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Structure and antitumor activities of the water-soluble polysaccharides from *Ganoderma tsugae* mycelium

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Abstract

Six water-soluble polysaccharides coded as GTM1 to GTM6 were extracted sequentially from the mycelium of *Ganoderma tsugae* with 0.2 M sodium phosphate buffer solution at 25, 40, and 80 °C, water at 120 °C, 0.5 M sodium hydroxide at 25 and 65 °C. The chemical structures were determined by using IR, EA, GC and ^{13}C NMR. The weight-average molecular mass (M_w) was characterized by size exclusion chromatography combined with laser light scattering (SEC-LLS). The results indicated that the samples GTM1 and GTM2 were heteropolysaccharide–protein complexes with the protein content of 13.5 and 20.1%, and apparent-mean M_w of 62.8×10^4, 81.8×10^4, respectively. GTM3 and GTM4 contained (1→3)-β-D-glucans and (1→4)-α-D-glucans, while GTM5 and GTM6 were mainly a (1→6)-branched (1→3)-β-D-glucan. Two peaks were found in the SEC patterns of the four samples GTM1 to GTM4, representing fractions 1 composed of branched (1→4)-α-D-glucan with high M_w and fraction 2 composed of galactose, glucose and mannose with relatively low M_w. With the progress of isolation, the content of fraction 2 decreased from 90.2 to 57.1%, accompanying with enhancing antitumor activity. The polysaccharides GTM1, GTM2 and GTM3 had significantly higher antitumor activity against solid tumor Sarcoma 180 with the inhibition ratio beyond 50%. The results suggested that the effects of the moderate content of galactose and bound protein, as well as relatively lower M_w, on the improvement of antitumor activities of polysaccharides could not be negligible.

Keywords: *Ganoderma tsugae* mycelium; Polysaccharide; Water-soluble; Structure; Antitumor activity

1. Introduction

Ganoderma tsugae, one of the famous traditional Chinese medicines, has long been used to promote health and longevity in China and other Asian countries. Recently, this fungus has attracted much attention because its polysaccharides have been demonstrated to have remarkable antitumor activities, which were manifested by enhancing the host mediated mechanisms including increasing IL-2 production and stimulation of cytotoxic T lymphocytes, NK activity and antibody production (Shiao, Lee, Lin, & Wang, 1994; Su, Shiao, & Wang, 1999; Wu, Shi, & Kuo, 2001). However, the wild fruiting body of *G. tsugae* is scarce, and it usually takes several months to cultivate the fruiting body. Therefore, many attempts have been made to obtain bioactive substances, especially polysaccharides, from the mycelium cultured by submerged fermentation, which takes only 2 weeks to complete a cultivation and is easier to control the product quality (Yang, Ke, & Kuo, 2000; Yang & Liau, 1998). It has been reported that three antitumor heteropolysaccharide–protein complexes with the molecular mass between $1.0–1.6\times10^4$ have been isolated from the mycelium of *G. tsugae* (Zhang et al., 1994). Sone, Okuda, Wada, Kishida, and Misaki (1985) have obtained a branched (1→3)-β-D-glucan with high antitumor activity from the culture filtrate of

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G. lucidum mycelium. In our previous work, a β-D-glucan-protein complex with molecular mass of 64.6×10^4 was extracted from the *G. lucidum* mycelium (Chen, Zhang, Yu, & Zhu, 2000) Interestingly, a water-insoluble (1→3)-α-D-glucan isolated from the fruiting body of *G. lucidum* with alkali solution did not have antitumor activity, but its sulfated derivative showed antitumor activity (Zhang, Zhang, Zhou, Chen, & Zeng, 2000) It is noted that the correlation of structure of such fungal polysaccharides to antitumor activity is not fully understood. In particular, few data have been published on the secondary structure such as molecular mass, conformation and solution properties, which have been proved to have a significant effect on the anti-tumor activities (Calazans, Lima, Franca, & Lopes, 2000; Yoshioka, Uehara, & Saito, 1992) In this work, we attempted to investigate systematically the chemical structure, solution properties and antitumor activities of six water-soluble polysaccharides isolated from the *G. tsugae* mycelium.

2. Materials and methods

2.1. Materials

The strain of *G. tsugae* was supplied by the Laboratory of Applied Mycology in Central China Agriculture University. Standard monosaccharides were Sigma products and used without further purification. All other reagents were of analytical grade made in China.

2.2. Isolation and purification of polysaccharides

The mycelium of *G. tsugae* was obtained through submerged cultivation, which was as described elsewhere (Peng, Zhang, Zeng, & Xu, 2003). The *G. tsugae* mycelium was defatted using Soxhlet extraction with ethyl acetate and acetone for 8 h, respectively. The resulting mycelia were powdered and then immersed in 0.2 M sodium phosphate buffer (pH 7.0) at 25 °C overnight before being centrifuged to give the supernatant. The supernatant was subjected to the Sevag (Staub, 1965) method to remove free proteins, and 30% H_2O_2 to decolorize, then dialyzed using regenerated cellulose tube (M_w cut-off 8000, Union Carbide USA) against tap water for 5 days and distilled water for 3 days, finally concentrated by rotary evaporation at reduced pressure below 45 °C and lyophilized to give the colorless sample GTM1. Subsequent isolation of polysaccharides with phosphate buffer at 40 and 80 °C, distilled water at 120 °C as well as 0.5 M sodium hydroxide aqueous solution at 25 and 65 °C were carried out as shown in Scheme 1. Six water-soluble polysaccharides obtained were coded as GTM1, GTM2, GTM3, GTM4, GTM5 and GTM6.

GTM4 (25 mg) was further purified by dissolution in 0.2 M sodium chloride and application to a column (50× 2.0 cm) of DEAE-Sepharose CL-6B. After loading with sample the column was washed with 0.2 M sodium chloride at an elution rate of 42 mL/h. A differential refractive index detector (RI-150) was used on line. Two peaks were found in the elution pattern. Fractions corresponding to the first peak was collected, dialyzed, concentrated and lyophilized to give a sample of GTM4-F1 (5.0 mg).

2.3. Characterization

^{13}C NMR spectra were recorded on an INOVA-600 spectrometer (Varian Inc., USA) at 150 MHz in D_2O for GTM3, GTM4 at 20 °C and for GTM4-F1 at 70 °C, in DMSO-d6 for GTM5 and GTM6 at 60 °C, using acetone (δ 31.45) as an internal reference. The parameters used were as following: the number of scans was 6080; spectra width was 37700 Hz and line broadening 4.0 Hz. The N element content was measured with an elemental analyzer (Heraeus Co., Germany). Infrared spectroscopy of the samples was recorded on Nicolet 170SX FTIR in the range of 400–4000 cm^{-1} with DGTS detector and DMNIC 3.2 software. Gas chromatography (GC) of the alditol acetates derivatives of the saccharides according to the literature (Englyst, Quigley, & Hudson, 1994) was carried out on a HP 6890 instrument (Hewlett Packard, USA) with an DB-225 column (15 m×0.25 mm) at temperatures programmed from 180 to 220 °C at 4 °C/min.

2.4. SEC-LLS measurements

Size exclusion chromatography combined with laser light scattering (SEC-LLS) measurements were performed on a multi-angle laser photometer ($\lambda=633$ nm, DAWN-DSP, Wyatt Technology Co., USA) combined with a P100 pump equipped with TSK-GEL G6000PWXL and G4000PWXL column (7.8 mm×300 mm) and differential refractive index detector (RI-150) at 25 °C. The eluent was 0.2 M sodium chloride at a flow rate of 1.0 mL/min. All the solutions used were first filtered with a sand filter and then with a 0.20 μm filter (Whattman, UK). Astra software was utilized for data acquisition and analysis. The refractive index increments (dn/dc) were determined by using an Optilab refractometer (OPTILAB-DSP, Wyatt Technology Co., USA) at 25 °C. The values of dn/dc at 633 nm obtained were 0.149 for GTM1, 0.145 for GTM2, and 0.141 mL/g for GTM3, GTM4, GTM4-F1, GTM5 and GTM6, respectively.

2.5. Viscometry

Viscosities of polysaccharides in 0.2 M NaCl sodium chloride were determined at 25 °C using an Ubbelohde viscometer. The kinetic energy correction was always negligible. Huggins and Kraemer plots were used to estimate the intrinsic viscosity [η].