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Hypoglycemic Activity of Grifola Frondosa Mycelium Polysaccharide and its Elementary Chemical Properties

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ABSTRACT

Grifola frondosa is a kind of precious edible and medicinal fungus. In this study, we used a solid-state fermentation method to grow *Grifola frondosa* mycelium. *Grifola frondosa* mycelium polysaccharide (GFP) was obtained by solid state fermentation, it was taken orally by alloxan induced diabetes mice in order to evaluate its hypoglycemic activity. After 28 days of GFP gavage in mice, the results showed GFP significantly reduced blood glucose and increased glucose tolerance in diabetic mice. And the low dose treatment has the best hypoglycemic effect. Further purification of GFP by DEAE Sephadex A-50 and Sephadex G-200 chromatography revealed that the main component was GFP-1. FT-IR spectroscopy and 1H NMR spectroscopy were used to investigate the structure of the GFP-1, and results revealed that it contained α-configuration and β-configuration. Our work suggested GFP might be used as a functional hypolipidaemic ingredient.

Keywords: Grifola frondosa; Solid State Fermentation; Polysaccharide; Hypoglycemic Activity; Chemical properties; AUC: Area Under the Curve

Abbreviations: DM: Diabetes Mellitus; PDA: Potato Dextrose Agar; NCG: Normal Control Group

Introduction

Diabetes Mellitus (DM) is a chronic endocrine and metabolic disease caused by genetic factors, immune disorders, microbial infections and their toxins, free radical toxins, mental factors, and other pathogenic factors that act on the body. A series of metabolic disorders such as sugar, protein, fat, water, and electrolytes, are caused by loss of insulin function and insulin resistance. Once the diabetes is out of control, it can cause many complications which would result in failure of the kidneys, eyes, feet and other parts, these diseases can hardly be cured. In addition, diabetes is an independent cause of coronary heart diseases, and complications of macrovascular disease are the main cause of death in patients with diabetes [1]. Currently, the main treatment of diabetes is taking oral hypoglycemic drugs. However, most of them have inescapable side effects on the human body, such as liver damage, gastrointestinal reactions and lactic acidosis. In the past few decades, fungal polysaccharides have been found to play a role in regulating blood glucose [2]. Therefore, it can be used as an adjunct in the treatment of patients with diabetes, which has stimulated scientists to pay special attention for it [3]. Polysaccharides are usually the main bioactive components of edible fungi and are natural macromolecular polymers. In the last decade, studies have shown that fungal polysaccharides generally have immunoregulatory, antioxidant, anti-tumor, anti-inflammatory and anti-fatigue effects. At present, many natural polysaccharides have been studied and proved to be safe and effective therapeutic drugs [4].

Grifola frondosa is a popular edible fungus in Asia due to its flavor, taste, and it has been proved to have positive biological effects in vivo for a long time [5]. Moreover, several studies have in-

dicated that G. frondosa polysaccharides own many significantly biological activities, such as hypoglycemic effects [6,7], anti-tumor [8,9], hypolipidemic effects [10], anti-oxidation [11,12], anti-virus [13], anti-inflammatory [14], immunomodulatory [4], etc. The production time of G. frondosa fruit body directly affect its market price. Furthermore, the long solid cultivation period, large area, and unstable production are not conducive to the development of G. frondosa. Therefore, the first report of liquid fermentation of G. frondosa emerged in 1986 [15]. Later, Suzuki I successfully cultivated G. frondosa mycelia in a similar method [16]. A large number of studies have focused on the production of bioactive compounds with submerged culture [17,18]. However, few previous studies have reported on bioactive polysaccharides produced by G. frondosa mycelia grown in solid state fermentation. In this study, liquid strains were used as solid-state fermentation strains, and herbaceous plants (Miscanthus floridulus) were used as the medium for solid-state fermentation of G. frondosa, and its hypoglycemic activity was investigated. A water-soluble crude polysaccharide was extracted from G. frondosa mycelia and further purified, and the structure of the main component GFP1 of G. frondosa polysaccharide was characterized.

Materials and Methods

Preparation of g. Frondosa mycelium polysaccharide

G. frondosa strain was obtained from the Centre of Fungal Research (Fujian Agriculture and Forestry University, Fuzhou, China). G. frondosa strain was inoculated on Potato Dextrose Agar (PDA) culture medium, and placed in 25 ℃ constant temperature incubator for 7 d. The seed cultures medium contained following components (in g/L): dextrose 30%, $MgSO_4$ 0.05%, KH_2PO_4 0.3%, peptone 0.5%, Vitamin B1 1mg, and the loading volume was 100 mL in 250 mL conical flask. Then, the seed culture medium was subjected to sterilization for 30 min at 121℃. After cooled, the mycelium in the PDA was inoculated to seed cultures medium for 3 d to obtain liquid strain. The liquid strain was inoculated to solid-state fermentation medium, which composed of 70% Miscanthus floridulus, corn flour 13%, wheat bran 15% and gypsum powder 2%, water content 60%. Solid-state fermentation was carried out in dark environment at 25 ℃ for 30 d.

The G. frondosa solid fermentation mycelium was dried in an oven at 70 ℃ and crushed in a mini-grinding machine. G. frondosa polysaccharide was extracted by a microwave oven at 450 W for 4 min, and maintained extraction for 4.5 h, solid-liquid ratio 1:45. Polysaccharide solution is filtrated by vacuum filtration. Three volumes of ethanol were slowly added to the polysaccharide solution and kept at 4 ℃ for 10 h. Precipitate was collected by centrifugation (2650×g, 10 min), then it was lyophilized (Tokyo Rikakaico. Ltd., Tokyo, Japan). Finally, crude G. frondosa mycelium polysaccharide (GFP) was obtained (polysaccharide content >70%).

Hypoglycemic Activity of Gfp In Vivo

Sixty male ICR mice were adaptively fed in the experimental environment for 3 days. All mice were given a normal diet and water. After 24 hours of fasting in all mice, ten of them were randomly selected as Normal Control Group (NCG) and injected with 0.9% saline, and another 50 were intraperitoneally injected with freshly prepared alloxan (2%) at a dose of 200 mg / kg. After 72 h, they were fasted for 5 h, and their blood was taken from the tail for determining blood glucose levels. The mice whose blood glucose levels higher than 11 mmol/L would be used as diabetes mice models. These model mice were randomly divided into 5 groups of 10 each. The information of group was listed in Table 1. These mice were taken metformin or GFP by gavage daily for 28 consecutive days. The body weight of the mice was measured every 4 days, and all the mice were fasted for 5 h before weighing and blood collection. On the 9^{th}, 18^{th} and 27^{th} day, blood was drawn from the tail vein of the mice. Blood glucose level was measured with a blood glucose meter (Yicheng Bioelectronics Technology Co., Ltd., Beijing, China). Blood glucose level and the decreasing ratio of blood glucose were compared among the groups. The decreasing ratio of blood glucose (DR) was calculated according to the following formula:

$$DR = \frac{L0 - L27}{L0} \times 100\%$$

a) DR—Decreasing ratio of blood glucose;

b) L0—blood glucose level of 0 day;

c) L27—blood glucose level of 27 d.

On the 28^{th} day, all groups were fasted for 4 h, then gavaged with a 250 mg/mL glucose solution at a dose of 2 g/kg. The blood glucose levels were determined after 0 min, 30 min and 120 min respectively, and the Area Under the Curve (AUC) was calculated according to the following formula:

Area Under the Curve (AUC) = 0.25×(blood glucose level of 0h + 4×blood glucose level of 0.5h + 3×blood glucose level of 2h). All mice were handled and euthanized according to ethical guidelines of College of Food Science, Fujian Agriculture and Forestry University.

Separation and Purification of Gfp

The GFP solution was deproteinized according to the Sevag method , and the ratio of GFP solution to the Sevag reagent (chloroform: butanol= 4:1, v/v) was 4:1. The mixture of the Sevag reagent and GFP solution was vigorously shaken for 30 min in a shaker. After that, it was placed in a separatory funnel to remove the denatured protein (middle layer) and the chloroform (bottom). This operation should be repeated for several times until the denatured

protein no longer appears in the middle layer. The deproteinized polysaccharide solution and the pretreated macroporous adsorption resin AB-8 (Huafu Chemical Industry Co., Ltd., Langfang, China) were mixed and shaken in a shaker at 120r/min for 5 h so that the adsorption resin could fully adsorb the pigment. This operation was repeated for several times until the color of solution was hardly seen. The solution is dialyzed with 14kD dialysis bags (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) to remove low molecular weight impurities and residual pigments. The solution was purified with a DEAE Sephadex A-50 chromatography (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) column. Thus, pure polysaccharide solution was collected and concentrated.

Purity Identification of Polysaccharide

The preliminary purification polysaccharide solution was scanned in full wavelength to observe whether there is protein absorption zone and pigment absorption zone. The solution was further purified and separated with a Sephadex G-200 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) column to determine the purity of the polysaccharide after purification by DEAE Sephadex A-50 [19]. The main purified single-component polysaccharides GFP-1 was concentrated and freeze-dried.

Fourier Transform Infrared (Ft-Ir) Analysis of Gfp-1

A certain amount of GFP-1 was mixed with dried KBr powder, and then they were pressed into tablets after ground. The tablet was scanned in the range of 4000 cm^{-1} -400 cm^{-1} by fourier transform infrared spectrometer (Thermo Nicolet Co., Ltd., America).

1H NMR Analysis of GFP

GFP-1 was dissolved in 0.5 ml of deuterated methanol (Cambridge Isotope Laboratorics Co., Ltd., America) and lyophilized for 3 times. The 1H NMR spectra were recorded on a 400 MHz Bruker Biospin spectrometer (Avance III Bruker Biospin, Rheinstetten, Germany) at 30°C [20].

Statistical Analysis

Data was expressed as the mean ± S.E. and analyzed by oneway ANOVA followed by Tukey's test by using SPSS 13.0 software (International Business Machines Corporation, Chicago, USA). $P<0.05$ was considered statistically significant.

Results and Discussion

Hypoglycemic Activity of Gfp in Vivo

After making model by alloxan, the mice showed typical symptoms of diabetes, such as polydipsia, polyphagia, polyuria, and weight loss in different extent [21]. After 27 days of feeding, the weight increased nearly 7 g in normal control mice, which indicated the experimental environment and the other conditions are suitable for mice growth. The results shown in Table 2 indicate that in the 0th day, the body weight of the normal control group was significantly higher than that of the other five hyperglycemia groups, which is consistent with the symptoms of diabetes. The weight of the high-dose group was significantly lower than that of the metformin group ($P<0.05$), indicating that the high-dose treatment was inferior to the low-dose and medium-dose. Table 3 shows that there was no significant difference among the model group, metformin group and three GFP group on the 0th day. After 9 days of continuous treatment with different doses of GFP, the blood glucose levels of the three different doses of GFP treated group decreased by 1.77 mmol/L, 1.35 mmol/L and 1.43 mmol/L, respectively, as compared with 0th day. However, there was no significant difference among the three groups.

Table 1: The grouping of experimental animals.

Groups	Drugs	Dose
Normal	0.9% saline	0.3 ml
Model	0.9% saline	0.3 ml
Metformin	Metformin	200 mg/kg
Low dose	GFP solution	100 mg/kg
Medium dose	GFP solution	200 mg/kg
High dose	GFP solution	400 mg/kg

Table 2: Effect of GFP on the weights of diabetic rats (g).

Group	Day 0	Day 4	Day 8	Day 12	Day 16	Day 20	Day 24
Normal	33.24± 2.08a	34.84± 2.00a	35.30± 2.56a	37.48± 2.65a	38.54± 3.50a	39.17± 2.83a	40.08± 3.24a
Model	20.97± 4.79b	22.87± 3.71b	24.80± 4.01bc	25.41± 4.43bc	25.87± 5.21bc	23.58± 5.65cd	26.18± 6.02c
Metformin	23.85± 3.39b	25.91± 3.90b	28.62± 3.90bc	28.26± 3.77b	29.19± 3.65b	29.29± 3.51b	32.64± 3.22b

Low dose	23.85± 4.18b	26.09± 2.30b	26.09± 2.74bc	27.48± 2.74bc	28.27± 2.37bc	26.81± 3.57bc	29.69± 3.00bc
Medium dose	22.19± 3.74b	24.01± 4.61b	25.21± 5.14bc	25.82± 4.36bc	25.63± 5.38bc	27.87± 4.29bc	29.03± 2.73bc
High dose	22.90± 2.67b	22.38± 1.81b	22.76± 2.31c	22.63± 3.03c	23.48± 3.06c	21.03± 3.25d	24.68± 4.21c

Note: The data are expressed as the mean ± SD. The results of LSD analysis of a single-factor complete randomized trial, a, b, c, d, e different letters in the same column indicate significant differences (P<0.05, n=10).

Table 3: Effect of GFP on blood glucose of diabetic rats (mmol/L).

Groups	Day 0	Day 9	Day 18	Day 27	HR %
Normal	5.73± 0.52b	6.75± 0.33b	6.50± 0.25d	6.23± 0.67e	—
Model	18.41± 5.83a	17.57± 4.99a	18.7± 1.27a	20.28± 2.78a	—
Metformin	18.54± 5.16a	15.27± 3.35a	11.04± 4.47c	8.90± 4.73de	51.99
Low dose	18.13± 5.56a	16.36± 4.85a	14.31± 3.80bc	12.01± 3.84bcd	33.74
Medium dose	18.56± 5.02a	17.21± 4.08a	16.18± 3.99ab	14.61± 3.87bc	21.29
High dose	18.84± 5.56a	17.41± 4.94a	16.21± 4.99ab	14.91± 5.23b	20.85

Note: The data are expressed as the mean ± SD. The results of LSD analysis of a single-factor complete randomized trial, $^{a, b, c, d, e}$ different letters in the same column indicate significant differences (P<0.05, n=10).

The normal group showed a slight increase in blood glucose, but it was in the normal range. After 18 days of treatment, the blood glucose level of the low-dose group was significantly different from that of the model control group (P<0.05). As compared with the 0th day, the blood glucose level of the low-dose group decreased by 3.82 mmol/L, and the rate reached 21.07%.

There was no significant difference between the medium dose group and the high dose group. After 27 days, the blood glucose levels of all treated groups were further decreased, and there was a significant difference between the treated groups and the model group (P<0.05). However, no significant difference was found among three GFP groups. The blood glucose level of low-dose group decreased by 6.12mmol/L, and its hypoglycemic rate reached 33.74%. The blood glucose level of the GFP low-dose group was close to the diagnostic criteria. These results show that the low dose of GFP has significant hypoglycemic effect. On the 28th day, the effect of GFP on glucose tolerance and AUC in diabetic mice was studied. The effect of GFP on the glucose tolerance and AUC of diabetic rats is showed in Table 4. From 0h to 2h, the blood glucose levels were much lower in GFP group than that of model group, and the lowest blood glucose level was observed in low-dose GFP group. The AUC of the low dose group is the minimum in all treatment groups, which was significantly lower than that of the other groups (P<0.05). Interestingly, the AUC of GFP medium dose group and GFP high dose group was significantly lower than model group (P<0.05). This is consistent with the conclusions of the blood glucose test results that the hypoglycemic effect of the low dose treatment is the best.

Table 4: Effect of GFP on the glucose tolerance of diabetic rats (mmol/L).

Group	Glucose tolerance (mmol/L)			AUC
	0 h	0.5 h	2 h	
Model	20.33±3.43	21.05±1.73	19.95±1.29	41.095±2.301a
Low dose	12.07±4.52	12.72±1.64	10.61±4.39	23.695±3.015b
Medium dose	14.58±1.40	16.58±2.37	13.98±2.01	30.710±1.384cd
High dose	14.91±3.32	17.01±0.75	16.23±0.92	32.910±2.374cd

Note: The results of ANOVA analysis, $^{a, b, c, d}$ different letters in the same column indicate significant differences (P<0.05, n=10).

Purification of Crude Gfp

Figure 1 shows the purification curve of GFP by DEAE Sephadex A-50. After orderly eluted by distilled water and NaCl solutions (0.1 mol/L, 0.3 mol/L), three kinds of polysaccharide component was obtained, named GFP1, GFP2, and GFP3, respectively. The GFP1 were located in the 4th to 20th tubes, GFP2 were located in 30th to 59th tubes, and GFP3 were located in the 63rd to 91st tubes. A higher concentration of NaCl solution (0.5 mol/L) was sequentially employed to elute until 100th tube, but there was no more polysaccharide components. The Figure 1 shows that the main component of GFP is GFP-1. Afterwards, the content of protein in GFP1 was determined by Uv-vis scanning at wavelength of 200-700 nm (Figure 2), and then the purity of GFP1 was identified by Sephadex G-200. The Uv-vis scanning curve of GFP1 is showed in Figure 2. There was no absorbance peak at 250-300 nm, indicating that GFP1 contained no nucleic acid or protein components. The absorbance value of GFP1 was closed to zero in wavelengths of 300-700 nm, which suggested that few pigment was in GFP1. Therefore, it could be initially determined that GFP1 was pure. Figure 3 shows the purification curve of GFP1 by Sephadex G-200. As for GFP1, only one absorption peak in tubes 2-15 was observed, indicating that GFP1 was a single-component polysaccharide. Therefore, the structure of GFP1 was further analyzed using FT-IR spectroscopy and 1H NMR spectroscopy.

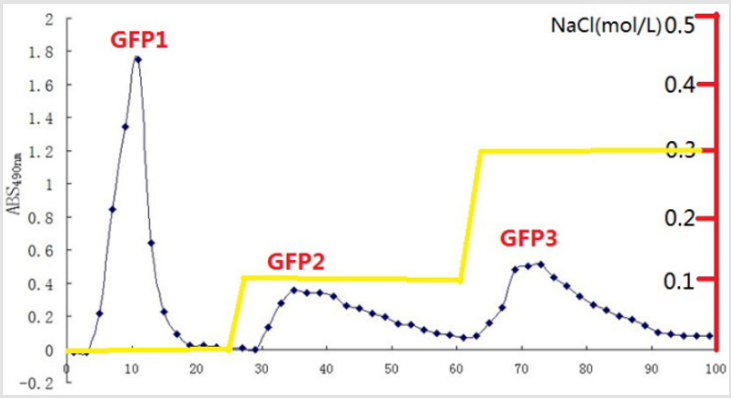

Figure 1: Purification curve of GFP by DEAE Sephadex A-50.

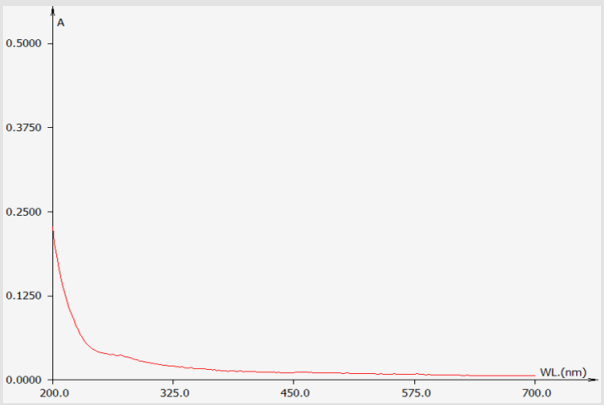

Figure 2: Uv-vis scanning curve of GFP1.

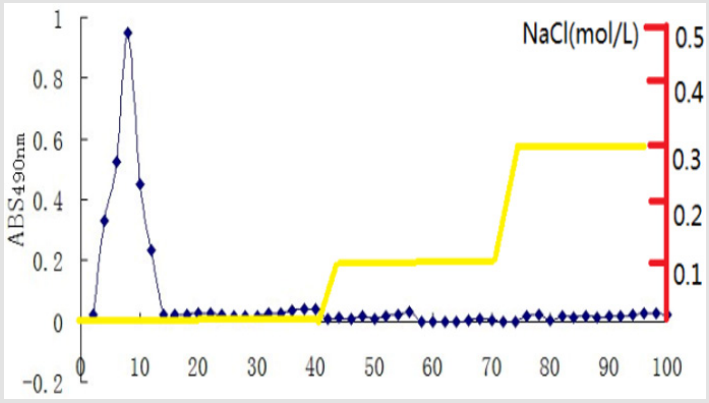

Figure 3: Purification curve of GFP1 by Sephadex G-200.

Ft-Ir Spectroscopy Analysis

The FT-IR spectrum of GFP1 is showed in Figure 4A. The absorption peaks in the range of 800-975 cm^{-1} indicated the existence of α- and β-configurations. Peaks at 1200-1000 cm^{-1} indicated the stretching vibration of C-O-H in carboxyl and ether bond C-O-C in pyran ring. The absorption between 1800 and 1600 cm^{-1} can be attributed to vibration of C=O and C=C, while absorption between 3010 and 2850 cm^{-1} were designated as the stretching vibration of $-CH_2$ and $-CH_3$ [22]. The stretching vibration of O-H and N-H was generally in the range of 3700-3100 cm^{-1}, showing a broad absorption peak, which indicated the existence of intermolecular and intramolecular hydrogen bonds [23]. The absorption peak of the free hydroxyl is generally in the range of 3650-3580 cm^{-1}, of which shape is sharp and there is no interference of other absorption peak. However, since the hydroxyl group is a strongly polar group and the association phenomenon is remarkable. The absorption peak of the O-H moves to the low wave-number, and a broad and strong absorption peak appeared in the range of 3500-3200 cm^{-1}. Therefore, the absorption peak at 3409 cm^{-1} indicated the existence of O-H, and the absorption peak at 2935 cm^{-1} indicated the existence of C-H [24].

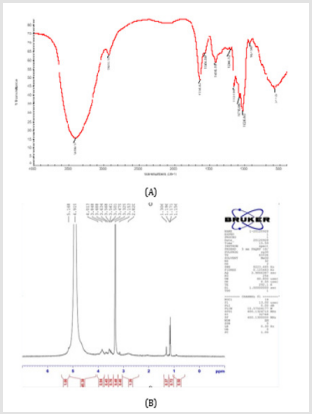

Figure 4: FT-IR spectrum (A) and ^1H NMR spectrum (B) of GFP1.

There was no absorption at 1700-1775 cm-1, indicating that there was no carboxyl in it. As a result, GFP1 did not contain glucuronic acid and it was a kind of neutral sugar. The absorption peak at 1636 cm^{-1} was the stretching vibration of C=O, and the range of 1026-1204 cm^{-1} was the stretching vibration of C-O-C and C-O. A weak absorption peak near 927 cm^{-1} was the absorption peak caused by the symmetrical stretching vibration of C-C and C-O in arabinose furan rings [25].

^{1}h Nmr Spectroscopy Analysis of Gfp1

The 1H NMR spectrum was mainly used to solve configuration of glycosidic bond, and the signal of polysaccharides was mainly in the narrow range of δ3.0-5.5 ppm. The 1H NMR spectrum of GFP1 is showed in Figure 4B. The δ value of anomeric hydrogen in α-configuration glycoside is greater than 5.0 ppm, thus δ5.168 ppm was the chemical shift of H-1, indicating that GFP1 had the glycosidic bond of α-configuration. The δ4.919 ppm was the signal of

β-configuration glycosidic bond, and δ3.5-4.0 ppm was the signal of the sugar ring proton [26,27], while the δ2.6-3.2 ppm was the signal of carbohydrates, indicating that the sample contains carbohydrates.

Conclusion

In our study, the GFP from the mycelia of G. frondosa cultured by solid state fermentation has hypoglycemic property. The results showed that the low-dose of GFP showed significant hypoglycemic activity. Moreover, pure GFP1 was separated by column chromatography and structurally analyzed by FT-IR and 1H NMR, suggesting that GFP1 had the glycosidic bond of α-configuration and β-configuration. However, it is necessary to study the key mechanisms of GFP1, GFP2 and GFP3 hypoglycemic activity, respectively. In addition, their molecular structure and monosaccharide composition need to be researched in the future.

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Conflict of Interest

All authors have no competing interest to declare.

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