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Full Length Research Paper

In vitro antioxidant properties of polysaccharides from *Armillaria mellea* in batch fermentation

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Antioxidant properties of exopolysaccharides (EPS) and intracellular polysaccharides (IPS) obtained respectively from mycelia and filtrates of submerged culture by *Armillaria mellea* in a 20-L stirred tank bioreactor were investigated. Effective production of EPS and IPS is available by submerged culture of *A. mellea* with respective number average molecular weights and protein/polysaccharide ratios as 7.68×10^6 Da and 7.68% as well as 5.65×10^6 Da and 5.26%. Both EPS and IPS exhibit powerful antioxidant activities by conjugated diene method, chelating effect on ferrous ions and scavenging effect on superoxide anion, as evidenced by their quite low EC_{50} values (< 5 mg/mL). Results confirmed that fermented *A. mellea* polysaccharides are potential antioxidant sources of both healthy medicine and food industries.

Key words: *Armillaria mellea,* intracellular polysaccharide, exopolysaccharide, antioxidant activity.

INTRODUCTION

Oxidation is essential to most living organisms for the production of energy to fuel biological processes (Sun and Kennedy, 2010; Soares et al., 2009). However, humans and other living organisms breathe oxygen to produce energy by metabolic processes, but the byproducts such as oxygen-derived free radicals and other reactive oxygen species (ROS) released by physiological processes (Ferreira et al., 2009; Shu and Lung, 2008; Song and Yen, 2002) bring about oxidative damages to initiate many diseases and degenerative processes in aging (Tsai et al., 2007; Mau et al., 2002; Liu et al., 1997). Many ROS-induced diseases related to pathological effects such as DNA damages, carcinogenesis, cancer, rheumatoid arthritis and cell degeneration associated with aging have been proven to inflict human life damages (Sun and Kennedy, 2010; Ferreira et al., 2009; Shu and Lung, 2008).Some enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase produced by organisms can protect almost all organisms from oxidative damages but these enzymes are frequently insufficient to prevent complete damages (Fang et al., 2002; Yang et al., 2002). Moreover, unbalanced mechanism of human antioxidant protection can result in many diseases and aging (Lung et al., 2010). Therefore, it is necessary to develop some potential supplements of free radical-scavenging antioxidants from some sources such as oilseeds, cereal crops, vegetables, fruits, leaves, roots, spices, herbs, and mushrooms to reduce oxidative damages (Ferreira et al., 2010, 2009; Lee and Yoon, 2009; Luo and Fang, 2008; Tseng et al., 2008; Wang and Luo, 2007).

Mushrooms, popular in Chinese medicine and food fields, are highly medicinal and nutritious. Recently, many compounds with antitumor and antioxidant potential have been isolated and identified from medicine, edible and wild mushrooms (Ferreira et al., 2010, 2009; Wasser, 2002). Moreover, these mushrooms have currently received great attention due to the therapeutic effects such as antitumor (Ferreira et al., 2010; Zhang et al., 2010), immunomodulating (Lung and Huang, 2011; Wasser, 2002; Ohno et al., 2001) and antioxidant activities (Lung et al., 2010; Ferreira et al., 2009; Shu and Lung, 2008). More evidences have been reported that many potential antioxidants can be isolated from mushroom sources. In spite of the many studies on antioxidant properties

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Abbreviations: EPS, Exopolysaccharides; IPS, intracellular polysaccharides; **BHA,** butylated hydroxyanisole; **EDTA,** Ethylenediaminetetra acetic acid; **AOA,** antioxidant activity; **DPPH,** 1, 1-diphenyl-2-picrylhydrazyl.

of mushrooms, little research has been done on antioxidant properties of submerged culture by medicinal mushroom *Armillaria mellea*. *A. mellea*, also called honey mushroom, belongs to the Tricholo-mataceae family and is an edible and medicinal mushroom. Its growth is slow and has a strong symbiotic relationship with *Gastrodia elata*, known as Tian Ma of the Orchidaceae family. It has been used as a traditional medicine in Asia to treat various human medical diseases such as headache, insomnia, neurasthenia, palsy, dizziness, numbness in limbs, infantile convulsion and microbial infectious diseases (Lung and Tsai, 2009; Yang et al., 1984). It has been reported that some bioactive components from *A. mellea* have been isolated and characterized (Sun et al., 2009; Wu et al., 2007; Gao et al., 2001; Momose et al., 2000; Kim and Kim, 1999; Kim et al., 1999), and possess potential biological activities (Sun et al., 2009; Kim et al., 1999, 2008; Ng et al., 2007; Wu et al., 2007; Yang et al., 1984). Among all bioactive components from *A. mellea*, polysaccharides are especially important due to their potential application in immune-therapeutic potential against tumors (Sun et al., 2009; Moradali et al., 2007; Li et al., 2005; Shamtsyan et al., 2004). Submerged culture is an efficient method to produce polysaccharides by many mushrooms (Lung and Huang, 2010, 2011; Shu and Lung, 2004). Antioxidant activity is one of the most important bioactivities of poly-saccharides from various mushrooms. It was reported that antioxidant properties of mushroom polysaccharides are related to their molecular weight and protein/ polysaccharide ratios (Lung and Huang, 2011; Lung and Tsai, 2009; Shu and Lung, 2008; Liu et al., 1997). Several researchers have found that many types of polysac-charides produced by submerged cultures of mushrooms possess effective antioxidant properties (Sun et al., 2010; Soares et al., 2009; Luo and Fang, 2008; Tseng et al., 2008; Tsai et al., 2007; Wang and Luo, 2007; Liu et al., 1997). However, there is so far no further information about the antioxidant properties of the polysaccharides produced by submerged culture of *A. mellea*. Therefore, it is essential to evaluate the antioxidant properties of polysaccharide, EPS and IPS, produced effectively from *A. mellea* submerged cultures for possible food supple-ment or pharmaceutical agent developments.

Evaluation of the antioxidant properties of EPS and IPS from *A. mellea* by submerged cultures is engaged in a 20-L stirred-tank bioreactor with several other comple-mentary test systems for *A. mellea* polysaccharides to investigate antioxidant activities by conjugated diene method, reducing power, scavenging ability on radicals, chelating ability on ions and superoxide anion scavenging.

MATERIALS AND METHODS

Microorganism and seed culture

A. mellea BCRC 36362 was obtained from Bioresources Collection and Research Center in Hsinchu, Taiwan. The culture was maintained in a solid culture medium with the following compositions (g /L): malt extract, 20; glucose, 40; peptone, 1 and agar, 20. The sub-culture was conducted by transferring grown mycelia to a fresh nutrient agar medium every month. The three-week-old cells grown on the media agar plate were collected with 25 mL sterilized water mixed by mycelia. 20 mL collected mycelia were then transferred to 250-mL seed culture flasks containing 50 mL culture medium (g/L) composed of PDB (potato dextrose broth), 24; thiamine, 0.01; KH_2PO_4, 1.5 and $MgSO_4$, 0.75. The seed culture was incubated at 28°C on a rotary shaker at 125 rpm for 7 days.

Culture conditions of *A. mellea*

A. mellea submerged culture products used in this study were obtained in the laboratory. The fermentation of *A. mellea* proceeded in a 20-L stirred tank bioreactors filled with 12 L culture medium and 5% (v/v) inoculums derived from seed cultures. The culture medium in the bioreactor was composed of 40.0 g/L glucose, 3.0 g/L yeast extract, 4.0 g/L KH_2PO_4 and 2.0 g/L $MgSO_4$. The stirred tank bioreactor culture was operated at 22°C, 1vvm (volume of aeration per volume of bioreactor per minute) aeration rate, 150 rpm agitation speed and controlled pH 4.0 for 14 days. The pH of culture medium was automatically controlled by adding 1 N HCl or 1 N NaOH. Mycelia were separated from fermented broth by centrifugation (4°C, 8000×g for 15 min), then washed with distilled water and finally freeze-dried to powders. Biomass concentration was determined in dry weight per unit volume. Residual sugar in the supernatant was determined by the dinitrosalicylic acid method (Miller, 1959). The polysaccharide concentration in the supernatant was determined by phenol-sulphuric acid assay (Dubois et al., 1956). All experiments were performed in three duplicates (n=3).

Preparation of polysaccharide samples

Intracellular polysaccharide (IPS) was isolated from cultured mycelia of *A. mellea*. Cultured mycelia were extracted with boiling water for 1 h and then filtered through filter paper (Whatman No 1). Filtrates were precipitated with four volumes of 95% (v/v) ethanol, and left overnight at 4°C. Precipitated polysaccharides were centrifuged at 10,000×*g* for 10 min, and the supernatant was discarded. The polysaccharide precipitate was redissolved in deionized water and pretreated by membrane filtration (MWCO 8 kDa). The polysaccharide solution (Mw > 8 kDa) were precipitated with four volumes of 95% (v/v) ethanol, and stood overnight at 4°C. The precipitated polysaccharides were isolated by centrifuge at 10,000×*g* for 10 min, and then the supernatant was discarded. The precipitate was lyophilized into powders. The powder was redissolved in deionized water to a concentration of 50 mg/mL and stored at 4°C for further use.

Exopolysaccharides (EPS) were isolated from the mycelia-free cultured broth of *A. mellea* by precipitation with four volumes of 95% (v/v) ethanol. The sequent isolation process was the same as that of the IPS. Dried EPS powders were dissolved in deionized water to a solution of 50 mg/mL concentration and stored at 4°C for further experiments.

Determination of polysaccharide molecular weight and protein content in polysaccharide

Molecular weights of polysaccharides were determined by gel permeation chromatography (GPC) of Waters (Milford, MA, USA) 600E system equipped with a GPC column (Shodex OHpak SB-804HQ) and a model 410 RI detector. All chromatographic data were processed by Millennium (Milford, MA, USA) software.

Polyethylene glycol (PEG) standards (Polymer Laboratories, Church Stretton, UK) with narrow polydispersity and with molecular weights ranging from 1.9 to 1260 kDa constructed a calibration curve. Polysaccharide samples for molecular weight testing were pretreated by membrane filtration (MWCO 8 kDa) before injection. Deionized water was used as the mobile phase at a flow rate of 0.6 mL/min. Protein contents in polysaccharides were determined by the Lowry method (Lowry et al., 1951).

Antioxidant activity

The antioxidant activity assay was examined by the conjugate diene method (Lingnert et al., 1979). Each polysaccharide sample (0–20 mg/mL) of 0.5 mL was mixed with 2.5 mL 0.01 M linoleic acid emulsion in 0.2 M phosphate buffer (pH 6.5), and then placed in darkness at 37℃ to accelerate oxidation. After incubation for 15 h, 6 mL of 60% methanol in deionized water was added to the mixture, and the absorbance of the mixture solution was measured at 234 nm in a spectrophotometer (JASCO V-530, Japan). The antioxidant activity (AOA) was calculated with the following equation:

AOA (%) = [(ΔA_{234} of control - ΔA_{234} of sample)/ ΔA_{234} of control] × 100.

AOA value of 100% corresponds to the strongest antioxidant activity. EC_{50} value in mg extract/mL expresses the effective concentration at which the antioxidant activity is 50% and is obtained by linear regression interpolation. Butylated hydroxyanisole (BHA), α-tocopherol, and ascorbic acid were used for comparison. Each value was presented as mean ± standard deviation (n=3) of triplicate measurements.

Reducing power

The reducing power was determined with the method described by Oyaizu (1986). Each polysaccharide sample (0–20 mg/mL) in methanol and deionized water (2.5 mL) was mixed with 2.5 mL 200 mM sodium phosphate buffer at pH 6.6 and 2.5 mL of 1% (w/v) potassium ferricyanide, and the mixture was incubated at 50℃ for 20 min. After 2.5 mL of 10% (w/v) trichloroacetic acid was added, the mixture was centrifuged at 200×g for 10 min. The upper layer (5 mL) was mixed with 5 ml of deionized water and 1 ml of 0.1% (w/v) ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. EC_{50} value in mg extract/mL expresses the effective concentration at which the absorbance is 0.5 in the reducing power assay and is interpolated by linear regression. Ascorbic acid and α-tocopherol were used as standards. Data were presented as mean ± standard deviation (n=3) of triplicate measurements.

$DPPH^{\cdot}$-scavenging activity

A total of 4 mL polysaccharide samples of various concentrations (0–20 mg/mL) were mixed with 1 mL methanolic solution containing 1 mM DPPH radicals and resulted in a final concentration of 0.2 mM DPPH radicals. The mixture was shaken vigorously and left to stand for 30 min in darkness. DPPH radical reduction was determined by measuring the absorbance at 517 nm against a blank (Shimada et al., 1992). The scavenging ability was expressed as follows:

[(ΔA_{517} of control - ΔA_{517} of sample)/ ΔA_{517} of control]×100.

EC_{50} value in mg extract/mL expresses the effective concentration at which DPPH radicals are 50% and are obtained by linear regression interpolation. BHA, α-tocopherol and ascorbic acid were used as standards. Data were expressed by triplicate measurement with standard deviation.

Chelating effects on ferrous ions

Chelating ability was examined according to the method of Dinis et al. (1994). Each polysaccharide sample (0-20 mg/mL, 1 mL) was mixed with 3.7 mL methanol and 0.1 mL 2 mM ferrous chloride. The mixture was then reacted with ferrozine (0.2 mL, 5 mM) for 10 min. Each value was expressed by triplicate measurement with standard deviation. With the absorbance reading at 562 nm (A_{562}), chelating activities on ferrous ions were calculated by the following equation:

Chelating effect (%) = [1-(ΔA_{562} of sample)/(ΔA_{562} of control)] ×100%.

A lower absorbance indicates a higher chelating power. EC_{50} value in mg extract/mL expresses the effective concentration at which ferrous ions are chelated by 50% and are obtained by linear regression interpolation. Ethylenediaminetetraacetic acid (EDTA) and citric acid were used as standards. The assay was performed in three duplicates and expressed as mean ± standard deviation (n=3)

Scavenging effects on superoxide anions

Scavenging capacity of polysaccharide samples on superoxide anions was assayed by the method of Robak and Gryglewski. (1988), in which reduction of nitro blue tetrazolium (NBT) was measured and superoxide anions were generated in the PMS-NADH system. Identical volumes of the sample, 30 μM phenazine methosulfate (PMS), 338 μM dihydro nicotineamide adenine dinucleotide (NADH) and 72 μM NBT in 0.1 M phosphate buffer of pH 7.4 were mixed and incubated for reaction at the ambient temperature for 5 min. The absorbance was measured at 560 nm against blank samples. The scavenging capability to superoxide radicals was calculated as follows:

[(A_{560} of control - A_{560} of sample)/ A_{560} of control]×100.

EC_{50} value in mg extract/mL expresses the effective concentration at which the scavenging superoxide anion activity is 50% and is interpolated by linear regression. Ascorbic acid was used for comparison. The results were expressed as mean ± standard deviation by triplicate measurement.

RESULTS AND DISCUSSION

Fermentation results of *Armillaria mellea* polysaccharides

Polysaccharides (EPS and IPS) by *A. mellea* submerged culture were produced in a 20-L stirred tank bioreactor after 14 days, and their fermentation results are listed in Table 1. The maximum EPS and IPS productions are 588.30 and 704.54 mg/L, respectively, which correspond to the EPS productivity of 42.02 mg/L/day and the IPS

Table 1. Fermentation results of exopolysaccharide (EPS) and intracellular polysaccharide (IPS) from submerged culture of *Armillaria mellea*.

Sample	Q_P[a] (mg/L/d)	P_{max}[b] (mg/L)	P_{max} content[c] (mg/g)	$Y_{P/X}$[d] (mg/g)	$Y_{P/S}$[e] (mg/g)	t[f](d)
EPS[g]	42.02	588.30 ±17.5[i]	-	40.35	32.47	14
IPS[h]	50.33	704.54 ±18.3	54.04 ±3.9	-	30.63	14

[a]Q_P: polysaccharide productivity (mg/L/day); [b]P_{max}: maximum polysaccharide product concentration (mg/L); [c]P_{max} content: IPS content in dry cell (mg IPS/g biomass); [d]$Y_{P/X}$: specific product yield (mg EPS/g biomass); [e]$Y_{P/S}$: product yield (mg EPS or IPS/g glucose); [f]t: culture time (day); [g]EPS: exopolysaccharide; [h]IPS: intracellular polysaccharide. [i]Data are expressed as mean ± standard deviation (n = 3).

Table 2. Number average molecular weight (Mn) and protein content of exopolysaccharide (EPS) and intracellular polysaccharide (IPS) from submerged culture of *Armillaria mellea*.

Sample	Number average molecular weight (Mn) (Da)	protein/polysaccharide (%, w/w)
EPS	7.68×10^6	7.68
IPS	5.65×10^6	5.26

productivity of 50.33 mg/L/day. The production yield ($Y_{P/S}$, mg EPS/g glucose) of EPS is 32.47 mg/g and its specific product yield ($Y_{P/X}$, mg EPS/g biomass) is 40.35 mg/g. In addition, the P_{max} content (mg IPS/g dry mycelia weight) and production yield ($Y_{P/S}$, mg IPS/g glucose) of IPS are 54.04 and 30.63 mg/g, respectively. These results confirm the effective production of EPS and IPS by submerged culture of *A. mellea* in a 20-L stirred tank bioreactor.

Molecular weight and protein content of *Armillaria mellea* polysaccharides

Polysaccharides isolated from higher fungi such as mushrooms are usually associated with protein as complexes. Protein in polysaccharides seems to play an important role in bioactivities. The biological properties of polysaccharides are closely related to the chemical structure and molecular weight (Lung and Huang, 2011; Lung and Tsai, 2009; Ohno et al., 2001). Polysaccharides with high molecular weight and protein in polysaccharide seem to have relatively high biological activities (Lung and Huang, 2011; Lung and Tsai, 2009; Ohno et al., 2001). Recently, antioxidant properties of polysaccharide from mushroom sources have been extensively studied. Although, the antioxidant mechanism of polysaccharides are not fully understood, many reports have proposed correlations among antioxidant properties of polysaccharides and the corresponding chemical components, molecular weights, structures, conformations and protein/ polysaccharide ratios. Molecular weight and protein content of EPS and IPS are shown in Table 2. The number-average molecular weight (Mn) and protein/ polysaccharide ratios (%, w/w) of EPS are 7.68×10^6 Da and 7.68% and those of IPS are 5.65×10^6 Da and 5.26% respectively. Both EPS and IPS exhibit high number-average molecular weights (Mn) and protein/ polysaccharide ratios. In addition, polysaccharides (EPS and IPS) reveal good antioxidant properties in all tested systems (Table 3). Therefore, the good antioxidant properties of EPS and IPS might be related to the high number-average molecular weights (Mn) and protein/ polysaccharide ratios.

Antioxidant activity

Antioxidant activities of EPS and IPS isolated from mycelia and broth of *A. mellea* batch cultures were evaluated by the conjugated diene method and their dose-course profiles are illustrated in Figure 1. Antioxidant activities of EPS and IPS exhibit different patterns and positively correlate with concentrations (Figure 1). At 10 mg/mL, antioxidant activities of EPS and IPS are 74.32 and 65.24%, respectively. EPS is higher in antioxidant activity than IPS for all tested concentrations. However, BHA, α-tocopherol and ascorbic acid show higher antioxidant activities of 98.89, 95.95 and 90.22% at 10 mg/mL, respectively. The analysis principle of antioxidant activity is principally the estimation of the inhibition ability of linoleic acid peroxidation. Antioxidant activity of antioxidants might be attributed to some mechanisms such as preventing chain initiation, binding transition metal ion, decomposing peroxides, preventing continued hydrogen abstraction, reducing ability and scavenging free radical (Gülçin et al., 2003; Mau et al., 2002). Therefore, the antioxidant activities of EPS and IPS in this study might partially be as a result of hydroperoxide reduction, free radical inactivation, or forming metal ions complex, or the combination thereof.

Reducing power

Reducing powers of EPS and IPS from *A. mellea*

Table 3. EC_{50} values of exopolysaccharide (EPS) and intracellular polysaccharide (IPS) from submerged culture of *Armillaria mellea*.

Antioxidant property	EC_{50} (mg/mL)						
	Polysaccharide		Standard				
	IPS	EPS	BHA	Ascorbic acid	α-Tocopherol	EDTA	Citric acid
Antioxidant activity	4.66±0.12	3.45±0.03	0.066±0.004	1.64±0.06	0.078±0.004	-	-
Reducing power	4.52±0.08	7.28±0.16	-	0.23±0.02	0.25±0.03	-	-
Scavenging effect on DPPH radicals	7.61±0.22	6.11±0.13	0.033±0.003	72.85±3.25	0.26±0.02	-	-
Chelating effect on ferrous ions	0.12±0.01	3.04±0.05	-	-	-	0.048±0.005	66.90±3.25
Scavenging effect on superoxide anion	2.91±0.11	1.09±0.02	-	0.49±0.04	-	-	-

EC_{50} value: The effective concentration at which the antioxidant activity was 50%; the absorbance was 0.5 for reducing power; the DPPH radicals were scavenged by 50%; the ferrous ions were chelated by 50%; and the superoxide anion were scavenged by 50%, respectively. EC_{50} value was obtained by interpolation from linear regression analysis. BHA, Butylated hydroxyanisole; EDTA, Ethylenediaminetetraacetic acid.

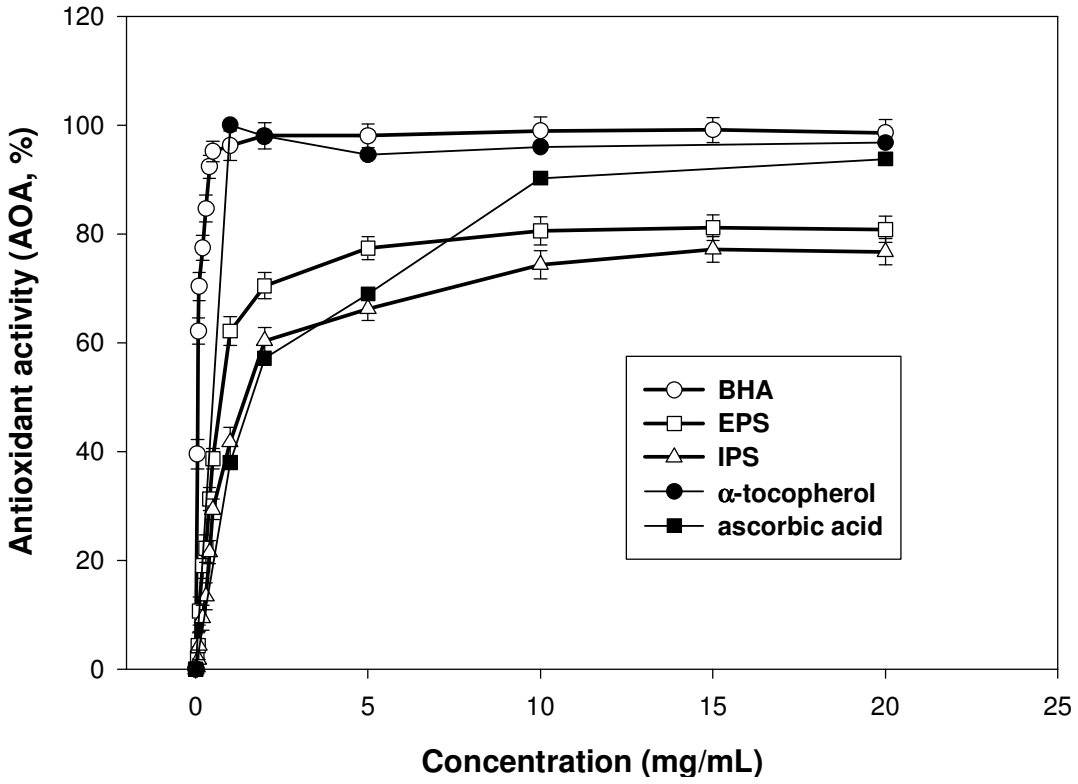

Figure 1. Antioxidant activities of exopolysaccharide (EPS) and intracellular polysaccharide (IPS) from submerged culture of *Armillaria mellea*. Each value is expressed as mean ± standard deviation (n = 3). BHA: butylated hydroxyanisole.

submerged culture determined at 700 nm are depicted in Figure 2. Reducing powers of EPS and IPS increase rapidly with the concentration increases. Reducing powers of EPS and IPS are 0.72 and 1.52 at 10 mg/mL, and 1.33 and 2.53 at 20 mg/mL. EPS is lower in reducing power than IPS. Ascorbic acid and α-tocopherol exhibit excellent reducing power of 1.00 and 1.03 at 0.5 mg/mL, respectively. Shimada et al. (1992) reported that reducing powers of various extracts might be due to hydrogen-donating. Our data on the reducing capacities of EPS and IPS suggest that reductone-associated and hydroxide groups of polysaccharides can act as electron donors to

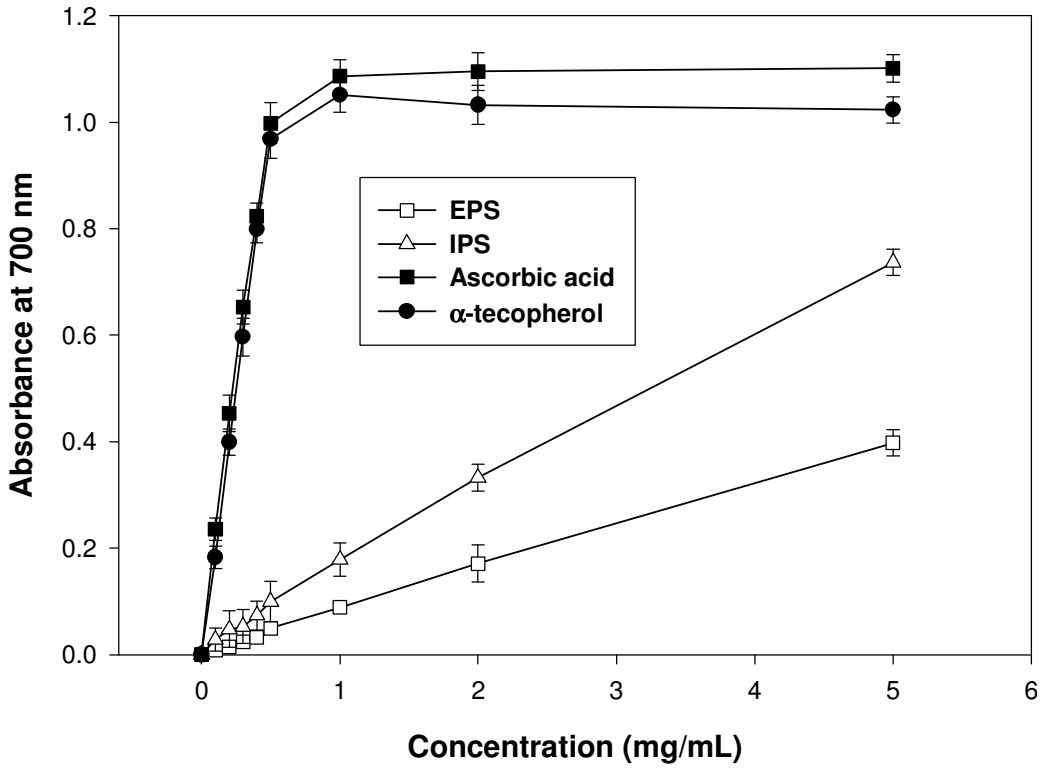

Figure 2. Reducing power of exopolysaccharide (EPS) and intracellular polysaccharide (IPS) from submerged culture of *Armillaria mellea*. Each value is expressed as mean ± standard deviation (n = 3).

react with free radicals to form stable products and thereby terminate radical chain reactions. The reducing capacity is a significant index of antioxidant activity (Meir et al., 1995). Various mechanisms related to antioxidant activities include chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Gülçin et al., 2003). Furthermore, fermented EPS and IPS of *A. mellea* might contain reductones which can react with certain precursors of peroxides to prevent peroxide formation.

Scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The free radical-scavenging activities of polysaccharide samples are determined by the method of Shimada et al. (1992). DPPH method is usually used to evaluate antioxidant activities of various natural compounds by reducing stable DPPH radicals to yellow-colored diphenylpicrylhydrazine. DPPH radical scavenging is readily responsible for the hydrogen-donating efficiency of antioxidants. Scavenging DPPH radical activities of EPS and IPS are shown in Figure 3 with compared results to the control standards of BHA, α-tocopherol, and ascorbic acid. The higher the concentration, the higher the scavenging abilities of EPS and IPS used in the test. At 10 mg/mL, scavenging effects of EPS and IPS are 81.43 and 66.44%, respectively; however, those of EPS and IPS are 84.41 and 76.07% at 20 mg/mL. α-tocopherol has a scavenging effect of 39.57% at 10 mg/mL and scavenging effects of BHA and ascorbic acid are 89.54 and 97.42% at 0.5 mg/mL, respectively. Scavenging effects of EPS are higher than those of IPS. EPS and IPS are higher in scavenging effects than α-tocopherol but lower than BHA and ascorbic acid. Wong and Chye (2009) reported that antioxidant components with high hydrogen-donating abilities can effectively scavenge free radicals, which are the major propagator of auto oxidation chain of fat, thereby terminating the chain reaction. Therefore, EPS and IPS might be potent scavengers with antioxidative activities attributed to proton-donating abilities. EPS and IPS are rich in reductones to react with radicals to stabilize and terminate radical chain reactions.

Chelating effects on ferrous ions

Ferrozines can easily react with ferrous ions to form red Fe^{2+}-ferrozine complexes. The addition of chelating agents in the reaction solution interferes with the formation of complexes and results in complex reduction.

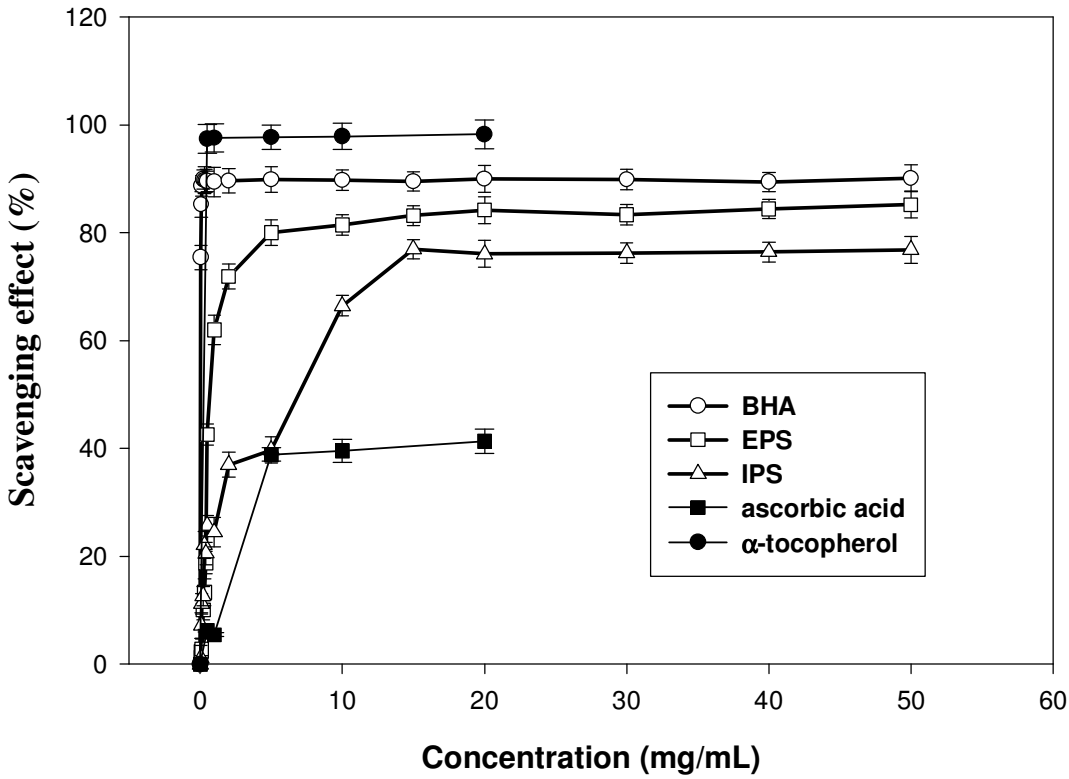

Figure 3. Scavenging effect of exopolysaccharide (EPS) and intracellular polysaccharide (IPS) from submerged culture of Armillaria mellea on 1, 1-diphenyl-2-picrylhydrazyl radical. Each value is expressed as mean ± standard deviation (n = 3). BHA: butylated hydroxyanisole.

The chelating abilities of polysaccharides can be estimated by concentration measurements of the complex. Chelating effect on ferrous ions is one important antioxidant activity mechanism. In addition, chelating abilities are found to be related to the concentration of catalyzing transition metals in lipid peroxidation and this indicates that higher chelating ability brings about lower concentration of catalyzing transition metals. Figure 4 shows the chelating the effects of EPS, IPS, EDTA and citric acid on ferrous ions. EPS, IPS and EDTA excluding citric acid are found to have higher potent chelating abilities on Fe^{2+} and are concentration dependent from 0 to 5.0 mg/mL. At 1.0 mg/mL, chelating abilities of EPS and IPS are 73.83% and 85.77%, respectively but rise to 88.58% and 92.18% at 5.0 mg/mL. However, EDTA exhibit excellent chelating abilities of 92.55% at 1.0 mg/mL and 93.62% at 5.0 mg/mL. Ferrous ions are known as the most effective prooxidant among various species of metal ions due to the high reactivity, which accelerates lipid oxidation by breaking down hydrogen and lipid peroxidase to reactive free radicals via the Fenton type reaction. Metal chelating activity is claimed as one antioxidant mechanism, since it reduces the concentration of the catalyzing transition metal in lipid peroxidation (Qiao et al., 2009). Therefore, IPS and EPS from *A. mellea* in submerged culture are good sources of chelators for ferrous ions.

Scavenging effects on superoxide radical

Superoxide radical is a highly toxic species generated by numerous biological and photochemical reactions (Banerjee et al., 2005) and has been thought to be one of the extremely harmful reactive oxygen species that induce oxidants in biomolecules. Superoxide radical is also a precursor that can convert superoxide and H_2O_2 to more reactive species such as hydroxyl radical. Superoxide radicals in this study are originated in PMS/NADH system and assayed by the reduction of NBT. As shown in Figure 5, the scavenging abilities of EPS, IPS and ascorbic acid on superoxide radicals are obviously correlated well with concentration increases. EPS leads IPS in superoxide radical scavenging activity for all concentrations. At 500 µg/mL, superoxide radical scavenging activities of EPS and IPS are 25.69 and 9.74%, respectively but at this concentration, ascorbic acid reaches a considerably high superoxide radical scavenging activity of 50.93%. EPS is apparently higher effective in superoxide radical scavenging than IPS. Although, superoxide radical is a weak oxidant, it decomposes to a stronger reactive oxidative species, such as singlet oxygen and hydroxyl radical with greater oxidative and oleophilic ability than the precursor to initiate lipid peroxidation for longer period (Sun and

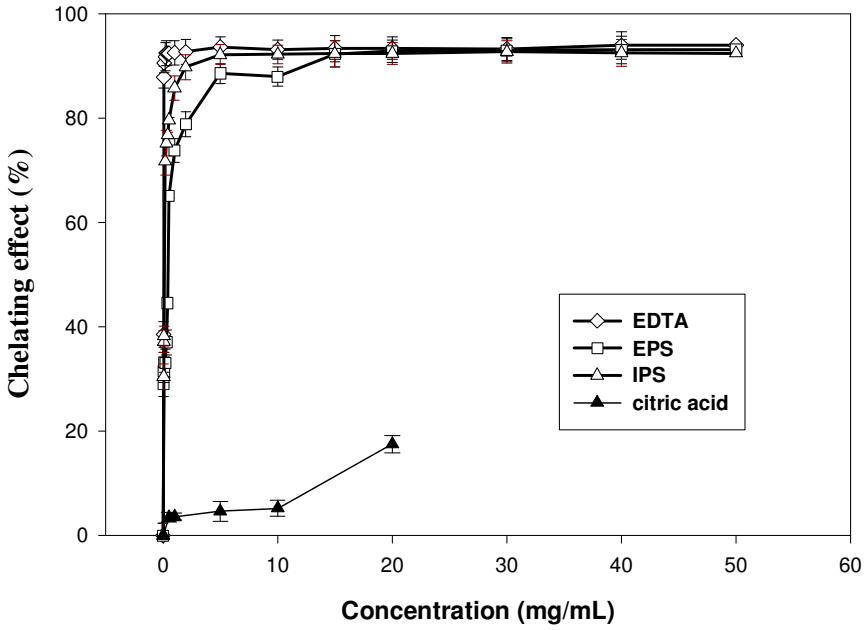

Figure 4. Chelating effect of exopolysaccharide (EPS) and intracellular polysaccharide (IPS) from submerged culture of *Armillaria mellea* on ferrous ions. Each value is expressed as mean ± standard deviation (n = 3). EDTA: ethylenediaminetetraacetic acid.

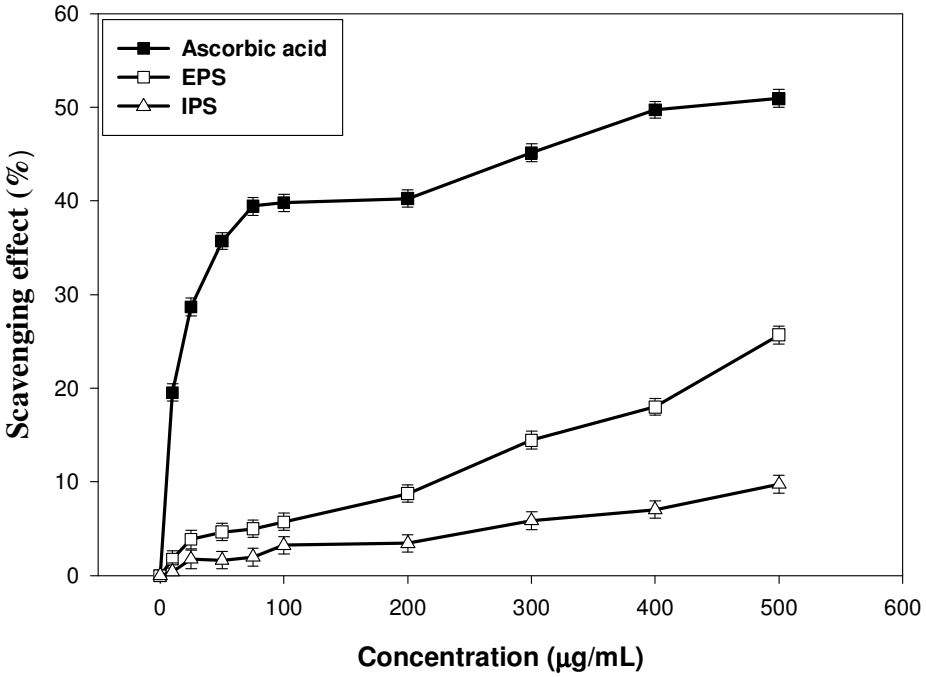

Figure 5. Scavenging effect of exopolysaccharide (EPS) and intracellular polysaccharide (IPS) from submerged culture of *Armillaria mellea* on superoxide anion. Each value is expressed as mean ± standard deviation (n = 3).

Kennedy, 2010). Superoxide radical and its derivatives can cause damages to DNA and cell membrane. These results clearly indicate that the antioxidant activities of all samples are related to the abilities of scavenging superoxide radicals. It is of vital importance to scavenge superoxide radicals.

EC_{50} values in antioxidant properties

The antioxidant properties assayed herein are summarized in Table 3 and the results are calculated and expressed as EC_{50} values for comparison. High EC_{50} values correspond to be weak antioxidant properties. Values below 10 mg/mL are considered as effective antioxidant activities (Liang et al., 2009). EC_{50} values of EPS and IPS by the conjugated diene method are 3.45 and 4.66 mg/mL, respectively. EPS and IPS are effective in antioxidant activities as evidenced by their low EC_{50} values under 5.0 mg/mL. EC_{50} values of BHA and α-tocopherol are less than 0.08 mg/L, whereas that of ascorbic acid is 1.64 mg/mL.

EC_{50} values of EPS and IPS concerning reducing power are 7.28 and 4.52 mg/mL, respectively. The reducing power of IPS is more effective than that of EPS. Ascorbic acid and α-tocopherol have excellent reducing powers of 0.25 and 0.23 mg/mL, respectively.

EC_{50} values obtained for EPS and IES in DPPH radical scavenging are 6.11 and 7.61 mg/mL, respectively. Both values are below 10 mg/mL, and indicate the high effectiveness in DPPH radical scavenging. BHA and α-tocopherol exhibit powerful scavenging DPPH radical effects with respective EC_{50} values of 0.033 and 0.26 mg/mL, but ascorbic acid is weak in DPPH radical scavenging because EC_{50} is over 20 mg/mL.

For chelating effects on ferrous ions, EC_{50} values of EPS and IES are 3.04 and 0.12 mg/mL, respectively. EPS is apparently less effective in chelating effect than IPS. EC_{50} value of EDTA is 0.048 mg/mL and citric acid over 20 mg/mL. Therefore, IPS is more effective in chelating effect and remotely comparable with EDTA.

EC_{50} values of EPS and IPS concerning scavenging activity on superoxide radicals are 1.09 and 2.91 mg/mL, respectively, and both scavenging activities are excellent due to their low EC_{50} below 5 mg/mL. Ascorbic acid shows an exceptional scavenging activity for the EC_{50} value of 0.49 mg/mL. The scavenging activity of EPS on superoxide radical is apparently comparable to ascorbic acid.

In this study, all EC_{50} values of EPS and IPS related to antioxidant properties are below 10 mg/mL, indicating the effective antioxidant properties for both EPS and IPS. As compared with IPS, EPS with higher number-average molecular weight (Mn) of 7.68×10^6 Da and protein/polysaccharide ratios of 7.68% is superior in antioxidant activity by the conjugated diene method, scavenging effect on DPPH radicals and superoxide anion. However, IPS is dominant in reducing power and chelating effect on ferrous ions for its number-average molecular weight (Mn) of 5.65×10^6 Da and protein/polysaccharide ratios of 5.26%. Antioxidant properties of polysaccharides seem to be related to their number-average molecular weight and protein/polysaccharide ratios. Song et al. (2010) pointed out that higher antioxidant activities appeared when the molecular weight and protein content increased. The results suggested that polysaccharides isolated from submerged cultures of *A. mellea* were found to have different *in vitro* antioxidant properties owing to their molecular weight and protein/polysaccharide ratios.

Conclusion

EPS and IPS are isolated from broth and mycelia of *A. mellea* by submerged culture in a 20-L stirred tank bioreactor with experiment results clearly demonstrating that EPS and IPS are highly effective in antioxidant property as evidenced by their quite low EC_{50} values. Therefore, EPS and IPS from *A. mellea* by submerged culture can be developed as new antioxidants for potential applications in pharmaceutical and food industries. Up till now, the antioxidant mechanism of *A. mellea* polysaccharides is not fully clear yet and it is an absolute necessity for the development of the correlation between the chemical characteristics and the antioxidant property of polysaccharide isolated from submerged culture of *A. mellea* for further investigation.

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