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Full Length Research Paper

Structure and immunological activity of a novel polysaccharide from the spores of *Ganoderma lucidum*

Lixia Zhang[1]*, Yajun Zhang[2] and Liping Zhang[3]

[1]School of Life Science, Daqing Normal College, Daqing 163712, China.
[2]College of Life Science, Zhongkai University of Agriculture and Engineering, Guangzhou 5510225, China.
[3]Laboratory of Biochemistry and Molecular Biology, School of Life Science, Northeast Normal University, Changchun 130024, China.

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The water-soluble polysaccharide (GLP) was obtained from the spores of *Ganoderma lucidum* by hot water extraction and gel chromatography. The molecular weight was estimated to be 143 KD determined by using HPGPC. Structure features of GLP were investigated by a combination of chemical and instrumental analysis. The results indicated that GLP consisted of a backbone composed of (1→6)-linked-α-d-glucopyranosyl, (1→3)-linked-β-d-glucopyranosyl and (1→3,6)-linked-β-d-glucopyranosyl residues in the ratio of 1:2:1, and terminated with one single terminal (1→)-β-d-glucopyranosyl at the O-6 position of (1→3,6)-linked-β-d-glucopyranosyl, along the main chain. Preliminary tests *in vitro* showed that GLP has stimulating effects on murine lymphocyte proliferation induced by concanavalin A (ConA) and lipopolysaccharide (LPS) in a dose-dependent manner. It is a possible potential immunopotentiating agent for use in functional foods or medicine against both pathogens and cancer.

Key words: *Ganoderma lucidum*, polysaccharide, structure, lymphocyte proliferation.

INTRODUCTION

Recently, many polysaccharides have been isolated from some natural materials including mushrooms, fungi, yeasts, algae, lichens and plants, and their biological activities have attracted more attention in the biochemical and medical areas due to their immunomodulatory and anti-cancer electrochemotherapy (ECT) (Ooi and Liu, 2000; Wasser, 2002). *Ganoderma lucidum* (Fr.) Karst (Ganodermataceae), basidiomycetous fungi, has been used as a medical remedy in traditional Chinese medicine and in many Asian countries during the past two millennia (Su et al., 1999, 2001). This edible mushroom was thought to preserve human vitality and their ability to inhibit cancer was observed in promoting longevity (Shiao et al., 1994). It had been reported that the polysaccharides distilled from *G. lucidum* have the functions of anti-tumor, anti-inflammation, anti-radiation and immunomodulation (Chen, 2000; Zhang and Chen, 1997; Bao and Wang, 2002; Bao et al., 2001). Guo et al. (2009) isolated a water-soluble polysaccharide named GSG from the spores of *G. lucidum*. GSG is characterized to be a branched glucan that contains several different kinds of linkages. So far, there is no information published about the structural elucidation of the water-soluble polysaccharide isolated form the spores of this fungus. Therefore, this paper was concerned with the isolation, structure of a novel water-soluble polysaccharide from the spores of *G. lucidum.* Its chemical structures were characterized, and its immunomodulatory activities were reported for the first time. The result of this study introduced *G. lucidum* as a possible valuable source for β-d-heteroglycan which helped to exhibit unique immunomodulatory properties *in vitro* by Concanavalin A (ConA) or lipopolysaccharide (LPS) induced lymphocyte proliferation test.

*Corresponding author. E-mail: zhanglx573@yahoo.com.cn. Tel: +86-459-85510177.

Abbreviation: HPGPC, High performance gel permeation chromatography; **GSG,** the spores of *Ganoderma lucidum* polysaccharide; **GLP,** *Ganoderma lucidum* polysaccharide; **CGLP,** crude polysaccharide of *Ganoderma lucidum*.

METHODS

Materials and chemicals

The spores of *G. lucidum* used in this experiment were purchased from Jilin drugstore. A voucher specimen identified by Prof. Hongxing Xiao, a faculty member of School of Life Science, Northeast Normal University, Changchun, China.

Sepharose CL-6B and DEAE-Sephadex A-25 was purchased from Pharmacia Biotech. Trifluoroacetic acid (TFA) and Me_2SO were purchased from E. Merck. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Concanavalin A (ConA) and lipopolysaccharide (LPS) were from Sigma Chemical Co. Medium RPMI-1640 was purchased from Gibco Invitrogen Co. The complete RMPI-1640 medium, used for immunological tests, was supplemented with penicillin 100 IU/ml, streptomycin 100 µg/ml, and 10% newborn bovine serum, pH 7.4. All other reagents were of grade AR.

General methods

The specific rotation was determined at 20 ± 1℃ with a WZZ-T1 Polarimeter (Shanghai Physical Optics Instrument Co.). UV-Vis absorption spectra were recorded with a Shimadzu MPS-2000 spectrophotometer. GC was performed on a Vavian 3400 instrument (Hewlett-Packard Component, USA) equipped with SE-30 column (50 mm × 0.20 mm × 0.2 to 5 µm). The column temperature was maintained at 120℃ for 2 min, and then increased to 250℃ for 3 min at a rate of 8℃/min. Gas chromatography-mass spectrometry (GC-MS) was done on a HP5890 (II) instrument (Hewlett-Packard Component, USA) with an HPS quartz capillary column (25 m × 0.22 mm × 0.2 nm), and at temperatures programmed from 120 to 140℃ at 1℃/min. The FT-IR spectra (KBr pellets) were recorded on SPECORD in a range of 400 to 4000 cm^{-1}. Total carbohydrate content was determined by the Dubois's method (Dubois et al., 1956), using d-glucose as the standard. Protein was measured by the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard. Dialysis was carried out by using dialysis tubing (Spectra/Por MWCO: 500).

Extraction and purification of polysaccharide

The spores of *G. lucidum* (500 g) were extracted with 3 volume of 70% EtOH at 80℃ for 20 h under reflux to remove lipid, and the supernatant was removed. The residue was then extracted with 20 volume of distilled water at 80℃ for 3 times and 1 h for each time. After centrifugation (3000 *g* for 10 min, at 20℃), the supernatant was concentrated 10-fold, and precipitated with 95% EtOH (1: 4, v/v) at 4℃ for overnight. The precipitate collected by centrifugation was suspended in distilled water to remove the protein by the Sevag method (Sun et al., 2008), and exhaustively dialyzed against water for 2 days. Then the concentrated dialyzate was precipitated with 3 volumes of 95% EtOH. The precipitate was washed with absolute ethanol, acetone and ether, respectively (Chi et al., 2007). The washed precipitate was the crude polysaccharide, named as CGLP (3.3 g).

The CGLP (600 mg) was purified on the auto liquid chromatographic fractionation apparatus (MF99-1) made in shanghai city of China. The CGLP (600 mg) was dissolved in distilled water, centrifuged, and then the supernatant was applied to a column of DEAE-Sephadex A-25 equilibrated with 0.9% NaCl. After loading with sample, the column was eluted with different concentrations of NaCl aqueous solution (0.15 and 3.9 M) stepwise at 8 ml/12 min. Test tubes (100 containing 8 ml eluant each) were collected using an automated step-by-step fraction collector. Total carbohydrate and protein content of each tube were measured by Dubois's and Lowry's method, respectively. The eluted solution was only separated into one fraction, and then purified by gel-permeation chromatography on a Sepharose CL-6B column (90 × 2 cm), loading 100 mg the above-purified fraction for each run. The column was eluted with 0.9% NaCl with a flow rate of 0.5 ml/min. Fractions (test tube Nos. 38 to 41) containing a large amount of sugar were applied to a Sephadex G-25 column to remove salts, and freeze dried to obtain purified polysaccharide (353 mg), named as GLP.

Monosaccharide composition, properties and molecular weight determination

Gas chromatography (GC) was used for identification and quantification of the monosaccharides. GLP was hydrolyzed with 2 M TFA at 110℃ for 4 h (Honda et al., 1981). The monosaccharides were conventionally converted into the alditol acetates as described (Sun et al., 2008) and were analyzed by GC as foresaid. The absolute configurations of the monosaccharides were determined as described by Vliegenthart et al. using (+)-2-butanol (Oades, 1967; Johnes and Albersheim, 1972).The average molecular weight of GLP was determined by high-performance gel-permeation chromatography (HPGPC) (Zhang et al., 2009), which was performed on a SHIMADZU HPLC system fitted with one TSK-gelG3000PWXL column (7.8 × 300 mm TOSOH, Japan) connected to a Shimadzu HPLC system. Twenty microliters of sample (5 mg/ml) was injected, eluted with 0.2 M NaCl at a flow rate of 0.6 ml/min and monitored using a refractive index RID-10A detector (Shimadzu, Tokyo, Japan). The molecular mass was estimated by reference to a calibration curve made from a set of Dextran T-series standards of known molecular mass (T-700, 580, 470, 350, 280, 50, 25, 12, 10, sigma).

Partial acid hydrolysis

The GLP (150 mg) was hydrolyzed with 0.05 M trifluoroacetic acid (4 ml), maintained at 80℃ for 30 h, centrifuged. After TFA was removed by evaporation, the remains were dialyzed with distilled water for 48 h, and then the solution was diluted in the sack with ethanol. After hydrolyzation, the precipitate and supernatant in the sack and the fraction out of sack were dried and analyzed by GC as the alditol acetate. The precipitate, in the sack, was subjected to monosaccharide composition analysis of backbone and methylation analysis (Sun et al., 2008).

Periodate oxidation-Smith degradation

For analytical purpose, 150 mg of the polysaccharide was dissolved in 25 ml of distilled water and 25 ml of 30 mmol/l $NaIO_4$ was added. The solution was maintained at 4℃ for 7 days in darkness, and 0.1 ml aliquots were withdrawn at 3 to 6 h intervals, diluted to 25 ml with distilled water and read in a spectrophometer at 223 nm (Linker et al., 2001). Excess periodate was decomposed by the addition of ethylene glycol (2 ml). The solution of periodate product (2 ml) was sampled to calculate the yield of formic acid by 0.01 M NaOH. The rest was dialyzed against distilled H_2O for 24 h. The solution was concentrated and reduced with $NaBH_4$ (80 mg), and the mixture was left for 24 h at room temperature, neutralized to pH 6.0 with 50% acetic acid, dialyzed as described previously, and was concentrated to a volume (10 ml). One-third of solution described previously was freeze dried and analyzed with GC. Others were added to the same volume of 1 M sulfuric acid, maintained for 40 h at 25℃, neutralized to pH 6.0 with barium carbonate, and filtered. The filtrate was dialyzed as foresaid, and the content out of the sack was desiccated for GC analysis; the content inside was diluted with ethanol, and after centrifugation, the supernatant and

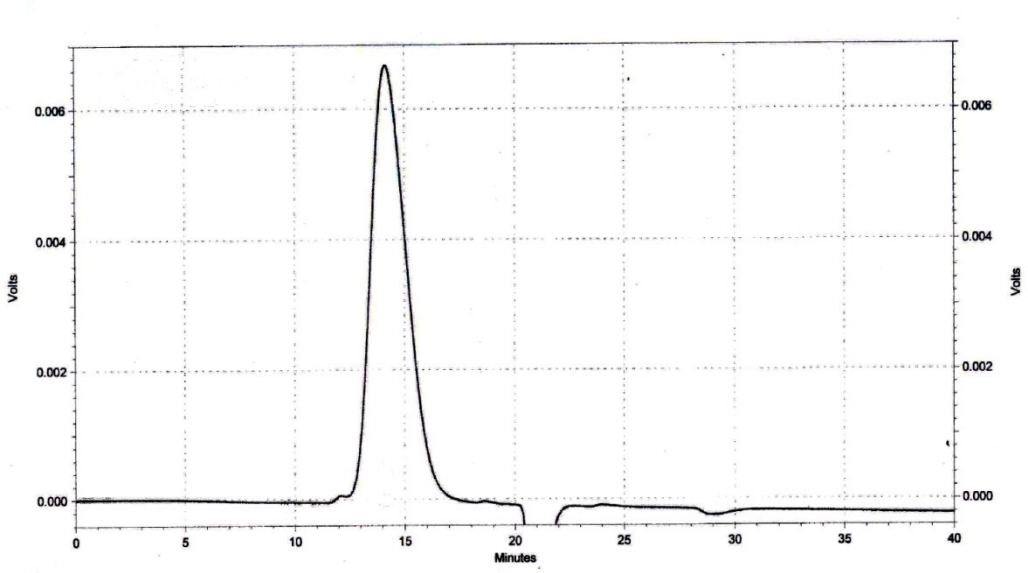

Figure 1. Profile of GLP in HPGPC with 0.05 mol/LNa_2SO_4 at 0.8 ml/min.

precipitate were also dried out for the GC analysis.

Methylation analysis

The sample (20 mg) was methylated thrice according to Needs and Selvendran (1993). Complete methylation was confirmed by the disappearance of the OH band (3200 to 3700 cm^{-1}) in the IR spectrum. The methylated products were hydrolyzed, reduced and acetylated as described by Sweet et al (1975). The partially methylated alditol acetates were analyzed by GC-MS under the same chromatographic conditions as aforementioned.

NMR spectroscopy

For NMR measurements, GLP was dried in a vacuum over P_2O_5 for several days, and then exchanged with deuterium (Dabrowski, 1994) by lyophilizing with D_2O for several times. The deuterium-exchanged polysaccharide (50 mg) was put in a 5-mm NMR tube and dissolved in 0.7 ml of 99.96% D_2O. Spectra were recorded with a Bruker AV-400 spectrometer. The ^1H and ^{13}C NMR spectra were recorded at 50℃. Acetone was used as an internal standard (δ 31.09 ppm) for the ^{13}C spectrum. The ^1H NMR spectrum was recorded by fixing the HOD signal at δ 4.54 ppm at 50℃.

Immunobiological activity assay

Male Kunming mice (GradeII, 8 to 12 weeks old) were purchased from the Pharmacology Experimental Center of Jilin University and were acclimatized for 1 week prior to use. All mice were housed under the standard conditions at 24 ± 1℃, with humidity of 50 ± 10%, and a 12/12 h light/dark cycle. Rodent laboratory chow pellets and tap water were supplied *ad libitum*. All the procedures conducted by the Institute for Experimental Animals of Jilin University were carried out in strict accordance with the PR China legislation on the use and care of laboratory animals, and were approved by the university committee for animal experiments.

Spleen cells of mice were obtained by gently teasing the organ in RPMI-1640 medium. To isolate mononuclear cells, 5 ml aliquots of the spleen cell suspension were layered onto 2.5 ml aliquots of a polysucrosesodium ditrizoate solution, and were centrifuged at 3000 rpm for 20 min at room temperature. Mononuclear cells were gently removed from the interface between medium and histopaque, and were transferred to a sterile container and washed with RPMI-1640. At last, the cells were resuspended in 5 ml RPMI-1640 medium, and cell counts were done. An aliquot of 100 µl of splenocytes mixed with the polysaccharide (50, 100, 200, 400 µg/ml, final concentration) was seeded into each well of a 96-well plate in the presence of ConA (5.0 µg/ml) or LPS (10.0 µg/ml). After preincubation for three days at 37℃ in a humidified 5% CO_2 incubator, 10.0 µl of 0.4% MTT was added into each well (Jiao et al., 2009). The plate was incubated for another 4 h, and then a total of 150 µl Me_2SO was added to the culture and homogenized for at least 10 min to fully dissolve the colored material. The absorbance at 570 nm was measured on an ELISA reader (Model 680, Bio-RAD Instruments). The control experiments were performed without the polysaccharide. Each experiment was performed in triplicate.

RESULTS AND DISCUSSION

Isolation, purification and structural analysis of polysaccharides

The GLP showed a single and symmetrically sharp peak, indicating its homogeneity on HPGPC (Figure 1). According to the retention time, its molecular weight was estimated to be 1.43×10^5 Da, and it showed an $[\alpha]_D^{20}$ = +33.7 (*c* 0.5, H_2O). It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. Total carbohydrate content was determined to be 96.7%. The GLP was composed of only d-glucose as detected by GC (Figure 2). The absolute configuration of the monosaccharides were determined by the GC

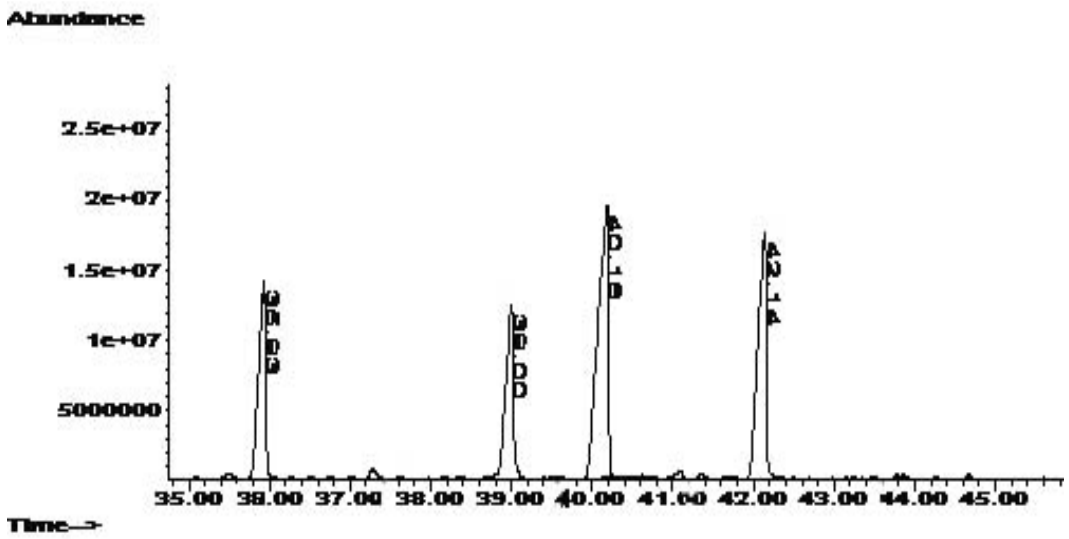

Figure 2. G. C. of methylated GLP.

Table 1. The data of UV analysis, IR analysis and NMR analysis of GLP.

Assay	Peaks or signals at:
UV analysis (nm)	210
IR analysis (cm^{-1})	3426.01, 2927.45, 1636.73, 890.75
^{1}H NMR analysis (ppm)	5.11, 5.06, 5.03, 5.01
^{13}C NMR analysis (ppm)	105.56, 105.40, 104.22, 101.30, 78.56, 68.72, 67.25, 65.31,60.11

Table 2. The results of methylation analysis of GLP.

Peak no.	Methylated sugar	Molar ratio	Main fragments(m/e)
1 (Residue-A)	2,3,4,- Me_3- Glcp	1	43,45,71,87,101,117,129,145,161,205
2 (Residue-B)	2,4,6,- Me_3- Glcp	2	43,45,87,101,117,129,161
3 (Residue-C)	2, 4,- Me_2- Galp	1	43,87,99,101,117,129,161,189
4 (Residue-D)	2,3,4,6- Me_4- Glcp	1	43,45,87,117,129

examination of acetylated (+)-2-octyl glycosides, and showed that all have D configurations. The FT-IR spectra of GLP are shown in Table 1. The bands in the region of 3426.01 cm^{-1} are due to the hydroxyl stretching vibration of the polysaccharides. The bands in the region of 2927.45 cm^{-1} are due to C-H stretching vibration, and the bands in the region of 1636.73 cm^{-1} are due to associated water. Moreover, the characteristic absorptions at 890.75 cm^{-1} in the IR spectra indicated that β-configurations is existing in GLP (Zhang, 1999).

The GC-MS results (Table 2) indicated that the backbone chains are mainly (1→6)-linked-β-d-glucopyranosyl (Residue-A), (1→3)-linked-β-d-glucopyranosyl residues (Residue-B) and (1→3,6)-linked-β-d-glucopyranosyl (Residue-C). The side chains attached to the O-6 position of Residue-B contained single terminal (1→)-β-d-glucopyranosyl (Residue-D) groups. According to the peak areas, four types of residues are in the ratio of 1:2:1:1. This was also in accordance with the results of the periodate oxidation and Smith degradation. Supporting the results of methylation analysis, GC of the products that was obtained from Periodate oxidation-Smith degradation only showed the presence of glycerol.

In the anomeric region of the ^{1}H NMR spectrum (Table 1) of GLP, four signals occurred at δ 5.11, δ 5.06, δ 5.03 and δ 5.01 ppm, which were assigned as Residue-A, Residue-B, Residue-C and Residue-D, respectively. And

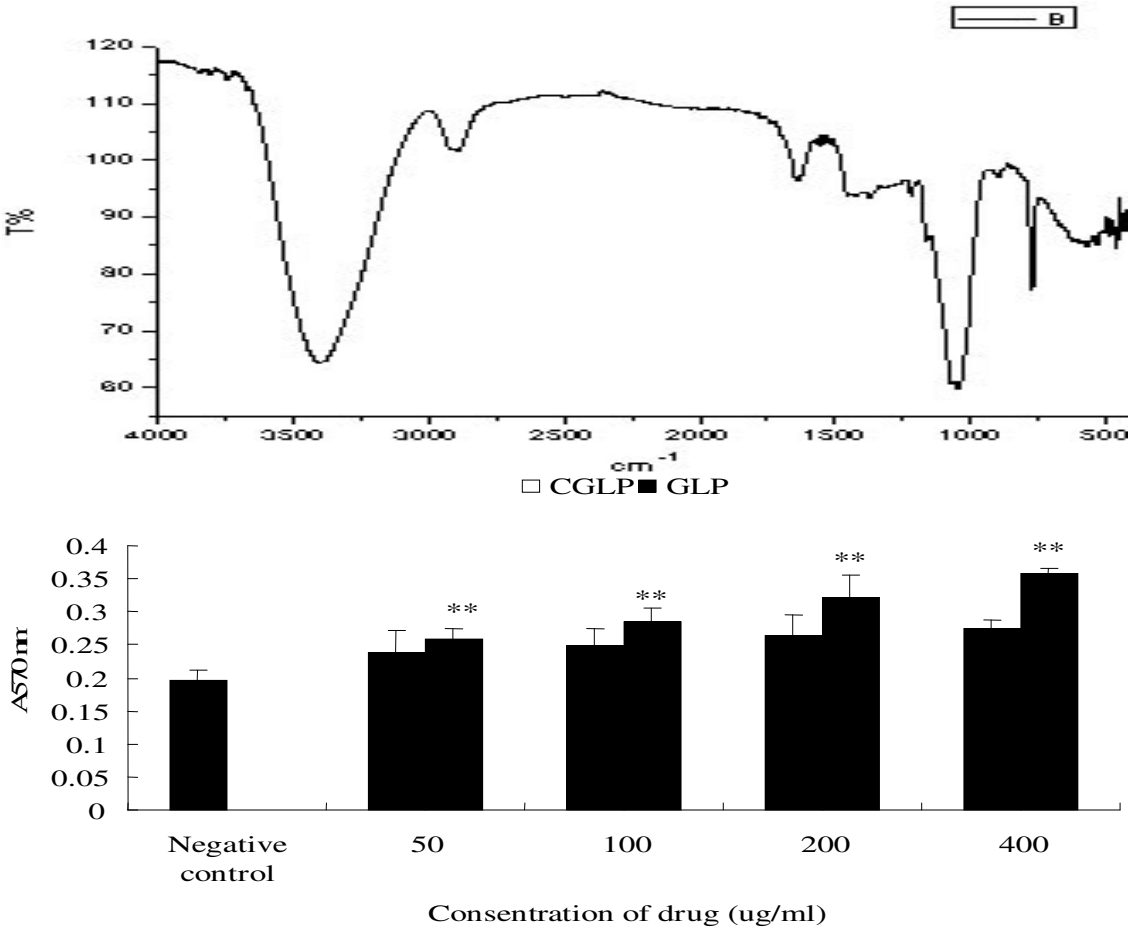

Figure 3. Effect of polysaccharides CGLP and GLP on lymphocyte proliferation. Spleen cells were stimulated by different concentration of the polysaccharides CGLP and GLP. Proliferation activity was expressed at 570 nm. Values are means±S.D. **P<0.01 *vs*. negative control.

accordingly, in the anomeric region of the ^{13}C NMR spectrum, four carbon resonances appeared at *δ* 104.22, *δ*101.3, *δ* 105.40 and *δ* 105.56 ppm. All the results confirmed the presence of four sugar residues and their configurations: Residues-A, Residues-B, Residues-C and Residue-D are form of β-configuration, consistent with GC and FT-IR data. In the high magnetic field, the *δ* 78.31 signal should come from C-3 resonance of Residue-C. C-6 chemical shifts of Residue-A, Residue-B, Residue-C and Residue-D occurred at *δ* 67.79, *δ* 68.29, *δ* 69.11, *δ* 69.88, respectively. All the NMR chemical shifts were compared with the literature values (Hua et al., 2004; Ishurd et al., 2004; Cui et al., 2007; Zhao et al., 2006).

The structure of an immunoregulatory polysaccharide from the spores of *G. Lucidum,* by means of chemical analyzes and NMR spectroscopy was studied for the first time. The core structure of GLP can be demonstrated as follows:

A C B C D

→6)- β-D-Glcp-(1→3)- β-D-Glcp-(1→3)- β-D-Glcp-(1→3)-β-D-Glcp-(1→ 6→1β-D-Glcp

Immunological activity of GLP

Lymphocyte proliferation is a crucial event in the activation cascade of both cellular and humoral immune responses. In order to investigate a possible immunomodulatory effect of the polysaccharides, contaminant endotoxins were removed from the polysaccharide preparations by affinity chromatography in a polymyxin B-coupled column. Spleen lymphocyte proliferation induced by ConA *in vitro* may be used as a method to evaluate T lymphocyte activity, while that induced by LPS may be used to examine B lymphocyte activity (Jiao et al., 2009). As observed in Figure 3, when ConA or LPS was added as mitogen for lymphocytes, GLP could significantly increase lymphocytes proliferation ($P < 0.05$ or $P < 0.01$) especially at the concentration of 200 ug/ml. However, CGLP has nothing to do with lymphocytes proliferation *in vitro*. Immunobilological activity assay showed that GLP could increase the ConA or LPS-induced lymphocytes proliferation *in vitro*, while CGLP has no stimulating effects on murine lymphocyte

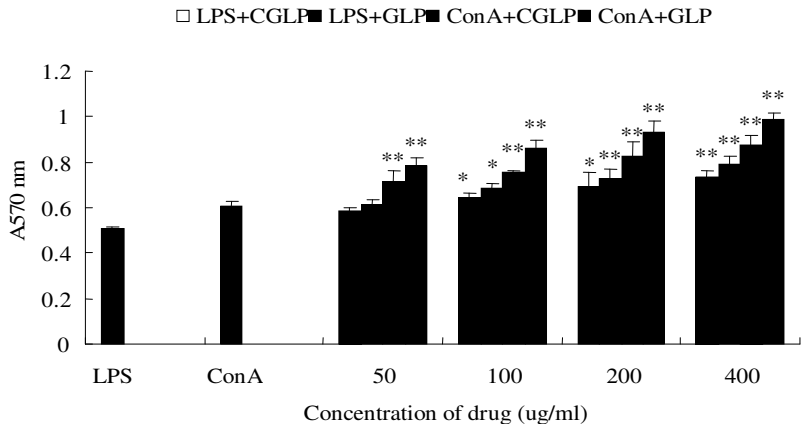

Figure 4. Effect of polysaccharides CGLP and GLP on ConA-induced or LPS-induced lymphocyte proliferation. Proliferation activity was expressed at 570 nm. Values are means±S.D. *P<0.05; **P<0.01 *vs.* ConA or LPS, respectively.

Proliferation (Figure 4). Therefore, we can draw a conclusion that CGLP or GLP is possible potential immune potentiating agent for use in health-care food or medicine. Based on these findings, the separation of active components from the spores of *G. Lucidum* and elucidation of mechanisms responsible for its activities should deserve an in depth research in the near future.

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