Structure and bioactivities of a polysaccharide isolated from *Ganoderma lucidum* in submerged fermentation

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ABSTRACT

In this study, a *Ganoderma lucidum* polysaccharide GLP-1–1 was isolated from a culture broth with Mw of 22014 Da. Monosaccharide contained glucose, mannose, and galactose with mole percentages of 92.33%, 7.55%, and 0.22%, respectively. Moreover, FTIR and methylation analysis were conducted to characterize the structural properties of GLP-1–1. The results of antioxidant activity analysis showed that GLP-1–1 had a great DPPH and ABTS radical scavenging activity. Meanwhile, GLP-1–1 also exhibited anti-tumor activity to A431 and MDA-MB-231 cells, and inhibitory rates were dose-dependent. During culturing with GLP-1–1, the G1/G0 cell percentage of A431 cells was increased from 48.64% to 84.52%, and the G1/G0 cell percentage of MDA-MB-231 cells was increased from 57.14% to 73.48%. Therefore, the anti-tumor activity of GLP-1–1 may be caused by inducing the G1/G0 arrest of tumor cells.

INTRODUCTION

*Ganoderma lucidum* is a valuable medicinal mushroom and has been used in China and other Asian countries for over 2000 y for improving health and longevity. The extracts from the sporophore and mycelium of *G. lucidum* contain polysaccharides, proteoglycans, triterpenoids, and other active compounds, which have been used to treat many diseases. Among these active compounds, *G. lucidum* polysaccharide is one of the most studied biologic compounds and has several bioactivities, including antioxidant, anti-tumor, and immunomodulatory activities.

Recent researchers focused on the extraction of *G. lucidum* polysaccharide from sporophores by various methods, and extraction conditions were optimized to enhance the polysaccharide production. The extracted polysaccharide was further used to detect the bioactivities. However, the polysaccharide and other biologic compounds in *G. lucidum* sporophores were easily influenced by cultivation conditions and their place of origin. Submerged fermentation was commonly used to produce bioactive products in industry. Recently, Peng et al. reported the monosaccharide composition of *G. lucidum* polysaccharide was influenced by culture conditions and carbon sources. Therefore, the development of fermentation technology and further understanding of polysaccharide synthesis process may be helpful for the mass production of biologic *G. lucidum* polysaccharide in submerged fermentation.

In this study, *G. lucidum* obtained by submerged fermentation were purified, and the structural properties of isolated polysaccharide were investigated. Moreover, the antioxidant and anti-tumor activities of the isolated polysaccharide were also studied. Meanwhile, the possible reason behind polysaccharide anti-tumor activity was analyzed by detecting the cell cycle of used tumor cells.

RESULTS AND DISCUSSION

**Isolation and purification of *G. lucidum* EPS**

The obtained crude *G. lucidum* EPS was purified by a DEAE Fast Flow column, and eluted by distilled water and various concentrations of NaCl solutions. The EPS (GLP-1) eluted by distilled water was the dominant polysaccharide, after comparing with other EPS eluted by different concentrations of NaCl (Fig. 1A). GLP-1 was further purified by a Sephadex G-75 gel fil-
Molecular weight and monosaccharide composition of G. lucidum polysaccharide

Dextrans and GLP-1–1 were determined by HPGFC, and a sharp peak of GLP-1–1 was shown in Fig. 2, indicating a homogenous polysaccharide component after purification. Various dextrans with different molecular weights were used as standard to calculate the equation. According to the equation, the molecular weight of GLP-1–1 was calculated by the retention time and was 22014 Da (Fig. 2). The monosaccharide composition of GLP-1–1 was analyzed by gas-liquid chromatography. The results showed that the Glc was the main monosaccharide in GLP-1–1, and mole percentage of Glc exceeded 90% (Table 1). Peng et al.9,10 reported that carbon sources and culture conditions in submerged fermentation, all influenced the mole percentage of monosaccharides in G. lucidum EPS. In this study, the carbon source for the G. lucidum culture was glucose, and thus the dominant monosaccharide of the main polysaccharide, GLP-1–1, was Glc, which exceeded 90%.

FTIR and methylation analysis

The organic groups in GLP-1–1 were analyzed by FTIR spectroscopy, and the results are shown in Fig. 3. In a previous study, Ross et al.14 summarized the functional groups and absorptions of polysaccharides from several other studies. The spectra of GLP-1–1, shown in Fig. 3, indicate the possible functional groups. The absorption peak of around 3401 cm⁻¹ can be attributed to -OH stretching in sugar residues.15 Peaks at 2863 cm⁻¹ may represent the stretching vibration of C-H. The absor- bances at around 1653 cm⁻¹ and 1385 cm⁻¹, were
associated with the carboxyl group, and a stretching vibration of $C = O$. GLP-1–1 also showed the following absorption peaks: 1313 cm$^{-1}$ could be attributed to carboxylic acid (C-O stretch); 1149 cm$^{-1}$ corresponded to C-O-C and the -OH bending vibration in the pyran structure; 1047 cm$^{-1}$ represented the bending vibration of hydroxyl groups; 903 cm$^{-1}$ and 869 cm$^{-1}$ may be associated with the $\alpha$ or $\beta$ configurations of glycosidic linkages.

Different methylated sugars of *G. lucidum* EPS were identified by retention times and mass spectra in GC-MS, as shown in Table 2. The result showed that the detected methylated sugars contained mannose and glucose, and no galactose was detected in GC-MS. Mannose had only one linkage pattern of Man (1- and was the terminal sugar in GLP-1–1. The most abundant sugar in GLP-1–1 was glucose and had 6 different linkages. The result indicated that glucose was the backbone and branching residue sugar in GLP-1–1.

Antioxidant activity

As the biologic macromolecules, polysaccharides from different resources had several activities, and antioxidant activity of polysaccharides was always detected. In this study, DPPH and ABTS radical scavenging activity and reducing power analysis were investigated to evaluate *G. lucidum* EPS. In radical scavenging activity assay, the activity of GLP-1–1 all achieved the highest activity of 89.02% (DPPH) and 69.53% (ABTS), and maintained a steady level while increasing to 2000 μg/mL (Fig. 4A and B). Wu et al. isolated 3 polysaccharides from purple sweet potato, and all 3 polysaccharides exhibited different DPPH and ABTS scavenging activity. As the dominant polysaccharide, GLP-1–1 had a great antioxidant activity, and was the main active component in *G. lucidum* polysaccharide.

Table 1. Monosaccharide composition of different fractions of *G. lucidum* EPS.

| Fraction | Glc | Man | Gal |
|----------|-----|-----|-----|
| GLP-1–1  | 92.23 | 7.55 | 0.22 |

Table 2. Methylation analysis of GLP-1–1.

| Peak No. | Methylated sugar | Linkage type | Relative mole ratio |
|----------|------------------|--------------|---------------------|
| 1        | 2,3,4,6-Me4-Man   | Man (1-      | 14                  |
| 2        | 2,3,4,6-Me4-Glc   | Glc(1-      | 4                   |
| 3        | 3,4,6-Me4-Glc     | −2|Glc(1-     | 8                   |
| 4        | 2,3,6-Me3-Glc     | −4|Gl(1-      | 28                  |
| 5        | 2,3,4-Me3-Glc     | −6|Glc(1-      | 8                   |
| 6        | 2,3-Me2-Glc      | −4,6|Glc(1-   | 2                   |
| 7        | 3,4-Me2-Glc      | −2,6|Glc(1-   | 2                   |

Figure 3. FTIR spectra of GLP-1–1.

Figure 4. Antioxidant activity analysis of GLP-1–1 and vitamin C was used as the positive control. (A) Scavenging DPPH radical activity; (B) Scavenging ABTS radical activity. All data in figure were presented as mean ± SD, n = 3.


**Anti-tumor activity**

Anti-tumor activity of *G. lucidum* polysaccharide against several tumor cell lines *in vivo* and *in vitro* was reported by many studies.\(^2,5,13\) In this study, the anti-tumor activity analysis of *G. lucidum* EPS was performed by detecting the inhibitory rate of A431 and MDA-MB-231 cells (Fig. 5). The anti-tumor activity of GLP-1–1 against A431 and MDA-MB-231 cells was dose-dependent, and the inhibitory rate increased in a concentration range of 100 – 500 μg/mL. The highest inhibitory rates of GLP-1–1 to A431 and MDA-MB-231 were 60.38% and 48.34%, respectively. This result indicated GLP-1–1 was the main active component of *G. lucidum* polysaccharides. Similarly, anti-tumor activity against 3 hepatocarcinoma cell lines (HepG2, BEL-7402, Huh-7) of the *G. lucidum* polysaccharide was reported.\(^4\) Moreover, the *G. lucidum* polysaccharides also exhibited great anti-tumor activity in the gastric rat model.\(^2\) Combined with the results of this study, *G. lucidum* polysaccharides showed the potential to develop as an effective drug for cancer treatment.

**Cell cycle analysis of A431 and MDA-MB-231 cells**

*In vivo* experiments, *G. lucidum* polysaccharides suppressed the growth and induced the apoptosis of tumor cells, by enhancing an immune response.\(^2\) Moreover, T and B lymphocyte proliferation was also enhanced by *G. lucidum* polysaccharides.\(^7\) Zhang et al.\(^13\) reported that polysaccharides only exhibited anti-tumor activity as the existence of functional p53 protein in various human carcinoma cell lines. In this study, the percentage of cells in the G1/G0 phase was increased after incubating with 2000 μg/mL GLP-1–1 for 48 h (Fig. 6). Comparing with control, G1/G0 cell percentage of A431 raised approximately 73.77%, and 28.6% for MDA-MB-231. This result suggested that the inhibited growth of tumor cells may be caused by the arrest of the G1/G0 phase of A431 and MDA-MB-231 cells. Similarly, the G1 phase arrest of HepG2 induced by isolated *G. lucidum* polysaccharide was also reported, and caused the apoptosis of HepG2 cells.\(^4\) Moreover, Wang et al.\(^19\) regarded the cell cycle arrest as an extrinsic apoptotic pathway in the *Boschniakia rossica* polysaccharide study. Therefore, anti-tumor activity of GLP-1–1 in this study may be achieved by inducing a G1/G0 phase arrest.

**Materials and methods**

**G. lucidum culture and EPS preparation**

The fungal mycelium of *G. lucidum* was preserved by our laboratory and maintained in potato dextrose agar (PDA) at 4°C. The seed and fermentation medium of *G. lucidum* were composed of: 20 g/L glucose, 5 g/L peptone, 10 g/L wheat bran, 3 g/L KH₂PO₄, and 2 g/L MgSO₄·7H₂O (pH 6.6). In submerged fermentation, mycelia squares from PDA slants were inoculated to the seed medium and cultured at 30°C and 150 rpm for 2 weeks. The seed culture medium containing *G. lucidum* mycelia was inoculated to 150 mL fermentation medium and then cultured at 30°C and 150 rpm for 7 d.

The culture broth from the *G. lucidum* fermentation medium was obtained by centrifuging to remove...
mycelia, and crude EPS was collected by precipitating with 3 volumes of 95% (v/v) ethanol overnight. The obtained crude EPS was dissolved in distilled water, and insoluble compound in solution was removed by centrifuging. Crude EPS in solution was concentrated by lyophilization for further research.

**Purification of crude *G. lucidum* EPS**

The deproteinated EPS was purified by DEAE fast flow column, and eluted by distilled water and 0.5 mol/L NaCl solution. Different fractions were collected with 5 ml per tube, and the major fractions were desalted. Then, different fractions were concentrated by lyophilization for further purification in Sephadex G75 column. After loading EPS, different fractions were eluted by distilled water, and collected fractions were lyophilized for further research. All fractions collected during purification were detected by phenol-sulfuric acid method at 490 nm.

**Molecular weight determination**

The molecular weight of *G. lucidum* polysaccharide was determined by high-performance gel filtration chromatography (HPGFC) (Waters 600) with Ultrahydrogel™ Linear (7.8 × 300 mm). The column was eluted by 0.1 M NaNO₃ at a flow rate of 0.9 mL/min. Various dextrans with different molecular weights were used as standards, and the molecular weight of GLP-1–1 was calculated according to the equation.

**Monosaccharide composition**

Twenty mg crude *G. lucidum* EPS was hydrolyzed by 2 mL of 1 mol/L H₂SO₄ at 100°C for 2 h and then neutralized with BaCO₃. The obtained monosaccharide was concentrated by lyophilization and reacted with 20 mg hydroxylamine hydrochloride, 0.5 mL pyridine, and 0.5 mL acetic anhydride. The type and content of monosaccharide was detected by gas-liquid chromatography and inositol was used as internal standard.

**Fourier transformation infrared (FT-IR) spectroscopy analysis**

Crude *G. lucidum* EPS was grounded with KBr and pressed into a pellet. The obtained pellets were used for FTIR analysis (Nicolet Nexus 470), and FTIR spectra of GLP-1–1 were shown in the range of 400 to 4000 cm⁻¹.

**Methylation**

*G. lucidum* polysaccharide in this study was methylated according to Ciucanu method reported by Needs and Swlvendran previously. The methylated sample were further hydrolyzed, reduced, and peracetylated, and then analyzed by Shimadzu GCMSQP2010 system equipped with a capillary column as described by Wang et al.

**Antioxidant activity analysis in vitro**

The DPPH and ABTS radical scavenging activity was performed according to the method reported previously. Vitamin C was used as the positive control in this experiment.
**Anti-tumor activity in vitro**

Two human tumor cell lines, epidermoid carcinoma A431 and breast carcinoma MDA-MB-231, were used to analyze the anti-tumor activity of *G. lucidum* EPS. Tumor cells were cultured by Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (Gibco), 100 μg/mL streptomycin, and 100 U/mL penicillin. Cells (1 × 10⁴ cells/well) were seeded in a 96-well microplate and cultured at 37°C, in 5% CO₂, for 24 h. After by medium with EPS for 48 h, MTT method was performed to detect the inhibitory rate of tumor cells, comparing with cells cultured without EPS.

**Cell cycle analysis**

A431 and MDA-MB-231 cells were cultured with 2000 μg/mL GLP-1–1 for 48h in a 6-well microplate, and culture conditions were the same as described above. Two tumor cells incubating without EPS were used as controls. Cells were collected and fixed with 70% ethanol in PBS. Then, cells were suspended by 0.5 mL PBS containing propidium iodide (50 mg/mL) and DNase-free RNase (100 mg/mL). The treated cells were analyzed by FACSCalibur (Becton Dickinson), and percentages of cells in different phases were calculated by obtained data.

**Statistical analysis**

Data were expressed as mean ± SD, and analyzed using the software program GraphPad Prism 5.0. Differences with *p* < 0.05 were considered significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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