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Extraction, purification, characterization and hypoglycemic activity of a polysaccharide isolated from the root of *Ophiopogon japonicus*

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ABSTRACT

In this research, a water-soluble polysaccharide (OJP1) extracted with hot water from the roots of *Ophiopogon japonicus* which is a traditional Chinese medicinal herb was precipitated with 95% ethanol and purified by DEAE-52 cellulose anion-exchange and Sephadex G-100 gel filtration chromatography. The high performance gel permeation chromatography (HPGPC) analysis showed that the average molecular weight (Mw) of OJP1 was 35.2 kDa. Monosaccharides analysis revealed that the OJP1 is composed of Ara, Glc, Gal with a relative molar ratio of 1:16:8. Pharmaceutical experiments showed OJP1 can significantly reduce blood glucose level, increase the insulin level and remediating destruction of pancreatic islets in STZ-induced diabetic rats compared with the diabetic control group. The study shows that OJP1 has an anti-diabetic effect on STZ-induced diabetic rats, and has potential use as an anti-diabetic agent.

1. Introduction

Diabetes is a hereditary, chronic metabolic disease characterized by hyperglycaemia that results from absolute or relative deficiency of insulin secretion, impaired insulin action, or both. It is a group of heterogeneous, autoimmune, hormonal and metabolic disorders, often accompanied by hyperphagia (obesity), a selective loss of pancreatic islet β-cell mass, high blood glucose level, and microvascular complications (Adikwu, Yoshikawa, & Takada, 2004; Yamac et al., 2008). It is the third most life threatening disease whose mortality is right after cancer and cardiovascular disease. Consequently, diabetes is rapidly emerging as a global health care problem that threatens to reach pandemic levels by 2030; the number of people with diabetes worldwide is projected to increase from 171 million in 2000 to 366 million by 2030 (Lin, Jiang, Hu, Qiao, & Tuo, 2007; Wild, Roglic, Green, Sicree, & King, 2004). Research and development of drugs against diabetes and its complications have been getting more and more attentions. However, currently available drugs for diabetes mellitus have a number of limitations such as adverse effects, limited efficacy and high rates of secondary failure. Therefore, there is a strong incentive to develop new hypoglycemic agents, and the search for appropriate hypoglycemic agents has recently focused on many plants used in traditional medicine (Kim et al., 2006; Li, Zheng, Bukuru, & De Kimpe, 2004; Lo, Tsai, Wasser, Yang, & Huang, 2006; Kesari, Gupta, & Watal, 2005).

Ophiopogon japonicus (Thunb.) Ker-Gawl, widely distributed in South-east Asia, is a traditional Chinese medicine used to treat cardiovascular and chronic inflammatory diseases for thousands of years (Xiao, 2002), and has been confirmed in various experiments as having anti-inflammatory, anti-arrhythmia, and microcirculation improvement, etc. (Zhou et al., 2003; Huang & Ni, 2003). Chemical studies have shown that this plant includes saponins, polysaccharide and homoisoflavonoidal compounds (Kou et al., 2005). The polysaccharides isolated from the roots of *O. japonicus* have been reported to exhibit a variety of biological activities, including immunostimulation, anti-ischaemia, inhibiting platelets aggregation, hypoglycemic, etc. (Zheng, Feng, Xu, Lin, & Chen, 2009; Fan & Zhang, 2006). However, there are few reports on its hypoglycemic properties, and the structure and function of these polysaccharides have never been well characterized. Chen, Qian, and Wang (1998) reported a crude *O. japonicus* polysaccharide without further purification had significant antagonistic effect on the experimental hyperglycemias in normal and alloxan-induced diabetic mice.

In the present study, we isolated, purified and characterized the composition of the polysaccharide (designated OJP1 below) from *O. japonicus* and investigated its hypoglycemic effect on streptozotocin-induced diabetic rats. The results showed that OJP1 (Mw = 35.2 kDa) was comprised of Ara, Glc and Gal in the ratio of 1:16:8. Pharmaceutical experiments indicated that OJP1 can signif-

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icantly reduce blood glucose level and increase the insulin level in streptozotocin-induced diabetic rats, and has potential use as an anti-diabetic agent.

2. Experimental

2.1. Materials and chemicals

The roots of *O. japonicus* were collected in Zhejiang province (China). Sephadex G-100, DEAE-cellulose, glucose, galactose, arabinose, rhamnose, mannose, lactic acid, trifluoroacetic acid (TFA), streptozotocin (STZ) were purchased from Sigma. RPMI-1640 medium (Gibco). Blood Glucose Meters was purchased from Life scan (Milpitas, CA, USA). Insulin kits were purchased from Changfeng Biotechnology Co. Ltd. (Zhejiang, China). Immunohistochemistry kits were purchased from Zhongshan Goldenbridge Biotechnology Co. Ltd. (Beijing, China). All the other chemicals used were of analytical grade.

2.2. Experiment animal

Sprague–Dawley rats (body weight 250 ± 20 g) used for experiments were purchased from Experimental Center, Wenzhou Medical College, Wenzhou, Zhejiang, China. The rats were acclimatized for 1 week before being used for the experiment. Before and during the experiment the rats were housed under controlled environmental conditions of temperature (22 ± 2 °C) and a 12 h light and dark cycle, and maintained on (unless otherwise stated) standard food pellets and tap water ad libitum.

All animal (used in this experiment) handling procedures were performed in strict accordance with the P.R. China legislation the use and care of laboratory animals, with the guidelines established by Institute for Experimental Animals of Wenzhou Medical College, and were approved by the College committee for animal experiments.

2.3. Experimental design

The diabetic rats were induced by the intraperitoneally (i.p.) injection of STZ freshly at a dose of 60 mg/kg body weight. Three days after STZ treatment, sera were collected for measurement of blood glucose from the tail vein. The rats that were marked hyperglycemia (the blood glucose level > 16.7 mmol/L) were used as the diabetic rats for further study.

The STZ-induced diabetic rats (mentioned above) were randomly divided into four groups (8 rats per group), and normal rats were used as the control.

Group I ($n = 8$): normal control (NC), normal rats were allowed to free access to a normal diet and treated with saline for 28 days.

Group II ($n = 8$): diabetic control (DC), the diabetic rats were allowed to free access to a normal diet and treated with saline for 28 days.

Group III ($n = 8$): OJP1-150, the diabetic rats were put on a normal diet and treated with 150 mg/kg of OJP1 for 28 days.

Group IV ($n = 8$): OJP1-300, the diabetic rats were put on a normal diet and treated with 300 mg/kg of OJP1 for 28 days.

Group V ($n = 8$): Metformin, the diabetic rats were put on a normal diet and treated with 200 mg/kg of metformin for 28 days.

On the last day of experiment, the animals were deprived of food overnight and sacrificed by cervical dislocation. Blood was collected in polystyrene tubes without the anticoagulant. Serum was immediately separated by centrifugation at 3000 rpm at room temperature for 10 min. Samples were stored at −70 °C until assayed.

The pancreas were removed quickly, cleaned and washed in ice-cold normal saline for biochemical study.

2.4. General methods

Evaporation was performed at around 45 °C under reduced pressure. The products were dried by lyophilization. Gas chromatography (GC) (Shimadzu GC-2010) equipped with RTX-50 column (30.0 m × 0.25 mm × 0.25 μm) and flame-ionization detector (FID). The operation was performed using the following conditions: column temperature was programmed from 140 °C (maintained for 2 min) to 170 °C at a rate of 6 °C/min, and increased to 173 °C at a rate of 0.2 °C/min, then increased to 233 °C at a rate of 6 °C/min, held for 40 min at 233 °C; the rate of N_2 carrier gas was 1.0 ml/min; injection temperature was 250 °C; detector temperature was 300 °C. Gas chromatography–mass spectrometry (GC–MS) was run on the instrument Shimadzu GCMS-QP2010 (Shimadzu, Japan) and equipped with RTX-50 column (30 m × 0.25 mm × 0.25 μm), and at temperatures programmed from 140 °C (maintained for 2 min) to 250 °C (kept for 20 min) at a rate of 3 °C/min. Nitrogen was the carrier gas. The polysaccharide was monitored by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.5. Isolation and purification of the polysaccharide

The roots of *O. japonicus* were soaked with 95% ethanol to remove the pigments and small lipophilic molecules. The residue was then extracted with 10 vol. of distilled water at 90 °C for 3 h thrice. All water-extracts were combined, filtrated, concentrated, and precipitated with 95% EtOH (1:4, v/v) at 4 °C for overnight. The precipitate was collected by centrifugation and deproteinated by Sevag method (Staub, 1965). Finally the supernatant was lyophilized to give crude polysaccharides.

The crude polysaccharides were purified by DEAE-52 cellulose and Sephadex G-100, and the main polysaccharide fraction (OJP1) was collected and lyophilized. OJP1 was used for further study.

2.6. Homogeneity and molecular weight

The homogeneity and molecular weight of OJP1 was evaluated and determined by high performance gel permeation chromatography (HPGPC). The sample solution was applied to Waters High Performance Liquid Chromatography (HPLC) equipped with a TSK-GEL G3000 SWXL column (7.8 mm × 300 mm), eluted with 0.1 mol/L Na_2SO_4 solution at a flow rate of 0.4 ml/min and detected by a Waters 2414 Refractive Index Detector. The columns were calibrated with Dextran T-series standard of known molecular weight (200,000, 70,000, 40,000, 10,000, 5000 Da). The molecular weight of OJP1 was estimated by reference to the calibration curve made above.

2.7. Analysis of monosaccharide composition

The monosaccharide of OJP1 was analyzed by GC. OJP1 was hydrolyzed with 2 M TFA (2 ml) at 120 °C for 2 h. After removing TFA with methanol, the hydrolyzed product was reduced with $NaBH_4$ (50 mg), followed by neutralization with dilute acetic acid and evaporated at 45 °C. The reduced products (alditols) were added with 1 ml pyridine and 1 ml acetic anhydride in a boiling water bath for 1 h. The acetylated products were analyzed by GC.

2.8. Methylation analysis

OJP1 (20 mg) was methylated three times according to the method of Needs and Selvendran (1993). The methylated products were extracted by chloroform and examined by IR spectroscopy. The absence of the absorption peak corresponding to hydroxyl indicated the complete methylation. The product was hydrolyzed using

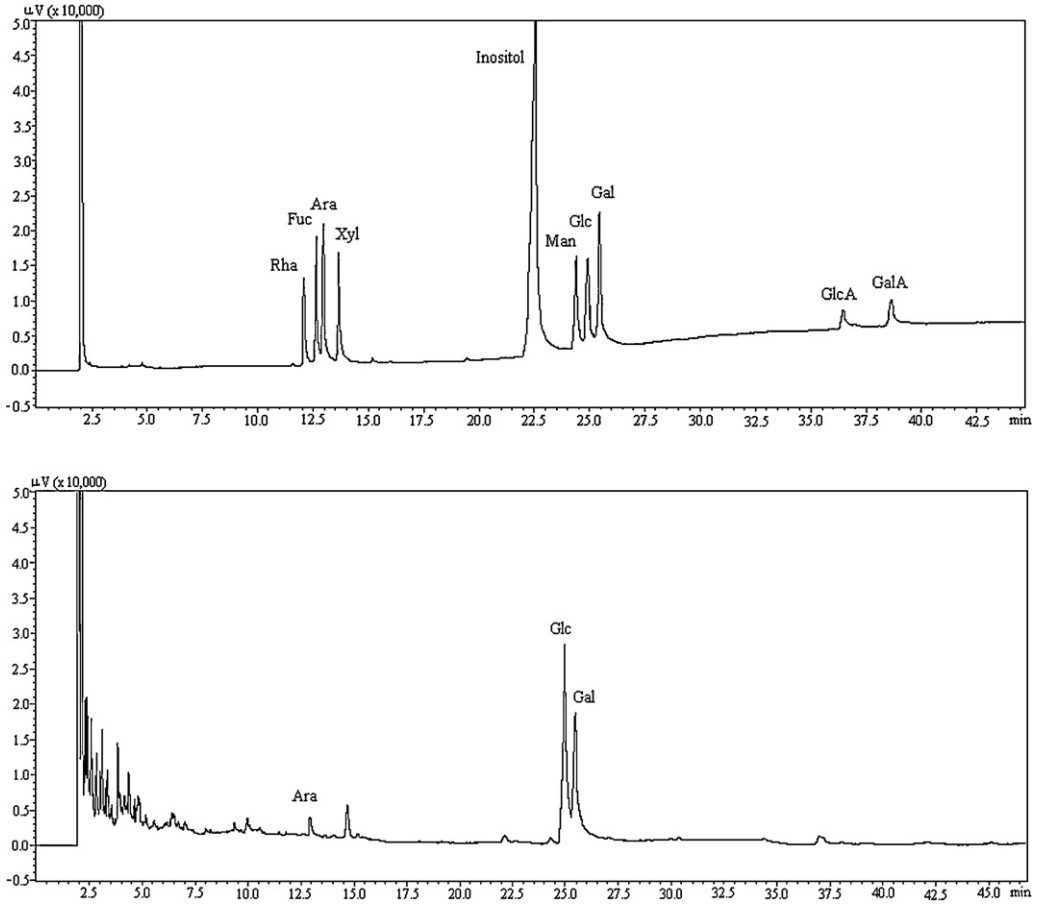

Fig. 1. GC profile of OJP1 ((A) standard monosaccharides, peaks from left to right: Rha, Fuc, Ara, Xyl, Inositol, Man, Glc, Gal, GlcA, GalA; (B) monosaccharide composition of OJP1, peaks from left to right: Ara, Glc, Gal).

2 M TFA, followed by reduction using $NaBH_4$ and finally acetylated with acetic anhydride. The partially methylated alditol acetates were analyzed by GC–MS.

2.9. Biochemical assays

Blood glucose and insulin levels were measured with commercial kits. In brief, glycemia was assessed using a One Touch II Meter. Insulin was determined by an enzyme-linked immunosorbent assay using a commercial kit, based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the insulin molecule.

2.10. Pancreas histology

Tissues were fixed in 10% neutral formalin for at least 24 h and processed for light microscopic study. Each piece of pancreas embedded in paraffin blocs was sectioned at 5 μm in length before applying on slides and stained with H&E for histopathological examination by using light microscopy. The total operative procedures complied with the standard protocols.

2.11. Statistical analysis

All results were presented as mean ± SD Data were analyzed by one-way ANOVA using SPSS and Dunnett's test. *P* values less than 0.05 were considered significant.

3. Results and discussion

3.1. Isolation and characterization of OJP1

The water-soluble crude polysaccharides were obtained from the root of *O. japonicus* by hot water extraction, ethanol precipitation, deproteinized, and lyophilized. The extracts were purified through with DEAE-cellulose column and Sephadex G-100 column. The main fraction was collected and named as OJP1 for further structure characterization and bioactivity assay. OJP1 has no absorption at 280 and 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid.

The average molecular weight of OJP1 was determined as 35.2 kDa by HPGPC. Results from phenol–sulfuric acid assay showed that OJP1 contained 98.5% carbohydrate.

OJP1 was hydrolyzed by TFA into individual monosaccharides that were further reduced and acetylated for GC analysis. The results showed that OJP1 is composed of Ara, Glc, Gal with a relative molar ratio of 1:16:8 (Fig. 1).

As shown in Fig. 2, the IR spectrum of OJP1 revealed a typical major broad stretching peak around 3422.99 cm^{-1} for the hydroxyl group, and the small band at around 2934.69 cm^{-1} was attributed to the C–H stretching and bending vibrations. The relatively strong absorption peak at around 1630.66 cm^{-1} reflects the absorption of the C=O group that is part of glycosides (Zhu et al., 2010). The wave number between 950 and 1200 cm^{-1} is often called the fingerprint of molecules because it allows the identification of major chemical groups in polysaccharides: the position and intensity of the

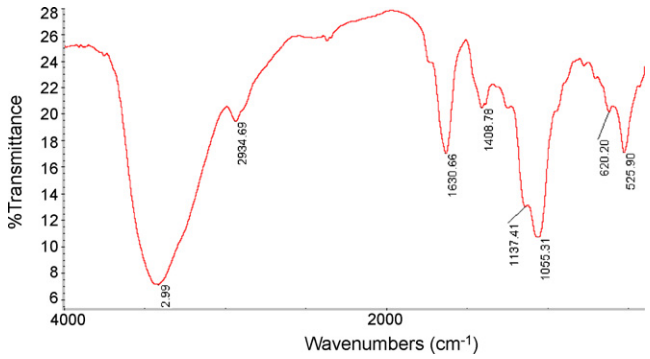

Fig. 2. IR spectrum of the polysaccharide of OJP1 isolated from the roots of *O. japonicus*.

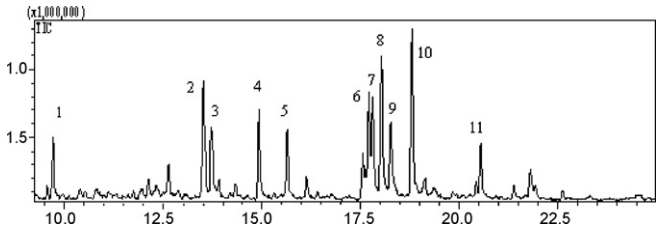

Fig. 3. The gas chromatogram of the products derived by methylated of the OJP1: **1**: 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methyl-arabinose; **2**: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucose; **3**: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-galactose; **4**: 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-glucose; **5**: 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-glucose; **6**: 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucose; **7**: 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-galactose; **8**: 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-glucose; **9**: 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-galactose; **10**: 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl-glucose; **11**: 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-galactose.

bands that are specific for each polysaccharide (Fellah, Anjukandi, Waterland, & Williams, 2009). This region is dominated by ring vibrations overlapped with stretching vibrations of (C–OH) side groups and the (C–O–C) glycosidic band vibration. The absorptions at 1055.31 and 1137.41 cm^{-1} indicated a pyranose form of sugars (Zhao, Kan, Li, & Chen, 2005). The bands in the range of 350–600 cm^{-1} are assigned to skeletal modes of pyranose rings (Yang & Zhang, 2009). On the basis of the aforementioned results, it can be concluded that OJP1 was composed of pyranose form sugars.

The fully methylated OJP1 was hydrolyzed with acid, converted into alditol acetates and analyzed by GC–MS (Fig. 3). The results indicate that the polysaccharide exhibit a highly complex nature (Table 1). The ratios of methylated fragments were calculated based on the areas of the methylated products and corrected using the effective-carbon response method (Sweet, Shapiro, & Albersheim, 1975). The GC–MS results (Table 1) indicated that 2,3,4-Me-Glc (1,6-linked Glc), 2,3,6-Me-Glc (1,4-linked Glc), and 2,3-Me-Glc (1,4,6-linked Glc) were major components of the backbone structure, part of Glc and Gal were distributed in branches, and residues of branches terminated with Ara, Glc and Gal.

Table 1
The results of methylation analysis of OJP1.

Peak No.	Methylated sugar[a]	Molar ratio	Linkages types
1	2,3,5-Me-Ara	2	Ara-(1→
2	2,3,4,6-Me-Glc	5	Glc-(1→
3	2,3,4,6-Me-Gal	4	Gal-(1→
4	2,4,6-Me-Glc	4	→3)-Glc-(1→
5	3,4,6-Me-Glc	3	→2)-Glc-(1→
6	2,3,6-Me-Glc	5	→4)-Glc-(1→
7	2,3,6-Me-Gal	2	→4)-Gal-(1→
8	2,3,4-Me-Glc	7	→6)-Glc-(1→
9	2,3,4-Me-Gal	4	→6)-Gal-(1→
10	2,3-Me-Glc	7	→4,6)-Glc-(1→
11	2,4-Me-Gal	4	→3,6)-Gal-(1→

[a] 2,3,4,6-Me-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-glucose, etc.

3.2. Hypoglycemic activity of OJP1

Streptozotocin is one of the most commonly used substances to induce diabetes in the rat. This toxin causes the death of pancreatic β-cells by alkylation of DNA resulting in reduced synthesis and release of insulin (Montilla et al., 2004).

The effects of OJP1 on blood glucose level in STZ-induced diabetic rats were shown in Table 2. The blood glucose level in normal rats maintained constant during 4 weeks and was significantly ($P < 0.01$) lower than those of STZ-induced diabetic rats of the rest four groups. The daily administration of OJP1 (150, 300 mg/kg) in STZ-induced diabetic rats caused a significant reduction in the blood glucose level when compared with the diabetic control group ($P < 0.05$). At fourth week, the mean decrease percentage of blood glucose levels caused by OJP1 at the dose of 150 mg/kg was 32.9%.

Table 2
The effect of OJP1 on blood glucose levels in STZ-induced diabetic rats.[a]

	Blood glucose (mmol/l)				
	0 Days	7 Days	14 Days	21 Days	28 Days
Normal control	4.45 ± 0.28	5.37 ± 0.30	4.83 ± 0.60	5.78 ± 0.39	5.30 ± 0.41
Diabetic control	19.25 ± 1.56[b]	20.47 ± 2.16[b]	23.97 ± 2.57[b]	21.15 ± 2.10[b]	22.43 ± 2.18[b]
OJP1-150	20.03 ± 2.07[b]	15.72 ± 1.98[c]	14.45 ± 2.43[c]	12.83 ± 2.54[c]	13.45 ± 2.69[c]
OJP1-300	20.22 ± 0.79[b]	19.68 ± 2.22	18.58 ± 3.79[d]	15.92 ± 4.35[c]	17.32 ± 3.83[d]
Metformin	19.82 ± 1.95[b]	16.82 ± 2.72[d]	15.67 ± 3.02[c]	14.25 ± 2.90[c]	15.45 ± 3.09[c]

[a] Data represent mean ± SD ($n = 8$ for each group).
[b] $P < 0.01$ (compared with the normal control).
[c] $P < 0.01$ (compared with the diabetic control).
[d] $P < 0.05$ (compared with the diabetic control).

Table 3
The effect of OJP1 on insulin levels in STZ-induced diabetic rats.[a]

	Normal control	Diabetic control	OJP1-150	OJP1-300	Metformin
Insulin (IU/ml)	18.24 ± 1.22	9.79 ± 0.62[b]	20.79 ± 1.42[b,c]	11.44 ± 0.86[b,d]	17.70 ± 1.03[c]

[a] Data represent mean ± SD ($n = 8$ for each group).
[b] $P < 0.01$ (compared with the normal control).
[c] $P < 0.01$ (compared with the diabetic control).
[d] $P < 0.05$ (compared with the diabetic control).

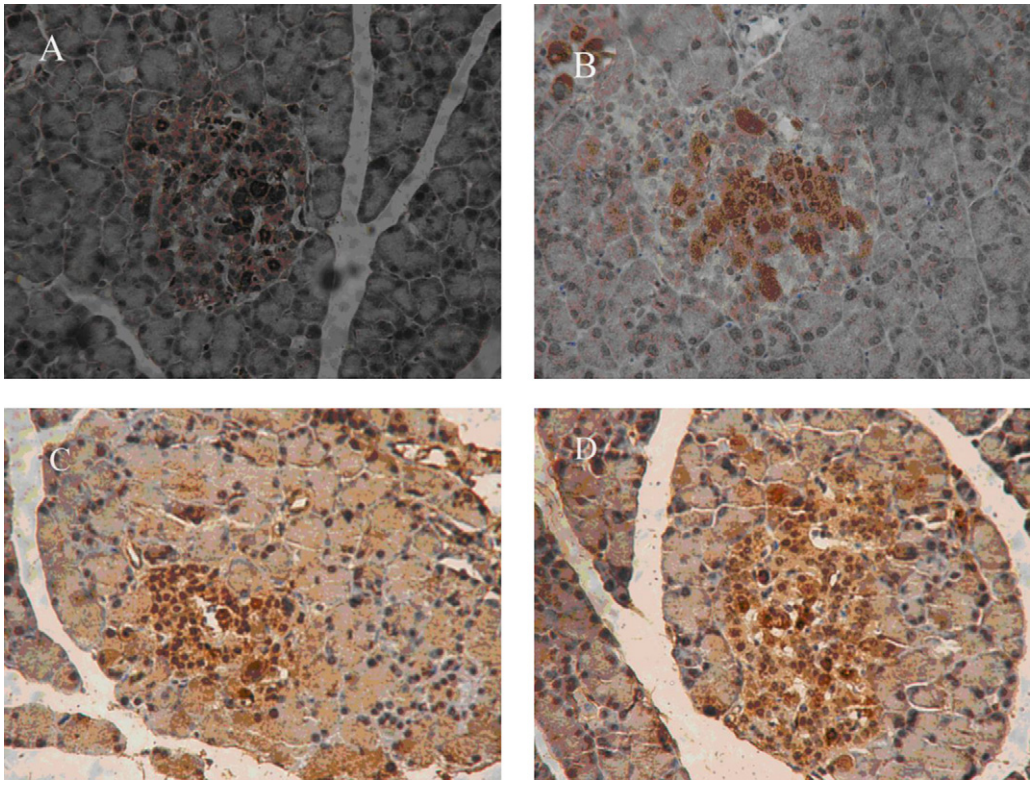

Fig. 4. Effects of OJP1 on pancreas of STZ-induced diabetic rats: (A) nondiabetic animals given saline as the negative control, (B) diabetic animals given saline as the diabetic control, (C) diabetic animals given OJP1 at a dose of 150 mg/kg, and (D) diabetic animals given OJP1 at a dose of 300 mg/kg. Magnification ×200.

The results showed that lower dose (OJP1-150) was a little more effective in reducing blood glucose level than Metformin on STZ-induced diabetic rats. However, no significant differences were observed between OJP1-150 group and Metformin group.

The effects of OJP1 on insulin level in STZ-induced diabetic rats were shown in Table 3. The insulin level in diabetic control rats was significantly ($P < 0.01$) lower than that of normal control rats. After 4 weeks, insulin levels increased significantly in OJP1-traeted groups and metformin-treated group ($P < 0.05$).

Pancreatic β cells are highly specialized cells which are responsible for producing all of the insulin required by an organism to maintain glucose homeostasis. Defects in development, maintenance, or expansion of β-cell mass can result in impaired glucose metabolism and diabetes (Ackermann & Gannon, 2007).

As shown in Fig. 4, the results of immunohistochemical staining of the pancreatic tissues showed that strong insulin antigen positivity was detected in the β-cells of the islets in healthy rats, and islets maintained a normal rounded appearance (Fig. 4A). In contrast, there was weak insulin immunoreactivity in a few β-cells in the islet of diabetic control rats (Fig. 4B), many of the groupings of insulin-positive cells had lost any of the appearance of an islet structure and were seen as single cells and the islet was disorganized. OJP1 treatment considerably increased the insulin antigenesity of diabetic islet β-cells and had a normal appearance, suggesting the possibility of β-cell proliferation or regeneration by OJP1 therapy (Fig. 4C and D). The results also showed that the number of cells increased significantly in OJP1-treated groups, and OJP1 seemed to have the potential of remediating destruction of pancreatic islets.

Treatment of STZ-induced diabetic rats with OJP1 can significant prevented the development of diabetes. More interesting, the hypoglycemic effect of OJP1 was not dose-dependent, and the mechanism is not clear yet. We postulate that the 300 mg dose may be an overdose, and further studies will be carried out. Although the specific mechanism of hypoglycemic activity of OJP1 cannot be determined from the present study, the effects on blood glucose and insulin and the effects on pancreatic β cells could all be the indirect result of OJP1's action.

4. Conclusion

This study has demonstrated that OJP1 isolated from the root of *O. japonicus* is a heteropolysaccharide consisting of Ara, Glc, Gal with a relative molar ratio of 1:16:8, and 1,6-linked Glc, 1,4-linked Glc and 1,4,6-linked Glc were major components of the backbone structure, part of Glc and Gal were distributed in branches, and residues of branches terminated with Ara, Glc and Gal.

The pharmacological assays suggest that OJP1 (150, 300 mg/kg) administered orally in STZ-induced diabetic rats can significantly reduce blood glucose levels, increase the insulin levels, remediating destruction of pancreatic islets and the damage of pancreatic β-cells. It could be concluded that OJP1 may be considered as a potential candidate for developing a new anti-diabetic agent. The further studies on the mechanism of hypoglycemic activity are undergoing and will be reported in due course.

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