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Full Length Research Paper

Purification, composition analysis and antioxidant activity of different polysaccharides from the fruiting bodies of *Pholiota adiposa*

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Three water-soluble polysaccharide fractions (PAP1-1, PAP1-2 and PAP2-1) were isolated and purified from the fruiting bodies of *Pholiota adiposa* by diethylaminoethanol (DEAE) Sepharose fast flow column and superdex^{TM} 200 column chromatography on an ÄKTA Purifier system. Results of high-performance size-exclusion chromatography (HPSEC) indicated that the average molecular weights of PAP1-1, PAP1-2 and PAP2-1 were 2.3×10^6 Da, 8.8×10^3 Da and 2.1×10^6 Da, respectively. Monosaccharide compositions analysis revealed that PAP1-1 and PAP2-1 were both mainly composed of glucose, and PAP1-2 was composed of rhamnose and glucose with a mole ratio of 1:3.61. The evaluation of antioxidant activity suggested that PAP1-1 with β-configuration glycosidic bond exhibited stronger scavenging activity of superoxide radical and hydroxyl radical than PAP1-2 and PAP2-1 contained both α- and β-configuration glycosidic bond, and should be explored as a novel potential antioxidant. Available data obtained with *in vitro* models suggested that antioxidant activity of polysaccharide may be related to the configuration of glycosidic bond.

Key words: *Pholiota adipose,* polysaccharide, purification, antioxidant activity.

INTRODUCTION

Oxidation is essential to many organisms for the production of energy to fuel biological processes. However, excessive oxygen free radicals are involved in onset of many diseases such as cancer, aging, atherosclerosis, reperfusion injury and hepatic injury (Ke et al., 2009; Luo and Fang, 2008; Zha et al., 2009; Zou et al., 2008). In order to reduce damage to the human body, synthetic antioxidants are used widely at present.

However, recent research suggested that synthetic antioxidants were responsible for liver damage and carcinogenesis (Grice, 1988; Qi et al., 2005). Therefore, enhancement of body's antioxidant defences through natural and safe antioxidants would seem to provide a reasonable and practical approach to reduce the oxidative stress to human body. Published data indicated that natural polysaccharides and their conjugates extracted from plants and fungus in general possessed potential and potent antioxidant activities and could be explored as novel potential antioxidants (Chen et al., 2008b; Ge et al., 2009; Luo et al., 2010; Matkowski et al., 2008; Sun et al., 2010). *Pholiota adiposa* is an edible as well as a medicinal mushroom cultured in China and Japan. It belongs to *Agaricales* (Strophariaceae), which is rich in protein, essential amino acids, dietary fiber, trace elements, vitamins and carbohydrates (Hui et al., 2003). The polysaccharides extracted from the fruiting bodies of *P. adiposa* have the functions of antitumor (Jiang et al., 2007), fatigue resistance (Shimizu et al., 2003), antimicrobial (Dulger, 2004) and antioxidation (Deng et

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Abbreviations: DEAE, diethylaminoethanol; **HPSEC,** high-performance size-exclusion chromatography; **FT-IR,** Fourier transform infrared.

al., 2011; Ji et al., 2007). However, up to now, no detailed investigation has been conducted on composition characterization and antioxidant activities of different polysaccharides isolated from the fruiting bodies of *P. adiposa*. Therefore, the aim of present study is to characterize the compositions of polysaccharides extracted from the fruiting bodies of *P. adiposa* and evaluate the antioxidant activities of these polysaccharides *in vitro* by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging assay, superoxide radical scavenging assay and hydroxyl radical scavenging assay.

MATERIALS AND METHODS

The fruiting bodies of *P. adiposa* were cultivated and collected from Taishan Medical University, Taian, Shandong Province, China.

Diethylaminoethanol (DEAE) Sepharose fast flow column and superdex^{TM} 200 column were purchased from Amersham (Sweden). Dextran standards of T-2000, 500, 70, 40, 20, 10 were purchased from Pharmacia Co. Ltd. DPPH radicals and trifluoroacetic acid (TFA) were purchased from Sigma Co. The standard monosaccharides (d-glucose, d-mannose, l-rhamnose, d-Ribose, d-xylose, d-sorbitol, d-mannitol, l-arabinose and inositol) were purchased from Chinese Institute for the Control of Pharmaceutical and Biological Products. Hydroxylamine hydrochloride, pyridine, ascorbic acid, acetic anhydride, NaCl, petroleum ether, ethanol, ethyl acetate, acetone, methanol, deoxyribose, ethylene diamine tetra-acetic acid (EDTA), phenazine methosulfate (PMS), dihydromicotineamidadenine dinucleotide (NADH), ferrous ammonium sulfate, H_2O_2, thiobarbituric acid (TBA), nitroblue tetrazolium (NBT), trichloroacetic acid, Tris–HCl buffer and all other reagents used were of analytical grade.

Extraction of polysaccharide

The dried fruiting bodies of *P. adiposa* were cut into smaller pieces and further ground into powder by a mill, then extracted with petroleum ether and ethanol at 65°C for 5 h under reflux to remove lipid. The residue was then extracted with double-distilled water at 100°C for three times and 3 h for each time. All water-extracts were combined, concentrated in a rotary evaporator under reduced pressure at 50°C and filtered. Then the filtrate was precipitated with five volumes of dehydrated ethanol at 4°C overnight, followed by centrifugation at 5000 rpm for 20 min. The precipitate was dissolved in double-distilled water and deproteinized using the Sevag reagent (Navarini et al., 1999). After removal of the Sevag reagent, the aqueous fraction was precipitated again with five volumes of dehydrated ethanol at 4°C overnight. The precipitate was collected by centrifugation, washed successively with ethyl acetate and acetone, and then dissolved in water and lyophilized to yield the crude polysaccharides (PAP). The extract yield of crude polysaccharides was 3.56%.

Isolation and purification of polysaccharide

The freeze-dried sample (PAP) was purified on an ÄKTA Purifier system (Amersham Pharmacia Biotech, Sweden) equipped with a pump P-900, a pH/C-900 monitor and a Frac-950 collector. PAP was dissolved in distilled water, centrifuged, and then the supernatant was applied to a DEAE Sepharose fast flow column(1.6 cm × 10 cm) equilibrated with distilled water about 60 mL.

After loading with sample, the column was eluted with distilled water and a continuous gradient of NaCl aqueous solution (from 0 to 2 M) about 40 mL, then 40 mL 2 M NaCl aqueous solution at a flow rate of 0.6 mL/min, respectively. Different fractions (5 mL in each tube) were collected using the Frac-950 collector and monitored by the phenol–sulfuric acid method at 490 nm (Dubois et al., 1956). The elution profile detected by the phenol–sulfuric acid assay showed two big elution peaks namely as PAP1 and PAP2, respectively. The two fractions were further purified on a Superdex^{TM} 200 column (1 cm × 30 cm) with distilled water at a flow rate of 0.5 mL/min. Different fractions (1 mL in each tube) were collected using the Frac-950 collector and monitored by the phenol–sulfuric acid method at 490 nm. Finally, three polysaccharide fractions, PAP1-1, PAP1-2 and PAP2-1, were obtained, dialyzed with distilled water, and lyophilized to give white purified polysaccharide fractions.

Molecular weight determination

The homogeneity and the average molecular weight (Mw) of polysaccharide fractions was determined by high-performance size-exclusion chromatography (HPSEC) (Wei and Fang, 1989), which was performed on a Agilent 1200 high performance liquid chromatography (HPLC) system (Agilent Technologies, USA) fitted with one TSK-G4000PWXL column (7.8 mm ID × 30.0 cm L) and an Agilent ELSD detector. The operation was performed using the following conditions: mobile phase: distilled water; flow rate: 1 mL/min; column temperature: 40°C; injection volume: 20 µL; running time: 20 min. A sample (5 mg) was dissolved in the distilled water (0.5 mL) and centrifuged (10,000 rpm, 5 min), and 20 µL of supernatant was injected in each run. The molecular mass was estimated by reference to a calibration curve made under the conditions described above from a set of Dextran T-series standards of known molecular mass (T-2000, 500, 70, 40, 20, 10).

Monosaccharide composition

Monosaccharide compositions of polysaccharides were determined by gas chromatography (GC), which was performed on a GC2010 instrument (Shimadzu, Japan) fitted with DB-23 column (30 m × 0.25 mm × 0.25 µm) (Agilent Technologies, USA). First, the polysaccharide (5 mg) was hydrolyzed with 2 M TFA at 110°C for 3 h in a sealed glass tube. The resulting solution was concentrated *in vacuo* and the excess of acid was removed by repeated co-distillations with dehydrated methanol. Then the hydrolyzed products were prepared for acetylation. The acetylation was carried out with 10 mg of hydroxylamine hydrochloride and with 0.5 mL of pyridine by getting heated in a water bath for 30 min at 90°C. After incubation, the mixture was cooled at room temperature, and then 0.5 mL of acetic anhydride was added and mixed thoroughly by vortexing. The tube was sealed and incubated in a water bath for another 30 min at 90°C. After cooling, the derivatives were loaded onto a DB-23 capillary gas chromatography column equipped with flame-ionization detector (FID), using inositol as the internal standard. Alditol acetates of standard monosaccharides (glucose, mannose, mannitol, rhamnose, Ribose, sorbitol, xylose, and arabinose) with inositol (2 mg) as the internal standard were prepared and subjected to GC analysis separately in the same way. The operation was performed using the following conditions: carrier gas (high-purity nitrogen): 1 mL/min; injection temperature: 250°C; detector temperature: 250°C; column temperature: 220°C; injection volume: 0.5 µL; split ratio: 20:1.

Analysis of uronic acid contents

Uronic acid contents were determined by measuring the

absorbance at 525 nm using the m-hydroxybiphenyl colourimetric procedure and with d-glucuronic acid as the standard (Blumenkrantz and Asboe-Hansen, 1973).

Infrared (IR) spectral analysis

The IR spectra of the polysaccharides were determined using a Fourier transform IR spectrophotometer (FTIR) (PerkinElmer, USA). The purified polysaccharides were ground with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 4000-500 cm^{-1} (Kumar et al., 2004).

Assay for antioxidant activities

DPPH scavenging assay

The DPPH radicals scavenging activity of the purified polysaccharides were measured according to the method of Liu and Zhao (2006) with some modifications. Samples were dissolved in distilled water at 0 (control), 0.05, 0.1, 0.25, 0.5, 1, 2, or 4 mg/mL. The 0.2 mmol/L solution of DPPH in 95% ethanol was prepared daily before UV measurements. The reaction mixture contained 2 mL of 95% ethanol, 2 mL of freshly prepared DPPH and 1 mL of each sample solution. The solution was left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank, using ascorbic acid as a positive control. The antioxidant activity of the purified polysaccharides was evaluated according to the following formula:

Scavenging rate (%) = [1- (Ai - Aj)/Ac] × 100%

Where, Ac is the absorbance of DPPH solution without sample (2 mL DPPH + 3 mL of 95% ethanol); Ai is the absorbance of the test sample mixed with DPPH solution (1 mL sample + 2 mL DPPH + 2 mL of 95% ethanol) and Aj is the absorbance of the sample without DPPH solution (1 mL sample + 4 mL of 95% ethanol).

Superoxide radical scavenging assay

The superoxide radical scavenging activity of the purified polysaccharides was evaluated according to the method detailed by Stewar and Beewley (1980) with a minor modification. Samples were dissolved in distilled water at 0 (control), 0.05, 0.1, 0.25, 0.5, 1, 2, or 4 mg/mL. The sample solution (0.1 mL) was mixed with 1 mL of 16 mM Tris–HCl (pH 8.0) containing 557 µM NADH, 1 mL of 16 mM Tris–HCl (pH 8.0) containing 45 µM PMS, and 1 mL of 16 mM Tris–HCl (pH 8.0) containing 108 µM NBT. After 5 min of incubation at 25°C, the absorbance was measured at 560 nm using ascorbic acid as a positive control. The capability of scavenging the superoxide radical was calculated using the following equation:

Scavenging rate (%) = (1- As/Ac) × 100%

Where, Ac is the absorbance of the blank and As is the absorbance of the test sample mixed with reaction solution.

Hydroxyl radical scavenging assay

Scavenging effects of the purified polysaccharides on hydroxyl radicals were performed, as described by Halliwell et al. (1987). Samples were dissolved in distilled water at 0 (control), 0.05, 0.1, 0.25, 0.5, 1, 2, or 4 mg/mL. The sample solution (0.1 mL) was mixed with 0.6 mL of reaction buffer [0.2 M phosphate buffer (pH 7.4), 2.67 mM deoxyribose, and 0.13 mM EDTA]; 0.2 mL of 0.4 mM ferrous ammonium sulfate, 0.05 mL of 2.0 mM ascorbic acid, and 0.05 mL of 20 mM H_2O_2 was then added to the reaction solution. The reaction solution was incubated for 15 min at 37°C and then 1 mL of 1% TBA and 1 mL of 2.0% trichloroacetic acid were added to the mixture. The mixture was boiled for 15 min and cooled on ice. The absorbance of the mixture was measured at 532 nm using ascorbic acid as a positive control. The hydroxyl radical scavenging activity was expressed as:

Scavenging rate (%) = (1-As/Ac) × 100%

Where, Ac is the absorbance of the blank and As is the absorbance of the test sample mixed with reaction solution.

RESULTS

Isolation and purification of polysaccharides

PAP was isolated from the hot-water extract of the fruiting bodies of *P. adiposa* by a yield of 3.56%. ÄKTA Purifier system was successfully deployed to purify PAP. According to the charge difference, two fractions of PAP1 and PAP2 were isolated from distilled water elute and NaCl elute by the means of ion-exchange chromatography on a DEAE Sepharose fast flow column, respectively (Figure 1). On account of molecular weight difference, different charged polysaccharide was further purified by size-exclusion chromatography on Superdex^{TM} 200 column, giving three homogeneous fractions of PAP1-1, PAP1-2 and PAP2-1 (Figure 2).

Molecular weights, monosaccharide compositions and uronic acid contents of purified fractions High-performance size-exclusion chromatography (HPSEC) has been shown to be an effective method to determine homogeneity and molecular weights of polysaccharide fractions.

The purified fractions of PAP1-1, PAP1-2 and PAP2-1 showed a single symmetrical narrow peak on HPSEC, indicating that no other polysaccharide was present in the sample (Figure 3), and the average molecular weights (Mw) of PAP1-1, PAP1-2 and PAP2-1 were estimated to be 2.3×10^6 Da, 8.8×10^3 Da and 2.1×10^6 Da, respectively, in reference to Dextran T-series standard samples of known molecular weights (T-2000, 500, 70, 40, 20, 10).

The uronic acid contents and monosaccharide compositions of the purified polysaccharide fractions were determined by m-hydroxybiphenyl colourimetric procedure and GC.

The result indicates that contents of uronic acid in PAP1-1, PAP1-2 and PAP2-1 were 1.08, 1.73 and 1.44%, respectively. The results of monosaccharide compositions showed that PAP1-1 and PAP2-1 were only composed of glucose (Glc) and PAP1-2 was mainly composed of rhamnose (Rha) and glucose (Glc), with a mole ratio of 1:3.61.

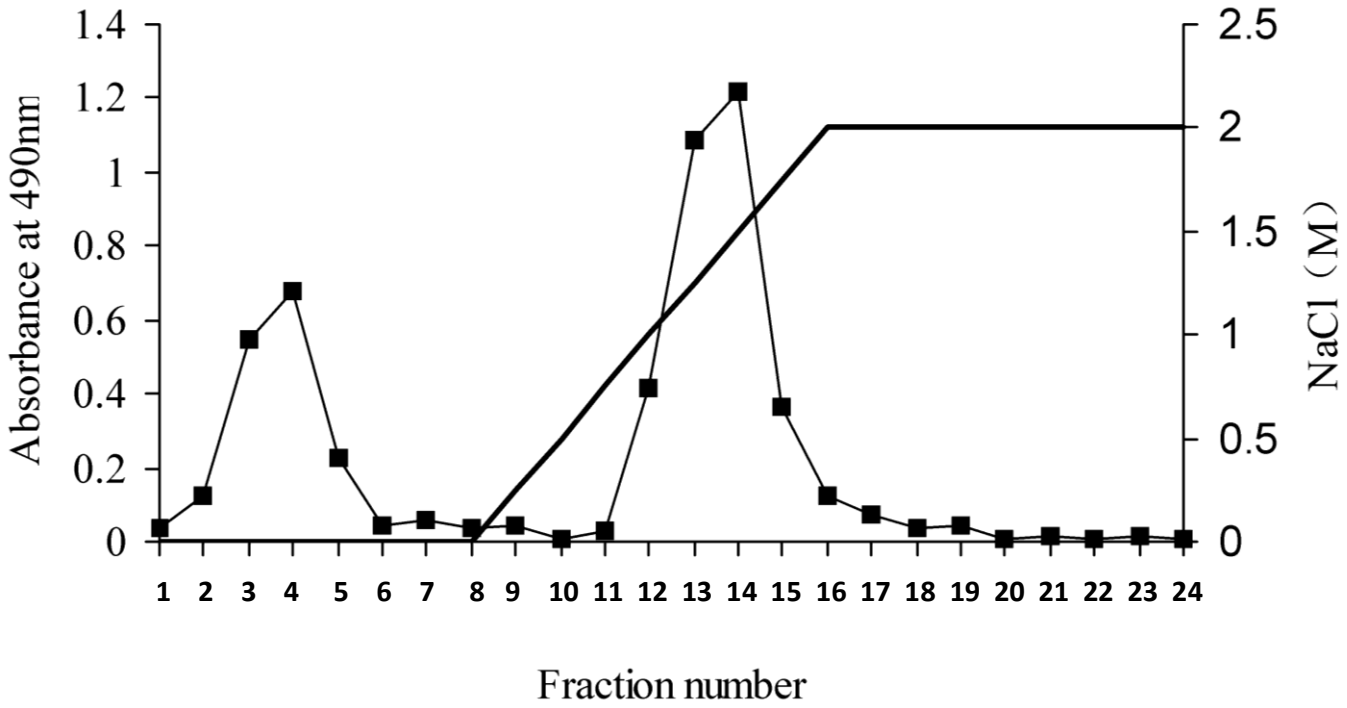

Figure 1. The profile of PAP isolated from the fruiting bodies of *P. adiposa* on a DEAE Sepharose fast flow column eluted with distilled water and continuous gradient of NaCl aqueous solutions (from 0 to 2 M) at a flow rate of 0.6 mL/min.

IR spectra of purified fractions

The IR spectra of PAP1-1, PAP1-2 and PAP1-2 are shown in Figure 4. All samples exhibited an absorption peak between 3600 and 3200 cm^{-1} characteristic of hydroxyl groups and a weak band at ~ 2930 cm^{-1} attributed to the C–H stretching vibrations. The band at 1624.03 cm^{-1} (PAP1-1), 1636.70 cm^{-1} (PAP1-2) and 1641.74 cm^{-1} (PAP2-1) were due to the bound water (Zhao et al., 2005). The absorption band at 1423 cm^{-1} (PAP1-1), 1401 cm^{-1} (PAP1-2) and 1369 cm^{-1} (PAP2-1) were represented C–H bending vibrations. Each particular polysaccharide had a specific band in the 1200-1000 cm^{-1} region. This region is dominated by ring vibrations overlapped with stretching vibrations of (C–OH) side groups and the (C–O–C) glycosidic bond vibration. The absorptions at 1025, 1078 and 1151 cm^{-1} (PAP1-1), at 1027, 1077 and 1151 cm^{-1} (PAP1-1), at 1022, 1078 and 1150 cm^{-1} (PAP2-1), indicated a pyranose form of sugars (Zhao et al., 2005). The characteristic absorptions around 849 cm^{-1} and 919 cm^{-1} in the IR spectra indicated that α- and β-configurations were simultaneously (Barker et al., 1954). The absorption bands at 860 cm^{-1}, 931 cm^{-1} (PAP1-2) and 860 cm^{-1}, 930 cm^{-1}（PAP2-1）indicated that PAP1-2 and PAP2-1 contained both α- and β-type glycosidic bond in their structure. A characteristic peak at around 930 cm^{-1} was found in PAP1-1, indicating the β-configuration in pyranose form.

Antioxidant activity analysis

Effect of scavenging DPPH radicals

The DPPH free radicals are a stable free radical, which has been widely accepted as a tool for estimating the free radical-scavenging activities of antioxidants (Hu et al., 2004; Leong and Shui, 2002). Figure 5 shows the DPPH radical scavenging activity of crude polysaccharides (PAP), the purified polysaccharides (PAP1-1, PAP1-2 and PAP2-1) and ascorbic acid. The results indicate that PAP1-1, PAP1-2 and PAP2-1 exhibited very low radical scavenging activity at every concentration point. Compared to these purified polysaccharide fractions, PAP exhibited a relatively high level of radical scavenging activity (53.2% at the concentration 1.0 mg/mL), but significantly lower than ascorbic acid (94.8% at the concentration 1.0 mg/mL).

Scavenging effects of polysaccharides on superoxide radicals

Superoxide radicals are generated in a PMS/NADH system for being assayed in the reduction of NBT. The scavenging ability of purified polysaccharides on superoxide radicals with ascorbic acid as a positive control is shown in Figure 6. The results indicate that PAP1-1 had more scavenging activities of superoxide

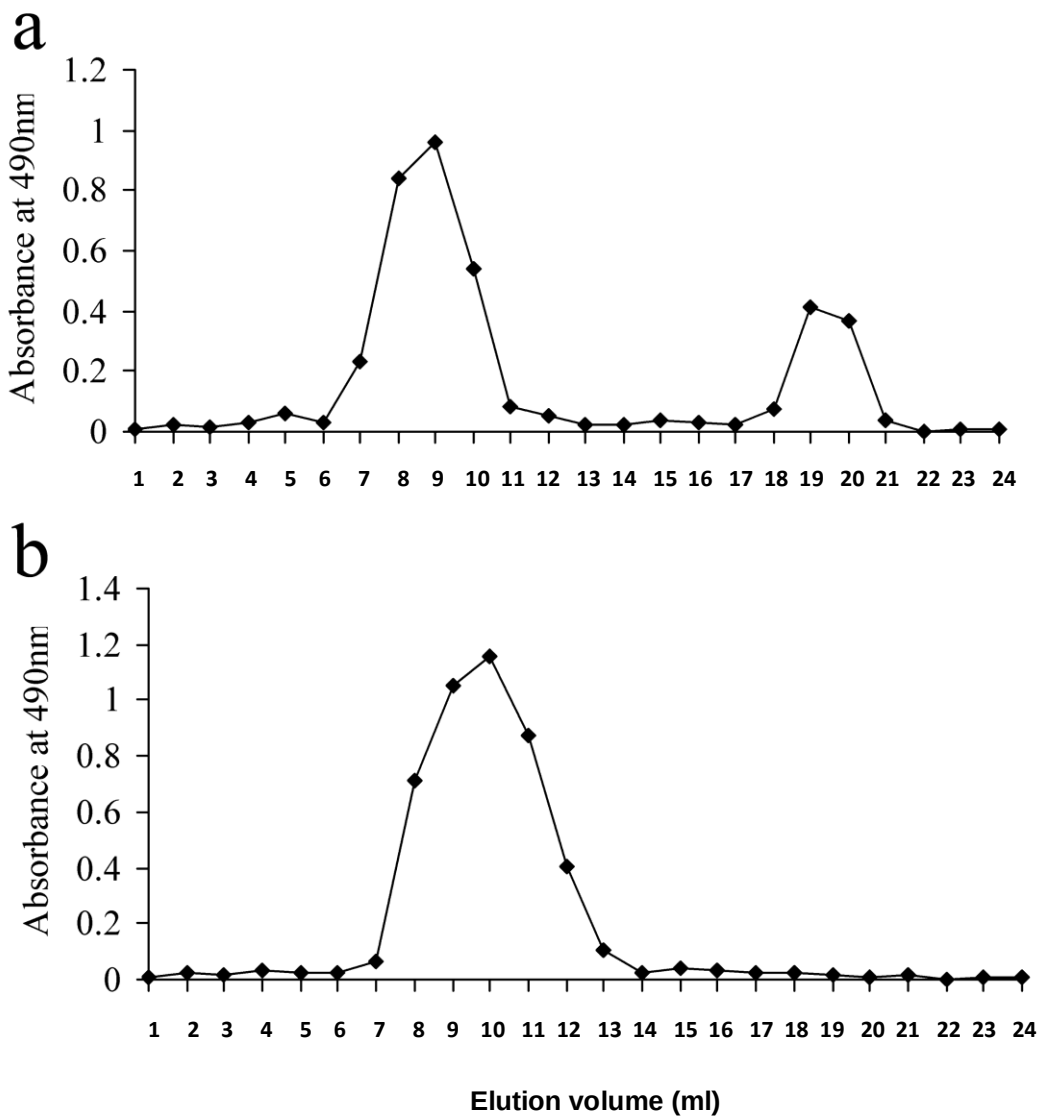

Figure 2. Superdex^{TM} 200 column chromatogram of PAP1 (a) and PAP2 (b) eluted with distilled water at a flow rate of 0.5 mL/min.

radical (49.3% at the concentration 2.0 mg/mL) than PAP and two other polysaccharide fractions. However, the radical scavenging activity of PAP1-1 was far lower than that of ascorbic acid used in this study. Their scavenging ability on superoxide radicals decreased in the order of ascorbic acid > PAP1-1 > PAP >PAP2-1> PAP1-2.

Scavenging effects of polysaccharides on hydroxyl radicals

Hydroxyl radicals are mainly responsible for the oxidative injury of biomolecules (Ke et al., 2009). The scavenging effects of purified polysaccharide fractions and ascorbic acid on hydroxyl radicals are shown in Figure 7. Their scavenging activity increased gradually with increasing concentration of polysaccharides. Moreover, PAP1-1 showed pronounced high radical scavenging activity, which was close to that of ascorbic acid. The scavenging activity of PAP1-1 was 75.1% at the concentration 4.0 mg/mL, and it was the strongest among all the others. Their scavenging activity on hydroxyl radicals decreased in the order of ascorbic acid > PAP1-1 > PAP >PAP2-1> PAP1-2. These results indicate that PAP1-1 had strong scavenging activity on hydroxyl radicals and should be explored as potential antioxidants.

DISCUSSION

In recent years, fungal polysaccharides have received considerable attention due to their various potential

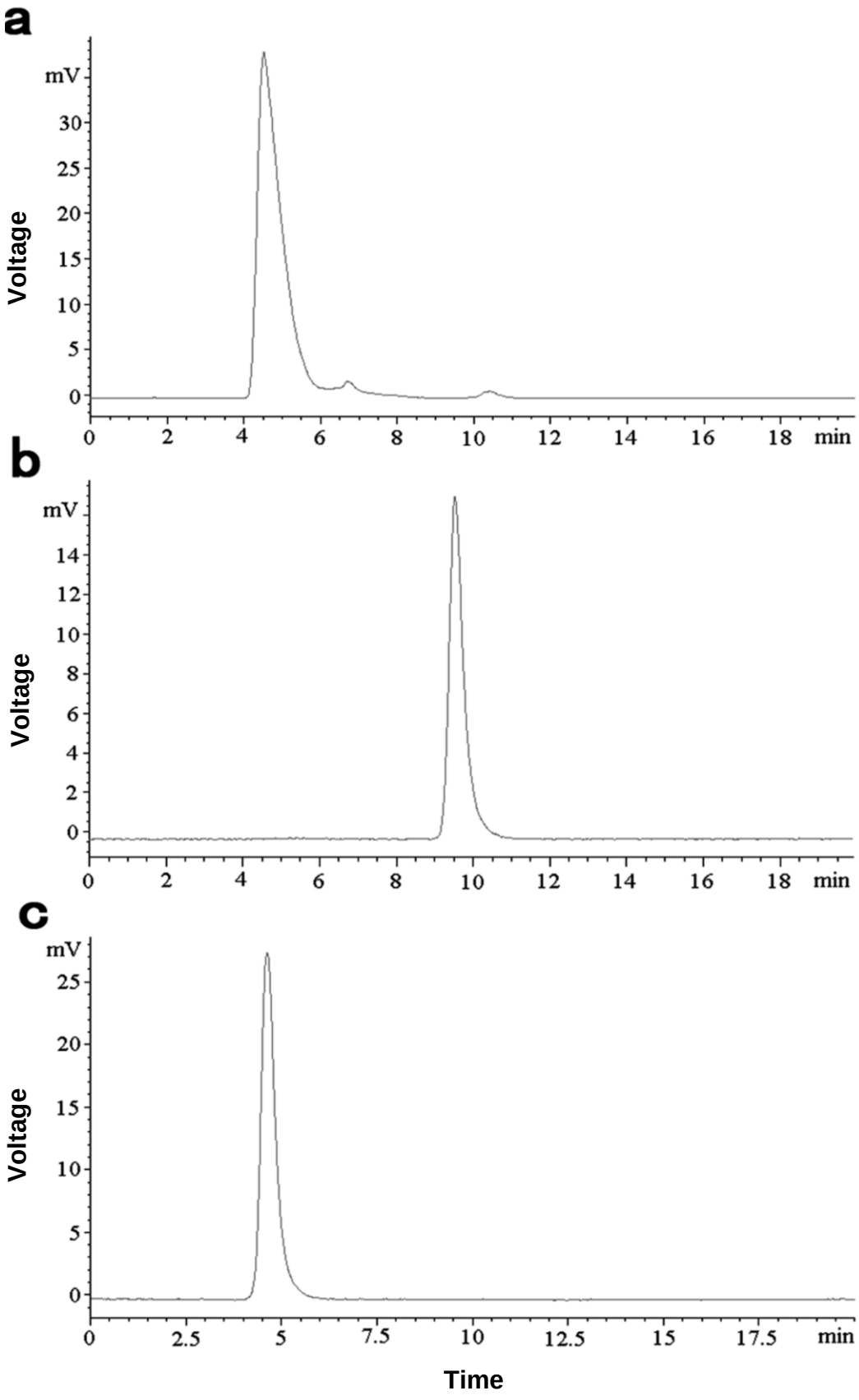

Figure 3. Profile of the purified polysaccharide fractions on TSK-G4000PWXL column with distilled water at 1 mL/min. (a) PAP1-1; (b) PAP1-2 and (c) PAP2-1.

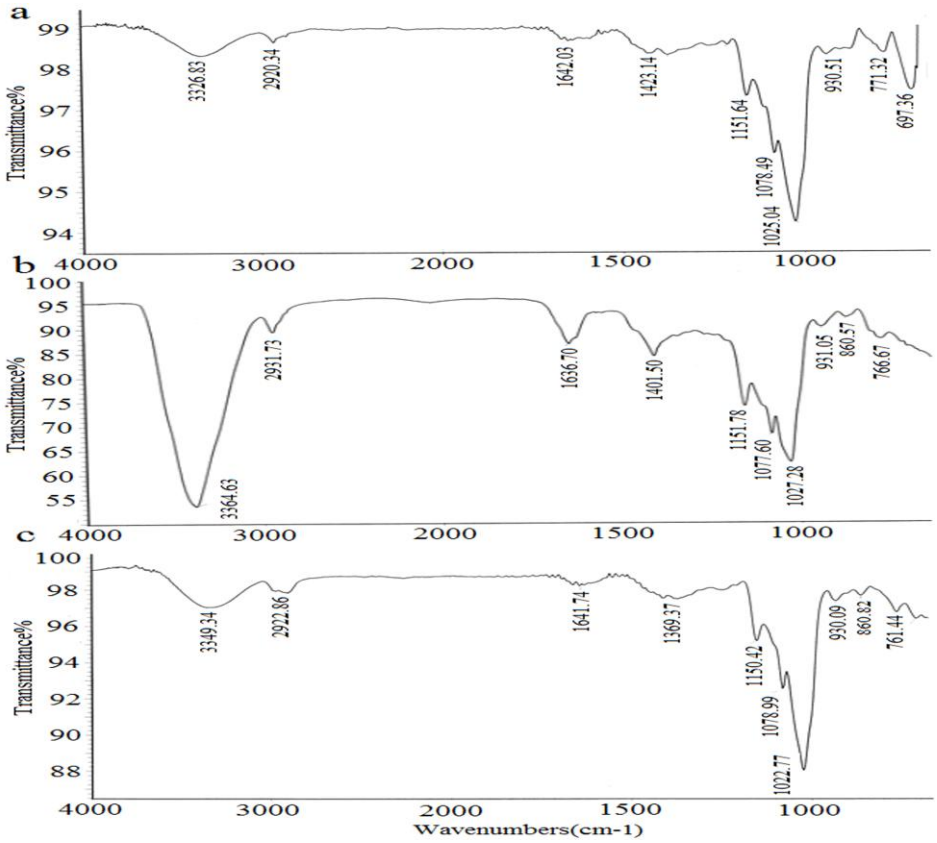

Figure 4. FTIR spectra of the purified polysaccharide fractions. (a) PAP1-1; (b) PAP1-2 and (c) PAP2-1.

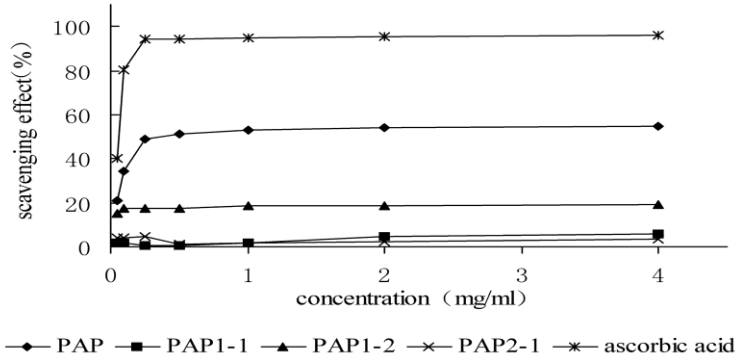

Figure 5. Scavenging effects of polysaccharides on DPPH radicals with ascorbic acid as a positive control.

biological activities that could be applied to healthcare foods or medicine, especially antioxidant, immunostimulatory and antitumor effects (Chen et al., 2008b; Huang et al., 2007; Sun et al., 2008). *P. adiposa* is one kind of medicinal and edible mushroom with high economic values in recent years. In this experiment, we first reported the extraction and purification of different fractions of polysaccharides from the fruiting bodies of *P. adiposa*. The PAP contained predominantly three polysaccharide fractions (PAP1-1, PAP1-2 and PAP2-1). Furthermore, the antioxidant activities of PAP, PAP1-1, PAP1-2 and PAP2-1 were evaluated *in vitro*; the results indicate that the purified polysaccharide fractions exhibited less scavenging activity for DPPH radical than PAP. The reason for this could be that PAP was rich in antioxidant components, such as amino acids, ascorbic

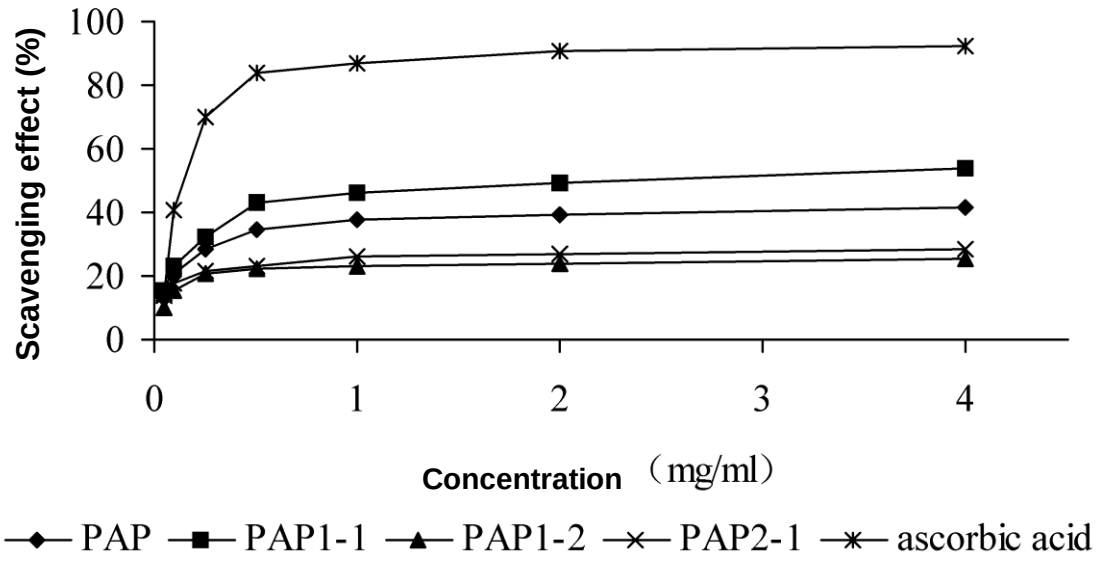

Figure 6. Scavenging effects of polysaccharides on superoxide radicals with ascorbic acid as a positive control.

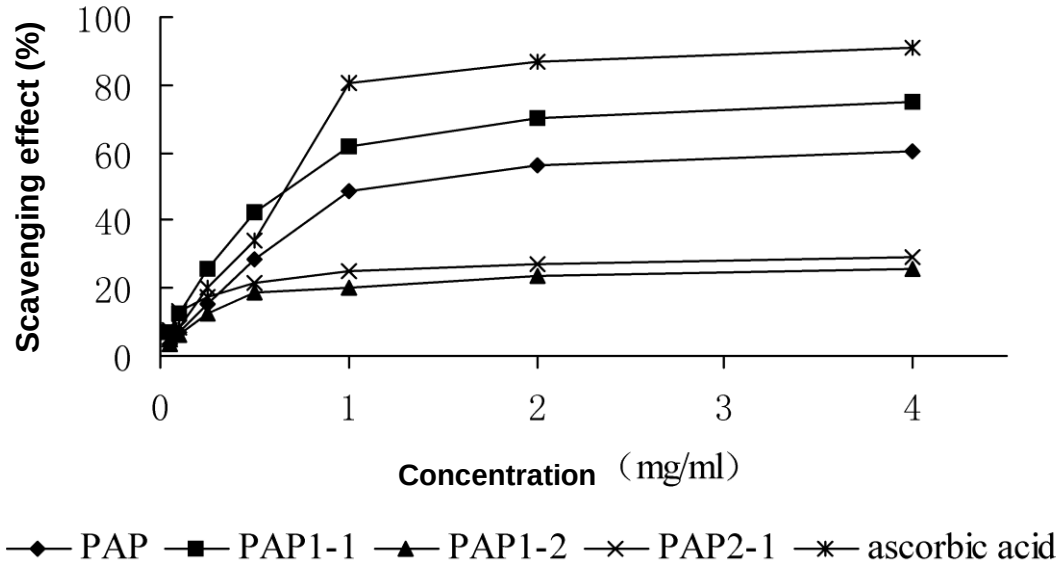

Figure 7. Scavenging effects of polysaccharides on hydroxyl radicals with ascorbic acid as a positive control.

acid, thiamine, nucleotide, nicotinic acid, organic acids and microelements, which contributed to their antioxidant properties. Antioxidant test *in vitro* also showed that PAP1-1 possessed stronger scavenging activity of superoxide radical and hydroxyl radical, especially scavenging activity of hydroxyl radical may be comparable to ascorbic acid.

In the Infrared spectra analysis, we found PAP1-1 was composed of β-configuration in pyranose form sugars; on the contrary, PAP1-2 and PAP2-1 contained both α- and β-configuration in pyranose form sugars. Antioxidant results showed that PAP1-1 with β-configuration glycosidic bond exhibited stronger antioxidant activity than PAP1-2 and PAP2-1 contained both α- and β-configuration glycosidic bond. The results suggest that the configuration of glycosidic bond of polysaccharide played an important role on antioxidant activity. The results are similar to Luo's reports that polysaccharides with β-configuration in pyranose form sugars exhibited stronger biological activity (Luo et al., 2010).

Results of HPSEC indicated that the molecular weights of three polysaccharide fractions were 2.3×10^6 Da, 8.8 ×

10^3 Da and 2.1 × 10^6 Da, respectively. The molecular weight of polysaccharides played an important role on their bioactivity; polysaccharide with lower molecular weight showed stronger antioxidant activity (Chen et al., 2008a; Luo et al., 2010). In this work, the molecular weight of PAP1-2 was the minimal among the three polysaccharide fractions, but it did not exhibit stronger antioxidant activity. Besides, uronic acid was an effective indicator of antioxidant activity of polysaccharide, the higher the content of uronic acid, the stronger the antioxidant activities of polysaccharides (Sun et al., 2008). In this experiment the contents of uronic acid in PAP1-1, PAP1-2 and PAP2-1 were all very low, but PAP1-1 exhibited stronger antioxidant activity. The results stated above indicate that the antioxidant activity of polysaccharide is usually influenced by various factors combined rather than one single factor. Therefore, it is important to define the complete structure of the polysaccharide and the structure-function relationship, which will certainly present a good opportunity to elucidate the biological roles of polysaccharide and develop potential antioxidant agent based on the three-dimensional structures.

Conclusion

According to the results stated above, it could be concluded that the water extracting PAP of *Pholiota adiposa* contained three major polysaccharide fractions (PAP1-1, PAP1-2 and PAP2-1) purified by DEAE Sepharose fast flow column and superdexTM 200 column chromatography. Antioxidant test *in vitro* showed that PAP exhibited strong scavenging activity for DPPH radical, but PAP1-1 was confirmed having stronger scavenging activity of superoxide radical and hydroxyl radical, which was close to the configuration of glycosidic bond. Based on the above studies, further investigation of its antioxidant activities *in vivo* and detailed structural characterization of PAP1-1 will be carried out in our later work.

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