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Preliminary characterization and anti-hyperglycemic activity of a pectic polysaccharide from okra (*Abelmoschus esculentus* (L.) Moench)

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ABSTRACT

Okra is widely used as vegetable, food ingredient, and traditional medicine in China for various applications. The main healthy properties are accepted to originate from the high polysaccharide content of okra. In this study, a homogenous pectic polysaccharide WOP-2 was extracted, isolated, and purified from okra by DEAE-cellulose and Sepharose CL-6B column, and its molecular weight was estimated to be 580 kDa. Monosaccharide composition was analyzed to be Rha (21.4%), GalA (34.9%), Gal (29.6%), GlcA (4.5%), Glc (5.9%), and Ara (3.7%) by HPLC with PMP derivatization. Combined with NMR analysis, WOP-2 was elucidated to be a rhamnogalacturonan I backbone with type-II arabinogalactan side-chains substituted partly at O-4 of Rha*p*. WOP-2 exhibits significant anti-hyperglycemic activity on STZ-induced diabetic mice by inhibiting the lipid peroxidation chain reaction probably. Overall, these results provide new insight into structure–activity relationships of polysaccharide from okra and provide impetus towards the development of polysaccharide-based therapeutics on diabetes.

1. Introduction

The fruits of *Abelmoschus esculentus* (L.) Moench, also known as okra, are widely grown in Asia, the Middle East, and Africa. Okra is often used as vegetable and folk medicine, owing to its high amount of acidic polysaccharides named as pectin-like mucilages (Lengsfeld, Titgemeyer, Faller, & Hensel, 2004). The structural element of okra pectic polysaccharide contains a rhamnogalacturonan I (RG-I) domain with a 5.5% (w/w) degree of acetylation as described by Tomoda, Shimada, Saito, and Sugi (2008). Sengkhamparn et al. described the different pectin fractions: Hot buffer soluble solids (HBSS), chelating agent soluble solids (CHSS), dilute alkaline soluble solids (DASS), and concentrated alkaline soluble solids (CASS) were obtained by the sequential extraction (Sengkhamparn, Bakx et al., 2009; Sengkhamparn, Verhoef et al., 2009). The structures of the pectin fractions were elucidated by MS and NMR, and the results showed that HBSS fraction consisted of an acetylated RG-I domain backbone with oligogalactan side chains, while CHSS consisted of homogalacturonan (HG) and RG-I domains with longer oligogalactan side chains (Sengkhamparn, Bakx et al., 2009; Sengkhamparn, Verhoef et al., 2009).

Pectin is the most structurally complex family of polysaccharide in plant. Pectin usually contains four domains: homogalacturonan (HG), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), and xylogalacturonan (XGA) (Ridley, O'Neill, & Mohnen, 2001). The HG domain backbone consists of a linear polymer of 1,4-linked α-**D**-galacturonic acid (Gal*p*A) residues partially methyl esterified at the C-6 position and O-acetylated at the O-2 or O-3 positions. RG-I has a backbone of alternating α-**L**-rhamnose (Rha*p*) and α-**D**-Gal*p*A repeating units [-4)-α-**D**-Gal*p*A-(1,2)-α-**L**-Rha*p*-(1]$_n$, which is highly branched with α-**L**-arabinose (Ara*f*)- and β-**D**-galactose (Gal*p*)-rich side chains attached at the C-4 position of Rha residues. RG-II is the most structurally diverse pectin, consisting of a 9 or 10 α-(1 → 4)-**D**-Gal*p*A unit HG backbone with four different oligosaccharide chains attached at C-2 or C-3 positions of the GalA residues (Perez, 2003; Ridley, O'Neill, & Mohnen, 2001; Yapo, 2011). In recent years, various groups have demonstrated that pectin serves several kinds of physiological functions, such as anti-diabetes, reducing serum cholesterol, inhibiting lipase, anti-oxidant, and immunomodulation activities (Gao et al., 2015; Maxwell, Belshaw, Waldron, & Morris, 2012).

In 2015, ~415 million people suffered from diabetes mellitus (DM), and the number will be expected to grow to ~640 million by 2040. DM has become one of the most challenging medical issues worldwide, which is a multifactorial metabolic disorder characterized by hyperglycemia due to defects in insulin secretion (Guo et al., 2017; Zhao,

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Son, Kim, Jang, & Lee, 2007). Diabetic nephropathy and diabetic secondary infection are the common complications of diabetes (Badal & Danesh, 2015; Gilbert, 2015). Other studies also demonstrated that diabetes can easily cause kidney disease, and abnormal immune function (Piccoli et al., 2015). Although okra has a long history of use as a Chinese food and traditional medicine, the anti-hyperglycemic activity of its polysaccharides is yet unknown. The polysaccharide derived from plants are accepted to be endowed with low toxicity, and it do not cause significant side effects. In this study, we extracted, isolated, and purified a homogenous pectic polysaccharide from okra. The preliminary structure and bioactivity activity of the pectic polysaccharide on the anti-hyperglycemic had been elucidated.

2. Experimental methods

2.1. Plant materials, chemicals, and animals

The okra pods, *A. esculentus* (L.) Moench, (6–9 cm in length) were grown in Guizhou Province, China, collected at a local market in June 2015. Various dextrans (670 kDa, 270 kDa, 80 kDa, 25 kDa, and 1 kDa), monosaccharides Mannose (Man), Rhamnose (Rha), Glucuronic acid (GlcA), Galacturonic acid (GalA), Glucose (Glc), Galactose (Gal), Xylose (Xyl), Arabinose (Ara), Fucose (Fuc), as well as ascorbic acid, 1,1-Diphenyl-2-picrylhydrazy (DPPH), NADH, nitro blue tetrazolium salt (NBT), horseradish peroxidase, phenazine methosulfate (PMS), streptozotocin (STZ) were all purchased from Sigma, Inc. Sepharose CL-6B was purchased from Amersham Pharmacia Biotech. Glucose assay kit, blood SOD (superoxide dismutase) assay kit, MDA (Malondialdehyde) assay kit, and insulin assay kit were all purchased from Guangru Biological Technology (Shanghai, China). Other reagents were of analytical grade made in China.

Male ICR mice (18–22 g, 5-week old) were purchased from Experimental Animal Centre of the Third Military Medical University. Housing and breeding of the animals were performed in strict compliance with Animal Care and Use Guidelines in China, and use of laboratory animals and their experimental use was approved by the animal Ethics Committee of Zunyi Medical College (SYXK 2014-003).

2.2. Preparation of crude polysaccharide

The crushed okra pods were first exhaustively extracted with 95% ethanol using Soxhlet extractor for 12 h to remove hydrophobic substances, and then the residue was dried at 25 °C. The residue (500 g) was extracted with 10 L distilled water at 100 °C for 4 h and filtered through four sheets of gauze. The solid material was extracted twice again under same conditions. The extraction filtrates were combined and concentrated to 750 mL, and treated with 15% trichloroacetic acid at 4 °C for 4 h for protein removal. After neutralization and centrifugation, the 95% ethanol was added to the supernatant up to 80% to precipitate the crude polysaccharides (WOP) which was lyophilized by the vacuum belt freeze dryer.

2.3. Separation and purification of crude polysaccharide

The lyophilized WOP (10 g) was dissolved in water (100 mL) and loaded on a DEAE-Cellulose column (8.0 cm × 20 cm, 50 μm, Cl^-) which was eluted in succession with dH_2O (2 L), 0.3 M NaCl (2 L), and 0.5 M NaCl (2 L) at a flow rate of 13.0 mL/min. The appropriate fractions were combined, dialyzed and lyophilized to obtain the fractions WOP-N, WOP-1, and WOP-2. WOP-2 was further purified by Sepharose CL-6B gel filtration chromatography (1.5 × 90 cm) and eluted with 0.15 M NaCl at a flow rate of 0.15 mL/min. The phenol-sulfuric method was used to determine the total carbohydrate content (Zhang et al., 2016).

2.4. Sugar composition analysis

Polysaccharide (2 mg) was hydrolyzed with anhydrous methanol containing 2 M HCl at 80 °C for 16 h and then with 2 M TFA at 120 °C for 1 h. Released monosaccharides were derivatized by using 1-phenyl-3-methyl-5-pyrazolone (PMP), and the derivatives were analyzed by high performance liquid chromatography (LC-20AT, Shimadzu) with a Shimadzu Inertsil ODS-3 column (4.6 × 250 mm) and an ultraviolet detector. The column was eluted with 82.0% PBS (0.1 M, pH 7.0) and 18.0% acetonitrile (v/v) at a flow rate of 1.0 mL/min and monitored by UV absorbance at 245 nm (Zhang et al., 2016).

2.5. Homogeneity and molecular weight distribution analysis of WOP-2

Molecular weights were determined by gel-permeation chromatography on a TSK-gel G-4000PW_{xl} column (7.8 × 300 mm, TOSOH) equipped with an Agilent 1100 HPGPC system and Refractive Index Detector (RID). The column was pre-calibrated by using dextrans of known molecular weights (670 kDa, 270 kDa, 80 kDa, 25 kDa, and 1 kDa). The molecular weight of the WOP-2 was calculated using linear regression analysis. The sample solution (5 mg/mL) was injected and eluted with 0.2 M NaCl at a flow rate of 0.5 mL/min.

2.6. NMR spectra

^{13}C nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AV600 spectrometer. The polysaccharide sample (20 mg) was dissolved in D_2O (1 mL, 99.8%) with overnight stirring at room temperature.

2.7. Antioxidant activity of WOP-2 in vitro

2.7.1. Superoxide radical-scavenging activity

The superoxide radical-scavenging activity was detected by the method according toLiu, Ooi, and Chang (1997). WOP-2 solution 1 mL (0–4.0 mg/mL) was mixed with 1 mL NBT (200 μM), 1 mL NADH (200 μM), and 1 mL PMS (80 μM). After the mixture solution was reacted at 37 °C for 5 min, a value of $A_{560\ nm}$ was recorded. Distilled water and ascorbic acid were used as negative and positive control, respectively. Superoxide radical-scavenging activity (%) = $[1 - (A_{s1} - A_{s2})/A_c] \times 100$, where A_c is the absorbance value of negative control; A_{s1} is the absorbance value of the test sample mixed with reaction solution; A_{s2} is the absorbance value of the sample only.

2.7.2. Hydroxyl radical-scavenging activity assay

The hydroxyl radical-scavenging activity was detected by the Fenton reaction (Giese et al., 2015), with slight modification. Briefly, WOP-2 solution 1.0 mL (0–4.0 mg/mL) was mixed with 1.0 mL H_2O_2 (0.025%, w/v), 1.0 mL sodium salicylate (9 mM), and 1.0 mL $FeSO_4$ (9 mM). And then the mixture solution was reacted at 37 °C for 0.5 h. Distilled water and ascorbic acid were used as negative and positive control, respectively. Finally, a value of $A_{562\ nm}$ was recorded of the mixture solution. Hydroxyl radical-scavenging activity (%) = $[1-(A_{s1}-A_{s2})/A_c)] \times 100$, where A_c is the absorbance value of negative control; A_{s1} is the absorbance value of the test sample mixed with reaction solution; A_{s2} is the absorbance value of the sample only.

2.8. Anti-hyperglycemic activity of WOP-2 in vivo

2.8.1. Streptozotocin-induced diabetic mice model

After a 12-h fasting, mice in diabetes group were induced by intraperitoneal injection of streptozotocin (STZ) (100 mg/kg dissolved in 0.1 M pH 4.5 citrate buffer), while control group was treated with citrate buffer. After seven days, fasting blood glucose (FBG) of the mice was detected by using glucose assay kit according to the manufacturer's instructions. The STZ-induced mice with high blood glucose levels

(> 11.1 mM) were selected as diabetic mice for further study.

2.8.2. Hypoglycemic effects of WOP-2 by intra-gastric administration

STZ-induced diabetic mice and normal mice were both randomly divided into five groups (10 mice per group), and subsequently administered intra-gastrically with WOP-2 dissolved in distilled water at doses of 50 and 150 mg kg^{-1} day^{-1}. After 10 days of consecutive administration, the body weights and FBG of mice were measured.

2.8.3. Mechanism of the hypoglycemic effect of WOP-2 in STZ-induced diabetic mice

The STZ-induced diabetic mice were randomly divided into two groups (10 mice per group). The WOP-2 group was administered intragastrically with WOP-2 (50 mg kg^{-1} day^{-1}) dissolved in distilled water. The control group (diabetes) and normal group (normal) received distilled water. After 10 days of consecutive administration, the mice were sacrificed by cervical dislocation. Blood samples were collected in polystyrene tubes without anticoagulant. Serum was immediately separated by centrifugation (1000*g*, 10 min), and stored at −20 °C for the measurement of SOD, MDA, and insulin according to the manufacturer's instructions.

2.9. Statistical analysis

All data were expressed as the mean ± standard deviation (SD) from 3 independent experiments performed in triplicate, and the statistical significance of difference using Student's *t*-tests and one-way ANOVA analysis were conducted to detect significant differences between treatments as a whole. The value of $P < .05$ was considered statistically significant.

3. Results and discussion

3.1. Isolation and purification of WOP-2

The crude polysaccharide WOP was obtained (yield 7.9% of dried material) by hot water extraction from dried Okra (*Abelmoschus esculentus* (L.) Moench) previously submitted to ethanol extraction of hydrophobic substances. It was firstly fractionated by anion-exchange chromatography on a DEAE–cellulose column, eluted stepwise with sequential elution with distilled H_2O, 0.3 M and 0.5 M NaCl to yield WOP-N (21.7%), WOP-1 (16.3%) and WOP-2 (47.2%), respectively (Fig. 1A). The fraction of WOP-2 was further purified by using gel permeation chromatography on Sepharose CL-6B as shown in Fig. 1B.

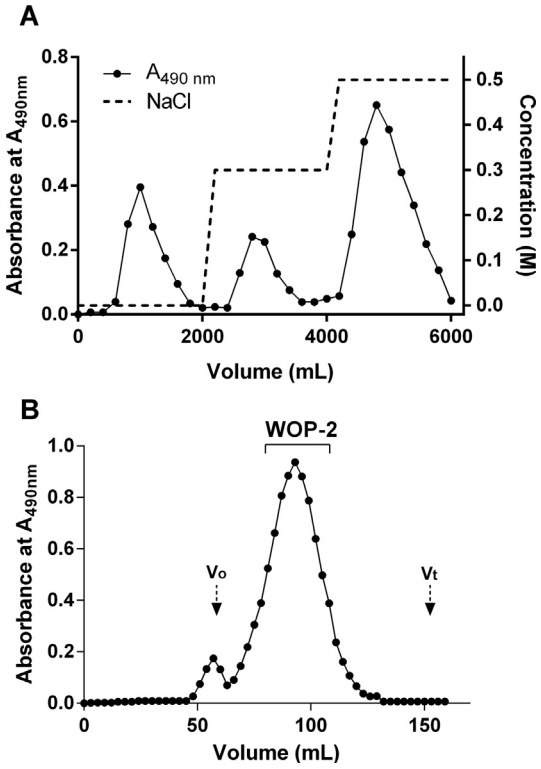

Fig. 1. Elution profile of WOP on DEAE–cellulose (A) and elution profile of sub-fraction WOP-2 eluted from a Sepharose CL-6B column (B). V_o, void volume; V_t, total volume.

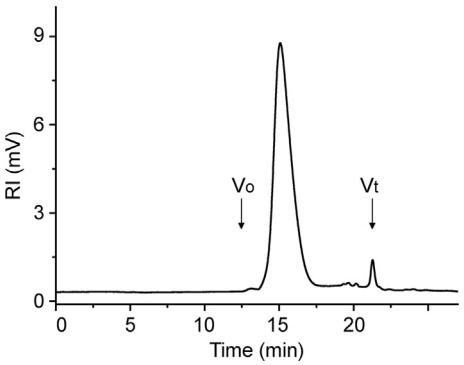

Fig. 2. The homogeneity and molecular weight of WOP-2. V_o, void volume; V_t, total volume.

3.2. Homogeneity and molecular weight

The homogeneity and molecular weight of WOP-2 were determined by HPGPC on TSK-gel G-4000PW_{xl} (Fig. 2). According to the HPGPC data, the profile of WOP-2 showed a single and symmetrical narrow peak, indicating that WOP-2 was a homogeneous and highly pure polysaccharide. HPGPC analysis revealed that the molecular weight (Mw) of WOP-2 was 580 kDa, a high molecular weight polysaccharide. A similar result was reported for a hot buffer extracted fraction HBSS, whose molecular weight was much more than 100 kDa (Sengkhamparn, Verhoef et al., 2009).

3.3. Monosaccharide composition determination

The monosaccharide composition of WOP-2 was determined by HPLC method based on PMP pre-column derivatization as shown in Fig. 3. WOP-2 was mainly composed of Rha (21.4%), GalA (34.9%), and Gal (29.6%), with traces of GlcA (4.5%), Glc (5.9%), and Ara (3.7%). The ratio of Rha/GalA is 0.61, falling in RG-I type range from 0.05 to 1.0 as defined bySchols and Voragen (1996), which suggested that WOP-2 is a RG-I domain-rich pectic polysaccharide. In addition to RG-I type domain, the value for Rha/GalA is 0.61, lower than 1.00, which indicated that oligogalacturonic acid units might be present in the RG-I domain. The ratio of (Gal + Ara)/Rha for WOP-2 is 1.56, suggesting that the length of the side chains was short attached to 4-O-Rha in RG-I backbone (Ferreira, Mafra, Soares, Evtuguin, & Coimbra, 2006; Sengkhamparn, Verhoef et al., 2009).

3.4. Structural features of WOP-2

The structure of WOP-2 was further analyzed by ^{13}C NMR spectrum (Fig. 4). NMR spectrum of WOP-2 was very complicated due to the complex structure of RG-I domain. RG-I domain is mainly composed of a backbone with alternating α-**L**-Rha*p* and α-**D**-Gal*p*A residues, and with α-**L**-Ara*f*- and β-**D**-Gal*p*-rich side chains attached via 4-O-Rha (Yapo, 2011). Most of the signals from C-2 to C-5 could not be assigned accurately. However, some characteristic signals, such as C-1 and C-6 resonances of α-**L**-Rha*p* and α-**D**-Gal*p*A, could be clearly observed and assigned. The C-1 signals for (1 → 2)-linked and (1 → 2,4)-linked α-

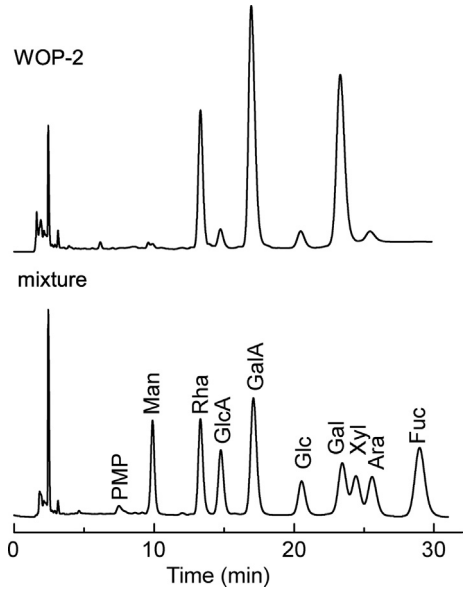

Fig. 3. Monosaccharide composition analysis of WOP-2 by HPLC with PMP derivatization.

Rha*p* residues appeared at 98.1 and 97.2 ppm, respectively, as did their C-6 signals at 16.1 and 16.3 ppm (Zhang et al., 2012). The resonance signals from the carboxyl carbons of un-esterified GalA residues were at 174.1 ppm, and the esterification of GalA residues was also confirmed by the resonance signal from the methyl group at 52.7 ppm and methyl ester carbonyl carbons at 172.2 ppm (Yu et al., 2010). The complexity of these broad signals from the carboxyl carbons of GalA residues in WOP-2 were also observed in other pectic polysaccharides, which might be due to the influence of neighboring free and esterified GalA residues. In addition, according to NMR analysis, the signals from methyl carbons of acetyl groups in O-2 or O-3 of GalA were clearly observed at 19.7 ppm. Based on the ratios of the signals from carboxyl groups, the degree of acetylation is relatively high while the degree of methyl esterification is relatively low. Thus, WOP-2 possessed NMR resonances characteristic of RG-I type backbone.

The NMR signals from C-1 of Gal residues were clearly identified. Signals at 104.3, 103.4, 102.8 and 102.2 ppm represented terminal β-Gal*p*, β-(1 → 3,6)-, β-(1 → 3)-, and β-(1 → 6)-linked β-Gal*p*, respectively, which suggested that the branched chains in RG-I domain of WOP-2 might contain type II arabinogalactan (AG-II) (Zhang et al., 2012). In AG-II domain, GlcA residues usually present as molecular terminals. In this study, several NMR signals characteristic of Glc*p*A at 58.7, 99.5, and 175.3 ppm were observed for methyl group linked to C-4, C-1 of the terminal non-reducing, and C-6 of Glc*p*A, respectively, which suggested that WOP-2 contained 4-O-Me-β-Glc*p*A and was consistent with the monosaccharide composition of WOP-2 (4.5% of GlcA).

3.5. Anti-hyperglycemic activity

After injection with STZ, diabetic mice had remarkably high baseline fasting blood glucose (FBG) levels compared to the normal group ($P < .01$) as shown in Fig. 5A, which indicated that the diabetic mice model was successful. The pectic polysaccharide fraction WOP-2 at dose of 50 and 150 mg/kg decreased FBG levels significantly after 10 days of treatment by intra-gastric administration compared to the control group. The effects of WOP-2 on body weights in mice were shown in Fig. 5B. After 10 days of treatment, body weights of WOP-2 groups were significantly elevated compared to that of diabetic mice group.

Antioxidants are able to inhibit the peroxidation chain reaction related to the destruction of β cells, which might provide protection against the development of diabetes and its complications. Peroxidation reactions are generally approved as a potential causative factor in the development of diabetes and its complications. To investigate the conceivable mechanism of the anti-hyperglycemic activity, serum insulin, malondialdehyde (MDA), and superoxide dismutase (SOD) activity were studied. As shown in Fig. 6, serum insulin and SOD activity were decreased significantly in STZ-induced mice, compared to normal group mice, while MDA level were increased significantly. After treatment with WOP-2 at dose of 50 mg/kg, as compared to STZ-induced diabetic mice, polysaccharide-treated mice had lower MDA level, and higher serum insulin and SOD activities, which was one of the conceivable mechanism for WOP-2 on anti-diabetic activity.

In addition, peroxidation reactions are also associated with a reduction in antioxidant capacity on diabetes, which could generate the deleterious compounds of free radicals. The superoxide, one of the precursors of the singlet oxygen and hydroxyl radicals, and the hydroxyl radicals, one of the most harmful reactive oxygen species, which could both result in cell death and tissue damage. In this study, superoxide radical-scavenging activity and hydroxyl radical-scavenging activity of WOP-2 were investigated *in vitro*. As shown in Fig. 7, the superoxide and hydroxyl radical-scavenging activities of WOP-2 exhibited a dose-dependent manner. Although the radical-scavenging activities of WOP-2 were weaker than ascorbic acid at the lower concentrations, the activities enhanced rapidly as the concentrations continuously increased and were closed to ascorbic acid, which indicated that the pectic polysaccharide fraction WOP-2 had a remarkable activity on scavenging superoxide and hydroxyl radicals attributed to the high acetylated groups probably. The results were consistent with the previous papers: the high acetylated polysaccharides in superoxide scavenging activity showed stronger antioxidant activity as reported by Chen et al. (2014) and Song et al. (2013).

Diabetes mellitus is one of the major chronic disorder worldwide, caused by either insufficient secretion of insulin or insulin resistance. Because the therapeutic drug usually has side effects, pectic polysaccharides with low toxicity have been focused on the development of a new-type anti-diabetic agent from dietary and medicinal plants. Polysaccharides from the cultured mycelium of *Cordyceps sinensis*,

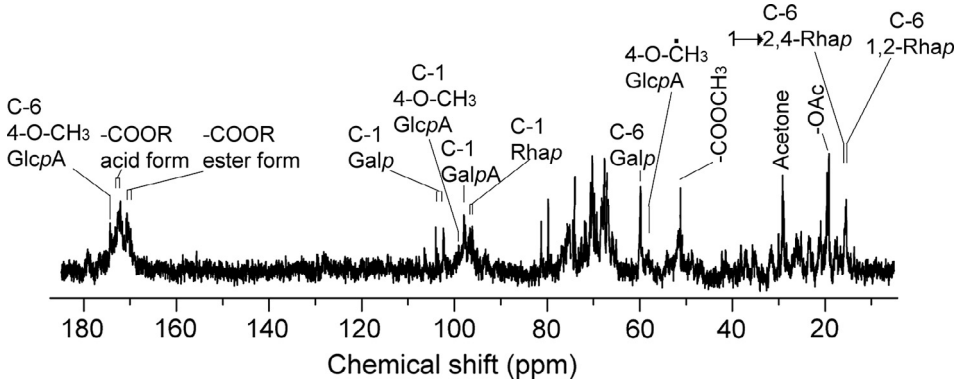

Fig. 4. NMR spectrum of the pectic polysaccharide fraction WOP-2.

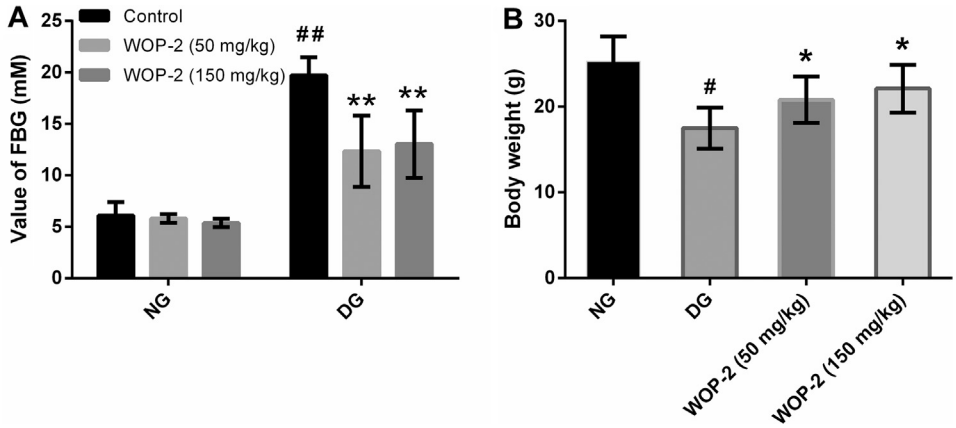

Fig. 5. The effects of WOP-2 on fasting blood glucose levels (A) and body weights (B) in mice by intra-gastric administration. Data are presented as mean ± SD. Compared with the normal group, # means $P < .05$ and ## means $P < .01$. Compared with the diabetes group, * means $P < .05$ and **means $P < .01$. NG means the normal group and DG means the diabetes group.

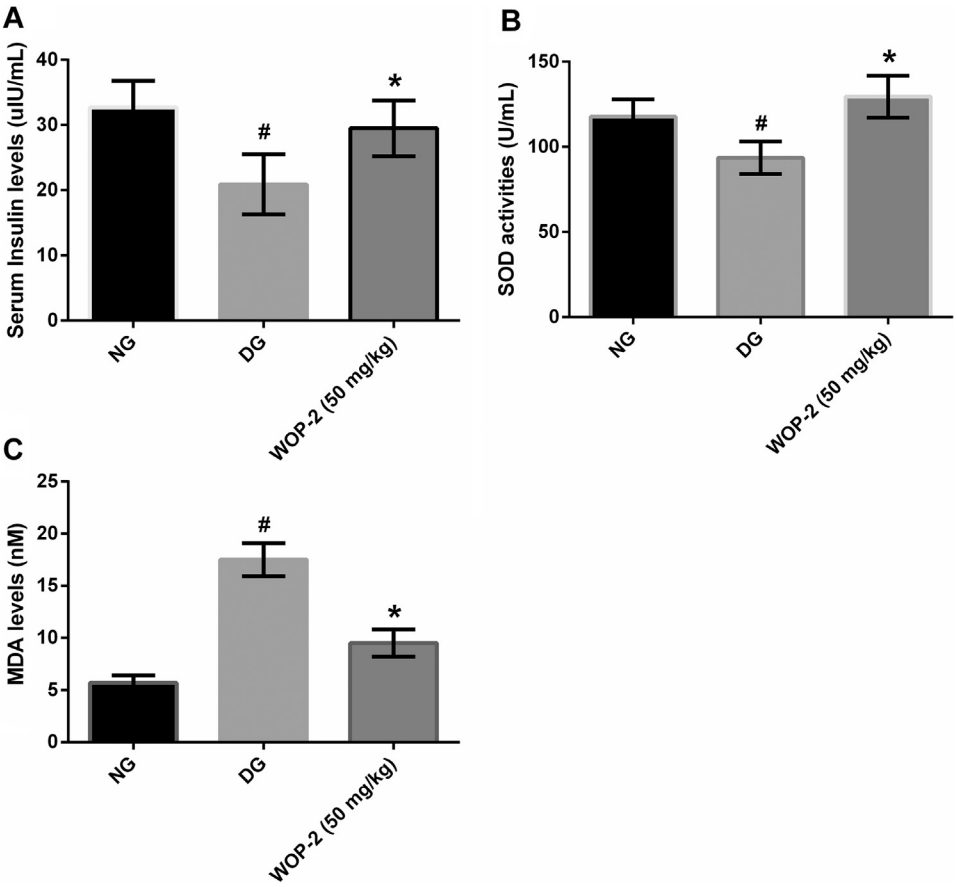

Fig. 6. The effects of WOP-2 on serum insulin level (A), SOD (B), and MDA (C) in diabetic mice by intra-gastric administration. Data are presented as mean ± SD. Compared with the normal group, # means $P < .05$. Compared with the diabetes group, * means $P < .05$. NG means the normal group and DG means the diabetes group.

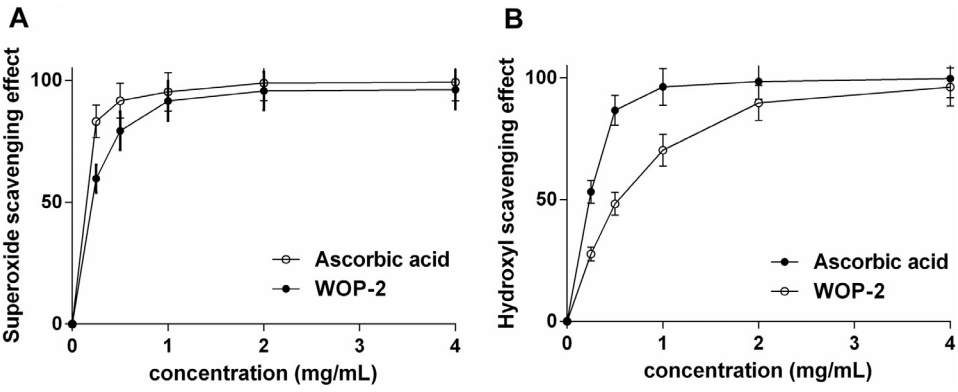

Fig. 7. Antioxidant effects analysis of WOP-2. (A) Scavenging effects on superoxide; (B) Scavenging effects on hydroxyl radicals.

Panax ginseng C. A. Meyer, and American ginseng berry were all found to have a significant anti-hyperglycemic activity (Kiho, Ookubo, Usui, Ukai, & Hirano, 1999; Sun et al., 2014; Xie, Wu, Mehendale, Aung, & Yuan, 2004). It has also been reported that polysaccharides having strong antioxidant potential might have a role to prevent the development of diabetes (Li et al., 2006). Thus, polysaccharides could be one of ideal anti-diabetic drugs, and the anti-hyperglycemic properties of the pectic polysaccharide fraction WOP-2 highlights the application as a therapeutic agent for diabetes.

4. Conclusion

In this study, a pectic polysaccharide fraction WOP-2 was extracted, isolated and purified from okra, which was identified to contain RG-I domain. The backbone of WOP-2 was speculated to be →4)-α-**D**-Gal*p*A-(1→2)-α-**L**-Rha*p*-(1→, which was partly substituted at O-4 of Rha*p* by side chains, such as AG-II. Its molecular weight was estimated to be 580 kDa, approximately. After treatment with WOP-2, in STZ-induced diabetic mice, the serum insulin and SOD activities increased and the level of MDA decreased, significantly. In addition, WOP-2 also showed a remarkable activity on antioxidant activity *in vitro*. Our finding suggested that WOP-2 exhibits significant anti-hyperglycemic activity on STZ-induced diabetic mice by inhibiting the peroxidation chain reaction probably, and WOP-2 could be a potential novel therapeutics to diabetes.

Conflict of interest

The authors confirm that there are no conflicts of interest.

Acknowledgments

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