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

Hsueh-Ting Wang a, Li-Chan Yang b, Hui-Ching Yu a, Miaw-Ling Chen c, Huei-Ju Wang d, Ting-Jang Lu a,∗

a Graduate Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan
b School of Pharmacy, Department of Pharmacy, China Medical University, Taichung, Taiwan
c Department of Nutrition and Health Sciences, Chang Jung Christian University, Tainan, Taiwan
d Department of Applied Science of Living, Chinese Culture University, Taipei, Taiwan

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)-Galp and (1,4)-GalA backbone; (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.

Copyright © 2017, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
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 xylglucan-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 NaNO3 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 methanolized 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
loop, and an electrochemical detector with a gold working electrode (E1 = 0.05 V, 0.48 s; E2 = 0.80 V, 0.18 s; E3 = −0.30 V, 0.36 s). A CarboPac PA1 (4 mm × 250 mm) analytical column (Dionex Corp., Sunnyvale, CA) with a guard column (4 mm × 50 mm) was used. For separation of neutral monosaccharides, the eluent (10 mM NaOH containing 2 mM barium acetate, or 19 mM NaOH containing 1 mM barium acetate) was applied at a flow rate of 0.5 mL/min (19 mM NaOH containing 1 mM barium acetate was use to separating sugars containing 1 mM barium acetate) was applied at a flow rate of 1 mL/min. Data were collected and analyzed using the Metrordata™ IC Net 2.1 software package (Metrohm).

2.2.3. Structural characterization
Linkage analysis was performed through methylation, which was performed with methyl iodide (Sigma–Aldrich, St. Louis, MO) in DMSO (Sigma–Aldrich) and sodium hydroxide (Wako, Osaka, Japan). After methylation, derivatives were hydrolyzed with 2 M TFA, then reduced and acetylated, and processed as described by Pettolino et al. [21]. Acidic polysaccharides were reduction with sodium borodeuteride and CMC (1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimide metho-p-toluenesulfonate) before methylation. The partially methylated alditol acetates were identified by gas chromatography-mass spectrometry (GC-MS) using a Agilent Technologies 6890N gas chromatograph on a DB-5MS column. The injector temperature was maintained at 300 °C, using the following temperature program: 14 min 110 °C, the gradient was warmed to 179 °C at 0.75 °C/min; when it reached 179 °C, the temperature was increased to 300 °C at a rate of 20 °C/min. Helium was used as the carrier gas (1.0 mL/min, constant flow). The mass spectrometer was an Agilent Technologies 5975C at ionization potential 70 eV. Partially O-methylated alditol acetates were identified from m/z of their positive ions, by comparison with standards prepared as described by Wang et al. [22]; myo-inositol was used as an internal control.

Enzyme-linked immuno sorbent assay (ELISA) was performed to detect the different characteristic structures. Different antibodies were used to retain different epitopes; carbohydrate recognition rat monoclonal antibodies L2M, L5M, LM5, LM6, LM7, LM10, LM19, LM20, and JIM7 (purchased from PlantProbes, Leeds, UK) were used to detect β-linked glucuronic acid, linear tetrasaccharide in (1-4)-β-D-galactan, linear pentasaccharide in (1-5)-α-L-arabinan, partially methylsterified epope of homogalacturonan, (1-4)-β-D-xylan, linear trisaccharide in (1-4)-α-D-galacturonan, linear tetrasaccharide in methyl-(1-4)-α-D-galacturonan, and linear hexasaccharide with (1-4)-α-GaLa-[MeGalA]_n GalA, respectively. Each sample was dissolved in phosphate buffered saline (PBS), pH 7.4, and transferred in 100 μL aliquots to a 96-well plate. The samples were allowed to coat the plate wells for 16 h at 4 °C. After coating, each well was rinsed four times with 300 μL distilled water and blocked for 1 h at 4 °C using 200 μL PBS containing 3% (w/v) non-fat bovine milk powder. The plate was washed four more times with distilled water. Then, 100 μL primary antibody (diluted 20-fold in PBS) was added and incubated for 2.5 h at room temperature. The plate was washed six times with distilled water followed by the addition of 100 μL anti-rat IgG coupled with horseradish peroxidase (HRP, Sigma), diluted 2000-fold in PBS containing 1% (w/v) milk powder with incubation for 1.5 h, followed by another six washes. The plates were developed by adding 150 μL 3.3',5,5'-tetramethylbenzidine liquid substrate (Sigma; diluted two-fold in distilled water) for 10 min. The reaction was stopped by adding 50 μL of 2 M H2SO4. The results were measured at 450 nm with a UV-Vis ELISA reader [23]. 1H NMR and 13C NMR spectra were recorded with using a BRUKER AVIII-500MHZFT-NMR spectrometer, operating at 500 MHz.

2.3. Macrophage stimulating activity assay

2.3.1. TNF-α release activity
The murine macrophage cell line RAW 264.7 (Bioresource Collection and Research Center) was cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), high glucose (4.5 g/L) and 2 mM glutamine at 37 °C under 5% CO2. Cells were seeded into 96-well culture plates (Nunc, Roskilde, Denmark) at a density of 1 × 10^4 cells/well, and left to adhere for 3 h. After removing the medium, cells were subsequently incubated in media containing each polysaccharide sample for 48 h. The concentrations of TNF-α in the cultured supernatants were assayed by DuoSet ELISA Development Systems (R&D Systems, Minneapolis, MN) following the manufacturer’s protocol.

2.3.2. Gene reporter platforms luciferase assays
The RAW 264.7 macrophage cells transfected with pGL4.32-COX-2 were used to conduct the Gene Reporter Platform luciferase assay as described in the literature, and kindly provided by Dr. Hsieh [17].

2.3.3. Inhibition of F3-FA induced TNF-α production using pattern-recognition receptor antibodies
To determine the role of Toll-like receptor 2 (TLR2), Toll-like receptor 4 (TLR4), Dectin-1, and Complement receptor 3 (CR3) in TNFα production, the RAW 264.7 cells cultivated in 24-well tissue culture plates were pretreated with TLR2-specific mAb mT2.4 (sc-73361, Santa Cruz Biotechnology, Dallas, TX), TLR4-specific mAb MTS510 (sc-13591, Santa Cruz), Dectin-1-specific mAb 2A11 (GTX41467, GeneTex, Irvine, CA), or CR3 (CD11b, GTX42473, GeneTex) mAb at a concentration of 10 μg/mL for 1 h. The RAW 264.7 cells were then treated with F3_FA (at 10 μg/mL) or a medium for 24 h. After incubation, the levels of TNF-α in the supernatants were measured using commercial ELISA kits.

2.3.4. Fucosidase treatment
Aliquots (3 μg) of polysaccharide sample in 50 mM citrate buffer (pH 5.5) were treated with 125 mUnit α-1,2,4,6-fucosidase (New England Biolabs, Hitchin, UK) at 37 °C for 24 h. To inactivate the enzyme, the sample and enzyme mixture was heated at 65 °C for 10 min. Dialysis and ethanol precipitation were used before the enzyme activity studies.
2.4. Statistical analysis

For fraction analyses, data are presented as the means ± standard deviations. Differences among the variants were analyzed using one-way analysis of variance with the Dunnett test; p < 0.001 was considered statistically significant. We used an two sample t-test (F < 0.001) to evaluate the effect of fucose on bioactivity.

3. Results and discussion

3.1. Fractionation of the bioactive polysaccharides

The bioactive polysaccharides in the A. blazei fermentation product were fractionated through an activity-guided strategy and a fucose-containing polysaccharide fraction was obtained. The yield of crude polysaccharide was 14% on dry basis. The crude polysaccharide was further fractionated according the differences of their charge density and molecular size. According to charge differences, we used three different stepwise gradients of NaCl solution (0, 0.1, and 0.2 M) to separate and collect three polysaccharide fractions (F1, F2, and F3) on a DEAE-650M column (Fig. 1). The yields of F1, F2 and F3 were 37%, 37% and 14% on the carbohydrate content basis of crude polysaccharide. The bioactivity of each polysaccharide fraction was evaluated for its capability to stimulate TNF-α secretion in the macrophage cell line RAW 264.7. We incubated RAW 264.7 cells with different doses (1, 5, 10, 20, 50, 100 μg/mL) of polysaccharide fractions and the TNF-α levels were monitored at 48 h (Fig. 2). The TNF-α levels increased significantly in a dose-response manner from 0.09 ng/mL (control) to 1.93 and 2.10 ng/mL, respectively, for the crude polysaccharide and the F3 fraction (both at 100 μg/mL concentration). Both the protein and uronic acid content of each fraction were monitored for the DEAE-column separation. These results indicated that the F3 fraction was an acidic polysaccharide fraction with proteins (with a higher negative charge density) and was the major bioactive polysaccharide fraction in the A. blazei fermentation product. The sugar composition of each polysaccharide fraction was analyzed and the results shown in Table 1. Galactose and xylose (with small amount of mannose, which could not be well separated and quantified in our HPAEC analysis) were the major sugar compositions of the F3 fraction as well as the F1 and F2 fractions. In addition, the F3 fraction also contained 11.7% arabinose (with a small amount of rhamnose, which could not be well separated and quantified in our HPAEC analyses) and 22.2% uronic acids with few proteins. A significant content difference of fucose, arabinose, glucose, and uronic acids was observed among F3 and F1 and F2. The F3 also contained more protein content as noted above.

Fucose has an important role in suppressing cancer growth [16]; thus, we paid attention to the content of this sugar in the fractions. The sugar composition of each polysaccharide fraction was analyzed and the content of fucose correlated positively with the tendency of TNF-α stimulating activity on RAW 264.7 cells. The results indicated that F3 contained 2.6% fucose, which was the highest content among the levels of the fractions from DEAE separation. DEAE-650M is a weak anion exchanger for biomolecule fractionation and purification. A similar DEAE column has been used to separate a neutral polysaccharide fraction consisting of glucose, mannose, and galactose without uronic acid from the fruiting body of A. blazei, which showed an inhibitory effect on the growth of osteosarcoma cell lines [24]. In contrast, the fucose-containing polysaccharides of F3 were a stronger negative charged fraction on the DEAE column.

The polysaccharides in the F3 fraction were further separated on a Toyopearl HW-65F gel filtration chromatographic column according their molecular weight difference (Fig. 3), and the sugar composition and bioactivity of the fractions were analyzed (Table 1, Fig. 4). F3_FA, F3_FB, and F3_FC represented three fractions with distribution coefficients 0–0.4, 0.4–0.58, and 0.58–1, respectively. The content ratio of F3_FA, F3_B and F3_C was 1:1.2:1.7 on the carbohydrate content basis of F3 fraction. F3_FA was the largest molecular weight fraction. The polysaccharides contained fucose (6.4%), arabinose (15.5%), xylose (14.7%), and less amount of mannose, glucose,
and mannose with significantly higher amount (25.0% in molar ratio) of uronic acids. The uronic acid was further identified as galacturonic acid. F3_FA was the highest among fucose fractions (6.4%) and showed activity toward stimulating RAW 264.7 macrophages to significantly increasing the release of TNF-α (Fig. 4).

Fucose-containing glycans have an important role in bioactivity and they have been associated with the activities that induce antibodies against tissue damage [15,25]. A fucose-containing glycoprotein fraction has been isolated from the medical mushroom *Ganoderma lucidum* (Reishi) [14]. However, in contract to those found in *G. lucidum*, the active component isolated in this study was acidic polysaccharides without polypeptides yielding a reaction in the Bradford assay.

### 3.2. Chemical characteristics of F3_FA

To understand the structural characteristics of the fucose-containing polysaccharides, the F3_FA fraction was subjected to high-performance size-exclusion chromatography, GC-MS, and

| Fractions | Molar percentage (%) | Uronic acid (%) |
|-----------|----------------------|-----------------|
| P/C       | Fuc | Ara/Rha | Gal | Glc | Man/Xyl |         |
| F1        | 1.5 ± 0 | 0.5 ± 0.1 | 47.5 ± 9.4 | 3.0 ± 0.5 | 47.7 ± 9.9 | ND |
| F2        | 0.1 ± 0.2 | 3.9 ± 0.3 | 32.3 ± 4 | 4.4 ± 0.3 | 59.3 ± 4.4 | 2.1 |
| F3        | 2.6 ± 0.1 | 11.7 ± 0.2 | 32.1 ± 1.3 | 9.8 ± 0.7 | 43.8 ± 1.0 | 22.2 |
| F3_FA     | 6.4 ± 0.1 | 15.5 ± 0.0/5.1 ± 0.2 | 28.5 ± 0.5 | 2.1 ± 0.3 | 2.7 ± 0.1/14.7 ± 0.2 | 25.0 |
| F3_FB     | 3.3 ± 0.3 | 14.7 ± 0.9 | 36.3 ± 1.0 | 5.0 ± 0.1 | 40.6 ± 0.2 | 15.2 |
| F3_FC     | 1.1 ± 0.2 | 3.9 ± 0.1 | 21.3 ± 0.3 | 23.2 ± 0.2 | 50.5 ± 0.3 | 15.4 |

*Fig. 3* – Gel filtration chromatogram of the acidic polysaccharides F3 fraction obtained from submerged fermentation of *Agaricus blazei* Murill. Pullulan molecular-weight standards and glucose were used to construct a calibration curve for molecular weight determination.

*Fig. 4* – Dose-dependent effects of TNF-α stimulating activity of fucose-containing acidic polysaccharide fractions from submerged fermentation of *Agaricus blazei* Murill on the macrophage cell line RAW 264.7 (Each value presents Mean ± SD, n = 4).

*Fig. 5* – Chromatogram of the fucose-containing acidic polysaccharides F3_FA fraction from submerged fermentation of *Agaricus blazei* Murill. Analytical conditions: column: TSK-Gel G4000PW–G3000 PW, 70°C. Flow rate: 0.5 mL/min. Eluent: 0.3 N NaNO₃/0.02% NaN₃.
and confirmation by carbohydrate epitope-recognition test against monoclonal antibodies and NMR spectroscopy.

The HPSEC chromatogram of the F3_FA fraction revealed a single symmetrical peak, indicating the polydispersity and the even distribution of the polysaccharide molecules. The weight-average molecular weight of F3_FA was 3.5 × 10^5 Da (Fig. 5). The sugar composition of F3_FA was mainly galactose, galacturonic acid, arabinose, xylose, fucose, and rhamnose (Table 1). The GC-MS results (Table 2) indicated that the backbone chain consisted of (1,4) linked Galp and (1,4) linked Galp, (1,2)-Xyl, and that (1,2)-Rha might present in the backbone chain or might be linked to the 6-O position of galactose as a side chain; additionally, (1,5)-Ara might serve as a side chain and terminal fucose may be involved in the polysaccharides. NMR data was used to support the above results (Table 3) with assignments based on published data. The signals at Δ 4.5–4.6 and Δ 5.0–5.2 showed that F3_FA demonstrated both α- and β-type configurations, with the signals at Δ 1.1–1.3 being characteristic of the H6 of L-fucose and Δ 4-rhamnose methyl group and the 13C signal at approximately Δ 175.0 was characteristic of carboxylic acid (C=O) (Fig. 6). The signals at Δ 4.58 (H1, 3.63 (H2), 3.73 (H3), 4.11 (H4), and 3.66 (H5) ppm in the 1H NMR spectrum and at Δ 104.4 ppm in the 13C NMR spectrum were assigned to the C1 carbon attributed to (1,4)-β-Galp [26,27]. The signals at Δ 5.02 were assigned to H1 of (1,4)-α-Galpα, Δ 3.60–4.7 were attributed to H2 – H5 of the α-Galpα, and the 13C Δ 174.7 ppm signal was characteristic of carboxylic acid (C=O) [28,29]. The signal at Δ 65.19 was attributed to the H1 of terminal residues of Araf, that at Δ 5.09 was attributed to H1 of (1,5)-Araf, and those at Δ 3.73–4.25 were attributed to H2–H5 of the Araf residues (Table 3) [27,30]. An acidic fraction from A. blazei showed...
activity in RAW 264.7 cells of which the main sugar composition was glucose [12]; in contrast, F3_FA consisted of galactose and glucuronic acid.

The structures were also confirmed by the carbohydrate epitope recognition test against monoclonal antibodies, in which we used eight different monoclonal antibodies through ELISA assays to recognize specific sugar linkages for the aforementioned main sugar composition (Table 4). The results indicated that (1, 4)-\(\beta\)-D-galactan, (1, 5)-\(\alpha\)-L-arabinan, and (1, 4)-\(\alpha\)-D-galacturonan existed in F3_FA. Monoclonal antibodies can recognize specific epitopes and this technique has been used to screen and interpret the complexity of pectin structures [31]. The rhamnogalacturonan I (RGI) domains of pectic polysaccharides contain arabinogalactan type I, arabinogalactan type II, and galactan, which are highly branched structures with neutral sugars whose side chains are complex structurally and highly bioactive [32–34]. To our surprise, the carbohydrate epitope recognition test against monoclonal antibodies through ELISA assays indicated that the F3_FA structural characteristics had high similarity with those of RGI.

The signals of the fucose and deoxy sugar were apparent in the NMR spectra, the signals at \(\delta 1.24\) were characteristic of the H6 of L-fucose methyl group, and the signals at \(\delta 5.12, \delta 3.80–3.85, \delta 4.19\) were attributed to H1, H2/H4, and H5 of terminal \(\alpha\)-L-fucp residues [13]. The \(\alpha\)-fucosyl residues were exclusively linked to the non-reducing termini. A bioactive polysaccharide fucogalactan has been isolated and characterized from cold-water extracts of A. bisporus, A. brasiliensis and Lactarius rufus [35]. The fucogalactan is a (1,6)-\(\alpha\)-D-galactan with \(\alpha\)-\(\alpha\)-fucosyl branches linked on O-2. In comparison, the fucose-containing polysaccharides in the F3_FA fraction had different characteristics in sugar composition (content of uronic acid and other neutral sugar) and anomeric linkages of galactose backbone, although they all had the \(\alpha\)-\(\alpha\)-fucosyl branches.

### 3.3. Trials of bioactivity

#### 3.3.1. Effect of fucose on bioactivity

The terminal \(\alpha\)-fucosyl residues play an important role on the bioactivity of F3_FA polysaccharides toward stimulating the RAW 264.7 cell-line to release TNF-\(\alpha\). Removal of terminal \(\alpha\)-fucosyl residues by using enzymatic digestion caused a loss of F3_FA fraction activity. The results of a comparison experiment indicated that the TNF-\(\alpha\) stimulating activity of F3_FA at the concentration of 10 mg/mL became insignificant (unpaired t-test; \(F < 0.001\)) after the polysaccharides were pre-treated with \(\alpha\)-1,2,4,6-fucosidase (Fig. 7). L-Fucose has been found in some biologically relevant glycans from mushrooms [13–15,35,36]. The fucose-containing polysaccharides obtained in this report share similar non-reducing terminal \(\alpha\)-fucose features as those found in G. lucidum and other Agaricus spp, although the backbone sugar composition and linkage were different.

| Epitope structure for carbohydrate antigen Response | Antibody | LM2 | LM5 | LM6 | LM7 |
|---------------------------------------------------|----------|-----|-----|-----|-----|
| \(\beta\)-linked glucuronic acid NO                | LM2      | YES |     |     |     |
| Linear tetrasaccharide in (1,4)-\(\beta\)-D-galactan YES | LM6      |     | YES |     |     |
| Linear pentasaccharide in (1,5)-\(\alpha\)-L-arabinan YES | LM7      |     |     | Partially methylesterified epitope of homogalacturonan NO |}

| Epitope structure for carbohydrate antigen Response | Antibody | LM19 | LM20 | JIM7 |
|---------------------------------------------------|----------|------|------|------|
| (1,4)-\(\beta\)-D-xylan NO                        | LM10     | YES  |     |     |
| Linear trisaccharide in (1,4)-\(\alpha\)-D-galacturonan YES | LM19     |     | YES |     |
| Linear tetrasaccharide in methyl-(1,4)-\(\alpha\)-D-galacturonan NO | LM20     |     |     | Linear hexasaccharide with (1,4)-\(\alpha\)-GalA-[MeGalA]$_n$-GalA NO |
3.3.2. Gene reporter platforms luciferase assays and inhibition of cytokine production using pattern-recognition receptor antibodies

NF-κB activates a number of cytokines and is a key transcription factor involved in F3_FA-induced TNF-α expression; moreover, TLR 4 binding affects bioactivity. Cyclooxygenase-2 (COX-2) is a transcriptional target of nuclear factor kappa B (NF-κB) and a gene reporter platform was established to determine mediated immuno-modulatory activity. In this study, we utilized RAW 264.7 macrophage cells transfected with pGL4.32-COX-2 to evaluate immuno-modulatory activity. The results show that F3_FA significantly stimulated RAW 264.7 macrophage cells transfected with pGL4.32-COX-2 even at a concentration of 1 μg/mL (Fig. 8). Moreover, poly saccharides mediated active macrophages through recognition specific receptors [37–40]. As fucose-containing polysaccharides from A. blazei Murill, their mechanism of immune response has rarely been studied. Thus, this study investigated whether these receptors are involved in the F3_FA-induced production of TNF-α. The results show that treatment with anti-TLR4 mAb (10 μg/mL) significantly blocked F3_FA induced TNF-α. Cells treated with anti-TLR2 mAb (10 μg/mL), anti-Dectin-1, and anti-CR3 mAb (10 μg/mL) failed to inhibit F3_FA-induced TNF-α secretion (Fig. 9). Based on above results we suggest that NF-κB is a key transcription factor involved in F3_FA-induced TNF-α expression and that (F3_FA)-(TLR4) binding affects F3_FA-induced TNF-α production.

4. Conclusion

Polysaccharides from Agaricus spp have drawn considerable attentions as have traditional medicinal mushrooms, e.g. Ganoderma lucidum, for their immunomodulating and antitumor activities. Numerous bioassays have been conducted in vivo and in vitro to confirm their efficacy. However, the mechanism of their activities is still not fully understood. A major obstacle is the difficulty in obtaining a standardized polysaccharide with high purity for bioassays. (1, 6)-β-D-glucans and fucogalactans from fruiting bodies of Agaricus blazei and Agaricus bisporus, respectively, are two exceptional examples for their feature of highly homogeneous repeating pattern. In this study, we selected a profiling fractionating and activity-guided strategy to observe the spectra of polysaccharide extracts of fermentation product including mycelia and broth. The aforementioned two polysaccharides did not exist in a substantial amount in our tested samples; a complex fucose-containing acidic polysaccharide-enriched fraction was obtained instead. Acidic polysaccharides of
fruiting bodies of *A. blazei* have been reported by Mizuno et al. (with similar sugar composition of arabinose, galactose, xylose, and uronic acids with relatively little amount of mannose and fucose) [41]. They did not further investigate the acidic polysaccharide fraction, which was masked by a large quantity of glucans, nor was the acidic moieties of the polysaccharide examined, although significant antitumor activity was observed. Notably, although the structure information of the fucose-containing polysaccharides is not completely revealed in the current study, it provides new information regarding the polysaccharide profile from *A. blazei* mycelium fermentation biomass, which is commonly used in functional food products. The identification of the acidic polysaccharide fraction, with terminal α-L-fucose as essential activity key residues, provides a new clue for further study.

In summary, polysaccharides exhibit beneficial immunomodulatory activity [42], antitumor activity [43], and antitumor activity [40]; the immunomodulatory capabilities of *A. blazei* Murill have also been demonstrated [10]. Moreover, the biological properties of *A. blazei* Murill polysaccharides cannot be attributed to only glucan [1,12], thus, the present study characterizes a bioactive acidic fucose-containing polysaccharide, F3_FA, derived from submerged fermentation of *A. blazei* Murill. The structure of F3_FA is similar to that of RGI, with a 3.5 × 10^5 Da. molecular weight. F3_FA is a group of heteropolysaccharides consisting of (1→3)-b-D-galactan, (1→4)-α-D-galacturonan, (1→3)-α-L-arabinan, (1→2)-Xyl, (1→2)-Rha, and terminal α-Fuc structures, and contains arabinose, galactose, glucose, fucose, mannose, rhamnose, xylose, galacturonic acid, and glucuronic acid. Although the F3_FA immunomodulatory mechanism remains unclear, we demonstrated that the fucose component of this compound is involved in its bioactivity, and that the NF-κB transcription factor and TLR4 affect TNF-α expression, F3_FA thus acts as a biological response modifier [44]. It is still not clear that occurrences and functions of the fucose-containing polysaccharides in the tissue of *Agaricus blazei* Murill, though the information is important for product development. The fucose-containing polysaccharides could be one of components in the cell wall material of the mushrooms. The existence of fucose can be a useful marker for designing scale-up fermentation strategy and downstream processing for producing the bioactive polysaccharides from the mushroom in future studies.

**Acknowledgements**

This work was financially supported in part by NSC 94-2321-B-002-007 and NSC 99-2313-B-002 -017 -MY3 from the National Science Council, Taiwan, ROC. The authors thank Professor Chin-Hang Shu, Department of Chemical and Materials Engineering, National Central University, Taiwan, for providing *Agaricus blazei* Murill fermentation product.

**References**

[1] Mizuno T, Inagaki R, Kanao T, Hagiwara T, Nakamura T, Ito H, et al. Antitumor-activity and some properties of water-insoluble hetero-glycans from “Himenomatsutake”, the fruiting body of *Agaricus-blazei* Murill. Agric Biol Chem 1990;54:2897—905.

[2] Mizuno M, Morimoto M, Minato K, Tsuchida H. Polysaccharides from *Agaricus blazei* stimulate lymphocyte T-cell subsets in mice. Biosci Biotechnol Biochem 1998;62:434—7.

[3] Fujimiy A, Suzuki Y, Oshiman K, Kobori H, Moriguchi K, Nakashima H, et al. Selective tumoricidal effect of soluble proteoglycan extracted from the basidiomycete, *Agaricus blazei* Murill, mediated via natural killer cell activation and apoptosis. Cancer Immunol Immun 1998;46:147—59.

[4] Gonzaga ML, Menezes TM, de Souza JR, Ricardo NM, Freitas AL, et al. D. A. Analgesic activity of a glucan polysaccharide isolated from *Agaricus blazei* Murill. Int J Carbohydr Chem 2013, ID846565. Available at: http://dx.doi.org/10.1155/2013/846565.

[5] Mizuno M, Minato K, Ito H, Kawade M, Terai H, Tsuchida H. Anti-tumor polysaccharide from the mycelium of liquid-cultured *Agaricus blazei* murill. Biochem Mol Biol Int 1999;47:707—14.

[6] Chung HY, Cho YJ, Kim T. Isolation and characterization of a water-soluble polysaccharide from the mycelia of solid cultured *Agaricus blazei* Murill. Food Sci Biotechnol 2005;14:259—62.

[7] Schepetkin IA, Quinn MT. Botanical polysaccharides: macrophage immunomodulation and therapeutic potential. Int Immunopharmacol 2006;6:317—33.

[8] Shu C-H, Wen B-J, Lin K-J. Monitoring the polysaccharide quality of *Agaricus blazei* in submerged culture by examining molecular weight distribution and TNF-α release capability of macrophage cell line RAW 264.7. Biotechnol Lett 2003,25:2061—4.

[9] Wasser S. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. Appl Microbiol Biotechnol 2002;60:258—74.

[10] Tomsik P, Soukup T, Cermakova E, Micuda S, Niang M, Carbonero ER, Cordova MM, Baggio CH, et al. *Agaricus bisporus* fucogalactan: structural characterization and pharmacological approaches. Carbohyd Polym 2013;92:184—91.

[11] Wang YY, Kho KH, Chen ST, Lin CC, Wong CH, Lin CH. Studies on the immuno-modulating and antitumor activities of Ganoderma lucidum (Reishi) polysaccharides: functional and proteomic analyses of a fucose-containing glycoprotein fraction responsible for the activities. Bioreg Med Chem 2002;10:1057—62.

[12] Liao SF, Liang CH, Ho MY, Hsu TL, Tsai TI, Hsieh YSY, et al. Immunization of fucose-containing polysaccharides from Reishi mushroom induces antibodies to tumor-associated Globo H-series epitopes. Proc Natl Acad Sci U S A 2013;110:13809—14.

[13] Tomski P, Soukup T, Cermakova E, Micuda S, Nigg N, Sucha L, et al. L-rhamnose and L-fucose suppress cancer growth in mice. Cent Eur J Biol 2011;6:1—9.

[14] Wang CH, Lin JH, Lu TJ, Chiang AN, Chiu ST, Chen YA, et al. Establishment of reporter platforms capable of detecting NF-
kappa B mediated immuno-modulatory activity. J Agric Food Chem 2013;61:12582–7.

[18] Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350–6.

[19] Blumenkr N, Asboehan G. New method for quantitative-determination of uronic acids. Anal Biol 1973;54:484–9.

[20] Bradford MM. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biol 1976;72:248–54.

[21] Pettolino FA, Walsh C, Fincher GB, Bacic A. Determining the polysaccharide composition of plant cell walls. Nat Protoc 2012;7:1590–607.

[22] Wang ZF, He Y, Huang LJ. An alternative method for the determination of uronic acids. Anal Biol 1973;54:484.

[23] Zhang H, Li W-J, Nie S-P, Chen Y, Wang Y-X, Xie M-Y. A polysaccharide from Prunus dulcis. Carbohydr Polym 2012;87:1620–7.

[24] Wu B, Cui JC, Zhang CG, Li ZH. A polysaccharide from Himematsutake, the fruiting body of Agaricus blazei Murill. Agric Bio Chem 1990;54:2889–96.

[25] Guryanov O, Gorshkova T, Kabel M, Schols H, van Dam JEG. Structural characterization of tissue-specific galactan from flax fibers by 1H NMR and MALDI TOF mass spectrometry. Russ J Bioorg Chem 2006;32:558–67.

[26] Willfor S, Sjoholm R, Laine C, Holmbom B. Structural features of water-soluble arabinogalactans from Norway spruce and Scots pine heartwood. Wood Sci Technol 2002;36:101–10.

[27] Yang C, Gou Y, Chen J, An J, Chen W, Hu F. Structural characterization and antioxidant activity of a pectic polysaccharide from Codonopsis pilosula. Carbohydr Polym 2013;98:886–95.

[28] Zhang H, Li W-J, Nie S-P, Chen Y, Wang Y-X, Xie M-Y. Structural characterization of a novel bioactive polysaccharide from Ganoderma atrum. Carbohydr Polym 2012;88:1047–54.

[29] Dourado F, Cardoso SM, Silva AMS, Gama FM, Coimbra MA. NMR structural elucidation of the arabinan from Prunus dulcis immunobiological active pectic polysaccharides. Carbohydr Polym 2006;66:27–33.

[30] Köhler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 1975;256:495–7.

[31] Mohnen D. Pectin structure and biosynthesis. Curr Opin Plant Biol 2008;11:266–77.

Abbreviation list of carbohydrates

**Araf**: arabinofuranose

**Arap**: arabinopyranose

**Fucp**: fucopyranose

**Galp**: galactopyranose

**GalpA**: galacturonpyranic acid

**Glcp**: glucopyranose

**Manp**: mannopyranose

**Rhap**: rhamnopyranose

**Xylp**: xylopyranose

T- or t-: a linkage indicator for terminal sugar residues