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Antioxidant Capability of Polysaccharides Fractionated from Submerge-Cultured *Agaricus blazei* Mycelia

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Five polysaccharide fractions (AB-1, AB-2, AB-3, AB-4, and AB-5) were obtained after a systemic solvent extraction and precipitation of *Agaricus blazei* mycelia with yields of 5.20, 9.03, 2.84, 17.47, and 0.44%, respectively. Among which, AB-1 and AB-3 demonstrated great DPPH• radical scavenging ability around 85.0 and 72.0%, respectively, at a concentration of 5 mg/mL. The ferrous ion chelating powers were even more excellent at a concentration of 1 mg/mL, reaching almost over 99.65% for fractions AB-2, AB-3, and AB-4 as compared to the reference control of citric acid (35%); at a dosage of 5 mg/mL, fraction AB-1 even showed 105% in efficiency. In terms of the absolute chelating power (ACP), the mole numbers of ferrous (Fe^{2+}) ions chelated were inversely related with the mean molecular mass of the polysaccharides in each fraction. In addition, gel permeation chromatography analysis showed that the more potent fractions were residing in those fractions with lower molecular masses, such as fractions AB-1 (757 kDa), AB-2 (887 kDa), and AB-4 (631 kDa) etc., and surprisingly, the free radical scavenging capability was also not closely correlated with the mean molecular masses, alternately being well-associated with the solubility of polysaccharides.

KEYWORDS: ***Agaricus blasei* Murill; mycelia; polysaccharides; GPC; DPPH; Fe^{2+}; ACP_{50}**

INTRODUCTION

Agaricus blasei Murill (ABM), popularly named Brazil mushroom, is one species of edible mushrooms. Recently, much of the literature was cited with its unique biological activity as both a nutraceutic and a supplementary medicine. The constituents isolated from its fruit bodies consist of steroids (*1*), lipids (*2*), polysaccharide−protein complexes (*3*, *4*), and polysaccharides (*5*−*7*), etc.; among them, the unique characteristics of its polysaccharides have drawn much attention. Polysaccharide (fraction FIII-2-b) from the fruiting bodies of *A. blazei* has been reported to possess antitumor activity against Sarcoma 180-bearing mice (*7*). These results seem to show that the polysaccharides from *A. blazei* initiate antitumor activity through a modulation of the immune response system in tumor-bearing mice.

A previous report (*3*) has indicated that macrophage activation and an alteration of the third component are necessary for the induction of an antitumor effect of polysaccharide isolated from *A. blazei* mycelium. In addition, hot water extract from mycelia has shown an enhanced c-Jun/AP1 expression in the human breast cancer cell line MCF7 (*8*). Apparently, no matter what polysaccharides are isolated from the mycelium or fruiting body of *A. blazei*, they all have the immune system modulating and antitumor activity in living organisms.

A variety of methods have been developed to isolate the anticancer polysaccharides from the fruiting bodies, mycelia, and liquid media of mushrooms. Most of these involved simple extractions with hot water (*9*, *10*); the drawback thus has been suspected to be an incomplete extraction with loss of some active fractions, or more crucially, some tightly bound forms unextractable by such a simple treatment (*11*). Mizuno obtained five fractions from the ABM mycelia by their uniquely developed isolation process, which included serial treatments with hot water, ammonium oxalate, 5% NaOH, acetic acid, and ethanol. Each fraction had a characteristic structure, molecular mass, monosaccharide moiety, and biological activity (*11*), indicating that different solvent systems may yield quite different bioactive polysaccharide fractions. On the basis of such findings, to our knowledge, no gel permeation chromatography (GPC) had been applied in such related investigations. In this study, we tried to develop our unique systemic solvent extraction scheme, which involved serial fractionations using hot water, isoelectric

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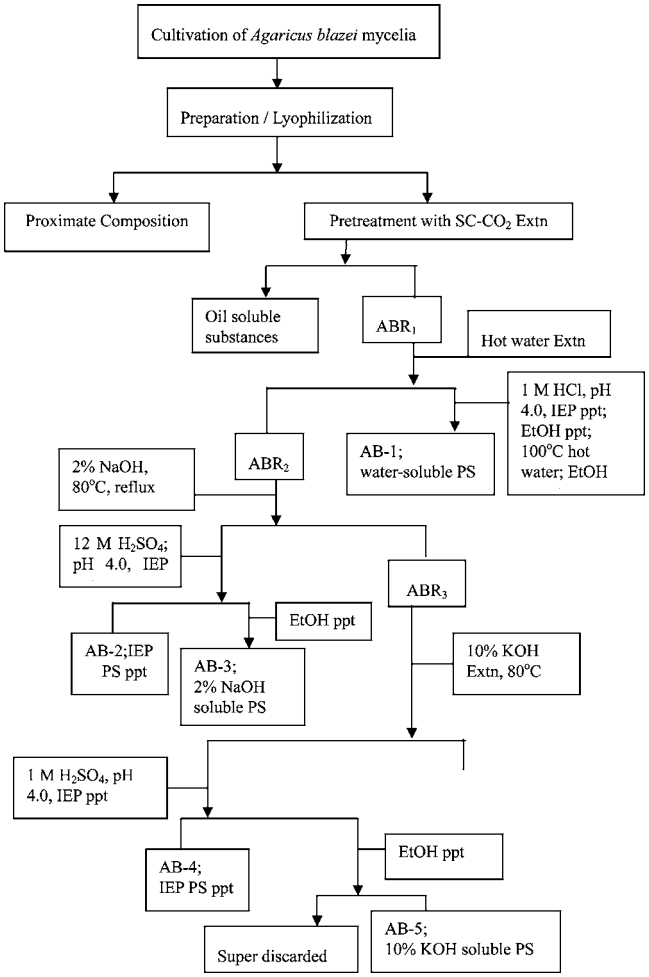

Figure 1. Flowchart for preparation of polysaccharide fractions from *A. blazei* mycelia.

precipitation, 5% NaOH, 10% KOH, and/or then combined with isoelectric precipitation; the fractions obtained were further analyzed by their molecular masses using the GPC technology and finally tested with their physiochemical properties, molecular size determination, and their associated antioxidant capabilities.

MATERIALS AND METHODS

Pretreatment and Extraction of AB Mycelia. The flowchart for the whole protocol of fractionation of polysaccharides from ABM mycelia used in this investigation is illustrated in **Figure 1**.

Microorganism and Cultivation. *A. blazei* ATCC 76739 was grown according to the previously modified procedure (*12*). In brief, *A. blazei* was transferred from a 7 day old seeding mycelium in a PDA medium, in which a piece of 0.5 cm × 0.5 cm of mycelial lump was inoculated into a 500 mL flask. A total of 250 mL of liquid culture (pH 4.0) that comprised (%, w/w) 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 2% glucose was used for the mycelial culture. After cultivation at 25 °C for 7 days, the inoculum (4.5 mL) was added to 1 L of the same medium in a 2 L flask for another seeding culture. The cultivation was continued under the same conditions, and 18 mL of the inoculum was added to 150 L of medium (pH 4.0), which had been previously placed in a 200 L stirred fermentor. The 150 L medium (pH 4.0) comprising (%, w/w) 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1% glucose (pH 4.0) was used for mycelial cultivation and to prepare the mycelial samples. The operational conditions (temperature, 28 °C; duration, 10 days; aeration rate, 150 vvm; and shaking rate, 70 rpm) were maintained until the residual glucose content reached 200 ppm. Thereafter, the mycelia were filtered off, and the residue was rinsed with deionized water for several times and then lyophilized for further tests.

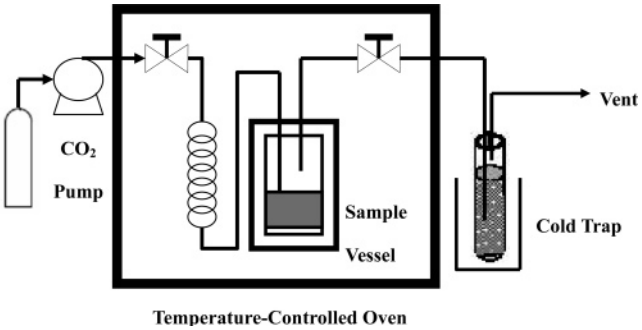

Figure 2. Apparatus for supercritical fluid carbon dioxide extraction (SC−CO_2E).

Pretreatment of the Mycelial Powder. The entire flowchart of analyses is shown in **Figure 1**. Prior to the extraction of polysaccharides, a supercritical fluid carbon dioxide (SC−CO_2) (99.5% in purity) extraction (**Figure 2**) (*13*) was performed to remove some inherently existing oil soluble substances to minimize the interference on the extractability of polysaccharides. Briefly, 100 g of the lyophilized mycelial powder was weighed and added with 5% *n*-hexane to serve as a modifier. The extraction was carried out at 60 °C and 5000 psi in a SCF extraction apparatus (ISCO SFX 2-10, Isco, Lincoln, NE) attached with a modified extraction vessel (**Figure 2**). In the beginning, a dynamic continuous extraction was adopted at a flow rate of 1 mL SC−CO_2/min for 1 h and then followed by a static extraction for additional 1 h. The oil soluble extracts were collected in 95% ethanol. The residue (ABR_1) that remained in the extraction vessel was used in the subsequent experiments (**Figure 1**).

Proximate Composition Analysis. Crude protein, total crude lipids, ash, fiber, and moisture content in freeze-dried mycelia were determined according to the AOAC official procedures (methods 984.13, 43.275, 968.08, 991.43, and 950.46.B, respectively) (*14*). The conversion factor used for nitrogen to crude protein was 6.25. The total crude lipids content was obtained from 1 h of hexane extraction. The ash content was calculated from the weight of the sample after burning at 550 °C for 4 h. The moisture content was measured, based on a sample in an oven at 105 °C until a constant weight was obtained. The total carbohydrate excluding crude fiber was calculated by the difference. All of the calculations were carried out on a dry weight basis of freeze-dried mycelia.

Polysaccharide Fractionation. *Water Soluble Fraction.* Dong et al. (*10*) were followed for the preparation of water soluble polysaccharides. ABR_1 (100 g) was extracted with reflux three times with 2000 mL of double distilled water (DDW) at 90 °C with constant stirring at 400 rpm for 2 h. The extracts were filtered with aspiration after cooling, and the residue (ABR_2) was kept for further experimentation. To the filtrate 1 M HCl was added to adjust the pH to 4.0, and then, a 2-fold volume of ethanol (95%) was added to precipitate the water soluble polysaccharides, which were collected and further purified in 400 mL of hot water (100 °C). Finally, the water soluble polysaccharides were precipitated on addition of a 3-fold volume of ethanol (95%) and then collected and lyophilized (AB-1) (**Figure 1**).

NaOH (2%) Extractable Fraction. The residue ABR_2 was added with 1000 mL of 2% NaOH and extracted three times with constant stirring at 400 rpm and reflux. The extracts were filtered with aspiration after cooling, and the residue (ABR_3) was kept for subsequent experimentation. The filtrate was collected and adjusted to pH 4.0 with 12 M H_2SO_4 and left to stand overnight. The sediment was collected, dialyzed, and lyophilized to recover the isoelectric precipitate (AB-2). The supernatant was treated with a 3-fold volume of ethanol (95%), and the precipitate was collected, dialyzed, and lyophilized to obtain the 2% NaOH extractable polysaccharides (AB-3) (**Figure 1**).

KOH (10%) Extractable Fraction. The residue (ABR_3) was added with 1000 mL of 10% KOH and extracted three times with constant stirring at 400 rpm and reflux at 80 °C. The extracts were filtered with aspiration after cooling, while the residue was discarded. The filtrate

was collected and adjusted to pH 4.0 with 12 M H_2SO_4 and left to stand overnight. The sediment was collected, dialyzed, and lyophilized to recover the isoelectric precipitate (AB-4). The supernatant was treated with a 3-fold volume of ethanol (95%) to precipitate the polysaccharides, which were collected, dialyzed, and lyophilized to obtain the 10% KOH extractable polysaccharides (AB-5) (**Figure 1**). The total carbohydrate and protein contents in each fraction were then determined by using the phenol−H_2SO_4 method (*16*) and the bicinchoninic acid (BCA) method using the BCA protein assay kit (#23227, Pierce, Rockford, IL), respectively.

GPC Sieving. To each, 20 mg of the fractions AB-1, AB-2, AB-3, AB-4, and AB-5, respectively, was added with 1 mL of NaOH, and DDW was added to obtain each with a final volume of 5 mL to facilitate the dissolution. The solutions were centrifuged at 2500 rpm for 10 min to precipitate those insoluble fractions. The supernatants were decanted, and 3 mL of each was eluted with a 0.05 N NaOH (containing 0.02% of NaN_3) solution on a Sephadex G-100 column (2.5 cm × 100 cm) at a flow rate of 0.5 mL/min. The eluents were collected by a fraction collector (ISCO Retriever 500, Isco., Lincoln, NE), each 6 mL in a tube. The entire course of collection was made up to a total of 100 tubes. The molecular mass distribution and mean molecular mass were determined by a standard curve established by standard dextrans (Sigma, St. Louis, MO) having known molecular masses (8.8, 40, 500, 2000, and 5000−40000 kDa, respectively). From the data obtained, the average molecular mass was calculated by linear correlation between the logarithm of the molecular mass of the standards and the ratios of their elution volumes to the void volume of the column (*16*).

Chromatograms of Carbohydrate and Peptido-Moiety Content from Each Collected Fraction. After the polysaccharide fraction (AB-1−AB-5) separated from the Sephadex G-100 column, each collection was monitored for the content of carbohydrate and peptido-moiety. The phenol−sulfuric acid colorimetric method cited by Dubois et al. (*15*) was followed for the determination of carbohydrate. In essence, each 1 mL of the polysaccharide fraction was added with 1 mL of phenol solution (5%); after it was thoroughly mixed, 5 mL of sulfuric acid was added and shaken well. After the solution cooled at room temperature, the absorbance (optical density, OD) was measured at 490 nm. The determination of peptido-moiety in polysaccharide fraction was performed by Coconnier et al. (*17*). Briefly, the absorbancea at 280 nm and at room temperature of the five polysaccharide eluents (AB-1, AB-2, AB-3, AB-4, and AB-5; refer to **Figure 1**) obtained from GPC were directly read. The greater the intensity of OD_{280nm} means that there might be, yet not definitely, more peptido-moiety present in the polysaccharide molecules.

Analyses of the Antioxidant Capability. *Scavenging Capability for DPPH Radicals.* The method reported by Shimada et al. (*18*) was adopted for measurement of free radical scavenging capability. To each 4 mL of sample extract, 1 mL of freshly prepared methanolic DMSO solution of DPPH (0.5 mM) was added, mixed well, and then let stand for 30 min. The absorbance was taken at 517 nm using a spectrophotometer (BioMate 5, Thermo Electron Corporation, San Jose, CA). DHA was used as a reference control. The lower the absorbance, the more potent is the scavenging capability.

Relative Chelating Power (RCP) for Ferrous (Fe^{2+}) Ions. To each 1 mL of the sample extract, 3.7 mL of methanol and 0.1 mL of $FeCl_2 \cdot H_2O$ (2 mM) were added, mixed well, and let stand for 30 s, and then, 0.2 mL of ferrozine (5 mM) was added, after shaken well to facilitate the mixture, let stand for 10 min, and then, the absorbance was immediately read at 562 nm. Citric acid was used as a reference control. The lower the absorbance, the more potent is the relative chelating power.

Absolute Chelating Power (ACP) for Ferrous (Fe^{2+}) Ions. ACP was defined as the mole numbers of ferrous ions chelated by per mole of polysaccharide, i.e.,

$$\text{ACP} = (\text{mole numbers of ferrous ions chelated})/(\text{mole numbers of chelating polysaccharide}) \quad (1)$$

On the basis of the data obtained from the RCP, the weight of polysaccharide in each fraction was divided by the mean molecular weight of that fraction to obtain the mean mole number of the polysaccharide in the same fraction (N_p). Similarly, the mole number of ferrous (Fe^{2+}) ion (N_{Fe}) was obtained by dividing the weight of ferrous (Fe^{2+}) ion present by the atomic weight of Fe. Thus, the ACP is expressed as

$$\text{ACP} = (N_{Fe})/(N_p) \quad (2)$$

To obtain a more accurate index with a better linearity for a universal comparison, we proposed to use the half ACP (ACP_{50}) as the measuring index, by which, ACP_{50} was defined as an index of a definite status that the mole numbers of a polysaccharide required to chelate a half mole number of a given amount of ferrous (Fe^{2+}) ions used in the experimentation system. Obviously, the lower the ACP_{50} values, the more powerful is the related polysaccharide with respect to the chelating ability.

Table 1. Yield, Carbohydrate, and Protein Content of Polysaccharides from Mycelia of *A. blazei* by Systemic Solvent Extractions Combined with Various Other Treatments

extract	yield[a]	carbohydrate[b]	protein[c]
AB-1	5.20	81.18	16.49
AB-2	9.03	23.42	60.28
AB-3	2.84	69.25	28.03
AB-4	17.47	55.39	41.08
AB-5	0.44	58.90	31.27

[a] Weight-based percentage of polysaccharides extracted from 100 g of lyophilized mycelial powder of *A. blazei.* AB-1, the 3-fold ethanol precipitate from hot water extracts; AB-2, the isoelectric precipitate from 2% NaOH extracts; AB-3, the 3-fold ethanol precipitate from 2% NaOH extracts; AB-4, the isoelectric precipitate from 10% KOH extracts; and AB-5, the 3-fold ethanol precipitate from 10% KOH extracts. [b] Carbohydrate (%, w/w) in each extract was measured by the phenol–H_2SO_4 method. [c] Protein (%, w/w) in each extract was determined by BCA protein assay.

RESULTS AND DISCUSSION

Proximate Composition. The crude composition in ABM was shown to be abundant mostly in total carbohydrate (42.06 ± 0.08%) and the second most in crude protein (29.13 ± 0.41%), while the crude fat, crude fiber, and crude ash contributed 12.63 ± 0.51, 11.28 ± 0.07, and 5.07 ± 0.01%, respectively. The analysis of total carbohydrate content in mycelia was consistent in the basic composition of cultivated mushrooms, which mostly existed in total carbohydrate (*19*).

Polysaccharide Fractionations. The polysaccharide content obtained from AB by different fractionation methods (refer to the flowchart in **Figure 1**) is indicated in **Table 1**. The total yield of the polysaccharide of AB mycelia is about 34.98 (% w/w). The fraction AB-4 (the isoelectric precipitate from 10% KOH extracts) was the most abundant in yield (17.47%), as compared to the fraction A-5 (the 3-fold ethanol precipitate from 10% KOH extracts), which yielded only 0.44%; the second in abundance (9.03%) was found in fraction AB-2 (the isoelectric precipitate from 2% NaOH extracts), while moderate contents 5.20 and 2.84% were found in fractions AB-1 (the 3-fold ethanol precipitate from hot water extracts) and AB-3 (the 3-fold ethanol precipitate from 2% NaOH extracts), respectively.

GPC Sieving. Figure 3 shows a calibration curve of standard dextrans with known molecular masses ranging from 8.8, 40, 500, 2000, and 5000 to 40000 kDa established by GPC on Sephadex G-100. The extremely high correlation coefficient R^2 of 0.9845 indicates a highly significant accuracy of the GPC analysis to be quite excellently feasible for measuring the molecular masses of different polysaccharides. **Figure 4** is the GPC elution pattern of fractions AB-1−AB-5, respectively. The average molecular masses of five polysaccharide fractions calculated from the calibration curve (**Figure 3**) are shown in

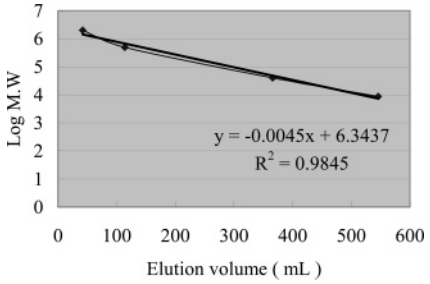

Figure 3. Calibration curve of a standard curve using dextrans with known molecular masses (ranging from 8.8, 40, 500, 2000, 5000, and 40000 kDa) established by GPC on Sephadex G-100; column, 2.5 × 100 cm; flow rate, 0.5 min/mL; and 6 mL/tube.

Table 2. These polysaccharides, shown with a distribution of the average molecular masses from 631 to 1318 kDa, was suggested to be different from a previous report in a distribution of 7.74−575 kDa, which was prepared from water soluble polysaccharides of *A. blazei* using different temperatures (*20*).

Peptido-Moiety in Various Polysaccharide Fractions. The various peptide contents in each polysaccharide fraction (AB-1−AB-5) as determined by direct reading of the absorbance at 280 nm were shown in **Figure 4**. The peptide content was extremely significant in fraction AB-2 (**Figure 4**) as compared to the fractions AB-1, AB-3, AB-4, and AB-5. Fraction number 20s of the latter showed a slighter absorbance at 280 nm, however, which obviously might be with much smaller amounts. The direct photometric reading of the absorbance at 280 nm has long been used for determination of proteins; yet, the chromophores that responsd to such a spectrometric measurement include only the minor amino acids tryptophan, tyrosine, and phenylalanine. In essence, the absorbance at 280 nm determines the true amount of these three amino acid contents, rather than the whole protein. Naturally, one would expect a more intense absorbance in proteins with a higher content of these three amino acids than those totally without or merely with minute amounts of them. Thus, one can anticipate nearly zero absorbance even though there should be a tremendous quantity of proteins present. A purified fraction of soluble proteoglucan, HM3-G, having a molecular mass of 380 kDa and a composition of more than 90% glucose, obtained from the fruiting bodies of ABM, had been shown to possess significant tumoricidal activity (*4*). Further analysis of the peptide content is obviously alternative interesting research work that remains ahead.

Analysis of Antioxidant Capability. *Scavenging Capability for DPPH Radicals.* A remarkable excellent scavenging capability on DPPH radicals at a dosage of 1 mg/mL (reaching 72%) was found with the fraction AB-3 (the 3-fold ethanol precipitate from 2% NaOH extracts) as compared with the control BHA (100%) regarding the low dosage ranges used (**Figure 5**). More significant and effective radical scavenging capability (130%) was also found with the fraction AB-1 (the 3-fold ethanol precipitate from hot water extracts) at a higher dosage of 5 mg/mL. Comparable results with the fraction AB-3 were found to be 85 and 72% at the dosages of 5.0 mg/mL for both fractions AB-1 and AB-3, respectively (**Figure 5**). In contrast, the fraction obtained from the isoelectric precipitate from 10% KOH extracts (AB-4) demonstrated insignificant free radical scavenging capability among all dosages tested, although having the largest percent yield (17.47%) of polysaccharides (**Table 1**). In contrast, fractions AB-2 and AB-5 (**Figure 5**), among all dosages tested, demonstrated the least scavenging capability among five fractions. Normally, the antioxidants else reported are both thermal

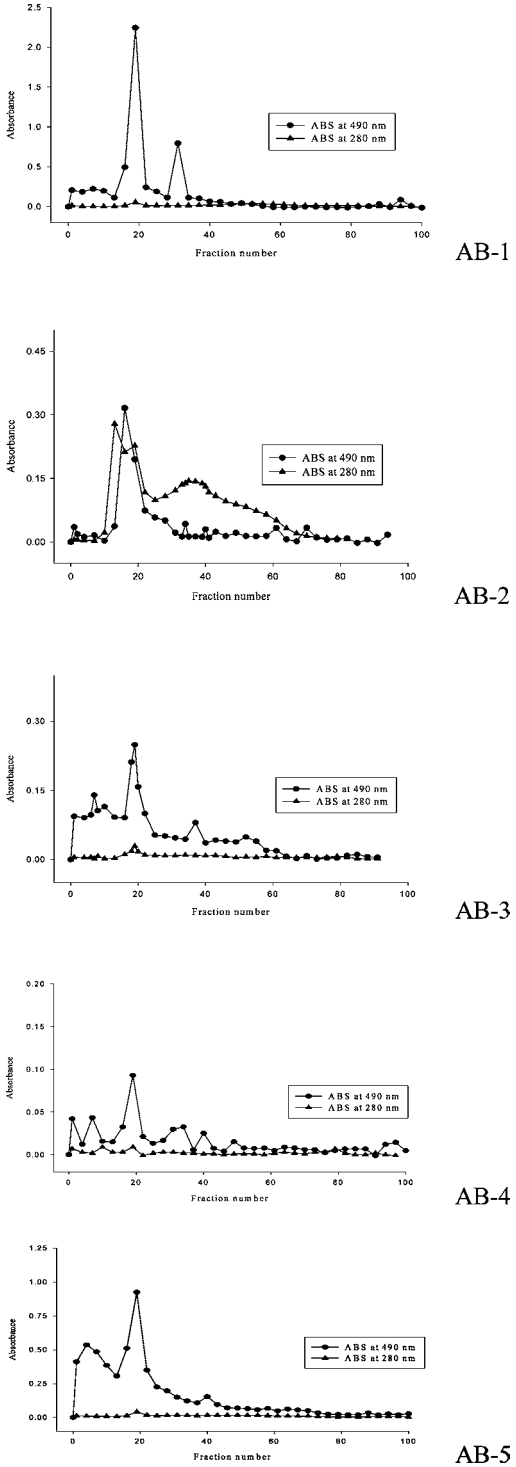

Figure 4. GPC sieving of the polysaccharide fractions of AB-1, AB-2, AB-3, AB-4, and AB-5 obtained from *A. blazei* mycelia on Sephadex G-100.

labile and susceptible to oxidation by atmospheric oxygen. However, in this study, the different fractions of polysaccharides still retained powerful antioxidant activity even after a period of 120 min of boiling treatment, which is consistent with the cited characteristics (*21*).

RCP for Ferrous (Fe^{2+}) Ions. Taking citric acid as the reference control, below a concentration of 1 mg/mL, the fractions AB-3 and AB-4 were shown to exhibit excellent chelating capabilities as compared to citric acid (**Figure 6**). Under a similar condition, the fraction AB-1 had only 72% capability as that of citric acid; however, above a concentration of 5 mg/mL, it revealed an activity of 105%, even far beyond

Table 2. Values of ACP, ACP_{50}, and the Mean Molecular Mass of Polysaccharides in Fractions AB-1–AB-5 Obtained from *A. blazei* Mycelia

fraction	ACP × 10^{-6} (mol/mol)	ACP_{50} (*p* mole)[a]	mean molecular mass (kDa)[b]
AB-1	333.56	81.53	757
AB-2	310.15	6657.45	887
AB-3	191.52	226.39	1318
AB-4	399.82	50.50	631
AB-5	206.50	4.09	1226

[a] ACP, expressed as the mole numbers of polysaccharide required to chelate the mole numbers of ferrous (Fe^{2+}) ions present in experimentation. ACP_{50}, expressed in *p* mole numbers of polysaccharides required to chelate the half amount of ferrous ions present in experiment. [b] By gel permeation chromatographic (GPC) sieving.

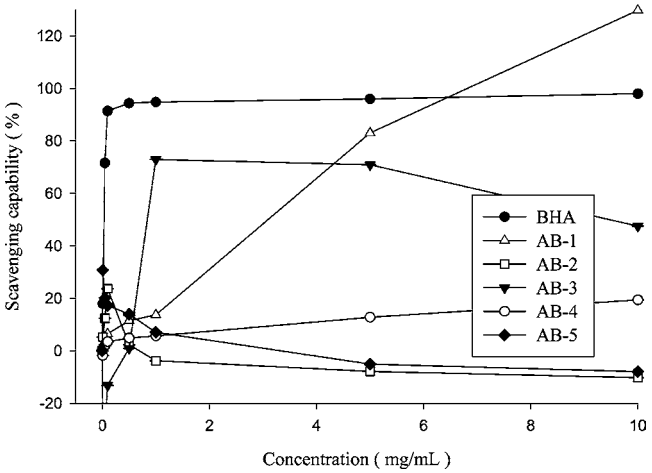

Figure 5. Scavenging capability of different polysaccharides fractionated from *A. blazei* mycelia for DPPH radicals. BHA was used as a reference control. Values are expressed in means ± SD ($n = 3$).

that of citric acid (35%) (**Figure 6**), i.e., AB evidenced a more powerful chelating capability than the citric acid that has been conventionally considered as a potent reference chelating agent.

ACP for Ferrous (Fe^{2+}) Ions. It was noted that the chelating power of a polysaccharide for Fe^{2+} ions increased in linearity with the mole numbers of polysaccharide (**Figures 6** and **7**). Fractions AB-1 and AB-4 were the most prominent in term of ACP, having values of 333.56 × 10^{-6} and 399.82 × 10^{-6}, respectively (**Table 2**). In reality, the ACP values are inversely related with the mean molecular mass, as can be seen from fractions AB-3 and AB-5 that possessed the larger mean molecular masses (1318 and 1226 kDa, respectively), yet with the lowest corresponding values of ACP (191.52 × 10^{-6} and 206.5 × 10^{-6}, respectively) (**Table 2**). As the values of ACP are unable to reflect well the inherent chelating capability of each fraction (Figure. 6), to express more pertinently for such an evaluation, we proposed herein a quantifying method named ACP_{50}, which was defined as the *p* mole numbers of polysaccharide required for chelating half the amount of ferrous (Fe^{2+}) ion present in the experimentation as mentioned in the above (**Figure 7**). Apparently, the lower the values of ACP_{50}, the more powerful chelating capability the polysaccharide can possess. As can be seen, the fraction AB-5 (ACP_{50}, 4.09 *p* moles) was the most prominent, and the fraction AB-4 (ACP_{50}, 50.50 *p* moles), the next. In contrast, the fraction AB-2 (ACP_{50}, 6657.45 *p* moles) was shown to be the worst (**Table 2**).

Moreover, although both the free radical scavenging capability (**Figure 5**) and the chelating power (**Figures 6** and **7**) were found to be dose-dependent, a declining tendency was noted at higher dosages (curves AB-3, AB-2, and AB-5, and probably at higher dosages of AB1 and AB-4 in **Figure 5**, data not shown; and curves AB-2, AB-3, AB-4, and AB-5 in **Figure 6**). Such phenomena are considered probably resulting from two reasons: the limitation of solubility and the association by hydrogen bonding, once the concentration of polysaccharide is increased. The diminished solubility would result in the aggregation of the polysaccharide molecules to form invisible colloidal particles, while the inter- and intramolecular hydrogen bonding can cause reduction of available hydroxyl groups; otherwise, they can be active for free radical scavenging and ferrous ions chelation under a reasonably low concentration. Thus, theoretically,

$$\mathrm{ACP} = k_1 N_o \tag{3}$$

where N_o is the available number of hydroxyl groups at infinitely dilution and *k* is a proportional constant. Normally, two hydroxyl groups are needed for the chelation of a single ferrous (Fe^{2+}) ion (**Figure 7**).

In fact, as stated above, the concentration of available hydroxyl groups for the free radical scavenging capability (**Figure 5**) or the ferrous ion chelating power (**Figures 6** and **7**) is easily expressed by the polynomial equation

$$N = k_2 C + aC^2 + bC^3 + ... \tag{4}$$

in which k_2 is a proportional constant for conversion of the polysaccharide concentration (*C*) to the number of available hydroxyl groups (*N*) and the coefficients *a* and *b* are negative in magnitude in mathematical sense. On differentiation, eq 4 gives

$$\mathrm{d}N/\mathrm{d}C = k_2 + 2aC + 3bC^2 + ... \tag{5}$$

where the term on the lefthand side, d*N*/d*C*, describes the concentration-dependent molar change of available hydroxyl groups. At infinite dilution, eq 5 becomes

$$\mathrm{d}N/\mathrm{d}C = k_2 \tag{6}$$

which claims the maximum concentration of hydroxyl numbers on each polysaccharide molecule, which chemically is considered a constant, i.e., N_o as indicated in eq 3. Furthermore, from eqs 3 and 5, we have

$$\mathrm{d(ACP)}/\mathrm{d}C = k_1(\mathrm{d}N/\mathrm{d}C) = k_1(k_2 + 2aC + 3bC^2 + ...) \tag{7}$$

$$= K + a'C + b'C^2 + ... \tag{8}$$

where $K = k_1k_2$, $a' = 2k_1a$, and $b' = 2k_1b$.

Equations 7 and 8 state that the change of ACP depending on the polysaccharide concentration is dependent on the available number of hydroxyl groups. Thus, the concentration-dependent deviations of the free radical scavenging capability (**Figure 5**) or RCP (**Figure 6**) (or ACP, **Figure 7**) resulting from all possible related causes as above-mentioned are easily assessable.

In summary, different fractions behave quite differently from each other in view of solubility, dosages that have to be administered, the free radical scavenging capability, and the RCP

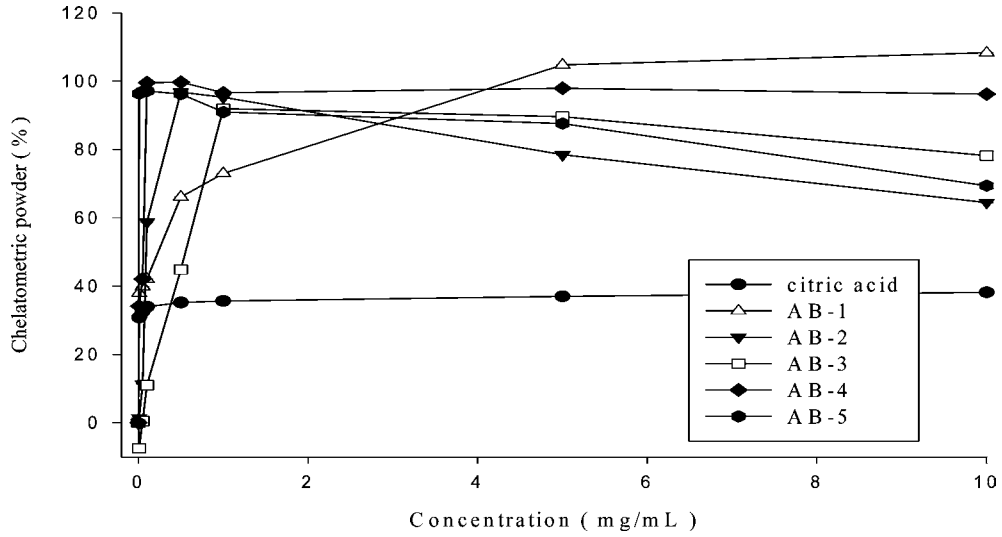

Figure 6. Relative chelating power of different polysaccharides fractionated from *A. blazei* mycelia for ferrous (Fe^{2+}) ions. Citric acid was used as a reference control. Values are expressed in means ± SD ($n = 3$).

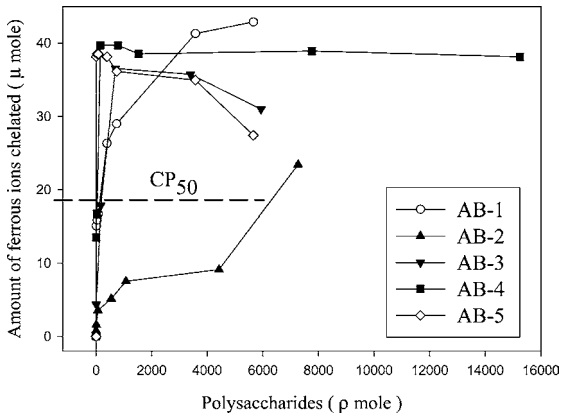

Figure 7. Absolute chelating power of different polysaccharides fractionated from *A. blazei* mycelia for ferrous (Fe^{2+}) ions. Values are expressed in means ± SD ($n = 3$).

Table 3. Ranking of the Biological Activity vs Different Fraction

	fraction/ranking				
parameter	AB-1	AB-2	AB-3	AB-4	AB-5
solubility	1, excellent	5, poor	2, moderate	3, poor	4, poor
free radical scavenging capability[a]	1, excellent	5, poor	2, moderate	3, poor	4, poor
chelating power[b]	1, excellent	5, moderate	3, moderate	2, good	4, moderate
ACP_{50}	3, good	5, poor	4, moderate	2, good	1, excellent
p mole[c]	81.53	6657.45	226.39	50.50	4.09

[a,b] Data refer to **Figures 5** and **6** with the significances of tested concentrations at 0.1–10 mg/mL, respectively. [c] Data refer to **Table 2**.

as well as the ACP. **Table 3** summarizes the raking from our experimental results.

Obviously, the free radical scavenging capability was in good correlation with the solubility as compared to the chelating power of polysaccharides (**Table 3**). Fraction AB-5 showed excellent ACP_{50}, but its drawback is poor solubility, poor free radical scavenging capability, and moderate chelating power. Apparently, the active functionality inherently borne in each individual structure and their actual availability (eqs 7 and 8) must act as the major roles in these respects. In summary, considering the free radical scavenging capability and the chelating power for ferrous (Fe^{2+}) ion, the polysaccharide fraction AB-1 (the hot water soluble fraction) in *A. blazei* showed excellent biological activities. The fraction AB-3 [the NaOH (2%) soluble fraction] was the next. At 5 mg/mL, their free radical scavenging capabilities reached over 72% in comparison with the reference control BHA (100%). In contrast, although its chelating power for ferrous (Fe^{2+}) ion was found abundant in all fractions including AB-2 [the fraction NaOH (2%) isoelectric precipitate] to AB-5 [KOH (10%) isoelectric precipitate for AB-4; 10% KOH soluble fraction for AB-5], being over 99.65% as compared to the reference control citric acid (35%) at a concentration of 1 mg/mL, the fraction AB-1 can be the most potent (105%) among all if the concentration used were raised to above 5 mg/mL. The hot water soluble preparation of the liquid cultured *A. blazei* mycelia was reported to exhibit antitumor active polysaccharides against Sarcoma 180, which has been identified to be a glucomannan with a backbone of β-1,2-linked D-mannopyranosyl units with β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl residues as a side chains (*22*). Polysaccharide of the fruit bodies of *A. blazei*, prepared by repeated extractions with hot water, was shown in majority to possess a distinct structure of 1,6-β-glucan. In contrast, the cold NaOH extracts contained polysaccharides with highly branched 1,3-β-glucan segments (*23*). Whether these structures can be related to the scavenging capability for DPPH radicals or the chelating power for ferrous (Fe^{2+}) ions remains to further studies. However, our preliminary results suggest that different activities from these fractions apparently can be associated with their different molecular structures. The GPC patterns showed that the more potent fractions were those with lower molecular masses: AB-1 (757 kDa), AB-2 (887 kDa), and AB-4 (631 kDa) as compared to those polysaccharides as commonly encountered in all biosystems. However, in view of ACP_{50}, the fraction AB-5 has indicated the least ACP_{50} at 4.09 *p* moles; apparently, the active functionality on each individual structure plays an important role for biological activity in addition to the limitation of solubility.

In conclusion, the hot water soluble polysaccharide fraction (fraction AB-1) of *A. blazei* mycelia can be an excellent and potent antioxidant, while the KOH (10%) extractable polysaccharides (fraction AB-5) exhibit the most powerful chelating capability, which is inversely related with the mean molecular

mass of the polysaccharides in each fraction. In contrast, the free radical scavenging ability is closely related with the solubility of polysaccharides. All together, the interfractional difference of the antioxidant activity can be ascribed to the differences in solubility, the mean molecular mass, and the actual availability of the functionality in each individual structure.

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