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Purification, structural elucidation, and anti-inflammatory activity of xylosyl galactofucan from *Armillaria mellea*

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ABSTRACT

A xylosyl 1,3-galactofucan (**AMPS-III**) was isolated and identified as a novel anti-inflammatory agent from an edible fungus, *Armillaria mellea*. The characteristics chemical structure of **AMPS-III** including the linkages of compositional monosaccharides and structure of the repeat unit were depicted and elucidated by proton, carbon and two-dimensional nuclear magnetic resonance techniques. **AMPS-III** was chemically proposed to have a partial 4-*O*-xylosylated 1,3-linked α-D-galactosyl-interlaced α-L-fucan composed of a pentadecasaccharide repeat unit with a molecular mass approximately 13 kDa. **AMPS-III** significantly suppressed the release of tumor necrosis factor-α (TNF-α) and cytokine monocyte chemotactic protein-1 (MCP-1) in RAW264.7 macrophages and EAhy926 following LPS and TNF-α induction. The results provide helpful evidences for application of **AMPS-III** as anti-inflammatory food supplements.

1. Introduction

Armillaria mellea (formerly named *Armillaria mellea*), an edible fungus species, has long been used as a traditional Chinese herb medicine. It is known to improve health and prevent various diseases, such as insomnia, pain, and neurasthenia [1]. The extract of *A. mellea* exhibits anti-oxidative [2], anti-inflammatory [3], and immunomodulatory activities [4]. *A. mellea* can also induce maturation of human dendritic cells [5,6]. The chemical constituents isolated from *A. mellea* include sesquiterpenoids [7], steroids [1], triterpenoids [8], adenosine and resin acids [7].

Gastrodia elata (it is pronounced as tianma in Chinese) is an orchid species, and its root part is an important traditional Chinese medicine, Rhizoma Gastrodiae. Gastrodin, one of the active constituents of *G. elata*, exhibits anticonvulsant and sedative activities [9]. *A. mellea* has a subtle symbiotic relationship with *G. elata* as its nutrition and energy provider [10,11]. Such a delicate relationship has attracted great interest of us. A number of studies suggested that *A. mellea* possesses similar pharmacological properties to those of *G. elata*, and the bioactive constituents of *G. elata* were proved to be mainly from its symbiotic fungus *A. mellea* [12–14]. For example, the anti-proliferative activity of armillaridin, a melleolide isolated from *A. mellea*, is confirmed to be one of the major anti-tumor constituents of *G. elata* extracts [15,16]. Furthermore, *A. mellea* was reported to be used as an alternative medicinal material to *G. elata* [13].

Polysaccharides (PS) from mushrooms have a wide range of pharmacological activities, such as immunomodulation, antitumor, hepatoprotection, and anti-inflammation [17]. Inflammatory cells produce cytokines, such as interleukin (IL)-1, -6, cytokine monocyte chemotactic protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α). TNF-α and IL-6, have been known to play important roles in inflammatory responses, apoptosis, angiogenesis, cell adhesion and transformation [18].

A number of studies suggest polysaccharides as the key components of the anti-inflammatory-activity of *A. mellea* [19]. Therefore, it is of high importance to isolate and elucidate the chemical structure of the PS (especially the bioactive ones) from the entitled fungus. In this study, mycelial culture was chosen as the source of polysaccharides for the advantages of fast growing in a short period of time, high yield of polysaccharides as compared that with fruiting body, ease of quality control, ease of manipulation of polysaccharide production and capability of large scale production. Assessing the potential usage of *A. mellea*, in the present study we conducted in vitro mycelial culture from *A. mellea*, identified one of its major constituent in the active fraction

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chemically via the use of 1D and 2D NMR techniques and investigated its anti-inflammatory bioactivities.

2. Material and methods

2.1. General

A. mellea was provided courtesy of Dr. Tun-Tschu Chang [20], Division of Forest Protection, Taiwan Forestry Research Institute, Taipei, Taiwan. All reagents were purchased from Sigma Chemical (St. Louis, MO, USA), except where specified. LC-grade organic solvents were purchased from Merck (Darmstadt, Germany).

2.2. Liquid culture

Fungi were maintained and cultured with Cheng's method [21]. *A. mellea* were maintained on potato dextrose agar (PDA) slants and transferred to fresh medium at 3-week intervals. In each pasteurized Petri dish, 25 mL of PDA medium (39 g/L) was used and incubated at 28°C for 19 days. The mycelia on the media surface were transferred to 800-mL culture flasks containing 100 mL of 24 g/L potato dextrose broth and 20 g/L glucose at pH 5.6 for 49 days. Polysaccharides were isolated from 49-day-old cultures. Mycelia were harvested and rapidly washed with 1 L of 250 mM NaCl under aspiration to remove contaminating extracellular polysaccharides. Samples were then lyophilized (VirTis, FM 25EL-85, Sp Scientific), and stored at 4 °C. Mycelia were then lyophilized, and stored at 4 °C, and the dry weight of mycelia was measured. Growth media for the solid and liquid cultures were purchased from Sigma (Saint Louis, MO, USA).

2.3. Isolation and purification of polysaccharide

Lyophilized mycelia of the *A. mellea* were extracted twice with 80 °C water in a 1: 100 (w/w) ratio for 6 h. The extracts were cooled and four volumes of 95% ethanol were added, than allowed to precipitate overnight at 4 °C. The precipitated polysaccharides were collected by centrifugation and lyophilized, resulting in a crude brownish polysaccharide. After being dissolved in 150 mM NaCl and 10 mM NaH_2PO_4 at pH 6.8 buffer, 40 mg of the polysaccharide was fractionated into fractions I–V (see Fig. 1).

2.4. Homogeneity and molecular weight

The polysaccharide of ***A. mellea*** was fractionated by size-exclusion column chromatography (SEC). Forty milligrams of the lyophilized AMPS was dissolved in 5 mL of a buffer containing 150 mM NaCl and 10 mM NaH_2PO_4 at pH 6.8 and chromatographed on a column of Fractogel BioSec 103 × 1.5 cm (Merck) which had been equilibrated with the buffer as mentioned above. Fractions (2.8 mL/tube) were collected. The eluted fractions were assayed for hexose by the phenol-sulfuric acid method [22]. Briefly, 200 μL 5% phenol was added into 200 μL eluted fraction, following by addition of 1 mL 37% sulfuric acid and gently mix at room temperature for 30 min. The sugar containing fraction exhibited an orange color and the absorbance at 488 nm was recorded. AMPS was resolved into five fractions (AMPS-I–V; the amount of **AMPS-III** was 6.0 mg) (Fig. 1). A calibration curve was constructed using a series of molecular weight standards, dextrans (Sigma-Aldrich) and galactose with molecular weights of 69.8, 40.0, 10.5, 0.18 kDa. A regression equation was determined between the log [Mw] (Y) and fraction number (X) to be Y = 9.435–0.136 X; R^2 = 0.9956436814. The molecular weight of **AMPS-III** was determined by mean value of molecular weight from the fraction number 37 to 42 detected by the phenol-sulfuric acid method. **AMPS-III** was further characterized. The molecular weight homogeneity of AMPS-**III** was confirmed with high-performance size-exclusion chromatography (HPSEC) using a Viscotek model 301 coupled with SEC columns (G4000PW_{XL} 7.8 × 300 mm and

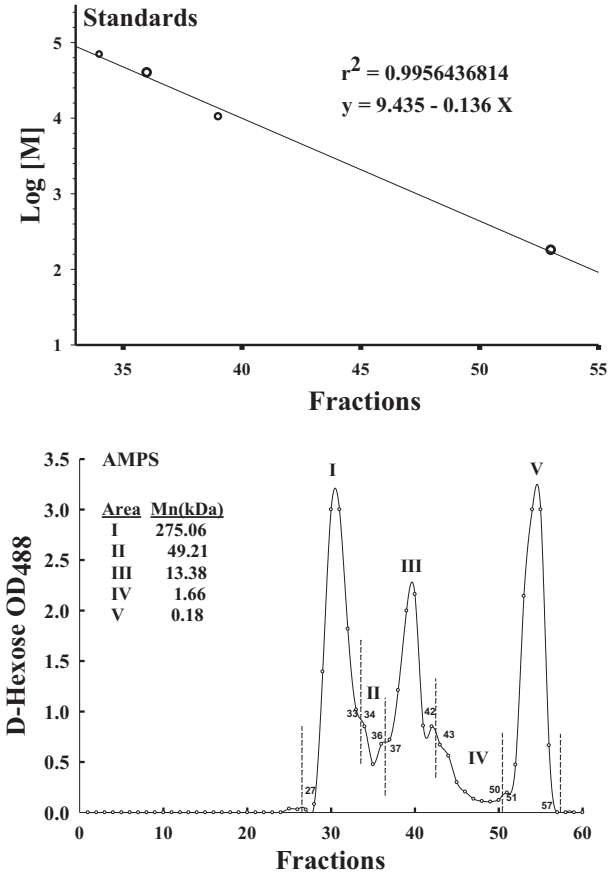

Fig. 1. Fractionation of polysaccharides of *A. mellea*. Fractions (2.8 mL/tube) were collected. Fractionation of polysaccharide by gel filtration column chromatography into Fr-I (27–33), -II (34–36), -III (37–42), -IV (43–50), and -V (51–57).

G3000PW_{XL} 7.8 × 300 mm, Viscotek) with de-ionized H_2O as the eluent at a flow rate of 0.5 mL/min.

2.5. Monosaccharide analysis

Acid hydrolysis of polysaccharides is carried out as follows. One milligram of lyophilized polysaccharide was hydrolyzed with 4.95 N trifluoroacetic acid (TFA) at 80 °C in a heating block for 24 h. The sugar composition of polysaccharides (PS) was determined following acid hydrolysis of PSs, and carried out according to Cheng's method [5].

2.6. Cell culture

The murine macrophage cell line RAW264.7 and Ea.hy926 endothelial cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells are cultured in RPMI 1640 medium (GIBCO Inc., NY, USA) supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin and 10% fetal bovine serum (FBS; GIBCO Inc., NY, USA). The cells were incubated in an atmosphere of 5% CO_2 at 37 °C and sub-cultured every 3 days. Cell viability and cell numbers were determined by the Trypan Blue dye-exclusion method.

2.7. Cytokine secretion

To study the effects of fractionated PS on bacterial LPS-induced inflammation in RAW264.7 macrophage cytokine production, an in vitro assay was performed. Tumor necrosis factor-α (TNF-α) plays an important role in inflammation. Production of TNF-α was induced by bacterial LPS. RAW264.7 macrophages (1.0×10^5 cells/mL) were incubated with PS at a concentration of 500 μg/mL for 6 h, and finally the macrophages