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A novel process for isolation and purification of the bioactive polysaccharide TLH-3′ from *Tricholoma lobayense*

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ABSTRACT

Our previous studies have revealed that the *Tricholoma lobayense* polysaccharide TLH-3, which has an IC50 value for scavenging superoxide radicals comparable to that of Vitamin C (VC), could be prepared by hot water extraction and column chromatography. However, this method is tedious and inefficient. In this study, high-pressure homogenization (HPH) was used to extract polysaccharides from *T. lobayense* Heim. The yield of 17.3% using HPH was higher than the 12.3% yielded by traditional extraction. Moreover, the operation combined quaternary ammonium salt precipitation with ultrafiltration (QASP-UF) to purify the crude polysaccharides successfully, which provided a higher TLH-3′ yield, a larger separation volume, and a shorter purification time than that of column chromatography. The whole process for TLH-3′ preparation presented here exhibited significant advantages of quicker processing, low consumption, high efficiency, and flexible compatibility with follow-up studies. Based on a characterization of molecular weight, Fourier Transform Infrared Spectroscopy (FT-IR) analysis, and monosaccharide composition analysis, it can be concluded that TLH-3 and TLH-3′ are the same polysaccharide. Antioxidant activity was tested to prove that the TLH-3′ prepared in this study could scavenge DPPH and superoxide radicals, with IC50 values of 111.7 μg/mL and 160.0 μg/mL, respectively. Its outstanding antioxidant ability was comparable to that of ascorbic acid.

1. Introduction

Edible fungi, which are widely distributed in China and Japan [1] and are famous for their abundant nutritive and medicines value [2], have long been cultivated and studied. The fungi have been widely used as folk medicines and healthy foods [3]. Natural polysaccharides found in the edible fungi have attracted increasing attention due to their multiple bioactivities, pharmacological activities, and safety values [4].

Tricholoma lobayense Heim is a nutritious and valuable precious edible fungus under development for its pharmacological potential. Proteoglycans from *T. lobayense* Heim have been proven to exhibit excellent immune regulation and anti-tumor activities [5]. Our previous study of *in vitro* antioxidant activities revealed that TLH-3 had the strongest antioxidant activity amongst the *T. lobayense* polysaccharides, with an IC_{50} value of 126 μg/mL which is comparable to that of ascorbic acid [6]. TLH-3 has shown great potential for applications in various fields such as natural non-toxic anti-oxidants, foods, pharmaceuticals and cosmetics. However, a tedious process is usually needed to ultimately produce the polysaccharide.

Hot water extraction has traditionally been used for the extraction of crude polysaccharides. Further purification of the polysaccharides can be accomplished by column chromatography [7]. Li et al. [8] demonstrated that *Ganoderma capense* polysaccharides could be extracted by boiled water and purified by column chromatography (DEAE Sepharose CL-6B). Hot water extraction at 60 °C has also been used for *Pleurotus cornucopiae* polysaccharide extraction, with subsequent purification by column chromatography (DEAE-52 cellulose) [9]. Our previous study also showed that TLH-3 could be successfully prepared by hot water extraction and column chromatography. However, this method consumes a high level of starting material, involves an relatively long extraction time, and has a low extraction yield [10]. The development of a feasible, efficient, economic and easily handled protocol for separating bioactive TLH-3 is still greatly demanded.

Abbreviations: DPPH, 1,1-diphenl-2-picryldydrazyl; ELSD, evaporative light scattering detector; FT-IR, Fourier transform IR spectroscopy; CTAB, hexadecyl trimethyl ammonium bromide; HPLC, high-performance liquid chromatography; HPH, high-pressure homogenization; MWCO, molecular weight cut off; PMP, 1-phenyl-3-methyl-5-pyrazolone; QASP, quaternary ammonium salt precipitation; TFA, trifluoroacetic acid; UF, ultrafiltration.

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The major objective of this research was to isolate TLH-3 quickly and efficiently. In this study, according to a preliminary study on the characterization of the polysaccharides TLH-1, TLH-2 and TLH-3, new technologies such as the high-pressure homogenization (HPH) method and ultrafiltration (UF) were used to isolate the polysaccharide TLH-3′. Such a protocol not only made the extraction simple and efficient but also retained the bioactivities of the extracted polysaccharides. The polysaccharide TLH-3′ is expected to exhibit excellent activity for a wide range of applications in numerous fields.

2. Materials and methods

2.1. Materials

T. lobayense Heim was obtained from the Panji Shenshan edible fungus cooperative (Huainan, Anhui Province, China).

1,1-Diphenl-2-picryldydrazyl (DPPH), vitamin C (VC), gallic acid and hexadecyl trimethyl ammonium bromide (CTAB) were obtained from China National Medicines Corporation, Ltd. All other chemicals and solvents used in this study were analytical grade.

2.2. Extraction of polysaccharides by HPH and conventional extraction

The HPH [11] equipment was obtained from AH-PILOT (ATS Engineering Inc., Canada). The stalks of *T. lobayense* were dried to a constant weight, then crushed over an 80 mesh sieve. The dried *T. lobayense* powder (100 g) was mixed with 3500 mL of distilled water, and extracted two times by HPH at 80 MPa. Then, the supernatant was evaporated *in vacuo*, concentrated, and precipitated with 4 equivalents of ethanol [12]. The resulted precipitates were collected, dissolved in distilled water, and subjected to the Sevag method to remove protein [13]. After the protein was completely removed, the supernatant was concentrated, dialyzed, and lyophilized. The yield of the *T. lobayense* polysaccharide extract by HPH was found to be 17.3 ± 0.2%. The total saccharide content was 99.2%. The UV spectrum of the extract showed no absorption at 260 and 280 nm for nucleic acid and protein, respectively.

Conventional extraction was carried out according to a previously reported method [14]. The dried *T. lobayense* powder (100 g) was mixed with 3500 mL of water at 83 °C and incubated for 2.5 h. After centrifugation the supernatant was concentrated by evaporation *in vacuo*, precipitated by ethanol, re-dissolved, subjected to protein removal, dialyzed, and freeze-dried to obtain *T. lobayense* polysaccharides. The yield of *T. lobayense* polysaccharides by hot water extraction was found to be 12.3 ± 0.2%. The total saccharide content of the extract was 98.5%. The UV spectrum of the extract showed no absorption at 260 and 280 nm, suggesting no nucleic acid or protein content.

2.3. Isolation and purification of TLH-3 by QASP-UF and column chromatography

The dried polysaccharide extract (100 g) was dissolved in 5000 mL distilled water, to which 5000 mL 10% hexadecyl trimethyl ammonium bromide (CTAB) solution was then added. After incubating the solution overnight, the precipitate was collected and re-dissolved with 10% NaCl. Then, 3 to 4 equivalents of ethanol were added to precipitate polysaccharides from the solution. The residue was lyophilized in a freeze-dryer to yield *T. lobayense* polysaccharides (TLH-S′). Finally, the dried TLH-S′ polysaccharides were dissolved and diluted to 2 mg/mL, which was then used for further purification of TLH-3′ by ultrafiltration membranes with a MWCO of 10 kDa. The resulting solution was concentrated, dialyzed, and lyophilized to obtain TLH-3′ powder.

Ion-change chromatography and gel filtration column chromatography were also used for the purification of the polysaccharide [15]. DEAE ion-exchange was first used to obtain TLH-1 and TLH-S. The column was first eluted with distilled water, followed by elution with a linear concentration gradient of NaCl from 0 to 1 mol/L. Further separation was performed to obtain TLH-2 and TLH-3 from polysaccharide TLH-S by gel Superdex-75 column chromatography (2.6 × 50 cm), eluted with deionized water at a flow rate of 0.5 mL/min.

2.4. Measurement of molecular weights of TLH-3 and TLH-3′

High Performance Liquid Chromatography (HPLC) was used for determining the molecular weight of TLH-3 and TLH-3′ [16]. Standard dextrans 2000, T-10, T-40, T-70 and T-100 were detected by ELSD-HPLC, and retention times were plotted against the logarithms of their respective molecular weights. TLH-3 and TLH-3′ polysaccharides (4 mg each) were separately dissolved in distilled water (2 mL) and centrifuged. Then, each supernatant was subjected to ELSD–HPLC and eluted at a fixed flow rate (1 mL/min) by distilled water. The retention times of the polysaccharides were then plotted on the same graph as the standards, and their molecular weights could be determined.

2.5. Infrared spectroscopic analysis

The IR spectra of the polysaccharides were determined using Fourier Transform IR Spectrophotometry (FT-IR). The purified polysaccharides were ground with KBr powder and then pressed into pellets for FT-IR measurement in the frequency range of 4000–400 cm^{-1}.

2.6. Analysis of monosaccharide composition

The monosaccharide compositions of TLH-3 and TLH-3′ were analyzed by HPLC. Each polysaccharide (10 mg) was dissolved in 5 mL 2 mol/L trifluoroacetic acid (TFA) at 110 °C in a sealed-tube for 8 h. The resultant solution was concentrated using a rotary vacuum evaporator at 50 °C and excess acid was removed until the obtained hydrolysate was neutral. After that the hydrolysate was added 1 mL distilled water, at which point it was considered ready for the following experiments.

PMP derivatization of monosaccharides was carried out according to the method of Lv et al. [17] with proper modifications. Briefly, standard monosaccharides and the hydrolyzed samples were mixed with 50 μL of a methanol solution of 1-phenyl-3-methyl-5-pyrazolone (PMP, 0.5 mol/L) and 50 μL NaOH(0.3 mol/L), and incubated at 70 °C for 30 min. Then, the reaction mixture was neutralized with 50 μL of 0.3 mol/L HCl. The resulting products were analyzed using HPLC coupled with UV detection. The operation conditions for the HPLC were as follows: the analytical column was an Agilent ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, particle size 5 μm). The flow rate, column temperature, and wavelength for UV detection were 1.0 mL/min, 25 °C, and 245 nm, respectively. The mobile phase A was a mixture of phosphate buffer (50 mmol/L) – acetonitrile (85:15, v/v) and the mobile phase B was a mixture of phosphate buffer (50 mmol/L) – acetonitrile (60:40, v/v). The analysis was using a gradient elution of 15–23–15% phase B by a linear from 0–20–35 min.

2.7. Antioxidant activity

2.7.1. Assay of DPPH radical scavenging

DPPH scavenging activity was measured according to the method of Yao et al. [18] with some modifications. TLH-3 and TLH-3′