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Structure and bioactivities of a galactose rich extracellular polysaccharide from submergedly cultured *Ganoderma lucidum*

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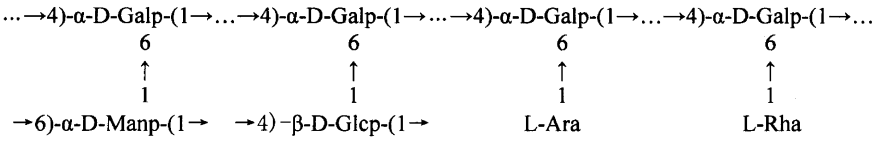
Abstract

A galactose rich extracellular polysaccharide (GLP-2), which is composed of galactose, mannose, glucose, arabinose and rhamnose in the molar ratios 103:17:12:10:3, was isolated from the submerged culture broth of a basidiomycete *Ganoderma lucidum*. Its structure was evaluated with chemical techniques, NMR and IR spectroscopy and ESI-MS experiments and the following structure was suggested:



The bioactivity tests showed that GLP-2 enhanced the T and B lymphocyte proliferation at polysaccharide concentrations of 10 and 50 μg/mL and exhibited lymphocyte activity at concentrations of 100 and 200 μg/mL *in vitro*. The test in mice also showed that GLP-2 significantly enhanced the T and B lymphocyte proliferation and antibody production and increased the mass of spleen tissue at a dose of 25 or 75 mg/kg, but did not show the same effect at a high dose of 150 or 300 mg/kg. However, GLP-2 did not significantly influence the serum IgG and C-3 levels in mice. GLP-2 also showed a hepatoprotective activity in liver injury mice induced by Bacille Calmette-Guérin and lipopolysaccharide.

Keywords: *Ganoderma lucidum*; Polysaccharide; Structure; Immunoregulation; Hepatoprotection

1. Introduction

Ganoderma lucidum (Fr.) Krast (Polyporaceae), a basidiomycete, is a traditional Chinese medicine which is usually used for strengthening the bodies resistance to diseases. In east-Asian countries, *G. lucidum* is also used as health food, especially the submergedly cultured mycelia and the cultured broth. Recent literature showed that *G. lucidum* immune regulating (Zhang, Tang, Martin, Werner, & Fan, 2002), anti-tumor (Wang et al., 1997), anti-virol (Sahar et al., 1998; Kim, Eo, Oh, Lee, & Han, 2000) and hepatoprotective activities (Back, Jung, Kim, & Cho, 1999). It has been reported that *G. lucidum* contains polysaccharides and protein bound polysaccharides. Literature showed that the polysaccharides were the dominant biologically active substances in *G. lucidum*. The biological activities of these polysaccharides also included immune

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regulation, antitumor and other activities (Chen et al., 2004; Miyazaki & Nishijima, 1981). Most reported polysaccharides from *G. lucidum* are dominantly composed of β- or α-D-glucose residues in the main chain, sometimes accompanied with galactose, mannose, rhamnose, arabinose and fucose residues in side chain (Bao, Liu, Fang, & Li, 2001; Bao, Wang, Dong, Fang, & Li, 2002; Wang et al., 2002; Berovič et al., 2003). These studies describe polysaccharides from fruiting bodies or spores. There are only a few reports on the biologically active polysaccharides from submergedly cultured broth (Berovič et al., 2003). The immunoregulation activities of polysaccharides include immunostimulation and immunosuppression. However, their immunostimulation activities have attracted more attention. In viral hepatitis, sensitive response of T lymphocytes is one of the causes of induced liver cell injury (Vento, 1986). Therefore, immunosuppressants are used to treat viral hepatitis in medical practices.

In this study, an extracellular polysaccharide, which mainly contained galactosyl residues, was isolated from submerged culture broth of *G. lucidum*. And the immunoregulation activities and hepatoprotective activity of this polysaccharide were investigated.

2. Materials and methods

2.1. Materials

The strain of *G. lucidum* CCGMC 5.616 was purchased from China General Microbiological Fermentation Collection Center (Beijing, China).

Concanavalin A (ConA) and lipopolysaccharide (LPS) were obtained from Sigma and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Fluka. Medium RPMI 1640 was purchased from Gibco Laboratories. Cyclophosphamide (CP) was purchased from Shanghai Hua Lian Pharmaceutical Co. Ltd. Biphenyldicarboxylate (DDB) was purchased from Jiangsu Huanghe Pharmaceutical Co. Ltd. Bacille Calmette-Guérin (BCG) was purchased from Shanghai Institute of Bioproducts.

2.2. General methods

HPSEC was performed with a Waters 600 apparatus, using a Waters 2410 RI detector. GC was done with a Shimadzu-9A apparatus equipped with an OV1701 column (30 m × 0.32 mm). GC-MS was performed with a Finnigan MD-800 instrument fitted with HP-1 column, EI 70 eV. ESI-MS spectra were obtained with a VG Quattro MS/MS spectrometer.

2.3. Isolation and purification

Cultivation medium contained (g l^{-1}): glucose, 40; peptone, 4; yeast extract, 2; KH_2PO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 0.75; vitamin B_1, 0.01. *G. lucidum* was grown in a 250 mL flask containing 70 mL medium at 30 °C for 7 days with shaking at 150 rpm. After cultivation, the cultured media were centrifuged at 3000*g* to remove the mycelia. The aqueous fraction obtained from centrifugation was concentrated at reduced pressure and 45 °C and then precipitated at 4 °C for 12 h with ice cold ethanol to 30% ethanol concentration. The precipitate was removed by centrifugation and the supernatant was precipitated with ethanol at a concentration of 60%. The precipitate consisted of crude polysaccharides and was recovered by centrifugation and isolated later.

The precipitated crude polysaccharides were resolved, dialyzed and concentrated to a small volume. The polysaccharide solution was treated with Sevag agent (chloroform: n-butanol = 1:4) to remove proteins. The aqueous phase was distilled to remove residual n-butanol and concentrated. The polysaccharides in the concentrated solution were fractionated over a DEAE-Sephacel (2.6 × 100 cm) column applying a gradient of buffer containing A (citric acid – NaH_2PO_3, pH 8.0, 0.02 M) and B (citric acid – NaH_2PO_3, pH 4.0, 0.02 M and 1 M KCl) as shown in Table 1. The fractions was monitored for carbohydrate content by the phenol sulfuric acid reaction and for peptide content by UV absorption at 280 nm. The dominant fraction P-1 (Fig. 1) was pooled, dialyzed, concentrated and then further fractionated with gel filtration chromatography on Sephadex G200 eluted with 0.1 M NaCl. Fractions were collected according to the carbohydrate profile, concentrated, dialyzed and lyophilized to yield GLP-1, GLP-2 and GLP-3 (Fig. 2).

2.4. Homogeneity and molecular weight of GLP-2

Homogeneity and molecular weight measurement was carried out by HPSEC on a Waters Ultrahydrogel TM Linear 7.8 × 300 mm column, eluting with 0.10 mol/L $NaNO_3$ at a flow rate of 0.9 mL/min. The eluates were monitored by refractive index detection and by UV detection at 280 nm. The column was pre-calibrated using a standard dextran series of different molecular weights (2000, 400, 150, 60, 10, 1.5 and 0.3 kDa).

2.5. Composition and D,L-configuration

The sugar composition of GPL-2 was determined by TLC of the acid hydrolysates and by GC analysis of the

Table 1
Elution gradient on DEAE column

Elution volume/mL	A*/%	B**/%
0	100	0
40	100	0
450	0	100
550	0	100

* Citric acid – NaH_2PO_3 (pH 8.0, 0.02 M).
** Citric acid – NaH_2PO_3 (pH 4.0, 0.02 M) + 1 M KCl.

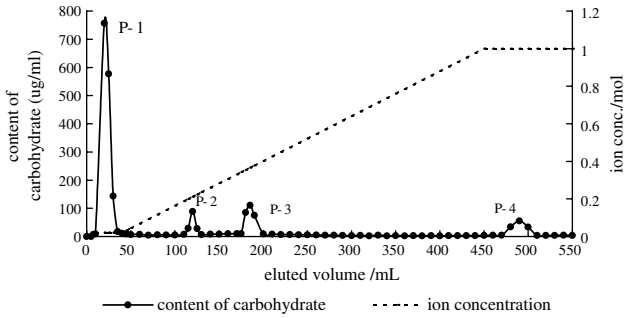


Fig. 1. DEAE elution profile of polysaccharides from submergedly cultured *Ganoderma lucidum*.

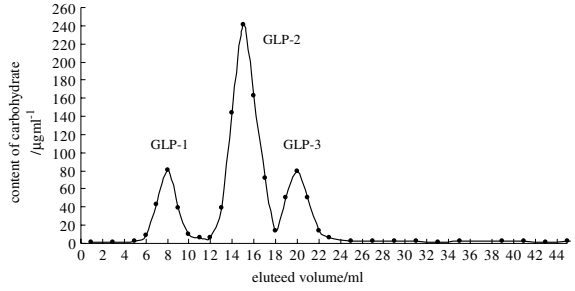


Fig. 2. Carbohydrate profile on G200.

alditol acetates derivatives as described by Li et al. (Li, Wu, & Zhang, 1982). The configurations of the sugars were identified by GC analysis of their TMSi(-)-2-butylglycosides derivatives (Gerwig, Kamerling, & Vliegenthart, 1978).

2.6. Glycosyl linkage composition analyses

GLP-2 was methylated by the modified Hakomori method (Ciucanu & Kerek, 1984). The permethylated polysaccharide was hydrolyzed, reduced, acetylated and then analyzed by GC-MS. The partially methylated alditol acetates were identified by their fragment ions in EI–MS and by relative times in GC, and the molar ratios were estimated from the peak areas and response factors (Sweet, Shapiro, & Albersheim, 1975). Moreover, GLP-2 was partially hydrolyzed by 0.05 M TFA at 100 °C for 1 h. After cooling, concentration and dialysis, the retentate was isolated on a Sephadex G-10 column (100×1.6 cm) to give a degraded polymer PA. The glycosyl residue composition of PA was determined by GC analysis. PA was methylated and its glycosyl linkage composition was determined as described above. Two oligosaccharides (O-1 and O-2) isolated from the partially hydrolyzed GLP-2 on the Sephadex G-10 column were also determined their glycosyl residue compositions and glycosyl linkage by the methods described above.

2.7. Immunological assays

Three months old male Kun Ming mice weighing 20 ± 2 g were obtained from Jiangxi Medical Institute, Nanchang, China. For the tests *in vitro*, mouse splenocytes were incubated in the medium with the polysaccharide samples (content 10–200 μg/mL) and mitogen ConA (5.0 mg/mL) or LPS (20 mg/mL). After incubation for 40 h at 37 °C in a humidified 5% CO_2 atmosphere, T and B lymphocyte proliferations were assayed with the MTT method (Heek, Reimann, Kabelitz, Hardt, & Wagner, 1985; Zhou, Shen, & Zhao, 1986).

For the *in vivo* tests, mice obtained as described above were divided randomly into five groups of 10 animals each: normal control (receiving no GLP-2, but given saline instead), GLP-2 25, 75, 150 and 300 mg/kg. GLP-2 or saline was given to the mice by intraperitoneal injection for 4 successive days. Mice were sacrificed on day 5 and their spleens were removed to obtain single cell suspensions. The cells were suspended in an RPMI 1640 medium and the cell content was 5×10^6 cells/mL. The lymphocyte proliferation and antibody production of spleen cells were measured by the MTT method and quantitative hemolysin spectrophotomic assay, respectively (Xiang & Li, 1993). Serum IgG and C-3 levels were also measured.

2.8. Protection from BCG/LPS-induced hepatotoxicity in mice

Mice obtained as described above were randomly divided into six groups of 10 animals each. The six groups were used as one control group, one model group, two positive reference groups and two sample groups. The control group was given 30 mL/kg day saline (i.p.) each day. In the other five groups, each animal was treated with 0.2 mL BCG (contained 5×10^6 bacteria, i.v) at the beginning, and then on each day of the successive 9 days, the model group was received 30 mL/kg day saline (i.p.), the positive reference groups were given either 200 mg/kg day DDB (i.p.) or 20 mg/kg day CP (i.p.), the sample groups were given GLP-2 100 or 300 mg/kg day (i.p.). On day 10, each animal except in the control group was given 7.5 μg LPS (i.v.). Sixteen hours after giving LPS, blood of all animals (including control group) was withdrawn from eye socket. All blood samples were centrifuged at 3000*g* and 4 °C for 10 min to obtain the serum. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with VENUSLEON-180 Eos bravo biochemistry measuring system (Hospitex Diagnostics Landao Laboratories LTD, Italy).

3. Results and discussion

3.1. Isolation and characterization

Crude polysaccharides were precipitated with ethanol from the centrifuged culture broth of *G. lucidum*. The precipitate was fractionated on a DEAE–cellulose column with a gradient elution of buffer solution (citric acid/NaH_2PO_3 and KCl), leading to the isolation of four polysaccharide sub fractions P-1, P-2, P-3 and P-4 (Fig. 1). P-1

was the dominant fraction and was further fractionated on a Sephadex G200 column by eluting with 0.1 M NaCl, resulting in three sub fractions GLP-1, GLP-2 and GLP-3 (Fig. 2). GLP-2 was dominant one among the three fractions. HPSEC analysis showed that GLP-2 was a homogeneous polysaccharide and its molecular weight was ca. 2.2×10^4 Da based on the calibration curve of the elution times of standard Dextrans on HPSEC.

Sugar composition analysis showed that GLP-2 was composed of Gal, Man, Glc, Ara and Rha in the molar ratios of ca. 103:17:12:10:3. D,L-configuration analysis showed that all glycosyl residues in GLP-2 were D except rhamnose and arabinose which were L. No absorption at 280 nm indicated that GLP-2 did not contain any peptide.

The results of glycosyl linkage composition analysis (Table 2) indicated that GLP-2 was a branched polysaccharide. The high content of 1,4-linked galactopyranosyl residues suggested that the backbone chain of GLP-2 contained the 1,4-linked galactosyl residues. The content of 1,4,6-trisubstituted galactosyl residues was about 20% in all galactosyl residues, and it indicated that the backbone chain was branched at O-6 of some galactosyl residues. Methylation analysis results showed that L-rhamnosyl and L-arabinosyl were linked as side residue on the backbone chain, while α-D-mannosyl and β-D-glucosyl were linked as side chains on the backbone chain.

The hydrolysis of GLP-2 with TFA gave a degraded polymer PA, oligosaccharide (O-1 and O-2) and monosaccharides. PA was a homogeneous polymer (Mr 1.2×10^4) measured by HPSEC. Glycosyl linkage analysis of PA (Table 2) showed that it was only composed of 1,4-linked galactosyl residues. Rhamnose, arabinose, mannosyl and glucosyl residues and 1,4,6-linked galactosyl residues were not present in PA. O-1 was a trisaccharide composed of mannose and O-2 was a tetrasaccharide contained only glucose, while the monosaccharide fraction contained rhamnose, arabinose, glucose and galactose. These results indicated that the backbone was composed of only galactosyl residues and rhamnose and arabinose were linked to O-6 of the galactosyl residues, while mannose and glucose were linked to O-6 of the backbone residues as side chain composed of three or more glycosyl residues.

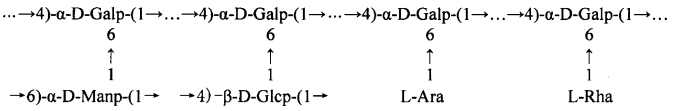
Table 2
Glycosyl linkage compositions of GLP-2, PA[a], O-1[a] and O-2[a] glycans

Components	Molar percent				Linkage
	GLP-2	PA	O-1	O-2	
2,3,4,6-Me_4-Gal	0	2			Gal_p-(1→
2,3,6-Me_3-Gal	56	98			→4)-Gal_p-(1→
2,3-Me_2-Gal	15				→4,6)-Gal-(1→
2,3,4,6-Me_4-Man	4		30		Man_p-(1→
2,3,4-Me_3-Man	8		70		→6)-Man_p-(1→
2,3,4,6-Me_4-Glc	2			25	Glcp-(-1→
2,3,6-Me_3-Glc	6			75	→4)-Glc_p-(1→
2,3,4-Me_3-Ara	7				Ara-(1→
2,3,4-Me_3-Rha	2				Rha-(1→

[a] PA, O-1 and O-2 are the polymer generated by partial acid hydrolysis of GLP-2.

The NMR spectrum (Table 3) conformed the results of methylation analysis described above. The signals from rhamnosyl, arabinosyl, glucosyl, mannosyl residues and O-6 substituted galactosyl residues did not appear in the NMR spectrum of the partial hydrolyzed product PA, while the signal for 1,4-linked galactosyl residues became stronger.

Therefore, the results described above suggested that GLP-2 had a backbone composed of 1,4-linked α-D-galactopyranosyl residues with branches comprised of terminal arabinose and rhamnose, 1,6-linked α-D-mannopyranosyl and 1,4-linked β-D-glucopranosyl residues linked to O-6 of 1,4-linked galactosyl residues. The structural feature may be described as below.



3.2. Immunoregulating ability of GLP-2

The effects of GLP-2 on ConA- or LPS-induced lymphocytes proliferation were tested *in vitro* (Table 4). The polysaccharide at low concentrations of 10 and 50 μg/mL showed an activity in enhancing the T and B lymphocyte proliferation. Moreover, when the concentration of GLP-2 was more than 100 μg/mL, the polysaccharide GLP-2 began to exhibit a lymphocyte suppressive activity. No observed toxicity of GLP-2 was measured in the experimental concentration range.

Table 3
Characteristic signals in ^1H and ^{13}C NMR spectra of GLP-2

Chemical shifts (H/C) (ppm)	Hydrogen/Carbon assignments
4.89/98.45	H1/C1 of α-D-Gal(1 → 4)-
4.97/101.79	H1/C1 of α-D-Man(1 → 6)-
5.04/102.66	H1/C1 of β-D-Glc(1 → 4)-
70.06	C4 of O-4 substituted β-D-Glc
69.99	C4 of O-4 substituted α-D-Gal
68.81	C6 of O-6 substituted α-D-Gal
66.56	C6 of O-6 substituted α-D-Man
2.08/20.62	H6/C6 of α-L-Rha

Table 4
Effects of the polysaccharide GLP-2 on ConA- or LPS-induced lymphocyte proliferation in mouse splenocytes *in vitro*

Samples	Concentration (μg/mL)	Lymphocyte	
		T cell (A_{570})	B cell (A_{570})
Control		0.54 ± 0.04	0.45 ± 0.03
GLP-2	10	0.58 ± 0.02	0.49 ± 0.04
	50	0.68 ± 0.03**	0.57 ± 0.02**
	100	0.50 ± 0.03	0.40 ± 0.03
	200	0.39 ± 0.02**	0.30 ± 0.02**

Results are shown as means ± SD based on four independent experiments.
** $p < 0.01$, significantly different from the control.

Table 5
Effects of intraperitoneal injections of GLP-2 on the spleen mass, lymphocyte production, antibody production, serum IgG and C-3 levels in mice

Samples	Dose (mg/kg)	Mass of spleen (mg/g)	Antibody (A_{520})	IgG (mg/mL)	C-3 (mg/mL)	Lymphocyte	
						T cell (A_{570})	B cell (A_{570})
Control		6.53 ± 0.84	0.75 ± 0.03	11.3 ± 2.1	1.4 ± 0.3	0.63 ± 0.02	0.70 ± 0.01
GLP-2	25	$8.54 \pm 1.38^{*}$	$1.35 \pm 0.02^{**}$	11.6 ± 1.8	1.8 ± 0.4	$0.97 \pm 0.01^{***}$	$0.95 \pm 0.02^{***}$
	75	$9.63 \pm 1.62^{**}$	$1.42 \pm 0.02^{**}$	12.6 ± 2.0	1.7 ± 0.3	$0.89 \pm 0.02^{**}$	$0.85 \pm 0.01^{**}$
	150	7.32 ± 1.22	0.80 ± 0.02	12.2 ± 3.5	1.3 ± 0.3	0.74 ± 0.01	0.76 ± 0.02
	300	6.87 ± 1.02	0.79 ± 0.03	12.0 ± 2.4	1.5 ± 0.5	0.65 ± 0.02	0.71 ± 0.03

Results are represented as the means ± SD ($n = 10$) from ten mice in each group.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significantly different from the control.

The immunoregulating activity of GLP-2 was further investigated *in vivo*. The results are shown in Table 5. The results showed that, at a dose of 25 or 75 mg/kg, GLP-2 significantly enhanced the T and B lymphocyte proliferation and antibody production and increased the mass amount of spleen tissue in the tested mice, but did not significantly influence the serum IgG and C-3 levels. However, at a high dose of 150 or 300 mg/kg, it did not show significant effect on the T and B lymphocyte proliferation, antibody production, mass amount of spleen tissue as well as serum IgG and C-3 levels.

These results suggested that GLP-2 had immunoregulatory activities. It was evident that GLP-2 had immunostimulation activity at low dosages and had immunosuppressive activity at high dosages in view of the lymphocyte proliferation (T and B cells) and the production of antibodies against sheep red blood cells (SRBC) in mice. However, GLP-2 had no noticeable effect on serum IgG and complement (C-3) levels at the tested dosage range.

3.3. Hepatoprotective ability of GLP-2

Bacille Calmette-Guérin (BCG) and lipopolysaccharide (LPS) induced liver injury model in mice was widely used for assessment of hepatoprotective activities of drugs (Nagai, Yakuo, & Yamada, 1988). In our present study, the hepatoprotective activity of GLP-2 was assessed with this model. The results are shown in Table 6. Significant increase of ALT and AST level in mice serum showed immunity injury in model group mice successfully induced by BCG + LPS, while the levels of ALT and AST in GLP-2 group at 300 mg/kg dosage were significantly lower ($p < 0.01$) than the model group as showed in Table 6. Cyclophosphamide (CP) and Biphenyldicarboxylate (DDB) tested simultaneously as positive references. Clearly, the effect of GLP-2 at the dosage of 300 mg/kg was comparable with that of DDB at the test dosage.

Attracting by BCG and LPS can induce sensitive immunological response which will lead to leakage of liver cell. Large amount of ALT and AST released to serum is signal of liver cell leakage. GLP-2 could suppress the immunity reaction and decrease the leakage of liver cells, as a result the amount of ALT and AST in serum decreased. It may be concluded that GLP-2 has protective activity from mice liver injury induced by BCG + LPS.

Table 6
The protective effects of GLP-2 on mice immunity liver injury induced by BCG + LPS ($\bar{x} \pm s$, $n = 10$)

Group	Dosage (mg/kg day)	ALT (U/L)	AST (U/L)
Control		64.51 ± 37.42	81.1 ± 31.3
Model		264.8 ± 44.5^{a}	301.3 ± 56.3^{a}
CP	20	98.4 ± 28.4^{b}	85.8 ± 36.5^{b}
DDB	200	155.1 ± 54.2^{b}	221 ± 59.23^{b}
GLP-2	100	232.0 ± 43.0	246.2 ± 40.3
	300	150.0 ± 40.3^{b}	181.6 ± 51.1^{b}

[a] $p < 0.01$ compared with control.
[b] $p < 0.01$ compared with model.

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