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### Recommended Citation

Wang, H.-T.; Yang, L.-C.; Yu, H.-C.; Chen, M.-L.; Wang, H.-J.; and Lu, T.-J. (2018) "Characteristics of fucose-containing polysaccharides from submerged fermentation of *Agaricus blazei* Murill," *Journal of Food and Drug Analysis*: Vol. 26 : Iss. 2 , Article 40.

Available at: <https://doi.org/10.1016/j.jfda.2017.07.006>

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## Original Article

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



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## ARTICLE INFO

## Article history:

Received 25 June 2017

Received in revised form

24 July 2017

Accepted 24 July 2017

Available online 18 August 2017

## Keywords:

*Agaricus blazei* Murill

Acidic polysaccharide

Fucose

Galactan

TNF- $\alpha$  stimulation

## 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  $3.5 \times 10^5$  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)-GalAp 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- $\alpha$  cytokine stimulating activity of the polysaccharides in a RAW 264.7 macrophage cell-line test, whereas NF- $\kappa$ B and TLR4 affected the polysaccharide-induced TNF- $\alpha$  production.

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<http://dx.doi.org/10.1016/j.jfda.2017.07.006>

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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  $\alpha$ -(1,4; 1,6) linkages,  $\alpha$ -(1,4) linkages, and  $\beta$ -(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,  $\beta$ -(1,6) glucan, and  $\beta$ -(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- $\alpha$  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- $\alpha$ . 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 $\times g$  for 10 min) to remove insoluble materials. The supernatant was applied to a DEAE-650M (Toyopearl, Tokyo, Japan) column (2.6 cm  $\times$  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  $\times$  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  $\mu$ L sample loop, and an OPTILAB DSP interferometric refractometer (P10 cell, 690 nm, Wyatt Technology Co., Santa Barbara, CA) with the temperature controlled at 35  $^{\circ}$ C. The samples were analyzed by TSK-gel columns (7.8 mm  $\times$  300 mm), PW-4000, PW-3000 connected with TSK-gel PW guard column, and eluted with 0.3 N NaNO<sub>3</sub> at a flow rate of 0.5 mL/min at 70  $^{\circ}$ 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  $^{\circ}$ 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  $^{\circ}$ 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  $\mu$ L

loop, and an electrochemical detector with a gold working electrode ( $E_1 = 0.05$  V, 0.48 s;  $E_2 = 0.80$  V, 0.18 s;  $E_3 = -0.30$  V, 0.36 s). A CarboPac PA1 (4 mm  $\times$  250 mm) analytical column (Dionex Corp., Sunnyvale, CA) with a guard column (4 mm  $\times$  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 used to separate sugars with similar capacity factors Ara/Rha and Man/Xyl). For separation of acidic monosaccharides (galacturonic acid and glucuronic acid), the eluent (75 mM NaOH, 150 mM sodium acetate, and 1 mM barium acetate) was applied at a flow rate of 1 mL/min. Data were collected and analyzed using the Metrodata™ 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 reduced with sodium borodeuteride and CMC (1-cyclohexyl-3-(2-morpholino-ethyl)carbodiimide metho-p-toluenesulfonate) before methylation. The partially methylated alditol acetates were analyzed by gas chromatography-mass spectrometry (GC-MS) using an 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 immunosorbent assay (ELISA) was performed to detect the different characteristic structures. Different antibodies were used to retain different epitopes; carbohydrate recognition rat monoclonal antibodies LM2, LM5, LM6, LM7, LM10, LM19, LM20, and JIM7 (purchased from PlantProbes, Leeds, UK) were used to detect  $\beta$ -linked glucuronic acid, linear tetrasaccharide in (1-4)- $\beta$ -D-galactan, linear pentasaccharide in (1-5)- $\alpha$ -L-arabinan, partially methylesterified epitope of homogalacturonan, (1-4)- $\beta$ -D-xylan, linear trisaccharide in (1-4)- $\alpha$ -D-galacturonan, linear tetrasaccharide in methyl-(1-4)- $\alpha$ -D-galacturonan, and linear hexasaccharide with (1-4)- $\alpha$ -GalA-[MeGalA]<sub>n</sub>-GalA, respectively. Each sample was dissolved in phosphate buffered saline (PBS), pH 7.4, and transferred in 100  $\mu$ L 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  $\mu$ L distilled water and blocked for 1 h at 4 °C using 200  $\mu$ L PBS containing 3% (w/v) non-fat bovine milk powder. The plate was washed four more times with distilled water. Then, 100  $\mu$ L 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  $\mu$ L 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  $\mu$ L 3,3',5,5'-tetramethylbenzidine liquid substrate (Sigma; diluted two-fold in distilled water) for 10 min. The reaction was stopped by adding 50  $\mu$ L of 2 M H<sub>2</sub>SO<sub>4</sub>. The results were measured at 450 nm with a UV-Vis ELISA reader [23]. <sup>1</sup>H NMR and <sup>13</sup>C 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- $\alpha$ 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% CO<sub>2</sub>. Cells were seeded into 96-well culture plates (Nunc, Roskilde, Denmark) at a density of  $1 \times 10^4$  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- $\alpha$  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- $\alpha$ 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 TNF $\alpha$  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  $\mu$ g/mL for 1 h. The RAW 264.7 cells were then treated with F3\_FA (at 10  $\mu$ g/mL) or a medium for 24 h. After incubation, the levels of TNF- $\alpha$  in the supernatants were measured using commercial ELISA kits.

### 2.3.4. Fucosidase treatment

Aliquots (3 mg) of polysaccharide sample in 50 mM citrate buffer (pH 5.5) were treated with 125 mUnit  $\alpha$ -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.



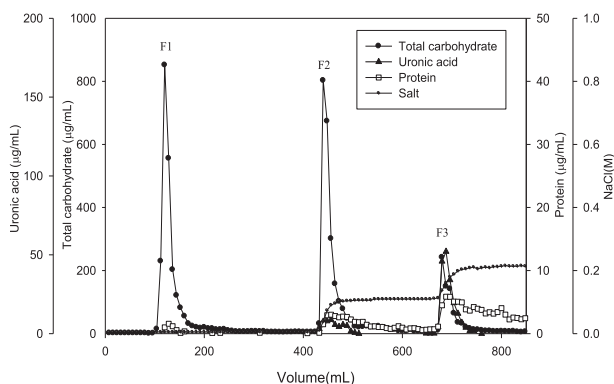
## 2.4. Statistical analysis

For fraction analyses, data are presented as the means  $\pm$  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 a two sample t-test ( $F < 0.001$ ) to evaluate the effect of fucose on bioactivity.

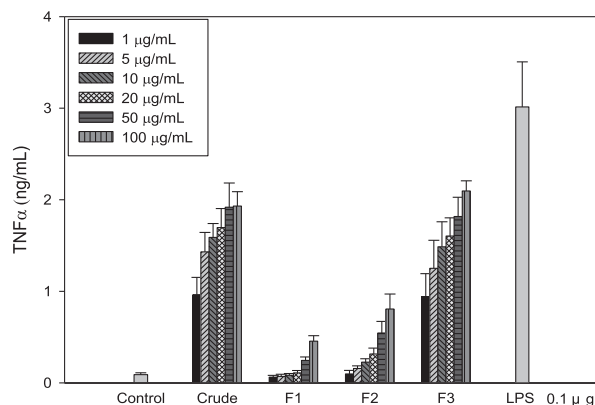
## 3. Results and discussion

### 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 to 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- $\alpha$  secretion in the macrophage cell line RAW 264.7. We incubated RAW 264.7 cells with different doses (1, 5, 10, 20, 50, 100  $\mu\text{g}/\text{mL}$ ) of polysaccharide fractions and the TNF- $\alpha$  levels were monitored at 48 h (Fig. 2). The TNF- $\alpha$  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  $\mu\text{g}/\text{mL}$  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



**Fig. 1** – Anion-exchange chromatogram of water-soluble crude polysaccharide from submerged fermentation of *Agaricus blazei* Murill on a DEAE-650M column. Starting buffer: 20 mM Tris buffer (pH 7.8). The column was eluted stepwise with 0, 0.1 and 0.2 M NaCl in the starting buffer.



**Fig. 2** – Dose-dependent response of TNF- $\alpha$  stimulating activity of crude polysaccharide from submerged fermentation of *Agaricus blazei* Murill and its fractions from DEAE-column separation on the macrophage cell line RAW 264.7 (Each value presents Mean  $\pm$  SD,  $n = 4$ ).

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 in 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- $\alpha$  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 to 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 rhamnose, glucose,

**Table 1 – Sugar composition of the polysaccharide fractions from submerged fermentation of *Agaricus blazei* Murill.**

Fractions	Molar percentage (%) <sup>a</sup>						Uronic acid (%) <sup>b</sup>
	P/C <sup>c</sup>	Fuc	Ara/Rha	Gal	Glc	Man/Xyl	
F1	<0.01	1.5 ± 0	0.5 ± 0.1	47.5 ± 9.4	3.0 ± 0.5	47.7 ± 9.9	ND <sup>d</sup>
F2	0.01	0.1 ± 0.2	3.9 ± 0.3	32.3 ± 4	4.4 ± 0.3	59.3 ± 4.4	2.1
F3	0.10	2.6 ± 0.1	11.7 ± 0.2	32.1 ± 1.3	9.8 ± 0.7	43.8 ± 1.0	22.2
F3_FA	<0.01	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 <sup>e</sup>
F3_FB	0.01	3.3 ± 0.3	14.7 ± 0.9	36.3 ± 1.0	5.0 ± 0.1	40.6 ± 0.2	15.2
F3_FC	0.03	1.1 ± 0	3.9 ± 0.1	21.3 ± 0.3	23.2 ± 0.2	50.5 ± 0.3	15.4

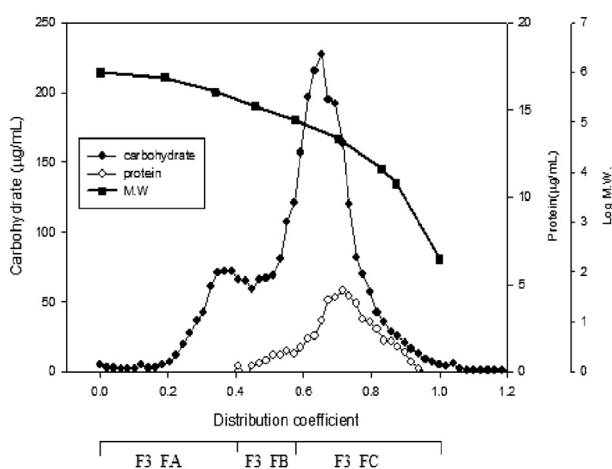
<sup>a</sup> Analysis by high-performance anion-exchange chromatography.

<sup>b</sup> Determined by colorimetric method using galacturonic acid as standard.

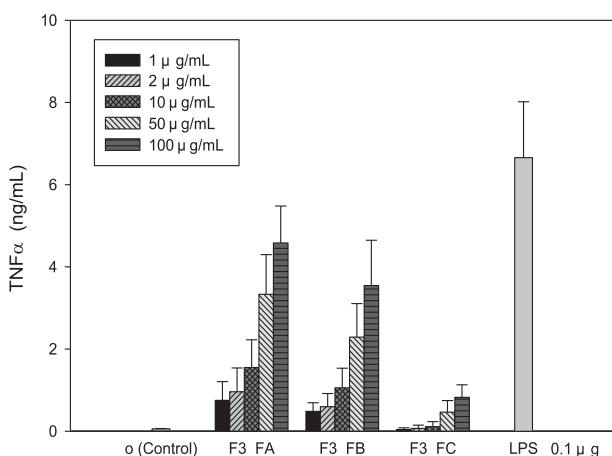
<sup>c</sup> P/C parameter represents protein/ carbohydrate weight ratio of crude polysaccharide and its fractions.

<sup>d</sup> Not detected.

<sup>e</sup> Contains 25.0 ± 0.2% galacturonic acid and traces of glucuronic acid.



**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.**



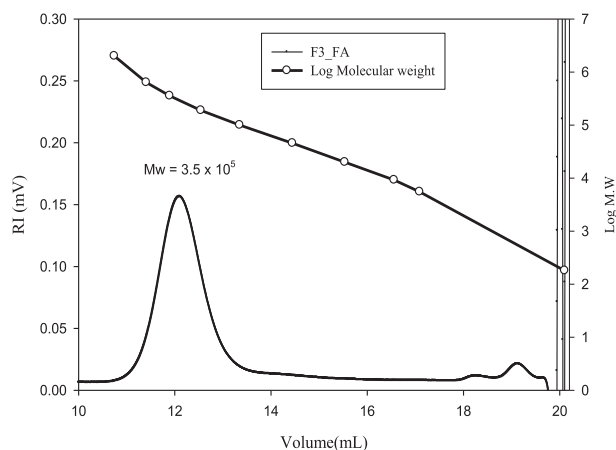
**Fig. 4 – Dose-dependent effects of TNF- $\alpha$  stimulating activity of fucose-containing acidic polysaccharide fractions from submerged fermentation of *Agaricus blazei* Murill on the macrophage cell line RAW 264.7 (Each value presents Mean  $\pm$  SD, n = 4).**

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- $\alpha$  (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 contrast 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,



**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 NaNO<sub>3</sub>/0.02% NaN<sub>3</sub>.**

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 weigh-average molecular weight of F3\_FA was  $3.5 \times 10^5$  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 GalAp. (1,2)-Xyl, and that (1,2)-Rha might present in the backbone chain or might be jointed 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  $\delta$  4.5–4.6 and  $\delta$  5.0–5.2 showed that F3\_FA demonstrated both  $\alpha$ - and  $\beta$ -type configurations, with the signals at  $\delta$  1.1–1.3 being characteristic of the H6 of L-fucose and L-rhamnose methyl group and the  $^{13}\text{C}$  signal at approximately  $\delta$  175.0 was characteristic of carboxylic acid (C=O) (Fig. 6). The signals at  $\delta$  4.58 (H1), 3.63 (H2), 3.73 (H3), 4.11 (H4), and 3.66 (H5) ppm in the  $^1\text{H}$  NMR spectrum and at  $\delta$  104.4 ppm in the  $^{13}\text{C}$  NMR spectrum were assigned to the C1 carbon attributed to (1,4)- $\beta$ -Galp [26,27]. The signals at  $\delta$  5.02 were assigned to H1 of (1, 4)- $\alpha$ -GalpA,  $\delta$  3.60–4.7 were attributed to H2 – H5 of the  $\alpha$ -GalpA, and the  $^{13}\text{C}$   $\delta$  174.74 signal was characteristic of carboxylic acid (C=O) [28,29]. The signal at  $\delta$  5.19 was attributed to the H1 of terminal residues of Araf, that at  $\delta$  5.09 was attributed to H1 of (1,5)-Araf, and those at  $\delta$  3.73–4.25 were attributed to H2–H5 of the Araf residues (Table 3) [27,30]. An acidic fraction from *A. blazei* showed

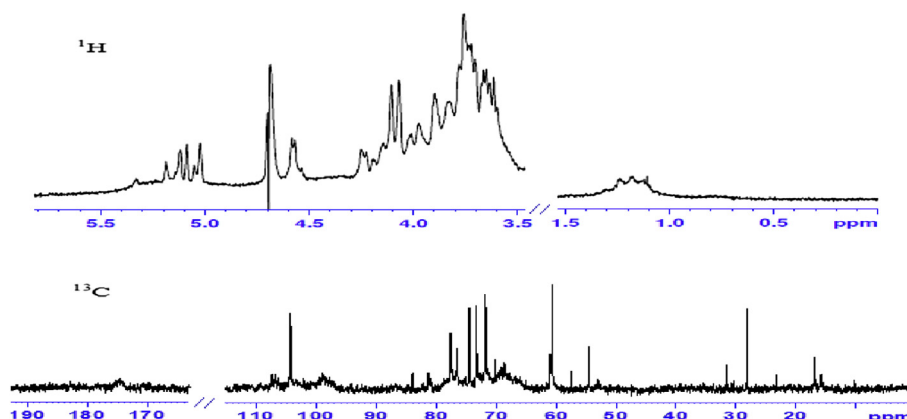
**Table 2 – Results of methylation analysis of polysaccharide F3\_FA fraction from submerged fermentation of *Agaricus blazei* Murill.**

Glycosyl residues	Partially O-methylated alditol acetates	Retention index	Deduced linkage	Sugar composition (%)	Characteristic ions
Galactose (28.7%)	2,3,4,6-Me <sub>4</sub> -Gal	1818	t-Galp	4.5	102, 145, 118, 129, 101
	2,3,6-Me <sub>3</sub> -Gal	1897	1-4Galp	22.1	118, 113, 102, 87, 99, 233
	2,3-Me <sub>2</sub> -Gal	2024	1-4,6Galp	2.1	118, 85, 102, 127, 99, 261
	2,3,4-Me <sub>3</sub> -Gal	1956	1-6Galp	trace	118, 102, 99, 129, 87
Galacturonic (24.5%)	2,3,6-Me <sub>3</sub> -Gal	1897	1-4GalAp	24.5	118, 115, 102, 87, 101, 235
	2,3,5-Me <sub>3</sub> -Ara	1610	t-Araf	3.5	118, 129, 102, 161, 87
Arabinose (15.6%)	2,3,4-Me <sub>3</sub> -Ara	1647	t-Arap	3.8	118, 117, 101, 102, 161
	2,3-Me <sub>2</sub> -Ara	1744	1-5Araf	5.4	118, 129, 87, 102, 189
	3-Me-Ara	1848	1-2,4Arap	1.9	118, 85, 127, 99, 59
	2-Me-Ara	1834	1-3,4Arap	1.0	129, 130, 87, 88, 189, 190
	2,4-Me <sub>2</sub> -Ara	1714	1-3Araf	trace	118, 87, 113, 99, 59, 129
	3,4-Me <sub>2</sub> -Xyl	1748	1-2Xylp	14.8	117, 130, 88, 101, 190
Fucose (6.4%)	2,3,4-Me <sub>3</sub> -Fuc	1679	t-Fucp	6.4	131, 102, 118, 89, 115, 162, 72
Rhamnose (5.1%)	3,4-Me <sub>2</sub> -Rha	1747	1-2RhAp	5.1	131, 89, 130, 88, 100, 190, 115
	2,3,4-Me <sub>3</sub> -Rha	1645	t-RhAp	trace	131, 102, 118, 89, 115, 162, 72
Mannose (2.7%)	4,6-Me <sub>2</sub> -Man	1976	1-2,3Manp	2.7	129, 161, 128, 86, 262
	2,3,4,6-Me <sub>4</sub> -Man	1795	t-Manp	trace	102, 145, 118, 129, 101
	6-Me-Man	2028	1-2,3,4Manp	trace	129, 140, 87, 115, 185, 157, 98, 99
Glucose (2.1%)	2,6-Me <sub>2</sub> -Glc	1973	1-3,4Glc	2.1	118, 129, 87, 143, 59
	2,3,4,6-Me <sub>4</sub> -Glc	1790	t-Glcp	trace	102, 145, 118, 129, 101
	3,4,6-Me <sub>3</sub> -Glc	1887	1-2Glcp	trace	129, 130, 161, 88, 100, 190, 87, 101, 145
	2,3,4-Me <sub>3</sub> -Glc	1917	1-6Glcp	trace	118, 102, 99, 129, 87
	2,3-Me <sub>2</sub> -Glc	2016	1-4,6Glcp	trace	118, 85, 102, 127, 99, 261

**Table 3 –  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR chemical shifts of the significant signals in spectra for polysaccharide F3\_FA fraction from submerged fermentation of *Agaricus blazei* Murill.**

Recognized to	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6	Ref. <sup>a</sup>
$\beta$ -Gal-(1-4)	4.58/104.4	3.90/72.0	3.78/74.1	4.07/–	3.60/–	3.80/–	[13,27,45]
$\beta$ -Gal-(1-6)	4.58/104.4	3.63/–	3.73/–	4.11/–	3.66/–	–	[26,27]
$\beta$ -Gal-(1-4) $\alpha$ -GalpA (-1)	–	–	3.90/–	–	–	–/70.3	[13,27]
T-Araf	5.02/98.5–99.5	3.60/71.9–72.1	3.83/73.3	4.25/79.9–80.5	4.69/71.8	–/174.7	[28,29]
5)-Araf-(1-3,5)-Araf-(1-2,3,5)-Araf-(1-	5.19/107.5	4.14/81.4	3.98/–	4.07/–	3.73, 3.83/–	–	[27,30]
T- $\beta$ -Xyl	5.09/–	4.14/84.0	4.05/–	4.23/–	3.83, 3.90/–	–	[30]
T- $\alpha$ -Fucp	5.12/–	–	4.11/–	–	3.83/–	–	[30]
	–	–	–	4.25/–	3.83, 3.90/–	–	[30]
	4.58/97.5–98.0	3.28/75.0–75.5	3.50–3.60/75.0–75.5	3.78/77.7	4.07(eq), 3.42(ax)/–	–	[46]
	5.12/103.9	3.80–3.85/71.9–72.1	3.80–3.85/71.0–71.5	3.80–3.85/74.5	4.19/69.9	1.24/16.79	[13]

<sup>a</sup> References used for comparison of chemical shifts.



**Fig. 6** – NMR spectra of the fucose-containing polysaccharides F3\_FA fraction from *Agaricus blazei* Murill mycelial fermentation biomass.

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, and  $\delta$  4.19 were attributed to H1, H2/H4, and H5 of terminal  $\alpha$ -L-fucp residues [13]. The L-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  $\alpha$ -L-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  $\alpha$ -L-fucosyl branches.

### 3.3. Trials of bioactivity

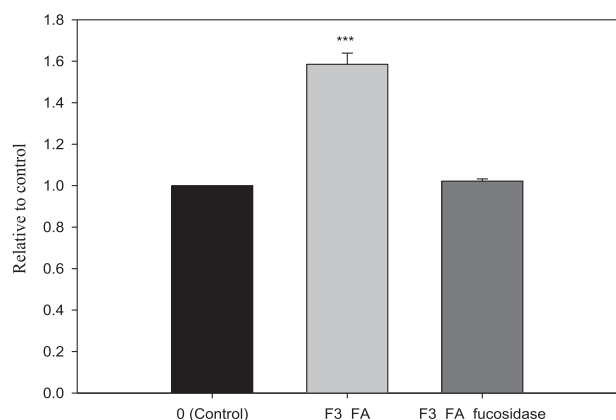
#### 3.3.1. Effect of fucose on bioactivity

The terminal L-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 L-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  $\mu$ g/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 L-fucose features as those found in *G. lucidum* and other *Agaricus* spp, although the backbone sugar composition and linkage were different.

**Table 4** – Carbohydrate epitope recognition test of polysaccharide F3\_FA fractions from submerged fermentation of *Agaricus blazei* Murill against monoclonal antibodies through enzyme-linked immunosorbent assays.

Antibody	LM2	LM5	LM6	LM7
Epitope structure for carbohydrate antigen	$\beta$ -linked glucuronic acid	Linear tetrasaccharide in (1,4)- $\beta$ -D-galactan	Linear pentasaccharide in (1,5)- $\alpha$ -L-arabinan	Partially methylesterified epitope of homogalacturonan
Response	NO	YES	YES	NO
Antibody	LM10	LM19	LM20	JIM7
Epitope structure for carbohydrate antigen	(1,4)- $\beta$ -D-xylan	Linear trisaccharide in (1,4)- $\alpha$ -D-galacturonan	Linear tetrasaccharide in methyl-(1,4)- $\alpha$ -D-galacturonan	Linear hexasaccharide with (1,4)- $\alpha$ -GalA-[MeGalA] <sub>n</sub> -GalA
Response	NO	YES	NO	NO

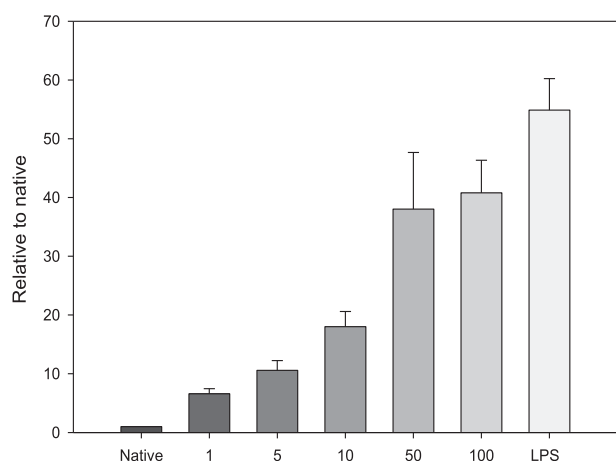




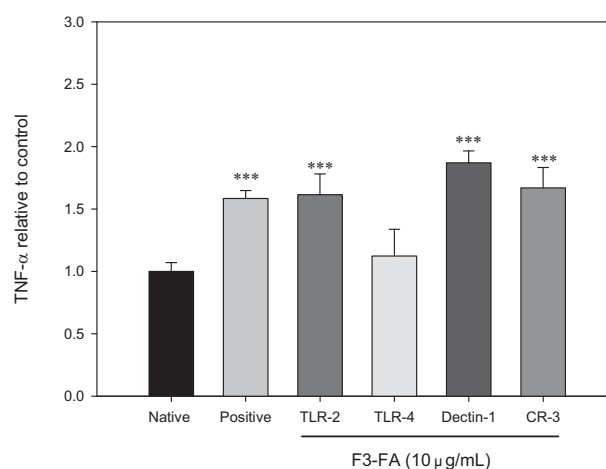
**Fig. 7 – Effect of fucose on stimulating activity of F3\_FA polysaccharide fraction to RAW 264.7 TNF- $\alpha$  expression (The values are presented as the mean  $\pm$  SD,  $n = 3$ ; \*\*\*F as compared to control as analyzed by two sample t-test  $<0.001$ ).**

### 3.3.2. Gene reporter platforms luciferase assays and inhibition of cytokine production using pattern-recognition receptor antibodies

NF- $\kappa$ B activates a number of cytokines and is a key transcription factor involved in F3\_FA-induced TNF  $\alpha$  expression; moreover, TLR 4 binding affects bioactivity. Cyclooxygenase-2 (COX-2) is a transcriptional target of nuclear factor kappa B (NF- $\kappa$ B) 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  $\mu$ g/mL (Fig. 8). Moreover, polysaccharides mediated active macrophages through recognition specific receptors [37–40]. As fucose-containing polysaccharides from *A. blazei* Murill, there mechanism of immune response has rarely been studied. Thus, this study



**Fig. 8 – Dose-dependent manner of the effect of F3\_FA polysaccharide fraction on reporter gene platform COX 2-luciferase-based assay systems (The values are presented as the mean  $\pm$  SD,  $n = 3$ ).**



**Fig. 9 – The effects of pattern-recognition receptors on TNF- $\alpha$  production in RAW 264.7 cells treated with F3\_FA polysaccharide fraction (10  $\mu$ g/mL). RAW 264.7 cells were incubated without polysaccharides as native, incubated with F3\_FA polysaccharide fraction (10  $\mu$ g/mL) alone as positive and incubated with various function-blocking antibodies specific to TLR2, TLR4, Dectin-1, and CR3 in the presence of F3\_FA (10  $\mu$ g/mL). (The values are presented as the mean  $\pm$  SD,  $n = 3$ . \*\*\* $p < 0.001$  as compared to native as analyzed by Dunnett's test.)**

investigated whether these receptors are involved in the F3\_FA-induced production of TNF- $\alpha$ . The results show that treatment with anti-TLR4 mAb (10  $\mu$ g/mL) significantly blocked F3\_FA induced TNF- $\alpha$ . Cells treated with anti-TLR2 mAb (10  $\mu$ g/mL), anti-Dectin-1, and anti-CR3 mAb (10  $\mu$ g/mL) failed to inhibit F3\_FA-induced TNF- $\alpha$  secretion (Fig. 9). Based on above results we suggest that NF- $\kappa$ B is a key transcription factor involved in F3\_FA-induced TNF- $\alpha$  expression and that (F3\_FA)-(TLR4) binding affects F3\_FA-induced TNF- $\alpha$  production.

## 4. Conclusion

Polysaccharides from *Agaricus* spp have drawn considerable attentions as have traditional medicinal mushrooms, e.g. *Ganoderma lucidum*, for their immunomodulating and anti-tumor 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)- $\beta$ -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  $\alpha$ -L-fucose as essential activity key residues, provides a new clue for further study.

In summary, polysaccharides exhibit beneficial immunoactivity [42], anticancer 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 polysaccharides, F3\_FA, derived from submerged fermentation of *A. blazei* Murill. The structure of F3\_FA is similar to that of RGI, with a  $3.5 \times 10^5$  Da. molecular weight. F3\_FA is a group of heteropolysaccharides consisting of (1-4)- $\beta$ -D-galactan, (1-4)- $\alpha$ -D-galacturonan, (1-5)- $\alpha$ -L-arabinan, (1-2)-Xyl, (1-2)-Rha, and terminal  $\alpha$ -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- $\kappa$ B transcription factor and TLR4 affect TNF- $\alpha$  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.

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### Abbreviation list of carbohydrates

Araf: arabinofuranose  
 Arap: arabinopyranose  
 Fucp: fucopyranose  
 Galp: galactopyranose  
 GalpA: galacturonopyranic acid  
 Glcp: glucopyranose  
 Manp: mannopyranose  
 Rhap: rhamnopyranose  
 Xylp: xylopyranose  
 T- or t-: a linkage indicator for terminal sugar residues