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Production, fractionation, characterization of extracellular polysaccharide from a newly isolated *Trametes gibbosa* and its hypoglycemic activity

Yuping Ma[a], Duobin Mao[b], Lujing Geng[b], Zheng Wang[b], Chunping Xu[b,*]

[a] China Tobacco Henan Industrial Co. Ltd., Zhengzhou, Henan 450016, PR China
[b] College of Food and Biological Engineering, Zhengzhou University of Light Industry, Zhengzhou, Henan 450002, PR China

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ABSTRACT

The submerged fermentation for extracellular polysaccharide (EPS) production from *Trametes gibbosa* was optimized. An optimal medium for EPS production was obtained through central composite design (CCD) as follows: 53.12 g/L maltose and 4.21 g/L polypeptone in distilled water. Furthermore, four groups of EPSs (designated as Fr-I, Fr-II, Fr-III and Fr-IV) were obtained from the culture filtrates by size exclusion chromatography (SEC), and their molecular characteristics were examined by a multiangle laser-light scattering (MALLS) and refractive index (RI) detector system. The weight-average molar mass of Fr-I was determined to be 3.872×10^5 g/mol and its molecular shape was revealed to be a rigid rod in an aqueous solution. Finally, the hypoglycemic effect of the EPS, investigated in streptozotocin induced diabetic mice, decreased plasma glucose, total cholesterol and triacylglycerol concentrations by 17.4%, 14.0% and 12.6%, respectively. The results indicate the potential of this EPS to prevent hyperglycemia in diabetic patients.

1. Introduction

The polysaccharides were a group of high value biopolymers with wide various applications in food, pharmaceutical industries (Poli et al., 2010; Zhong & Tang, 2004). Much interest has been focused on extracellular polysaccharide (EPS) produced by medical fungi due to their biological and pharmacological activities including anti-tumor, antioxidant, hypoglycemic activities, etc. Compared with EPS extraction from cultivating fruiting body of fungus, submerged culture of fungi obviously gave rise to potential advantages of effective EPS production in a compact space and shorter time (Wasser, 2002). Meanwhile, EPS obtained from submerged culture always exhibited the similar biological activity as that of the EPS extraction from fruit body (Kim et al., 2001).

Trametes gibbosa, commonly known as the 'lumpy bracket', is a polypore mushroom that causes a white rot. It grows on beech stumps and the dead wood of other hardwood species. *T. gibbosa* has been reported to possess antitumor activity as a traditional Chinese medicine (Huang, 1998). Ga and Kaviyarasana (2011) investigated the methanolic and water extract of *T. gibbosa* fruit body has antimicrobial and antioxidant activities. Czarnecki and Grzybek (1995) isolated two polysaccharide fractions (registered as TG-1 and TG-2 from the fruit bodies and their antiinflammatory and vasoprotective activities was confirmed. However, to the best of our knowledge, the EPS production by submerged culture of *T. gibbosa*, its molecular characteristics and bioactivity had not been demonstrated.

Though much information on bioactivities of EPS was available, the molecular weight, structure and chain conformation had not been fully investigated due to the small amount of yield and the difficultly in fractionating. Zhang, Zhang, Cheung, and Ooi (2004) reported that the molecular weight was very important to the bioactivity of *Pleurotus* polysaccharide. Hence, data of detailed molecular characterization were critical to elucidate the relationship between physiochemical properties and physiological functions.

Response surface methodology (RSM) was a mathematical/statistical based technique which was useful for analyzing the effects of several independent variables on the response. RSM had been successfully used to produce enzymes, biomass, and metabolites (Kamimura, Medieta, Rodrigues, & Maugeri, 2001; Malinowska, Krzyczkowski, Lapienis, & Herold, 2009). Usually, it applies an experimental design such as central composite design (CCD) to fit a second-order polynomial equation by regression analysis of the experimental data. The equation was used to describe how the test variables affect the response and determine the interrelationship among the variables.

In the present study, a novel *T. gibbosa* was isolated and identified, and submerged culture conditions of *T. gibbosa* were optimized for the production of EPS by RSM. Furthermore, the EPSs fractions

* Corresponding author. Tel.: +86 371 6355 6212; fax: +86 371 6355 6627.
E-mail addresses: c.p.xu@zzuli.edu.cn, xuchunping05@163.com (C. Xu).

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were isolated by gel filtration chromatography and the molecular features were investigated by a SEC/MALLS system. Finally, hypoglycemic effect of *T. gibbosa* EPS was studied in male Kunming mice.

2. Materials and methods

2.1. Isolation and identification of fungus

While searching fungus-pathogenic organisms, a white-rot fungal fruit body was isolated from stumps of beech in our campus. The isolated strain was phylogenetically identified by ITS-5.8S rDNA sequencing analysis. The chromosomal DNA of the strain was isolated from the fresh mycelium using a CTAB method (Reski, Wehe, Hadeler, Marienfeld, & Abel, 1991). The resulting genomic DNA was amplified using 2× Taq MasterMix (Beijing CoWin Biotech Co., Ltd., Beijing City, China) and primers ITS1(5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) on an Applied Biosystems Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA) under the following conditions: 94 °C 5 min (1 cycle); 94 °C 45 s, 55 °C 50 s, 72 °C 60 s (33cycles); 72 °C 5 min (1 cycle). The PCR products were sequenced in both directions by Sangon Biotech Co., Ltd. (Shanghai, China). The obtained nucleotide sequence of the ribosomal sequence was compared with those of GeneBank using the NCBI Blast program, and sequence homology was comparatively analyzed using the Clustal X program (Glass & Donaldson, 1995).

2.2. Microorganism and growth conditions

T. gibbosa was collected in our laboratory and was used throughout this study. Stock cultures were maintained on potato dextrose agar (PDA) slant. Slants were incubated at 26 °C for 8 d and stored at 4 °C, and sub-cultured every one month. The seed culture was grown in a 250 mL flask containing 50 mL of GP medium (0.3% peptone, 3% glucose) at 26 °C on a rotary shaker incubator (150 rpm) for 4 d. Flask culture experiments were performed in 250 mL flasks containing 50 mL media for 10 days after inoculating with 4% (v/v) of the seed culture. *T. gibbosa* was initially grown on PDA medium in a Petri dish, and transferred into the seed medium by punching out 5 mm of the agar plate culture with a self-designed cutter (Park, Kim, Hwang, & Yun, 2001).

2.3. Analytical methods

Samples collected from shake flasks were centrifuged at 9000 × *g* for 15 min, and the resulting supernatant was filtered through a membrane filter (0.45 μm, Millipore). The dry weight of mycelium was measured after repeated washing of the mycelial pellet with distilled water and drying at 70 °C for overnight to a constant weight. The resulting culture filtrate was mixed with four times volume of absolute ethanol, stirred vigorously and kept overnight at 4 °C, thereafter, centrifuged at 10,000 rpm for 15 min to obtain EPS after discarding the supernatant. The precipitate of EPS was lyophilized for further bioactivity analysis, or the precipitate of EPS was redissolved in distilled water and the concentration of the EPS was determined by phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). And each experiment was performed tripartite.

2.4. Purification of EPS

The ethanol precipitates of the crude EPS treated with Sevag reagent (1:4 n-butanol/chloroform, v/v) to remove most of proteins. After removing the proteins and Sevag reagent by centrifugation, the water phase was dialysed against distilled water and lyophilized to yield polysaccharide. The EPS were dissolved in 0.2 M NaCl buffer, and loaded onto a Sepharose CL-6B column (2.4 cm × 100 cm, Sigma Chemical Co., St Louis, MO). The column was eluted with the same buffer at a flow rate of 0.6 mL/min. The eluent was collected by a fraction collector (5 mL eluent/tube). The protein and sugar concentrations of each collector tube were measured. Protein concentration was determined according to Bradford method (Bradford, 1976). The total sugar content in the EPS was determined by phenol-sulfuric acid method using glucose as the standard. The protein moiety in the EPS was monitored by measuring the absorbance at 280 nm, whilst the carbohydrate moiety was monitored at 490 nm (Dubois et al., 1956).

2.5. SEC/MALLS analysis

The molecular weights of the EPS were estimated by SEC coupled with MALLS Dawn DSP detector (Wyatt Technology, Santa Babara, CA) and a refractive index (RI) detector (Optilab rEX, Wyatt Technology, Santa Barbara, CA). The EPS samples were dissolved in a 0.1 M PBS buffer (pH 6.8) containing 0.04% diaminotetraacetic acid–disodium salt (Na_2EDTA) and 0.01% sodium azide and filtered through 0.25 μm filter membranes (Millex HV type, Millipore Co., Bedford, MA) prior to injection into the SEC/MALLS system. The chromatographic system consisted of a degasser (Degasys, DG-1200, uniflow, HPLC Technology, Macclesfield, UK), a SSI 222D pump (Scientific Systems, State College, PA, USA) single-piston isocratic, pulse-dampened HPLC pump (Model 590 Programmable Solvent Delivery Module, Waters Co., Milford, MA), an injection valve (Rheodyne, Inc., Cotati, CA) fitted with a 500 μL loop, and two SEC columns (Shodex OH Pack SB-803 and 805 HQ, JM Science Inc., Buffalo, NY) connected in series. The flow rate was 0.75 mL/min and the injection volume and concentration was 100 μL and 2 mg/mL, respectively. During the calculation of molecular weights of each EPS, the value of d*n*/d*c* (specific refractive index increment) was used from the data in literature (Jumel, Fiebrig, & Stephen, 1996), in which the estimated d*n*/d*c* was 0.14 mL/g. Calculations of molecular weight and root mean square (RMS) radius of gyration for each EPS were performed by the Astra 4.72 software (Wyatt Technology). The RMS radii of each polysaccharide were determined from the slope by extrapolation of the first-order Debye plot (Wyatt, 1993).

2.6. Animal experiments and induction of diabetes

A total 24 5-week-old male Kunming mice was obtained from Henan Experimental Animal Center (Zhengzhou, China) were housed in individual stainless steel cages and acclimatized with free access to food and water for at least 1 week in an air conditioned room (23 ± 2 °C with 55 ± 5% humidity) under a 12:12-h light–dark cycle. The mice were fed with a commercial pellet diet (Henan Experimental Animal Center) throughout the experimental period.

Mouse were adapted for 7 days in the growth room and fasted for 12 h. Diabetes was induced by intramuscular injection of streptozotocin (Sigma Chemical Co., St. Louis, MO) dissolved in 0.1 M sodium citrate buffer (pH 4.5) at a dose of 50 mg/kg body weight (Bolkenta, Yanardağb, Tabakoğlu-Oğuza, & Özsoy-Saçan, 2000; Hwang & Yun, 2010). Two days after injection of diabetogenic agent (streptozotocin), fasting blood glucose was determined before administration of the EPS and the mice with more than blood glucose >300 mg/dL were included in the group of diabetics. All procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" approved by Zhengzhou University of Light Industry.