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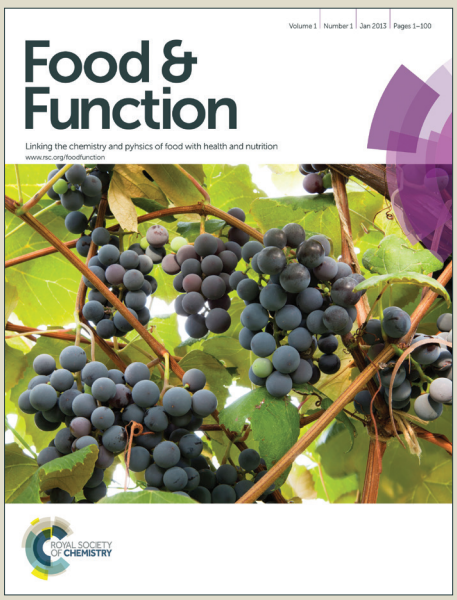

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Hypoglycemic effects of *Grifola frondosa (Maitake)* polysaccharides F2 and F3 through improvement insulin resistance in diabetic rats

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Short title: Hypoglycemic effect of F2 and F3

Abstract

Our laboratory has previously demonstrated that *Grifola frondosa* polysaccharides (GFPs) showed hypoglycemic effects. This study aimed to investigate which polysaccharide-enriched fraction in GFPs was the main active constituents, and to disclose their hypoglycemic mechanism. F2 and F3 were obtained from GFPs and their hypoglycemic effects were investigated. Fasting serum glucose (FSG), fasting serum insulin (FSI) levels, homeostasis model assessment of insulin resistance (HOMA-IR) were measured, and the hepatic mRNA levels of insulin receptor (IR), insulin receptor substrate-1 (IRS-1), proteintyrosine phosphatase-1B (PTP1B), phosphatidylinositol 3-kinase (PI3K), Akt/protein kinase B (PKB) were determined by quantitative polymerase chain reaction (qPCR). The activity of IR, IRS-1 were determined by enzyme-linked immunosorbnent assay (ELISA), and their phospho-protein levels were analyzed with westbloting. F2 and F3 significantly decreased FSG, FSI and HOMA-IR compared with diabetic control group (P<0.05). F2 and F3 increased the activity and mRNA levels of IR，and the latter also increased the mRNA levels of IRS-1. As for the protein levels of phospho-IR and IRS-1, both F2 and F3 increased the protein levels of IR (Try 1361), but decreased IRS-1 (Ser307). In PI3K/Akt pathway, F3 increased the mRNA levels of PI3K and Akt, however F2 inhibited PTP1B expression. F2 and F3 are presumed to improvement insulin resistance triggered by reactivating IR and IRS-1.

Keywords *Grifola frondosa* polysaccharides, hypoglycemic activity; hypoglycemic mechanism, type 2 diabetes

Introduction

Type 2 diabetes is a global public health crisis that threatens the economies of all nations, particularly developing countries. [1, 2] In aggregate, there is a pressing need to develop novel modalities for the treatment of diabetes to stem the spread of this global epidemic. Medicinal mushrooms have been valued as a traditional source of natural bioactive compounds over many centuries and have been targeted as potential hypoglycemic and anti-diabetic agents.[3] However, there is insufficient evidence to draw definitive conclusions about the efficacy of individual medicinal mushrooms for diabetes.[4] Thus, a great of interest has been focused on the mushrooms treatment of diabetes.

Grifola frondosa (maitake) has been confirmed to contain substances with antidiabetic activity, and it was found to lower blood sugar due to the presence of an α-glucosidase inhibitor.[5] Data suggest that MT-α-glucan has an anti-diabetic effect on KK-Ay mice, which might be related to its effect on insulin receptors (i.e., increasing insulin sensitivity and ameliorating insulin resistance of peripheral target tissues).[6] A lot of researches focused on *G. frondosa* SX -fraction which is a bioactive glycoprotein with molecular weight of 20 KD, that has exhibited hypoglycemic activity in diabetic mice and in clinical studies of type 2 patients.[7-11] The action of SX is presumed to be associated with the activation of an impaired insulin signal transduction pathway through high glucose or under a hyperglycemic milieu, thereby ultimately facilitating glucose uptake.[12] Apart from the fruit bodies, fermented *G. frondosa* rich in vanadium (GFRV) also significantly induced decreases of the blood glucose levels in hyperglycemic mice,[13] and its submerged culture mycelium and broth improved glycemic responses in diabetic rats with significant decreases in

postprandial blood glucose levels and serum triglyceride levels.[14]

Consistent with the above reports, our laboratory previously demonstrated that *G. frondosa* polysaccharides (GFPs) showed hypoglycemic effects with lowering the diabetic blood serum glucose level by 50.09% in type 2 diabetic mice induced by streptozotocin (STZ) injection combination high fat diet fed.[15]

In the present study, our aim was to determine which polysaccharide-enriched fraction in GFPs was the main active constituents and investigate the key hypoglycemic mechanisms. And then, this study investigated the effect of fractions on insulin signal pathway to explore possible underlying molecular mechanisms for their action.

Type 2 diabetes is a heterogeneous disorder characterized by hyperglycemia and insulin resistance. Nevertheless, insulin resistance would block the insulin signal pathway and insulin receptor (IR), insulin receptor substrate-1 (IRS-1) would be inactivated. The phosphatidylinositol 3-kinase- serine/threonine kinase Akt (PI3K-Akt) pathway plays a pivotal role in insulin signal transduction.[16] The IR is a heterotetrameric glycoprotein consisting of two α-subunits and two β-subunits.[17] The binding of insulin to the α-subunits of IR induces a conformational change that leads to trans-autophosphorylation of tyrosine residues on the β-subunits, activating their tyrosine kinase activity.[18] One such tyrosine residue phosphorylated, serves as a binding site for the phosphotyrosine binding domains of IR IRS-1, whose tyrosine residues are then phosphorylated.[19] This tyrosine-phosphorylated IRS-1 acts as a docking site/molecule that binds to and activates PI3K, which in turn activates serine/threonine kinase Akt.[20] Activated Akt ultimately promoted glucose uptake and

lipid synthetic.[21] As a negative regulated factor, protein tyrosine phosphatase-1B (PTP1B) interacts with and dephosphorylates the IR as well as the IRS-1. This is rather a simplified scheme of the insulin signal transduction pathway, which is triggered by activation of the IR (Fig. 1). Thus, one rational approach to overcoming such insulin resistance would be by reactivating the IR/IRS to successfully execute the entire signal transduction pathway.

To explore such a mechanism, the effects of F2 and F3 on mRNA levels, protein activity and phospho-protein levels of IR, IRS-1, PTP1B, PI3K and Akt which are key elements involved in insulin signal pathway were assessed using quantitative polymerase chain reaction (qPCR), enzyme-linked immunosorbnent assay (ELISA), and western-blotting.

MATERIALS AND METHODS

Extraction and purification of polysaccharides

The dried fruiting bodies of *G. frondosa* 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 (not exceeding 60°C) and precipitated with four volumes of absolute ethanol at 4°C overnight. The resulting precipitate was dispersed in water, dialyzed, and lyophilized to yield the polysaccharides-enriched fraction, named GFPs.

GFPs was applied onto a column of DEAE Sepharose Fast Flow chromatography, equilibrated with Tris-HCl (10mmol L^{-1}, pH 8.0), followed by 0.1M NaCl in Tris-HCl (10mmol L^{-1}, pH=8.0), followed by 0.5M NaCl in Tris-HCl (10mmol L^{-1}, pH=8.0) at the same rate. Fractions were assayed for carbohydrate by the phenol-sulfuric acid method [22] and for protein by the absorbance at 280nm. The concentration of the

fractions was adjusted to 5 mg ml^{-1} or 10 mg ml^{-1}.

Compositional analysis of F2 and F3

The neutral polysaccharides content of F2 and F3 were determined by the phenol-sulfuric acid method using dextran as the standard.[22, 23] The monosaccharide composition was analyzed by gas chromatography-mass spectrometry.[24] Total protein content was determined by the Lowry method with bovine serum albumin as the standard.[25] The composition of amino acids in the protein hydrolysate were analyzed by reversed-phase high performance liquid chromatography with a HP1050 analyzer.[26]

Animals

Six-week-old male SD rats (140±20g) and a standard pellet diet were provided by Guangdong Province Experimental Animals Center (Production Certificate No. scxk (Yue) 2008–0002. Quality Certificate No. 20121209. Experimental Animals License No. syxk (Yue) 2008-0011). The rats had free access to standard pellet diet and water. The rats 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 Ethical Committee of the Center for Disease Control and Prevention of Guangdong Province (Approval ID: 20091224).

Induction of diabetes and assessment of hypoglycemic activity

The rats were adapted for 3 days and then fed a high-fat diet.[27] After 4 weeks, the rats were fasted overnight before an intraperitoneal (i.p.) injection of freshly prepared STZ [Sigma, 35 mg kg^{-1} body weight (BW), dissolved in citrate buffer, pH 4.5]. Rats with fasting serum glucose (FSG) levels > 10.0 mmol L^{-1} were considered to be diabetic and were used in the study.

For the experiment, rats were randomly divided into the following groups (8 rats/group): (1) normal control group, (2) diabetic control group, (3) F2 low dose-treated diabetic group (50 mg kg^{-1} d^{-1}), (4) F2 high dose-treated diabetic group (100 mg kg^{-1} d^{-1}), (5) F3 low dose-treated diabetic group (50 mg kg^{-1} d^{-1}) and (6) F3 high dose-treated diabetic group (100 mg kg^{-1} d^{-1}). The rats of normal/diabetic control groups were intragastrical administration (i.g.) with saline. All rats were given free access to drinking water and the respective pelleted diet for 14 days. BW gain, food intake, water intake were determined every day. Once a week, the rats were fasted for 5 h and FSG levels were determined.

At the end of the study, rats were fasted and blood samples were withdrawn from the orbital sinus, after which the rats were anaesthetized and sacrificed by cervical decapitation. The livers were quickly removed, snap-frozen, and stored at –70 °C.

Biochemical analyses

Serum was separated by centrifuging blood samples at 1000 ×*g* for 10 min at 4°C. FSG levels were determined using a commercially available assay kit (Jiancheng Bioengineering Institute, Nanjing, China) based on the glucose oxidase method. [28] Fasting serum insulin (FSI) was determined by 125I-labled insulin radioimmunoassay kit (Beijing beifang Biotech Institute, Beijing, China). Homeostasis model assessment-insulin resistance (HOMA-IR) index was calculated to measure the insulin sensitivity of rats fed experimental diets using the following formula[29]: $HOMA-IR=(FSI\times FSG)\div 22.5$. Serum total cholesterol (TC), triglyceride (TG), high density lipoprotein-cholesterol (HDL-c), low density lipoprotein-cholesterol (LDL-c) were measured using commercial kits (BioSino Bio-technology and Science Inc, Beijing, China).

Total RNA extraction

Each liver sample was immediately homogenized in physiological saline with a PRO200 homogenizer (PRO Scientific Inc., Oxford, CT, USA). Total RNA was extracted using an RNAprep Pure Tissue Kit (Tiangen Biotech Ltd., Beijing, China), with a DNaseI digestion step. The total RNA concentration was determined by measurement the absorbance at 260 nm.

Semiquantitative SYBR Real-time PCR

Total RNA was reverse-transcribed with a random-primer first-strand cDNA Kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. PCR was performed in a real-time thermal cycler using an SYBR Green kit (Takara) as follows: 40 cycles of denaturation at 95°C for 5 sec and annealing/elongation at 60°C for 30 s. The primer sequences of IR, IRS-1, PTP1B, PI3K, Akt are shown in Table 1. The SYBR Green assay was used to detect products from the reverse-transcribed cDNA sample. The mRNA signals were normalized to the GAPDH mRNA signals in each group. PCR reactions were performed in duplicate for each sample. Relative gene expression was determined as previously described by Livak and Schmittgen.[30]

ELISA analysis of IR, IRS-1 Proteins

The activity of IR and IRS-1 in liver supernatant homogenate were determined using a commercially available ELISA kit (Westang Biotech Co., Ltd., Shanghai, China).

Western-blotting analysis of IR (Try1361) and IRS-1 (Ser307)

Liver supernatant homogenate containing 20 mg protein was run on SDS-PAGE and transferred electrophoretically onto the Nitrocellulose (NC) membrane. The NC membrane was blocked for 2 h at room temperature and then incubated with anti-IR (Try1361) and IRS-1 (Ser307) polyclonal antibody (Abcam Inc. Cambridge, MA, USA) overnight at 4°C, and then with anti-rabbit IgG conjugated with

horseradish-peroxidase. Finally, the NC membranes were washed for 30 min with wash solution, and the immunoreactive lanes on the NC membrane were detected by the enhanced chemiluminescence's method and digitalised by the BandScan software version 5.0.

Statistical analysis

All data were expressed as means ± standard deviation (SD). Differences between groups were determined by analysis of variance followed by one-way least significant difference tests. Statistical differences were considered significant at $P < 0.05$. SPSS software version 21.0 was used for all analysis.

Results

Fractionations of the GFPs

The three peak fractions were obtained, as shown in Fig. 2 by DEAE Sepharose Fast Flow chromatography (Fig. 2). Fraction 1 (the first peak) was eluted by Tris-HCl, Fraction 2 (the second peak) was eluted by 0.1 mol L^{-1} NaCl in Tris-HCl and Fraction 3 (the third peak) was eluted by 0.5 mol L^{-1} NaCl in Tris-HCl. Fraction 2 and fraction 3 were observed to be associated with the protein.

Compositional analysis of F2 and F3

The total polysaccharides and proteins content in F2 were 62.5%, 37.5%, and they were 78.3% and 21.7% in F3 respectively (Table 2). It can be seen that glucose, mannose, galactose, xylose, arabinose, rhamnose and ribose were the major monosaccharide of the polysaccharides moiety in F2. However, ribose, arabinose, xylose were the major monosaccharide of the polysaccharides moiety in F3. Sixteen kinds of amino acids constituted the protein moiety, of which the major amino acids in F2 and F3 were proline, glutamic acid, alanine, arginine, valine, lysine, leucine,

histidine, isoleucine, phenylalanine, tyrosine, serine and *et al*.

Effects of F2 and F3 on BW gain, food intake, water intake and food efficiency ratio

From Table 3, the BW gain of diabetic rats administered F2 (100 mg kg^{-1} d^{-1}) significantly decreased compared with diabetic control (P<0.05). Daily food intakes of fractions-administered rats were similar with those of control rats. Daily water intakes of diabetic rats were significantly increased compared with those of normal control rats (P<0.01), whereas water intakes of diabetic rats administered F2 (50 mg kg^{-1} d^{-1}) and F3 (100 mg kg^{-1} d^{-1}) significantly increased compared with diabetic control (P<0.05). The food efficiency ratios representing BW gain relative to food intake were the same for fractions-administered rats, and the ratios of each group were not different significantly.

Effects of F2 and F3 on FSG, FSI and HOMA-IR index

As shown in Fig. 3A, there were no significant differences in FSG levels between the fraction-treated groups and the diabetic control group at the start of the study. After 1 week, however, FSG in F3-treated (50 mg kg^{-1} d^{-1}, 100 mg kg^{-1} d^{-1}) group was significantly lower (P < 0.05) compared with the diabetic control group. FSG levels in F2-treated group was also decreased, but the difference was not statistically significant. After 2 week, the significant decrease of FSG were observed in both of F2 (100 mg kg^{-1} d^{-1}) and F3-treated (50 mg kg^{-1} d^{-1}, 100 mg kg^{-1} d^{-1}) groups (P < 0.05) compared with the diabetic control group.

At the end of the experiment, the FSI levels were significantly higher in diabetic control rats than in normal rats (P < 0.01), consistent with the characteristics of type 2

diabetes (Fig. 3B). However, compared with the diabetic control group, the FSI levels in fractions-treated groups were significantly lower ($P < 0.05$). Fig. 3C showed F2 and F3 significantly lowered HOMA-IR index ($P < 0.01$).

Effects of F2 and F3 on fasting serum lipids levels

The plasma lipids are usually raised in diabetes, and such an elevation represents a risk factor for coronary heart disease. Hypercholesterolemia and hypertriglyceridemia have been reported to occur in STZ induced diabetic rats and a significant increase observed in our experiment was in accordance to those studies. As shown in Table 4, oral administration of F2 or F3 (50 mg kg^{-1} d^{-1}, 100 mg kg^{-1} d^{-1}) significantly reduced the TC and TG in serum compared with diabetic control rats. We have also observed that oral administration of F3 (50 mg kg^{-1} d^{-1}) significantly decreased LDL-c compared with diabetic control group. However, no significant change in HDL-c level was shown in diabetic control group compared with the normal.

Effects of F2 and F3 on the activity of hepatic IR and IRS-1

The activity of IR and IRS-1 were significantly reduced in diabetic control rats and these lowered levels of IR were enhanced significantly ($P < 0.01$) in F2 and F3-treated rats, and these lowered levels of IRS-1 were enhanced significantly ($P < 0.01$) in F3-treated rats (Fig. 4).

Effects of F2 and F3 on mRNA levels of IR, IRS-1, PTP1B, PI3K and Akt

Hepatic mRNA levels of IR, IRS-1, PTP1B, PI3K and Akt were similar in the normal and diabetic control groups (Fig. 5). It is interesting to note that F3 increased the mRNA levels of IR, IRS-1, PI3K and Akt compared with diabetic control rats ($P < 0.01$), and F2 only increased the mRNA levels of IR, IRS-1. As for negative factor of PTP1B, F2 was capable of decreasing the higher mRNA levels of diabetic rats, but F3

could not do it.

Western blotting Analysis of IR (Try1361) and IRS-1 (Ser307).

From Fig. 6, compared with normal control rats, the IR (Try1361) protein levels of diabetic control in the livers were significantly decreased by 90 % ($P< 0.01$). While the diabetic rats were treated by F2 and F3, the IR (Try1361) protein levels in the livers were increased compared with that of the diabetic control rats ($P< 0.01$). As for IRS-1 (Ser307), compared with normal control rats, the protein levels of diabetic control in the livers were significantly increased by 74 % ($P< 0.01$), but F2 and F3 can decreased the protein level ($P<0.01$).

Discussion

The hypoglycemic agents from mushroom almost were polysaccharides or polysaccharides-protein/peptide complex.[31, 32] Lei found that *G. frondosa* MT-α-glucan has an anti-diabetic effect on KK-Ay mice.[33] *G. frondosa* SX -fraction is a bioactive glycoprotein with molecular weight of 20 KD, that has exhibited hypoglycemic activity in diabetic mice and in clinical studies of type 2 patients.[7-9, 11, 34] As for *Ganoderma lucidum,* Ganoderan A was composed of rhamnose, galactose and glucose with molecular weight of 23KD, ganoderan B was composed of protein; mannose, glucose, galacturonic acid with molecular weight of 7.4KD, ganoderan C was composed of glucose and galactose with molecular weight of 5.8KD.[35, 36,37]. Consistence with the previous report, our lab successfully isolated *G. lucidum* polysaccharides F31 which seemed to be a polysaccharides-protein/peptide complex with the weight-average molecular weight of 15.9 KD. In the present study, we isolated two fractions of polysaccharides-protein/peptide complex from *G. frondosa.* As for the structures of main chain and branches, it needs to be studied further. To date, the relationship between the structural feature and anti-hyperglycemic activity

was not clear. So, further analysis study on polysaccharide F2 and F3 structure would contribute to the structure-activity relationship.

In this study, diabetic rats were induced by STZ injection combination with high-fat dietary feeding, and the character of hyperglycemia, hyperinsulin and insulin resistance was similar to type 2 diabetes. In a state of insulin resistance, glucose uptake and utilization are dramatically decreased, and skeletal muscle becomes metabolically inflexible, unable to switch between glucose and fatty acid use.[38] We found that F2 and F3 lowered FSG, FSI, improved insulin resistance in diabetic rats (Fig. 3). The results were consistence with our previous report that GFPs lowered hyperglycemia by counteracting insulin resistance.[15] As we know, type 2 diabetes is related to significant cardiovascular morbidity and mortality by modulation of lipid profiles. Dyslipidemia, which occurs in approximately 50% of patients with type 2 diabetes, results in cardiovascular complications by elevated triglyceride levels, low levels of HDL-c, and high rise of LDL-c.[39] Lipids play an important physiological role in skeletal muscle, heart, liver and pancreas. Deregulation of fatty acid metabolism is the main culprit for developing insulin resistance and type 2 diabetes.[40] In the present study, associated with the corrected glycemia, TC, TG and LDL-c in plasma were reduced during the period of F2 and F3 administration (Table 4). So F2 and F3 lowed FSG, associated with lowed lipid accumulation in serum may be a result of improvement insulin sensitivity.

As an important clue that F2 and F3 improvement insulin resistance, this study investigated the effect of fractions on insulin signal pathway to explore possible underlying molecular mechanisms. The PI3K-Akt pathway plays a pivotal role in insulin signal transduction.[16] Liver is a major site of glucose metabolism in response to insulin.[41] So the key components, IR, IRS-1, PTP1B, PI3K and Akt in liver which

play a pivotal role in the insulin signaling pathway were investigated in the present study.

Insulin acts by binding to its cell surface receptor, thus activating the IR's intrinsic tyrosine kinase activity, resulting in IR autophosphorylation and phosphorylation of several substrates. Tyrosine phosphorylated residues on the receptor itself and on subsequently bound receptor substrates provide docking sites for downstream signalling molecules.[42] While the phosphorylation of IRS-1 on tyrosine residue is required for insulin-stimulated responses, the phosphorylation of IRS-1 on serine residues has a dual role, either to enhance or to terminate the insulin effects.[21] However, serine phosphorylation of IRS1 at Ser307 is a. negative regulatory sites S307.[43] That is to say, and serine phosphorylation of IRS-1(Ser307) would impair its tyrosine phosphorylation, then attenuate the rest of the PI3K-Akt signal pathway, whereas tyrosine-phosphorylated IR would facilitate the pathway. In this study, we found IR (Try1361) phosphorylation levels decreased and the IRS-1 (Ser307) phosphorylation levels increased in diabetic rats (Fig. 6). While the diabetic rats were treated by F2 and F3, the IR (Try1361) protein levels in the livers were increased and IRS-1 (Ser307) phosphorylation levels decreased. That is to say, such inactivation of IR and IRS-1 was reversed or reactivated by F2 and F3, presumably aiding the occurrence of successive signaling events. These results suggest that the signal pathway being impaired with high blood serum glucose could be reactivated or turned on by F2 and F3.

Actually, activated IRS is responsible for activation of PI3K, which in turn promotes serine/threonine phosphorylation of Akt for its activation. Akt is also

required for the insulin regulation of gluconeogenesis and glucose release in the liver. Interestingly, we found that the mRNA levels of PI3K and Akt in the insulin signaling transduction was significantly improved in F3-treated rats (Fig. 5). It is presumed that the PI3K-Akt pathway was activated by F3.

Our findings of F2 and F3 improvement resistance are in accordance with *G. frondosa* SX. The hypoglycemic action of SX is presumed to be associated with activation such an impaired insulin signal transduction pathway of IR, IRS, and Akt, thereby ultimately facilitating glucose uptake.[12]

Protein tyrosine phosphatase 1B (PTP1B) plays an important role in the negative regulation of insulin signal transduction pathway and has emerged as novel therapeutic strategy for the treatment of type 2 diabetes.[44, 45] PTP1B interacts with and dephosphorylates the IR as well as the IR substrate (IRS). If PTP1B were over expressed, then most of the IRS would be dephosphorylated and a series of enzymes such as PI3K and Akt participating in the process of glucose uptake would be inactivated since the insulin transduction pathway is blocked. Recent gene knockout studies in mice identify PTP1B as a promising target for anti-diabetes/obesity drug discovery.[46] That is to say, the inhibition of PTP1B has emerged as an attractive therapeutic strategy to treat type 2 diabetes and obesity. In this study, it is interesting that F2 inhibited mRNA levels of PTP1B significantly ($P<0.05$) but F3 did not decrease the levels (Fig. 5). There is little report about PTP1B inhibitor from edible fungi. A novel PTP1B activity inhibitor named Fudan-Yueyang-Ganoderma luciden (FYGL),[47-50] screened from *G. lucidum* also have anti-diabetic properties in animal models of type 2 diabetes.

The present study showed F2 and F3 increased the activity and the mRNA levels of IR and IRS-1 (Fig. 5). F2 and F3 increased the protein expression of IR (Try1361) but decreased the protein of IRS-1 (ser307) (Fig. 6). In PI3K/Akt pathway, F3 increased mRNA levels of PI3K and Akt, however F2 inhibited mRNA levels of PTP1B. These results suggest that *Grifola frondosa* polysaccharides F2 and F3 may specifically target the insulin signal pathway, and, in particular, F3 may be activated the IR and IRS therein that trigger the subsequent PI3K/Akt signaling events, however F3 inhibited the mRNA levels of PTP1B. Their improvement of insulin resistance action mode is summarized in Fig. 1.

However, as for functional foods, safety is the most important factor. From Table 3, daily food intakes and food efficiency ratios of fractions-administered rats were similar with those of control rats. Intragastrical (i.g.) administration of F2 and F3 would not resulted in any adverse effects and poor food intake, in further, the hypoglycemic effect of F2 and F3 was not due to poor food intake. These data suggested F2 and F3 may be non-toxicity to diabetic rats.

Conclusions

In conclusion, F2 and F3 decreased FSG levels through improvement insulin sensitivity by increased protein levels of phospho-IR (Try 1361) and decreased phospho-IRS-1 (Ser307). These results demonstrate that *G. frondosa* polysaccharides is useful as potential functional food ingredients for the prevention and treatment of type 2 diabetes.

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Figure captions

Fig 1. Action mechanism of F2 and F3 on insulin signal transduction.

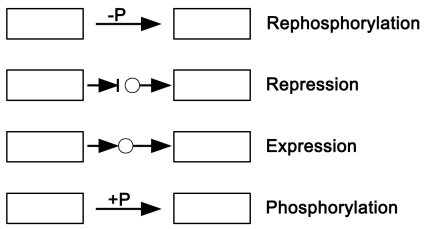

Fig. 2 Fractions of GFPs by DEAE Sepharose Fast Flow chromatography. (Fractions were analyzed by measuring the absorbance at 490nm for polysaccharides and at 280nm for the proteins).

Fig. 3 (A) Effects of F2 and F3 on FSG, (B) Effects of F2 and F3 on fasting insulin levels, (C) Effects of F2 and F3 on HOMA-IR index in diabetic rats**.** (Values represent means ± SD (n=8). $^{*}P < 0.05$, $^{**}P < 0.01$ vs diabetic control and $^{\alpha\alpha}P < 0.01$ vs normal group)

Fig. 4 Effects of F2 and F3 on the protein activity of hepatic IR and IRS-1. (Values represent means ± SD (n=8). $^{*}P < 0.05$, $^{**}P < 0.01$ vs diabetic control and $^{\alpha\alpha}P < 0.01$

vs normal group)

Fig. 5 Effects of F2 and F3 on mRNA expression of IR, IRS-1, PI3K, Akt and PTP1B. (Values represent means ± SD (n=8/group). *$P < 0.05$, **$P < 0.01$ vs diabetic control and $^{\alpha\alpha}P < 0.01$ vs normal control).

Fig. 6 (A) Effects of F2 and F3 on protein expression of IR（Try1361）, (B) Effects of F2 and F3 on protein expression of IRS-1（Ser307）. (Values represent means ± SD (n=8/group). *$P < 0.05$, **$P < 0.01$ vs diabetic control and $^{\alpha\alpha}P < 0.01$ vs normal control).

Tables

Table 1 Sequences of primers in this study

Gene	Primer sequence		Amplicon (bp)	Accession No.
GAPDH	Forward	5′-CCGCATCTTCTTGTGCAGTG-3′	250	NM_017008.4
	Reverse	5′-TCCCGTTGATGACCAGCTTC-3′		
IR	Forward	5′- TTCATTCAGGAAGACCTTCGA -3′	258	NM_017071.2
	Reverse	5′- AGGCCAGAGATGACAAGTGAC -3′		
IRS-1	Forward	5′- AGAGTGGTGGAGTTGAGTTG -3′	277	NM_012969.1
	Reverse	5′- GGTGTAACAGAAGCAGAAGC -3′		
PI3K	Forward	5′- GAAGGCAACGAGAAGGA -3′	213	XM_008760659.1
	Reverse	5′- CGTCAGCCACATCAAGTA -3′		
Akt	Forward	5′- ACCTCTGAGACCGACACCAG -3′	133	XM_006240631.2
	Reverse	5′- AGGAGAACTGGGGAAAGTGC -3′		
PTP1B	Forward	5′- TGCACAGCATGAGCAGTATG -3′	133	XM_006235639.2
	Reverse	5′- TGTGCCTTTTGTTCCTCC -3′		

Table 2 Chemical composition of polysaccharides and proteins in F2 and F3

F2		F3	
Polysaccharides and protein	Composition (%, w w^{-1})	Polysaccharides and protein	Composition (%, w w^{-1})
polysaccharides (62.5 %)[a]		polysaccharides (78.3 %)[e]	
Glucose	26.74[b]	Ribose	74.73[f]
Mannose	22.79	Arabinose	14.20
Galactose	16.76	Xylose	11.08
Xylose	16.02		
Arabinose	14.29		
rhamnose	2.05		
Ribose	1.35		
Amino acid (37.5 %)[c]		Amino acid (21.7 %)[g]	
Proline	9.55[d]	Proline	5.84[h]
Glutamic acid	4.26	Alanine	3.59
Alanine	4.14	Glutamic acid	2.01
Arginine	2.90	Arginine	1.44
Valine	2.87	Valine	1.35
Lysine	2.75	Tyrosine	1.22
Leucine	2.53	Leucine	1.20
Histidine	2.16	Phenylalanine	1.10
Isoleucine	1.92	Histidine	1.07
Phenylalanine	1.78	Lysine	1.02
Tyrosine	1.62	Serine	0.97
Serine	1.08	Isoleucine	0.85
Aspartic acid	<0.05	Aspartic acid	<0.05
Glycine	<0.05	Glycine	<0.05
Methionine	<0.05	Methionine	<0.05
Threonine	<0.05	Threonine	<0.05

[a]Percentage of polysaccharides to total F2
[b]Percentage of polysaccharides in F2
[c]Percentage of proteins to total F2
[d]Percentage to total amino acids in F2
[e]Percentage of polysaccharides to total F3
[f]Percentage of polysaccharides in F3
[g]Percentage of proteins to total F3
[h]Percentage to total amino acids in F3

Table 3 Effect of F2 and F3 on BW gain, food intake, water intake and food efficiency ratio in type 2 diabetic rats after treatments for 14 days [a]

Group	Dose (mg $kg^{-1}d^{-1}$)	BW gain (g day^{-1})	Food intake (g g^{-1} day^{-1})	Water intake (ml g^{-1} day^{-1})	Food efficiency ratio[b]
Normal Control	/	4.50±0.60	0.06±0.01	0.10±0.01	0.15±0.02
Diabetic Control	50	3.41±0.48	0.06±0.01	0.21±0.05$^{\alpha\alpha}$	0.17±0.02
F2	50	3.68±0.45	0.07±0.01	0.24±0.07$^{\alpha\alpha}$*	0.15±0.02
F2	100	2.98±0.61*	0.06±0.01	0.21±0.05$^{\alpha\alpha}$	0.14±0.02
F3	50	3.29±0.48	0.07±0.01	0.22±0.06$^{\alpha\alpha}$	0.14±0.02
F3	100	3.41±0.41	0.07±0.02	0.24±0.07*	0.13±0.02

[a] Values are means ± SE (n=11).

[b] BW gain/food intake

*P<0.05, ** P<0.01, compared with that in diabetic control rats

$^{\alpha\alpha}P$<0.05, compared with that in control rats

Table 4 Effects of F2 and F3 on TG, TC, HDL-c and LDL-c in type 2 diabetic rats

Group	Dose (mg $kg^{-1}d^{-1}$)	TG	TC	HDL-c	LDL-c
Normal	/	1.10±0.35	1.71±0.24	1.17±0.16	0.36±0.07
DM rats	50	6.33±1.88$^{\alpha\alpha}$	6.14±1.92$^{\alpha\alpha}$	1.35±0.27	1.91±0.68$^{\alpha\alpha}$
F2	50	3.62±0.46*	3.95±1.51	1.32±0.22	1.71±0.28
F2	100	3.60±0.82*	3.24±1.27*	1.23±0.16	1.33±0.26
F3	50	2.52±1.09**	2.52±1.01**	1.13±0.15*	0.80±0.46*
F3	100	4.01±0.95*	3.74±0.69*	1.20±0.27	1.59±0.42

Values represent means±SD (n=8). *P < 0.05, **P < 0.01 vs diabetic control and $^{\alpha\alpha}P$ < 0.01 vs normal group)

TG:Triglyceride, TC: Total cholesterol, HDL-c: High density lipoprotein-cholesterol, LDL-c: Low density lipoprotein -cholesterol

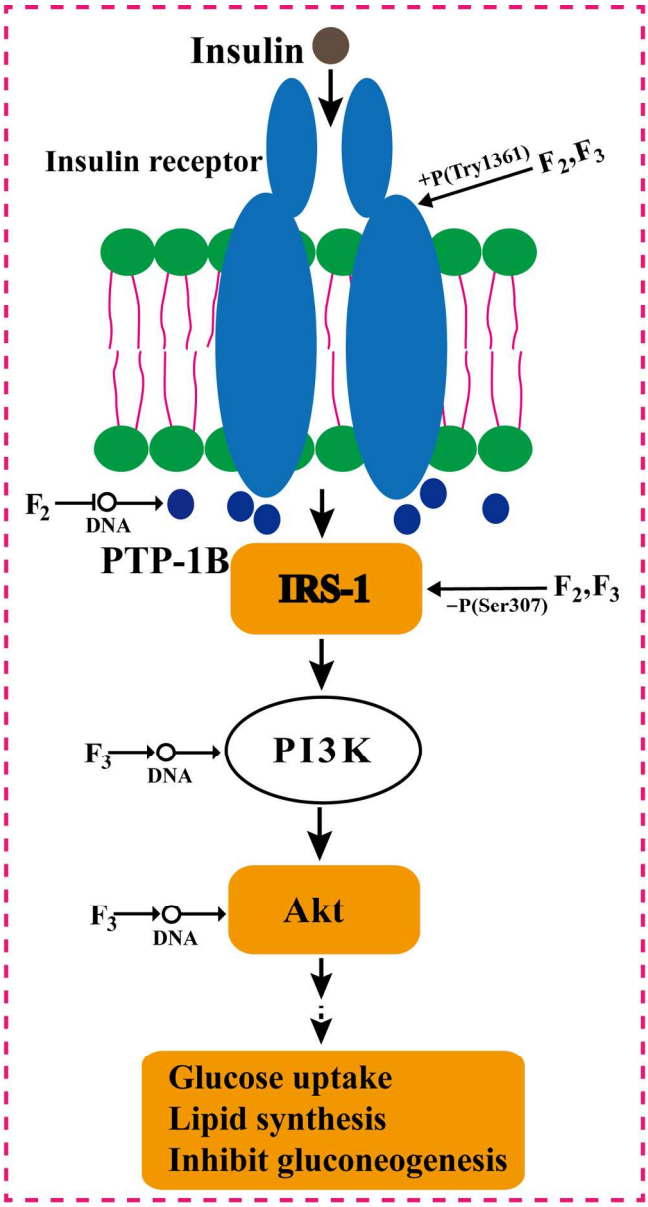

Fig 1. Action mechanism of F2 and F3 on signal transduction in insulin action.
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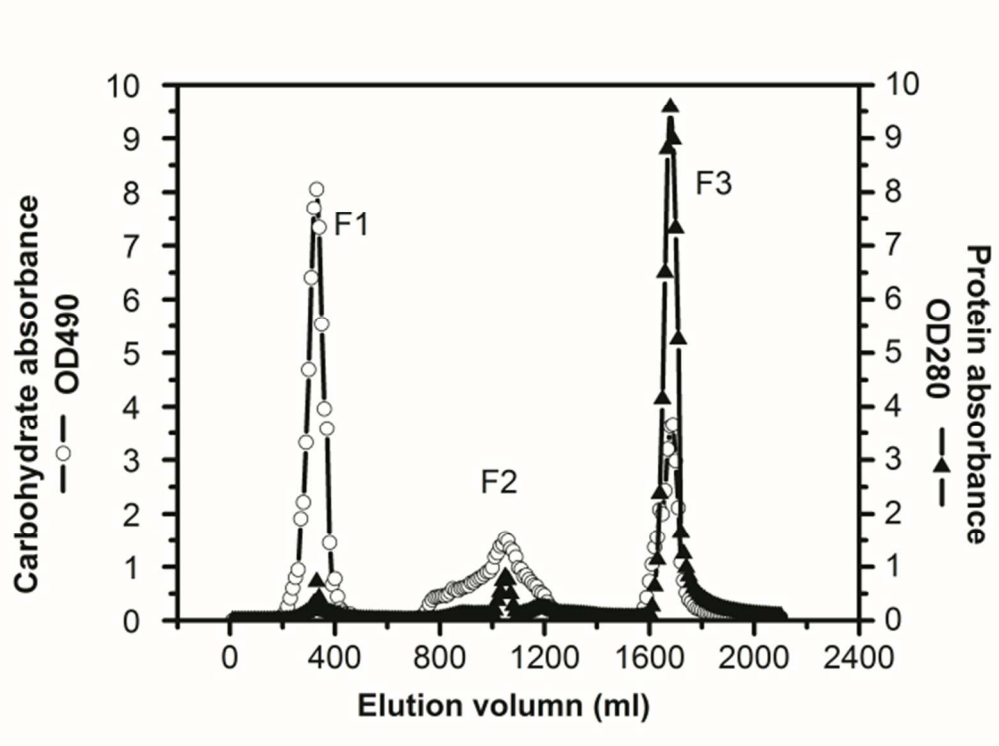

Fig 2 Fractions of GFPs by DEAE Sepharose Fast Flow chromatography.(Fractions were analyzed by measuring the absorbance at 490nm for polysaccharides and at 280nm for the proteins).
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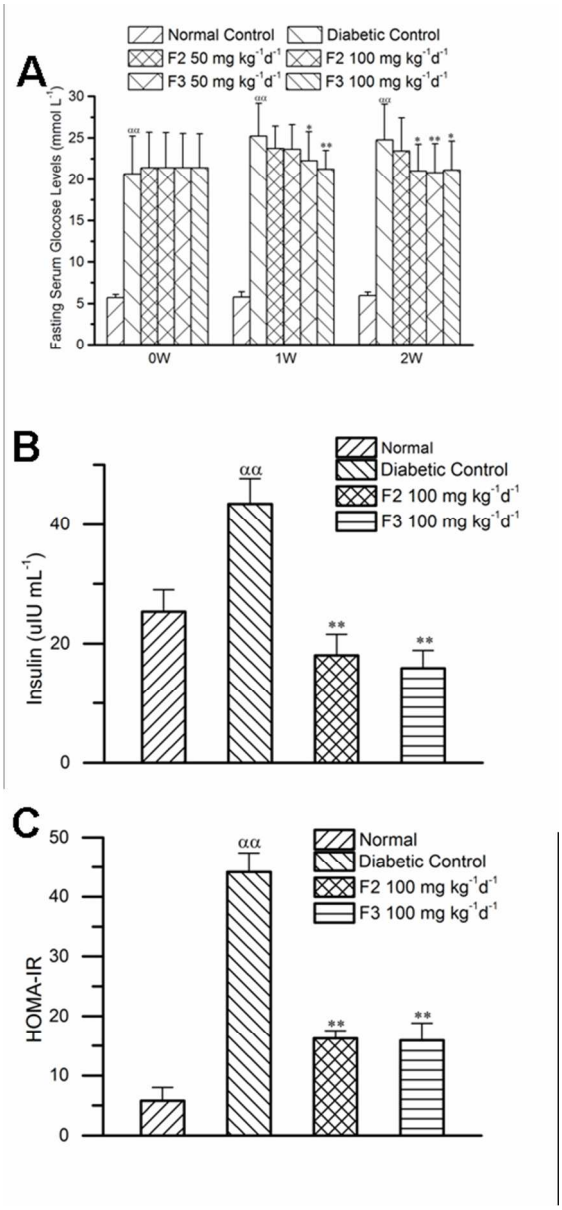

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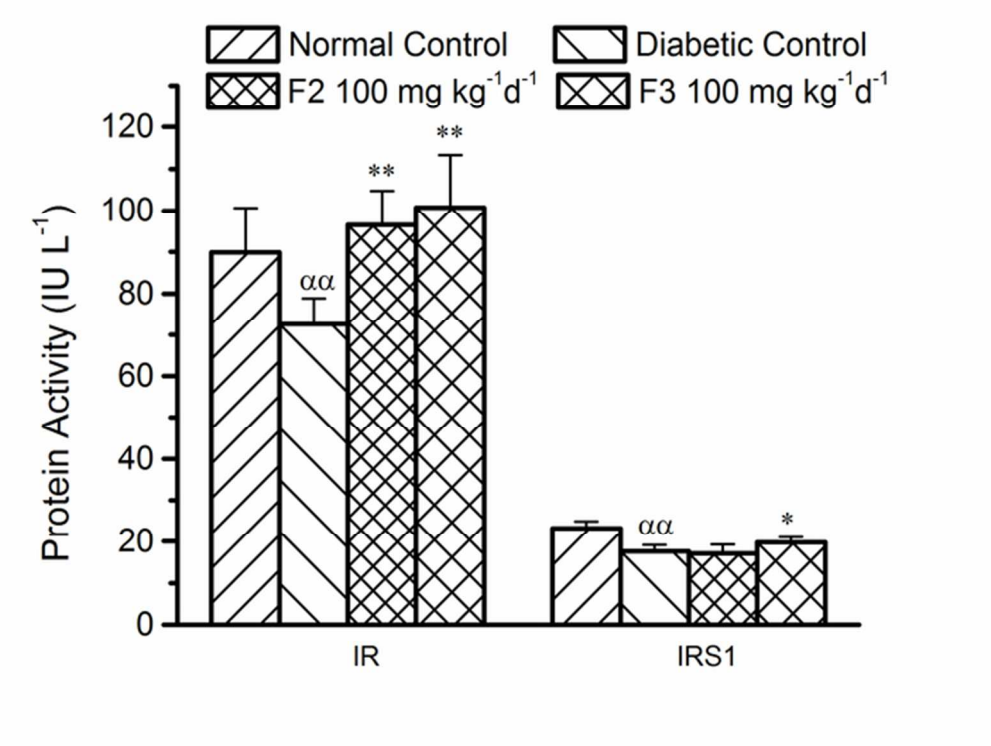
83x180mm (150 x 150 DPI)



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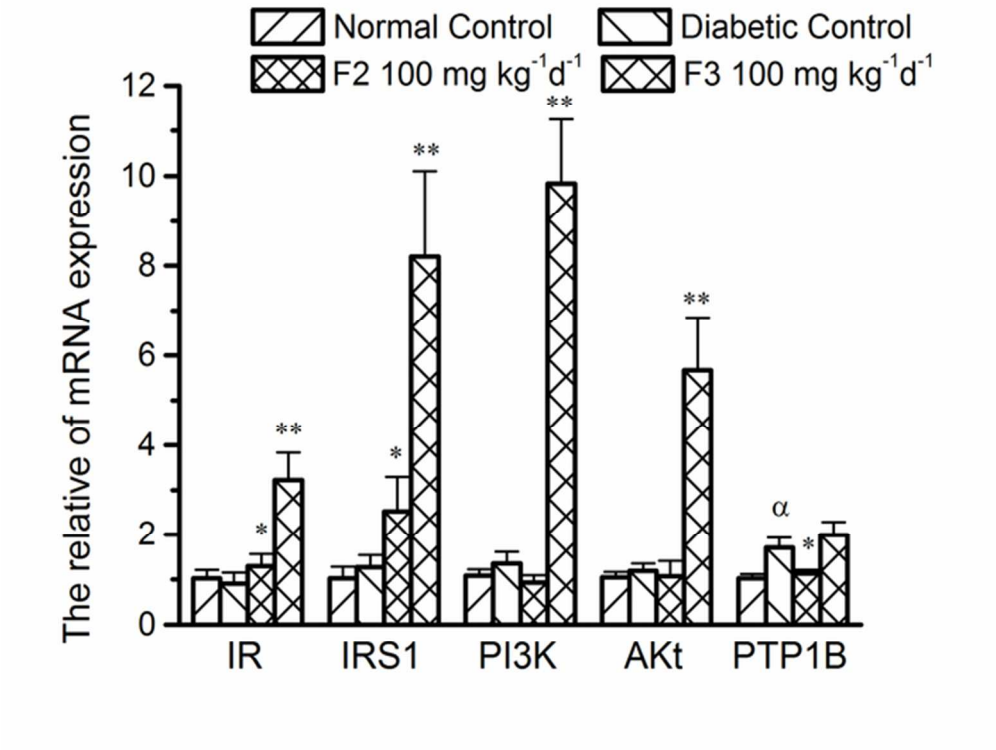

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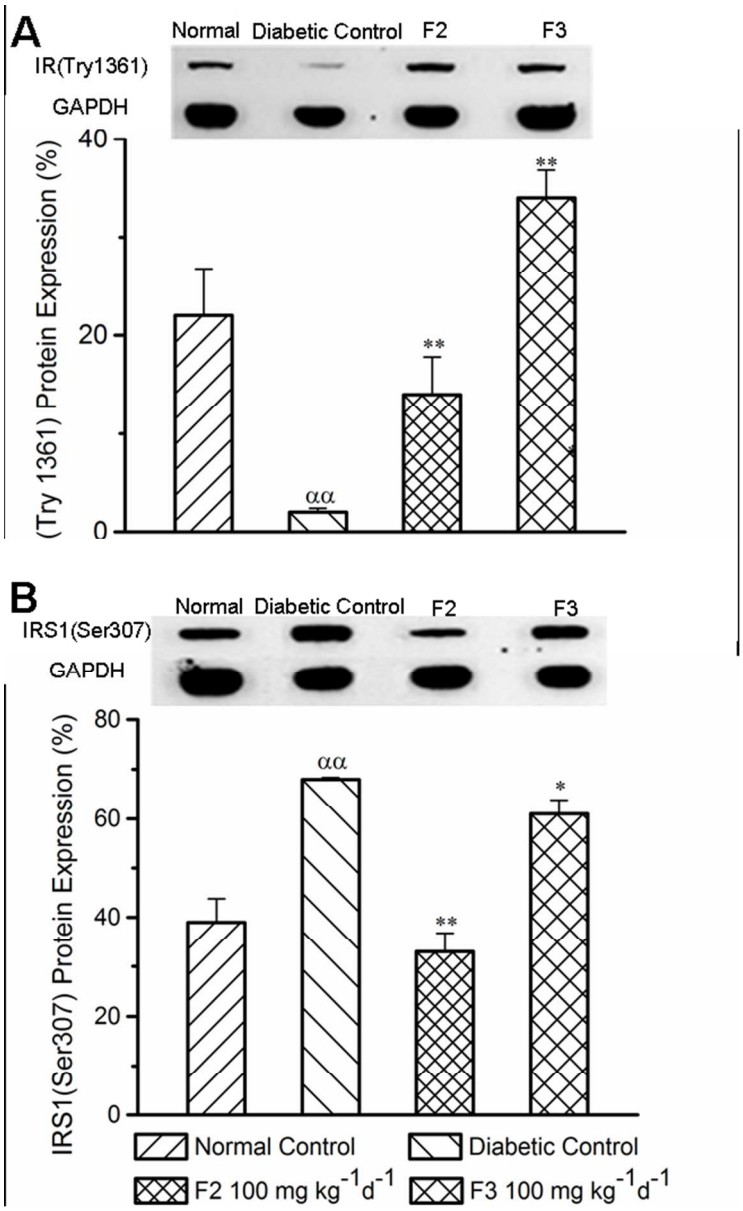

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110x180mm (150 x 150 DPI)