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Antidiabetic activity of Ganoderma lucidum polysaccharides F31 down-regulated hepatic glucose regulatory enzymes in diabetic mice

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ABSTRACT

Ethnopharmacological relevance: *Ganoderma lucidum* (Lin Zhi) has been used to treat diabetes in Chinese folk for centuries. Our laboratory previously demonstrated that *Ganoderma lucidum* polysaccharides (GLPs) had hypoglycemic effects in diabetic mice. Our aim was to identify the main bioactives in GLPs and corresponding mechanism of action.

Materials and methods: Four polysaccharide-enriched fraction were isolated from GLPs and the antidiabetic activities were evaluated by type 2 diabetic mice. Fasting serum glucose (FSG), fasting serum insulin (FSI) and epididymal fat/BW ratio were measured at the end of the experiment. In liver, the mRNA levels of hepatic glucose regulatory enzymes were determined by quantitative polymerase chain reaction (qPCR) and the protein levels of phospho-AMP-activated protein kinase (*p*-AMPK)/AMPK were determined by western blotting test. In epididymal fat tissue, the mRNA and protein levels GLUT4, resistin, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC1) were determined by qPCR and immuno-histochemistry. The structure of polysaccharide F31 was obtained from GPC, FTIR NMR and GC–MS spectroscopy,

Results: F31 significantly decreased FSG ($P < 0.05$), FSI and epididymal fat/BW ratio ($P < 0.01$). In liver, F31 decreased the mRNA levels of hepatic glucose regulatory enzymes, and up-regulated the ratio of phospho-AMP-activated protein kinase (*p*-AMPK)/AMPK. In epididymal fat tissue, F31 increased the mRNA levels of GLUT4 but decreased fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC1) and resistin. Immuno-histochemistry results revealed F31 increased the protein levels of GLUT4 and decreased resistin.

Conclusion: Data suggested that the main bioactives in GLPs was F31, which was determined to be a β-heteropolysaccharide with the weight-average molecular weight of 15.9 kDa. The possible action mechanism of F31 may be associated with down-regulation of the hepatic glucose regulated enzyme mRNA levels via AMPK activation, improvement of insulin resistance and decrease of epididymal fat/BW ratio. These results strongly suggest that F31 has antidiabetic potential.

1. Introduction

Type 2 diabetes mellitus (DM) is a metabolic disease characterized by persistent hyperglycemia (Ros et al., 2015). Despite great efforts that have been made in the understanding and management of diabetes, its prevalence continues to grow. Recent discoveries have opened up an exciting opportunity for developing new types of therapeutics from mushrooms to control DM and its complications (De Silva et al., 2012; Lo and Wasser, 2011).

To date, more and more active components including polysaccharides and their protein complexes, dietary fibers, and other compounds extracted from fruiting bodies, cultured mycelium, or cultured broth of mushrooms have been reported as to having anti-hyperglycemic activity (Lo and Wasser, 2011). Literature suggests that natural

Abbreviations: **ACC1**, acetyl-CoA carboxylase 1; **AMPK**, AMP-activated protein kinase; **BW**, body weight; **DM**, diabetes mellitus; **ELISA**, enzyme-linked immunosorbent assay; **FAS**, fatty acidy synthase; **FBPase**, fructose-1,6-bisphosphatase; **FSG**, fasting serum glucose; **FSI**, fasting serum insulin; **FTIR**, Fourier transform infrared; **GC–MS**, gas chromatography mass spectrometry; **GLUT4**, glucose transporter type 4; **GP**, glycogen phosphorylase; **GPC**, gel permeation chromatography; **G6Pase**, glucose-6-phosphatase; **H & E**, Hematoxylin & Eosin; **NMR**, nuclear magnetic resonance; **PEPCK**, phosphoenolpyruvate carboxykinase; **STZ**, streptozotocin

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extracts from edible fungi (mushrooms), including Fraction X (FXM) (Manohar et al., 2002), MT-α-glucan (Lei et al., 2007), F2 and F3 (Xiao et al., 2015) from *Grifola frondosa*, an acidic polysaccharide (TAP) isolated from *Tremella aurantia* (Kiho et al., 2000b, 1995), and water-soluble polysaccharides from *Auricularia auricular* (Yuan et al., 1998) have anti-diabetic properties in animal models of type 2 diabetes.

Ganoderma lucidum is a basidiomycete white rot fungus which was called Ling Zhi in China, has been used for treatment of several diseases, such as hepatitis, hypertension, chronic bronchitis, bronchial asthma, cancer and others for thousands of years, as reported in Shen Nong's Materia Medica (Boh et al., 2007). As a natural traditional medicine, *G. lucidum* has been used to treat DM (recognized as "Xiao Ke Zheng" in ancient China) in folk (Li et al., 2004; Teng et al., 2011). However, there is insufficient evidence to draw definitive conclusions about the efficacy of *G. lucidum* for DM (Lo and Wasser, 2011). In the past several decades, well-designed trails was contributed to clarify the efficacy and mechanism of *G. lucidum* for DM. Extracts of *G. lucidum* have been recognized as an alternative adjuvant treatment for DM (Ma et al., 2015). Ganoderan A, B and C isolated from this fungus showed activity in type 1 diabetic mice (Hikino et al., 1985; Tomoda et al., 1986). Ganoderan B was reported to increase blood insulin levels and accelerate glucose metabolism by potentiations of the activities of hepatic key enzymes participating in the carbohydrate metabolism (Hikino et al., 1989). *G. lucidum* water extract can also provide beneficial effects in treating type 2 diabetes mellitus by lowering the serum glucose levels through the suppression of the hepatic PEPCK gene expression (Seto et al., 2009). In addition, as a novel PTP1B activity inhibitor, Fudan-Yueyang-*Ganoderma luciden* (FYGL) screened from *G. lucidum* also have anti-diabetic properties in animal models of type 2 diabetes (Pan et al., 2013; Teng et al., 2012; Wang et al., 2012).

Our laboratory has previously demonstrated that *G. lucidum* polysaccharides (GLPs) had hypoglycemic effects in diabetic mice, which was induced by streptozocin (STZ) injection combination with high fat fed diet [18]. The hypoglycemic effects may be associated with decreased mRNA levels of several key hepatic glucose regulatory enzymes [glycogen phosphorylase (GP), fructose-1,6-bisphosphatase (FBPase), phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase, including G6Pase-catalytic protein and a specific transporter named G6Pase-T1)] involved in gluconeogenesis and/or glycogenolysis.

In the present study, four polysaccharide-enriched fractions named F21, F22, F31 and F32 were isolated from GLPs. Our aim was to identify the main bioactives and corresponding mechanism of action.

2. Materials and methods

2.1. Extraction and purification of polysaccharides

The dried fruiting bodies of *G. lucidum* were homogenized to a fine powder. The powder was mixed with distilled water at a ratio of 1:20 (w/v) and extracted at approximately 80 °C. The mixture was filtered and centrifuged at 5000*g* for 10 min at 4 °C. The resulting supernatant was concentrated under a reduced pressure at (not exceeding) 60 °C and then precipitated with four volumes of absolute ethanol at 4 °C overnight. The precipitate obtained was dispersed in water, dialyzed, and lyophilized to yield the polysaccharides-enriched fraction, named GLPs.

The procedure of isolation and purification of polysaccharides is described in Fig. 1. GLPs was applied onto a column of DEAE Sepharose Fast Flow chromatography, equilibrated with Tris-HCl (10 mmol/l, pH 8.0), followed by 0.1 mol/l of NaCl in Tris-HCl (10 mmol/l, pH=8.0), followed by 0.5 mol/l of NaCl in Tris-HCl (10 mmol/l, pH=8.0) at the same rate. Fractions were assayed for carbohydrate with the phenol-sulfuric acid method (Dubois et al., 1956) and for protein using the absorbance method at 280 nm.

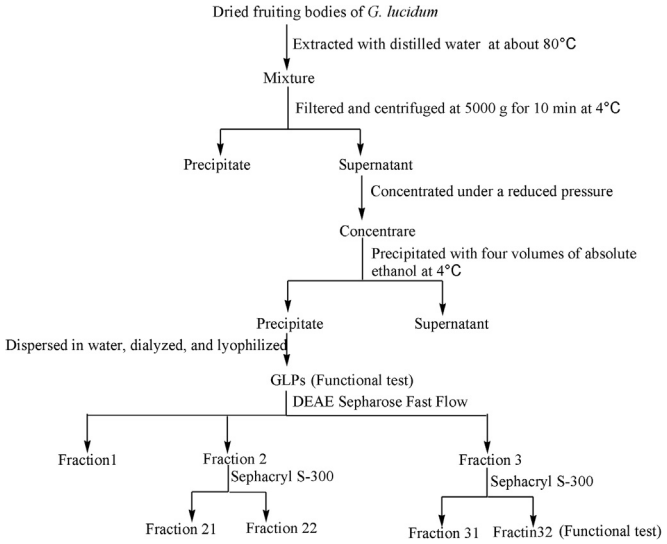

Fig. 1. Isolation and purification procedure of polysaccharide fraction of F21, 22, 31 and 32..

The three peak fractions were obtained by DEAE Sepharose Fast Flow chromatography (Fig. 2A). F1 (the first peak) was eluted by Tris-HCl, F2 (the second peak) was eluted with 0.1 mol/l NaCl in Tris-HCl, and F3 (the third peak) was eluted by 0.5 mol/l NaCl in Tris-HCl.

F2 and F3 were applied onto Sephacryl S-300 chromatography separately, equilibrated with H_2O, followed by H_2O at the same rate. Two peak fractions (named F21 and F22) were obtained by Sephacryl S-300 from Fraction 2 (Fig. 2B). Two peak fractions (named F31 and F32) were obtained by Sephacryl S-300 from fraction 3 (Fig. 2C). The carbohydrate content of the fractions was determined by the phenol-sulfuric acid method. The concentration of the four fractions was adjusted to 5 mg/ml.

2.2. Screening of hypoglycemic fractions from GLPs in diabetic mice

2.2.1. Screening of hypoglycemic fractions in diabetic mice induced by STZ injection combination with high fat diet (i.p.)

Six-week-old male Kunming mice (24–26 g) and a standard pellet diet were provided by Guangdong Province Experimental Animals Center (Production Certificate No. scxk (Yue) 2008–0002. Quality Certificate No. 0058563. Experimental Animals License No. syxk (Yue) 2008-0011). The mice had free access to standard pellet diet and water, and were maintained under a constant 12 h light/dark cycle and an environmental temperature of 21–23 °C. All animal procedures complied with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Committee of the Center for Disease Control and Prevention of Guangdong Province (Approval ID: 20091224).

The mice were adapted for 7 days and then fasted overnight before an intraperitoneal (i.p.) injection of freshly prepared STZ [Sigma, 60 mg/kg body weight (BW), dissolved in citrate buffer, pH 4.5]. Then the mice were fed a high-fat diet (Srinivasan et al., 2005). After 4 weeks, the mice were fasted for 5 h and fasting serum glucose (FSG) levels were determined. Mice with FSG levels > 10.0 mmol/l were considered to be diabetic and were used in the study.

For the experiment, mice were randomly divided into the following groups (8 mice/group): (1) normal control group, (2) diabetic control group, (3) F21 diabetic group (50 mg/kg/d), (4) F22 diabetic group (50 mg/kg/d), (5) F31 diabetic group (50 mg/kg/d) and (6) F32 diabetic group (50 mg/kg/d). The mice were injected (i.p.) with fractions or saline (normal/diabetic control groups). All mice were given free access to drinking water and the respective pelleted diet for 7 days. At the end of the study, mice were fasted, weighed and blood