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# A galactomannoglucan derived from *Agaricus brasiliensis*: Purification, characterization and macrophage activation via MAPK and IκB/NFκB pathways

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ABSTRACT

In this study, a novel galactomannoglucan named as TJ2 was isolated from *Agaricus brasiliensis* with microwave extraction, macroporous resin, ion exchange resin and high resolution gel chromatography. TJ2 is composed of glucose, mannose and galactose in the ratio 99.2:0.2:0.6. Infrared spectra (IR), methylation analysis and nuclear magnetic resonance spectra indicated that TJ2 mainly contained a β-(1 → 3)–linked glucopyranosyl backbone. Interestingly, TJ2 significantly promoted RAW264.7 cell proliferation, and was able to activate the cells to engulf *E. coli*. In addition, TJ2 induced the expression of Interleukin 1β (IL-1β), Interleukin 6 (IL-6), tumor necrosis factor α (TNF-α) and cyclooxygenase-2 (Cox-2) in the cells. TJ2 also promoted the production of nitric oxide (NO) by inducing the expression of inducible nitric oxide synthase (iNOS). Moreover, TJ2 is a potent inducer in activating the mitogen-activated protein kinase (MAPK) and inhibitor of nuclear factor-kappa B (IκB)/nuclear factor-kappa B (NFκB) pathways.



## 1. Introduction

*Agaricus brasiliensis* is originally native to southern Brazil and produced on an industrial scale in some countries such as China and Japan nowadays (Kuroiwa et al., 2005; Wasser et al., 2005). It is one of the most important edible and medicinal mushrooms in the world (Akiyama et al., 2011). In recent years, it has been demonstrated that the mushroom exhibits various bioactivities, for example, immunoregulation (Goncalves et al., 2011), anti-cancer (Ishii et al., 2011; Kaneno et al., 2004), liver protection (Al-Dbass, Al-Daihan, & Bhat, 2012), anti-diabetes (Oh et al., 2010) and antioxidant activity (Carneiro et al., 2013; Kozarski, Klaus, Niksic, & Jakovljevic, 2011; Stojković et al., 2014). Thus, it shows the great potential of being developed as a novel functional food additive for health care.

*Agaricus brasiliensis* contains a variety of chemical compounds including polysaccharides, proteins, lipids, and sterols (Jia et al., 2013; Kawagishi et al., 1988; Takaku, Kimura, & Okuda, 2001). Among them, polysaccharides are commonly believed to be the main component responsible for the various bioactivities (Liu, Miao, Wen, & Sun, 2009). Studies have shown that *Agaricus brasiliensis* polysaccharides exert certain immunostimulation activities (Cui et al., 2013; Wang, Fu, & Han, 2013). For example, the polysaccharide extracts from *Agaricus brasiliensis* play a crucial role in stimulating immunity and induce the expression of IL-1β, IL-6, TNF-α and IFN-γ in murine immune cells (Huang et al., 2012; Yamanaka et al., 2013). However, the immuneregulatory mechanism of the polysaccharides, especially their structural characteristics key to the bioactivity, is still vague.

In the present study, we isolated and purified a polysaccharide designated as TJ2 from *Agaricus brasiliensis*. Its purity, monosaccharide composition, and structure characters were determined with high performance size-exclusion chromatography, GC–MS, IR and NMR. The effect of TJ2 on proliferation and phagocytosis of RAW264.7 macrophages were assayed to evaluate its immunoregulatory activities. Moreover, the relevant mechanisms, such as cytokine production, NO production and signaling pathway mediating the events, were investigated.

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## 2. Materials and methods

### 2.1. Materials and reagents

*Agaricus brasiliensis* (strain AbML11) was from Jinsheng agricultural technology development company (Tianjin, China). It is identified by Doctor Junbo Xie, and the voucher specimen was deposited in the College of Biotechnology and Food Science, Tianjin University of Commerce. Dextran standards were obtained from Ampolymer (Mentor, USA). Chromatographic pure water was from J. T. Baker (Center Valley, USA). Glucose was purchased from Troody Technology, Ltd. (Shanghai, China). Rhamnose was purchased from the National Institute for the Control of Pharmaceutical and Biological Products. Mannose, arabinose and galactose were obtained from Lanji Technology Development, Co. (Shanghai, China). HPLC grade acetonitrile and methanol were from Merck (Darmstadt, Germany). DMEM culture medium, fetal bovine serum (FBS), Trypsin-EDTA, Trizol reagent, Penicillin-Streptomycin and FITC-E.coli particles were purchased from Gibco/Invitrogen (Eugene, USA). 3-(4,5-dimethylthiazol-2-yl) – 2,5-diphenyltetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) and the Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthyl)-ethylene diamine dihydrochloride in 5% phosphoric acid] were obtained from Sigma (St. Louis, MO, USA). FastStart Universal SYBR Green Master (Rox) was obtained from Roche (Indianapolis, USA) and the High Capacity cDNA Reverse Transcription Kit was from Applied Biosystems Pty, Ltd (Foster City, CA, USA). The protein extraction commercial kit was obtained from Sangon Biotechnology, Ltd (Shanghai, China), and the polyvinylidenedifluoride (PVDF) membrane was from Millipore (Billerica, USA). The ECL™ primer western blotting detection reagent was from Pierce (Rockford, USA). The antibodies to p-ERK, p-IκB, iNOS and β-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA). Cox-1 and Cox-2 antibodies were purchased from Cayman (Ann Arbor, USA). Raw 264.7 cells were obtained from ATCC (Manassas, USA).

### 2.2. Isolation and purification of TJ2

The freeze-dried mushrooms were crushed and mixed with distilled water at a ratio of 1/50 (*W*/*V*, g/mL). The crude polysaccharide was then extracted with the microwave method (640 W, 5 min). Through macroporous resin (AB-8, The Chemical Plant of NanKai University, China) and ion exchange resin (D280, the Chemical Plant of NanKai University, China) columns, the pigment and the proteins were removed from the extracting solution. After dialysis (molecular weight cut-off 10,000 Da), the crude polysaccharide was further separated and purified through a Sephacryl™ S-300 High Resolution gel column (80 cm × 3.0 cm) with ultrapure water as eluent. A main single fraction (TJ2) was obtained and then freeze-dried.

The purity of TJ2 polysaccharide was determined by using the Agilent 1100 Series system (Agilent Technologies, USA) equipped with an Alltech3300 ELSD (Grace Technologies, USA). The sample was assayed using an Agilent PL aquael-OH column (4.6 × 150 mm, 3 μm) with the temperature at 25 °C. Isocratic elution was applied with water (HPLC grade, 100%). The flow rate was 0.8 mL/min, and the injection volume was 20 μL. The temperature of the ELSD drift tube was 75 °C, and the gas flow rate was 1.8 L/min. Each fraction was dissolved in water (1 mg/mL), and then measured under the chromatographic conditions described above.

### 2.3. Characterization of the polysaccharide

The molecular weight of TJ2 polysaccharide was determined with the same HPLC-ELSD method described above. The linear regression was calibrated by using dextrans (DXT 91K-1900K) as standards. A standard curve was prepared based on the retention time (RT) *versus* the log molecular weight.

Monosaccharide composition was determined by using HPLC with precolumn derivatization (Song & Du, 2012). Briefly, the polysaccharide was hydrolyzed with 4 M trifluoroacetic acid (TFA) at 120 °C for 4 h. After being dried with $N_2$, the residue was dissolved in 0.3 M NaOH and derivatized with 0.5 M 1-phenyl-3-methyl-5-pyrazolone (PMP, methanol solution) at 70 °C for 100 min. Then, 0.3 M HCl was sucked to neutralize the solution, and an equal volume of chloroform was added and shaken. After layering, the supernatant was filtered with a 0.45 μm microporous membrane. Then, it was analyzed by HPLC using a SUPEL COSIL $LC_{18}$ column (4.6 × 150 mm, 3 μm) (Agilent Technologies, USA) with a mobile phase consisting of phosphate buffer-acetonitrile (83:17, v/v) at a flow rate of 0.5 mL/min. The UV detection wavelength was 250 nm. The monosaccharide composition of the polysaccharide was identified by comparing retention times with those of PMP-labeled standard monosaccharides (glucose, galactose, rhamnose, arabinose, mannose), and the content of each monosaccharide was calculated using the corresponding peak areas and response factors.

Methylation analysis was performed according to the reported method with some modification (Ciucanu & Kerek, 1984). Briefly, the polysaccharide was O-methylated with NaOH in DMSO –MeI. After being treated with 50% sulfuric acid (1 h, 0 °C), the resulting solution was diluted with $H_2O$ to 5.0% (v/v) and hydrolyzed at 100 °C for 18 h. The solution was neutralized with $BaCO_3$ and filtered. The filtrate was evaporated with $N_2$ to a small volume. The partially O-methylated aldoses were converted to alditol derivatives with $NaBD_4$. The sample was analyzed by Trace DSQ -II GC–MS (Themo, USA), and the TR-5MS column was programmed as 160–210 °C at 5 °C/min, and then 210–260 °C at 8 °C/min.

Infrared spectra (IR) spectrum was performed by using a NEXUS 670 FT-IR (Thermo Nicolet, USA) spectrophotometer in the range of 4000–400 $cm^{-1}$. The $^1H$ NMR and $^{13}C$ NMR spectra were recorded on a Brucker Avance 600 MHz spectrometer (Brucker, Germany). Dimethyl Sulfoxide-D6 (DMSO-D6) was used as the solution.

### 2.4. Cell proliferation assay

The viability of RAW264.7 cells was determined using the colorimetric CellTiter 96 aqueous cell proliferation assay (MTS) according to the instruction provided by the manufacturer (Promega). Briefly, cells ($1 \times 10^4$ cells per well) were seeded in 96 wells plates. One day after seeding, they were treated with or without different concentrations of TJ2 and 1 μg/mL of LPS for 24 h. At the end of incubation, 50 μL CellTiter 96 Aqueous reagent (40% v/v dilution in 1 × PBS) was added to each well. Plates were incubated at 37 °C for 2 h, and absorbance was measured at 490 nm with a 96-well plate reader (model Spectra Max 340; Molecular Devices).

### 2.5. Reverse transcription PCR (RT-PCR) and Real-time quantitative PCR (qPCR)

After the cells were treated with 1 μg/mL LPS or various concentrations of TJ2 for 24 h, the total RNAs were isolated with Trizol reagent (Invitrogen, USA). The Superscript One-Step RT-PCR kit (Invitrogen, USA) was used for RT-PCR. cDNA synthesis was performed with the following condition: 55 °C for 30 min (1 cycle), and 94 °C for 2 min (1 cycle). The condition of PCR amplification is: denaturing at 94 °C (15 s), annealing at 55 °C (30 s), and extension at 72 °C for 1 min (25 cycles), then at 72 °C for 8 min. The PCR primers are designed as the previous reports (Yi et al., 2013). Cox-2 sense: 5′ GCA AAT CCT TGC TGT TCC AAT C 3′, antisense: 5′ GGA