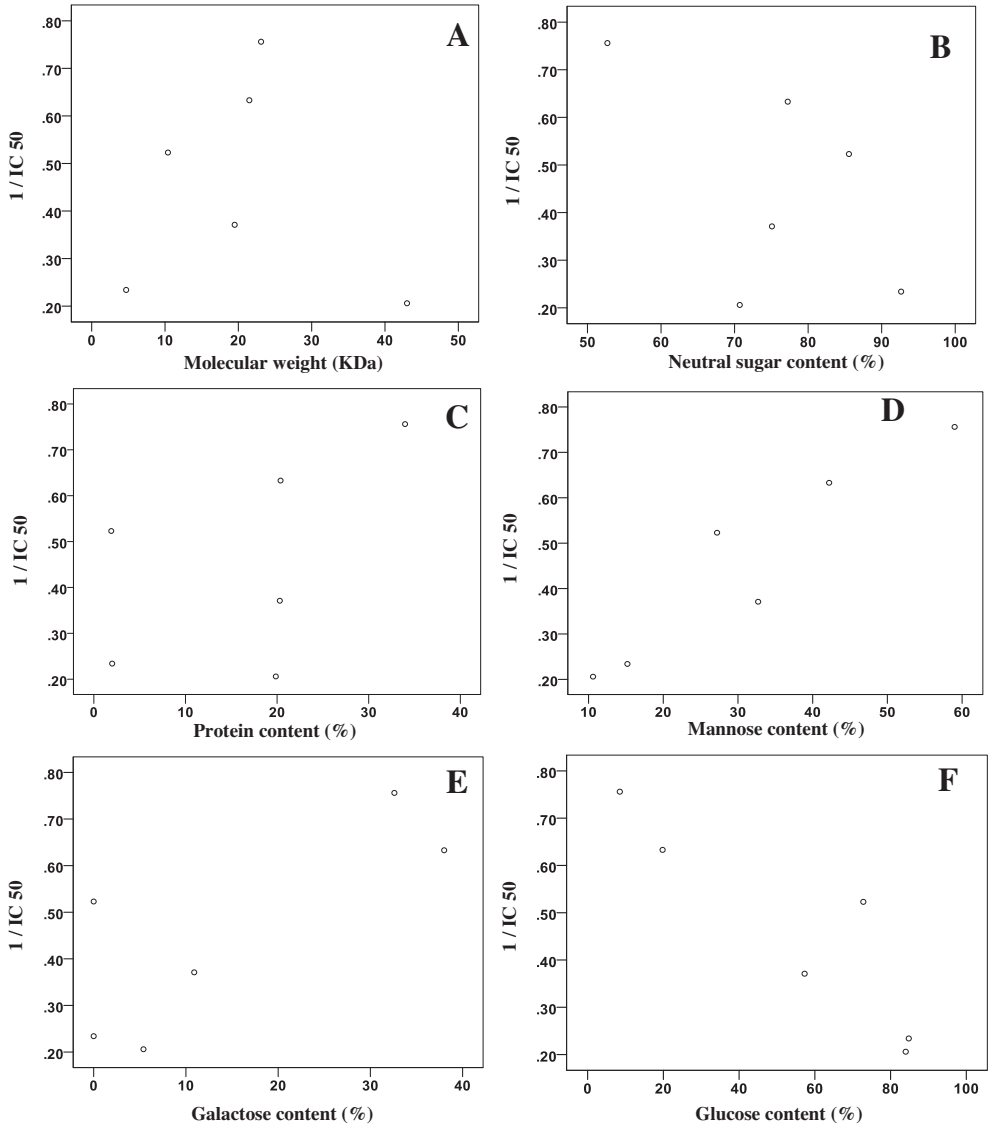

Fig. 2. Scatter diagram for OH $1/IC_{50}$ values and (A) molecular weight, (B) neutral sugar content, (C) protein content, (D) mannose content, (E) galactose content and (F) glucose content in EPS and IPS.

Table 4
Correlation analysis of antioxidant activity and monosaccharide composition.

	Mannose	Galactose	Glucose
The regression equation	$Y = 0.090 + 0.012X$	$Y = 0.304 + 0.010X$	$Y = 0.785 - 0.006X$
Pearson correlation	0.942**	0.780	−0.905*
Significant (2-tailed)	0.005	0.067	0.013

* Correlation is significant at $p < 0.05$.
** Correlation is significant at $p < 0.01$.

immunomodulation. Polysaccharides with lower neutral sugar content and higher protein content had better hydroxyl radical scavenging activity (Fig. 2-B, Fig. 2-C). The antioxidant functions of protein might be attributed to their constituent amino acids, which were capable of donating protons electron-deficient radicals (Elias, Kellerby, & Decker, 2008; Pérez, Iglesias, Pueyo, González, & de Lorenzo, 2007). A strong correlation was found between hydroxyl radical scavenging activity and monosaccharide composition (Fig. 2-D, Fig. 2-E, and Fig. 2-F).

Pearson correlation analysis test and linear regression analysis were used to analyze the relationship between the antioxidant activity and monosaccharide composition of polysaccharides (Table 4). The antioxidant activity showed significant correlation with mannose content ($p < 0.01$) and glucose content ($p < 0.05$). Galactose content was not correlated with antioxidant activity ($p > 0.05$). High correlation coefficients were found between antioxidant activities and monosaccharide composition (mannose content, $r = 0.942$; glucose content, $r = -0.905$), indicating the influence of mannose and glucose on the radical scavenging activity was significant. The content of mannose had positive influence on the activity, while the influence of glucose was negative. The result was similar to Xiang's report that polysaccharide with higher mannose content exhibited better radical scavenging activities (Huang et al., 2013; Xiang, Li, & Xu, 2012).

5. Conclusion

Gradient ethanol precipitation was proved to be a simple and feasible method for isolation and fractionation of polysaccharide. The polysaccharides with larger molecular weight, lower neutral sugar and higher protein content were precipitated first, followed by those with lower molecular weight, higher neutral sugar, and lower protein content. Polysaccharide fractions with a relatively high protein content, low neutral sugar content and molecular weight about 10–20 kDa exhibited better bioactivity. High correlation was found between the hydroxyl radical scavenging activity and contents of mannose and glucose, suggesting monosaccharide composition had significant influence on the activity of polysaccharide.

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