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Structure elucidation and immunomodulatory activity of a β-glucan derived from the fruiting bodies of *Amillariella mellea*

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ABSTRACT

A novel polysaccharide AAMP-A70 (5.6 kDa) has been purified from the fruiting bodies of Amillariella mellea. Compositional analysis and 1H and 13C NMR spectra indicate that AAMP-A70 is a branched β-glucan with a main chain that consists of β-D-(1→6) linked Glcp residues substituted at O-3 by β-Glcp or α-D-(1→6)-linked Galp side chains. AAMP-A70 increases macrophage phagocytosis and secretion of NO, ROS, TNF-α, IL-6 and IL-1β. Mechanistically, AAMP-A70 promotes degradation of IκB-α and nuclear translocation of the NF-κB p65 subunit, and enhances phosphorylation of MAPKs. In particular, the function blocking antibody to TLR2 substantially suppresses TNF-α and IL-6 production. Our data demonstrate that AAMP-A70 activates macrophages via NF-κB/MAPK signaling pathways and the TLR2 receptor. Overall, AAMP-A70 may serve as a good food supplement to enhance immunity.

1. Introduction

Mushrooms contain many bioactive, non-toxic compounds that make them potential sources for pharmaceutical drugs and functional foods. Polysaccharides are found mainly in the cell walls of fungi, in particular in the fruiting bodies where relatively large quantities (about 20% of the biomass) are found (Dong, Jia, & Fang, 2006; Ji et al., 2007). *Amillariella mellea* belongs to the *Basidiomycetes*, *Armillaria* family, which is a traditional Chinese medicine and edible fungus with extensive distribution in China. Previous studies have shown that the polysaccharide from *A. mellea* has potential biological activities, such as anti-inflammatory activity (Chang, Lur, Lu, & Cheng, 2013), antitumor activity (Wu et al., 2012), antioxidant activity (Zhang et al., 2015), and immunological activity (Sun, Liang, Zhang, Tong, & Liu, 2009). However, its structural characterization, immunological activity, and mechanism of action are lacking.

Macrophages occupy a unique niche in the immune system in that they can not only initiate immune responses, but are also effector cells that contribute to fighting infection and inflammation. Macrophages also exert an important role as an interface between innate and adaptive immunity. They are responsible for processes such as antigen processing and presentation to antigen-specific T cells. Following activation, macrophages can induce expression of accessory and co-stimulatory molecules that promote sustained stimulatory interactions with T cells and the generation of adaptive immunity (Van den Bossche, O'Neill, & Menon, 2017). Hence, macrophages are thought to be the important target cells of some antitumor and immunomodulatory drugs.

We recently purified and identified a novel polysaccharide (AAMP-A70) from the fruiting bodies of *A. mellea*. The present study was designed to study the structure of AAMP-A70, evaluate its immunostimulatory activity on RAW264.7 cells and identify the molecular mechanism responsible for its immunostimulatory activity.

2. Materials and methods

2.1. Materials

Fruiting bodies of *A. mellea* were collected from Changbai mountain area in Jilin Province, PR China and identified using rDNA-ITS sequencing analysis. DEAE-cellulose was purchased from Amersham pharmacia Biotech (Uppsala, Sweden). Standard monosaccharides, gel filtration standard dextrans, LPS and MTT were purchased from Sigma (Sigma Aldrich, St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) (high glucose medium and fetal bovine serum) were purchased from Gibco (Gibco, Grand Island, NY, USA). Penicillin/streptomycin was obtained from the Tina JinHao Yang Biological Manufacture Co., Ltd. (Tianjin, China). ELISA kits for mouse TNF-α, IL-

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1β and IL-6 were acquired from Boster Biological Technology (Wuhan, China). Fluorescent microspheres were from Molecular Probes (Thermo Fisher Scientific, USA). Antibodies against Dectin-1, TLR2 and TLR4 were purchased from Abcam (Cambridge, UK). Other Antibodies were purchased from Cell Signaling (Danvers, MA, USA). The ECL Plus Western Blotting Detection Kit was purchased from Beijing Saizhi Technology Co., LTD (Beijing, China). BAY11-7082, SP600125, U0126 and SB203580 were obtained from Selleck (Shanghai, China). Trypsin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Amersco (Amersco LLC, OH, USA). All of the other reagents were of analytical grade or better.

2.2. Extraction and isolation of the polysaccharides

The fruiting bodies were first extracted with distilled water at 100 °C for 4 h. This procedure was repeated twice, and the resulting residues were dried and extracted using 0.5 M NaOH with trace amounts of $NaBH_4$ at 80 °C for 3 h × 2 and 2 h × 1 (material-liquid ratio is 1:20 w/v each). Extracts were neutralized with glacial acetic acid, concentrated under vacuum at 60 °C, and 95% ethanol was added at a final concentration of 75% to precipitate the polysaccharides. The precipitate was collected by centrifugation (4000 rpm, 15 min), and vacuum dried yielding 12.8% (yield in relation to dry weight of initial material) of the total polysaccharide. The material was applied to a DEAE-cellulose column (8.0 × 20 cm, cl^-), eluted first with distilled water and then with 0.4 M NaCl solution to yield the neutral and acidic fractions (AAMP-N and AAMP-A, respectively) of the polysaccharide. The eluate was collected using an auto-collector and assayed by using the phenol-sulfuric acid method. AAMP-A was dissolved in distilled water and first precipitated by 50% EtOH, the supernatant was collected by centrifugation (10,000 rpm, 5 min). Then, 95% EtOH was added slowly to the supernatant until a final concentration of 70%, after centrifugation (10000 rpm, 5 min), the precipitate was collected and termed AAMP-A70 (26.8% of total polysaccharide). Our protocol for polysaccharide extraction and fractionation from *A. mellea* fruiting bodies is illustrated in Supplementary Fig. 1.

2.3. Analysis of chemical properties

Total carbohydrate content was determined by using the phenol-sulfuric acid method with glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Protein content was determined by using the Bradford assay with bovine serum albumin as the standard (Sedmak & Grossberg, 1977).

Monosaccharide composition was determined by using high performance liquid chromatography (HPLC) as described by Zhang (Zhang et al., 2009). Briefly, polysaccharide samples (2 mg) were initially hydrolyzed with 1 ml anhydrous methanol containing 2 M HCl at 80 °C for 16 h and then with 1 ml 2 M trifluoroacetic acid (TFA) at 120 °C for 1 h. After derivatization with 1-phenyl-3-methyl-5-pyrazo-lone (PMP), the derivatives were analyzed by HPLC.

Molecular weight distributions were determined by using gel-permeation chromatography on a TSK-gel G-3000PW_{XL} column (7.8 × 300 mm, TOSOH, Japan) coupled to a Shimadzu HPLC system as described by Zhang et al. (2009). The column was pre-calibrated by using standard dextrans (50 kDa, 25 kDa, 12 kDa, 5 kDa and 1 kDa) using linear regression.

2.4. Fourier transformed (FT-IR) spectroscopy analysis

Polysaccharides were ground with KBr powder and then pressed into a 1 mm pellet for FT-IR measurements. FT-IR spectra were obtained on a Nicolet 560 FT-IR spectrometer with DTGS detector in a range of 4000–400 cm^{-1}.

2.5. Methylation analysis

Methylation analysis was carried out according to the method of Needs and Selvendran (Needs & Selvendran, 1993). In brief, a sample (20 mg) was dissolved in DMSO (2 ml) and methylated by treatment with a suspension of NaOH/DMSO (2 ml) and iodomethane (4.0 ml). The reaction mixture was extracted with $CHCl_2$, and then the solvent was removed by vacuum evaporation. Complete methylation was confirmed by the disappearance of the –OH band (3200–3400 cm^{-1}) in the FT-IR spectrum. The per-O-methylated polysaccharide was hydrolysed subsequently by using HCOOH (85%, 1 ml) for 4 h at 100 °C and then CF_3COOH (2 M, 1 ml) for 6 h at 100 °C. The partially methylated sugars in the hydrolysate were reduced by using $NaBH_4$ and acetylated (Sweet, Albersheim, & Shapiro, 1975). The resulting alditol acetates were analyzed by GC–MS. The degree of branching value (DB) was obtained by using the following equation: DB = (NT + NB)/(NT + NB + NL), where NT, NB and NL represent the number of terminal, branched, and linear residues, respectively.

2.6. NMR analyses

^1H, ^{13}C, HSQC, and HMBC NMR spectra were recorded at 20 °C on a Bruker Avance 600 MHz spectrometer (Germany) with a Bruker 5 mm broadband probe operating at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR. Samples (20 mg) were dissolved in D_2O (0.5 ml) and centrifuged to remove excess sample. Data were analyzed using standard Bruker software.

2.7. Cell culture

The mouse macrophages cell line RAW264.7 was purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in DMEM high glucose medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated FBS. Cells were cultured at 37 °C in a 5% CO_2 incubator.

2.8. Cell viability assay

Cell viability was assessed using the MTT assay. RAW264.7 cells were seeded at a density of 2 × 10^5 cells/ml in a 96-well plate overnight and then treated with various concentrations of AAMP-A70 or 1 μg/ml of LPS for 24 h. The medium was then removed, and 100 μl/well of the MTT solution (0.5 mg/ml) was added. Following 4 h incubation, supernatants were discarded, and the resulting formazan was dissolved in 100 μl DMSO and the absorbance was measured at 570 nm using a microplate reader (Biotek, USA). Experiments were performed in triplicate and repeated at least three times. Cell proliferation is expressed as a percentage of the control set at 100%.

2.9. Measurement of NO production

RAW264.7 cells were seeded at a density of 8 × 10^5/ml and incubated with AAMP-A70 (0, 25, 50, 100 and 200 μg/ml) or LPS (1 μg/ml) for 24 h. After incubation, supernatants were collected and reacted with Griess reagent. The absorbance was monitored at 540 nm with a microplate reader (Biotek, USA). Sodium nitrite ($NaNO_2$) was used to generate a standard curve to calculate nitrite concentration. All experiments were performed in triplicate and repeated at least three times. NO production is expressed as percentage of the control set to 100%.

2.10. Measurement of reactive oxygen specie (ROS)

RAW264.7 cells at a density of 8 × 10^5/ml were incubated with AAMP-A70 (0, 25, 50, 100 and 200 μg/ml) or LPS (1 μg/ml) for 24 h.