Carbohydrate Polymers 98 (2013) 1524–1531

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Effects of extraction methods on the antioxidant activities of polysaccharides obtained from *Flammulina velutipes*

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ARTICLE INFO

Article history:
Received 8 May 2013
Received in revised form 25 June 2013
Accepted 22 July 2013
Available online 31 July 2013

Keywords:
Flammulina velutipes
Antioxidant activity
Polysaccharide
Extraction method

ABSTRACT

Four polysaccharides (CFP, UFP, MFP and EFP) were extracted from *Flammulina velutipes* using hot water, ultrasonic, microwave or enzymatic methods optimized by orthogonal test. Preliminary structural characterizations were conducted using physicochemical properties. Polysaccharides extracted by all four methods showed similar physicochemical characteristics and FT-IR spectra. However, SEM images of tissues of *F. velutipes* were significantly different. EFP demonstrated better antioxidant activities against hydroxyl radical as well as improved metal chelating activity. UFP showed higher DPPH scavenging activity, but CFP exhibited higher antioxidant activity in reducing power. Hence, these polysaccharides can be used as natural antioxidants in functional foods or medicine. Further experiments on the biological activities of these four polysaccharides are currently in progress.

1. Introduction

Antioxidant status in humans reflects the dynamic balance between the antioxidant defense and prooxidant conditions. When the mechanism of antioxidant protection becomes unbalanced by factors such as aging, impaired physiological functioning may occur, resulting in diseases and accelerated aging (Tiwari, 2004). It is widely accepted that appropriate supplementation with exogenous antioxidant may help reduce reactive oxygen species (ROS)-induced oxidant damage. Due to concern about the side-effects of some synthetic antioxidants, there is increasing interest in replacing synthetic antioxidants with natural antioxidants in the food, pharmaceutical and cosmetic industries (Chen, You, Huang, Yu, & Chen, 2010; Tannin-Spitz, Bergman, Van-Moppes, Grossman, & Arad, 2005).

Many reports have indicated that fungal polysaccharides have strong antioxidant activities in general and should be explored as novel potential antioxidants (Song, Zhang, Zhang, & Wang, 2010; Wang et al., 2010). The antioxidant activity of polysaccharides mainly depends on several structural parameters including sugar composition, molecular weight, type of glycosidic bond in the main chain, and the degree of sulfuric acid esterification (Lu, Wang, Hu, Huang, & Wang, 2008).

Flammulina velutipes is one of the most popular edible fungi in China and Japan because of its high nutritional value and attractive taste. It has been reported that both the fruiting bodies and the fungal mycelia of *F. velutipes* contain bioactive polysaccharides with beneficial immunomodulatory, anti-tumor, and biological activity on hepatocytes, as well as antioxidant activity (Leung, Fung, & Choy, 1997).

In this study, polysaccharides from *F. velutipes* were extracted using hot water, ultrasonic, microwave, and enzyme-based methods. Preliminary structural characterization of the four polysaccharides was then conducted *via* physicochemical property, Fourier transform infrared (FTIR) spectroscopy, and scanning electron microscopy analyses. Finally, the antioxidant activities of the extracted polysaccharides were determined against DPPH and hydroxyl radicals, in addition to assaying their metal chelating activity and reducing power. The main aim of this study is to investigate the application value of polysaccharides from *F. velutipes*.

2. Materials and Methods

2.1. Materials

The fruiting body of *F. velutipes* was obtained from the farm at the Zhejiang Academy of Agricultural Science. The mushroom were ground to fine powder, then defatted with petroleum ether and pretreated twice with 80% ethanol prior to experiments.

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0144-8617/$ – see front matter.
http://dx.doi.org/10.1016/j.carbpol.2013.07.052

1,1-Diphenyl-2- picrylhydrazxyl (DPPH), 3-(2-pyridyl)-5, 6-diphenyl-1, 2,4-triazine-4,4-disulfonic acid monosodium salt (ferrozine), butyl hydroxy anisole (BHA), trifluoroacetic acid (TFA), monosaccharide standards (D-galactose, D-arabinose, L-fucose, L-rhamnose, D-mannose, D-xylose, D-glucose) and EDTA were purchased from Sigma (St. Louis, MO, USA). Cellulase (160 U/mg) was supplied by Huzhou Lilai Chemical Reagent Co. Ltd. All other chemicals and solvents were of the highest commercial grade and obtained from Huadong Chemical Reagent Co. Ltd. (Hangzhou, China).

2.2. Extraction methods

2.2.1. Conventional solvent extraction (CSE) of polysaccharides from F. velutipes

Approximately 2.0 g of ground powder was mixed with distilled water in a round-bottom flask. The flask was placed into a HH-6 water bath (Guohua Wiring Company, Shanghai, China) and connected to the condenser. Extractions were performed under the conditions specified in Table 1a. The suspension was centrifuged (4000 × *g*, 15 min) and the insoluble part of the plant material was treated again as mentioned above. The supernatant was incorporated and concentrated to one-fifth of the initial volume using a rotary evaporator at 50 °C under vacuum. The supernatant was precipitated with four volumes of 95% (v/v) ethanol for 48 h at 4 °C. The precipitates were isolated by centrifugation (3000 × *g*, 10 min), washed with acetone and dried under reduced pressure at −40 °C to obtain crude polysaccharides (CFP).

2.2.2. Ultrasound-assisted extraction (UAE) of polysaccharides (UFP) from F. velutipes

Approximately 2.0 g of ground powder was mixed with distilled water in a conical flask and placed in an ultrasonic cleaner (KQ-500E, Kunshan Ultrasound Instrument Co., Ltd., Jiangsu, China, 40 kHz). The mixture was then extracted using various extraction times (30–50 min), ultrasonic power levels (100–300 W), solvent to raw material ratios (20–40 mL/g) and extraction temperature (30–50 °C). Extractions were carried out under the conditions specified in Table 1b. The subsequent processes were similar to those for CSE of polysaccharides.

2.2.3. Microwave-assisted extraction (MAE) of polysaccharides (MFP) from F. velutipes

MAE was carried out using a MARS-II (1000 W, 2450 MHz) microwave-accelerated reaction system from SINEO Microwave Chemistry Technology (Shanghai, China) with a power setting adjustable from 200 to 1000 W. It was equipped with ten 100-mL closed polytetrafluoroethylene (PTFF) vessels, a power sensor, a temperature sensor and a temperature controller. Approximately 1.0 g of ground powder was mixed with distilled water and then introduced into PFTE extraction vessels. Extractions were performed under the conditions specified in Table 1c. After extraction, the vessel was allowed to cool at room temperature. Subsequent procedures were similar to those for CSE of polysaccharides.

2.2.4. Enzymatic method for extraction (EAE) of polysaccharides (EFP) from F. velutipes

Cellulase was used to extract polysaccharide according to the method of Chen, Wang, Zheng, and Lin (1999). Samples were weighed in Pyrex glass bottles and the cellulase preparations were added. Distilled water was then added and the pH value was adjusted to 4.5. The flask was placed into a water bath and extractions were carried out under the conditions specified in Table 1d. Subsequent procedures were similar to those for CSE of polysaccharides.

The above crude polysaccharides were subjected to the Sevag method (4:1 chloroform: butyl alcohol) to remove free proteins. The deproteinated solution was re-precipitated with four volumes of 95% ethanol and used to investigate their structural characterizations and antioxidant activities.

2.3. Analytical methods

2.3.1. General methods

Neutral sugar content was examined by the phenol-sulfuric acid colorimetric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using glucose as a standard reference material. Protein content was determined by using the method detailed by Spector (1978) with bovine serum albumin as reference material. Monosaccharide composition analysis was conducted according to Sheng et al. (2007). The shape and surface characteristics of the samples after extraction (CSE, MAE, UAE or EAE) were measured by scanning electron microscope (Philips XL 30 ESEM, Philips, Holland).

2.3.2. Physicochemical property analysis

Physicochemical properties were determined using the following methods: phenol-sulfuric acid test (Dubois et al., 1956), α-naphthol reaction (Song, Li, & He, 2009), iodination reaction (Liu, Yan, & Han, 2012), Fehling's test (Song et al., 2009), carbazole reaction (Bitter & Muir, 1962), $FeCl_3$ reaction (Liu, Chou, Wang, Wu, & Zhang, 2011) and Coomassie brilliant blue reaction (Spector, 1978).

2.3.3. FTIR analysis

The organic functional groups of the four polysaccharides were identified using an FTIR spectrophotometer (FTIR-8400S, Shimadzu Co., Japan) in the 4000–400 cm^{-1} region *via* the KBr pressed-disk method.

2.4. Assay for antioxidant activity

2.4.1. DPPH radical scavenging activity

The method reported by Shimada, Fujikawa, Yahara, and Nakamura (1992) was adapted to measure free radical scavenging capability. To each 1 mL of sample solution at different concentrations (0.31–5.00 mg/mL), 1 mL of freshly prepared methanolic dimethyl sulfoxide (DMSO) solution of DPPH (0.325 mM) was added. This was mixed well and then left standing for 30 min at room temperature in the dark. The absorbance of the resulting solution was recorded at 517 nm. BHA was used as a reference material. All tests were preformed in triplicate. Scavenging activity was calculated as follows:

$$\text{Scavenging activity}\,(\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

where A_0 is the absorbance of the control (DPPH solution with no sample) and A_1 is the absorbance of the tested sample (DPPH solution with either sample or positive control).

2.4.2. Ferrous ion-chelating activity

The iron-chelating ability of all the samples was determined by chelation of ferrous ions by either the extracts or standards and estimation by the method described by Dinis, Maseira, and Almeida (1994). In this assay, 0.2 mL test samples at different concentrations (0.31–5.00 mg/mL) were added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured

Table 1a
Analysis of L9 $(3)^3$ test results of conventional solvent extraction (CSE).

No.	A, extraction temperature (°C)	B, extraction time (min)	C, ratio of water to raw material	Extraction yield (%)
1	75	60	20	6.98
2	75	90	30	7.21
3	75	120	40	7.95
4	85	60	30	7.46
5	85	90	40	9.37
6	85	120	20	8.24
7	95	60	40	9.68
8	95	90	20	8.65
9	95	120	30	8.96
K_1	7.38	8.04	7.96	
K_2	8.36	8.41	7.88	
K_3	9.09	8.38	9.00	
R	1.71	0.34	1.12	

R refers to the result of extreme analysis.
Extraction yield (%) = (polysaccharides weight/mushroom powder weight) × 100.

Table 1b
Analysis of L9 $(3)^3$ test results of ultrasound-assisted extraction (UAE).

No	A, extraction temperature (°C)	B, extraction time (min)	C, ratio of water to raw material	D, ultrasonic power (W)	Extraction yield (%)
1	30	30	20	100	8.76
2	40	30	30	200	10.58
3	50	30	40	300	11.46
4	30	40	40	200	10.23
5	40	40	20	300	10.35
6	50	40	30	100	9.56
7	30	50	30	300	10.98
8	40	50	40	100	9.77
9	50	50	20	200	9.36
K_1	9.99	10.27	9.49	9.36	
K_2	10.23	10.05	10.37	10.06	
K_3	10.12	10.04	10.48	10.93	
R	0.24	0.23	0.99	1.33	

R refers to the result of extreme analysis.
Extraction yield (%) = (polysaccharides weight/mushroom powder weight) × 100.

at 562 nm. The percentage inhibition of ferrozine–Fe^{2+} complex formation was determined using the following formula:

$$\text{Chelating activity}\,(\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100,$$

where A_0 is the absorbance of the control, and A_1 is the absorbance of the samples. The control contained $FeCl_2$ and ferrozine complex formation molecules. EDTA was used as positive control.

2.4.3. Reducing power

The reductive potential of all samples was determined by the method described by Oyaizu (1986). Different concentrations of test samples (0.31–5.00 mg/mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. Trichloroacetic acid (2.5 mL, 10%) was added to the mixture, which was then subjected to centrifugation (10 min, 1000 × *g*). The upper layer of solution (2.5 mL) was removed and mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm.

2.4.4. Hydroxyl radical scavenging assay

Hydroxyl radical-scavenging activity was measured according to Smirnoff and Cumbes's work (1989). For this, 0.5 mL $FeSO_4$ (1.5 mM) was mixed with 0.35 mL H_2O_2 (6 mM), 0.15 mL sodium salicylate (20 mM) and 1.0 mL sample (0.31–5.00 mg/mL), then

Table 1c
Analysis of L9 $(3)^4$ test results of microwave-assisted extraction (MAE).

No	A, extraction time(min)	B, extraction temperature (°C)	C, ratio of water to raw material	D, microwave power (w)	Extraction yield (%)
1	5	90	20	300	9.08
2	10	90	30	400	9.96
3	15	90	40	500	11.11
4	5	100	40	400	11.27
5	10	100	20	500	12.33
6	15	100	30	300	10.32
7	5	110	30	500	12.67
8	10	110	40	300	10.80
9	15	110	20	400	10.53
K_1	11.01	10.05	10.07	10.07	
K_2	11.03	11.31	10.98	10.59	
K_3	10.65	11.33	10.06	12.04	
R	0.38	1.28	0.92	2.03	

R refers to the result of extreme analysis.
Extraction yield (%) = (polysaccharides weight/mushroom powder weight) × 100.

Table 1d
Analysis of L9 $(3)^4$ test results of enzymatic method for extraction (EAE).

No	A, extraction time (min)	B, extraction temperature (°C)	C, ratio of water to raw material	D, cellulase concentration (wt.% of mushroom powder)	Extraction yield (%)
1	60	40	20	0.5%	7.26
2	60	50	30	1.0%	6.80
3	60	60	40	1.5%	7.94
4	120	40	30	1.5%	7.01
5	120	50	40	0.5%	7.45
6	120	60	20	1.0%	8.15
7	180	40	40	1.0%	7.71
8	180	50	20	1.5%	8.07
9	180	60	30	0.5%	8.90
K_1	7.33	7.33	7.83	7.87	
K_2	7.54	7.44	7.57	7.95	
K_3	8.23	8.33	7.70	7.67	
R	0.9	1.0	0.26	0.28	

R refers to the result of extreme analysis.
Extraction yield (%) = (polysaccharides weight/mushroom powder weight) × 100.

incubated at 37 °C for 1 h. The absorbance of the hydroxylated salicylate complex was measured at 562 nm. BHA was used as the positive control. The antioxidant activity was calculated with the following equation:

$$\text{Scavenging effect}\,(\%) = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

where A_0 was the absorbance of the solvent control, and A_1 is the absorbance of the test (sample or BHA).

2.5. Statistical analysis

All the experiments were carried out in triplicate and data were expressed as mean ± standard deviation. Statistical analysis was performed with one-way analysis of variance (ANOVA). Statistical significance of differences between the groups was assessed by Student's *t*-test. All the calculations were performed in the SAS 9.0. A level of $P < 0.05$ was taken as statistically significant.

3. Results and discussion

3.1. Optimization of the extraction conditions of the four methods

An orthogonal test was used to optimize the four extraction methods (CSE, UAE, MAE and EAE). Table 1 shows the polysaccharide yield obtained under the experimental conditions evaluated. With regard to CSE, extraction temperature had the highest *R* value (1.71) and exerted the greatest effect on the polysaccharide yield. The effects of the variables on extraction yields followed the order: extraction temperature > ratio of water to raw material > extraction time. With regard to UAE, ultrasonic power exhibited the greatest effect on the extraction yield, as evidenced by the highest *R* value (1.33), the effects of the variables on extraction yields followed the order: ultrasonic power > ratio of water to raw material > extraction temperature > extraction time. With regard to MAE, microwave power had the highest *R* value (2.03) and exerted the greatest effect on the polysaccharide yield, the effects of the variables on extraction yields followed the order: microwave power > extraction temperature > ratio of water to raw material > extraction time. With regard to EAE, extraction temperature and time exhibited higher *R* values (1.0 and 0.9, respectively). The effects of the variables on extraction yields followed the order: extraction temperature > extraction time > cellulase concentration > ratio of water to raw material.

As shown in Table 1, the optimum conditions for each process were: CSE at ratio of water to raw material of 40:1, an extraction temperature of 95 °C, and an extraction time of 90 min; UAE at ratio of water to raw material of 40:1, an extraction temperature of 40 °C, extraction power of 300 W and an extraction time of 30 min; MAE at ratio of water to raw material of 30:1, an extraction temperature of 110 °C, extraction power of 500 W, and an extraction time of 10 min; EAE at ratio of water to raw material of 20:1, an extraction temperature of 60 °C, an extraction time of 180 min, and an enzyme concentration of 1.0% (wt.% of mushroom powder).

Table 2
Comparison of the yields and basic physicochemical characteristics of polysaccharides of the four methods.

Method	CSE	MAE	UAE	EAE
Extraction temperature (°C)	95	110	30	60
Extraction power (W)	–	500	300	–
Extraction time (min)	90	10	40	180
Ratio of water to raw material (mL/g)	40	30	40	20
Yield (%)	9.68	12.67	11.46	9.02
Polysaccharide content (%)	58.63	52.58	60.10	64.31
Protein content (%)	1.64	3.86	1.36	0.96
Sugar components (mol%)				
L-Fucose	25.34	18.49	15.31	6.45
D-Mannose	18.20	20.54	24.35	19.81
D-Glucose	21.35	31.46	37.86	37.89
D-Galactose	35.11	29.35	22.48	35.85
Phenol-sulfuric acid test	+[a]	+	+	+
α-Naphthol reaction	+	+	+	+
Iodination reaction	–[b]	–	–	–
Fehling's test	–	–	–	–
Carbazole reaction	–	–	–	–
$FeCl_3$ reaction	–	–	–	–
Coomassie brilliant blue reaction	+	+	+	+

[a] Positive.
[b] Negative.

3.2. Physicochemical property analysis

Table 2 shows the efficiencies of CSE, MAE, UAE and EAE for extraction of polysaccharide from the fruit body of *F. velutipes*. MAE and UAE exhibited higher efficiencies with high yields and short extraction times, compared to CSE and EAE. Considerable pressure was built up inside the materials during the MAE process (Kratchanova, Pavlova, & Panchev, 2004). This high pressure drastically changed the physical properties of the cell wall, breaking down the structure of cells and improving the capillary-porous structure of treated tissues. High-intensity shock waves generate intense pressures, shear forces and temperature gradient due to the bubble of cavitation; indeed, this is responsible for the majority of physical, chemical and mechanical effects of ultrasound within a material, which can rupture plant cell walls and accelerate mass transfer into the solution, increasing the extraction efficiency (Chen

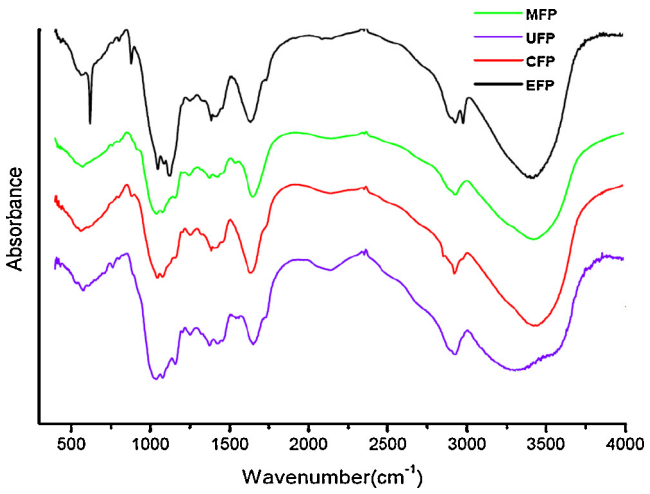

Fig. 1. Infrared spectra of the four polysaccharides from *Flammulina velutipes*.

et al., 2012). Enzymatic hydrolysis disrupts cell walls, accelerates the migration of target compounds from the material to the surroundings and increases the contact area; these effects enhance the polysaccharide extraction efficiency (Li, Zhang, Han, & Row, 2012). The heated solvent used in CSE diffused slowly through the cell walls, and dissolved and carried away the target compound. Thus, the microstructure of tissues was slightly damaged.

Neutral sugar content was highest in EFP (64.31%) then followed by UFP (60.10%), CFP (58.63%) and MFP (52.58%). Each extract contained protein and its concentration decreased in the following order: MFP > CFP > UFP > EFP. Analysis of sugar composition was performed on the four polysaccharides by GC indicated that they all consist of L-fucose, D-mannose, D-glucose and D-galactose. As shown in Table 2, the results from the phenol-sulfuric acid, α-naphthol, iodination, Fehling's, carbazole, $FeCl_3$, and Coomassie brilliant blue tests were similar for all four polysaccharides. These similarities indicated that the four extractions were polysaccharides that contained some proteins, but did not contain starch, reducing sugar, uronic acid, or polyphenols. In conclusion, the basic physicochemical properties of the four polysaccharides were similar.

3.3. FTIR analysis

FTIR spectroscopy is typically used for the qualitative measurement of organic functional groups, especially O—H, N—H, and C=O. Fig. 1 shows the FTIR spectra of the four polysaccharides from *F. velutipes*. There were stretching vibrations of O—H and saturated C—H at 3300–3500 and 2927–2930 cm^{-1}, respectively. Two amide bands, one at around 1640 cm^{-1} for Amide I (for C=O) indicated that the four polysaccharides had conjugated proteins. The group of bands extending from 1485 cm^{-1} to 1350 cm^{-1} were assigned to —CH ($O—CH_2$) flexural vibrations. The absorption band at 1000–1200 cm^{-1} suggested that the four polysaccharides contained pyranose monomers in their structures. The bands in the range of 350–600 cm^{-1} were assigned to skeletal modes of pyranose rings (Yang & Zhang, 2009).

3.4. SEM analysis

The extraction efficiency was related to physical changes in the cell wall of the plant tissue. The microstructures of *F. velutipes* tissue after extraction were observed by SEM image. The results in Fig. 2 showed that different extraction methods produced different physical changes. The cell wall of the *F. velutipes* tissue (Fig. 2A and D) was almost intact after treated by CSE and ESE. However, cell walls treated by UAE and MAE (Fig. 2B and C) were explosively damaged. It was in agreement with previous investigations (Balachandran, Kentish, Mawson, & Ashokkumar, 2006; Ying, Han, & Li, 2011). The SEM analysis provided strong evidence of the high polysaccharide extraction efficiencies of the UAE and MAE processes.

3.5. Antioxidant activity

3.5.1. DPPH scavenging effect

The DPPH radical is a stable free radical and can accept an electron or hydrogen radical to become a stable diamagnetic molecule. It has been widely accepted as a tool for estimating the free-radical scavenging activities of antioxidants (Hu, Lu, Huang, & Ming, 2004). This assay can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations (Sanchez-Moreno, 2002).

In this study, DPPH was used to determine the proton-scavenging activity of the four polysaccharides (CFP, UFP, MFP and EFP) extracted from *F. velutipes*. The dose–response curves for the four tested samples are shown in Fig. 3A. The four polysaccharides demonstrated clear scavenging activity at all tested concentrations, and their activity correlated well with concentration, increasing up to 5.0 mg/mL. UFP and MFP showed the higher activity. At 2.50 mg/mL, the scavenging activities of CFP, UFP, MFP, EFP and BHA were 58.50%, 66.75%, 64.34%, 57.93% and 99.82%, respectively. Ultrasonic and microwave treatments may induce the degradation of polysaccharide, which was supposed that the chemical groups in degrade sample have more chance to contact with radical because of the better water-soluble and more surface area (Zhang, Wang, Mo, & Qi, 2013). The effect of antioxidants on DPPH scavenging was thought to be due to their hydrogen-donating abilities. In this study, the tested samples showed scavenging activity on DPPH, which may be attributable to a strong hydrogen-donating ability.

3.5.2. Reducing power

A direct correlation between the antioxidant and reducing capacity has been reported (Amarowicz, Pege, Rahimi-Moghaddam, Barl, & Weil, 2004). Reducing properties are generally associated with the presence of reductones, which can donate a hydrogen atom and exert antioxidant action by breaking the free-radical chain (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, preventing peroxide formation (Duh, Du, & Yen, 1999). Thus, the reducing capacity of a compound or extract may be a significant indicator of its potential antioxidant activity (Fan, Li, Deng, & Ai, 2012). The reducing powers for all samples are shown in Fig. 3B, the absorbance was 0.058–0.174 for CFP, 0.068–0.158 for UFP, 0.053–0.126 for MFP and 0.054–0.099 for EFP. However, the reducing power of the four tested samples was much weaker when compared with the positive control, BHA. Reducing properties are generally associated with the presence of electron-donating groups or hydrogen atoms. These data on reducing power of the tested polysaccharides indicated that it could play a role in the antioxidant observed.

3.5.3. Metal chelating assay

Iron-chelating may produce important antioxidant effects by retarding metal-catalyzed oxidation (Kehrer, 2000). Effective Fe^{2+} chelators may also afford protection against oxidative damage by removing Fe^{2+} that may otherwise participate in HO·-generating Fenton-type reactions. Minimizing Fe^{2+} may also protect against oxidative damage by inhibiting the production of reactive oxygen species and lipid peroxidation. The Fe^{2+}-chelating capacity of the tested samples was determined by measuring iron–ferrozine complexes (Fig. 3C). The Fe^{2+} chelating effects of all

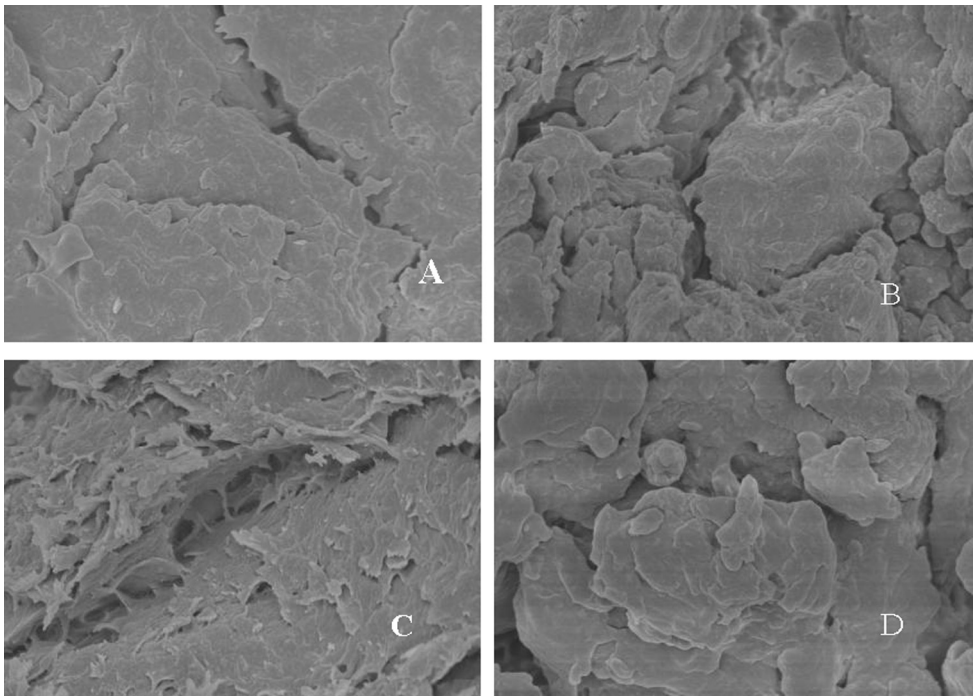

Fig. 2. Scanning electron micrographs of *Flammulina velutipes* after extraction (3000×): (A) treated by CSE (conventional solvent extraction); (B) treated by UAE (ultrasound-assisted extraction); (C) treated by MAE (microwave-assisted extraction) and (D) treated by EAE (enzymatic method for extraction).

polysaccharides were concentration-dependent. The Fe^{2+} chelating effect of EFP was the highest than the other polysaccharides, though all of them had lower activity than EDTA in the concentration range from 0.31 to 5.00 mg/mL. The chelating capability of the polysaccharides decreased as follows: EFP > CFP > MFP > UFP. Various polysaccharides exhibited remarkable binding capacity for Fe^{2+}, further demonstrating they had stronger antioxidant capability. Therefore, we hypothesize that the ferrous ions chelating

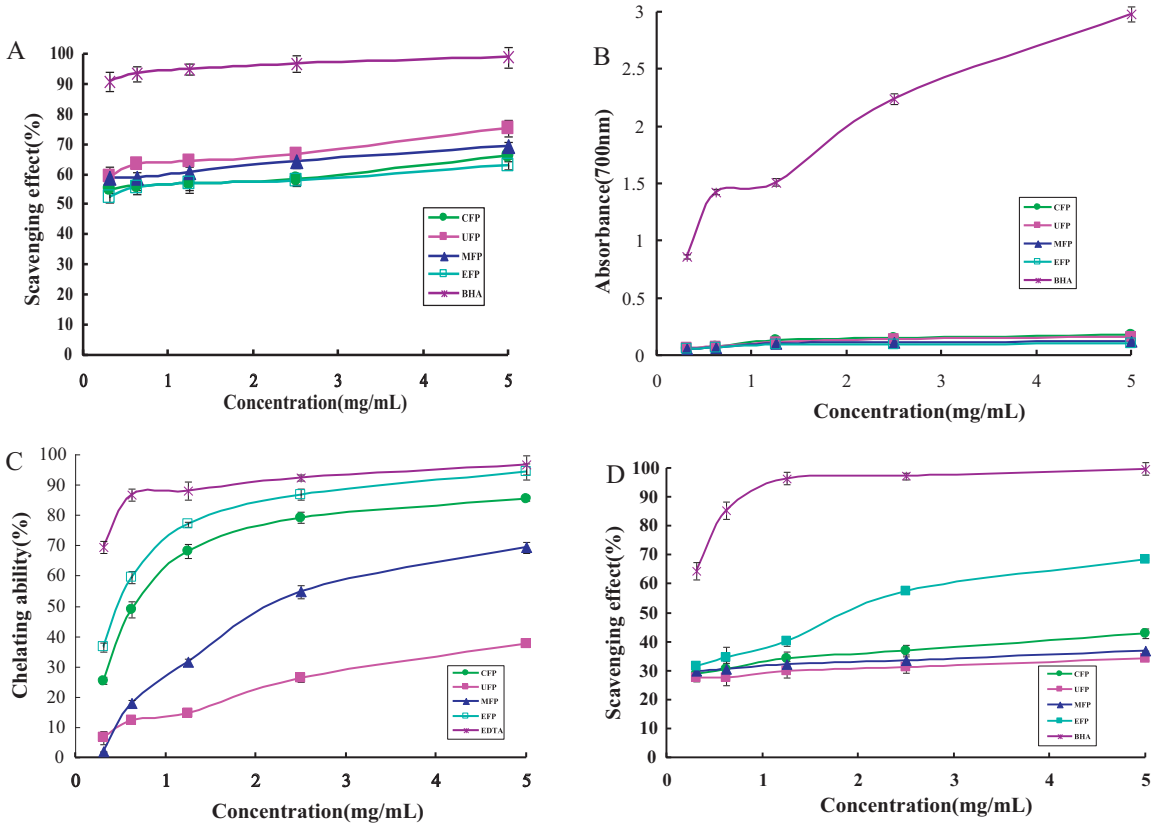

Fig. 3. Antioxidant activity of the four polysaccharides from *Flammulina velutipes*. (A) DPPH radical scavenging activity, (B) reducing power, (C) Fe^{2+} chelating activity, (D) hydroxyl radical scavenging activity. Data are the means of three replicates with standards deviations shown by vertical bars.

effects of the tested polysaccharides would be beneficial to protect against oxidative damage.

3.5.4. Hydroxyl radical scavenging activity

The mechanism of hydroxyl radical scavenging is related to the transition metal ions. In the absence of transition metal ions, hydrogen peroxide was fairy stable. However, hydroxyl radical act in superoxidation by hydrogen peroxide with metal ions, usually ferrous or copper. Molecules that can chelate iron and render them inactive in Fenton reaction may have scavenging ability against hydroxyl radical (Macdonald, Galley, & Webster, 2003). Fig. 3D shows the scavenging activities of CFP, UFP, MFP, EFP and BHA against the hydroxyl radical. The scavenging effects of the four polysaccharides were evident at all tested concentrations. EFP showed the highest scavenging effect compared to the others three polysaccharides. At 2.50 mg/mL, the scavenging activities of CFP, UFP, MFP, EFP and BHA were 36.87%, 31.21%, 33.40%, 57.39% and 97.20%, respectively. Interestingly, the hydroxyl radical scavenging activities of the four polysaccharides were similar to their metal chelating ability. These data showed that the outstanding activities of the four polysaccharides may be attributed to reducing the generation of hydroxyl radicals by chelating ferrous ion.

Among the reactive oxygen species, the hydroxyl radical is considered to be a highly potent oxidant that can react with most biomacromolecules functioning in living cells and induce severe damage to adjacent biomolecules (Halliwell, Murcia, Chirico, & Aruoma, 1995). Thus, removal of hydroxyl radical is important for antioxidant defense in cell or food systems, making hydroxyl radical scavenging is extremely important to antioxidant work. The hydroxyl radical scavenging activities of the tested polysaccharides suggested that their activity likely contributed towards observed antioxidant effects.

Previous studies have suggested that the antioxidant activities of natural polysaccharides might be related to their molecular weight, monosaccharide composition, and structure and conformation. Therefore, the antioxidant activities of polysaccharides are not a function of a single factor but a combination of several factors (Wang et al., 2012). *F. velutipes* polysaccharides have been demonstrated to exhibit antioxidant activities both *in vitro* and *in vivo* (Yang, Fang, Liang, & Hu, 2011; Zhang et al., 2003). In the present study, the antioxidant activities of the polysaccharides obtained using different extraction methods were compared using DPPH, hydroxyl, metal chelating activity and reducing-power assay. The DPPH-radical-scavenging activities of UFP and MFP were significantly greater than those of EFP and CFP. One possible mechanism is the degradation of polysaccharides and further changes in chemical structure induced by ultrasonic and microwave treatments (Yang, Zhao, Shi, Yang, & Jiang, 2008). However, UFP exhibit the weakest hydroxyl-radical-scavenging activity. This might be due to hydroxyl radical produced by acoustic cavitation in the extract due to ultrasound application (Chen et al., 2012). Because the physicochemical properties of all samples were similar, molecular weight and chemical structure might play an important role in their antioxidant activity. The mechanisms underlying the antioxidant activities of the tested samples should be investigated further.

4. Conclusions

In the present study, four polysaccharides from *F. velutipes* were extracted using hot water, ultrasonic, microwave and enzyme-based methods that were optimized by orthogonal test. Preliminary structural characterizations were conducted using physicochemical properties. Polysaccharides extracted by the four methods showed similar physicochemical characteristics and FT-IR spectra. However, SEM images of tissues from this mushroom were significantly different depending on the extraction method used. EFP demonstrated better antioxidant activities in hydroxyl and metal chelating activity assay, while UFP showed higher activity in DPPH scavenging ability. However, CFP exhibited higher antioxidant activity in terms of reducing power. These polysaccharides are clearly natural antioxidants and may be potential functional food ingredients. Further studies on the biological activities of the four polysaccharides are currently underway.

Acknowledgements

This research was supported by grants from Great Project of Science Technology Department of Zhejiang Province (No. 2011C12046), Project of Innovation and Promotion of Zhejiang Academy of Agriculture Science and the Project of Science and Technology of Jiaxing, Zhejiang Province (No. 2012AY1058). We acknowledge all staff for their valuable assistance in conducting this study.

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