JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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A Protein Tyrosine Phosphatase 1B Activity Inhibitor from the Fruiting Bodies of *Ganoderma lucidum* (Fr.) Karst and Its Hypoglycemic Potency on Streptozotocin-Induced Type 2 Diabetic Mice

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ABSTRACT: Inhibition of protein tyrosine phosphatase 1B (PTP1B) activity has been considered to be a promising therapy approach to treat type 2 diabetes. In this work, a novel PTP1B activity inhibitor, named *FYGL* (Fudan−Yueyang−*G. lucidum*), was screened from the fruiting bodies of *Ganoderma lucidum* and showed an efficient PTP1B inhibitory potency with IC_{50} = 5.12 ± 0.05 μg/mL. *FYGL* is a water-soluble macromolecular proteoglycan with a protein to polysaccharide ratio of 17:77 and a viscosity-average molecular weight (M_η) of 2.6×10^5. The type 2 diabetic mice treated orally by *FYGL* showed an obvious decrease in plasma glucose level compared with the diabetic controls without drug treatment, comparable with that of diabetic mice treated with metformin, a clinical drug. The toxicity of *FYGL* is very low. The results indicate that *FYGL* may serve as a drug candidate or a health-care food for diabetic therapy or protection.

KEYWORDS: *Ganoderma lucidum*, diabetes, protein tyrosine phosphatase 1B, inhibitor

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders induced by many etiologies and characterized by hyperglycemia. In recent years, DM has become a leading cause of morbidity. The World Health Organization estimated that diabetes is responsible for approximately 5% of all deaths worldwide and predicted a >50% increase in diabetes-related mortality in 10 years. The main pathogenesis of diabetes is lack of insulin secretion and insulin resistance.[1] Therefore, developing insulin-sensitive drugs is an important approach to treat this disorder.

Protein tyrosine phosphatases (PTPs), which dephosphorylate the phosphotyrosine residues of proteins, play an important role in the intracellular signal transduction process and metabolism. One important phosphatase, protein tyrosine phosphatase 1B (PTP1B), which is considered to be critical in the protein tyrosine phosphatases (PTPs) family, controls the insulin signaling pathway.[2,3] PTP1B has been considered to be a promising insulin-sensitive drug target for the prevention and treatment of metabolic disorders.[4] Although there are a number of reports on the design and development of synthetic PTP1B inhibitors and their extractions from plants,[5,6] most of them are small molecules.[7] Furthermore, there are only few reports on natural macromolecular compounds as PTP1B inhibitors.[8]

Ganoderma lucidum (*G. lucidum*), that is, "Lingzhi", is a well-known Chinese medicinal fungus and has long been used as a traditional Chinese medicine to promote health and longevity; it was called an "elixir" by ancient emperors. *G. lucidum* was recorded earliest in "Shen Nong's Materia Medica" in the Han Dynasty of China about 2000 years ago. In the past three decades, *G. lucidum* medicinal potencies on cancer, hypertension, oxidation, hypercholesterolemia, and immunological diseases have been demonstrated pharmacologically.[9−17] As the important biologically active components in *G. lucidum*, polysaccharides and proteoglycans are reported to have those roles.[15−17] The effects of the active extracts of *G. lucidum* on the plasma glucose level in vivo have also been investigated.[18−24] The studies indicated that the polysaccharides of *G. lucidum* can promote the release of serum insulin and decrease the plasma glucose level in vivo.[21−24] For instance, Zhang and Lin[24] reported that *G. lucidum* polysaccharides (*Gl*-PS) lowered dose-dependently in vivo the serum glucose levels of the normal fasted albino Swiss mice in 3 and 6 h after intraperitoneal administration of *Gl*-PS 100 mg/kg, meanwhile increased the circulating insulin levels in 1 h after administration. In addition, *Gl*-PS had no effect in vitro on the insulin content of pancreatic islets isolated from normal fasted albino Swiss mice, but stimulated insulin release after incubation with 5.6 mmol/L glucose. This result suggested that the hypoglycemic mechanism of *Gl*-PS is through the stimulation of insulin release from the islets directly. Although the evidence suggests that the extractions of *G. lucidum* are of therapeutic benefit for diabetes, the antihyperglycemic effects

Received: February 7, 2011
Revised: May 17, 2011
Accepted: May 17, 2011
Published: May 17, 2011

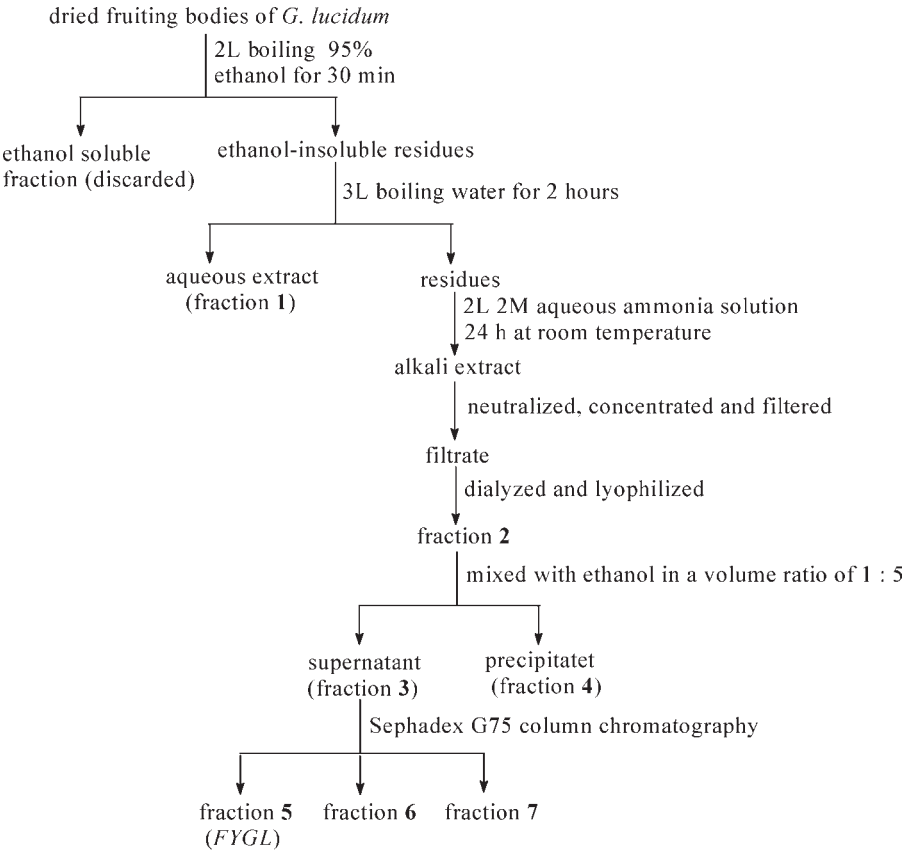

Figure 1. Screening procedure of PTP1B inhibitors from *G. lucidum*.

and mechanism of the extractions are still far from the sufficient investigation.

In the present work, we screened an extract, named *FYGL* (Fudan−Yueyang−*G. lucidum*), from the fruiting bodies of *G. lucidum*, which is capable of inhibiting PTP1B activity and decreasing the plasma glucose level in vivo. We studied the *FYGL* component and inhibition kinetics. Furthermore, the pharmacology and toxicity of the efficient extract were also investigated in vivo.

MATERIALS AND METHODS

Materials. All of the dried fruiting bodies of *G. lucidum* were purchased from Leiyunshang Pharmaceutical Co. Ltd. (Shanghai, China). PTP1B was donated by Shanghai Viva Biotech Co. Ltd. (Shanghai, China). Glucose, bluestone, potassium sodium tartrate, streptozotocin (STZ), Tris, *p*-nitrophenyl phosphate (*p*NPP), porcine insulin, and standard dextrans T-70, T-40, T-20, and T-10 were bought from Sigma Chemical Co (St. Louis, MO).

Ultraviolet Spectrum. The ultraviolet (UV) spectrum of the sample was recorded by a UV spectrometer (Lambda 35 UV, Perkin-Elmer, USA) for the polysaccharide analysis.[25] The polysaccharide derivatives were prepared by adding phenol and sulfuric acid to the samples, and then the formed derivatives were collected in a 1 cm quartz cell and their optical absorptions measured at a wavelength of 490 nm, using glucose as the standard.

Extraction and Isolation. The screening procedures referred to the literature[26] and are described as Figure 1. One hundred and sixty grams of dried fruiting bodies of *G. lucidum* was milled and then degreased with 2 L of 95% boiling ethanol with agitation for 30 min. The ethanol-soluble fraction was discarded, and 154 g of ethanol-insoluble residues was dried and then decocted in 3 L of boiling water with stirring for 2 h. After filtration of the decocted mixture, the aqueous extract was lyophilized, resulting in 12 g of fraction 1 (yield of 7.5%). Furthermore, the residues were treated with 2 L of 2 M aqueous ammonia solution with stirring for 24 h at room temperature, and then the mixture was filtered and the supernatant neutralized by 2 M acetic acid and then concentrated and filtered. The filtrate was dialyzed and lyophilized, resulting in 4.5 g of fraction 2 (yield of 2.8%). Subsequently, fraction 2 was dissolved in 450 mL of deionized water and then mixed with ethanol in a volume ratio of 1:5, and the supernatant was dialyzed and lyophilized, resulting in 2.4 g of fraction 3 (yield of 53%). The precipitate was lyophilized, resulting in 2.1 g of fraction 4 (with yield of 47%). Fraction 3 was dissolved in the deionized water and further fractionated by Sephadex G75 column chromatography with 2 M sodium chloride solution as the eluent. The eluates (6 mL/tube) were collected and characterized by the phenol−sulfuric acid method with ultraviolet (UV) absorption at wavelength of 490 nm.[25] There were 300 mL (50 tubes) of solutions collected in all, and the elution volumes of 91−126, 163−192, and 199−240 mL, which have relatively high UV absorption as shown in Figure 2A, were combined together, respectively, and then dialyzed and lyophilized, resulting in 1.54 g of fraction 5 (yield of 64%), 0.17 g of fraction 6 (yield of 7%), and 0.22 g of fraction 7 (yield of 9%).

PTP1B Inhibition Measurement. The inhibitory potencies of the seven fractions on the PTP1B activity were evaluated according to the method described in the literature.[27] Briefly, the PTP1B enzyme activity was measured by adding 50 μL of 1 mM *p*-nitrophenyl phosphate (*p*NPP, as substrate) and 50 μL of 100 μg/mL PTP1B in a buffer solution (pH 8.0) containing 50 mM Tris and 150 mM NaCl, with or without inhibitors. After incubation at 37 °C for 30 min, the enzyme reaction was terminated with 3 M NaOH. The amount of produced

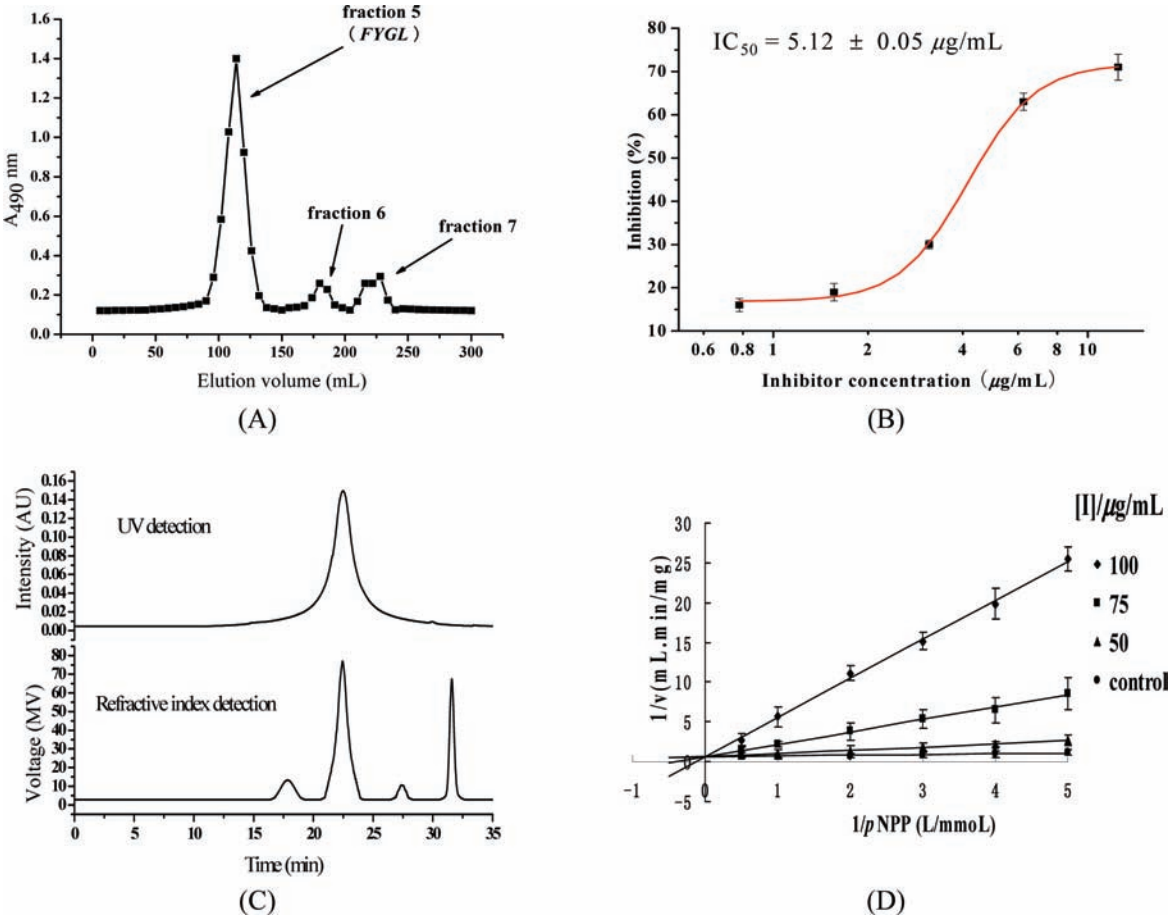

Figure 2. (A) Elution profiles for the isolation of *FYGL*; (B) *FYGL* inhibitory potency on PTP1B activity; (C) GPC of *FYGL* using UV and refractive index detectors (UV signal observed at λ = 254 nm); (D) Lineweaver–Burk plots of PTP1B enzyme reaction with the PTP1B substrate of *p*NPP at different concentrations [I] of the PTP1B inhibitor of *FYGL*, [I] = 0.0, 50.0, 75.0, and 100.0 μg/mL. Data are the mean ± standard deviation (n = 3).

p-nitrophenol was measured by UV absorbance at a wavelength of 405 nm with a microplate reader. The IC_{50} values referring to the concentration of the inhibitor required to decrease the initial PTP1B activity by 50% were used to evaluate the inhibitory potency of the inhibitors. The IC_{50} values for those seven fractions are summarized in Table 1. It is observed that the fraction 5 has the best inhibition potency of IC_{50} = 5.12 ± 0.05 μg/mL on the PTP1B activity as shown in Figure 2B. Therefore, we named fraction 5 as *FYGL*, with a yield of about 0.96% referred to the original dried fruiting bodies of *G. lucidum*. Following work will be focused on studies of *FYGL*.

Gel Permeation Chromatography (GPC). A GPC method is often used to detect the macromolecular homogeneity and molecular weight based on the volume exclusion theory. Therefore, the homogeneity of *FYGL* was detected by GPC using an Agilent 1100 GPC apparatus with a PL aquagel–OH mixed 8 μm column (300 × 7.5 mm, i.d.; Agilent) and 0.05 mol/L Na_2SO_4 eluent at a flow rate of 0.5 mL/min and a column temperature of 25 °C. The eluate was monitored by both UV and refractive index (RI) detectors. The UV signal was observed at λ = 254 nm. UV can detect organic compounds such as proteins and conjugate chemicals that absorb UV light, but does not detect those compounds without conjugate electrons, such as polysaccharides. The RI can detect most of the macromolecular chemicals even without conjugate electrons, such as polysaccharides, polyethylene, and polyethylene glycol. The double detectors of UV and refractive index can be used to analyze two motifs whether or not covalently bound in the studied samples. The GPC results of *FYGL* are shown in Figure 2C.

Table 1. PTP1B Inhibitory Potency, Total Polysaccharide, and Total Protein Contents of Different Fractions from the Fruiting Bodies of *G. lucidum*

fraction	PTP1B inhibitory potency/ IC_{50}[a] (μg/mL)	total polysaccharide content (%)	total protein content (%)
1	>300	36 ± 1	3.5 ± 0.8
2	71 ± 2	73 ± 2	5.1 ± 0.8
3	43 ± 2	57 ± 4	9.0 ± 0.7
4	94 ± 3	88 ± 3	2.9 ± 0.5
5 (*FYGL*)	5.12 ± 0.05	77 ± 3	16.8 ± 0.9
6	47 ± 3	49 ± 2	7.2 ± 0.7
7	82 ± 2	61 ± 4	3.6 ± 0.2

[a] IC_{50} refers to the concentration of inhibitor required to have a 50% inhibition of the initial one. IC_{50}, total polysaccharide and total protein contents are the mean ± standard deviation with n = 3.

Determination of Inhibition Kinetics and Its Parameters. *FYGL* aqueous solution at different concentrations of 0.0 (control), 50.0, 75.0, and 100.0 μg/mL was individually added to the reaction mixtures of 50 μL of 100 μg/mL PTP1B enzyme with various concentrations of PTP1B substrate of *p*NPP. The amount of the produced *p*-nitrophenol was measured by UV absorption at a wavelength of 405 nm with a microplate reader. The inhibition kinetics parameters were determined

using the Lineweaver−Burk plot,[28] $1/V$ versus $1/[S]$, shown in Figure 2D, where V is the enzyme reaction rate and $[S]$ is the concentration of the substrate.

Elements and Molecular Weight (M_η) Analysis. C, H, N, and S element contents in *FYGL* were determined by a Carlo Erba EA 1108 automatic elemental analyzer. Other elements (including Na, B, Ca, Cu, Fe, Mg, Mn, Si, Zn, and Se) were determined by a P-4010 inductively coupled plasma atomic emission spectrometer. The viscosity-average molecular weight of *FYGL* was determined by an Ubbelohde viscosimeter using standard dextrans T-70, T-40, T-20, and T-10, corresponding to the molecular weights of 70000, 40000, 20000 and 10,000, respectively, as the reference.

Infrared (IR) Spectrum. The IR spectrum of *FYGL* was recorded using a Fourier-transform infrared (FTIR) spectrometer (Nexus 5DXC FTIR, Thermo Nicolet, USA). The sample was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into a 1 mm thick pellet for IR measurement within the wavenumbers of 4000−400 cm^{-1} as shown in Figure 3.

Monosaccharide Component Analysis. The monosaccharide content in *FYGL* was determined after acid hydrolysis of the polysaccharides by trifluoroacetic acid (TFA).[29] The monosaccharide was separated by a CarboPac PA 10 anion-exchange chromatography column (length × diameter of 25 cm × 2 mm) using an isocratic elution consisting of 14 mmol/L sodium hydroxide at a flow rate of 0.20 mL/min. The monosaccharide was determined by the pulsed amperometric detector with an Au working electrode and an Ag/AgCl reference electrode. Standard sugars include galactose, rhamnose, glucose, arabinose, mannose, xylose, and fructose. The results are summarized in Table 2.

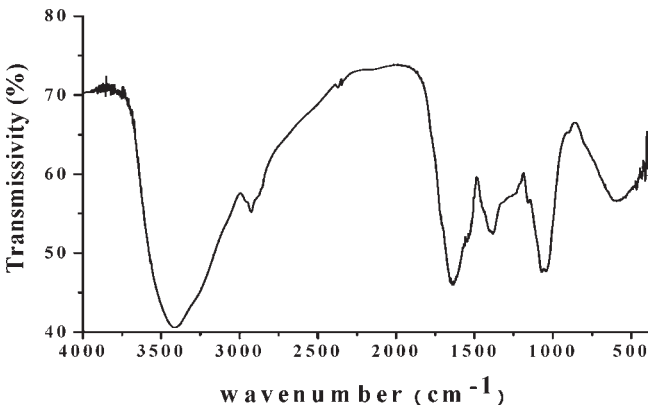

Figure 3. IR spectrum of *FYGL*.

Amino Acid Component Analysis. The amino acid content of *FYGL* was analyzed using an amino acid analyzer. The analysis procedure was similar to that reported by Cohen and Strydom.[30] The sample was vacuum-dried and placed in a hydrolysis vessel containing 18% HCl and 1% phenol. The vessel was purged with nitrogen gas and sealed under vacuum. The sample in the vessel was hydrolyzed at 110 °C for 24 h. After hydrolysis of the sample, the vessel was cooled and vacuum-dried to remove the residual HCl. The dried sample was dissolved in a citrate buffer of pH 2.2 and analyzed using a Hitachi L-8500 amino acid analyzer packed with a cation exchange resin and eluted with a series of buffers ranging from low (0.25 M sodium citrate, pH 3.05) to high (0.25 M sodium nitrate, pH 9.5) pH values. Detection of amino acids was carried out using postcolumn derivatization with *o*-phthalaldehyde, a fluorescent reagent that reacts with all of the amino acids except proline. For proline detection, the sample was treated with sodium hypochlorite, and the same procedure as that used for other amino acid analysis was carried out. The results are summarized in Table 2.

Polysaccharide Content Analysis. To correlate the polysaccharide content and PTP1B inhibition potency, the polysaccharide contents of the seven fractions extracted from the fruiting bodies of *G. lucidum* were analyzed by the Dubois phenol−sulfuric acid colorimetric method.[25] Briefly, the sample was dissolved in double-distilled water to form a 10 mg/mL stock solution. The stock solution was diluted with distilled water to concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 mg/mL, respectively. For every 0.5 mL of sample, 0.5 mL of 5% phenol color reagent was added. The mixture was vigorously stirred to form a homogeneous solution, and then 2.5 mL of sulfuric acid was rapidly added; the mixture was stirred thoroughly to produce an orange product, which was observed by UV absorption at a wavelength of 490 nm. The polysaccharide content was determined using standard glucose solution as the reference. The results are summarized in Table 1.

Protein Content Analysis. To correlate the protein content and PTP1B inhibition potency, the protein content of those seven fractions was determined by Lowry assay, using bovine serum albumin (BSA) as the standard reference.[31] Briefly, reagent R1 includes 4% (w/v) sodium carbonate and 0.2 M sodium hydroxide in the same volume, whereas reagent R2 includes 1% (w/v) bluestone and 2% (w/v) potassium sodium tartrate in the same volume. Reagent R comprises R1 and R2 in the ratio of 50:1. One milliliter of 50 μg/mL extraction or 1 mL of 100, 200, 300, 400, and 500 μg/mL standard BSA protein solution and 5 mL of reagent R were mixed together. The mixture solution was incubated for 10 min at room temperate, the Folin−phenol reagent was added for 30 min, and the solution was measured using UV at a wavelength of 650 nm. The results are summarized in Table 1.

Animal Trials. To test the hypoglycemic potency of *FYGL* on streptozotocin-induced type 2 diabetic mice, 60 male Chinese Kunming mice (i.e. *Mus musculus*), 8 weeks old and ∼20 g weight (from the

Table 2. Monosaccharide and Amino Acid Components[a] in *FYGL*

component	monosaccharide content (%)	component	amino acid content (%)	component	amino acid content (%)
glucose	78.12	Asp, Asn	13.03	Val	4.78
arabinose	9.72	Gly	10.96	Pro	4.11
xylose	7.50	Glu, Gln	10.15	Ile	3.84
rhamnose	2.26	Ala	8.72	Tyr	3.05
galactose	1.96	Ser	8.65	Arg	2.64
fructose	0.44	Thr	7.51	Lys	2.63
		Leu	5.98	His	2.10
		Phe	5.15	Cys	0.71
				Met	0.40

[a] Asp, aspartic acid; Asn, asparagine; Gly, glycine; Glu, glutamic acid; Gln, glutamine; Ala, alanine; Ser, serine; Thr, threonine; Leu, leucine; Phe, phenylalanine; Val, valine; Pro, proline; Ile, isoleucine; Tyr, tyrosine; Arg, arginine; Lys, lysine; His, histidine; Cys, cysteine; Met, methionine.

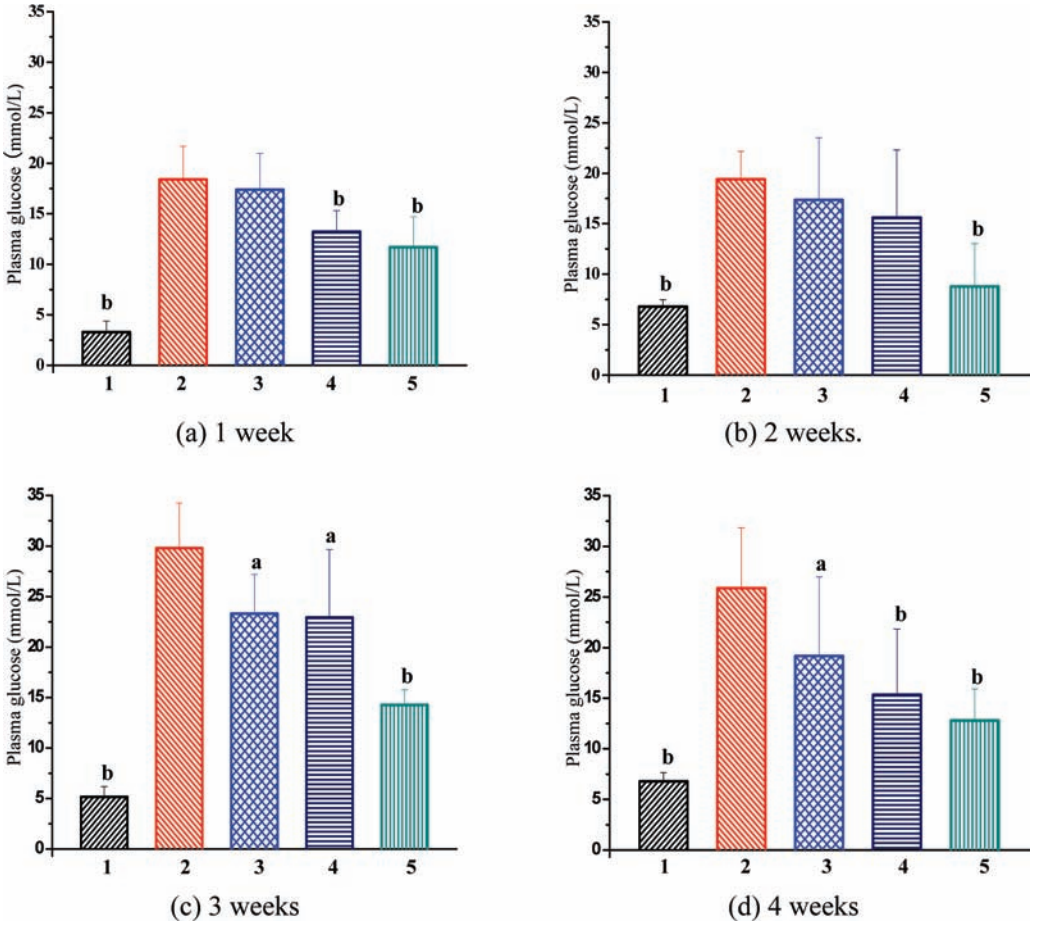

Figure 4. Plasma glucose level (mmol/L) of mice treated for 1 week (a), 2 weeks (b), 3 weeks (c), and 4 weeks (d): group 1, normal mice; group 2, STZ-induced diabetic mice (control); groups 3 and 4, STZ-induced diabetic mice treated with 50 and 150 mg/kg doses of *FYGL*, respectively; group 5, STZ-induced diabetic mice treated with 300 mg/kg dose of the clinical agent metformin. Data are the mean ± standard deviation (n = 10 for each group); a,$p < 0.05$, and b, $p < 0.01$, diabetes control group compared with the diabetes groups treated with drugs.

experimental animal center of Shanghai University of Traditional Chinese Medicine), were selected for the studies. All of the animal trial procedures instituted by the Ethical Committee for the Experimental Use of Animals in Shanghai University of Traditional Chinese Medicine were followed. Ten mice were fed normal foods under normal environment as a normal group, and the other 50 mice were housed 5 to a cage with a 12:12 h light/dark cycle at ambient temperature of 22−25 °C and fed a high-fat diet. After 5 weeks, the 50 mice were fasted for 12 h, but had free access to water, and then injected with STZ (40 mg/kg in 0.1 M citrate-buffered saline, pH 4.5) into the intraperitoneal to induce type 2 diabetes. The STZ-treated mice had free access to high-fat foods and water for 1 week and were subjected to 12 h of fasting. A total of 40 among the 50 STZ-treated mice showed fasting glucose levels of ≥ 11.1 mmol/L and were considered to be type 2 diabetic mice.[32]

Fifty animals, including 10 normal mice and 40 STZ-induced type 2 diabetic mice, were divided into five groups (numbered as groups 1−5) with 10 mice in each group. Group 1 are normal mice that were given 20 mL/kg physiological NaCl solution (vehicle); group 2 are diabetes control mice, which were also given 20 mL/kg physiological NaCl solution (vehicle). However, the mice in groups 3 and 4 were diabetic and orally treated with 50 and 150 mg/kg doses of *FYGL* once a day, respectively. The mice in group 5 were diabetic and treated with a 300 mg/kg dose of the clinical oral drug metformin once a day. All of the animals in the different groups were fed normal daily foods in addition to the given drugs during the trial. Blood samples were obtained from the tail vein 2 h before the oral administration of the drugs. Glucose concentration in the plasma was measured using glucose oxidase method (sensitivity of 0.1 mM, Sigma Diagnostics, St. Louis, MO). The results of the animal trials are shown in Figure 4.

Animal Toxicity Trials. The toxicity of *FYGL* was preliminarily evaluated by LD_{50} value (i.e., the drug dose leading to 50% lethality) for 20 male Chinese Kunming mice using the improved Karber's method.[33] The mice were divided into two groups with 10 mice in each group and administered *FYGL* orally once in dose of 6 or 3 g/kg, respectively, in different groups, and were observed for 5 days. Furthermore, the pathology of the nonproliferation tissues, including myocardium, kidney, pancreas, lungs, and brain, from the experimental mice was observed visually.

Statistical Analyses. Data are expressed as the mean ± standard deviation (SD). The statistical significance in the behavioral and biochemical effects was determined by one-way analysis of variance (ANOVA). A possibility of the p value of <0.05 or <0.01 was considered as a significant difference or very significant difference between the means.

RESULTS

PTP1B Activity Inhibition. The inhibitory potency of seven fractions screened from the fruiting bodies of *G. lucidum* on PTP1B activity was evaluated. From Table 1, comparing fractions

1 (IC_{50} > 300 μg/mL) and 2 (IC_{50} = 71 μg/mL) showed that fraction 2 had a lower IC_{50} value; hence, fraction 2 was further fractionated as shown in Figure 1, and fractions 3 and 4 were obtained. Comparing fractions 3 (IC_{50} = 43 μg/mL) and 4 (IC_{50} = 94 μg/mL) showed that fraction 3 had a better inhibitory potency; therefore, fraction 3 was further fractionated by Sephadex column chromatography, and three fractions, 5, 6, and 7, were obtained. Fraction 5, that is, *FYGL*, has the lowest IC_{50} value of 5.12 ± 0.05 μg/mL (Figure 2B) and was, therefore, interesting for us.

To the authors' knowledge, *FYGL* was first reported to have efficient PTP1B inhibitory potency. The following work will analyze its components and properties.

Homogeneity of *FYGL*. GPC analysis of *FYGL* in Figure 2C shows that there is only one peak detected by UV at a wavelength of 254 nm when the elution time is at 22.5 min, indicating that the peak is maybe attributed to the protein-included compound. Moreover, there are three peaks observed by RI detector. One of the peaks, appearing also at the elution time of 22.5 min, the same as that detected by UV, is a dominant component with a content of about 91%; that means the *FYGL* is a protein-covalently bonded compound, perhaps a proteoglycan, with a relatively high homogeneity and a wide molecular weight distribution of the analogues. The other two peaks at elution times of 17.8 and 27.5 min observed only by RI detector are maybe attributed to larger and smaller molecular weights of polysaccharides, respectively, without protein included. The peak at an elution time of 31.6 min is from solvent.

Inhibition Kinetics and Its Parameters. The inhibition kinetics of *FYGL* on PTP1B activity in vitro was investigated. Four Lineweaver−Burk plots are obtained as shown in Figure 2D. A common intercept of $1/V_{max}$ = 0.65 mL·min/mg on the *y*-axis can be observed for four lines as *FYGL* concentration, [I], increases from 0.0 to 100.0 μg/mL, indicating that the inhibition kinetics of *FYGL* is competitive with the substrate of the PTP1B.[28] The maximum enzyme reaction rate of V_{max} = 1.53 mg/mL·min is thus determined from the *y*-axis intercept of the $1/V_{max}$. K_m is a complex dissociation constant of the PTP1B with substrate *p*NPP, and $-1/K_m$ = 6.40 L/mmol is determined by the intercept on the *x*-axis at an inhibitor concentration of [I] = 0, that is, the control Lineweaver−Burk plot in Figure 2D, so the K_m is 0.156 mmol/L.

Elements and Molecular Weight (M_η) of *FYGL*. The elements in *FYGL* were analyzed as C, 38.94%; N, 4.14%; H, 5.18%; S, 1.61%; O, 45.29%; and other elements (including Na, B, Ca, Cu, Fe, Mg, Mn, Si, Zn, and Se), 4.84%.

The viscosity-average molecular weight, M_η, of *FYGL* is calculated by the Mark−Houwink equation as

$$[\eta] = KM^{\alpha} \quad (1)$$

where intrinsic viscosity $[\eta]_{15\,^\circ C}$ = 28.4 mL/g, constant α = 0.42, and constant K = 0.15 mL/g were determined by the experiment in this work; therefore, the viscosity-average molecular weight of $M_\eta = 2.6 \times 10^5$ was calculated.

IR Spectroscopy of *FYGL*. The IR spectrum of *FYGL* is shown in Figure 3. According to the literature reports on the IR spectrum of proteoglycan,[34,35] it is known that a broad band around 3420 cm^{-1} is a typical absorption of the hydroxyl groups of CH_2O-H, and a band around 2937 cm^{-1} is attributed to the C−H groups. The relatively strong absorption bands at 1620 and 1400 cm^{-1} are typical absorptions of amides I and II in the proteins, respectively. The polysaccharide has a typical absorption band in the region of 1200−1000 cm^{-1}. This region is dominated by the ring vibrations overlapped with stretching vibrations of C−OH side groups and C−O−C glycosidic groups. Thus, the IR spectrum proves that *FYGL* may be a proteoglycan.

Monosaccharide and Amino Acid Components in *FYGL*. Quantitative determination of monosaccharides shows that *FYGL* contains glucose, arabinose, xylose, rhamnose, galactose, and fructose (see Table 2), and the major monosaccharide is glucose.

Furthermore, the amino acid components were also analyzed. Nineteen amino acids are found in *FYGL*, except tryptophan, which was dissociated by hydrochloric acid when the sample was treated for measurement. The major amino acids in *FYGL* are aspartic acid, glycine, glutamic acid, alanine, serine, and threonine (see Table 2).

Correlation of Polysaccharide or Protein Contents in the Extracts with PTP1B Inhibition. Correlations of the polysaccharide or protein contents of seven fractions with PTP1B inhibition are compared in Table 1. A proportional correlation between the protein content and the inhibitory potency of IC_{50} on the PTP1B activity can be observed. The fraction containing the higher protein content demonstrates more efficient inhibitory potency. For example, except for fraction 1, which contains many other biologically active components (including triterpene and lucidenic acid in addition to the polysaccharide and protein),[36−38] interfering with the PTP1B inhibition results in this study, of the fractions 2−7, which contain dominantly the polysaccharide and protein, fraction 4 has the lowest protein content and the weakest inhibitory potency on PTP1B activity, whereas *FYGL* (fraction 5) has the highest protein content and the best inhibitory potency on PTP1B activity with IC_{50} of 5.12 ± 0.05 μg/mL.

Hypoglycemic Potency and Toxicity of *FYGL* in Vivo. The hypoglycemic potency of *FYGL* on the streptozotocin-induced type 2 diabetic mice is shown in Figure 4. It is found that the treatment of the STZ-induced type 2 diabetic mice with 150 mg/kg *FYGL* (group 4) results in the considerable decrease of the plasma glucose values ($p < 0.01$) after 4 weeks, compared with group 2 without drug. Furthermore, the decrease in the glucose value is dose-dependent when compared between group 3 (50 mg/kg dose of *FYGL*) and group 4 (150 mg/kg dose of *FYGL*) for 4 weeks. The decrease in the plasma glucose level in the *FYGL* treatment group is comparable with that in the metformin treatment group (group 5 with 300 mg/kg dose of metformin) within the statistical deviation range.

Furthermore, during the toxicity trial, 5 of the 10 mice administered orally the 6 g/kg dose of *FYGL* died, 3 in 2 h and 2 in 24 h; the remaining mice were healthy over the following 5 days. Meanwhile, the mice administered the 3 g/kg dose of *FYGL* were healthy during the 5 days of observation. Thus, the toxicity of *FYGL* is preliminarily evaluated as LD_{50} = 6 g/kg with 95% confidence limits of 4.8−7.4 g/kg calculated according to the improved Karber's method,[33] and the visual observations for the nonproliferation tissues, including myocardium, kidney, pancreas, lungs, and brain from the experimental mice, showed no lesions, indicating that *FYGL* has little toxicity and is well tolerated.

DISCUSSION

PTP1B Inhibitor and Antihyperglycemic Mechanism. Previous studies have demonstrated that PTP1B can be used as a drug target in the treatment of type 2 diabetic mellitus and

obesity based on the mechanism that if PTP1B activity and expression were inhibited, the tyrosine phosphorylation level of the insulin receptor β-subunit would be increased, leading to a decrease in plasma glucose.[39] Xia et al.[40] extracted a pectin polysaccharide, named SJB, from the alkali extract of mulberry leaves; SJB is a macromolecule with a molecular mass of 5.4×10^4, consisting of rhammose, arabinose, glucose, galactose, and galacturonic acid in the molar ratio of 1.00:1.26:0.66:2.04:0.95. The study of the hypoglycemic potency of SJB shows the inhibitory rate of SJB to PTP1B is 31.7% at the concentration of 20 μg/mL. Wu et al.[41] extracted an *Astragalus* polysaccharide, APS, with a molecular weight of 3.6×10^4, from the aqueous decoction. The APS consisting of glucose, galactose, and arabinose in a molar ratio of 1.75:1.63:1 can decrease the blood glucose level, PTP1B expression, and activity in the skeletal muscles of STZ-induced type 2 diabetes mellitus rats and, consequently, increase the tyrosine phosphorylation level of the insulin receptor β-subunit in the skeletal muscles of the rats. Similarly, in the present study, *FYGL* with a molecular mass of 2.6×10^5 can inhibit PTP1B activity with an IC_{50} of 5.12 ± 0.05 μg/mL and decrease considerably the fasting blood glucose level ($p < 0.01$) in vivo. We also found that the treatment of type 2 diabetic rats with a dose of 120 mg/kg *FYGL* for 30 days significantly decreased PTP1B expression and activity in the skeletal muscles in vivo, consequently increasing the tyrosine phosphorylation level of the insulin receptor β-subunit ($p < 0.05$) in the skeletal muscles. That means the *FYGL* or its hydrolyzed motifs may enter into the plasma and interact with PTP1B. The results will be reported in the next paper. The hypoglycemic mechanism of *FYGL* in vivo is like that of APS.

In addition, there are some other reports about the antihyperglycemic mechanisms of the extractions from plants. Seto et al.[42] found that a water extraction containing ganoderic acid A from *G. lucidum* markedly reduced the phosphoenolpyruvate carboxykinase (PEPCK) expression in obese/diabetic (*db*/*db*) mice as well as in lean (*db*/*m*) mice. The authors thought that the extraction suppressed the hepatic PEPCK gene expression, leading to the decrease in the serum glucose levels. Hikino et al.[43] reported that ganoderan B screened from *G. lucidum* could promote the release of serum insulin and decrease plasma glucose in vivo by stimulating the activities of hepatic glucokinase, phosphofructokinase, and glucose-6-phosphatase dehydrogenase, meanwhile inhibiting the activity of hepatic glucose-6-phosphate and glycogen synthetase. Lee et al.[44] found an *Agrocybe chaxingu* polysaccharide that can ameliorate STZ-induced diabetes by reducing the nitric oxide (NO) production and inhibiting the inducible NO synthase (iNOS) expression levels. Mao et al.[45] also investigated the hypoglycemic effect and mechanism of the *Astragalus* polysaccharide, APS, using diet-induced insulin-resistant C57BL/6J mice, skeletal muscle C2C12 cells, and endoplasmic reticulum (ER) stressed HepG2 cells. They found that insulin action in the liver of insulin-resistant mice was restored by administration of APS. APS enhances the adaptive capacity of the ER and promotes insulin signaling by inhibition of the expression and activity of glycogen synthase kinase 3 β (GSK3β).

Whether *FYGL* has other inhibition potency besides on the PTP1B is also of interest to us.

Interaction of PTP1B with *FYGL*. Drugs often require the highest binding affinity between the enzyme active site and the drug molecule.[46] The proteoglycan is generally formed by the protein backbone and the polysaccharide side chains.[47] Various biological activities of the proteoglycans depend on the interactions of the polysaccharide side chains with the surrounding proteins, such as glycosidase, proteases, growth factor, and receptors by the secondary bonding.[47] For instance, the open structural starch polysaccharides in cereals enable easy access to hydrolysis by α-amylases and are dissociated in the small intestine.[48] Li et al.[49] found a *Pholiota nameko* polysaccharide (PNPS-1) with M_w of 1.14×10^5, which can inhibit the bovine pancreas ribonuclease (RNase A) activity in a linear mixed-type inhibition mechanism, a combination of partial competitive and pure noncompetitive inhibition. In addition, Liu et al.[50] synthesized a small molecular PTP1B inhibitor containing oxalylarylaminobenzoic acid-based pharmacophore in a competitive inhibition kinetics. The X-ray crystal structure of PTP1B in the complex with the inhibitor shows that carboxylate groups of the inhibitor interact with Tyr20 and Arg254 of the PTP1B active sites by hydrogen bonding and that the hydroxyl groups of the inhibitor interact with Arg24 and Arg254 of the PTP1B by hydrogen bonding.

In the present study, *FYGL* is a water-soluble macromolecular proteoglycan with a protein to polysaccharide ratio of 17:77. This proteoglycan is maybe formed by the polypeptide as a core and the covalently bound polysaccharide side chains as a shell. The polysaccharide side chains have molecular weight dispersions observed by GPC. Although *FYGL* with an average molecular weight of 2.6×10^5 is larger than PTP1B at 5×10^4,[51] which makes it difficult to pass through the cell membranes, it could be dissociated by the glycosidase[52] to enter through the cells. Actually, we found that *FYGL* can interact with glycosidase in vitro (data not shown), suggesting that the *FYGL* is hydrolyzed by the glycosidase in the stomach and small intestine in vivo; consequently, the dissociated protein motifs in *FYGL* enter into the plasma through pinocytosis in the terminal position of small intestine[53] and then interact with the PTP1B, therefore inhibiting PTP1B activity in the skeletal muscle of the insulin target tissue in vivo. In addition, the polysaccharide motifs in the proteoglycan are able to prevent its protein motifs from the enzymolysis. The possible interactions of the protein motif of *FYGL* with PTP1B are maybe the carboxyl groups of the aspartic acid and glutamic acid in *FYGL* interacting with Tyr20, Arg24, and Arg254 in the PTP1B active sites by hydrogen bonding.[50] The detailed interaction mechanism will be investigated by hydrolyzing the protein or polysaccharide motifs from the *FYGL* and then interacting between the dissociated products and PTP1B in further work.

In conclusion, we screened the PTP1B inhibitors from the fruiting bodies of *G. lucidum* and demonstrated that one of the inhibitors, *FYGL*, is a novel macromolecular proteoglycan different from those previously reported[17,47,54] and efficient in PTP1B inhibition, which is first reported herein. *FYGL* has very low toxicity and is capable of a hypoglycemic effect for STZ-induced type 2 diabetic mice. In addition, *FYGL* is very stable in a normal environment without vacuum and light. The polysaccharide motif in the proteoglycan may prevent the protein motif from improper folding and enzymolysis and keep the protein stable.[55] Therefore, *FYGL* can be considered as a potential therapeutic candidate for the treatment of type 2 diabetes. We have applied for a Chinese patent and PCT for the protection of screening procedures, components, and usages of *FYGL*.[56] Further work on the *FYGL* molecular structure, its pharmacy, and interaction mechanism in vivo is ongoing in our laboratory.

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Funding Sources

This work was supported by the Natural Science Foundation of China (Nos. 20673022 and 21074025), the Soft Science Program of Jing-An district in Shanghai (No. 2008RZ002), and the Scientific Program of Shanghai Municipal Public Health Bureau (Nos. 2008220 and 2010231).

ABBREVIATIONS USED

DM, diabetes mellitus; PTPs, protein tyrosine phosphatases; PTP1B, protein tyrosine phosphatase 1B; STZ, streptozotocin; *p*NPP, *p*-nitrophenyl phosphate; *G. lucidum*, *Ganoderma lucidum*; *FYGL*, Fudan−Yueyang−*G. lucidum*; GPC, gel permeation chromatography; FTIR, Fourier-transform infrared; UV, ultraviolet; BSA, bovine serum albumin.

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