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Mushroom immunomodulatory proteins possess potential thermal/freezing resistance, acid/alkali tolerance and dehydration stability

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Abstract

Mushroom lectins have been reported as immunomodulatory proteins. To evaluate their functionalities, as affected by various industrial procedures, two mushroom proteins, including *Agaricus bisporus* lectin (ABL) and the immunomodulatory protein of *Auricularia polytricha* (APP), were treated by various methods, mimicking food processing procedures, in prior-to-cell experiments, and their macrophage-activating functionalities were determined in the induction of tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) productions by RAW264.7 cells *in vitro*. The remaining activities of ABL and APP, after autoclave treatment (121 °C, 15 min), were observed to be 77.4% and 80.7%, respectively, in their stimulations of TNF-α production by cells. Boiling (100 °C, 30 min) and freezing (−80 °C, 24 h) treatments did not reduce their effects on TNF-α and NO secretions, while treating with pH 2 and pH 13 buffers only resulted in insignificant decrease of the ABL- and APP-induced TNF-α and NO production. Moreover, ABL and APP also withstood vacuum dehydration with 96.5% and 84.6% of activities being retained, respectively, in their stimulations of TNF-α production. These findings revealed that ABL and APP had thermal/freezing-resistant, acid/alkali tolerance and dehydration stable properties, and that they were potential candidates, as stable immune stimulants, for health food and pharmaceutical utilization.

Keywords: Mushroom immunomodulatory protein; *Agaricus bisporus*; *Auricularia polytricha*; Stability

1. Introduction

Many components from mushrooms have been shown to be potential immunoregulatory agents that induce modulatory effects in immune function (Breene, 1990; Wasser & Weis, 1999). Fungal immunomodulatory proteins have also been found as immunostimulants in mushroom, which have attracted attention due to their pharmaceutical potential. Bioactive proteins, mostly identified as agglutinins, lectins or immunomodulatory proteins, constitute an important group of functional agents in mushrooms. Many mushroom proteins possess immunomodulatory and anti-tumor activities *in vitro* and *in vivo* (Wang, Ng, Liu, Ooi, & Chang, 1995; Yu, Fernig, Smith, Milton, & Rhodes, 1993). Several studies have demonstrated that some of the mushroom proteins, not only regulate immune cell growth, but also activate macrophages and lymphocytes (Hsu, Hsu, Lin, Kao, & Lin, 1997; Wang, Liu, Ng, Ooi, & Chang, 1996). It has also been shown that mushroom proteins are potent immune activators and tumor cell growth inhibitors, mediating their effects by regulating cytokine secretion and proliferation, and seem to be mitogen and immunomodulator with therapeutic potential (Ho, Sze, Shen, & Liu, 2004; Wang, Huang, & Chang, 2004; Ye et al., 2005; Zhao, Sun, Tong, & Qi, 2003). Numerous mushroom protein complexes have therefore been approved for clinical application in immunomodulation and cancer therapy (Fisher & Yang, 2002; Monro, 2003).

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Among abundant mushroom proteins, *Agaricus bisporus* lectin (ABL) is a well-documented mushroom protein because *A. bisporus* is a popular edible mushroom in western cuisines. ABL, which was first reported by Presant and Kornfeld (1972) and revealed erythrocyte-agglutinating activity, is a homotetramer with the molecular weight of 64,000 Da, composed of four apparently identical subunits (16,000 Da) that have quite similar carbohydrate-binding specificities (Sueyoshi, Tsuji, & Osawa, 1985). ABL has a sequence that does not show any significant similarities to any of the other lectins, that bind the T antigen, studied so far by X-ray diffraction (Crenshaw, Harper, Moyer, & Privalle, 1995). Additionally, ABL is also known as an immune modulator. ABL showed an anti-proliferative effect on diverse epithelial cell lines, including malignant cancer cells, by internalization without any apparent cytotoxicity (Parslew, Jones, Rhodes, & Sharpe, 1999; Yu et al., 1999). ABL also appeared to induce the up-regulation of IL-1β and TNF-α in splenocytes and macrophages and triggered the early lymphocyte activation cascades, resulting in a decline of $p56^{lck}$ and rapid expression of CD69 and CD25 (Ho et al., 2004; Ooi, Liu, Ooi, Ng, & Fung, 2002). Furthermore, *Auricularia polytricha* is another edible mushroom and is frequently consumed as a food and a traditional medicine in Asia. An immunomodulatory protein, APP, has been purified from the fruiting body of an edible *A. polytricha* by anion-exchange chromatography (Sheu, Chien, Chien, Chen, & Chin, 2004). APP is a simple protein with a molecular mass of 13.4 kDa with a pI of approximately 5.1. APP exhibits the ability to agglutinate mouse red blood cells and also to activate immune cells. APP activates RAW264.7 macrophages and induces nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) production by cells and presents no cytotoxicity *in vitro* (Sheu et al., 2004). TNF-α and NO are recognized as multifunctional cytokines and factors playing key roles in apoptosis and cell survival, as well as in inflammation and immunity. For all these reasons, immunomodulatory proteins from edible mushrooms, such as ABL and APP, are candidates as immune stimulants and display a great potential for use in health food and pharmaceutical applications.

Mushrooms have been valued by humankind as an edible and medical resource. The utilization and processing of mushrooms and their bioactive proteins usually include various extreme operations, such as thermal sterilization, freezing treatment, acid and alkali processing, and a dehydration procedure. Since the resistance of mushroom proteins to diverse treatments in food processing and pharmaceutical production for the regulation of cellular immune response remained unclear, it is of great importance to understand their remaining bioactivities after such ultimate processing operations. In the present work, we focussed on the abilities of ABL- and APP-activating murine macrophages to produce TNF-α and NO, and whether their activities were affected by handling in a comparable manner. The stability and tolerance of these two mushroom proteins when undergoing these processing operations were investigated for further utilization of the mushroom as a health food formula and dietary supplement.

2. Materials and methods

2.1. Materials and chemicals

Fruiting bodies of *A. bisporus* and *A. polytricha* were obtained from the local market. DEAE-52 cellulose was purchased from Whatman (Maidstone, Kent, UK) and Mono Q HR 5/5 columns were purchased from Amersham (Uppsala, Sweden). Dialysis membrane tubing (Spectra/Por 1, WMCO 6–8 kDa) was purchased from Spectrum (Rancho Dominguez, CA). Lipopolysaccharide (LPS) from *Escherichia coli* was obtained from Sigma Chemical (St. Louis, MO). All other chemicals were commercially available products of analytical grade.

2.2. Purifications of ABL and APP

ABL and APP were purified from *A. bisporus* and *A. polytricha* fruiting bodies according to the methods described by Irazoqui, Zalazar, Chiabrando, Romero, and Vides (1992) and Sheu et al. (2004), respectively. The fresh fruiting bodies were homogenized with cold 5% (v/v) acetic acid solution, including 0.05 M 2-mercaptoethanol. The homogenates were centrifuged and treated with ammonium sulfate to 95% of saturation to precipitate soluble proteins in the supernatant. The precipitates were collected and then dialyzed against 10 mM Tris–HCl buffer, pH 8.2 for 72 h with four changes of dialysis solution. The dialysate was fractionated on a DEAE-cellulose column (2.5 cm × 20 cm) that was previously equilibrated using 10 mM Tris–HCl buffer, pH 8.2. The column was eluted with 300 ml of 0–0.5 M NaCl in 10 mM Tris–HCl buffer, pH 8.2. The main active fractions were further purified by a fast protein liquid chromatography (FPLC) system with a Mono Q HR 5/5 column, which was previously equilibrated with 10 mM Tris–HCl buffer, pH 8.2. All purification processes were performed at 4 °C. Fractions containing ABL or APP were further collected for further experiments.

2.3. Electrophoresis and PAS staining

The purified proteins were routinely analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a Bio-Rad mini protein III gel apparatus to evaluate their identification and purity, using the method described by Laemmli (1970) and then the gels were stained with Coomassie Brilliant Blue R250. The molecular weights of the subunits were measured by using the low-range rainbow molecular weight markers (Amersham Biosciences, UK) consisting of the following proteins: ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), lysozyme (14,300), and aprotinin (6000). Apparent

molecular weights were determined by comparison with protein standards of known molecular weight. The polyacrylamide gels were further stained with periodic acid–Schiff's (PAS) reagent (Merck, Darmstadt, Germany) to determine the carbohydrate content according to the method of Zacharius, Zell, Morrison, and Woodlock (1969).

2.4. Determination of endotoxin levels (Limulus amoebocyte lysate assay)

Limulus amoebocyte lysate (LAL) reagents, endotoxin-free water, endotoxin-free pipette tips, and endotoxin-free reaction tubes were purchased from Charles River (Charleston, USA). Control standard endotoxin LPS from *E. coli* strain O55:B5 was obtained from Charles River Endosafe™ (Charleston, USA) and used as a reference LPS in the LAL assay. The LAL clot assay was performed in duplicate in test tubes to which 0.1 ml of protein sample (ABL and APP) and 0.1 ml of LAL reagent (Charles River Endosafe™, Charleston, USA) were mixed. After 1 h of incubation at 37 °C, the test tubes were examined by 180° inversion for the presence of a stable solid clot, which was considered to be a positive result. The sensitivity of the clotting assay was defined as the lowest concentration of LPS that still produced a positive LAL reaction. It was confirmed that the sensitivity of LAL clot assay was 0.015 EU/ml of endotoxin, by serial dilutions prior to use.

2.5. Physical and chemical treatments for ABL and APP

ABL and APP were treated with various operations, mimicking pharmaceutical and food processing procedures, in prior-to-cell experiments to characterize the stabilities of ABL and APP. To investigate their thermostability and freezing resistance, ABL and APP samples were parafilm-sealed in Eppendorf tubes and were further autoclaved at 121 °C for 15 min, or heated with boiling water at 100 °C for 30 min, or frozen at −80 °C overnight. To evaluate the acid/alkali tolerance of ABL and APP, the protein samples were dialyzed against equilibration buffers (50 mM sodium phosphate buffer, pH adjusted with HCl and NaOH to 2 and 13, respectively). After 2 h, the pH values of dialysates were measured using pH indicator paper (pH 0–14, Whatman International, Ltd., Maidstone, UK) to verify their equilibration and then dialyzed against phosphate-buffered saline buffer (PBS, pH 7.2) for 24 h. Moreover, the dehydration treatment was conducted by using a rotational-vacuum-concentrator (Sigma Laborzentrifugen GmbH, Germany) for 30 min/ml until the solutions were completely dried, and then the dehydrated protein powders were redissolved in PBS buffer. After these preparations, the protein concentration of each sample was determined according to the bicinchoninic acid method (Smith et al., 1985). According to the protein concentration of each sample, measured after various treatments, the pretreated ABL and APP were diluted at the indicated concentration with cell culture media for further cell experiments. For acid and alkali treatments, the pH values of final samples for cell experiments were confirmed again within a range of pH 6.5–8.0.

2.6. Cell culture and activation

RAW264.7, a murine macrophage cell line, was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 4 mM L-glutamine, 1.4 g/l of sodium bicarbonate, 4.5 g/l of glucose, 1 mM sodium pyruvate (Gibco/BRL Life Technologies, Eggenstein, Germany), and 10% (v/v) FBS (Hyclone, Logan, UT). RAW264.7 cells were maintained at 37 °C in a humidified incubator containing 5% CO_2 via weekly passage and cells were utilized for experimentation at 60–80% confluency. To investigate the effects of physical/chemical-treated ABL and APP on the RAW264.7 macrophages, the cells (2×10^5 cells per well) were incubated overnight at 37 °C and 5% CO_2 in 96-well plates (Costar; Corning Incorporated, Corning, NY) to facilitate attachment and spreading before experimentation. Cells were then stimulated with 100 ng/ml of LPS or treated with ABL or APP samples (0–40 μg/ml). After an additional culture for 20 h, supernatants were collected to determine their TNF-α and nitrite levels.

2.7. Determination of NO production

The nitrite concentration in the medium was measured as an indicator of NO production according to the colorimetric Griess reaction (Jun & Wennmalm, 1994). Briefly, cells were treated with different concentration of ABL or APP samples and then harvested culture media were reacted with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride, and 2.5% phosphoric acid) in 96-well microtitre plates (Greiner Bio-One GmbH, Frickenhausen, Germany) at room temperature for 10 min, and nitrite concentration was determined with an ELISA microplate reader (Hercules, CA), using absorbance at 540 nm in comparison with sodium nitrite ($NaNO_2$) as a standard. Background levels of nitrite were determined in cell-free DMEM, with or without the additives, and were subtracted from the total amount of nitrite formed.

2.8. Determination of TNF-α production

The levels of TNF-α were measured in cell culture media collected from treated cells using OptEIA murine TNF-α ELISA kits (BD PharMingen, San Diego, CA). Briefly, a 96-well ELISA plate (Costar; Corning Incorporated, Corning, NY) was coated with capture antibodies (anti-mouse TNF-α) diluted in coating buffer (sodium phosphate, pH 6.5) overnight at 4 °C. The wells were aspirated and washed three times with PBS containing 0.05% Tween 20. The plate was then blocked with PBS containing 10% FBS for 1 h at

room temperature. TNF-α was bound by incubating culture supernatants or serial standard diluents of recombinant mouse TNF-α for 2 h at room temperature. Subsequently, the wells were washed, and then the biotinylated mouse TNF-α mAb and avidin-HRP conjugate were placed in the wells for 1 h of incubation. After extensive washing, the substrate solutions (tetramethylbenzidine and hydrogen peroxide) were added to each well for 20 min. The absorbance was measured at 405 nm on a Bio-Rad-3550 microplate reader (Hercules, CA), and the TNF-α content was calculated by comparing recombinant mouse TNF-α as a standard.

2.9. Statistical analysis

Data are represented as means ± SD of three separate experiments performed in triplicate. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Duncan multiple comparisons test. Differences were considered as statistically significant when the P values were below 0.05 ($P < 0.05$).

3. Results

3.1. Preparation of ABL and APP

The proteins purified by FPLC anion-exchange chromatography were detected by electrophoresis analysis. The SDS/polyacrylamide gel showed that ABL gave a single band with an apparent molecular mass of 16 kDa (Fig. 1a, lane 1), and APP yielded an apparent molecular mass of 13.4 kDa (Fig. 1a, lane 2). Staining of the polyacrylamide gel, with periodic acid/Schiff's reagent, revealed that ABL and APP were both negative staining (Fig. 1b, lanes 4 and 5). These observations confirmed that the ABL and APP samples had the same characteristics as described in the literature (Irazoqui et al., 1992; Sheu et al., 2004) and also indicated that ABL and APP were simple proteins with little or no carbohydrate content. Subsequently, ABL and APP, previously treated by various procedures, were determined by polyacrylamide gel electrophoresis. As shown in Fig. 1c and d, ABL and APP had the abilities to avoid degrading through the foregoing processes.

3.2. Immunomodulatory activities of ABL and APP

To determine suitable concentrations of ABL and APP for further experiments, RAW264.7 macrophages were activated with 0–40 μg/ml solution of ABL or APP and the levels of TNF-α and nitrite in the culture soups were analyzed. This dose-response study revealed that ABL and APP could directly promote the TNF-α and NO production by RAW264.7 macrophages (Fig. 2). The predominant enhancements of TNF-α and NO levels were induced under the ABL and APP treatments at concentrations ranging from 2.5 to 40 μg/ml, respectively. ABL and APP caused rapid dose-dependent effects on TNF-α production. In the presence of ABL (10–40 μg/ml), the amounts of TNF-α secreted were up to $1.6–2.0 \times 10^5$ pg/ml and reached a plateau. APP was also demonstrated to have a similar tendency. In addition, there were significant

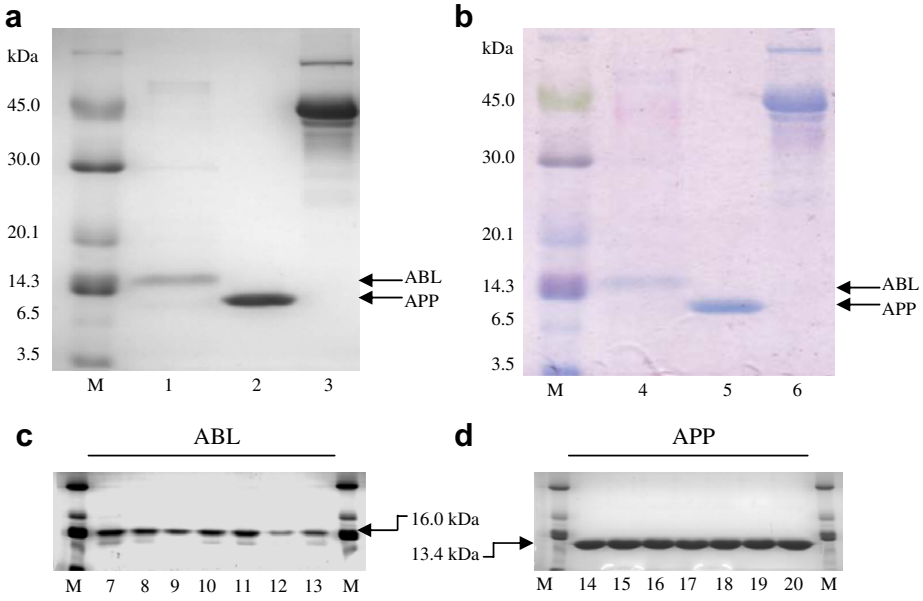

Fig. 1. SDS–PAGE of purified ABL and APP (a), and their remains after various treatments (c and d) and periodic acid/Schiff's (PAS) staining (b). The polyacrylamide gels were stained with Coomassie Brilliant Blue R-250 (a, c, and d) and Schiff's reagent (b). ABL (c) and APP (d) were pretreated by various procedures, including vehicle (untreated, lanes 7 and 14), freezing (−80 °C, lanes 8 and 15), boiling (100 °C, lanes 9 and 16), autoclaving (121 °C, lanes 10 and 17), different pH treatments (pH 2, lanes 11 and 18; pH 13, lanes 12 and 19), and dehydrating (lanes 13 and 20). Lane M, molecular mass markers; lanes 1, 4, and 7–13, ABL; lanes 2, 5, and 14–20, APP; lanes 3 and 6, ovalbumin as the molecular weight control.

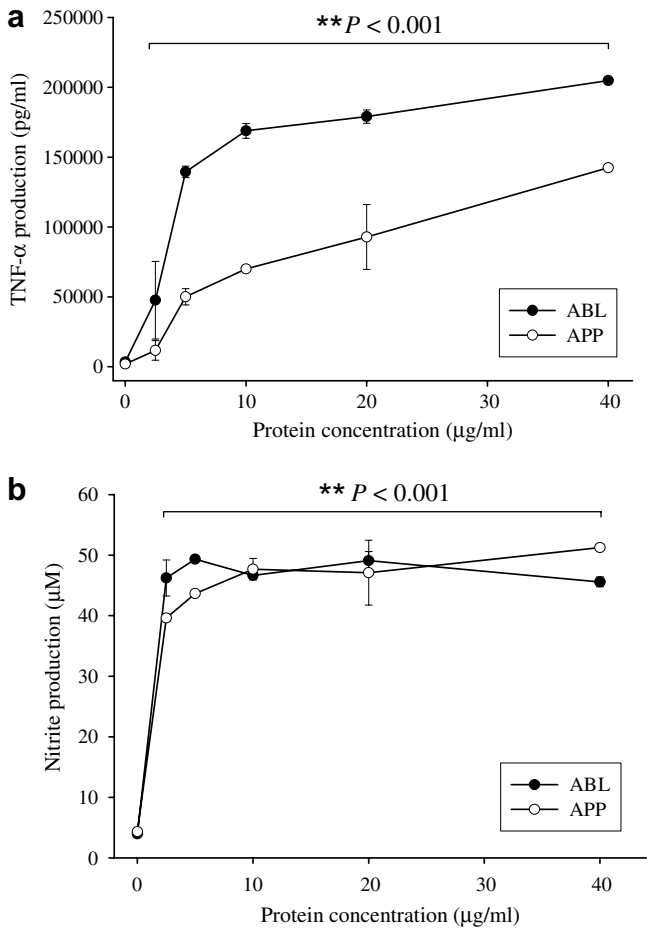

Fig. 2. ABL- and APP-promoted TNF-α production (a) and nitrite accumulation (b) in the culture supernatants of RAW264.7 macrophages. RAW264.7 macrophages were activated using ABL (0–40 μg/ml) or APP (0–40 μg/m1) alone for 20 h. The levels of TNF-α were measured using sandwich ELISA and the nitrite concentrations were determined from the Griess reaction. Results are expressed as means ± SE from three independent experiments ($n = 3$). A P value of <0.05 is considered to be significantly different from the culture media control ($^{**}P < 0.001$).

increases in nitrite accumulation by RAW264.7 macrophages treated with 2.5 μg/ml of ABL and APP alone. At higher concentrations, however, the abilities to promote NO accumulation of ABL and APP exhibited maxima. For both the ABL and APP, 10 μg/ml was chosen as the working concentration for the following experiments.

Moreover, ABL and APP, ranging from 2.5 to 40 μg/ml, exhibited negative results in the LAL clot assay (the absence of a stable solid clot) (data not shown). ABL and APP were found to be less than 0.015 EU/ml of endotoxin according to the sensitivity (0.015 EU/ml) of the gel-clot reagent. RAW264.7 macrophages could not induce TNF-α production in the presence of approximately 1.5 EU/ml of endotoxin of *E. coli* O55:B5 (Ochiai et al., 2002). Therefore, it was verified that the abilities of ABL and APP to induce TNF-α and NO production toward RAW264.7 macrophages did not result from the contamination of endotoxin.

3.3. Effects of temperature treatments on ABL and APP activities

Our results showed the immunomodulatory activities of ABL and APP. Here, we further investigated the thermal stability of ABL and APP. The results showed that autoclave treatments significantly decreased the production of TNF-α and NO for both ABL ($P < 0.001$) and APP ($P < 0.05$) but did not destroy all of their macrophage-activating activities (Fig. 3). After autoclaving at 121 °C for 15 min, ABL and APP still had 77.4% and 80.7% of their TNF-α promoting activities (Fig. 3, panels a and c), respectively, as compared to that of the vehicle. The boiling treatment, which could give a minor thermal breakage of proteins, yielded higher remaining activities of ABL and APP (92.3% and 99.9%, respectively). Moreover, ABL and APP showed resistance to freezing and thawing treatments, while remaining activities (89.4% and 98.8%), respectively, were observed for ABL and APP. Similar results (Fig. 3, panels b and d) for the temperature-stability of ABL and APP were obtained in the induction of NO generation by cells. The influence of temperature treatments on the protein activity (in stimulating NO production by cells) was inferior to that in TNF-α production for both ABL and APP. The entire following NO promoting activities of ABL and APP toward RAW264.7 cells remained above 89% after 121 °C, 100 °C, and −80 °C treatments. These results demonstrated that ABL and APP showed superior thermal and freezing resistances and kept most of their immunomodulatory activities toward RAW264.7 macrophages.

3.4. Effects of acid and alkali treatments on ABL and APP activities

To determine their stabilities and activities under ultimate pH conditions, the remaining activities of acid/alkali-treated ABL and APP are shown in Fig. 4. It was observed that ABL and APP exhibited superior protein stabilities at pH 2 and 13 (Fig. 4). When ABL and APP were exposed to acid or alkali conditions in prior-to-cell experiments, no significant reductions occurred in TNF-α and NO productions by protein-activated RAW264.7 macrophages ($P > 0.05$). Their macrophage-stimulating activities remained above 95% in all acid/alkali treatments. The results showed that ABL and APP resisted acid hydrolysis and alkali decomposition upon a short exposure to extreme pH conditions.

3.5. Effect of dehydration treatment on ABL and APP activities

Remaining activities, after the dehydration treatment, are shown in Fig. 5. After vacuum dehydrating, ABL was still able to activate RAW264.7 macrophages to secrete TNF-α and NO, and its activities remained above 96%. Similar results with APP promoting NO, were also

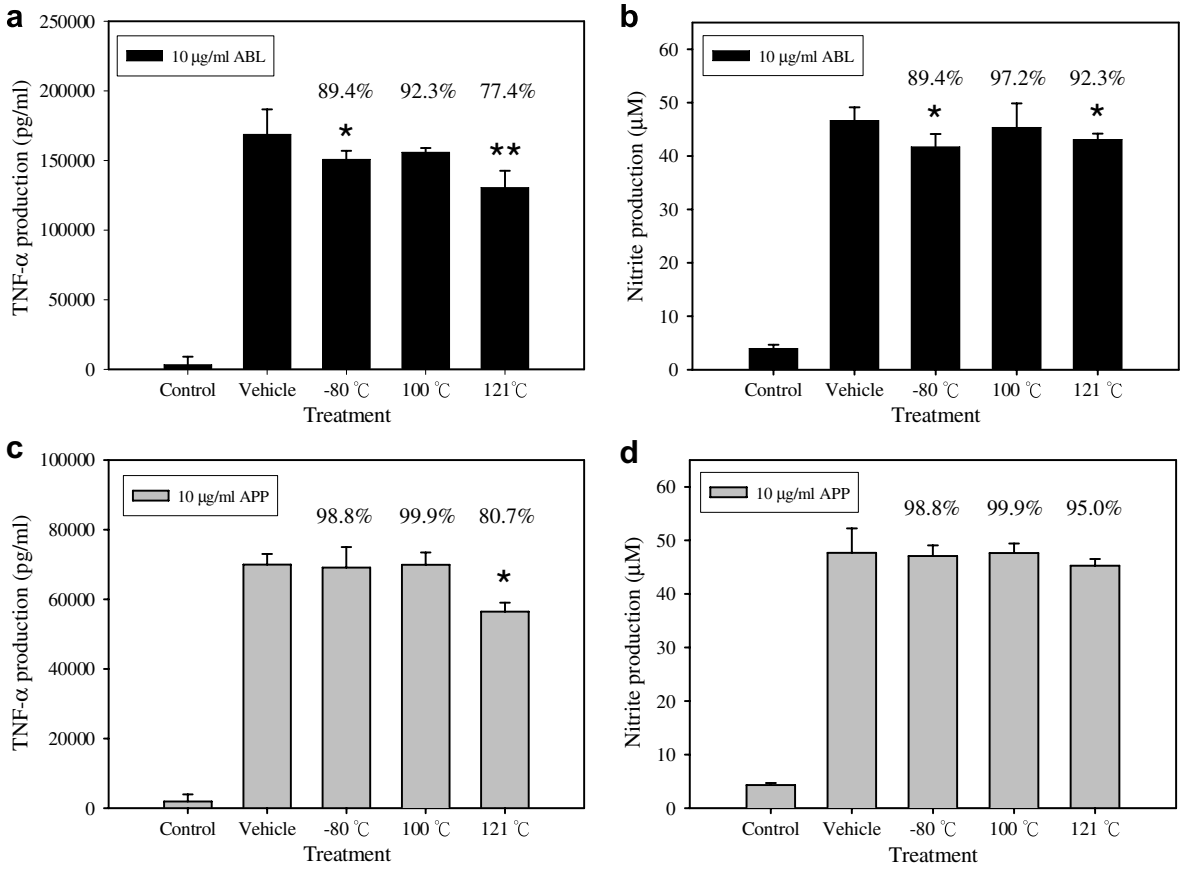

Fig. 3. Temperature resistance of ABL and APP in their induction of TNF-α production and nitrite accumulation in the culture supernatants of RAW264.7 macrophages. ABL (10 μg/ml) (a and b) and APP (10 μg/ml) (c and d) were pretreated by various temperatures (−80 °C, 100 °C, and 121 °C) and then incubated with RAW264.7 macrophages for 20 h later, followed by measuring the TNF-α and nitrite production. The percentages of remaining activities represent the relative activity compared to the vehicle (untreated ABL or APP). Cells cultured without stimulant in the culture supernatants were used as control. The levels of TNF-α were measured using sandwich ELISA and the nitrite concentrations were determined from the Griess reaction. Results are expressed as means ± SE from three independent experiments ($n = 3$). A P value of <0.05 is considered to be significantly different from the vehicle ($^{*}P < 0.05$; $^{**}P < 0.001$).

obtained. However, APP showed a significant ($P < 0.05$) decrease in TNF-α production from RAW264.7 cells, but it almost maintained its activity (84.6%) after the vacuum dehydration treatment. As indicated in Fig. 5, ABL and APP could resist dehydration processes.

4. Discussion

Many mushroom lectins and immunomodulatory proteins have been reported to exhibit various immune-activating functionalities. Unfortunately, general food proteins are usually sensitive and unstable to various processing conditions and therefore the remaining bioactivities and physiological effects of mushroom proteins are questionable. Thermal treatments, such as blanching, heating or sterilization, are usually applied for denaturing proteins in food processing. The results demonstrated that ABL and APP did not degrade through 100 °C boiling and 121 °C autoclaving treatments and maintained their activities up to 77–97% as compared with primordial induction (Figs. 1c,d and 3). Fermentation, salting and sugaring processes could lead to extreme acid or alkali conditions that could easily destroy the structure or decrease the activity of food proteins. Interestingly, ABL and APP retained above 94% of activities and showed superior resistance against pH 2 and pH 13 treatments (Fig. 4). In addition, ABL and APP also displayed tolerance to dehydration treatment (Fig. 5), which happens in dry foods and pharmaceutical products. The activities of ABL and APP when inducing TNF-α and NO from RAW264.7 macrophages were remarkable even though they were pretreated by thermal sterilization, freezing, acid/alkali and dehydration. Therefore, it is proposed that ABL and APP could withstand most food processing techniques and retain their immune-activating activities.

Edible mushrooms, which are usually consumed for both food and medical applications, may have important effects on health or even in treating diseases. The activities and processing properties of mushrooms are affected by the stabilities of their constituent compounds. The results of this study showed that ABL and APP displayed high stabilities upon industrial processing (e.g. heating, freezing, acid/

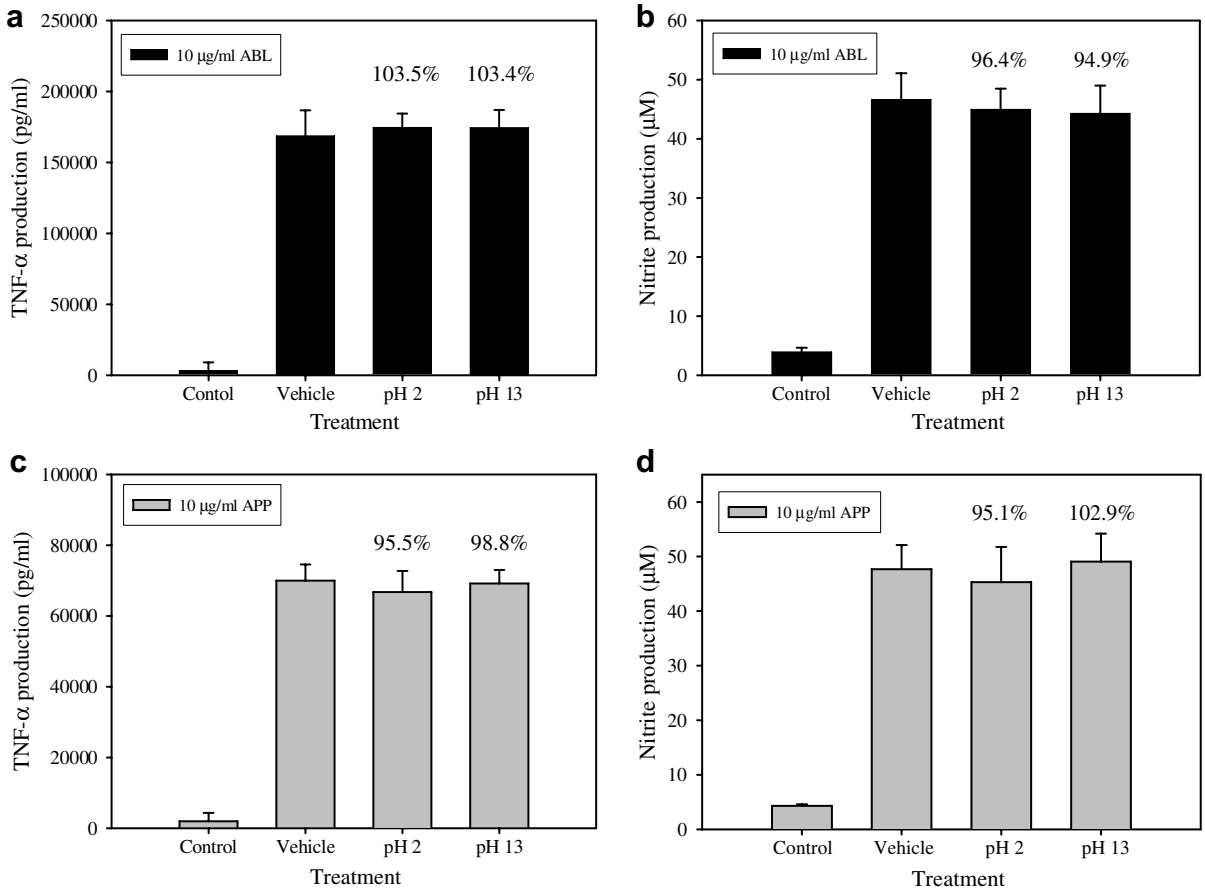

Fig. 4. Acid/alkali tolerance of ABL and APP in their induction of TNF-α production and nitrite accumulation in the culture supernatants of RAW264.7 macrophages. ABL (10 μg/ml) (a and b) and APP (10 μg/ml) (c and d) were pretreated by various pH values (pH 2 and pH 13) and then incubated with RAW264.7 macrophages for 20 h later, followed by measuring the TNF-α and nitrite production. The percentages of remaining activities represent the relative activity compared to the vehicle (untreated ABL or APP). Cells cultured without stimulant in the culture supernatants were used as control. Sandwich ELISA of TNF-α production and Griess reaction of nitrite accumulation were conducted. Results are expressed as means ± SE from three independent experiments ($n = 3$).

alkali treatment, and dehydrating), suggesting that these mushrooms and mushroom proteins could maintain their immunofunctionalities in almost all food types, e.g. canned, frozen, acidified, and dry foods. Moreover, these properties of ABL and APP would be helpful for applications in food processing and pharmaceutical manufacture. Furthermore, the compounds from *A. bisporus*, *A. polytricha*, and even other mushrooms, are valuable for the development of nutritious and medical materials.

It is known that many lectins, including concanavalin A (Schwarz, Puri, Bhat, & Surolia, 1993), pea seed lectin (Manoj, Srinivas, Surolia, Vijayan, & Suguna, 2000), lentil lectin (Schwarz et al., 1993), peanut lectin (Reddy, Bharadwaj, & Surolia, 1999) and legume lectin (Biswas & Kayastha, 2002), display superior thermal stability and some are structurally stable at temperatures above 100 °C (Biswas & Kayastha, 2002). For certain mushroom lectins, their hemagglutinating abilities are stable in the presence of acid/alkali solutions and temperature stress (Eifler & Ziska, 1980; Feng et al., 2006; Kobayashi et al., 2004; Wang et al., 1995). In addition, there is less effect, by ion suppression, on the hemagglutinating ability of the lectin from the toxic mushroom *Amanita pantherina* (Zhuang, Murata, Usui, Kawagishi, & Kobayashi, 1996). Although several reports have described the hemagglutinating stability of mushroom lectins, this study was the first one to demonstrate that ABL and APP retained high activities for the regulation of cellular immune responses after various food processing treatments.

The resistance and tolerance of ABL and APP against heating, freezing, acid, alkali and dehydration treatments, were attributed to their lectin-like structures. Recent thermodynamic studies on lectins have shown that the folding processes of these proteins were reversible and cooperative, and caused compact-protein structure (Manoj et al., 2000; Reddy et al., 1999). It has been reported that a change in pH was strong enough to break the weak non-covalent bonds between the two dimers of lectin, but it could not break the hydrogen bonds between the continuous beta sheets (Biswas & Kayastha, 2002). As described above, these results revealed that some lectins had overall stabilities depending on their conformational structures.

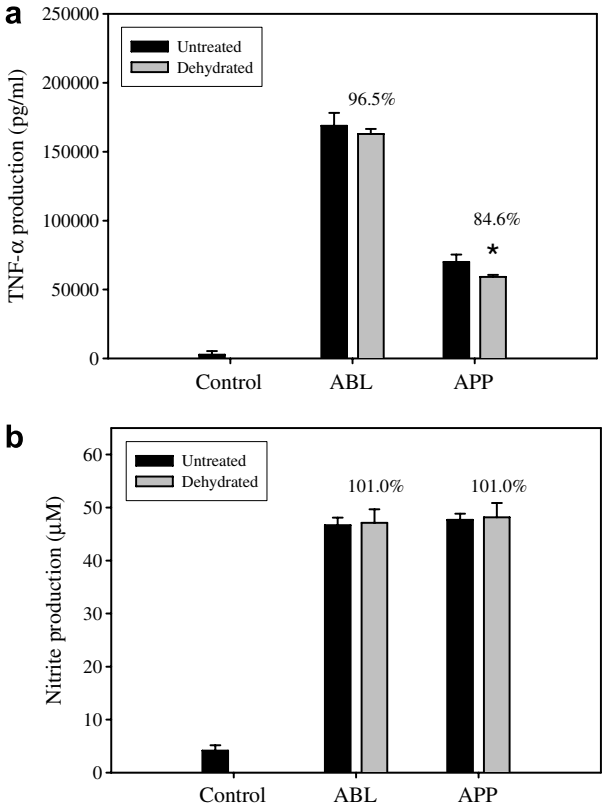

Fig. 5. Effects of dehydrating treatment on ABL and APP to induce TNF-α production and nitrite accumulation in the culture supernatants of RAW264.7 macrophages. ABL (10 μg/ml) and APP (10 μg/ml) were pretreated with or without vacuum-drying and then incubated with RAW264.7 macrophages for 20 h later, followed by measuring the TNF-α (a) and nitrite (b) production. Cells cultured without stimulant in the culture supernatants were used as control. The levels of TNF-α were measured using sandwich ELISA and the nitrite concentrations were determined from the Griess reaction. The percentages of remaining activities represent the relative activity compared to the vehicle (untreated ABL or APP). Results are expressed as means ± SE from three independent experiments ($n = 3$). A P value of <0.05 is considered to be significantly different from the untreated control ($^{*}P < 0.05$).

However, the structural and chemical differences of ABL and APP from other lectins have not been well studied. This demands further investigation.

Taken together, the foregoing results indicate that ABL and APP show commendable abilities to activate RAW264.7 macrophages producing TNF-α and NO. Furthermore, the bioactivities of ABL and APP show distinct stabilities at the same time. These findings contribute to the uses of mushrooms and their proteins in food processing and pharmaceutical production.

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