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Isolation and characterization of an immunomodulatory protein (APP) from the Jew's Ear mushroom *Auricularia polytricha*

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Abstract

A new immunomodulatory protein (APP) was purified from the fruiting body of an edible Jew's Ear mushroom, *Auricularia polytricha*, by extraction using 5% cold acetic acid in the presence of 0.1% 2-mercaptoethanol, followed by ammonium sulfate fractionation, DE-52 and MonoQ anion-exchange chromatography. As determined by electrophoresis techniques, the molecular mass of APP is around 13.4 kDa and its pI is approximately 5.1. APP is a simple protein without carbohydrate, and can agglutinate mouse red blood cells. APP alone activates murine splenocytes, markedly increasing their proliferation and gamma-interferon (IFN-γ) secretion, and presents no cytotoxicity in vitro. Although murine splenocytes are stimulated by the mitogen concanavalin A (ConA), APP suppresses their proliferation in a dose-dependent manner. APP (5–20 μg/ml) also enhances the production of both nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) by LPS-induced RAW 264.7 macrophages. These findings suggest that APP is an immune stimulant and can strengthen the immune response of its host.
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Keywords: Immunomodulatory protein; Jew's Ear mushroom; *Auricularia polytricha*

1. Introduction

Edible mushrooms are known to be a highly nutritious foodstuff, and to exhibit tonic and medicinal attributes in folk medicine. Recently, much attention has been paid to their therapeutic value, and more researchers are becoming interested in finding new functional compounds in mushrooms. The medicinal characteristics of some mushroom species, which have been thoroughly examined, include their antitumor, immunomodulating, antiviral, hypocholesterolemia, and hepatoprotective activities (Breene, 1990; Wasser & Weis, 1999). Various biologically active substances generated in mushrooms have been recorded in the literature, they include polysaccharides, terpenoids, polysaccharide-peptide complexes and proteins (Wasser & Weis, 1999; Wang, Ng, & Ooi, 1998).

Bioactive proteins, mostly identified as agglutinins, lectins or immunomodulatory proteins, constitute an important group of functional agents in mushrooms. In mushrooms, over 40 proteins, found not only in the fruiting body but also in the mycelium, have been investigated; they have been found to exhibit a range of chemical characteristics and various biological effects on immune cells (Guillot & Konska, 1997; Wang et al., 1998). Many mushroom proteins have been reported to activate lymphocytes and to stimulate cell proliferation and cytokine secretion in vitro (Wang et al., 1998); some mushroom proteins also activate immune cells and inhibit the growth of implanted tumor cells in vivo (Ikekawa et al., 1985; Wang, Liu, Ng, Ooi, & Chang, 1996). However, some others have are cytotoxic (Banchonglikitkul, Smart, Gibbs, Donovan, & Cook, 2002) or have in vivo immunosuppressive effects (Wang et al., 1998). Accordingly, mushroom proteins may affect the host's immune system, and are therefore thought to have potential in treating various disease states.

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The mushroom *Auricularia polytricha*, belonging to *heterobasidiae* of *basidiomycetes* and also called Jew's Ear, wood ear, red ear, black tree fungus or ear fungus, is frequently consumed as a food and a traditional medicine in the far east. Its nutritional value and taste components have been investigated (Mau, Wu, Wu, & Lin, 1998), and a few studies have reported its biological activity and active substances. Hammerschmidt (1980) and Hokama and Hokama (1981) reported that *A. polytrich* contains a platelet-aggregating inhibitor, which is present in the crude dialysates of aqueous *A. polytrich* extracts. Hokama, Cripps, Hokama, Abad, Sato, and Kimura (1983) reported a blastogenic inhibitory factor extracted from *A. polytrich*, which factor suppresses the incorporation of ^{3}H-thymidine in mitogen-induced blood mononuclear cells in vitro. This blastogenic inhibitory factor, which is a compound of low-molecular weight and can also be prepared from *A. polytrich* dialysates, is unrelated to the platelet-inhibiting compound and has not yet been isolated. Yagi and Tadera (1988) reported a galactose-binding lectin of *A. polytrich*, with a molecular mass of 23.0 kDa and a basic pI value. However, the physiological activity of this lectin is not clear. Additionally, the methanolic extract of *A. polytrich* has antioxidant activity and prevents lipid oxidation, scavenging radicals and chelating metal ions in vitro (Mau, Chao, & Wu, 2001). Furthermore, *A. polytrich* also exhibits antinociceptive characteristics. Subcutaneous injection of four compounds isolated from *A. polytricha* can reduce acetic acid-induced writhing in mice (Koyama et al., 2002). These data reveal that *A. polytricha* constituents have potential pharmacological applications.

This present work describes the isolation of a new hemoagglutinative protein (APP) from the fruiting bodies of *A. polytrich*. The biochemical characteristics of APP are determined and its immunomodulatory activities, modulating murine splenocytes and macrophage cells, are also demonstrated.

2. Materials and methods

2.1. Cell line and animal

Murine macrophage cell line RAW264.7 was purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 4 mM L-glutamine, 1.4 g/l sodium bicarbonate, 4.5 g/l glucose, 1 mM sodium pyruvate, and 10% (v/v) FBS (Gibco/BRL Life Technologies, Eggenstein, Germany). BALB/C mice, obtained from the animal centre of National Taiwan University (Taipei, Taiwan), were used to acquire splenocytes.

2.2. Materials and reagents

Fruiting bodies of the Jew's Ear mushroom, *A. polytricha*, were obtained from the local market in Taipei. DE-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, *E. coli*, Serotype 0.55:B5), sulfanilamide, naphthylethylenediamide dihydrochloride, concanavalin A (ConA), 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl) -2H-tetrazolium-5-carboxanilide (XTT) and phenazine methosulfate were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade.

2.3. Purification of APP

The activity of each purified fraction was determined by a significant increase ($p < 0.05$) in the production of nitric oxide (NO) production by LPS-stimulated RAW 264.7 macrophages. All purification steps were performed out at 4 °C. Fresh fruiting bodies of *A. polytricha* (1 kg) were homogenized with 3 l of cold 5% (v/v) acetic acid with 0.1% (v/v) 2-mercaptoethanol. The homogenates were centrifuged at 10,000*g* for 20 min and soluble proteins in the supernatant were precipitated by adding ammonium sulphate to 95% of saturation. The precipitates were stirred overnight, and then collected by centrifugation at 20,000*g* for 30 min. The pellets were then dialyzed against 5 l of 10 mM Tris buffer, pH 8.2 for 72 h with four changes of dialysis solution. The dialysate was first fractionated on a Whatman DE-52 cellulose column (2.5 cm × 20 cm) which was pre-equilibrated using 10 mM Tris buffer at pH 8.2. The fractions associated with the main peak were found to exhibit activity, and were further combined and purified on a Mono Q HR 5/5 column that had also been pre-equilibrated using 10 mM Tris buffer, pH 8.2. After the column was washed in 3 ml of the same buffer, two peaks were found when the column was eluted with 12 ml of 0–0.3 M NaCl in 10 mM Tris buffer, pH 8.2. The fractions, associated with both peaks, 2 and 3 in the chromatogram, were found to include APP, according to the following assays.

2.4. Electrophoresis and amino acid analysis

Active fractions were subjected to SDS/PAGE analysis to detect their purity. SDS/PAGE (12.5%, w/v) was performed on a Bio-Rad miniprotein III gel apparatus, following the methods of Laemmli (1970). Tricine-SDS–polyacrylamide gel electrophoresis was performed in 16.5% T and 3% C slab gels, according to the method of Schagger and von Jagow (1987). The gels were visualized by staining with Coomassie Brilliant Blue R-250 or

by the periodic acid/Schiff technique (Zacharius, Zell, Morrison, & Woodlock, 1969), to determine the carbohydrate content. Isoelectric focussing assay and silver staining were performed on precast PhastGel IEF pH 3–9 gels, using a Phastsystem (Amersham, Uppsala, Sweden), following the manufacturer's recommendations. The molecular mass and pI values of the proteins were calculated using a Syngene GeneGenius gel documentation system, with GeneTools software (Cambridge, UK). The protein concentration was determined according to the bicinchoninic acid method (Smith et al., 1985). Samples were taken for amino acid analysis using a Waters Pico-Tag system (Milford, MA). The values for threonine and serine were corrected for hydrolysis loss, by extrapolating to zero time relative to alanine.

2.5. Hemagglutination test

Various concentrations of APP in 50 μl were added to mixtures of 100 μl 1.5% mouse red blood cells in phosphate-buffered saline. The reaction mixtures were placed in 96-well microtitre plates. The plates were incubated at 37 °C, and the degree of hemagglutination was recorded after 1.5 h (Ko, Hsu, Lin, Kao, & Lin, 1995).

2.6. Activation of splenocytes and macrophages

Balb/c mice (eight weeks old) were killed by cervical dislocation and the spleens were removed using a sterile technique. Splenocytes were resuspended in DMEM supplemented with 10% (v/v) FBS. The cultures (5×10^5 cells/well in 96-well plates) were treated with various concentrations of APP or PBS in the presence or absence of ConA, and were cultured at 37 °C in air with 5% CO_2. After 72 h, the viability of the splenocytes was analyzed using XTT assay, and their proliferation of those was measured by both XTT assay and BrdU ELISA assay. The cultured soups were also collected to determine IFN-γ levels.

RAW 264.7 cells (5×10^5 cells/well in 96-well plates) were activated with various concentrations of APP (2.5–80 μg/ml) in the absence or presence of 1 μg/ml LPS, and were cultured at 37 °C in an atmosphere of 5% CO_2. After 24 h of incubation, the cultured soups were collected to evaluate the amount of NO and TNF-α produced by the macrophages.

2.7. Measurements of TNF-α and IFN-γ production

The amounts of mouse cytokines, TNF-α and IFN-γ, in culture supernatants were quantified by sandwich ELISA, using OptEIA mouse TNF-α/IFN-γ sets (BD PharMingen, San Diego, CA), respectively. Briefly, a 96-well ELISA plate (BD Falcon, San Diego, CA) was coated with a capturing antibody (Ab), anti-mouse TNF-α/IFN-γ monoclonal Ab (mAb), diluted in coating buffer (PBS, pH 6.5) overnight at 4 °C. After it was washed and blocked, TNF-α/IFN-γ was bound by incubating 50 μl culture supernatants diluted to 1/10 concentrations using assay diluent or serial standard dilutions of mouse rTNF-α/rIFN-γ for 2 h at room temperature. Subsequently, the wells were washed, and the biotinylated mouse TNF-α/IFN-γ mAb and avidin-HRP conjugate were placed in the wells for 1 h. After extensive washing, the samples were incubated in substrate solution (tetramethylbenzidine and hydrogen peroxide) for 20 min. After 50 μl stop solution (2 N H_2SO_4) was added, the OD was measured at 450/550 nm on a BioRad-3550 microplate reader (Hercules, CA). The TNF-α/IFN-γ content was calculated from the standard curve of mouse rTNF-α/rIFN-γ.

2.8. Measurement of NO production

The amount of NO released by macrophages was measured by determining the amount of accumulated nitrite (NO_2^-) in cell-free supernatants, using the colorimetric Griess reaction (Huygen, 1970). Briefly, 50 μl supernatant and serial dilutions of $NaNO_2$ standard solution were placed in a 96-well microtitre plate and then mixed with an equal volume of Griess reagent of 1% sulfanilamide, 0.1% naphthylethylenediamide dihydrochloride and 2.5% H_3PO_4. After the reaction products were incubated for 10 min at room temperature, their OD values were measured at 550 nm on a microplate reader, and the amount of accumulated nitrite in the samples was evaluated from the standard curve of $NaNO_2$.

2.9. Measurements of cell proliferation and viability

Cell respiration, as an index of cell proliferation and viability, was measured from the mitochondrial-dependent reduction of XTT to formazan (Ishiyama, Shiga, & Sasamoto, 1993). Briefly, the cells activated in a 96-well plate (10^6 cells/well) were incubated in 50 μl of XTT solution (XTT 1.0 mg/ml and phenazine methosulfate 15.3 μg/ml) at 37 °C for 3 h in the dark. The extent of XTT reduction was measured as the absorbance at 490 nm. The viability of cells was also measured as the release of lactate dehydrogenase (LDH) activity in APP treated cells, using a Roche LDH cytotoxicity detection kit (Mannheim, Germany).

The proliferation of activated mouse splenocytes was also measured by bromo-desoxyuridine (BrdU) uptake, using a commercial ELISA kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the cells were stimulated using APP and/or ConA in the presence or absence of APP for 72 h. The cultured soups were removed and a fresh culture medium, that contained 1.0 mg/ml of BrdU, was added to each well; the cells were then further incubated at 37 °C for 4 h. After the wells

were washed, the cells were fixed and the intracellular DNA was denatured using fixing/denature agent. Subsequently, the wells were washed, and the anti-BrdU-POD conjugate was added to each well for 1 h. After they were thoroughly washed, the samples were incubated using substrate solution for 20 min. After stop solution was added, the OD was measured at 450/550 nm using a microplate reader.

2.10. Statistical analysis

Each experiment was conducted in triplicate and performed three times. Results were expressed as means ± SE. Statistical comparisons were made by means of one-way analysis of variance (ANOVA), followed by a Duncan multiple comparisons test. Differences were considered significant when the p-values were below 0.05.

3. Results

3.1. Purification of APP

The purification of APP consisted of precipitation by ammonium sulphate and column chromatography on DEAE-52 cellulose and Mono Q. The activity of proteins was determined by measuring the increase in NO production by LPS-stimulated RAW 264.7 macrophages. In preliminary experiments, the active fractions of the isolated proteins were found in the flow-through fractions of a CM-52 cation-exchange chromatography, pre-equilibrated with 10 mM sodium acetate buffer, at pH 5.2 (data not shown). The data thus obtained suggest that the active protein of *A. polytricha* has an acid pI value of close to or below pH 5.2. Therefore, DE-52

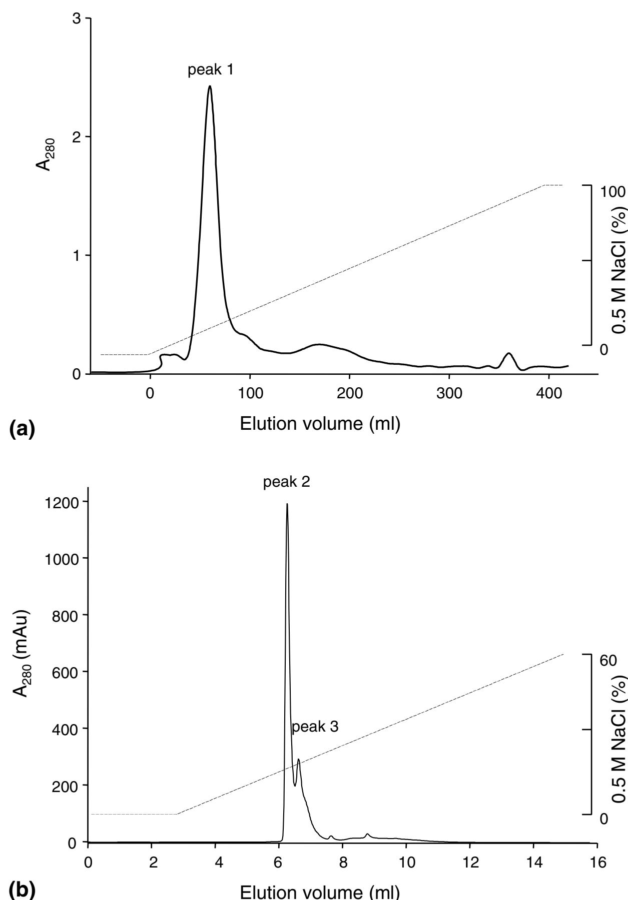


Fig. 1. Ion-exchange chromatographic purification of APP. (a) Elution profile of APP from a DE-52 cellulose column. (b) Mono Q column chromatogram of APP. Conditions are described in Section 2. The fractions associated with peaks 1, 2 and 3 exhibited activity that increased the production of NO production by LPS-stimulated RAW 264.7 macrophages. Electrophoretic analyses revealed that fractions of peaks 2 and 3 contained the same protein, APP.

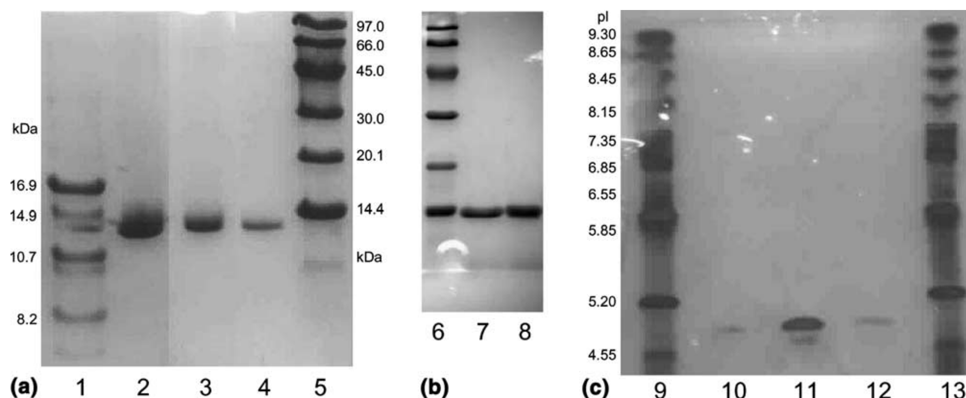


Fig. 2. Electrophoresis analysis of APP. APP, purified using ion-exchange chromatography, was analyzed by tricine-SDS–polyacrylamide gel electrophoresis (16.5% T and 3% C) (a), 12.5% SDS–PAGE (b) and isoelectric focussing (c). The polyacrylamide gels were stained with Coomassie Brilliant Blue R-250 (a, b), and the IEF precast gel was silver-stained (c). Lane 1, Amersham peptide markers: Globin (16.9 kDa), Globin I + II (14.9 kDa), Globin I + III (10.7 kDa), Globin I (8.2 kDa); lanes 2 and 10, active fractions obtained by DE-52 cellulose column chromatography; lanes 3 and 11, peak 2 fraction (APP) from MonoQ cation-exchange column chromatography; lanes 4 and 12, peak 3 fraction from MonoQ cation-exchange column chromatography; lanes 5 and 6, molecular mass markers: phosphorylase b (97.0 kDa), albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and lactoalbumin (14.4 kDa); lane 7, APP without 2-mercaptoethanol; lane 8, APP treated with 10% 2-mercaptoethanol.

anion-exchange was further performed for isolation. The elution profiles of proteins (Fig. 1, panel A) present a main peak (peak 1) after a linear-gradient elution using 0–0.5 M NaCl in 10 mM Tris buffer, pH 8.0. After their activity was confirmed, the fractions that corresponded to the peak were combined and purified using a Mono-Q anion exchange FPLC. APP was further eluted with a linear-gradient, using NaCl (0–0.3 M) and 10 mM Tris buffer, pH 8.0 (Fig. 1, panel B). Interestingly, the MonoQ chromatogram included two unseparated peaks (peaks 2 and 3). Further analyses by electrophoresis revealed that these two peaks were associated with the same protein, APP, with equal molecular weights and pI values (Fig. 2, lanes 3, 4 and 11, 12), and no other protein was observed. A total yield of 41 mg APP was finally obtained from 1.0 kg of fresh mushroom samples.

3.2. Characterization of APP

Following each purification step, various types of electrophoresis were performed to elucidate the biochemical characteristics of APP. Tricine-SDS/PAGE yielded a single band with an apparent MW of 13.4 kDa of the active fractions of the DEAE-purified peak 1, and the MonoQ purified peaks 2 and 3 (Fig. 2, bands 2–4). Treatment of APP with 2-mercaptoethanol also yielded an apparent MW of 13.4 kDa (Fig. 2, band 8). Isoelectric focusing on polyacrylamide in the pH range of 3–9 revealed a single band of APP with a pI of 5.1 (Fig. 2, band 11). Staining the polyacrylamide gel with periodic acid/Schiff reagent revealed that APP showed negative staining (data not shown). These observations showed that APP is a simple protein with very little or no carbohydrate content. The amino acid composition of APP (Table 1) indicated that APP comprises large quantities of threonine, but no histidine.

APP exhibited hemagglutinating activity toward mouse red blood cells at 4 μg/ml. The effects of various mono- and disaccharides on the hemagglutinating activity of APP were also examined. Hemagglutination was not inhibited by 0.1 M of D-glucose, D-galactose, D-fucose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine or lactose.

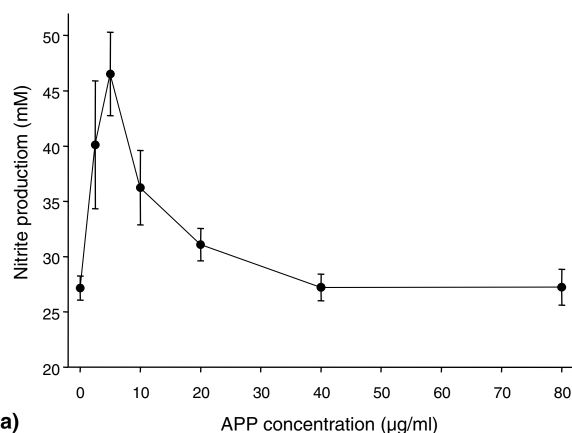
Table 1
Amino acid composition of APP and *A. polytricha* lectin (Hokama et al., 1983)

Amino acid	Content (%)	
	APP	*A. polytricha* lectin
Asp	7.06	11.11
Glu	4.73	7.58
Ser	6.78	5.05
Gly	8.14	10.10
His	0	0
Arg	1.93	6.06
Thr	11.5	10.10
Ala	5.86	11.62
Pro	5.57	9.60
Tyr	6.64	2.53
Val	9.83	9.60
Met	1.05	0
Cys	1.08	0
Ile	6.24	5.05
Leu	7.28	5.05
Phe	5.61	5.56
Lys	9.22	1.01
Trp[a]	1.51	2.53

[a] The tryptophan content was determined from absorbance at 280 and 288 nm in 6 M guanidine HCl.

3.3. APP enhances LPS-induced NO and TNF-α production by RAW 264.7 macrophages

RAW 264.7 cells were incubated with APP (0–80 μg/ml) and/or simultaneously stimulated with LPS, and the generation of nitrite ions and TNF-α in the cultured



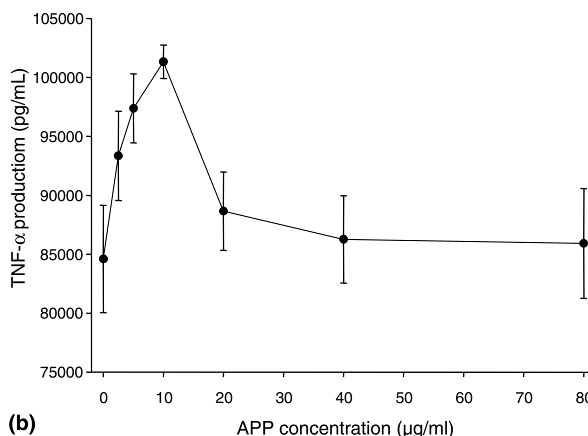
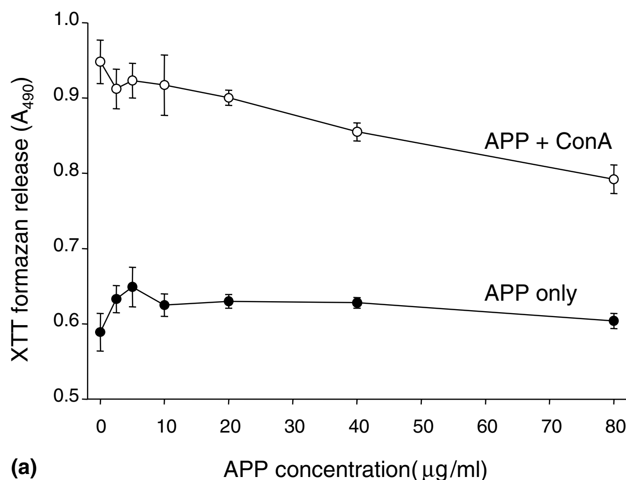


Fig. 3. APP promotes nitrite accumulation (a) and TNF-α production (b) in the culture supernatants of LPS-activated RAW 264.7 macrophages. RAW 264.7 macrophages were activated using LPS (1 μg/ml) and APP (0–80 μg/ml) for 20 h. The nitrite concentrations were determined from the Griess reaction (21), and the levels of TNF-α were measured using sandwich ELISA. Results are expressed as means ± SE; $n = 3$. The points at APP concentrations between 2.5 and 20 μg/ml differ significantly ($p < 0.05$) from those of the LPS-only treated sample.

soup were measured to evaluate the effect of APP on macrophages. The results (Fig. 3, panel A) indicate that APP can increase the accumulation of nitrite in the cultured medium of LPS-activated cells. The effective doses of APP were found to be 2.5–20 μg/ml, an increase of 14.5–71.3% over the NO production of the LPS-only controls ($p < 0.05$). Additionally, a similar effect of APP on TNF-α production by LPS-stimulated macrophages was observed (Fig. 3, panel B). Under treatment with APP, at concentrations ranging from 2.5 to 20 μg/ml, LPS-stimulated cells secreted more TNF-α (9.0–19.2%) than the LPS-only controls ($p < 0.05$). However, APP alone (2.5–80 μg/ml) did not impact NO or TNF-α production ($p > 0.05$, data not shown) by the macrophages that had not been stimulated by LPS. These results imply that APP synergistically promotes LPS-induced NO and TNF-α production, but not when alone, by RAW 264.7 macrophages.



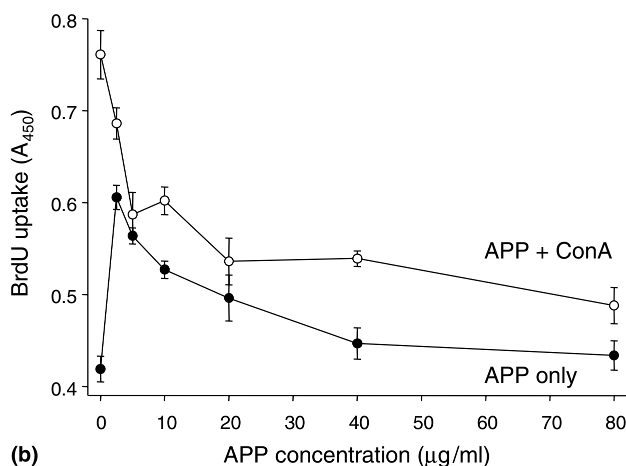


Fig. 4. Effects of APP on proliferation and viability of murine splenocytes treated and not treated with ConA. Cell proliferation and viability were measured by XTT metabolization (a) and BrdU incorporation (b). Murine splenocytes were cultured in vitro and activated using APP (0–80 μM) in the presence (○) or absence (●) of ConA (1 μg/ml) and for 72 h. Results are given as means ± SE; $n = 3$.

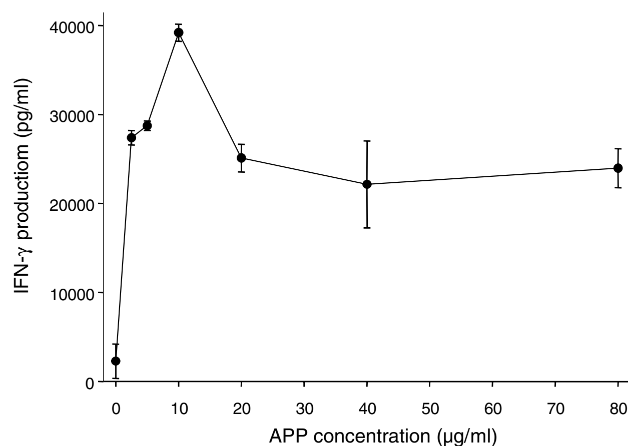


Fig. 5. APP promotes IFN-γ secretion in murine splenocytes. Mouse spleen cells were cultured with APP (0–80 μg/ml) for 72 h. The levels of IFN-γ were measured using sandwich ELISA. Results are expressed as means ± SE; $n = 3$. All points differ significantly from those obtained using the control ($p < 0.01$).

3.4. APP enhances cell proliferation and IFN-γ secretion in murine splenocytes

The respiration and mitogenesis of murine splenocytes, which were measured by means of measuring the mitochondrial-dependent reduction of XTT to formazan and the uptake of BrdU, respectively, were taken as indices of the proliferation and viability of APP-treated cells. APP (2.5–40 μg/ml) alone promotes the release of XTT formazan and the uptake of BrdU in murine splenocytes (Fig. 4) ($p < 0.05$). The LDH leakage of APP-treated cells was insignificant ($p > 0.05$) compared to the control (data not shown). Additionally, APP induces the IFN-γ secretion of murine splenocytes in vitro (Fig. 5). The most efficient concentration of APP was found to be 10 μg/ml, which significantly ($p < 0.05$) increases the secretion of IFN-γ (up to 40,000 pg/ml) by the cells. These results imply that APP shows no cytotoxicity and can activate the proliferation of murine splenocytes and can enhance the secretion of cytokine from the cells. Furthermore, APP was found to suppress cell proliferation in a dose-dependent matter when the cells were co-stimulated with mitogen ConA. In ConA-treated murine splenocytes, APP (2.5–80 μg/ml) not only reduced XTT metabolization (Fig. 4, panel A), but also inhibited cell mitogenesis (Fig. 4, panel B) of the cells, as determined by measuring the incorporation of BrdU. When the cells were treated with 80 μg/ml of APP, the uptake of BrdU was suppressed to 76.4% of that of the control.

4. Discussion

APP is not identical to any known protein derived from *A. polytricha*. Yagi and Tadera (1988) have reported a carbohydrate-containing lectin with a molecu-

lar mass of 23.0 kDa and a basic pI. APP, which has a MW of 13.4 kDa and is an acid protein with an isoelectric point of pH 5.1, contains little sugar in its moiety, as determined by periodate-Schiff staining. The hemagglutinative activities of these proteins also differ. The hemagglutinative activity of *A. polytricha* lectin was inhibited by D-galactose and lactose, but these sugars did not inhibit that of APP. Additionally, APP includes half-cystine and methionine (Table 1), but *A. polytricha* lectin lacks both of these amino acids and is, in this sense, much closer to the fungal immunomodulatory protein family (Ko et al., 1995; Lin, Hung, Hsu, & Lin, 1997). The observation that APP could agglutinate mouse red blood cells but that none of the six kinds of mono- and disaccharides examined could inhibit the hemagglutination, implies that these carbohydrates are probably not involved in hemagglutination.

APP displays potent immunomodulatory activity toward lymphocytes. APP alone significantly ($p < 0.05$) increases IFN-γ levels in the cultured soup of APP-treated murine splenocytes (Fig. 5), and also promotes the proliferation of the cells (Fig. 4). According to the results of XTT metabolization and LDH leakage, APP exhibits no cytotoxicity toward splenocytes. Moreover, APP was demonstrated to reduce the cell proliferation when murine splenocytes were stimulated with the mitogen ConA (Fig. 4). This inhibitory effect is dose-dependent and is consistent with the results of Hokama's study (Hokama et al., 1983), which described a blastogenic inhibitory factor in the crude dialysates of *A. polytricha*. Accordingly, APP is proposed to be the blastogenic inhibitory factor previously discussed by Hokama. The mitogen ConA is known to couple to a PZR receptor (Zhao, Guerrah, Tang, & Zhao, 2002) and induce intracellular signalling of the protein tyrosine kinase pathway (Matsuo, Hazeki, Hazeki, Katada, & Ui, 1996), which causes mitogenesis and other physiological changes in the cells. The suppression activity on the proliferation of the ConA-induced mouse splenocytes indicates that the target cells of APP may be similar to those of ConA, and APP might share the same signalling or mediators as are activated by ConA. The involvement of APP in signal transduction, leading to T cell activation, should be further studied.

However, APP also modulates LPS-induced macrophages in vitro. When RAW 264.7 cells were treated with the endotoxin LPS of *E. coli*, APP synergistically promoted nitrite accumulation and TNF-α production in the cultured medium (Fig. 3). In macrophages, Toll-like receptor 4 (TLR4) is the transmembrane component of the LPS signal transduction complex, which leads to the production of NO and TNF-α (Beutler, Du, & Poltorak, 2001; Yang, Zhu, Wang, & Jiang, 2002). APP alone cannot arouse the stimulation in RAW 264.7 macrophages without LPS induction, so TLR4 seems not to be the direct ligand of APP in the macrophage. Furthermore, the synergistic activity of APP, that affects NO/TNF-α production by LPS-induced macrophages, implies that APP can enhance the expression of downstream mediators that are generated by LPS activation and the TLR4 pathway.

APP displays marked activity that modulates lymphocytes and macrophages in various situations. APP promotes cell proliferation and IFN-γ secretion by lymphocytes, and increases NO and TNF-α production by LPS-induced macrophages. Accordingly, the authors posit that APP can effectively strengthen the immune response of its host. Further studies could be undertaken to clone the gene of APP and to study its molecular mechanism and effects in vivo to elucidate the natural effects of APP on the human body and other medicinal applications.

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