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Chemical Characterization and Anti-inflammatory Effect of Polysaccharides Fractionated from Submerge-Cultured *Antrodia camphorata* Mycelia

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Five polysaccharide fractions (AC-1, AC-2, AC-3, AC-4, and AC-5) were obtained after systemic solvent extractions and precipitations from *Antrodia camphorata* mycelia with yields of 2.92, 10.38, 1.65, 0.34, and 1.64%, respectively. Gel permeation chromatography (GPC) analysis showed that the distribution of mean molecular mass of the fractionated polysaccharides was in the range of 394−940 kDa. The proximate compositions from each polysaccharide fraction revealed that all fractions belonged to the category of glycoprotein, having ratios of carbohydrate/protein ranging from 0.29 to 10.79 (w/w). Glucose or galactose was the major monosaccharide in all fractions except fraction AC-2, which has a mean molecular mass of 394 kDa with lyxose as the most prominent constituent. In the evaluation of the DPPH$^{\bullet}$ radical scavenging capability, fraction AC-1 and AC-2 polysaccharides showed the better capabilities, around 74.5 and 50.5%, respectively, compared to the reference control of Trolox (87.5%) at a concentration of 1 μM. In testing with macrophage RAW264.7 cells, fraction AC-2 demonstrated a rather potent anti-inflammatory capability. Furthermore, the lipopolysaccharide-induced NO production and the protein expression by the inducible nitric oxide synthase (iNOS) gene were inhibited, respectively, in a dose-dependent (50−200 μg/mL) manner by fraction AC-2 polysaccharide.

KEYWORDS: ***Antrodia camphorata*; mycelia; polysaccharides; DPPH radicals; macrophage; nitric oxide; inducible nitric oxide synthase (iNOS)**

INTRODUCTION

Antrodia camphorata (AC; family Polyporaceae, Aphyllophorales) is a parasitic fungus on the inner cavity of the endemic species *Cinnamomum kanehirai* Hay (*1*). It recently has become popular as a folkloric remedy as well as a source of physiologically beneficial mushrooms in Taiwan. Much of the literature has described its unique biological activity as both nutraceutics and supplementary medicines. The constituents isolated from the fruit bodies of AC consist of steroids (*2*−*4*), triterpenoids (*5*), sesquiterpene lactone (*6*), and polysaccharides (*7*). In past years, many researchers have studied the extraction and isolation technology for mushroom metabolites that are capable of modulating the human immune system. A variety of bioactive metabolites were obtained from fruiting bodies, pure culture mycelia, and culture filtrate (cultured broth) (*8*). Polysaccharides extracted from cultured AC mycelia have been reported to possess potent anti-inflammatory activity (*9*). Although numerous polysaccharides have been fractionated from the mycelia of AC, the major active component of polysaccharides remained unknown. In the present study, we develop a unique systemic solvent extraction scheme that involved serial fractionations using hot water, isoelectric precipitation (IEP), 5% NaOH, and 10% KOH, combined with IEP; the fractions obtained were further analyzed by their molecular weights using the gel permeation chromatography (GPC) technology, and finally tests were performed on their physiochemical properties, molecular size distribution, and associated antioxidant and anti-inflammatory capabilities.

MATERIALS AND METHODS

Materials. LPS from *Escherichia coli* 055:B5 and anti-β-actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO). SuperSignal West Pico Chemiluminescent Substrate was obtained from Pierce (Rockford, IL). Anti-COX-2 antibodies were products of Santa Cruz (Santa Cruz, CA). Anti iNOS/NOS II antibodies were from Upstate (Lake Placid, NY). PRO-PREP Protein Extraction Solution was

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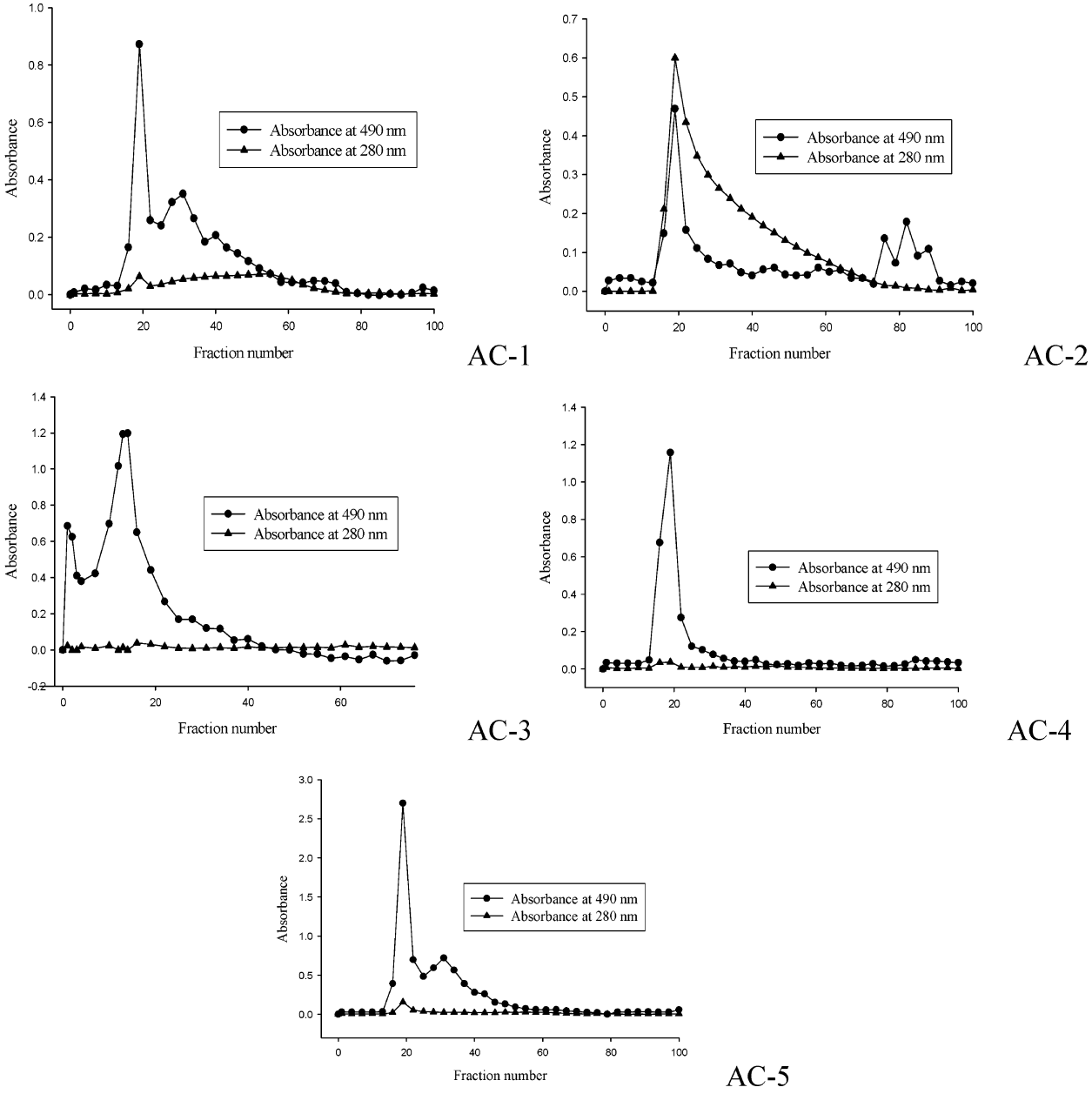

Figure 1. GPC sieving of the polysaccharide fractions of AC-1, AC-2, AC-3, AC-4, and AC-5 obtained from *A. camphorata* mycelia on Sephadex G-100.

supplied by iNtRON Biotechnology (Kyungki-Do, Korea). Bio-Rad protein assay kit was purchased from Bio-Rad (Hercules, CA). TRIzol reagent and SuperScript II were provided by Invitrogen (Carlsbad, CA).

Microorganism and Cultivation. *A. camphorata* BCRC 35398, purchased from the Bioresources Collection and Research Center in Food Industry Research and Development Institute (Hsinchu, Taiwan), was grown as previously reported (*5*). Briefly, a piece of 5 mm × 5 mm *A. camphorata* agar culture was inoculated into 1 L of the broth consisting of the following ingredients (g L^{-1}): glucose, 1.0; soybean powder, 0.5; peptone, 0.5; $MgSO_4$, 0.01. The pH was adjusted to 4.0 with 1 N HCl. The whole medium was placed in a 2 L Hinton flask and cultivated at 28 °C on a 100 rpm rotary shaker for 10 days. Thereafter, all of the grown broth was transferred into a 200 L fermentor containing 120 L of medium of the same ingredients and was incubated at 28 °C, with continuous agitation at 90 rpm and aeration at 60 L/min for 12 days. Thereafter, the mycelia were filtered off. The residue, after several rinses with deionized water, was lyophilized for further tests.

Pretreatment of the Mycelial Powder. Prior to the extraction of polysaccharides, a supercritical fluid carbon dioxide (SC-CO_2) (99.5% purity) extraction was carried out to remove some inherently existing oil-soluble substances to minimize their interference on the extractability of polysaccharides as in a previous paper (*10*). 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 to a 150 mL extraction vessel. In the beginning, a static extraction was adopted for 1 h and then followed by a dynamic continuous extraction at a flow rate of 1 mL of SC-CO_2/min for an additional l hr. The oil-soluble extracts were collected in 95% ethanol. The residue (ACR_1) remaining in the extraction vessel was used in the subsequent experimentations.

Proximate Composition Analysis. Crude protein, total crude lipids, ash, fiber, and moisture contents of the freeze-dried mycelia were determined according to the AOAC official procedures [methods 984.13, 43.275, 968.08, 991.43, and 950.46.B (*11*), respectively]. The conversion factor used for nitrogen to crude protein was 6.25. Total crude lipids content was obtained from 1 h of hexane extraction. Ash content was calculated from the weight of the sample prior to and after ignition at 550 °C for 4 h. Moisture content was measured in a oven at 105 °C until a constant weight was obtained. The total carbohydrate excluding crude fiber was calculated by difference. All of the calculations were carried out on a dry weight basis of freeze-dried mycelia.

Polysaccharide Fractionation and Proximate Composition. Five fractions (AC-1−AC-5) from mycelia of *A. camphorate* were prepared as previously reported (*10*) and as the following procedures. ACR_1 (100 g) was extracted with reflux three times with 2 L 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 (ACR_2) was kept for further experimentation. To the filtrate was added 1 M HCl 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 (AC-1). To the residue ACR_2 was added 1 L of 2% NaOH, and the mixture was extracted three times with constant stirring at 400 rpm and reflux. The extracts were filtered with aspiration after cooling, and the residue (ACR_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 (AC-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 (AC-3). To the residue (ACR_3) was added 1000 mL of 10% KOH, and the mixture was extracted three times with constant stirring at 400 rpm and reflux at 80 °C. The extracts were filtered with aspiration after cooling, whereas 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 (AC-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 (AC-5). The total carbohydrate and protein contents in each fraction were then determined according to the phenol−H_2SO_4 method (*12*) and Bradford method (*13*) by applying the Protein Assay Dye Reagent Concentrate (no. 500-0006, Bio-Rad Laboratories).

GPC Sieving. To each 20 mg of the fractions AC-1, AC-2, AC-3, AC-4, and AC-5, respectively, were added 1 mL of NaOH and DDW to make up a final volume of 5 mL to facilitate the dissolution; the mixture was centrifuged at 2500 rpm for 10 min to precipitate those insoluble fractions. The supernatants were separated by decantation, 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 × 100 cm) at a flow rate of 0.5 mL/min. Eluents were collected by a fraction collector (ISCO Retriever 500), each 6 mL in a tube. The collection was continued until a total of 100 tubes were reached. The molecular mass distribution and related mean molecular mass were determined by a standard curve established by authentic 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 the linear correlation between the log standard molecular mass and the ratios of their elution volumes to the void volume of the column (*14*).

Chromatographic Analysis of Carbohydrate and Peptido Moiety Contents. The polysaccharide fractions (AC-1−AC-5) were separated by a Sephadex G-100 column, and each fraction collected was monitored for the content of carbohydrate and peptido moiety. The phenol−sulfuric acid colorimetric method of Dubois et al. (*12*) was followed. In essence, to 1 mL of each fraction was added 1 mL of phenol solution (5%), followed by thorough mixing, and then 5 mL of sulfuric acid was added and shaken well; the mixture was cooled at ambient temperature, and the absorbance (optical density, OD) was read at 490 nm against a control. Determination of the peptido moiety in the polysaccharide fraction was done according to the Coconnier et al. method (*15*). Briefly, the absorbances of the five polysaccharide eluents (AC-1, AC-2, AC-3, AC-4, and AC-5, be referred to in **Figure 1**) obtained from GPC were directly read at 280 nm at ambient temperature. The greater the intensity of OD_{280nm} indicates that there might be, yet not definitely, more peptido moiety present in the polysaccharide molecules.

Monosaccharide in Fractionated Polysaccharide. Hydrolysis was performed with all fractions according to the method of Albersheim et al. (*16*). The liberated sugars were transformed into alditol acetates according to the method of Blakeney et al. (*17*) with the following modifications. In brief, 300 μL of 2 M TFA was added to 0.2 mg of sample and hydrolyzed at 121 °C for 3 h. The liberated neutral sugars were dried under nitrogen gas and then reduced with sodium borohydride (0.5 mL) for 90 min at 40 °C. On addition of glacial acetic acid (1 mL), the sugars were acetylated with acetic anhydride (2 mL) in the presence 1-methylimidazole (100 μL) for 10 min at ambient temperature. Water (5 mL) and dichloromethane (1 mL) were added to extract the derivatives. The bottom layer containing the derivatives was separated and transferred into a new tube and again dried under N_2 atmosphere. The residue was dissolved in 1 mL of acetone. An aliquot (1 μL) was injected into the gas chromatograph HP5890A series II (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector (FID). The identification and quantification of monosaccharide were made by comparison with the authentic standard. Peak areas of all identified sugar compounds were calculated and expressed in percentage.

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

extract[a]	yield[b]	carbohydrate[c]	protein[d]
AC-1	2.92	84.46	14.91
AC-2	10.38	22.13	77.51
AC-3	1.65	89.32	9.93
AC-4	0.34	54.61	45.15
AC-5	1.64	90.72	8.41

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

Antioxidant Capability. The method reported by Shimada et al. (*18*) was adopted for measurement of free radical scavenging capability. To each 4 mL of sample extract was added 1 mL of freshly prepared methanolic DMSO solution of DPPH (0.5 mM); this was mixed well and then allowed to stand for 30 min. The absorbance was taken at 517 nm using a spectrophotometer (BioMate 5, Thermo Electron Corp., San Jose, CA). Ascorbic acid and Trolox were used for comparisons. The lower the absorbance, the more potent is the scavenging capability.

Cell Culture and Cell Viability Assay. The murine macrophage cell line RAW 264.7 was cultured at 37 °C in a 5% CO_2 atmosphere in Dulbeco's modified Eagle's medium (DMEM) (GIBCO, Carlsbad, CA) supplemented with 10% FBS (Biowest, France). Cells were plated in a six-well plate at 2.8×10^6 cells per well and allowed to adhere to the plate overnight. The cultures were treated with fractions AC-1, AC-2, AC-3, AC-4, and/or AC-5 at concentrations of 0, 100, 200, 500, 1000, and 2000 μg/mL, respectively, from each fraction. Cell viability was monitored using a phase contrast microscopy. Then the suspension of cultured medium was separated, and 100 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (dissolved in phosphate-buffered saline at 0.5 mL/mL) was added. Further incubation for 2 h followed. The suspension was separated, and DMSO (100μL) was added to each well and mixed thoroughly. The absorbance in each plate was read on an ELISA reader (μQuant Microplate spectrophotometer, Bio-Tek) against a control at a wavelength of 570 nm.

Nitrite Determination. RAW 264.7 cells were cultured in a 24-well plate at a density of 5×10^5 cells/well 1 day before LPS treatment. Cells were treated with each polysaccharide fraction at dosage of 12.5, 25, 50, 100, or 200 μg/mL for 1 h before the introduction of 500 ng/mL LPS. After LPS treatment for 18 h, the amount of nitrite ion (NO_2^-) produced in the extracellular medium was determined by a colorimetric method on reaction with Griess reagent. The amount of nitrite ion (NO_2^-) as such relevantly produced has been treated as an indication of NO production. Thus, the supernatants obtained were added with

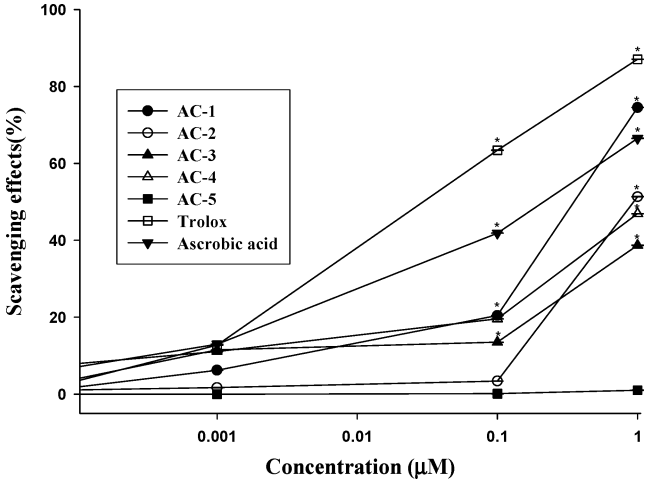

Figure 2. Scavenging capability of different polysaccharide fractions obtained from *A. camphorata* mycelia on DPPH radicals. Ascorbic acid and Trolox were used as reference controls. Data represent means ± SD of triplicate tests.

an equal volume of Griess reagent and incubated at ambient temperature for 10 min. Absorbance was read at 540 nm by using an ELISA plate reader (μQuant Microplate spectrophotometer, Bio-Tek); results were calibrated by comparison with known standard solutions of $NaNO_2$ (*19*).

Protein Extraction. RAW 264.7 cells were precultured in a 3.5-cm dish for cell lysates extraction 1 day before LPS treatment. The cells were treated with AC-1 or AC-2 polysaccharide 1 h prior to the introduction of 500 ng/mL LPS. Total cell lysates were prepared by lysing the cells in a buffered PRO-PREP protein extraction solution containing 10 mM NaF and 1 mM orthovanadate at 4 °C for 15 min and centrifuged at 7500*g* at 4 °C for 30 s. The supernatants containing protein extracts were stored at −80 °C for stabilization.

Western Blotting. Protein concentration was determined by using Bio-Rad protein assay reagent. Extracted protein (30 μg) was separated in 8% SDS-PAGE and transferred to PVDF membrane. After blotting, the membrane was incubated with specific primary antibodies overnight at 4 °C, then further incubated for 1 h with HRP-conjugated secondary antibody, and finally incubated with SuperSignal West Pico Chemiluminescent Substrate for 2 min. The amount of bound antibodies was detected by Kodak Digital Science (Image Station 4000MM).

RESULTS AND DISCUSSION

Proximate Composition. The mycelia of *A. camphorata* (ACM) was shown to be abundant mostly in total carbohydrate (39.27 ± 0.37%), and the second most abundant was crude protein (32.01 ± 0.38%), whereas crude fiber, crude fat, and crude ash contributed 10.07 ± 0.12, 6.67 ± 0.01, and 3.22 ± 0.01%, respectively. The total carbohydrate content in ACM was consistent with the fundamental composition usually found in most of the cultivated mushrooms (*20*).

Polysaccharide Fractionations. The polysaccharide contents obtained from ACM by different fractionation methods are indicated in **Table 1**. The total yield of the polysaccharide of ACM was about 16.93 (% w/w). The content in fraction AC-2 (the isoelectric precipitate from 2% NaOH extracts) was the most abundant (10.38%), compared to 0.34 and 2.92% in fractions AC-4 (the 3-fold ethanol precipitate from 2% NaOH extracts) and AC-1 (the 3-fold ethanol precipitate from hot water extracts), respectively, whereas only 1.65 and 1.64% were found in fractions AC-3 (the 3-fold ethanol precipitate from 2% NaOH extracts) and AC-5 (the 3-fold ethanol precipitate from 10% KOH extracts), respectively.

GPC Sieving. Figure 1 shows the GPC elution patterns of fractions AC-1−AC-5, respectively. A standard dextran calibration curve with known molecular masses ranging from 8.8, 40, 500, 2000, and 5000 to 40000 kDa established by GPC on Sephadex G-100 was first established, against which the average molecular masses of the five polysaccharide fractions were calculated. Results revealed a distribution of the average molecular masses from 394 to 940 kDs, from which various molecular ranges [AC-1 (508 kDa), AC-2 (394 kDa), AC-3 (677 kDa), AC-4 (940 kDa), and AC-5 (539 kDa)] were determined. These results were quite different from that previously reported (69−109 kDa) about the fraction obtained in the hot water (80 °C) soluble polysaccharide fraction of *A. cinnamomea* strain B85 (*21*).

Table 2. Monosaccharide Composition of Polysaccharide Fractions from *A. camphorata* Mycelia

	GC area (%)				
monosaccharide[a]	AC-1	AC-2	AC-3	AC-4	AC-5
rhamnose	10.52	2.69	8.06	4.84	4.27
fucose	6.93	–[b]	4.73	–	3.36
lyxose	7.36	48.93	15.87	–	5.02
arabinose	5.21	39.09	21.02	4.87	5.84
xylose	10.29	–	6.59	9.29	0.65
mannose	9.29	1.69	1.29	4.02	9.77
galactose	41.88	2.65	12.55	63.97	0.33
glucose	7.94	2.26	30.25	13.01	70.76
myo-inositol	0.67	–	–	–	–

[a] Identified with authentic compounds. [b] Not detected.

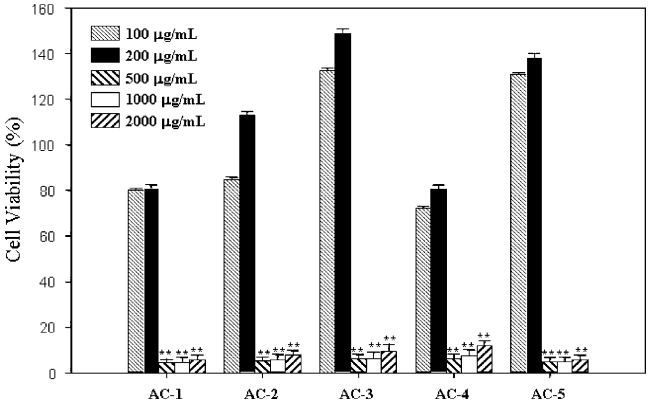

Figure 3. Effects of different polysaccharide fractions obtained from *A. camphorata* mycelia on cell viability of RAW 264.7 macrophages cells. Cells were treated with various concentrations of polysaccharides for 24 h, and then an MTT assay was performed as described in the text. Results are presented as mean ± SD of three independent experiments. **, statistically significant difference compared to untreated cells ($p < 0.001$).

Peptido Moiety and Monosaccharide Composition. The various peptide contents in each polysaccharide fraction (AC-1−AC-5) as determined by direct reading of the absorbance at 280 nm were demonstrated in **Figure 1**. The highest content of protein was observed in fraction AC-2 (77.51%), being significantly different from those in fractions AC-1, AC-3, AC-4, and AC-5 (**Table 1**). Similar results were found in *Agaricus blazei* in a previous paper (*10*). The direct photometric reading of the absorbance at 280 nm has long been used for the determination of proteins, yet the chromophores that respond to such a spectrometric measurement include only the minor amino acids tryptophan, tyrosine, and phenylalanine. However, the absorbance at 280 nm determines the whole protein still being used. The monosaccharide analysis (**Table 2**) showed that five different fractionated polysaccharides consisted of galactose (41.88%) in AC-1, lyxose (48.93%) in AC-2, glucose (30.25%)

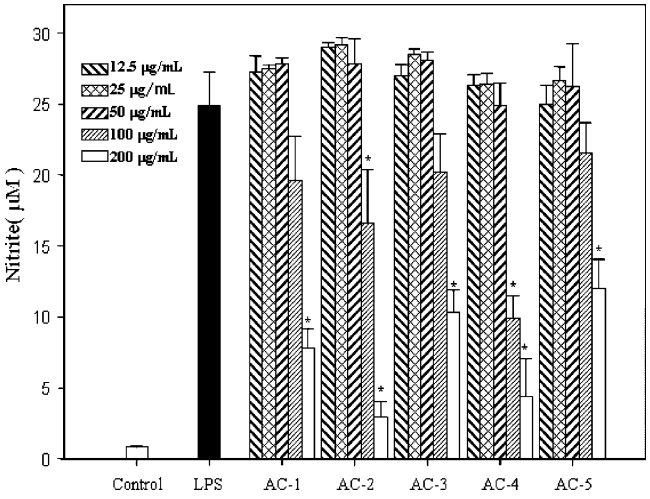

Figure 4. Effects of polysaccharide fractions obtained from *A. camphorata* mycelia on LPS-induced nitrite formation in RAW 264.7 cells. Measurements were performed in triplicate and are presented as mean ± SD. ∗, statistically significant difference compared to LPS-activated cells ($p < 0.05$). Cells were treated with or without indicated concentrations of tested fractions and LPS (500 ng/mL) for 24 h. Data represent means ± SE of triplicate tests.

in AC-3, galactose (63.97%) in AC-4, and glucose (70.76%) in AC-5, respectively, as the highest percentage in each fraction. The composition of monosaccharide in polysaccharide has been suggested with a different bioactivity. A purified fraction of soluble proteoglucan, HM3-G, having a molecular mass of 380 kDa and a composition of >90% glucose, obtained from the fruiting bodies of *A. blazei*, had been shown to possess significant tumoricidal activity (*22*).

Scavenging Capability for DPPH Radicals. As can be seen from **Figure 2**, fraction AC-1 (the 3-fold ethanol precipitate from hot water extracts) at 1 μM exhibited a remarkable scavenging capability on DPPH radicals, reaching 74.5% compared with the control Trolox (87.5%) in the low-dosage ranges. At the same dosage, fraction AC-2 (the isoelectric precipitate from 2% NaOH extracts) showed rather significant and effective radical scavenging capability (50.5%) and fraction AC-5 (the 3-fold ethanol precipitate from 10% KOH extracts), the least among the five (**Figure 2**).

Obviously, the biological activities of various fractions were neither closely associated with the mean molecular weight nor pertinently related with the proximate composition. In a previous paper, the hot-water soluble fraction of the mycelium of liquid-cultured *A. blazei* had been found to contain an antitumor active polysaccharide against Sarcoma 180, later identified to belong to a glucomannan having a main chain of β-1,2-linked D-mannopyranosyl residues and a side chain composed of β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl residues (*23*). In contrast, polysaccharides obtained by repeated extractions with hot water from the fruit bodies of *A. blazei* were shown to possess in majority a distinct structure of 1,6-β-glucan, whereas the cold NaOH extracts contained polysaccharides with a highly branched 1,3-β-glucan segment (*24*). Whether these structures can be related to scavenging capability for DPPH radicals remains to be determined in further studies. However, our preliminary results suggest that different bioactivities of these fractions apparently may be, in some respects, linked to their different molecular structures.

Effects of AC-1 and AC-2 on the Viability of RAW 264.7 Cells. After 24 h of treatment of mouse macrophage RAW 264.7 cells with AC-1, AC-2, and LPS, cell numbers were counted individually. **Figure 3** demonstrates that the viability of RAW 264.7 cells was not affected by either AC-1 at 100 μg/mL or AC-2 at 50 μg/mL. Only a minute decrease was found by AC-1 at 200 μg/mL or by AC-2 at 100−200 μg/mL. To summarize, no distinct cytotoxicity could be induced at dosages under 200 μg/mL by either AC-1 or AC-2. Thus, the anti-inflammatory test was carried out at dosages under 200 μg/mL.

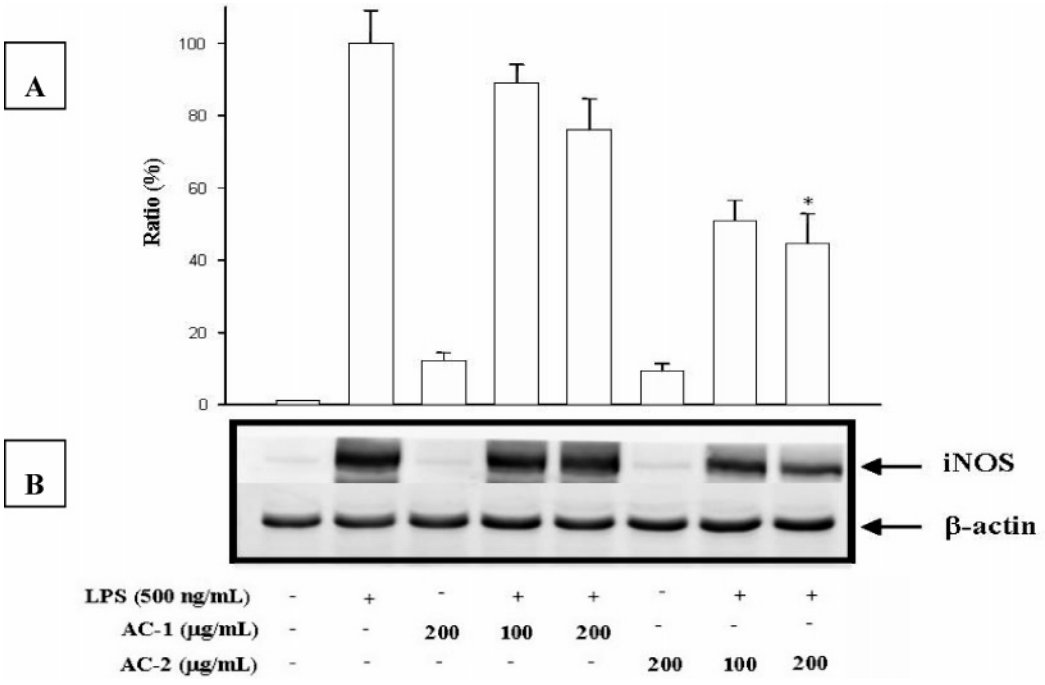

Figure 5. Effects of AC-1 and AC-2 on LPS-induced iNOS expression in RAW 264.7 cells: (**A**) The ratio of iNOS to β-actin expression observed with control is set at 1. ∗, the iNOS expression was <50% compared with LPS-treated group (100%). Data shown are means ± SD ($n = 3$). (**B**) The total cell lysates were extracted and analyzed by Western blotting as described under Materials and Methods. Data represent one of three similar results. RAW 264.7 cells were stimulated with LPS (500 ng/mL) in the presence or absence of different concentrations of AC-1 and AC-2 polysaccharides.

Effects of AC-1 and AC-2 on the LPS-Induced NO Production. NO synthesized by iNOS has been implicated as a mediator of inflammation. To study the anti-inflammatory effect of AC-1 and AC-2, the inhibitory effects of AC-1 and AC-2 on LPS-induced NO production were determined. NO production was greatly increased after LPS treatment for 24 h, as shown in **Figure 4**; only AC-2 but not AC-1 showed a dose-dependent reduction of NO production at dosages between 50 and 200 μg/mL, suggesting that fraction AC-2 may contain active polysaccharides which can be effective in the inhibition of LPS-induced NO production.

Effects of AC-1 and AC-2 on the LPS-Induced Expression of iNOS Protein and mRNA. Generally, iNOS is not present in the resting cells but can be induced by various stimuli. Increased expression of iNOS has been associated with inflammatory disorders (*25*). The LPS-induced NO production was reduced by AC-2; further testing was carried out for AC-1 and AC-2 by examining their suppressive effects on the LPS-induced iNOS protein and mRNA expressions. As shown in **Figure 5**, AC-2 but not AC-1 had dose-dependently inhibited LPS-induced iNOS protein (**Figure 5A**) and mRNA (**Figure 5B**) expression in RAW 264.7 cells, confirming that inhibition of AC-2 on LPS-induced NO production was due to the suppression of LPS-induced iNOS gene expression, accumulating the possibility that fraction AC-2 of *A. camphorata* could become an attractive candidate to be used as an adjuvant therapy for Gram-negative infections.

Conclusion. The present study is the first document which demonstrates that five polysaccharide fractions of *A. camphorata* possess different monosaccharide compositions and carbohydrate/protein ratios. By comparison of the yields, the scavenging capability against DPPH free radicals, and the inhibitory activities on LPS-induced nitrite formation in RAW 264.7 cells among fractions AC-1−AC-5, AC-2 was shown to be have the best nutraceutical potential. Fraction AC-2 at a concentration of 200 μg/mL showed distinct inhibitory activity on LPS (500 ng/mL)-induced iNOS expression. Such a result confirms the possible role of anti-inflammatory activity of *A. camphorata* as previously reported. However, further investigation is urged to elaborate a more detaiedl action mechanism.

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