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Structure, chain conformation and antitumor activity of a novel polysaccharide from *Lentinus edodes*

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ABSTRACT

A water-soluble polysaccharide LT1 was isolated from the basidiocarps of *Lentinus edodes* by hot water extraction and ethanol precipitations, further purified by gel chromatography. The Mw of LT1 was estimated to be 642 kDa by using HPGPC. Chemical and spectroscopic studies illustrated that LT1 has a backbone chain composed of 1→4-linked and 1→3-linked glucopyranosyl residues and has branches of single glucosyl stubs at C-6 of β-(1→4)-linked glucopyranosyl. AFM and Congo-red test revealed that LT1 existed as triple helix chain in 0.10 M NaOH solution or distilled water. Our studies showed that LT1 presented significant antitumor bioactivities on Sarcoma180 solid tumor cell implanted in BALB/c mice, which implies that LT1 could be potentially applied as a natural antitumor drug.

1. Introduction

Mushrooms are known for their nutritional and medicinal value because of various bioactive compounds they contain. *Lentinus edodes*, a valuable edible fungi, has been widely cultivated in China and other East Asian countries for centuries, and its importance was attributed to both its nutritional value and medical application [1]. Polysaccharides, vitamin, ergosterol and other important components were isolated from its basidiocarp, mycelium and culture medium. Recently, polysaccharides have attracted much attention from scholars as an important class of bioactive natural products. A wide range of polysaccharides have been found to possess a variety of bioactivities, such as antitumor activity [2–4], free radical scavenging activity [5–7], heparinoid activity [8] and immunomodulation [9]. There are many reports that focus on the isolation and structural analysis of several polysaccharides and polysaccharide–protein complexes extracted from *L. edodes*, some of which were particularly interesting because of their significant antitumor and immunoregulating activities [10–13]. For instance, Lentinan (Mw = 500 kDa), a biologically active polysaccharides extracted from *L. edodes* containing a β-(1→3)-linked glucosyl backbone with β-(1→6)-branched glucosyl side chains [13], has been used for adjuvent therapy to reduce the risk of cancer recurrence for years.

Most studies have shown that the bioactivities of polysaccharides are most closely related to their chemical composition, configuration and molecular weight, as well as their physical properties. Suarez et al. reported that the polysaccharides from *Chlorella pyrenoidosa* having higher molecular weight exhibited immunostimulatory effect, but the fractions of lower molecular weight did not [14]. However, further studies indicated that the chain conformation of polysaccharides was also important for interpretation of their bioactivities. Subsequent investigation revealed that the triple helix of medicinal mushroom β-(1→3) glucans is important for their immune-stimulating activity. When Lentinan was denatured with urea, DMSO, or NaOH, tertiary structure was lost as primary structure was retained, but antitumor properties were lowered with denaturation [15]. The same results, which confirmed the correlation between antitumor activity and triple helix structure, were obtained upon

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investigation of several other polysaccharides from *L. edodes* [16]. Ohono et al also found that the immuno-pharmacological activities of Schizophyllan in mice were conformation dependent [17]. Moreover, there are several polysaccharides from mushroom that have been used for the treatment of certain cancers that adopt triple-helical conformation in solution, including Schizophyllan, Scleroglucan, Lentinan, Curdlan, Cinerean and β-D-(1→3) xylan [18–22].

From what mentioned above, we can concluded that the physical properties, chemical structure and chain conformation are essential for the successful application of polysaccharides in the use of drug and food. In this study, a water-soluble polysaccharide was isolated from the basidiocarps of *L. edodes*, which has not been reported previously. We named it LT1. The present work is concerned with the isolation, structure characterization and evaluation of the antitumor activity to the S-180 cancer cells in vivo of LT1.

2. Experimental

2.1. Materials

L. edodes was collected from Fang County, Hubei Province, China. The material was identified at the Department of Traditional Chinese Medicine, Tongji Medical College at Huazhong University of Science and Technology.

Lentinan was produced by Nanjing Kanghai Pharmaceutical Company (State drug approval document number was H10950078). Sephadex G-200 was a Pharmacia Biotech product. DEAE cellulose, standard monosaccharides and a series of standard Dextrans of known molecular weight were purchased from Sigma-Aldrich Chemical Company. Other used reagents were of analytical grade.

2.2. Extraction, isolation and purification

The dried fruiting bodies of *L. edodes* were chopped to slices and reflux extracted with ethanol for 12 h to remove lipids. After filtration, the residue was air dried and extracted three times for 3 h each with boiling water. The combined extracts were concentrated in a rotary evaporator under reduced pressure at 60°C. Then the supernatant was subjected to Sevag method for four times to remove proteins, and treated with 30% H_2O_2 at 60°C for 6 h to decolorize the solution. 95% (v/v) alcohol was added to the resulting solution slowly, with stirring, until the concentration of the alcohol reached 75%. The mixture was kept overnight and centrifuged at 6000 rpm for 10 min to separate the supernatant and residue. The residue was dissolved in water and lyophilized.

The crude polysaccharides, termed LTP, were redissolved in ultra-pure water, centrifuged, and the supernatant (10 ml of 10 mg/ml) was loaded onto a DEAE-cellulose column (5×50 cm). After loading with sample, the column was eluted with 70 ml of 0.50 M NaCl solution at a flow rate of 2 ml/min. Each fraction with 2 ml of elute was collected. Based on phenol–sulfuric acid method, the main carbohydrate containing fraction was collected, dialyzed and lyophilized. The fraction was further purified using Sephadex G-200 column (5×50 cm), eluted with 0.05 M $NaHCO_3$ solution at a flow rate of 2 ml/min. The fraction forming the major peak was collected, dialyzed and lyophilized to obtain a white purified *L. edodes* polysaccharide (LT1).

2.3. Determination of molecular weight

The molecular weight of LT1 was determined by HPGPC on an Agilent-LC 1100 instrument (Agilent, USA), equipped with a TSK gel4000 PWXL column and eluted with 0.05 M Na_2SO_4 solution at a flow rate of 0.8 ml/min. Elution was monitored by an Agilent refractive index detector.

A series of solutions made from standard Dextrans were run under the same conditions and a standard curve linear over a wide range (10 kDa–1000 kDa) was obtained by plotting the elution volume versus the logarithm of the corresponding molecular weight.

2.4. Chemical structure of LT1

2.4.1. Infrared spectra

Infrared analysis of the sample was obtained by grinding a mixture of polysaccharide with dry KBr and then pressing in a mold. IR spectra were recorded on a Bruker-VERTEX 70 Fourier transform infrared spectrophotometer in a range of 4000–400 cm^{-1}.

2.4.2. Monosaccharide analysis

The LT1 was hydrolyzed with 2.0 M H_2SO_4 at 100°C for 4 h in a sealed glass tube. After neutralization with $BaCO_3$, the supernatant was collected and lyophilized. Then the hydrolyzates were converted into their corresponding acetylated aldononitriles and analyzed by GC using an Agilent 7890A instrument equipped with a HP-5 fused-silica capillary column (30 m×0.32 mm×0.25 um) and an Agilent 5975C MS detector. The temperature program was set as follows: the initial temperature of column was 50°C, increased to 75°C at 3°C/min, then to 300°C at 10°C/min, held for 20 min at 300°C. Sugar identification was done by comparison with reference sugars. The relative molar proportions were calculated by the area normalization method. As references, the following neutral sugars were converted to their acetylated derivatives and analyzed: D-Glc, D-Gal, D-Ara, L-Rha, D-Man, L-Fuc and D-xyl.

2.4.3. Periodate oxidation and Smith degradation

The 20.0 mg of LT1 was dissolved in 15 mM $NaIO_4$ solution and kept in the dark at 4°C, monitored with the absorbance at 223 nm every 3 h. The reaction was complete when absorbency did not decrease, then the ethylene glycol was added to decompose the excess $NaIO_4$. Consumption of $NaIO_4$ was measured by a spectrophotometric method and formic acid production was determined by titration with 0.01 M NaOH standard solution.

After dialyzing in distilled water (48 h), the periodate-oxidized polysaccharide was reduced with $NaBH_4$. The resulting solution, adjusted to pH 5.0 with 50% acetic acid, was dialyzed again and retentate was lyophilized. The product was then hydrolyzed with 1.0 M H_2SO_4 (60°C, 40 h). The PH was adjusted to 6.0 by $BaCO_3$, then the solution was filtrated by filter paper. The filtrate was lyophilized, acetylated and analyzed by GC–MS.

2.4.4. Methylation analysis

Polysaccharide (20 mg) was methylated with Ciucanu and Kerek method [23]. Complete methylation was confirmed by the disappearance of the –OH band (3200–3700 cm^{-1}) in the IR