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Characterization of three extracellular polysaccharides from *Shiraia* sp. Super-H168 under submerged fermentation

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ABSTRACT

Three extracellular polysaccharides (SP-1, SP-2 and SP-3) from *Shiraia* sp. Super-H168 under submerged fermentation were purified by using ethanol precipitation, chromatography separation on the DEAE-cellulose-32 column and the Sephadex G100 column. The molecular weights of SP-1, SP-2 and SP-3 were determined to be approximately 189 kDa, 130 kDa and 52 kDa, respectively. Monosaccharide analysis revealed that SP-1 was composed of mannose and glucose in molar ratios of 1:28.47, and SP-2 and SP-3 were both composed of mannose, glucose and galactose in molar ratios of 63.32:70.06:1 and 2.31:14.69:1, respectively. Three polysaccharides had very weak scavenging activity of superoxide radical (<3%), the hydroxyl radical scavenging activities of SP-1, SP-2 and SP-3 being 86.5%, 19.8% and 60.5%, respectively. SP-2 showed the strongest reducing power, and the reducing power of SP-3 was the weakest.

1. Introduction

Fungi, include mushroom, filamentous fungi and yeasts, are cell factories that have been used to produce solvents, antibiotics, enzymes, vitamins, amino acids, polymers as well as polysaccharides (Adrio & Demain, 2003). Researches had been undertaken on polysaccharides from medical mushroom. For example, some works have been done on media composition and culture condition of *Ganoderma lucidum* for polysaccharides production (Simonic et al., 2008; Zhong, Tang, & Fang, 2003). There have been also many works involving analysis of the structure of polysaccharide from several edible mushrooms (Ghosh et al., 2008; Mondal, Chakraborty, Pramanik, Rout, & Islam, 2004; Pramanik & Islam, 1999, 2000; Pramanik, Mondal, Chakraborty, Rout, & Islam, 2005; Roy, Maiti, Mondal, Das, & Islam, 2008; Smiderle et al., 2006; Vinogradov & Wasser, 2005; Yang, Ye, Zhang, Liu, & Tang, 2009). Mushroom polysaccharides had been found to be pharmaceutically important metabolites with antitumor and immunostimulating properties. It has been revealed by many researchers that mushroom polysaccharides had oxygen free radical scavenging activities (Li et al., 2007; Lung & Tsai, 2009; Wu & Hansen, 2008), which is influential for cellular components, such as lipids, proteins, nucleic acids and carbohydrates since these components may be damaged by reactions with oxygen free radicals, giving rise to metabolic and cellular disturbances to cause tumor promotion.

Shiraia bambusicola is a kind of bamboo parasitic mushroom. Its main component, Hypocrellin, had been traditionally used as medicinal agents for skin diseases, rheumatoid arthritis, gastric diseases in China for centuries (Chen et al., 1981). In our previous work, we had isolated a high-yielded hypocrellins producing stain, *Shiraia* sp. SUPER-H168 (Liang et al., 2009). It was found that this stain could also produce polysaccharides under submerged fermentation condition. The goal of this study is to isolate and purify extracellular polysaccharides, to analyze their chemical characteristics and to investigate their antioxidant properties.

2. Materials and methods

2.1. Strain and fermentation

Shiraia sp. SUPER-H168 was a stock culture of the Laboratory of Biochemistry, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu province, China.

The medium for fermentation contained the following components (per liter): 20 g glucose, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, 3 g beef extract, 10 g peptone, 0.2% triton X-100 and potato extract 200 g. Initial pH of the fermentation medium was adjusted to 6.5 with 1 N NaOH after autoclaving at 121 °C for 20 min. Fermentation were carried out at 30 °C on a reciprocal shaker at 200 rev/min for 72 h.

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2.2. Isolation and purification

The culture broth was centrifuged to remove the mycelia at 8000 rev/min for 5 min. The cell-free fermentation broth was concentrated 3-fold under a vacuum and precipitated with 3 volumes of 95% ethanol. The obtained precipitate was dissolved in distilled water, and Sevag method was used to deproteinize crude polysaccharide (Staub, 1965). After the Sevag reagent was removed, the aqueous fraction was dialyzed and precipitated again by adding 3-fold volume of 95% ethanol. The precipitate was washed successively with anhydrous ethanol, acetone and ethyl acetate, and then was dissolved in distilled water.

The crude polysaccharides solution was applied to DEAE-cellulose-32 (2.6 cm × 40 cm) column and washed with distilled water, 0.05 M, 0.1 M, 0.2 M, and 1.0 M NaCl sequentially at a flow rate of 60 ml/h for 120 min. The polysaccharides fractions were collected and loaded onto the Sephadex G100 column (1.6 cm × 60 cm), respectively. The column was then washed with distilled water. The polysaccharides fractions were pooled, dialyzed, concentrated by ultrafiltration and stored at −20 °C.

Total carbohydrate in the fractions of column chromatography was determined by the phenol–sulfuric acid assay (Dubois et al., 1956).

2.3. Determination of molecular weight

Purified polysaccharides were analyzed with Shodex OHpak KB-804 column (0.8 cm × 30 cm) (Showa Denko K.K., Tokyo, Japan) using distilled water as a mobile phase (column temperature, 50 °C; flow rate, 0.8 ml/min; injection volume, 20 μl). Dextrans of different molecular weight, T-500 (450 kDa), T-250 (250 kDa), T-150 (150 kDa), T-110 (110 kDa), T-70 (68.5 kDa), T-40 (44 kDa), T-20 (20 kDa) and T-10 (10 kDa) were used as standard. GPC software (Zhejiang University, China) was used to get the molecular weight data.

2.4. Infrared spectroscopy analysis

2 mg polysaccharide and 200 mg KBr were mixed and then pressed into pellets. Infrared spectroscopy was record with spectrometer (Thermo Nicolet NEXUS 670 FTIR) in the wavelength range 4000–400 cm^{-1} at a resolution of 4 cm^{-1}.

2.5. Analysis of monosaccharide compositions

Monosaccharide compositions of polysaccharides were determined by gas chromatography (GC) method (Lobas et al., 1994). 20 mg polysaccharide was hydrolyzed with 2 ml of 2 M trifluoroacetic acid at 120 °C for 6 h. Then, the hydrolyzed products were acetylated with 10 mg hydroxylamine hydrochloride and 0.5 ml pyridine by heating for 30 min at 90 °C. Reaction liquid was mixed with 0.5 ml acetic anhydride and heated for 30 min at 90 °C to get the final acetylated monosaccharide solution. The GC operation condition was: N_2, 1.0 ml/min; injection temperature: 240 °C; detector temperature: 240 °C; column temperature programmed, 160 °C for 2 min, then increased to 240 °C at 5 °C/min and finally holding for 5 min at 240 °C.

2.6. Superoxide radical scavenging activity of polysaccharides

The scavenging ability of polysaccharides was determined by pyrogallol method with some modifications (Qingming et al., 2010). Briefly, 2.99 ml Tris–HCl buffer (0.05 mol, pH 8.2), 0.01 ml HCl (10 mM) and 0.4 ml pyrogallol (3.0 mmol) were mixed and incubated at 25 °C. The auto oxidation rate of pyrogallol (ΔA_0) was calculated according to the absorbance changing of 4 min at 325 nm. 2.98 ml Tris–HCl buffer (0.05 mol, pH 8.2), 0.01 ml HCl (10 mM), 0.4 ml pyrogallol (3.0 mmol) and 0.01 ml polysaccharides solution were mixed to get the auto oxidation rate of pyrogallol (ΔA) at the same condition. Superoxide radical scavenging activity was defined as $(\Delta A_0 - \Delta A)/\Delta A_0 \times 100\%$.

2.7. Hydroxyl radical scavenging activity of polysaccharides

0.5 ml phosphate buffer (0.4 M), 0.5 ml phenanthroline (2.5 mM), 0.5 ml $FeSO_4$ (2.5 mM), 0.5 ml polysaccharide solution, and 0.25 ml H_2O_2 (20 mM) were mixed quickly and incubated at 40 °C for 30 min. Then absorbance of reaction mixture was detected at 520 nm. The ability to scavenge hydroxyl radicals was defined by $(A_p - A_1)/(A_2 - A_1) \times 100\%$, where A_1 was the absorbance of the control, A_p was the absorbance of the polysaccharide and A_2 was the absorbance of the solution without polysaccharide and H_2O_2.

2.8. Reducing power of polysaccharides

Reducing powers of polysaccharides were determined by potassium ferricyanide reduction method (Dorman & Hiltunen, 2004). In the experiment, 0.25 ml polysaccharides solution, 0.25 ml phosphate buffer (0.2 M, pH 6.6) and 0.25 ml potassium ferricyanide (1%, w/v) were mixed and incubated at 50 °C for 20 min. After being cooled to room temperature, 0.25 ml trichloroacetic acid (10%, w/v) were added to the reaction mixture, which was then centrifugated for 10 min with 3000 rpm at 4 °C. 0.5 ml supernate, 0.5 ml pure water and 0.1 ml $FeCl_3$ (0.1%, w/v) were mixed and reacted for 10 min. Then absorbance of reaction mixture, indicating the reduction power of polysaccharides, was detected at 700 nm against water as blank. Reductive power = $A_1 - A_2$, where A_1 was the absorbance of the sample and A_2 was the absorbance of the sample under identical conditions with water instead of $FeCl_3$ solution.

3. Results and discussion

3.1. Isolation and purification of the polysaccharides

The total extracellular polysaccharide production was about 1.35 g/l in the fermentation broth. The separation yield before column was about 85%. Fig. 1 shows the column separation results. Fig. 1a visualizes the result of DEAE-cellulose-32. Three polysaccharide fractions (DP-1, DP-2 and DP-3) were collected, and subsequently were load into Sephadex G100 and the results were shown in Fig. 1b–d, respectively. The contents of DP-1, DP-2 and DP-3 were 21.58%, 48.72% and 29.70%, respectively. It showed only one symmetrical peak for DP-3 from Sephadex G100 chromatography. Three purified polysaccharides (SP-1, SP-2 and SP-3) were collected and used to analyze their properties. The yield of SP-1, SP-2, and SP-3 was 81.0%, 77.9% and 93.6%, respectively.

3.2. Chemical characteristics

As calculated, the weight-average molecular weight of SP-1, SP-2 and SP-3 were approximately 189 kDa, 130 kDa and 52 kDa, respectively, according to the calibration curve with standard dextrans.

Infrared spectra of SP-1, SP-2, and SP-3, shown in Fig. 2a–c, respectively, were found to be very similar. The signals at 3600–3200 cm^{-1} (3387.13 of SP-1, 3405.16 of SP-2 and 3384.35 of SP-3) were generated from the stretch vibration of O–H, and the signals at 3000–2800 cm^{-1} (2921.94 of SP-1, 2925.32 of SP-2 and 2919.54 of SP-3) were from the stretch vibration of C–H. The signals at 1642.73 cm^{-1} (SP-1), 1639.71 cm^{-1} (SP-2) and

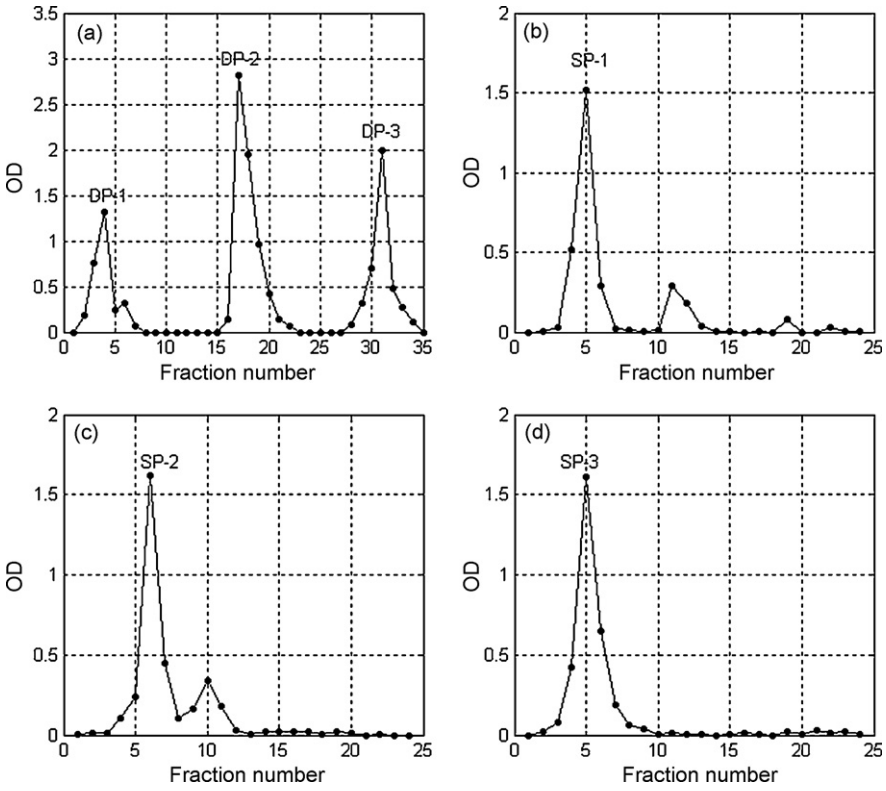

Fig. 1. Separation chromatography of polysaccharides. (a) Elution curve of the crude polysaccharides by a DEAE-cellulose-32 column. Three fractions (DP-1, DP-2, and DP-3) were collected from this column. (b) Elution curve of the DP-1 fraction by a Sephadex G100. The purified SP-1 was collected. (c) Elution curve of the DP-2 fraction by a Sephadex G100. The purified SP-2 was collected. (d) Elution curve of the DP-3 fraction by a Sephadex G100. The purified SP-3 was collected.

1638.41 cm^{-1} (SP-3) were attributed to the bound water. The signals at 1200–1000 cm^{-1} (1075.15 of SP-1, 1070.82 of SP-2 and 1075.30 of SP-3) were the results of the ring vibrations overlapped with stretching vibrations of (C–OH) side groups. The signals at 893.00 cm^{-1} (SP-1), 903.56 cm^{-1} and 813.64 cm^{-1} (SP-2) and 900.32 cm^{-1} (SP-3) were typical of a-dominating configuration in pyranose form.

The monosaccharide compositions of the polysaccharides are listed in Table 1, which shows that SP-1 was only composed of mannose and glucose, whereas SP-2 and SP-3 were both composed of mannose, glucose and galactose. However, galactose of SP-2 was present in very small amounts (about 0.74%, w/w). The amounts of mannose and glucose in SP-2 were approximately equal. In SP-3, glucose was the major composition, and its content was about 81.6% (w/w). DNP2-1, a polysaccharide from *Dendrobium nobile* Lindl, had the same monosaccharide compositions as SP-2 and SP-3 and similar infrared spectra (Luo et al., 2010).

Table 1
Monosaccharide compositions of SP-1, SP-2 and SP-3.

Polysaccharides	Monosaccharide	Molar ratio
SP-1	Mannose	1
	Glucose	28.47
SP-2	Mannose	63.32
	Glucose	70.06
	Galactose	1
SP-3	Mannose	2.31
	Glucose	14.69
	Galactose	1

Polysaccharides were hydrolyzed first. Subsequently, the monosaccharide composition was analyzed by gas chromatography using a fused silica capillary column (Na form, 300 mm × 0.25 mm, Supelco Inc., Bellefonte, PA).

3.3. Antioxidant properties

The results showed that three polysaccharides had very weak superoxide radical scavenging activities. No significant increase of the scavenging activity was observed at the polysaccharides concentration range of 3–5 mg/ml. The abilities of SP-1, SP-2 and SP-3 to scavenge superoxide radicals were only 2.9%, 1.5% and 2.7%, respectively, at 5 mg/ml.

Fig. 3 shows that there were significant differences among the hydroxyl radical scavenging activities of three polysaccharides. At the concentration of 6 mg/ml, the hydroxyl radical scavenging activates of SP-1, SP-2 and SP-3 were 86.5%, 19.8% and 60.5%, respectively. It is obvious that SP-1 has the highest scavenging activity.

The reducing power of polysaccharides is shown in Fig. 4. When the concentrations of polysaccharides varied from 1 mg/ml to 5 mg/ml, the absorbance of SP-1 varied from 0.234 to 0.322, the absorbance of SP-2 varied from 0.270 to 0.318, and the absorbance of SP-3 varied from 0.165 to 0.295. It was shown that SP-2 has the strongest reducing power, and SP-3 the weakest one. It was found that the reducing power of components might be related to their hydrogen-donating ability (Duh, Du, & Yen, 1999). The reducing power of these polysaccharides suggested that these polysaccharides could terminate radical chain reactions by reacting with free radicals to convert them to more stable products.

Superoxide radical and hydroxyl radical are the two most representative oxygen species which can be continually produced in tissues by the action of the mitochondrial electron transport system and of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in leukocyte and macrophages (Martinezcayuela, 1995). Organisms can develop several antioxidant defense systems, such as catalase for hydrogen peroxide, superoxide dismutase for superoxide, and glutathione peroxidases for hydrogen peroxide

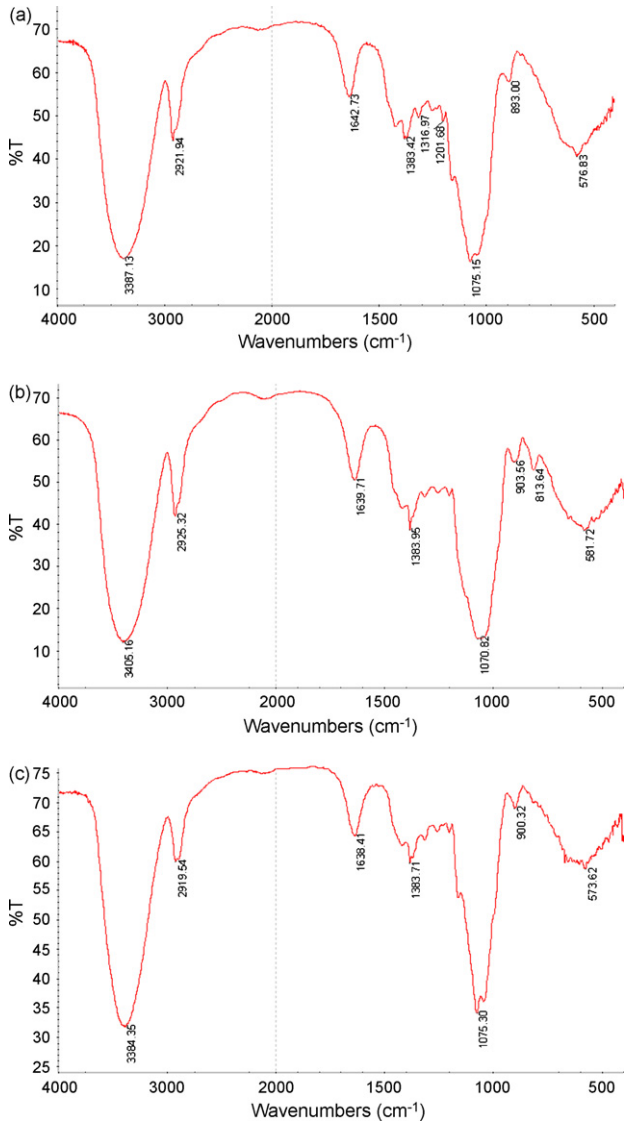

Fig. 2. Infrared spectra of the three purified polysaccharides. (a) Infrared spectra of SP-1. (b) Infrared spectra of SP-2. (c) Infrared spectra of SP-1.

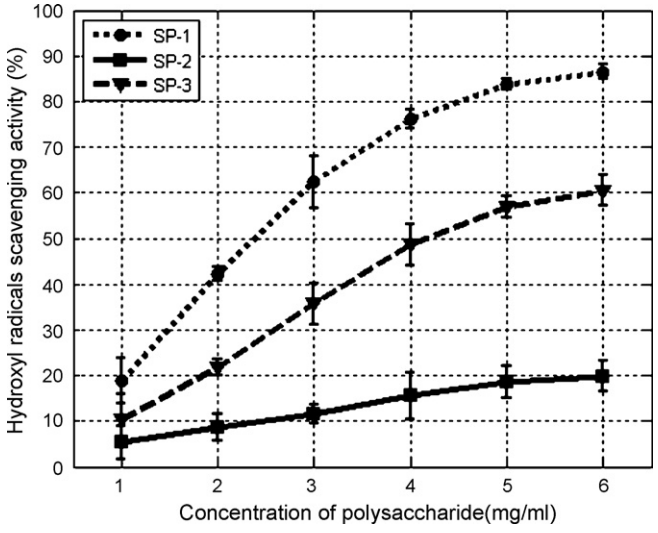

Fig. 3. The hydroxyl radical scavenging activities of the three polysaccharides. Each value is the mean ± SD of triplicate experiments.

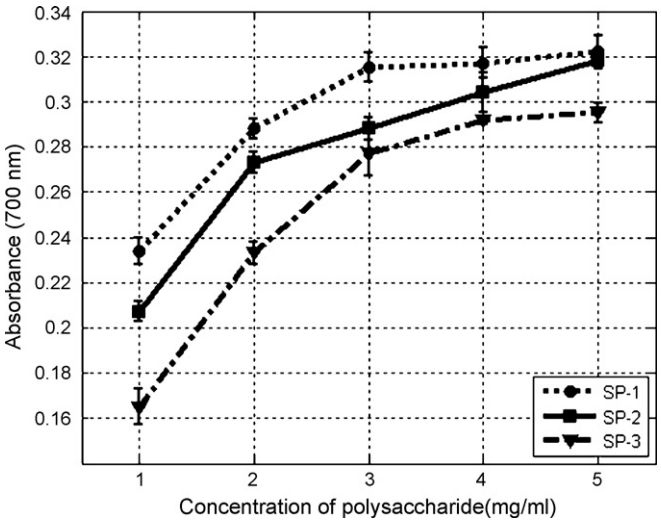

Fig. 4. The reducing power of the three polysaccharides. Each value is the mean ± SD of triplicate experiments.

and lipid peroxide in order to protect themselves from the damage of active oxygen species (Belozerskaya & Gessler, 2007; Cnubben, Rietjens, Wortelboer, van Zanden, & van Bladeren, 2001; Jaleel et al., 2009; Jiang & Zhang, 2004; Levine, Moskovitz, & Stadtman, 2000; Lyakhovich, Vavilin, Zenkov, & Menshchikova, 2006; Mahadik & Mukherjee, 1996; Moskovitz, 2005a,b; Trueba, Sanchez, & Giuliani, 2004). However, there is no specific defense mechanism against hydroxyl radicals. Hydroxyl radicals can react with any biological molecule, and it is the most damaging radicals within the body (Kuhn, 2003). The results showed that three polysaccharides had strong hydroxyl radical scavenging activities, but very weak superoxide radical scavenging activities. In particular, SP-2 had the highest activities, which might be related to its metabolite composition. In our previous work, we had reported that *Shiraia* sp. Super-H168 was a high yield hypocrellins production strain. Hypocrellins can generate singlet oxygen, superoxide when irradiated by light. These oxygen species in turn cause peroxidation of cell membrane lipids leading to changes in membrane fluidity and structure and death of the strain. It was found that hydroxyl radical could be derived from superoxide radical anion, and hydroxyl radical was the main product during irradiation of hypocrellins (Ou, Chen, Wang, Zhang, & Cao, 2002; Zou, An, Li, & Jiang, 1996). The resistance of strain to hypocrellins might be correlated with their ability to produce polysaccharides with strong hydroxyl radical scavenging activities.

4. Conclusion

Three polysaccharides (SP-1, SP-2 and SP-3) from submerged fermentation broth of *Shiraia* sp. Super-H168 were purified and characterized. They exhibited weak scavenging activity for superoxide radical, but significant scavenging activity for hydroxyl radical. It was also shown that the hydroxyl radical scavenging activity of SP-2 was the strongest among the three polysaccharides. As polysaccharides from mushroom often possess potent antitumor and immunomodulating properties (Wasser, 2002), further investigation to antitumor activity of these polysaccharides will be undertaken in our future work.

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