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The Hepatoprotective Effects of Polysaccharides Isolated from Submerged Fermentation of *Ganoderma lucidum*

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Abstract A neutral polysaccharide, GP, was isolated from a fermentation broth of *Ganoderma lucidum*. Acid hydrolysis and a paper chromatography analysis indicated that the polysacchride was composed of glucose, xylose, and mannose. The molecular weight was estimated to be 2.9×10^4. The oral administration of GP to mice showed that it can inhibit liver damage induced by GalN and CCl_4.

Key words: Neutral polysaccharide GP, *Ganoderma lucidum*, acid hydrolysis, paper chromatography, oral administration

Ganoderma lucidum has been regarded as an effective herbal medicine for treating various human diseases such as hepatopathy, chronic hepatitis, hypertension, and tumorigenic diseases [3, 13]. Recent studies have also demonstrated that the polysaccharides in *G. lucidum* have antitumor and antihypertensive activities, which would appear to be useful in immunotherapy as a new type of immunomodulatory agent [4, 15].

Due to its expected health benefits, *G. lucidum* has been cultivated in China, Korea, and Japan for hundreds of years. However, the traditional culture method, by which *G. lucidum* have normally been produced in solid cultures using substrates such as grain, sawdust, or wood, requires for a long time (about 6–9 months) to obtain the fruiting body and the yield is rather low. Therefore, many attempts have been made to produce bioactive compounds such as polysaccharides from submerged mycelia culture on a large scale [5,15-16]. When compared with the intracellular polysaccharides, the exopolysaccharides obtained from *G. lucidum* fermentation broths have several advantages as no low-yield-extraction process is necessary. An exopolysaccharide production system would appear to be move favorable for an industrial mycelial culture system.

Although many kinds of polysaccharides have been extracted from mycelia and fruiting body of *G. lucidum* [3, 4, 13], there are only few reports related to the exopolysaccharides from submerged fermentation broth [5]. Accordingly, in the current study, we isolated neutral polysaccharide from a submerged fermentation of *G. lucidum* and monitored its hepatoprotective effects on mice.

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Materials and Methods

Organism and Culture Conditions

G. lucidum GL-2 was selected from the fruiting body of a wild strain of *G. lucidum* and stored in our laboratory. The seed culture was grown in an optimized medium contained: sucrose 3.5%, peptone 0.5%, yeast powder 0.25%, KH_2PO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, and vitamin B_1 0.005%. The initial pH was adjusted to 6.0. The culture was maintained at 30°C in a rotary shaker (140 rpm) for 24 h, then 4% (v/v) of the culture was inoculated into a 100-l fermentor. The submerged media contained: sucrose 3.5%, metarose 0.5%, peptone 0.3%, yeast extract 0.2%, KH_2PO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, and vitamin B_1 0.005%. the fermentation was maintained at 30°C for 72 h. All the chemicals used were of analytical grade and obtained from commercial sources.

Fractionation and Purification of GP from Fermentation Broth

The fermentation broth was filtered and 3 volumes of alcohol were added to the filtrate. The precipitated polysaccharide was collected by centrifugation at 3,000 ×g for 10 min and then dried to remove any residual alcohol. The crude polysaccharide was dissolved in sodium phosphate buffer, dialyzed overnight, and then loaded on DEAE-cellulose

column. The neutral polysaccharide GP was eluted with 2 mM phosphate buffer, pH 7.0.

Determination of Molecular Weight

The molecular weight of the polysaccharide was estimated by gel filtration according to the Andrews method [1]. One mg of GP dissolved in 0.5 ml of cadoxen was applied to gel filtration column (TSK gel HW-65F, 0.5×15 cm) and eluted at 0.3 ml/min. Dextrans (molecular weights 12,000, 50,000, and 150,000) were used as the standards. The void volume was determined by the elution of blue dextran (molecular weight 2,000,000). The fractions were analyzed using the phenol-sulfuric acid method [2]. The molecular weight of GP-1, GP-2 was estimated by a calibration curve made by HPLC on TSK-GMPW column (0.75×30 cm) with 0.1 M NaCl as an eluant. The pullulans (Showa Denko, Japan) were used as a standard.

Monosaccharide Composition of Polysaccharide

The neutral polysaccharide GP (10 mg) was hydrolyzed with 20 ml of 2 M hydrochloric acid for 8 h at 105°C in a nitrogen-sealed screw-capped tube. The hydrolyzate was analyzed by paper chromatography and compared with standard monosaccharides.

Hepatoprotective Effects of GP Against D-Galactosamine-Induced Liver Damage in Mice

The mice were randomly assigned into three experimental groups of six mice each. Group 1 was given 0.2% CMC, while Groups 2 and 3 were given 250–500 mg/kg GP orally for 2 days. D-galactosamine at 500 mg/kg and 10 mg/kg endotoxin were injected intraperitoneally into each group 1 h after the last administration. Blood samples were collected from the orbital plexuses for an enzyme assay 10 h after the injection [11, 12].

Hepatoprotective Effects of GP Against Carbon Tetrachloride-Induced Liver Damage in Mice

The mice were randomly divided into four experimental groups of six mice each. Groups 1 and 2 were given 0.2% CMC, while Groups 3 and 4 were given 250, 500 mg/kg GP orally for 2 days. Eighteen ml of 0.01% carbon tetrachloride (CCl_4) was injected intraperitoneally into each group 1 h after the last administration. Blood samples were collected from orbital plexuses for an enzyme assay 24 h after the injection [11, 12].

Results and Discussion

A polysaccharide was isolated from the fermentation broth of *G. lucidum* (Fig. 1). When the crude polysaccharide was loaded onto DEAE-cellulose ion-exchange chromatography, the polysaccharide was fractionated into three kinds of

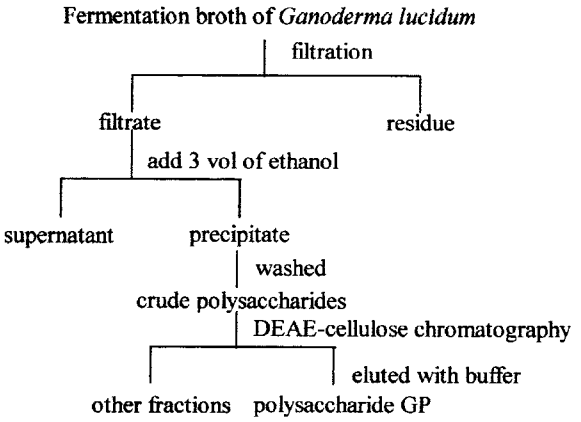

Fig. 1. Preparation scheme of polysaccharides from fermentation broth of *Ganoderma lucidum*.

polysaccharides. Figure 2 shows the elution chromatogram of the crude polysaccharide resulting from ion-exchange chromatography. The polysaccharide eluted with 2 mM sodium phosphate buffer, pH 7.0, was designated as the neutral polysaccharide (GP). After elution of the unbound component with the buffer, the retained components (GP-1 and GP-2) were eluted with the buffer containing 0.5 M and 1.0 M NaCl, respectively.

Further investigations were carried out using the neutral polysaccharide GP since its content was 74.1% for the three kinds of polysaccharides and GP exhibited stronger hepatoprotective activities. GP showed a broad peak on the gel filtration column and the molecular weight of the major component was estimated to be about 2.9×10^4. Moreover, the molecular weight of GP-1 and GP-2 was estimated to be about 2.1×10^5 and 1.4×10^6 kDa, respectively. Lee *et al.*

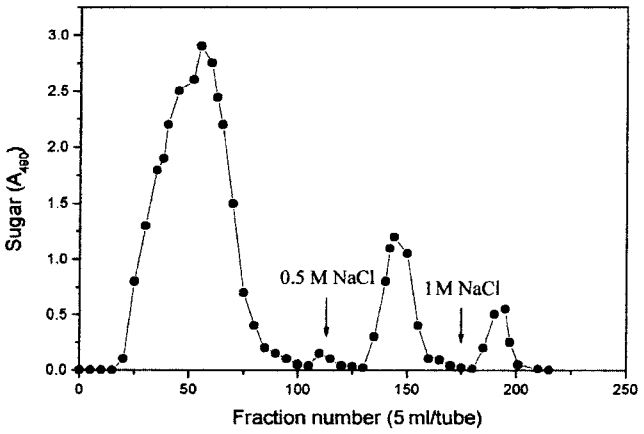

Fig. 2. Isolation of GP by DEAE-cellulose chromatography.
DEAE cellulose column (2.6×50 cm) was equilibrated with 2 mM sodium phosphate buffer, pH 7.0. After being loaded, the column was washed with the same buffer to elute the unbound polysaccharide GP, then the retained polysaccharides were eluted with 0.5 M and 1.0 M NaCl in the same buffer, respectively. The sugar was measured using the phenol-sulfuric acid method.

Table 1. Protective effect of GP in mice liver intoxicated by GalN and ET.

Group	GalN+ET	GP mg/kg	ALT	AST	MDA
			Unit/ml	Unit/ml	nmol/g of tissue
Normal	-	-	34.2±3.6	66.3±7.2	21.4±1.3
Control	+	-	68.6±5.5	110.2±18.3	62.2±2.5
1	+	250	49.7±4.2* (54.9%)	84.5±7.2* (58.5%)	45.7±3.6* (40.4%)
2	+	500	41.2±1.8** (79.7%)	77.3±7.1** (74.9%)	36.4±4.7** (64.2%)

The mice were administered with GP (250 and 500 mg/kg) orally, once a day for 7 days, then 500 mg/kg GalN and 10 mg/kg endotoxin (ET) (i.p.) were injected 1 h after the final administration of GP. Blood samples from the livers were collected 10 h after the injection. The results are expressed as a mean±SD, n=8. The values in parentheses are the percentage of protection calculated as 100×(values of control-values of sample)/(values of control-values of normal).

*P<0.05, **P<0.01; significant difference from control. Groups 1 and 2 contained 6 mice each and the dose of GP was different.

[5] previously reported on the isolation of an exopolysaccharide exhibiting antitumor activity from submerged cultivation of *G. lucidum*, and its molecular weight was found to be 1.2×10^6 kDa, which was similar to that of GP-2. Li *et al.* [6] also isolated five kinds of exopolysaccharides from fermentation broth of *G. lucidum*, and the molecular weight of the most abundant polysaccharide (60% of the total polysaccharides) was found to be 2.2×10^4, which was similar to that of GP.

Polysaccharide GP was found to consist of sugar and protein (data not shown). Paper chromatography showed that the polysaccharide was actually composed of glucose, xylose, and mannose. Lin [8] reported an exopolysaccharide composed of galactose, glucose, arabinose, and xylose, which was isolated from a fementation broth of GL 8801. Luo *et al.* [9] reported that a neutral polysaccharide composed of glucose, galactose, mannose, and xylose was isolated from the mycelia of *G. lucidum*. Song *et al.* [14] reported that rhamnose, arabinose, xylose, mannose, galactose, and glucose were found in the exopolymer produced from a submerged mycelial culture of *Ganoderma lucidum* WK-003. Thus, the composition of the polysaccharide obtained from a fermentation broth of *G. lucidum* would appear to be dependent on many factors such as the organism used, fermentation conditions, and isolation methods.

The effects of GP against hepatotoxicity in mice were investigated using D-galactosamine (GalN)-induced and carbon tetrachloride (CCl_4)-induced models, respectively. GalN-induced liver damage appears to be very similar to human viral hepatitis in their morphological and functional features. Thus, this is now accepted as one of the most authentic experimental animal models for liver damage [7]. Since it was previously reported that endotoxin can increase the degree of liver damage induced by GalN, endotoxin was applied along with GalN in the present study. In the GalN-treated controls, the activity of serum alanine transferase (ALT) and serum aspartic acid transferase (AST) increased significantly compared with the control group (Table 1). In contrast, pretreatment of GP significantly decreased these elevated activities when compared with the GalN control group. Similar results have also been reported where the glutamic pyruvic transaminase (GPT) activities in the serum of intoxicated Sprague-Dawley rats decreased from 871 to 263 Karnet unit with the oral administration of the exopolymer from a submerged culture of *G. lucidum* [14]. To evaluate the inhibitory effect of GP in GalN-induced lipid peroxidation, the concentration of malondialdehyde (MDA) in liver of one mouse from each group was determined (Table 1). The MDA concentration in the GalN-injected control group was 21.4±1.3 nmol, while in the group administered with 250 and 500 mg/kg of GP for 7 days, the MDA in their liver was lower by 40.4% and 64.2%, respectively. These results suggest that GP is a potent antioxidant agent in protecting the liver

Table 2. Protective effect of GP in mice liver intoxicated by CCl_4.

Group	0.01%CCl_4 ml/kg	GP mg/kg	ALT	AST	MDA
			Unit/ml	Unit/ml	nmol/g of tissue
Normal	-	-	32.2±3.5	62.1±6.6	23.7±1.9
Control	18	-	92.3±6.4	152.6±19.3	74.8±4.1
1	18	250	77.5±7.1 (24.6%)	92.5±11.2 (66.4%)	53.8±5.4 (41.1%)
2	18	500	61.2±5.4* (51.7%)	76.5±6.9* (84.1%)	40.6±4.3 (66.9%)

Mice were administered with GP (250 and 500 mg/kg) orally, once a day for 2 days. Eighteen ml of 0.01% carbon tetrachloride (CCl_4) was injected intraperitoneally into each group 1 h after the last administration. Blood samples from the livers were collected 24 h after the injection. The results are expressed as a mean±SD, n=6. The values in parentheses are the percentage of protection calculated as 100×(values of control-values of sample)/(values of control-values of normal).

*P<0.05, **P<0.01; significant difference from control. Groups 1 and 2 contained 6 mice each and the dose of GP was different.

from GalN-induced damage, which is similar to the role played by the polysaccharides isolated from the fruiting body of *G. lucidum*. Moreover, a similar result was also obtained from the CCl_4-induced model (Table 2). The mechanism of liver injury by CCl_4 is different from that induced by GalN, as liver injury due to CCl_4 is generally attributed to $\cdot CCl_3$ radicals [17]. These results imply that GP also has strong radical scavenging activities. Further characterization of GP and its other biological activities is currently in progress.

REFERENCES

1. Andrews, P. 1965. The gel-filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem. J.* **96:** 595–606.
2. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Roberts, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **28:** 350–356.
3. Jong, S. C. and J. M. Birmingham. 1992. Medicinal benefits of mushroom Ganoderma. *Adv. Appl. Microbiol.* **37:** 101–134.
4. Jong, S. C., J. M. Birmingham, and S. H. Pai. 1992. Immuno-modulatory substance of fungal origin. *J. Immunol. Immuno-pharmacol.* **11:** 115–122.
5. Lee, S. Y., T. S. Kang, S. O. Moon, I. D. Lew, and M. Y. Lee. 1996. Fractionation and antitumor activity of the water soluble exopolysaccharide by submerged cultivation of *Ganoderma lucidum* mycelium. *Kor. J. Appl. Microbiol. Biotechnol.* **24:** 459–464.
6. Li, P. Z. and K. Q. Zhang. 2000. Purification and its biological activities of exo-polysaccharide of *Ganoderma lucidum*. *Acta Microbiol. Sinica* **40:** 217–220.
7. Lim, H. K., H. S. Kim, H. S. Choi, S. W. Oh, and C. G. Jang. 2000. Effects of acetylbergenin against D-galactosamine-induced hepatotoxicity in rats. *Pharm. Res.* **42:** 471–474.
8. Lin, Y. H. 1997. Studies on extraction and structure of the polysaccharide from *Ganoderma lucidum* strain GL 8801 by liquid fermentation. *J. Fuzhou Univ.* **25:** 124–128.
9. Luo, L. X., S. Q. Zhou, and R. H. Yao. 1998. Analysis of the structure of polysaccharide from the fermentation broth of *Ganoderma lucidum*. *Analytical Lab.* **17(4):** 17–21.
10. Mizuno, T., G. Wang, J. Zhang, H. Kawagishi, T. Nishitoba, and J. Li. 1995. Reishi, *Ganoderma lucidum* and *Ganoderma tsugae*: Bioactive substances and medicinal effects. *Food Reviews International* **11:** 151–166.
11. Ohkawa, H., N. Ohishi, and K. Yagi. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95:** 351–358.
12. Reitman, S. and S. Frankel. 1957. A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *Am. J. Clin. Pathol.* **28:** 56–63.
13. Sone, Y., R. Okura, N. Wada, E. Kishida, and A. Misaki. 1985. Structure and antitumor activities of the polysaccharide isolated from fruiting body and the growing culture of mycelium of *Ganoderma lucidum*. *Agr. Biolog. Chem.* **49:** 2641–2653.
14. Song, H. C., B. K. Yang, K. S. Ra, D. H. Shon, E. J. Park, G. I. Go, and Y. H. Kim. 1998. Hepatoprotective effect of extracellular polymer produced by submerged culture of GL WK-003. *J. Microbiol. Biotechnol.* **8:** 277–279.
15. Tseng, T. C., M. S. Shiao, Y. S. Shieh, and Y. Y. Hao. 1984. Study on *Ganoderma lucidum* I. Liquid culture and chemical composition of mycelium. *Biotanical Bull. Academia Sinica.* **25:** 149–157.
16. Yang, F. C. and C. B. Liau. 1998. The influence of environmental conditions on polysaccharide formation by *Ganoderma lucidum* in submerged cultures. *Process Biochem.* **33:** 547–553.
17. Yuka, N., O. Takuo, and A. Hiroko. 1999. Effects of geraniin on the liver in rats III - correlation between lipid accumulation and liver damage in CCl_4-treated rats. *Nat. Med.* **53:** 22–25.