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Full Length Research Paper

In vitro antioxidant activities of polysaccharides from endophytic fungus *Fusarium oxysporum* Dzf17

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Three polysaccharides, namely exopolysaccharide (EPS), water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharide (SPS), from the endophytic fungus *Fusarium oxysporum* Dzf17 were investigated for their *in vitro* antioxidant activities. Among them, SPS was the most active antioxidant component, and WPS exhibited moderate antioxidant activity. The median effective concentration (EC_{50}) values of the polysaccharides were 162.38 µg/ml (for WPS), 63.37 µg/ml (for SPS) by DPPH radical scavenging activity assay, and 54.54 µg/ml (for WPS) and 44.91 µg/ml (for SPS) by using ferrous ions chelating activity assay. The polysaccharides from *F. oxysporum* Dzf17 could be an alternative source as the antioxidant components.

Key words: Antioxidant activity, polysaccharides, endophytic fungus, *Fusarium oxysporum* Dzf17.

INTRODUCTION

Fungi have been regarded as important resources of natural bioactive compounds with a variety of bioactivities, and have been widely applied in agriculture, medicine and food industry (Greve et al., 2010; Zhong and Xiao, 2009; Zhou et al, 2010). Plant endophytic fungi are a special group of fungi that reside within plant tissues intercellularly or intracellularly without causing any apparent symptoms of disease (Wilson, 1995). In the past two decades, many valuable bioactive compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities have been successfully obtained from the endophytic fungi (Kharwar et al., 2011; Verma et al., 2009; Zhao et al., 2011). These bioactive compounds could be mainly classified as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols and polysaccharides (Aly et al., 2010; Yu et al., 2010; Zhang et al., 2006). To the best of our knowledge, the antioxidant activities of the polysaccharides from endophytic fungi have been rarely reported, though there were some reports from other fungi (Liu et al., 1997; Ooi and Liu, 1999).

Fusarium oxysporum Dzf17 is an endophytic fungus isolated from the rhizomes of *Dioscorea zingiberensis*, a well known traditional Chinese medicinal herb indigenous to the south of China (Li and Ni, 2011; Zhang et al., 2009). Three polysaccharides, namely exopolysaccharide (EPS), water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharide (SPS), prepared from *F. oxysporum* Dzf17 were observed in our previous study to have enhancement effects on cell growth and diosgenin accumulation in *D. zingiberensis* cell cultures (Li et al., 2011). The purpose of this study was to investigate the antioxidant activities of three kinds of polysaccharides from the endophytic fungus *F. oxysporum* Dzf17 in order to provide fundamental data for the research and application of the polysaccharides from this fungus.

MATERIALS AND METHODS

General

The microplate spectrophotometer (PowerWave HT, BioTek Instruments, USA) was employed to measure the light absorption value. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (USA) in Beijing. 3-(2-Pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) was obtained from Johnson Matthey (UK) in Beijing. Butylated hydroxytoluene (BHT), ferrous chloride ($FeCl_2$), and ethylene diamine tetraacetic acid (EDTA) were

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bought from Beijing Chemical Company. All other chemicals and reagents were of analytical grade.

Endophytic fungus and culture conditions

The endophytic fungus *F. oxysporum* Dzf17 (GenBank accession number EU543260) was isolated from the healthy rhizomes of the medicinal plant *D. zingiberensis* C. H. Wright (Dioscoreaceae) in our previous study (Zhang et al., 2009). The living culture has been deposited in China General Microbiological Culture Collection Center (CGMCC) under the number CGMCC 2472. It was also maintained on potato dextrose agar (PDA) slants at 25℃, and in 40% glycerol at -70℃ at the Herbarium of the College of Agronomy and Biotechnology, China Agricultural University. The mycelia were grown in a 1000 ml Erlenmeryer flask containing 300 ml of liquid medium consisting of glucose (50 g/L), peptone (13 g/L), NaCl (0.6 g/L), K_2HPO_4 (0.6 g/L), and $MgSO_4•7H_2O$ (0.2 g/L). About 500 flasks were used. All flasks were maintained at 25°C on a rotary shaker at 150 rpm for 14 days. A total of 150 L of fermentation broth was harvested. The mycelia were separated from the supernatant by centrifugation at 7,741 ×g for 20 min. Mycelia were washed twice with deionized water, then lyophilized. About 600 g of mycelia in dry weight (dw) was obtained.

Preparation of polysaccharides

The preparation process of exopolysaccharide (EPS) has described in our previous study (Li et al., 2011). Briefly, the supernatant was concentrated under vacuum at 60℃ by a rotary evaporator to a proper volume and mixed with three volumes of 95% ethanol, then kept at 4ºC for 48 h. After that, the solution was centrifuged at 17,418 × g for 15 min, and the precipitate from ethanol dispersion was collected as crude EPS which was further subjected to deproteination with Sevag reagent (chloroform-*n*-butanol at 4:1, v/v), decolorization with H_2O_2, and re-movement of small molecular impurities by dialysis. Polysaccharide mixture with molecular weight greater than 8,000 to 14,000 Da was kept in dialysis tube. The carbohydrate content of EPS was measured by the method of anthrone-sulfuric acid spectrophotography (Wang et al., 2007), which involved sulfuric acid hydrolysis of the sample in the presence of anthrone agent at 100℃. The absorbance at 620 nm was measured and calibrated to carbohydrate content using glucose as a reference. After lyophilization, the purified EPS (31.98 g) was stored in a desiccator at room temperature.

Water-extracted mycelial polysaccharide (WPS) and sodium hydroxide-extracted mycelial polysaccharide (SPS) were also prepared according to our previous research (Li et al., 2011). Briefly, the lyophilized mycelia (600 g) were powdered in a high disintegrator, and then subjected to heat circumfluence extraction at 50℃ by 95% ethanol-petroleum ether at 1:1 (v/v) as the refluxing solvent to remove monosaccharide, disaccharide and lipid. The ratio of mycelia powder (g) to refluxing solvent (ml) was 1:5 (w/v). Defatted mycelial powder was obtained by centrifugation (7,741 × g, 20 min) and drying in an oven at 40℃ for 2 h, and then immersed in hot water at 90℃ for 2 h with the ratio of water (ml) to the material (g) as 30:1 (v/w). After that, centrifugation was carried out at 7,741 × g for 20 min to separate the residue and the supernatant. The supernatant was condensed to a certain volume under vacuum at 60℃, and then mixed with three volumes of 95% ethanol, then kept at 4℃ for 48 h. The following procedure for polysaccharide preparation and purification was the same as the treatments of exopolysaccharide (EPS). The gained polysaccharide (33.24 g) was named as water-extracted mycelial polysaccharide (WPS). The residue not containing WPS was further extracted with 10% sodium hydroxide (NaOH) solution at room temperature for 24 h. The remaining steps were the same as the treatments of EPS. The obtained polysaccharide (35.89 g) was designated as sodium hydroxide-extracted mycelial polysaccharide (SPS).

***In vitro* antioxidant activity assay**

The polysaccharides were subjected to a screening for antioxidant activity by two complementary tests, namely the DPPH radical scavenging assay and ferrous ions chelating assay, The free radical scavenging activity of different antioxidants was measured in terms of hydrogen donating or radical scavenging ability of the stable free radical DPPH (1,1-diphenyl-2-picryhydrazyl) (Ono et al., 2008). The scavenging activity of DPPH was measured according to the method reported by Qiao et al. (2009) with some modifications. Briefly, DPPH dehydrated alcohol solution (0.2 mg/ml, 100 µl) and polysaccharide water solution (100 µl) were added to each well of the microplate and mixed. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance was measured at 517 nm against a blank. Butylated hydroxy toluene (BHT) was used as the positive control. Lower absorbance of the reaction mixture indicates higher free-radical scavenging activity. All the tests were performed in triplicate and the graph was plotted with the mean values and standard deviations. The scavenging activity was calculated by the following equation:

$$\text{Scavenging activity (\%)} = [A_0-(A_1-A_2)]\times 100/A_0.$$

Where A_0 is the absorbance of DPPH solution without tested samples, A_1 is the absorbance of the sample, and A_2 is the absorbance of the sample under identical conditions as A_1 with water instead of DPPH solution.

The median effective concentration (EC_{50}) value was calculated using the linear relation between the effective probability and concentration logarithm according to the method of Sakuma (1998).

Metal ions chelating activity was determined according to the method of Wang et al. (2010) with some modifications. Briefly, polysaccharide solution (50 µl) was mixed with $FeCl_2$ solution (0.2 mg/ml, 30 µl), and shaken vigorously. The ferrozine solution (2 mg/ml, 70 µl) was then added to the reaction solution. The reaction mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture reached equilibrium, the absorbance of the solution was then measured at wavelength 560 nm using a microplate spectrophotometer. EDTA was used as the positive control. Lower absorbance of the reaction mixture indicates higher chelating activity. All the tests were performed in triplicate and the graph was plotted with the mean values and standard deviations. The ferrous ions chelating effect was calculated as the percentage (%) of inhibition of ferrozine-Fe^{2+} complex formation determined as:

$$\text{Chelating activity (\%)} = [B_0-(B_1-B_2)]\times 100/B_0.$$

Where B_0 is the absorbance of reaction solution without tested samples, B_1 is the absorbance of the sample and B_2 is the absorbance of the sample under identical conditions as B_1 with water instead of ferrozine solution.

The EC_{50} value calculation for ferrous ions chelating activity was the same as that for DPPH radical scavenging activity.

RESULTS AND DISCUSSION

DPPH radical scavenging activity

DPPH has been widely adopted as a reference for evaluating the free radical scavenging activities of the

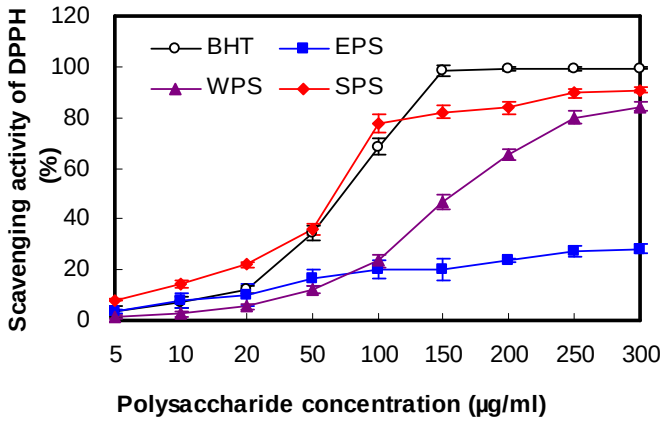

Figure 1. DPPH radical scavenging activity of the polysaccharides.

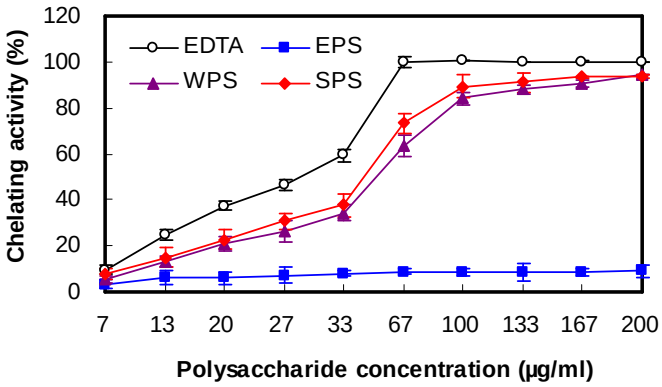

Figure 2. Ferrous ions chelating activity of the polysaccharides.

Table 1. EC_{50} values of the polysaccharides for antioxidant activity.

Assay	EC_{50} (μg/ml)			
	EPS	WPS	SPS	CK^+
DPPH scavenging activity	-	162.38a	63.37c	74.94b
Ferrous ions chelating activity	-	54.54a	44.91b	28.86c

The positive controls (CK^+) for DPPH scavenging and ferrous ions chelating assays are BHT and EDTA, respectively. '-' means that EC_{50} values cannot be obtained at the test concentrations. Different letters in each row indicate significant differences of the antioxidant activity for each assay at p = 0.0.5.

concentrations of 7 to 200 μg/ml, the chelating activity of natural compounds (Amarowicz et al., 2004). The DPPH radical scavenging effects of the polysaccharides EPS, WPS and SPS from *F. oxysporum* Dzf17 were presented in Figure 1. Among them, SPS exhibited the strongest scavenging DPPH activity at concentrations of 5 to 100 μg/ml, showing a good linear dependence between SPS concentration and DPPH scavenging activity. In contrast, WPS showed moderate, and EPS showed non-antioxidant activity. When WPS was at concentration of 300 μg/ml, the scavenging activity was 84.27%. The EC_{50} values (shown in Table 1) of WPS and SPS for antioxidant activity were 162.38 μg/ml and 63.37 μg/ml, respectively.

Ferrous ions chelating activity

Ferrous ions chelating activity was employed as another indicator to assess the quantity of the antioxidants (Lianhe et al., 2011; Xiao et al., 2011). In this research, the chelating activities of the polysaccharides from *F. oxysporum* Dzf17 on ferrous ions (Fe^{2+}) were investigated, which were presented in Figure 2. All the tested samples showed evident Fe^{2+} chelating activity in a concentration-dependent manner except EPS. At EPS varied only from 3.36 to 9.09%, which did not show its antioxidant activity. WPS or SPS showed stronger chelating activity than that of EPS, but slightly weaker than that of the positive control (EDTA). When the concentration of WPS was changed from 7 to 100 μg/ml, the chelating activity was rapidly increased from 5.59 to 84.07% showing a good linearity. With the concentration of WPS varied from 100 to 200 μg/ml, the chelating activity showed a gently increase with the value of 84.07 to 94.59%. The chelating activity of SPS exhibited the same trend but slightly stronger than that of WPS. The EC_{50} values (shown in Table 1) of WPS and SPS for antioxidant activity were 54.54 and 44.91 μg/ml, respectively.

In summary, this is the first report on the antioxidant activities of the polysaccharides from the endophytic fungus *F. oxysporum* Dzf17. Among three polysaccharides, SPS was the most active antioxidant component, WPS showed moderate, and EPS showed non-antioxidant activity. The antioxidant activity results of the polysaccharides obtained by two complementary assays were similar which indicated that they should have similar antioxidant mechanisms. DPPH is a stable free radical with a maximum absorption at 517 nm and can be readily scavenged by the antioxidants (e.g., phenolics, flavonoids, carotenoids and polysaccharides) which have hydrogen donating groups (Muller et al., 2011; Paixao et al., 2007; Qiao et al., 2009). Ferrozine quantitatively forms complexes with Fe^{2+}. In the presence of other chelating agents (e.g., polysaccharide), the complex formation is disrupted with the result that the red color of the complex was decreased (Yamaguchi et al., 2000). As the antioxidant mechanisms of polysaccharides are very complicated, other methods such as OH free radical scavenging and reducing powder assays should be employed in our further investigation (Huang et al., 2005). In our previous study, WPS was found to be the most effective polysaccharide to have enhancement effects on cell growth and diosgenin accumulation in *D. zingiberensis* cell cultures (Li et al., 2011). WPS showed

moderate antioxidant activity in this investigation which means that WPS should be studied in detail for its enhancing effect on secondary metabolite biosynthesis and antioxidant activity. The present study will provide additional data for supporting the utilization and development of the polysaccharides from *F. oxysporum* Dzf17 as the antioxidant components. Further studies to clarify other biological activities (e.g. immunoregulatory and antitumor activities) of the polysaccharides, their preparation on a large scale, composition including protein and carbohydrate percentage, antioxidant mechanisms, as well as the physiological and ecological roles of the polysaccharides on host plant cells are now in progress.

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