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Antitumor Activities of Spray-dried Powders with Different Molecular Masses Fractionated from the Crude Protein-bound Polysaccharide Extract of *Agaricus blazei* Murill

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Abstract In this study, we first prepared 3 kinds of powders with different molecular masses from the crude protein-bound polysaccharide extract of *Agraricus blazei* Murill through ultrafiltration, followed by spray-drying. Then, the antitumor activities of the powders were analyzed. Size exclusion chromatography coupled with a multi-angle laser-light-scattering system showed the 3 powders had the following molecular ranges: below 10 kDa (SD-1), 10 to 150 kDa (SD-2), and above 150 kDa (SD-3), representing peak molecular weights of 8.26×10^3, 9.65×10^4, and 5.94×10^6 g/mol, respectively. All the powders stimulated macrophage RAW264.7 cells to produce nitric oxide, of which SD-2 and SD-3 were superior to the crude extract powder (CP-SD), while SD-1 showed the lowest activity. Similar results were found for their cytotoxicities against human cancer cell lines (A549, MCF-7, and AGS), where the highest activity was obtained with the SD-2 treatment for 72 hr at 1,000 μg/mL. The MCF-7 cell line was less sensitive to the powders than the other cells. From this research we found that ultrafiltration, in combination with spray-drying, is applicable for preparing protein-bound polysaccharide powders with higher antitumor activities.

Keywords: *Agaricus blazei* Murill, protein-bound polysaccharide, antitumor activity, molecular weight distribution, ultrafiltration

Introduction

Agaricus blazei Murill, an edible mushroom native to southern Brazil, is widely regarded as an alternative medicine and health food, and its potential medicinal properties such as antitumor (1-3), immunomodulating (4-6), antimutagenic/antibacterial (7), antiviral (8), antiinflammatory (9), hypoglycemic (10), and antioxidant (11) activities are increasingly being reported. Considering the magnitude of human death from cancer and the adverse effects of traditional chemo- or radio-therapies, this mushroom's antitumor qualities are of great interest due to its relatively high activity and low side-effects (12). Many have reported that the nature of the antitumor activity is mainly from the water soluble polysaccharide-containing proteins (1-3, 13). However, the role of the lipid fraction (14), and the structural features of (1→6)-β-D-glucan and (1→4)-α-D-glucan, are also closely related to its antitumor activity (2, 3, 15), but to a smaller extent. Gonzaga *et al.* (16) reported that the β-glucans are more predominant than α-glucans, which may attribute to the antitumor activity. From intensive studies, these polysaccharides are known to control cancer via immunomodulation such as natural killer-cell activation (5, 6) and/or the production of cytokines in macrophages (4, 17), leading to minimal side effects for controlling cancer as opposed to chemo- or radio-therapies, which weaken immune cell activity (18-20).

Many sophisticated methods have been applied for fractionating and purifying the crude extract of *A. blezei* Murill for medicinal purposes, and to characterize its antitumor components (5, 16, 21). However, none of these methods seem to be applicable in the preparation of functional foods due to their cost and safety problems. Presently, for commercialized foods, *A. blezei* Murill has been processed into whole or freeze-dried fruiting bodies, teas, capsules, and concentrated liquid extracts, however, an antitumor activity requirement has not been satisfied. This drawback has led us to prepare fractions with higher antitumor activities that are suitable for food use.

A simple hot water extraction followed by precipitation with ethanol is often used for the fractionation of antitumoric protein-bound polysaccharides (22). However, a wide range of components are still retained by this method. Besides their fine structure, the molecular weight and/or molecular weight distribution of protein-bound polysaccharides are known to be important determinants of their functionality as well as physical properties. For example, β-glucans such as lentinan, schizophyllan, and grifolan can induce antitumor activity at molecular weights of more than $1\text{-}4\times10^4$ (23), approximately.

In this study, we ultrafiltered the crude extract of *A. blezei* Murill by molecular mass range, in order to prepare fractions with higher antitumor activities; the fractions were also spray-dried to present a practical use for further food processing. Finally, we compared their antitumor and immunomodulating activities.

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Materials and Methods

Materials The dried fruiting bodies of *A. blazei* Murill were obtained from Sunglim Farm Inc. (Gyungsan, Korea) and crushed into a powder of 60 to 100 size mesh using a mill, and were kept at -18°C until use.

Preparation and fractionation of crude extract and spray-drying The crude extract of *A. blazei* Murill was prepared, fractionated, and spray-dried according to previously stated methods (24, 25). Briefly, 1 kg of powder was extracted with 20 L of distilled water by heating at 121°C for 2 hr under 150 kPa of pressure, and the collected supernatant was centrifuged at 708×g after cooling at room temperature. After concentrating to 1/3 volume by vacuum evaporation, 4 volumes of 95% ethanol were added to the filtrate, which was kept at 4°C for 24 hr. The precipitate that formed was collected by centrifugation at 6,371×g and dissolved in distilled water to a concentration of 5°Bx. This solution was used for further experiments as the crude protein-bound polysaccharide fraction (CP).

The fractionation by molecular mass was carried out by ultrafiltration using polysulfone hollow fiber membranes with molecular weight cut-off values of 150 kDa and 10 kDa (Sunkyong Inc., Seoul, Korea). The CP fraction was first applied to a 150 kDa membrane at an operating pressure of 200 kPa, and in turn, the filtrate was taken through a 10 kDa membrane at 120 kPa. By the ultrafiltration processes, we obtained 3 fractions according to molecular weight range. The ranges were: below 10 kDa, between 10 to 150 kDa, and above 150 kDa. The 3 fractions and CP were concentrated to 20°Bx, and dried into powders by a B-191 spray drier (Lab Plant, Buchi Inc., Flawil, Switzerland) with a standard 0.7 mm nozzle. The feed rate, compressed air flow, and inlet temperature of the spray drier were 8 mL/min, 600 L/hr, and 200°C, respectively. The spray-dried CP powders, including the fraction below 10 kDa, the fraction between 10 to 150 kDa, and the fraction above 150 kDa, were designated as CP-SD, SD-1, SD-2, and SD-3, respectively.

Determination of molecular weight distributions The molecular weights of the protein-bound polysaccharide powders were estimated by size exclusion chromatography (SEC) coupled with a multi-angle laser-light-scattering system (MALLS, Down DSP; Wyatt Technology Co., Ltd., Santa Barbara, CA, USA) (25). The powders were dissolved in a phosphate/chloride buffer (ionic strength = 0.1, pH 6.8) containing 0.04% ethylenediaminetetraacetic acid-disodium salt and 0.01% sodium azide, and filtered through 0.22 μm membranes (Millex HV type; Millipore Corp., Bedford, MA, USA) prior to injection into the SEC/MALLS system. The chromatographic system consisted of a degasser (Degasys DG-1200, Uniflow; HPLC Technology, Macclesfield, UK), a high performance pump (Model 590 Programmable Solvent Delivery Module; Waters Corp., Milford, MA, USA), an injection valve (Rheodyne Inc., Cotati, CA, USA) fitted with a 100 μL loop, 3 SEC columns (Shodex Protein KW-802.5, 803, 804; Showa Denko K.K., Tokyo, Japan) connected in series, and an RI detector (410; Waters).

Chromatography was performed at room temperature. The flow rate was 0.8 mL/min, and the injection volume and concentration were 100 μL and 3 mg/L, respectively. The light scattering and RI signals were analyzed using the ASTRA software package (Wyatt Technology Co.) to calculate the weight-average molecular mass.

Assay of nitric oxide production in macrophages Nitric oxide (NO) production was estimated using the virus-transformed macrophage cell line RAW264.7 according to the method described by Kim *et al.* (22), with some modification. In brief, the RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 2 mM L-glutamine, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum (Hyclone, Logan, UT, USA). The cells were grown at 37°C and 5% CO_2 in fully humidified air, and subcultured 2 times a week; they were then seeded in 12-well plates at 1×10^5 cells/well. The cells were stimulated for 24 hr by adding the spray-dried powders at given concentrations. Lipopolysaccharide (LPS) was used for the comparison.

The synthesis of NO was determined by assaying the culture supernatants for nitrite, the stable reaction product of NO and molecular oxygen. After 100 μL of each culture supernatant was allowed to react with 100 μL of Griess reagent [1%(w/v) sulfanilamide, 0.1%(w/v) naphthylethylenediamine dihydrochloride, and 2.5%(w/v) phosphoric acid] at room temperature for 10 min, the optical densities of the assay samples were measured spectrophotometrically at 570 nm. The nitrite concentrations were calculated from a standard curve derived from the reaction of sodium nitrite under the assay conditions. Fresh culture medium served as the blank in all experiments.

Assay of antitumor activity The antitumor activities of the powders were analyzed using human cancer cell lines such as A549 (lung cancer), AGS (gastric cancer), and MCF-7 (breast cancer) obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). All the cell lines were incubated in RPMI 1640 medium supplemented with 10%(v/v) heat-inactivated fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a highly humidified atmosphere of 5% CO_2 in air at 37°C (26). The capacity of the powders to inhibit cancer cell growth was measured by a sulforhodamine B (SRB) assay (27). Immediately, adherent cells in 0.1 mL of media were placed into the wells of 96-well plates in a 5×10^4 cell/mL concentration and allowed to attach for 24 hr. The spray-dried powders, which had been dissolved in 100 μL of dimethyl sulfoxide to desired concentrations, were added to the wells, and the plates were incubated at 37°C for 24 and 72 hr. The cells were then fixed by gently adding 100 μL of cold 10% trichloroacetic acid (w/v) to each well, followed by incubation at 4°C for 1 hr. The wells were washed 5 times with deionized water and allowed to air-dry. Next, the cells were stained for 15 min by adding 100 μL of SRB solution [0.4% SRB (w/v) in 1% acetic acid (v/v)]. The wells were then quickly washed 5 times with 1% acetic acid (w/v) to remove any unbound dye, and air-dried. The bound dye was sufficiently solubilized with 100

μL of Tris buffer (10 mM, pH 10.5), and the optical density was measured at 540 nm using an ELISA reader (ELISA Processor II, Behring Co., Marburg, Germany). The results were expressed as the mean±SD of triplicate experiments.

Results and Discussion

Molecular weight distributions of spray-dried powders When the crude extract of *A. blazei* Murill is prepared by ethanol precipitation after a high-pressure water extraction, it is composed mainly of protein-bound polysaccharides with a wide range of molecular weights (28). For further fractionation by molecular weight an ultrafiltration process can be applied, which is suitable for separating natural polymers in a molecular range of 1 to 500 kDa, and has value in food processing for eliminating the toxicity problems pertaining to solvents and/or chemicals, as well as for economical efficiency, as compared to column fractionation (29, 30). After performing ultrafiltration, the fractions we obtained were dried into powders for the various advantages in food processing, such as extended storage, the reduction of transportation cost, and the diversification of food, etc. These powders were prepared by spray-drying, which is relatively quick, and in large scale applications the powder properties and low hygroscopic nature can be more easily controlled compared to freeze-drying (31, 32).

When the spray-dried powders with molecular ranges below 10 kDa (SD-1), 10 to 150 kDa (SD-2), and above 150 kDa (SD-3) were analyzed for their molecular weight distributions by the SEC/MALLS system (Table 1), all the powders showed an almost Gaussian distribution, and the peak molecular weights of SD-1, -2, and -3 were 8.26×10^3, 9.647×10^4, and 5.938×10^6 g/mol, respectively. These values are almost identical to those reported previously (25). The yields for SD-1, -2, and -3 were 13.4, 34.5, and 52.1%, respectively.

NO production in RAW264.7 cells Macrophages play an important role in the immune system and can directly induce tumoricidal activity through the release of several inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor-α (TNF-α), and also release NO when activated in a nonspecific manner by bacterial products such as LPS and muramyl dipeptide (33). NO is known to be an important messenger in diverse pathophysiological functions, including neuronal transmission, vascular relaxation, and immune modulation, and its cytotoxicity against tumor cells is also well-documented (34, 35).

Given the pivotal roles of macrophages in antitumor activity via the immune system, we compared the abilities of CP-SD, SD-1, SS-2, and SD-3 to produce NO in RAW246.7 macrophage cells. When the cells were cultured with the different powders at various concentrations, which were determined not to affect cell viability by a preliminary test (22), NO production increased beyond the control level in a concentration-dependent manner (Fig. 1), except for the treatment at the 10 μg/mL concentration. This suggests that the powders above the 10 μg/mL concentration activated the macrophages to produce NO irrelevant of their molecular weight distributions. The powders over 10 kDa (SD-2 and SD-3) further raised NO production compared to the powder (CP-SD) prepared from the crude extract, even though some treatments didn't show significant differences; for example, an increase of approximately 1.7 (SD-3) to 33% (SD-2) at 100 μg/mL, and 1.6 (SD-2) to 10.8% (SD-3) at the 500 μg/mL concentration. However, SD-1 didn't show a significant difference even at the highest concentration (1 mg/mL). These data suggest that the macrophages were activated mainly by protein-bound polysaccharides with molecular masses above 10 kDa, which is similar to the results of Yoshiyuki *et al.* (23). Also, during ultrafiltration, more active compounds were concentrated through the elimiation of less active ones below 10 kDa.

Table 1. MALLS analysis results for relevant molecular parameters of spray-dried powders with different molecular mass ranges prepared from *A. blazei* Murill[1)]

Parameter[2)]	SD-1 (error %)	SD-2 (error %)	SD-3 (error %)
M_n (g/mol)	7.58×10^3 (4.2)	9.069×10^4 (3.0)	4.259×10^6 (1.3)
M_w (g/mol)	8.26×10^3 (3.8)	9.647×10^4 (3.0)	5.938×10^6 (1.0)
M_z (g/mol)	8.74×10^3 (8.5)	1.027×10^5 (7.0)	7.392×10^6 (2.4)
M_w/M_n	1.09	1.064	1.394
R_n (nm)	61.8 (3.4)	81.2 (7.0)	14.3 (2.9)
R_w (nm)	66.2 (3.1)	82.0 (6.0)	19.1 (2.4)
R_z (nm)	72.7 (3.1)	82.9 (6.0)	23.1 (2.1)

[1)]SD-1, SD-2, and SD-2 represent the spray-dried powders with molecular weights below 10, 10 to 150, and above 150 kDa, respectively.
[2)]M_n, M_w, and M_z refer to number-, weight-, and z-average molecular weight, respectively. M_w/M_n represents the polydispersity ratio; R_n, R_w, and R_z refer to number-, weight-, and z-average root-mean-squared radius of gyration, respectively.

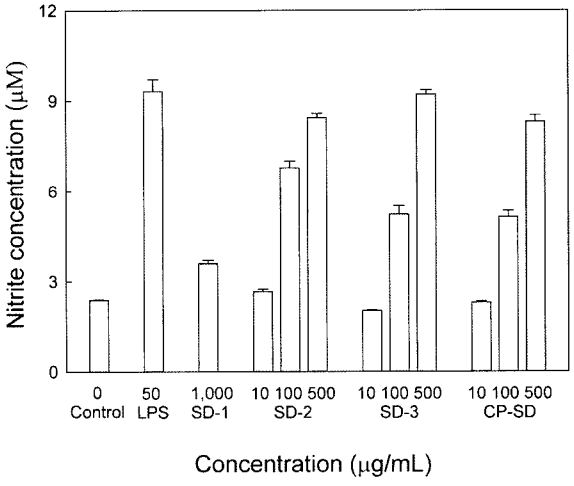

Fig. 1. Nitric oxide production in RAW264.7 cells treated with spray-dried powders of different molecular mass ranges prepared from *A. blazei* Murill. Control and LPS represent treatments without any stimulant with LPS (50 ng/mL), respectively, used for comparison.

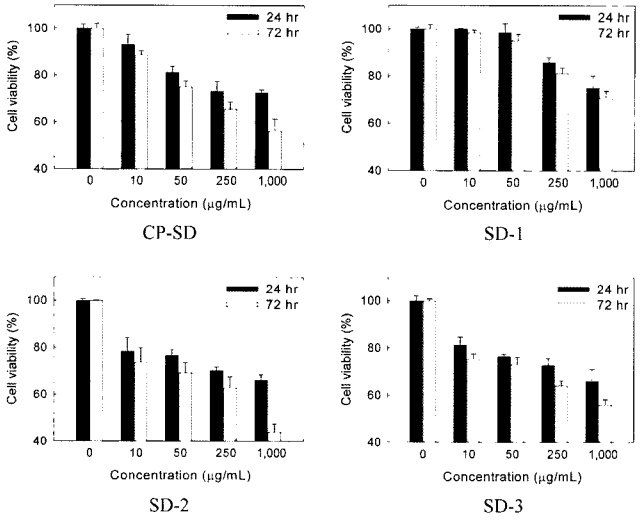

Fig. 2. Viability of A549 cells treated with spray-dried powders of different molecular mass ranges prepared from *A. blazei* Murill.

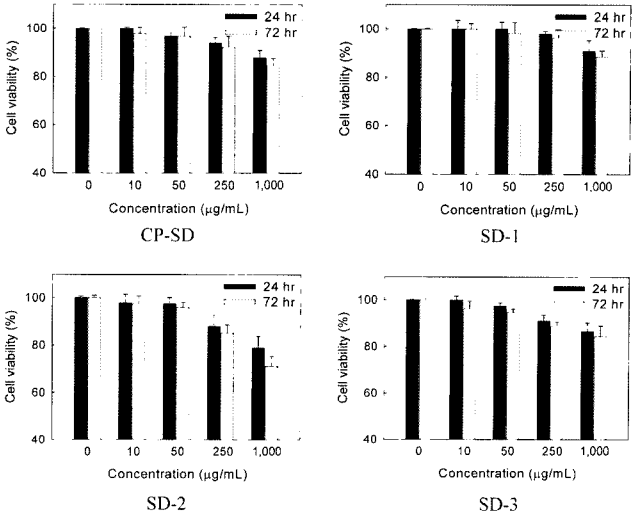

Fig. 3. Viability of MCF-7 cells treated with spray-dried powders of different molecular mass ranges prepared from *A. blazei* Murill.

Cytotoxicity against human cancer cell lines In addition to their immunoactivation properties, the cytotoxic effects of the powders against cancer cells were investigated by monitoring cell viability after exposing the cancer cells to the powders at given concentrations for 24 and 72 hr.

For the human lung cancer cell line A549, all of the powders showed cytotoxicity in dose- and time-dependent manners, and higher cytotoxicity was found in the treatments with SD-2 and SD-3 compared to the non-fractionated CP-SD, especially at the lower concentration of powder (Fig. 2). After the treatment of 10 μg/mL of powder for 72 hr, SD-2, and SD-3 reduced A549 viability by 26.4±6.2 and 24.6±2.2%, respectively, while CP-SD reduced viability 11.4±1.8%. The highest reduction (56.1± 3.5%) occurred with the 1,000 μg/mL SD-2 treatment for 72 hr. SD-1 presented an even lower activity than CP-SD, indicating that protein-bound polysaccharides below 10 kDa were ineffective at inactivating A549 cells. Similar to their immunoactivation, the stronger cytotoxicities of SD-2 and SD-3 against the A549 cells are likely from the exclusion of the ineffective smaller components of the crude extract. According to Choi *et al.* (28), who used whole water extracts of *A. blazei* Murill, the cytotoxicities of these powders seem to be related to the ability of the protein-bound polysaccharides to arrest the G2/M phase of the cell cycle and to induce apoptosis of the A549 cells.

Figure 3 shows the cytotoxic effects of the powders on the human breast cancer cell line MCF-7. These cells showed a similar declination in viability by the powder treatments as the A549 cells, but to a lesser degree. At 72 hr, the SD-2 treatment was most effective at inactivating the MCF cells, with 14.8±3.5 (250 μg/mL) and 28.8±4.2% (1,000 μg/mL) inhibition. SD-3 was better than the crude extract, but not significantly. SD-1, containing the smallest molecules, had the least cytotoxicity, just as in the A549 cells.

Similar cytotoxic effects were revealed in the human

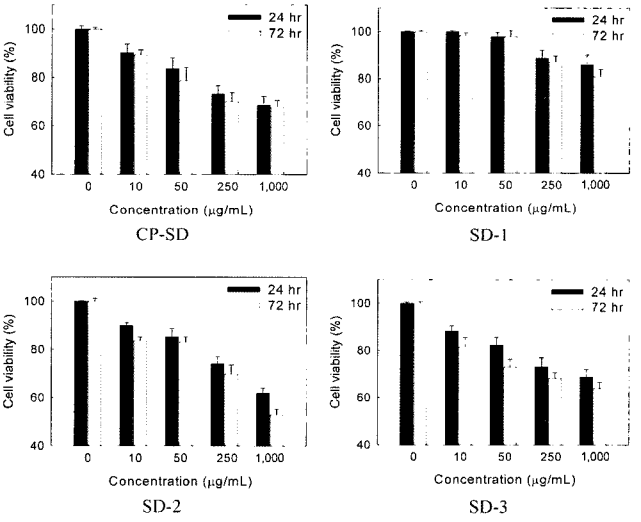

Fig. 4. Viability of AGS cells treated with spray-dried powders of different molecular mass ranges prepared from *A. blazei* Murill.

gastric cancer cell line AGS (Fig. 4), where the effects of the powders were generally lower than against the A549 cells, but higher than against the MCF-7 cells. The highest cytotoxicity was found with the SD-2 treatment of 1,000 μg/mL at 72 hr (47.1±2.5%), and the lowest was shown with SD-1.

In conclusion, it was possible to prepare protein-bound polysaccharide powders with higher antitumor activities through ultrafiltration followed by spray drying. When we compared antitumor activities, the SD-2 (molecular weight 10 to 150 kDa) and SD-3 (molecular weight above 150 kDa), were more effective than the CP-SD (crude extract not fractionated, that is, with an entire molecular weight range) with regards to NO production in macrophage RAW264.7 cells. These powders, especially SD-2, were

also superior at defeating human cancer cells such as A549, MCF-7, and AGS. Components with molecular weights below 10 kDa showed lower macrophage NO production as well as cancer cell cytotoxicity than CP-SD. The higher antitumor activities of SD-2 and SD-3 were presumed to be possible by the elimination of components below 10 kDa with ineffective activities.

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