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A polysaccharide from *Grifola frondosa* relieves insulin resistance of HepG2 cell by Akt-GSK-3 pathway

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Abstract *Grifola frondosa* is an important fungal research resource. However, there was little report about hyperglycemic activity of *Grifola frondosa* polysaccharide on insulin resistance *in vitro*. In this study, the hypoglycemic activity of a polysaccharide obtained from *Grifola frondosa* (GFP) on HepG2 cell and hpyerglycemic mechanism were investigated. The purity of the isolated polysaccharides was examined by HPLC. In this research, it was found that GFP enhanced the absorption of glucose of HepG2 cells in a dose dependent manner at 24 h of 30 ugmL^{-1}. GC-MS and FT-IR spectroscopy analysis results showed that glucose and galactose were the dominant monosaccharides in *GFP* and the major component of GFP was β-pyranoside. Western-blotting results showed that the HepG2 cell model treated with GFP activated the insulin receptor protein (IRS) in the cell membrane and increased phosphorylated-AktSer473 expression, which had an inhibition of glycogen synthase kinase (GSK-3). The down-regulation of GSK-3 stimulated synthesis of intracellular glycogen. The results above suggested that the GFP increased the metabolism of glucose and stimulated synthesis of intracellular glycogen through the Akt/GSK-3 pathway.

Keyworlds · *Grifola frondosa* polysaccharide · Hypoglycemic activity · Insulin resistance · Akt/p-Akt · GSK-3

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Introduction

Grifola frondosa, is an edible basidiomycete fungus belonging to the Polyporaceae family. It has been reported that fruit bodies and mycelium from liquid-cultured contain many biologically active compounds, and it has been a hot research subject with development potential as functional foods and physiologically beneficial medicine. [1, 5, 12].

According report, approximately 5 % of population worldwide are suffering by type 2 diabetes mellitus (T2DM) and the number of Type 2 diabetes is increasing by years [22], characterized by abnormalities in carbohydrate and lipid metabolism, which lead to postprandial and fasting hyperglycemia, dyslipidemia, and relative insulin secretion shortage [6]. At present, there are many medications for type2 diabetes, such as Rosiglitazone, Metformin, Acarbose and others. However, so many clinical researches have reported that long-term use of these medicines along with drug resistance afford side effects of drug (Ling Yi [18]).

Insulin-sensitive tissues, such as liver, fat, and muscle are typically involved in regulating whole body fuel metabolism [17]. In the insulin signaling pathway, glucose transport pathway of insulin signaling includes PI3K, Akt, glycogen synthasekinase-3β (GSK-3β), and glucose transport protein-4 (Glut-4). GSK-3 activity can be acutely activated by insulin signaling through insulin receptor substrate-1 B (Akt) to phosphorylate specific serine residues on the enzyme [4]. An additional substrate of GSK-3 is IRS-1, and phosphorylation of IRS-1 on serine/threonine residues leads to impairment of insulin signaling [7]. Therefore, GSK-3 can be a negative modulator of insulin action on GS and, potentially, on glucose transport activity.

Nowadays, it is a trendency of using natural products as an alternative treatment of type II diabetes for its safety and diversity [14]. It has been reported that rhein exerts anti-hypoglycemic effect through agitating PPAR-c [21]. And previous research has demonstrated the activities on insulin-

dependent diabetes mellitus (Hirotada [15]) and anti-diabetic activities in *Grifola frondosa* [16]. However, little research has been reported about hyperglycemic mechanism of *Grifola frondosa* polysaccharide in cell. In this study, the mechanism of hypoglycemic by polysaccharides of *Grifola frondosa* was investigated in insulin resistance model cells. The GFP (polysaccharides of *G. frondosa*) influenced the expression of Akt/GSK-3. The results demonstrated that GFP ameliorated insulin resistance probably by modulating Akt/GSK-3 pathway in HepG2 cells. Furthermore, GFP might be promising for clinical application to treat type 2 diabetes mellitus.

Materials and methods

Materials

Human liver cancer cells HepG2 was obtained from Academy Joint Institute of Tianjin (Tianjin, PR China). HepG2 cells were used in this study due to their common physiological function of glucose metabolism with normal hepatic cells [23]. HepG2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Hyclone) supplemented with 10 % fetal bovine serum (FBS, GIBCO), 50 U ml^{-1} penicillin (Solarbio) and 50 U ml^{-1} streptomycin (Solarbio). All cells were cultivated at 37 ◦C with 5 % CO_2. The p-Akt, Akt, G-6-p, GSK-3, β-actin antibodies, were purchased from Santa Cruz (California, USA).

Extraction and purification of G. frondosa polysaccharides

GFP was extracted from *G. frondosa* (Tianjin, China) and purified as described previously. [24]. Briefly, powder of *G. frondosa* was extracted twice with 10 volumes of water at 80 °C for 3 h, followed by centrifugatio. The concentrated supernatant was precipitated by 4 volumes of ethanol. There precipitate was redissolved and centrifuged. The supernatant was lyophilized to obtain crude polysaccharide. The crude product was further purified using Sephadex G-75, eluted with distilled water and monitored using the phenol–sulfuric acid method [10, 11]. The flow rate was 0.2 $mlmin^{-1}$.

The purity and molecular weight of GFP were determined by high performance liquid gel permeation chromatography (HPLC).

GC–MS analysis

GC analyses were performed on a Shimadzu gas chromatograph coupled to a Shimadzu spectrometer and provided with a split/splitless injection port. Helium was the carrier gas, at a linear velocity of 38 cms^{-1}. The injector temperature was 250 ◦C, and samples (1ul) were injected in the splitless mode. The temperatures of the ion source and the transfer line were 175 and 280 ◦C, respectively.

Fourier-transformed infrared spectroscopy

The major structural groups of purified *G. frondosa* polysaccharides were detected using Fourier-transformed infrared (FT-IR) spectroscopy. Sample for infrared analysis were obtained by grinding a mixture of 1 mg *G. frondosa* polysaccharides with 200 mg dry KBr, followed by pressing the mixture into a 16-mm-diameter mould. The FT-IR spectra were recorded in the region of 4,000–500 cm^{-1} on a VECTOR 22 FT-IR system (Bruker, Switzerland).

Periodate oxidation and Smith degradation

GFP (50 mg) dissolved in 25 ml of distilled water was mixed with 25 ml of 30 mM $NaIO_4$, and the mixture was kept in dark at 4◦C with occasional shocks. About 0.1 ml periodate-oxidized product were withdraw from the mixture at 12 h intervals and read in a spectrophotometer at 223 nm after it was diluted to 25 ml. Ethylene glycol (2 ml) was added to terminate the reaction after 5 days. About 2 ml periodate-oxidized product was used to calculate the yield of formic acid by 0.0005 M NaOH. After 0.1 g NaBH4 was added to periodate-oxidized product for 20 h, neutralized with 0.1 mol acetic acid, and then dialyzed. Then the solution mentioned above was freeze-dried and fully hydrolyzed for GC analysis.

HepG2 cells insulin resistance cell model

The insulin resistance cell model of HepG2 cells was induced as previous study described [3, 13, 19]. Briefly, HepG2 (3×10^5 $ml^{-1}well^{-1}$) were seeded into each well of a 96-well flat-bottomed culture plate in their exponential growth phase. After incubation for 24 h, culture medium with 10^{-6} $molL^{-1}$ insulin was added into each well. After incubation for 36 h, the medium was aspirated and each well was washed with PBS

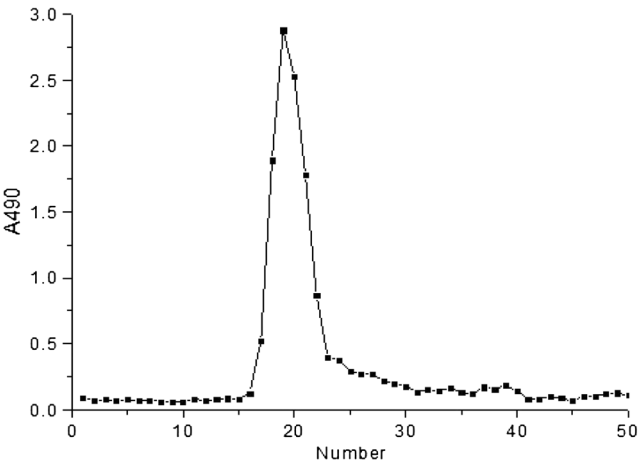

Fig. 1 Elution curve of crude G. frondosa polysaccharides on a column (1.6 cm×80 cm) of Sephadex G-75

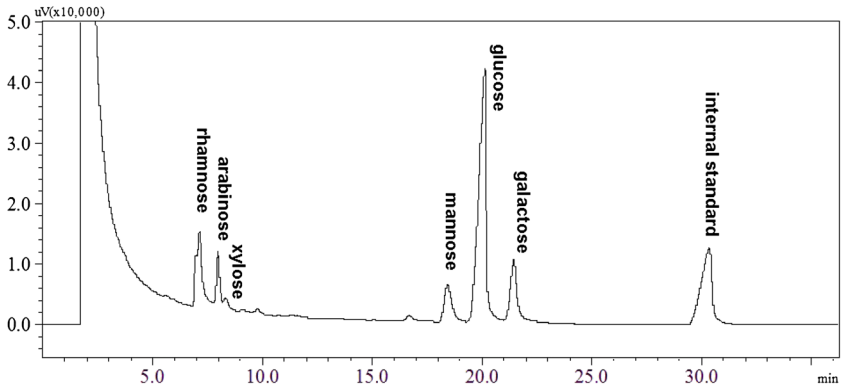

Fig. 2 Monosaccharide composition analysis of *G. frondosa* polysaccharides by gas chromatogram

once, then serum-free medium with or without GFP was added to cell for incubation.

Detection of glucose concentration in medium

In accordance with 2.4., insulin resistance model of HepG2 was established, medium collected at different time and detected by glucose test kit (Biovision Co.).

The detection of Intracellular glycogen content

In accordance with 2.6., HepG2 cell model was established, various concentrations of GFP were added into each well and each concentration was repeated in six wells. After incubation for a certain time, cells were collected for detecting by glycogen test kit (Solarbio Co.).

PAS stain

Briefly, the cell suspension (1×10^5 ml^{-1}) was inoculated on cover slips, which were partitioned previously into the wells of 6-well plates. After 36 h treated with insulin, HepG2 cells were treated with 30ugml^{-1} GFP for 12 h, 24 h, 36 h and 48 h, and then, pre-fixed with 2.5 % glutaraldehyde at 4 ◦C for 1 h. The cells were then rinsed thoroughly in PBS and incubated with periodic acid for 5–10 min, and washed with PBS for 3 times and incubated with schiff stain for 25 min, washed with distilled water for several times. And fluorescence microscope was investigated the changes of intracellular glycogen.

Detection of intracellular pyruvate kinase

After incubation for a certain time, cells were collected, and cell lysis buffer was added to cells for 15 min on ice, centrifuged at 5,000 r/min and 4 ◦C for 20 min. The supernatant was collected, enzyme activity was determined by kit (Sigma Co.) according to kit instructions.

Western blotting analysis

In order to investigate the mechanisms of relieving insulin resistance, the expression of relative glycometabolism protein

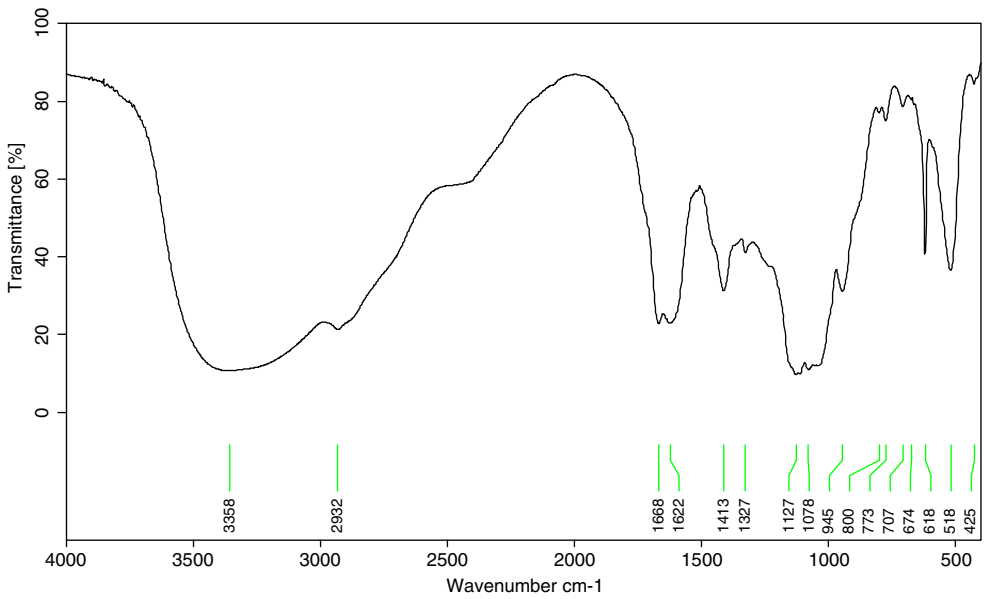

Fig. 3 FT-IR spectroscopy of Grey Polypore polysaccharides

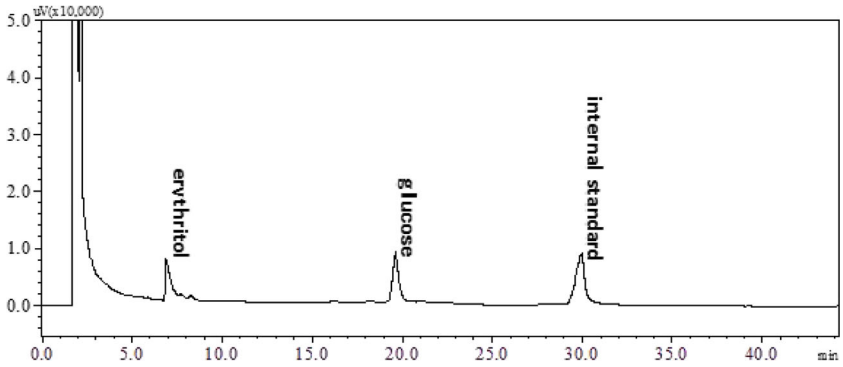

Fig. 4 GC-MS analysis of periodate-oxidized products

was detected by Western blot. Protein extracts of HepG2 cells treated with or without GFP were prepared by lysing cells in RIPA Lysis buffer with 1 % PMSF on ice for 15 min, after centrifugatio supernatant was collected. In brief, cell protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then blocked in a blocking buffer (Tris=20 mM, pH 7.6, NaCl=150 mM and Tween 20= 0.1 %) containing 5 % non-fat dry milk powder and incubated with the primary antibody overnight at room temperature. The nitrocellulose membranes were subsequently washed and incubated for 1 h at room temperature with the secondary antibodies conjugated with horseradish peroxidase (Amersham Pharmacia Biotech). Immunoreactive bands were detected by DAB kit (Zhongshan Goldenbridge, Beijing, China). Western blot analysis was carried out by the method as described previously [2].

Results

Purification of G. frondosa polysaccharides

The crude polysaccharide was prepared from the G. frondose by hot-water extraction, EtOH precipitation and dialysis. As Fig. 1 showed, *G. frondosa* polysaccharides was eluted as a single symmetrical peak corresponding to an average molecular weight of 4.53×10^4 as determined by gel filtration (Fig. 2).

GC–MS analysis of monosaccharides

By comparing retention times of unknown peaks with reference sugar standards and cochromatography with added standards (rhamnose, arabinose, xylose, mannose, glucose and galactose), six monosaccharides including rhamnose, arabinose, xylose, mannose, glucose and galactose were identified, with the retention times being 7.146, 7.975, 8.321,18.424, 20.146 and 20.457 min, respectively, and the molar ratio being 4.74:5:1:3.42:31.29:6.89. This results clearly demonstrated that both glucose and galactose were the dominant monosaccharides in *G. frondosa* polysaccharides.

FT-IR spectra analysis of G. frondosa polysaccharides

As shown in Fig. 3, the IR spectrum of GFP revealed a typical major broad stretching peak around 3,358 cm^{-1} for the hydroxyl group, and the small band at around 2,932 cm^{-1} was attributed to the C–H stretching and bending vibrations. The relatively strong absorption peak at around 1,622 cm^{-1} reflects the absorption of the C–O group that is part of glycosides [26].

Table 1 GC analysis result of completely acid hydrolysis, periodate-oxidized products and Smith degradation of GFP

Fractions	Molar ratios							
	Glycerol	Erythritol	Rhamnose	Arabinose	Xylose	Mannose	Glucose	Galactose
Completely acid hydrolysis			4.74	5	1	3.42	31.29	6.89
Periodate oxidized products								
Smith degradation	0	10.35	1.23	1			8.97	
Type of linkage								
(1→2) linked			×	×	×	×	×	×
(1→3),(1→2,3),(1→2,4) (1→3,4), (1→3,6),(1→2,3,6) linked			2.96	2.41	×	×	13.02	×
(1→4),(1→4,6) linked			1.77	2.59	1	3.42	18.26	6.89
(1→6) linked			×	×	×	×	×	×

The wave number between 950 and 1,200 cm^{-1} is often called the fingerprint of molecules because it allows the identification of major chemical groups in polysaccharides: the position and intensity of the bands that are specific for each polysaccharide [9]. The *G. frondosa* polysaccharides had IR bands at 1,000–1,100 cm^{-1}, 1,400–1,530 cm^{-1}, 2,900–3,000 cm^{-1}, and 3,100–3,500 cm^{-1}, which were distinctive absorptions of polysaccharides. *G. frondosa* polysaccharides exhibited a typical absorption of β configuration at 945 cm^{-1}. The adsorption peaks of 1,413 cm^{-1}, 1,127 cm^{-1} and 945 cm^{-1} suggested the polysaccharide consisted of pyranoside (Fig. 4).

Structural analysis

Results from periodate oxidation showed that 0.031 mmol per-iodated was consumed and no formic acid was produced, indicating the no monosaccharides which was 1→linked or (1→6)-linked.

The periodate-oxidized products were fully hydrolyzed and analyzed by GC analysis (Table 1). The existence of part of Glc revealed lots of Glc residues was (1→3)-linked, (1→2,3)-linked, (1→2,3,4)-linked that could not be oxidized. And another part of Glc was 1→4 or 1→4,6 linked. No Gly, Xyl, Man and Gal were observed and large amount of erythritol

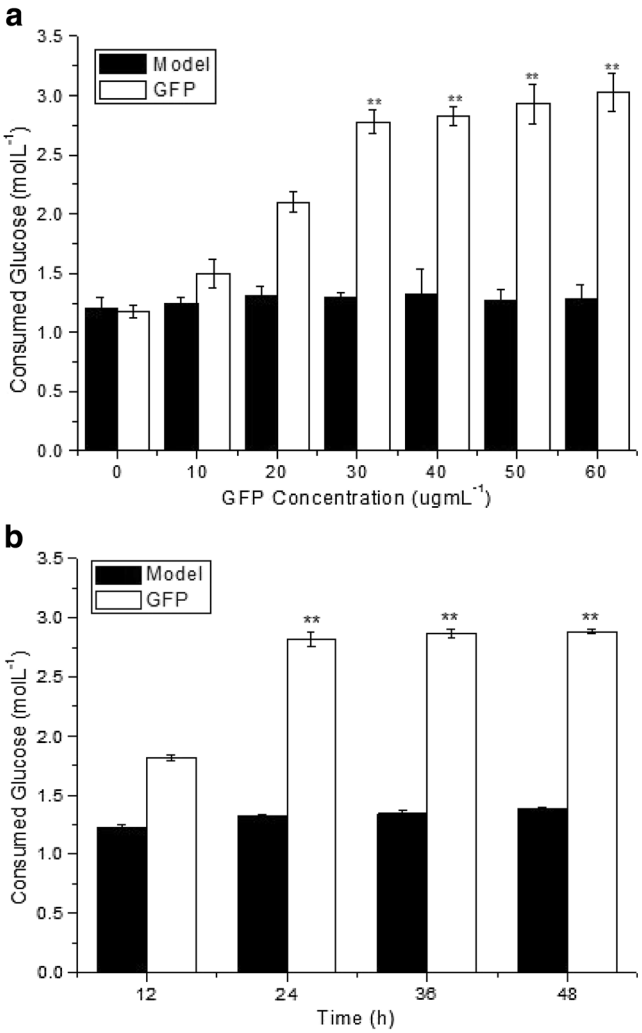

Fig. 5 **a** Dose-dependent effect of GFP on glucose consumption of HepG2, $^{**}P<0.01$, $^{*}P<0.05$ compared to control group. Data represent means ± SD of three independent experiments. **b** Glucose consumption of HepG2 treated with GFP, $^{**}P<0.01$,* $P<0.05$ compared to control group. Data represent means ± SD of three independent experiments

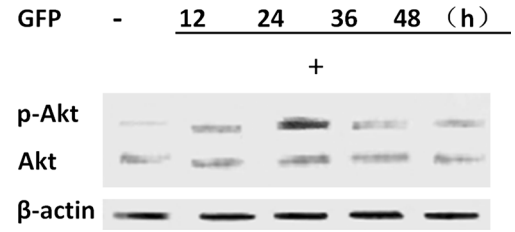

Fig. 6 Expression of Akt in HepG2 cells were evaluated by Western blot analysis. β-actin was used as an equal loading control

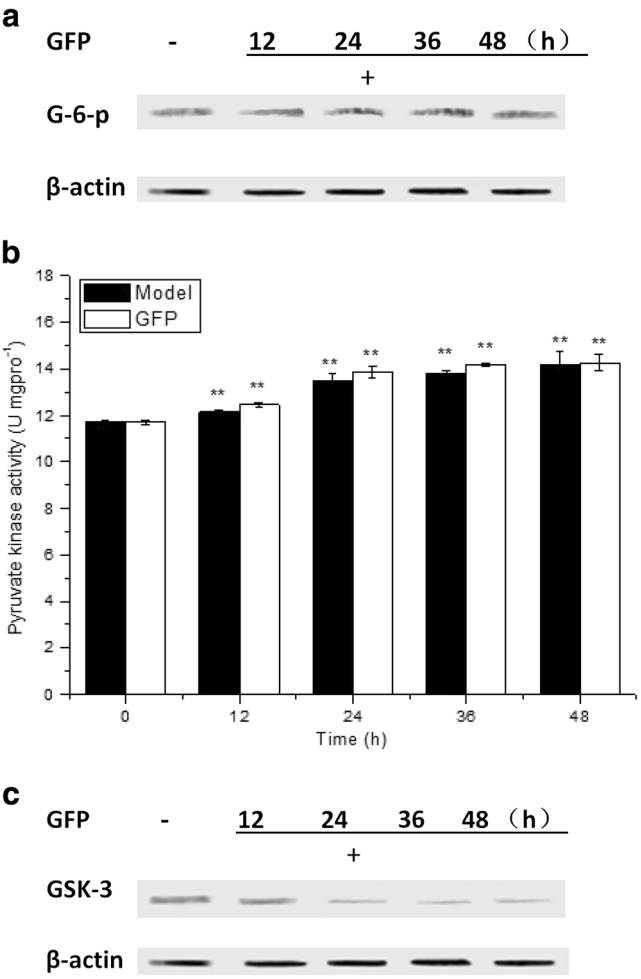

Fig. 7 **a** Expression of G-6-p in HepG2 cells were evaluated by Western blot analysis. β-actin was used as an equal loading control. **b** Effects of GFP on Pyruvate kinase activity in HepG2 cell model Data represent means ± SD of three independent experiments. **c** Expression of GSK-3 in HepG2 cells were evaluated by Western blot analysis. β-actin was used as an equal loading control

were obtained, demonstrating that Rha, Ara, Xyl, Man, Gal were all linkages as 1→4 or 1→4,6 linked which can be oxidized by periodate. Results of Smith degradation analysis were summarized in Tab. 1. There were large amounts of precipitation in the dialysis sack, demonstrated the backbone of GFP could not oxidized by HIO4.

The results from analysis of GC-MS, which were consistent with the completely acid hydrolysis, periodate oxidation and Smith degradation, indicated that (1,4)-linked Glc and (1,3)-linked Glc were possible the major components of the backbone structure; part of Rha, Ara, Glc may distribute in branches.

Effect of GFP on glucose consumption of HepG2 cell

As the Fig. 5a showed, GFP effected glucose consumption in a dose-dependent manner, with 129.17 % growth in concentration 30 $ugmL^{-1}$. With concentration increased, consumed glucose with no significant change. These results suggested that GFP had remarkably enhanced the absorption of glucose at 30$ugmL^{-1}$ *in vitro*.

As the Fig. 5b showed, the consumed glucose of model group didn't change significantly, which indicated that insulin resistance of model group could sustain for 48 h at least. And the glucose consumption of GFP treated group increased gradually from 1.87 $mmolL^{-1}$(12 h) to 2.88 $mmoLL^{-1}$(48 h). Compared with model group, the glucose consumption of GFP treated group increased 129.51 %. This showed that GFP significantly improved the insulin resistance, and improved the absorption of glucose from culture medium. The results showed that the GFP may activate the insulin related pathway to improve the ability of carbohydrate metabolism of HepG2 and ameliorated insulin resistance.

Effect of GFP on Akt expression

In order to investigate whether Akt is involved in GFP ameliorated insulin resistance, the effect of GFP on Akt in HepG2 cells was analyzed. As shown in Fig. 6, compared with the control group, the expression of Akt have no significant change, but the p-Akt increased significantly especially for 24 h. In short, the results indicated that the GFP may ameliorate insulin resistance of HepG2 through activating Akt pathway.

Effect of GFP on key enzyme

In order to investigate whether another pathway was involved in GFP ameliorated insulin resistance, we analyzed key

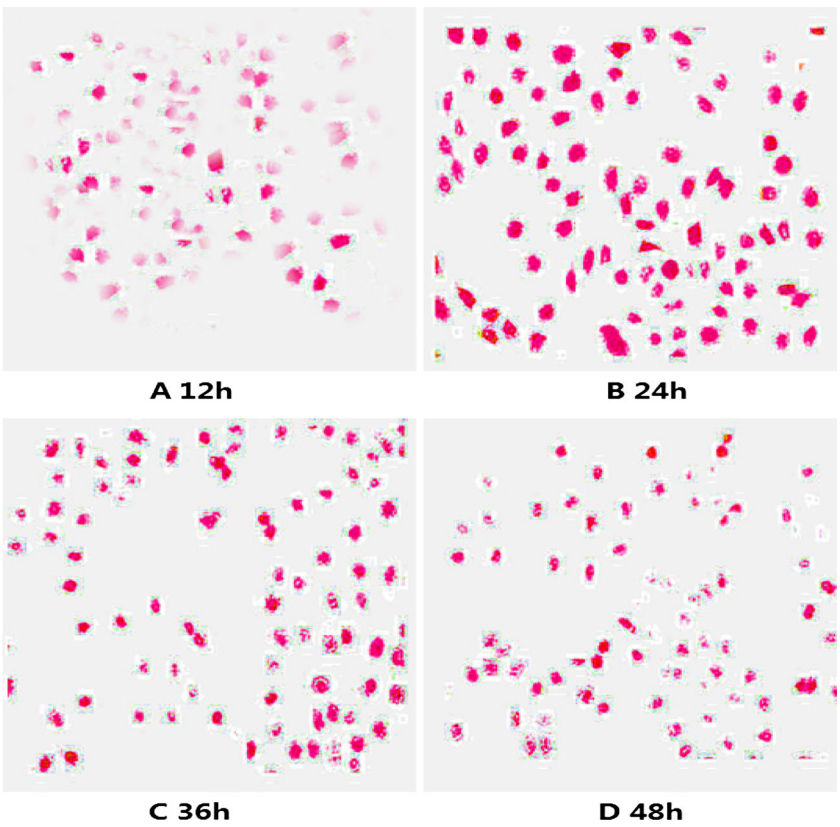

Fig. 8 Insulin resistance cell model of HepG2 Periodic acid-Schiff Staining (×100)

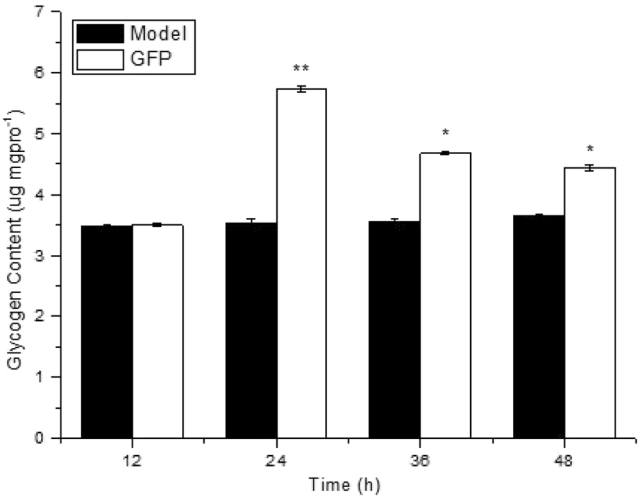

Fig. 9 Detection of intracellular glycogen in HepG2, $^{**}P<0.01$,* $P<0.05$ compared to control group. Data represent means ± SD of three independent experiments

proteins of gluconeogenesis by Western blot analysis. As the Fig. 7a showed, compared with normal control group, the expression of intracellular glucose 6 protein phosphatase of GFP treated group was no significant difference. It suggested that there was no significant inhibition on gluconeogenesis and intracellular glucose synthesis. Overall GFP didn't regulate metabolism process of gluconeogenesis to improve glucose metabolism.

In order to investigate whether glycolysis was involved in GFP ameliorated insulin resistance, pyruvate kinase activity was detected as a key factor in glycolysis irreversible reaction. As the Fig. 7b showed, the pyruvate kinase activity of GFP treated group showed a small increase in a time-dependent manner, which showed a same tendency with the model control group. These results suggested that the increase of pyruvate kinase activity may caused by cellular growth. Briefly, GFP didn't improve glucose metabolism through strengthening glycolysis.

GSK-3β is a key enzyme involved in glycogen metabolism, in the insulin signaling pathway controlled by insulin. In order to investigate whether glycogen synthesis was involved in GFP ameliorated insulin resistance; GSK-3 expression was detected after a different time exposure of GFP. As the Fig. 7c showed, after effecting time for 24 h, the expression of GSK-3 was significantly decreased that enhanced synthesis of intracellular glycogen. The results showed that GFP promoted glycogen synthesis through activating Akt pathway and mediating inhibition of glycogen synthase kinase 3 (GSK-3) expressions.

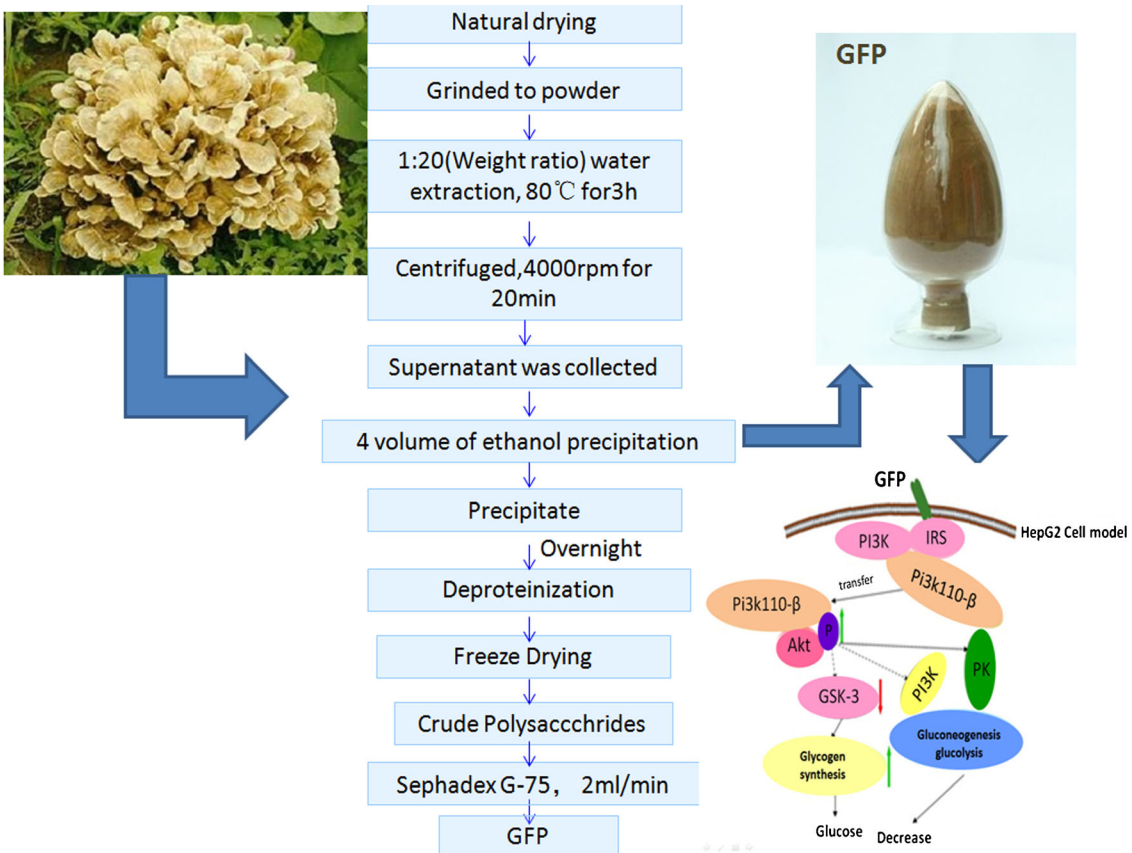

Fig. 10 The extraction process of GFP and the pathways involved in relieve insulin resistance effect of GFP in HepG2 cells

Detected the contents of intracellular glycogen

Liver plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification. In this study, periodic acid-schiff stain was adopted to detect changes of intracellular glycogen content.

As the Fig. 8 showed that, compared with control group, intracellular glycogen of GFP treated group changed obviously showed a remarkable increase. The intracellular glycogen of model group changed insignificantly, which showed the effectiveness of model. The results indicated that GFP ameliorated insulin resistance of HepG2 by enhance synthesis of intracellular glycogen.

In order to investigate intracellular glycogen content changes of GFP treated cell, intracellular glycogen was extracted for detecting. As the Fig. 9 showed, compared with model control group, the intracellular glycogen of GFP group increased significantly. The results suggested that GFP significantly enhanced synthesis of intracellular glycogen at 24 h, so GFP ameliorated insulin resistance of model control group through the pathway of enhancing synthesis of intracellular glycogen.

Discussion

In recent years, studies of the biological activities of polysaccharides have been of particular interest. However, little information of anti-diabetic activities was available regarding the water-insoluble polysaccharides from *G. frondosa*, only Kubo proved anti-diabetic activity of polysaccharide obtained from *G. frondosa in vivo* [16].

The serine/threonine kinase Akt, also known as protein kinase B (PKB), is a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli and is one of the most important and versatile protein kinases. Aberrant loss or gain of Akt activation underlies the pathophysiological properties of a variety of complex diseases, including type-2 diabetes and cancer [20]. The Akt signaling can regulate both of these processes. Particularly important in muscle and liver, Akt-mediated phosphorylation and inhibition of GSK-3 prevents it from phosphorylating and inhibiting its namesake substrate glycogen synthase, thereby stimulating glycogen synthesis [25]. Akt activation also increases the rate of glycolysis [8], and this probably contributes to the excessive flux through glycolysis in tumor cells.

In our research, GFP ameliorated insulin resistance may *via* active Akt-mediated phosphorylation and inhibit the expression of GSK-3. Synthesis of intracellular glycogen was enhanced as Fig. 8 and Fig. 9 showed. In summary, our result suggest that GFP ameliorated insulin resistance may *via* AKT/GSK-3 signal, possibly involving inhibiting the expression of GSK-3 as a result of enhancement of glycogen synthesis. A speculated schematic diagram of GFP ameliorated insulin resistance of cell is depicted in Fig. 10.

Our results provide the molecular basis for understanding the anti-diabetic effects of the active polysaccharides. GFP considered as potential source of lead molecules for anti-diabetic effects and should be studied further for the structural details by means of other analytical methods.

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