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Original Article

Characteristics of fucose-containing polysaccharides from submerged fermentation of *Agaricus blazei* Murill

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ABSTRACT

Fucose is one of important residues of recognition pattern for many immune cells. In this study, we characterized bioactive fucose-containing acidic polysaccharides from submerged fermentation of *Agaricus blazei* Murill. We obtained the polysaccharides through a cell-based activity-guided strategy, and used carbohydrate recognition monoclonal antibodies based Enzyme-Linked Immuno Sorbent Assay (ELISA) along with methylation and NMR analyses to investigate the structural characteristics of the polysaccharides. The polysaccharides had Mw of 3.5×10^5 Da. The major sugars were L-fucose, L-arabinose, D-galactose, D-xylose, and D-galacturonic acid in the molar ratio of 6.4, 15.5, 28.5, 14.7, and 25.0% with a small amount of D-glucose, D-mannose, L-rhamnose, and D-glucuronic acid. Results indicated that the bioactive polysaccharides consisted of a (1,4)-Gal*p* and (1,4)-GalA*p* back bone; (1,2)-Xyl and (1,2)-Rha might also comprise backbone or constitute side chain; linkage (1,5)-Ara and terminal fucosyl residues were also involved in the polysaccharides. Regarding bioactivity, removal of the terminal L-fucosyl residues reduced the TNF-α cytokine stimulating activity of the polysaccharides in a RAW 264.7 macrophage cell-line test, whereas NF-κB and TLR4 affected the polysaccharide-induced TNF-α production.

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1. Introduction

Agaricus blazei Murill is an edible mushroom that has become a functional food ingredient in Japan, Taiwan, and other Asian countries. Many studies have attributed the immunomodulating and anti-tumor activities of this fungus to its polysaccharides. For example, the structural features and functions of glucan- and xyloglucan-protein complexes, as well as glucans with α-(1,4; 1,6) linkages, α-(1,4) linkages, and β-(1,6) linkages isolated from fruiting bodies of *A. blazei* Murill have been studied [1–4]. In addition, polysaccharides produced by *A. blazei* Murill mycelial fermentation such as glucomannan, β-(1,6) glucan, and β-(1,3) glucan were also reported to have bioactivities [5,6].

Specifically, the isolated polysaccharides have been shown to stimulate macrophage proliferation, cytokine production, and phagocytosis [7]. In turn, the use of TNF-α released from the macrophage cell line RAW 264.7 as a bioactivity index to study the bioactivity of broth polysaccharides in a submerged culture of *A. blazei* has been described [8]. Other reports focused on the functions of neutral polysaccharides of the mushroom [9–11], with only little discussion of acidic polysaccharides [1,12]. The finding of fucogalactan in *Agaricus bisporus*, a related species, drew our attention [13], because fucose frequently plays an important role in bioactivities [14–16]. We thus hypothesized that the immunomodulating activities of *A. blazei* Murill may be attributed to specific fucose-containing polysaccharides. We therefore investigated the polysaccharide profile of *A. blazei* fermentation product, fractionated its crude polysaccharide, and characterized the structural features of the fucose-containing polysaccharides via a cell-based activity-guided strategy. Our bioactivity investigation utilized the polysaccharide-stimulated murine macrophage cell-line RAW 264.7 to measure the secretion of TNF-α. Moreover, a gene reporter platform and pattern-recognition receptor antibodies were adopted to understand the immuno-modulatory probability pathway [7,17]. Carbohydrate recognition monoclonal antibodies were also used in an Enzyme-Linked Immuno Sorbent Assay (ELISA) along with methylation and NMR analyses to investigate the structural characteristics of the polysaccharide.

2. Materials and methods

2.1. Polysaccharide preparation

2.1.1. Polysaccharide extraction

The product of submerged fermentation of *A. blazei* Murill was kindly provided by Prof. Chin-Hang Shu in the Department of Chemical and Materials Engineering at National Central University (Taoyuan, Taiwan) [8]. The yield of lyophilized powder from whole fermentation product is 580 mg/dL. The product containing both the mycelia and the broth were lyophilized and ground into powder. Then, 10 g of dry powder was extracted for 1 h in 250 mL boiling distilled water. The extract was filtered through Whatman No. 54 filter paper (GE Healthcare, Florham Park, NJ) under vacuum; residues were extracted sequentially in 150 mL and 100 mL of boiling distilled water for 0.5 h. All extracts were combined for rest of the study. The hot water extract was precipitated with four volumes of 95% ethanol (Taiwan Tobacco and Wine Corp., Taipei, Taiwan), to obtain crude polysaccharide.

2.1.2. Polysaccharide fractionation

The crude polysaccharides were re-dissolved in distilled water, and centrifuged (3000×*g* for 10 min) to remove insoluble materials. The supernatant was applied to a DEAE-650M (Toyopearl, Tokyo, Japan) column (2.6 cm × 30 cm). The DEAE column pre-equilibrated with 20 mM Tris, and eluted with different concentrations of NaCl solution (0, 0.1 and 0.2 M) in stepwise at a flow rate of 1 mL/min. The gradient was designed according our preliminary study to separate polysaccharides with different charge density and protein content (data not shown). Three fractions were collected by automatic fraction collector. Total carbohydrate, uronic acid, and protein contents were measured using the methods of Dubois [18], Blumenkrantz [19], and Bradford [20], respectively. Fraction "F3" was further fractionated via ascending gel filtration chromatography, performed on a Toyopearl HW-65F column (2.6 cm × 90 cm, Tosoh, Tokyo, Japan). The eluent for this fractionation was 50 mM NaCl aqueous (containing 1 mM NaOH) at a flow rate of 0.5 mL/min.

2.2. Characterization of polysaccharides

2.2.1. Molecular weight

The molecular weight and distribution were determined by high-performance size-exclusion chromatography (HPSEC). The system included an SSI single pump (Scientific System, Inc., State College, PA), a column oven (Super co-150, Enshine, Tainan, Taiwan) equipped with a Rheodyne injector (Cotati, PA), a 500 μL sample loop, and an OPTILAB DSP interferometric refractometer (P10 cell, 690 nm, Wyatt Technology Co., Santa Barbara, CA) with the temperature controlled at 35 °C. The samples were analyzed by TSK-gel columns (7.8 mm × 300 mm), PW-4000, PW-3000 connected with TSK-gel PW guard column, and eluted with 0.3 N $NaNO_3$ at a flow rate of 0.5 mL/min at 70 °C. The molecular mass was estimated by referencing a calibration curve made from pullulan standards (Shodex Standard P-series, Showa Denko, Kawasaki, Japan).

2.2.2. Sugar composition analysis

The polysaccharide samples were hydrolyzed to free sugars and the sugar composition was analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The hydrolysis procedure combined methanolysis and trifluoroacetic acid (TFA) hydrolysis. The polysaccharide sample (1–2 mg) was methanolyzed under vacuum in 1 mL of anhydrous 2 M HCl in absolute methanol, in a sealed hydrolytic tube at 80 °C for 12 h. The methanolysis reagent was evaporated and the methyl glycosides generated during methanolysis were further hydrolyzed with 2 M TFA at 100 °C for 1.5 h. TFA was removed via repeated evaporation under vacuum with HPLC-grade distilled water. The sugars in the hydrolyzate were analyzed using HPAEC-PAD. The HPAEC-PAD consisted of a Bioscan 817 Metrohm IC system (Metrohm, Herisau, Switzerland), including an IC pump 709, injection valve unit 812 with a 20 μL

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