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Full Length Research Paper

Isolation, purification and effects of hypoglycemic functional polysaccharides from *Inonotus obliquus*

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Inonotus obliquus **is generally used for the treatment of diseases such as cancers, angiocardiopathy and diabetes. However, few studies are available on its functional components. The objective of this study was to isolate and purify hypoglycemic functional polysaccharides from *I. obliquus* (HPIO) and study their hypoglycemic activities. *I. obliquus* powder was used to obtain crude polysaccharides at room temperature (HPIO-R) and at high temperature (HPIO-H) using diethylaminoethyl cellulose (DEAE) cellulose -52 column chromatography for purification. Streptozotocin (STZ)-induced hyperglycemic mice were used to evaluate the *in vivo* antihyperglycemic and antilipidperoxidative effects of HPIOs at each eluted level. The results show that HPIO was a uniform compound and HPIO (0.2 mol/L NaCl) had antihyperglycemic effects and regulated lipid metabolism.**

Key words: Hypoglycemic activity, polysaccharides, *Inonotus obliquus*, isolation, purification.

INTRODUCTION

Obesity, high blood glucose and diabetes which are the significant chronic diseases and causes of death in modern society and they are largely caused by diet. Worldwide, scientists have focused on how to reduce blood glucose levels (Bjorntorp et al., 1999). The worldwide incidence of diabetes mellitus is expected to continue growing by 6% annually and to become a leading cause of human death (Kang et al., 2008). Therefore, new drugs to manage this condition are needed.

Many drugs are available to manage diabetes; however, most are expensive and have side effects. To find new drugs and meet patient needs, scientists have studied herbs with no side effects. Studies have shown that fungi are highly edible and have medicinal value, especially *Inonotus obliquus*, a well-known medicinal plant traditionally used for antihyperglycemic effects.*I. obliquus* is a white rot fungus that grows under the bark of *Betula (birch), Ulmus, Alnus* and dry dead trees (Mao, 2000). It is a typical disease fungus of trees and is widely distributed over the latitude 45° N to 50° N area, such as in Northern Russia, Europe, China and Hokkaido, Japan. It has been used as a folk remedy to prevent digestive system diseases; cancers of the stomach, colon and liver; angiocardiopathy; diabetes; and viral diseases. Studies have shown that a glycoprotein and a water extraction of polysaccharides isolated from the fruiting bodies of *I. obliquus* have significant antihyperglycemic effects. In particular, an extract of *I. obliquus* can sustain antihyperglycemic effects for 48 h (Huang, 2002). Extracts from *I. obliquus* have protective and prosthetic effects on the pancreatic islands, and on hepatic and kidney injury in STZ-induced diabetic rats (Zhang et al., 2008). Studies have also shown that dry matter from the culture broth of *I. obliquus* and fruiting bodies, and sclerotia and polysaccharide extracts of *I. obliquus* have significant antihyperglycemic, antilipid-peroxidative, and antioxidant effects on diabetic mice (Sun and Ao 2008; Chen et al., 2006). Mizuno et al. 1999; Mizuno and Zhuang 2005) found that soluble or non-water-soluble

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Abbreviations: **HPIO,** Hypoglycemic functional polysaccharides from *I. obliquus;* **HPIO-R,** hypoglycemic functional polysaccharides at room temperature; **HPIO-H,** hypoglycemic functional polysaccharides at high temperature; **DEAE,** diethylaminoethyl cellulose; **STZ,** streptozotocin.

polysaccharides isolated from the scleritis and mycelia of *I. obliquus* showed antihyperglycemic effects. This was confirmed by further studies that concluded that trametenolic acid in the water extract of *I. obliquus* has an effect on non-insulin-dependent diabetes mellitus.

Most patients with diabetes have hypercholesterolemia and hyperglycemia and a higher risk of heart disease and dyslipidaemia. Diabetes is primarily characterized by fasting hyperglycemia and can lead to severe health problems (Tamrakar et al., 2008). While many drugs to prevent and treat diabetes are in trials, none are highly effective. Oral hypoglycemia agents can reduce blood sugar levels, but they have long-term toxic effects (Mitra et al., 1996).

Many new agents and methods have been used to treat diabetes. In recent years, mushroom polysaccharides have received attention and shown to have antitumor effects (Borchers et al., 1999; Leung et al., 1997). Previous studies found that *I. obliquus*, a kind of mushroom has therapeutic effects but the active ingredients have not been clearly elucidated.*I. obliquus* polysaccharides that might defend against cancers and obesity are needed; however, little research is available on the isolation and purification of *I. obliquus* polysaccharides and their hypoglycemic activity. In this article, a preparation method was established for purification of room-temperature and high-temperature extracts of crude polysaccharides (HPIO-R and HPIO-H) from *I. obliquus* fruiting bodies by DEAE cellulose-52 column chromatography. The hypoglycemic activity of different fractions was studied in mice, providing a theoretical and experimental basis for the application of *I. obiquus* extracts in treatment and further research.

MATERIALS AND METHODS

Plant material

I. obliquus harvested at Changbai Mountain, Jilin, China, were crushed and sieved by 40 mesh and stored in the laboratory.

Reagents

Metformin hydrochloride tablets were from Beijing Pharmaceutical Co., Ltd. (China; 0.25 g × 50 pieces/box). DEAE cellulose-52 column chromatography reagents were from Pharmacia Inc. (USA). Streptozotocin (STZ) was from Sigma-Aldrich Inc. (USA). All other chemicals were analytical grade. Assay kits for glucose oxidase, triglyceride (TG) and enzymatic determination of total cholesterol (TC) were purchased separately from Beijing Strong Biotechnologies, Inc. Beijing Kang Tai clinical reagent Co., Ltd. and BioSino Bio-technology and Science Inc.

Animals

Male ICR mice (20 ± 2 g) were from the Chinese Academy of Military Sciences Experimental Animal Center, license number SCXK (Army) 2002-001. All animal handling procedures were in strict accordance with the PR China legislation on the use and care of laboratory animals, with guidelines established by the Experimental Animal Center of Peking University.

Experimental procedures

Preparation of *I. obliquus* total crude polysaccharide extracts

The powdered fruiting bodies of *I. obliquus* were extracted with water at 30 times volume at 90°C for 3 h, and centrifuged at 4000 rpm for 20 min. The precipitated fraction was dried at 50°C and total polysaccharide determined. This was used to compare the antihyperglycemic effects to fractionated polysaccharide.

Preparation of crude polysaccharide from *I. obliquus* fruiting bodies

Polysaccharides at room temperature: For HPIO-R, *I. obliquus* powdered fruiting bodies were extracted with water at room temperature at 30 times volume for 48 h and centrifuged at 4000 rpm for 20 min. The aqueous phase was evaporated, reduced to an appropriate volume and mixed with absolute ethyl alcohol (1:4, v/v). The precipitated fraction was dried at 50°C and total polysaccharide was determined.

Polysaccharide at high temperature: For HPIO-H, after polysaccharide extraction at room temperature, the powder was washed three times with water at room temperature, then extracted with water at 90°C at 30 times volume for 3 h and centrifuged at 4000 rpm for 20 min. The aqueous phase was evaporated, reduced to an appropriate volume and mixed with absolute ethyl alcohol (1:4, v/v). The precipitated fraction was dried at 50°C and total polysaccharide was determined.

Isolation and purification of polysaccharide

HPIO-R and HPIO-H were dissolved in distilled water. And then they were loaded into an anion-exchange DEAE cellulose DEAE-52 column (2.6×25 cm), after that they were eluted stepwise with H_2O, 0.2 mol/L NaCl, 0.5 mol/L NaCl at 120 ml/h to give rise to three fractions. Polysaccharide was determined at 495 nm by using phenol-sulfuric acid method (Li et al., 1997). Fractions were collected, concentrated, and centrifuged. Precipitated polysaccharides were dialyzed for 24 h in distilled water, and then they were obtained for the subsequent studies.

Measuring *in vivo* antihyperglycemic effects of polysaccharide fractions

Establishment of the diabetes mice model

ICR mice were acclimatized under controlled conditions for 1 week before experiments. Mice were given intraperitoneal injections of freshly prepared STZ (35 mg/kg in 0.01 mol/L citrate buffer) 4 times per day for 3 days while normal control groups were injected with buffer only. On the sixth day, only water was offered to the animals for 6 h, then blood was collected from the tail vein, and glucose level measured.

Antihyperglycemic effects analysis

Mice with blood glucose levels above 12 mmol/L were deemed to be hyperglycemic and randomly divided into 10 groups with 12 animals in each group. Both the normal and diabetic control groups were fed a basal diet and sodium chloride solution. Model animals were fed with crude total polysaccharide (100 or 300 mg/kg body weight), HPIO-R elution fractions, or HPIO-H elution fractions (4.5 mg/kg body weight, in H_2O, 0.2 mol/L NaCl, 0.5 mol/L NaCl). The positive control group was given metformin hydrochloride tablets

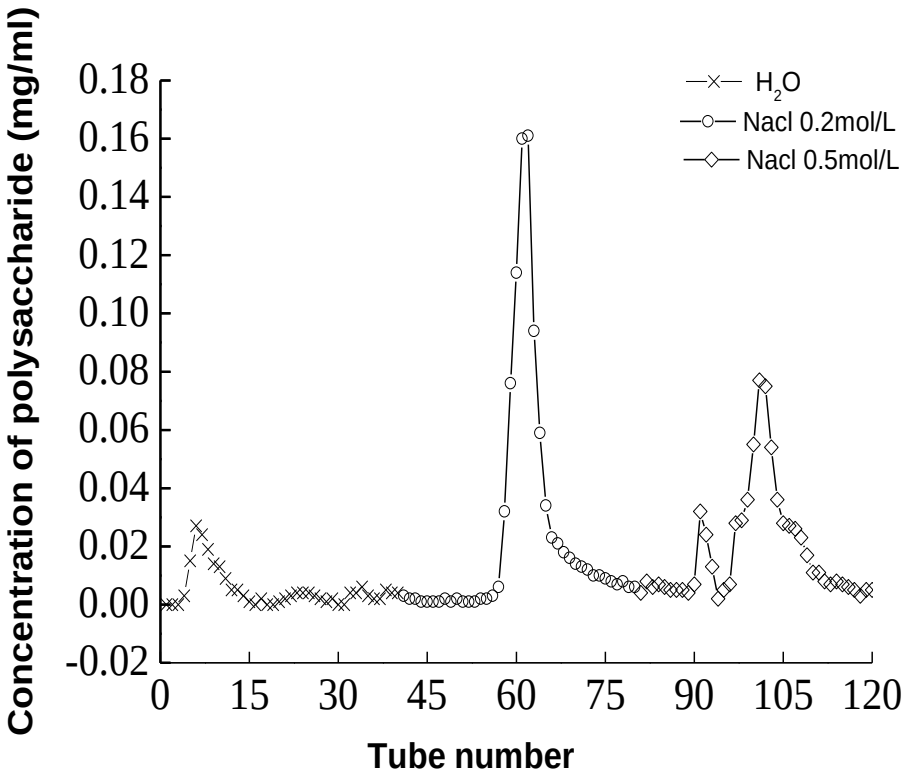

Figure 1. Elution profile of HPIO-R with the anion-exchange chromatography of DEAE-cellulose **DEAE-52**. Tubes (40 ml) of each fraction were assayed for polysaccharide at 495 nm by phenol-sulfuric acid method (Li et al., 1997).

(125 mg/kg body weight) for 21 days.During the experiments, fasting blood glucose levels and body weight were measured at weekly intervals for 21 days. Mice were sacrificed by cervical dislocation after the last administration, and then the blood was centrifuged to separate serum for measuring TG and TC levels. All of the fasting blood glucose, total cholesterol (TC) and triglyceride (TG) levels were measured following the instructions of commercial kits.

Statistical analysis

In the experiment, all the data was expressed as means ± standard deviation (Mean ± SD) and the statistical software statistical package for social sciences 17.0 (SPSS 17.0) was used for performing the statistical methods. Values of $P < 0.05$ used were considered to be significant.

RESULTS

HPIO was extracted from *I. obliquus* and isolated by DEAE cellulose-52 column chromatography.

HPIO-R was isolated by DEAE cellulose-52 column chromatography

Figure 1 shows the results of HPIO-R eluted with H_2O, 0.2 mol/L NaCl and 0.5 mol/L NaCl. As shown in Figure 1, polysaccharide was isolated in several fractions, in which water elution gave three peaks, with peak-I having a high polysaccharide content, and the others with a low content. NaCl (0.2 mol/L) isolated a complete and single fraction that might be a single compound. Multiple fractions were isolated by NaCl 0.5 mol/L; peak-II had the highest polysaccharide concentrations. However, the polysaccharide concentrations of all the peaks were little. And the eluted fractions for HPIO-R-H_2O-peak-I NaCl-peak-I, HPIO-R-0.2 mol/L NaCl and HPIO-R-0.5 mol/L NaCl-peak-II were collected and refrigerated respectively.

HPIO-H isolated by DEAE cellulose-52 column chromatography

Figure 2 shows the results of HPIO-H isolation. As shown in Figure 2, polysaccharide was isolated in several fractions with many peaks and complex compounds with water solution, in which Peak-I had a high polysaccharide content. NaCl 0.2 mol/L isolated a complete single fraction that might be a single compound.

Three fractions with small peaks were isolated with NaCl 0.5 mol/L. The main HPIO-H-H_2O-peak-I and HPIO-H-0.2 mol/L NaCl-peaks were collected and refrigerated respectively.

Our results show that fractions could be purified by DEAE-52 anion-exchange chromatography, and fractions

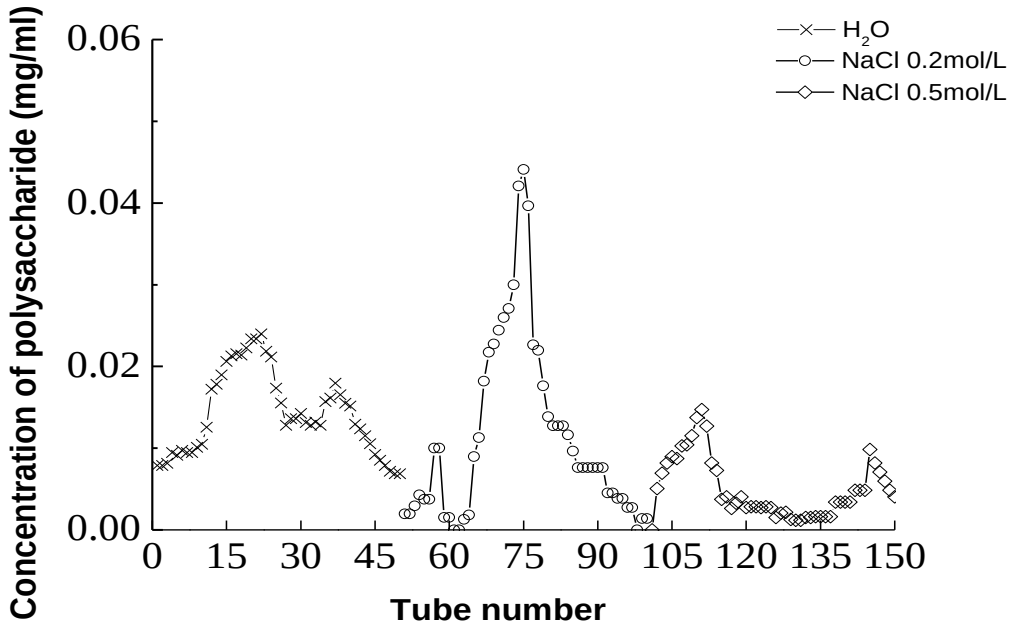

Figure 2. Elution profile of HPIO-H with the anion-exchange chromatography of DEAE-cellulose DEAE-52 Tubes (50 ml) of each fraction were assayed for polysaccharide at 495 nm by phenol-sulfuric acid method (Li et al., 1997).

Table 1. Effect of *I. obliquus* crude total polysaccharide and fractions on fasting blood glucose levels of STZ-induced diabetic mice.

Group	Number	Dose (mg/kg b.w.)	Blood glucose (mmol/L)			
			0 day	7 day	14 day	21 day
Normal control	12	0	7.74±5.3	8.72±1.24	9.96±1.8	9.70±1.70
Diabetic control	12	0	19.02±7.05*	22.81±2.02	34.25±1.94	19.50±1.86
Metformin	12	125	19.95±6.23*	18.24±3.95**	25.92±5.98**	16.62±3.2**
A	12	4.5	19.35±8.28*	20.76±4.33	26.54±7.44	17.92±3.70
B	12	4.5	18.7±7.26*	20.97±8.15	25.92±9.38	17.82±3.64
C	12	4.5	18.25±6.87*	20.83±5.73	30.58±6.28	17.73±4.04
D	12	4.5	18.24±7.49*	21.79±5.02	28.04±7.32	18.35±3.02
E	12	4.5	18.53±7.21*	18.59±7.69	28.25±10.78	14.9±5.26**
F	12	4.5	18.26±7.76*	22.64±7.23	30.42±5.5	18.53±2.46
H1	12	100	19.37±6.97*	24.31±3.82	32.69±4.73	17.76±2.41
H2	12	300	19.39±6.58*	16.56±5.53**	23.57±5.85**	18.60±2.53

Mean ± S.D; b.w., body weight. **P* < 0.05 vs. normal control group. ***P* < 0.05 vs. diabetic control group. A, B, C (HPIO-R elution fractions: H_2O, 0.2 mol/L NaCl, 0.5 mol/L NaCl); D, E, F (HPIO-H elution fractions: H_2O, 0.2 mol/L NaCl, 0.5 mol/L NaCl); H1, H2 (Crude total polysaccharide).

might include a single compound isolated by 0.2 mol/L NaCl.

Hypoglycemic activity of crude total polysaccharide and fractions

Effect of *I. obliquus* crude total polysaccharide and fractions from fasting blood glucose in STZ-induced diabetic mice

The antihyperglycemic effect of *I. obliquus* crude total polysaccharide and fractions on fasting blood glucose levels of STZ-induced diabetic mice are shown in Table 1. Mice in the diabetic modeling groups initially had significantly ($P < 0.05$) increased blood glucose levels compared to the normal control group, and Table 1 showed the establishment of diabetes mice model was succeeded. Compared to diabetic control mice, the blood glucose level of the H2 diabetic groups significantly decreased ($P < 0.05$) after 7 and 14 days. However, a rebound occurred after 21 days, while the blood glucose level of the E diabetic groups was significantly decreased ($P < 0.05$) after 21 days. Moreover, the blood glucose levels of the other diabetic groups were not significantly

Table 2. Effect of *I. obliquus* crude total polysaccharide and fractions on lipid profile of STZ-induced diabetic mice.

Group	Number	Dose (mg/kg b.w.)	TC (mmol L^{-1})	TG (mmol L^{-1})
Normal control	12	0	4.2±0.74	1.74±0.62
Diabetic control	12	0	4.05±0.8	4.11±3.24
Metformin	12	125	4.48±0.76	3.53±2.65
A	12	4.5	3.83±0.57	3.04±1.31
B	12	4.5	4.18±1.94	1.82±0.91**
C	12	4.5	4.86±0.66	3.01±0.99
D	12	4.5	4.21±0.71	3.09±0.9
E	12	4.5	4.15±0.81	2.1±0.98
F	12	4.5	4.69±1.1	2.61±0.99
H1	12	100	4.73±1.14	3.05±1.01
H2	12	300	5.58±1.33	3.16±1.56

Mean ±S.D; b.w., body weight; TC, total cholesterol; TG, triglyceride. *P < 0.05 vs. normal control group. **P < 0.05 vs. diabetic control group. A, B, C (HPIO-R elution fractions: H_2O, 0.2 mol/L NaCl, 0.5 mol/L NaCl); D, E, F (HPIO-H elution fractions: H_2O, 0.2 mol/L NaCl, 0.5 mol/LNaCl); H1, H2 (crude total polysaccharide).

Table 3. Effect of *I. obliquus* crude total polysaccharide and fractions on STZ-induced diabetic mice weight.

Group	Number	Dose (mg/kg b.w.)	b.w./g			
			0 day	7 day	14 day	21 day
Normal control	12	0	24.89±1.35	30.05±2.87	32.23±2.83	34.84±2.87
Diabetic control	12	0	23.41±1.78	25.15±1.73	27.51±1.92	29.22±2.55
Metformin	12	125	24.41±1.63	26.75±2.01	28.39±2.15	30.25±2.74
A	12	4.5	24.36±2.27	26.74±2.56	27.69±2.54	27.90±2.35
B	12	4.5	24.14±1.61	24.34±2.62	25.24±2.84	29.82±2.64
C	12	4.5	24.38±1.99	26.02±2.88	26.47±2.90	28.73±2.02
D	12	4.5	24.50±2.39	27.58±2.34	28.29±2.38	28.41±2.61
E	12	4.5	23.95±1.69	25.59±3.29	26.97±2.75	28.31±2.92
F	12	4.5	24.96±1.66	26.64±2.26	28.33±2.28	28.41±2.61
H1	12	100	24.46±1.35	26.41±2.32	26.33±2.13	26.10±1.35
H2	12	300	23.76±2.23	23.96±3.43	24.16±3.35	26.77±3.58

Mean ±S.D; b.w., body weight. *P < 0.05 vs. normal control group. **P < 0.05 vs. diabetic control group. A, B, C (HPIO-R elution fractions: 0 mol/L, 0.2 mol/L, 0.5 mol/l NaCl); D, E, F (HPIO-H elution fractions: 0 mol/L, 0.2 mol/L, 0.5 mol/L NaCl); H1, H2 (crude total polysaccharide).

decreased (P > 0.05) compared to diabetic control mice, except for the STZ+metformin group.

Effect of *I. obliquus* crude total polysaccharide and fractions on lipid profile of STZ-induced diabetic mice

TC and TG levels in serum were determined. Compared to diabetic control mice, the TG level of the STZ+ metformin group was not significantly decreased, while the TG level of the diabetic groups E and B decreased.

The B diabetic groups significantly decreased (P < 0.05) the TG level compared with the diabetic control groups (Table 2). The results show that *I. obliquus* extracts had antihyperglycemic effects and regulated lipid metabolism.

Effect of *I. obliquus* crude total polysaccharide and fractions on body weight of STZ-induced diabetic mice

As shown in Table 3, no obvious differences in body weight were seen between the different groups. The body weight of the diabetic groups was not significantly different than the diabetic control mice. Table 3 shows that *I. obliquus* extract had no side effects on the physiological condition of the mice.

DISCUSSION

In this study, the effects of DEAE-52 fractions of *I.*

obliquus in normal, diabetic normal and STZ-induced diabetic mice were evaluated. Traditionally, a small piece of *I. obliquus* (1 to 2 g) or one tablespoon of crushed extract in hot water was taken (Park et al., 2005). In this study, we showed that oral administration of 4.5 mg/kg HPIO isolated by NaCl 0.2 mol/L had a significant hypoglycemic or hypolipidemic effects *in vivo*. The body weight of the groups revealed no obvious differences. This meant that HPIO had no side effects on the physiological condition of the mice.

This amount of HPIO 0.2 mol/L NaCl elution might be reasonable compared to the dose recommended in traditional medicinal use.

In conclusion, our results found that DEAE-52 fractions of *I. obliquus* had significant antihyperglycemic effects in STZ-induced mice, as well as anti-TG effects, especially the fraction with a single peak isolated by NaCl 0.2 mol/L. Thus, *I. obliquus* extracts might have important pro-phylactic benefits to humans through their potential diabetes-preventing effects. However, more efforts are needed to characterize and evaluate the extract.

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