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Structure Characterization and Hypoglycemic Activity of a Glycoconjugate from *Atractylodes macrocephalae* Koidz

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ABSTRACT

Atractylodes macrocephalae Koidz is a traditional medicinal plant in China. We previously reported that a complex-polysaccharide fraction (AMP-B) isolated from the root of this plant showed potent hypoglycemic activity in alloxan-induced diabetic rats after oral administration, so we further isolated and purified the active component from AMP-B to study its structure and hypoglycemic activity. Using DEAE-cellulose and Sepharose CL-6B gel filtration chromatography, we obtained an active glycoconjugate (AMP-2) from AMP-B. The molecular weight of AMP-2 was estimated to be 56660 Da by MALDI-TOF-MS. AMP-2 contains 80.9% (w/w) carbohydrate and 19.5% protein. It is composed of L-rhamnose, L-arabinose, D-mannose, D-galactose, D-glucose and D-galacturonic acid in a molar ratio of 1.0: 3.0: 1.0: 3.5: 2.1: 3.0. Its structural features were elucidated by reduction of carboxyl-groups, enzymatic degradation and reductive alkaline degradation, methylation analysis, ^{1}H-NMR and ^{13}C-NMR. The results suggest that AMP-2 has the following residues: L-1,5-linked and rich terminal arabinose, D-1,2-linked, 1,4-linked and terminal galactose, L-1,2,4-linked rhamnose, D-1,2-linked and D-1,6-linked glucose, terminal D-mannose, and D-1,4-linked and terminal galacturonic acid. The protein fractions may be linked with L-1,2,4-linked rhamnose and D-1,6-linked glucose. AMP-2 showed a remarkable hypoglycemic activity in alloxan-induced diabetic rats after oral administration at a dose of 50 mg/kg. In conclusion, a highly branched carbohdyrate-conjugate obtained from the root of *Atractylodes macrocephala* has shown marked hypoglycemic activity, which may provide a practical quality control protocol for this herbal medicine.

Keywords: alloxan, antidiabetic, polysaccharide, protein-bound polysaccharide

INTRODUCTION

Diabetes, especially type II diabetes, has become a global public health problem of the 21st century. This disease not only severely compromises the daily quality of life, but also is an unbearable burden for the public healthcare system.

Due to the nature and complexity of diabetes and the lack of an effective cure, traditional herbal medicine, or Alternative Medicine as it is known in the scientific world, has been explored for potential ways to control, manage, and cure diabetes (Hu *et al*. 2003; Chau *et al*. 2006; Stone 2008). Extensive research has focused on exploring the hypoglycemic activity and the active compounds of various herbal plants (Langmead *et al*. 2001; Raskin *et al*. 2002; Dhiman *et al*. 2005). Among those identified molecules, polysaccharides as a group have shown some initial encouraging results (Paulsen 2002; Li *et al*. 2003; Hwang *et al*. 2005; Li *et al*. 2006). Lo discovered an acidic glucuronoxylomannan from an edible mushroom *Tremella mesenterica* that showed potent hypoglycemic activity in diabetic rats (Lo *et al*. 2006), while a polysaccharide from the fruiting bodies of *Cordyceps sinensis* significantly attenuated diabetes-induced weight loss, polydipsia, and hyperglycemia in nicotinamide- and streptozotocin-induced diabetic rats (Lo *et al*. 2004). An additional example is the antidiabetic effect of crude exo-polysaccharides produced by a medicinal mushroom, *Phellinus baumii* in streptozotocin-induced diabetic rats (Hwang *et al*. 2005).

Atractylodes macrocephalae Koidz is a traditional medical herb in China that possesses many clinical effects: (1) invigorating spleen and benefiting vital energy; (2) depriving dampness and promoting diuresis; (3) strengthening superficies of the brain, and (4) antipersperspiration (Chinese Pharmacopoeia 2000). Modern pharmacological studies showed that *A. macrocephalae* exhibited significant bioactivities such as antitumor, antidiabetic, antiinflammatory, antiaging and immunoregulation (Su 2008). The petroleum ether-ether (1:1) extract of *A. macrocephalae* exhibited significant inhibiting effects both on the ear edema induced by xylene and on the peritoneal capillary permeability induced by acetic acid in mice (Dong *et al*. 2008). A polysaccharide (AMP-1) isolated from the roots of the herb showed an antitumor effect, inhibiting the growth of Sarcoma 180 and Lewis pulmonary carcinoma implanted in mice (Shan *et al*. 2003a). We previously reported that a complex-polysaccharide fraction AMP-B isolated from the root of the herb showed potent hypoglycemic activity in alloxan-induced diabetic rats (Shan *et al*. 2003b). In this paper, we further isolated the active constituent from AMP-B, and studied its structural features and hypoglycemic activity.

MATERIALS AND METHODS

Materials

Dried roots (5 kg) of *A. macrocephalae* were purchased from Xinchang county of Zhejiang province, authenticated by professor Xiu-jia Zhou. A voucher specimen No. 13 was stored in the Herbarium of State Key Laboratory of Bio-organic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. The dry roots were pulverized and the powder was passed through an 8 mm-mesh sieve before use for aqueous extraction. Trifluoroacetic acid (TFA), Alloxan, and *N*-cyclohexyl-*N'*-(2-morpholinoethyl) carbodiimide methyl-*p*-toluenesulfonate (CMC) were purchased from Sigma-Aldrich; Pronase-E (70000 PUK/g) from Merck; Sepharose CL-6B and Sephadex G-75 from Pharmacia; and DEAE-cellulose from Shanghai Hengxin Chemical Reagent Corporation.

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Original Research Paper

General procedures

Total carbohydrates were measured by the phenol-H_2SO_4 method using glucose as standard (Dubois *et al*. 1956). Uronic acids were determined by the *m*-hydroxylbiphenyl method with minor modifications using glucuronic acid as standard (Blumenkrantz *et al*. 1973). The homogeneity of AMP-2 was confirmed by HPLC and capillary electrophoresis (CE). The molecular weight of AMP-2 was determined by MALDI-TOF-MS (Bruker® Reflex III). The sugar component was determined by capillary gas-liquid chromatography (GC) [3% OV-225 capillary column (0.32 mm × 30 m), Varian VISTA 402] of an alditol acetate derivative (Jacob 1987). The configurations of neutral sugars and neutral derivative of hexuronic acid were analyzed by GC of the corresponding TMSi-(-)-butylglycosides derivatives (Gerwig *et al*. 1978). Gas-liquid chromatography-mass spectroscopy (GC-MS) of the alditol acetates of the partially methylated fragments was performed on a Shimadzu QP 5000 Spectrometer (OV-17 capillary column, 0.30 mm × 25 m). ^{1}H NMR and ^{13}C NMR spectra were collected with a Bruker-MX-300 spectrometer. The sample was dissolved in D_2O at a concentration of 60 mg/mL. Protein content was measured by the method of Zhang *et al*. (1981) using bovine albumin as standard. The amino acid compositions of protein were determined with a Hitachi 835–50 amino acid analyzer after complete acid hydrolysis in 6 M HCl at 110°C for 18 h.

Isolation and purification an active component of glycoconjugate (AMP-2)

Fine pulverized dry roots (1.0 kg) of *A. macrocephala* were soaked in 10 L of distilled water for 24 h at room temperature with stirring. The liquid filtrate was collected and the solid residues were added with 10 L of fresh water and extracted for another 24 h under the same conditions. The liquid from the two extractions was combined and concentrated with a rotary evaporator to 2.0 L under diminished pressure at 50°C. Then six volumes of 95% ethanol were added to precipitate out the crude polysaccharides. The precipitates were collected with centrifugation, dissolved again in 2.0 L of distilled water, and dialyzed against water for 3 days. The dialyzed crude polysaccharide solution was lyophilized and yielded 12.3 g of brownish powder (termed AMP, yield 1.23%).

AMP (400 mg) was further fractionated on a DEAE–cellulose column (HCO_3^-, 3.0 × 30 cm) and eluted stepwise with H_2O, 0.25, 0.5, and 1.0 M of $NaHCO_3$. No carbohydrates were detected in the fractions eluted with 1.0 M of $NaHCO_3$ by the phenol-H_2SO_4 method. There were three main peaks fractions in AMP containing polysaccharides. Each peak fraction was pooled and lyophilized, which afforded AMP-A (60 mg), AMP-B (126 mg), and AMP-C (42 mg), respectively.

AMP-B (100 mg) was further purified on a Sepharose CL-6B size exclusion column (2.5 × 100 cm). Elution was carried out with 0.1 mol/L of NaCl solution at a flow rate of 0.3 mL/min. Fractions were monitored by the phenol-H_2SO_4 method for sugar moiety and UV absorbance at 280 nm for the protein component. A symmetrical peak was identified which contained a sugar moiety and showed protein absorbance at 280 nm. The fraction was collected and combined. The pooled fractions were concentrated, dialyzed against water, and lyophilized, which yielded 45 mg of a yellowish powder (termed AMP-2).

Reduction of carboxyl groups of AMP-2

Reduction of carboxyl groups in AMP-2 was carried out with *N*-cyclohexyl-*N*'-(2-morpholinoethyl) carbodiimide methyl-*p*-toluenesulfonate (CMC) and $NaBH_4$ according to Tayor and Courad (1972). Briefly, AMP-2 (50 mg) was dissolved in 50 mL distilled water, then 1.3 g of CMC was added. The pH of the reaction mixture was maintained at 4.75 by adding diluted hydrochloric acid (0.01 M). The activation reaction was allowed to proceed for 2 h at room temperature. The activated AMP-2 was then reduced with 30 mL of 2 M $NaBH_4$ for 1 h. The pH of the reaction mixture was maintained at a neutral pH (7.0) by adding 2.0 M HCl with stirring. The reaction product was dialyzed against water for 72 h and lyophilized. This process was repeated once under the same conditions to give a carboxyl-reduced product (termed AMP-2R).

Enzyme degradation of AMP-2

Pronase-E can be used to specifically cleave a protein-carbohydrate bond. Treatment of AMP-2 (100 mg) with Pronase-E (1%, g/g) was performed in 0.1 M Tris-HCl buffer, pH 8.0, containing 1.0 mM of $CaCl_2$ and a few drops of toluene. The mixture was stirred at 25°C for 2 h, followed by 48 h at 37°C with constant stirring. Then 0.5% Pronase-E (g/g) was added and the reaction was allowed to proceed for another 48 h at the same temperature. The reaction mixture was heated to 60°C and maintained at this temperature for 1 h to fully deactivate the enzyme. After centrifugation, the supernatant was dialyzed in distilled water and lyophilized (Zhang 1999). The lyophilized material was then re-suspended in H_2O and applied to a Sephadex G-75 column (2.0 × 80 cm). Target product was eluted with 0.1 M of NaCl. The sugar fractions were combined, dialyzed and lyophilized to give the Pronase-E degraded polysaccharide (termed AMP-2E) and further characterized below.

Cleavage of alkali-labile sugar-protein linkage of AMP-2E

AMP-2E (50 mg) was dissolved in 5 mL of 0.2 M NaOH containing 1.0 M $NaBH_4$ and incubated at 50°C for 72 h. The reaction mixture was then neutralized with 2.0 M of acetic acid. After concentration, the residue was loaded on to a Sephadex G-75 column (2.0 × 80 cm). Elution was carried out with 0.1 M NaCl at a flow rate of 0.3 mL/min. Fractions were monitored by UV absorption at 280 nm for the protein component and the phenol-H_2SO_4 method for carbohydrates. Fractions containing carbohydrates but no protein were collected, dialyzed, and lyophilized (termed AMP-2EE) (Chaplin *et al*. 1986).

Methylation analysis of AMP-2, AMP-2R and AMP-2EE

Methylation of free hydroxyl groups before complete hydrolysis provided an efficient way to analyze the connections among monosaccharides (Needs *et al*. 1993). Samples of AMP-2, AMP-2R, or AMP-2EE (10 mg each) in 2.0 mL of dimethyl sulfoxide were methylated under nitrogen by adding NaOH powder (100 mg) and methyl iodide (1.5 mL). The reaction mixture was incubated at 25°C for 2 h. Solvents were removed by evaporation. The methylated product was hydrolyzed with 90% formic acid (3 h at 100°C) or with 2 M TFA (6 h at 100°C). The partially methylated product in the hydrolysate was reacted with $NaBH_4$, was acetylated by acetic anhydride, and the resulting mixture of alditol acetates was analyzed by GLC and GC-MS (Sweet *et al*. 1975).

^{1}H, ^{13}C-Nuclear Magnetic Resonance

AMP-2 (50 mg) was exchanged with D_2O (99.8%) through repeated lyophilization to reduce the H_2O signals. At the end, the sample was dissolved in 0.5 mL of D_2O and ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker-AMX-300 spectrometer at room temperature.

Bioassay for hypoglycemic activity of AMP-2 in rats

Male Sprague-Dawley rats, aged 6-8 weeks (200 ± 20 g of body weight), were obtained from the Animal Center of Shanghai, Chinese Academy of Sciences. Alloxan-diabetic rats were prepared by an intravenous injection of alloxan (50 mg/kg, dissolved in saline) to the fasting rats (12 h). Plasma glucose was measured by an autoanalyzer (Basic-Plus, Agohuson–Gohuson Company, USA) using a blood sample from tail vein of rats. In addition to other diabetic features, rats with plasma glucose levels higher than 16.0 mmol/dL were considered as type 1-diabetes.

Rats used to study the hypoglycemic effects were divided into two batches. In the first batch, the alloxan-diabetic rats were randomized into four groups with 10 rats in each group. The first group was the untreated-diabetic control group. For the other three

groups (2, 3, 4), each group was treated with 100 mg/kg per day (p.o.) of AMP-A, AMP-B, or AMP-C. In the second batch, the alloxan-induced diabetic rats were divided into three groups with the first group as the untreated-diabetic control. The rats in the second group were treated with AMP-2 (50 mg/kg per day p.o) and that in the third group were treated with glibenclamide (as positive control drug group, 2.0 mg/kg per day p.o.). For all studies, dosing was carried out at day 15 after the injection of alloxan. All animal handling procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guideline of the Animal Welfare Act.

Statistical analysis

All the data were expressed with mean ± SE, and a Student's *t*-test was used for the statistical analysis. The values were considered to be different statistically when the p value was less than 0.05, and significantly different at $p<0.01$.

RESULTS

Isolation an active glycoconjugate AMP-2

Crude polysaccharides (AMP) from the roots of *A. macrocephalae* were extracted with water, then by ethanol precipitation and dialysis with a resultant yield of 1.23% (w/w). AMP was added to a DEAE-cellulose column, then eluted stepwise with water, 0.25 and 0.5 M $NaHCO_3$ to produce three absorption peaks. These peaks were AMP-A (11.2% yield), AMP-B (35.5% yield) and AMP-C (9.2% yield). The spectra are shown in **Fig. 1**.

AMP-B showed potent hypoglycemic activity. It was further purified on a Sepharose CL-6B column eluted with 0.1 M NaCl. A symmetrical peak (**Fig. 2**) was monitored with the phenol-H_2SO_4 method (saccharide) and absorbance at 280 nm (protein). The peak was collected, dialyzed and lyophilized to obtain a glycoprotein named AMP-2.

Identification the glycoconjugate AMP-2

The purity of AMP-2 was identified by HPLC and CE. As shown in **Figs. 3** and **4**, AMP-2 had highly homogeneous and symmetrical features. With this confirmed purity, we determined the molecular weight of AMP-2 to be 56,660 Da by MALDI-TOF-MS.

Physico-chemical property and structural characterization of AMP-2

The content of carbohydrate in AMP-2 was 80.9% (w/w) by phenol-H_2SO_4 measurement and its protein content was 19.5%. Sugar components of AMP-2 were analyzed by capillary gas-liquid chromatography of alditol acetate derivatives and were shown to be composed of L-rhamnose, L-arabinose, D-mannose, D-galactose, D-glucose, and D-galacturonic acid in a molar ratio of 1.0: 3.0: 1.0: 3.5: 2.1: 3.0. Complete acid hydrolysis of AMP-2 and amino acid analysis showed that its protein components were Asp 19.28%, Glu 18.63%, Gly 10.13%, Arg 8.83%, Ser 3.59%, Thr 3.59%, Lys 4.90%, Ala 4.90%, Val 4.90%, and Pro 3.59%. There were also trace amounts of Cys, Met, Ile, Leu, Tyr, Phe, Orn, and His residues.

^{1}H-NMR and ^{13}C-NMR spectra provide important structural information of the oligosaccharide components. In the anomeric region of the ^{1}H-NMR spectrum (**Fig. 5**), eight signals occurred at δ5.79, δ5.41, δ5.31, δ5.25, δ5.23, δ5.14, δ5.08 and δ5.00 ppm, and methyl protons of L-rhamnopyranosyl residues produced a signal at δ1.45 ppm. The anomeric regions of the ^{13}C-NMR spectrum (**Fig. 6**) contained eight signals. The signals at δ112.02-109.81 were assigned to the anomeric carbons of L-arabinofuranose, the signals at δ106.39 and δ105.94 were assigned to the anomeric carbons of D-galactopyranosyl residues, the signals at δ102.07-101.15 were assigned to D-galactopyranosyluronic acid residues, the signal at δ99.46 was assigned to L-rhamnopyranosyl residues, the signal at δ103.55 was assigned to D-glucopyranosyl residues, and the signal at δ95.08 was assigned to D-mannopyranosyl residues. The carbonyl signal at δ 177.61 was assigned to galacturonic acid, and the signal of methyl of L-rhamnopyranosyl residues was at δ 19.32 ppm.

Results of methylation analysis of AMP-2, AMP-2R and AMP-2EE are summarized in **Table 1**, which shows native AMP-2 to be composed of the following sugar residues: L-1,5-linked and rich terminal arabinose; D-1,2-linked,1,4-linked and terminal galactose; L-1,2,4-linked rhamnose; D-1,2-linked, D-1,6-linked glucose and terminal D-mannose residues. After the carboxyl reduction of AMP-2, there are terminal and D-1,4-linked galactose residues to generate in AMP-2R, which indicates that AMP-2 contains D-1,4-linked and terminal galacturonic acid residues.

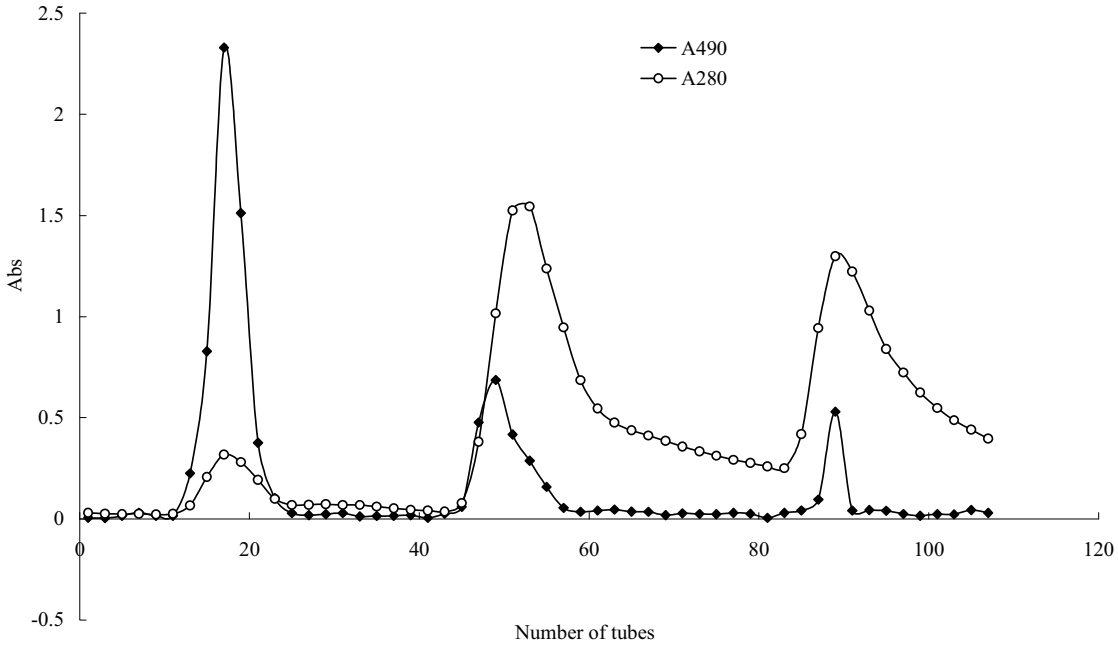

Fig. 1 Elution profiles of AMP on DEAE-cellulose column (3.0 cm × 30 cm). Elution was carried out with H_2O, 0.25 and 0.50 M $NaHCO_3$ solution. Solid line, 490 nm (saccharides); dotted line, 280 nm (protein).

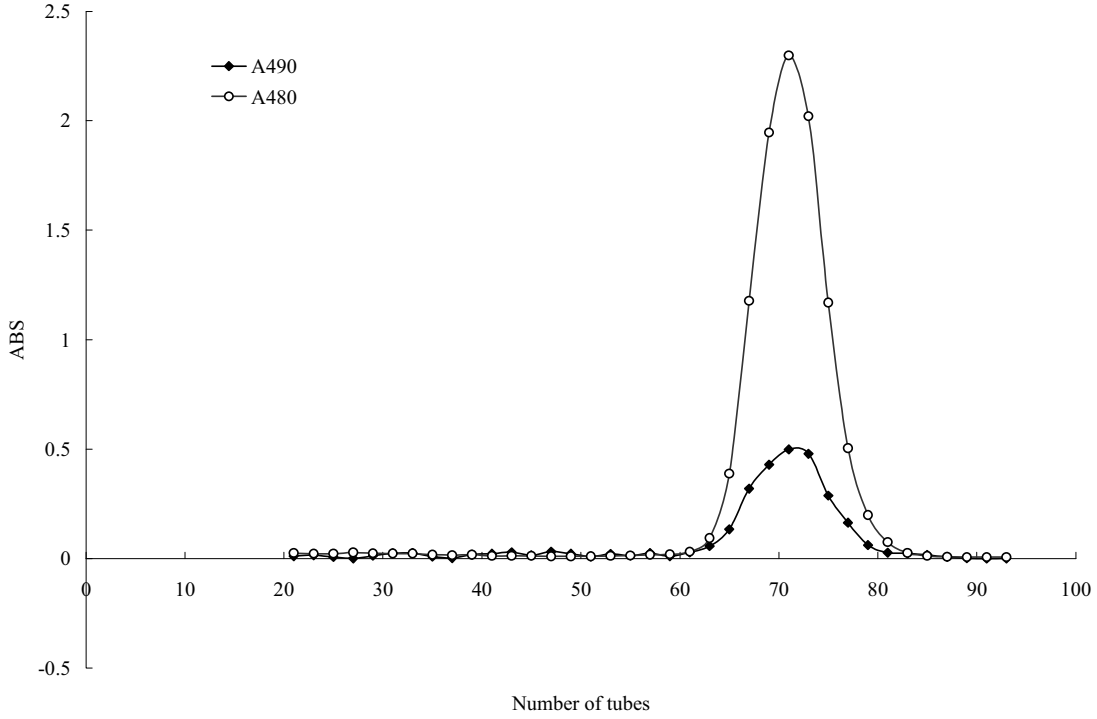

Fig. 2 Elution profile of Sepharose CL-6B column (2.5 × 100 cm) of AMP-B eluted with 0.1 M NaCl. Solid line, 490 nm (saccharides); dotted line, 280 nm (protein).

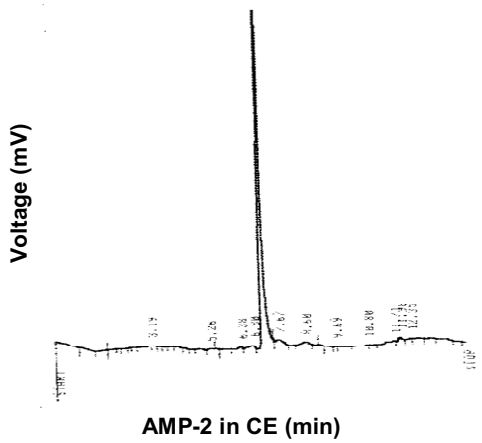

Fig. 3 Capillary electrophoresis of AMP-2 (carried out in 0.1 M H_3BO_3-KOH).

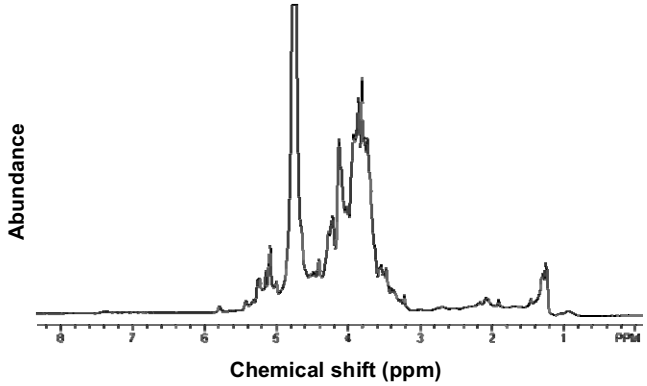

Fig. 5 ^{1}H-NMR spectrum of AMP-2 at 300 MHz (in D_2O).

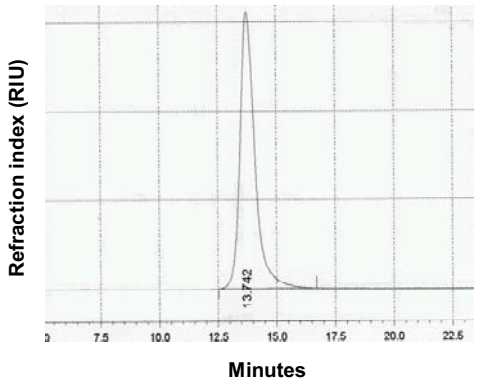

Fig. 4 HPLC chromatography of AMP-2 on a Bio-gel column, eluted with 0.1 M NaCl.

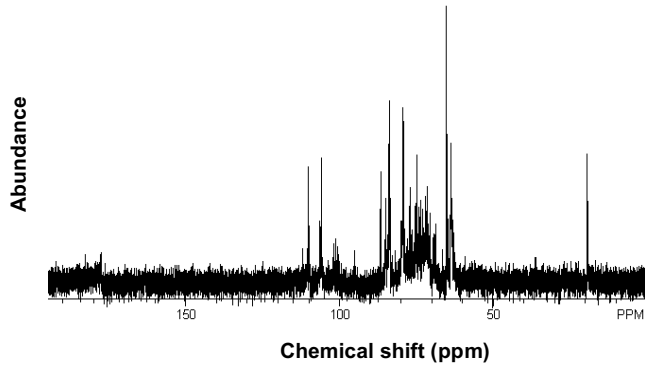

Fig. 6 ^{13}C-NMR spectrum of AMP-2 at 300 MHz (in D_2O).

After enzymatic degradation and reductive alkaline-degradation, carbohydrate fractions lost the L-1,2,4-linked rhamnose and D-1,6-linked glucose residues, suggesting that these residues are linked to the protein moiety. Additionally, we found that part of the terminal arabinose residues were lost after reductive alkaline-degradation and enzymatic degradation.

Hypoglycemic activity of AMP-2

The hypoglycemic activities of AMP-A, AMP-B and AMP-C were tested in a first batch. As shown in **Table 2**, the results showed that AMP-B markedly reduced blood glucose level in alloxan-induced diabetic rats at a dose of 100 mg/kg, while AMP-A and AMP-C showed no antihyperglycemic effect. In the second batch test, the hypoglycemic effect of AMP-2 is shown in **Table 3**. The untreated-dia-

Table 1 Methylation analysis of AMP-2, AMP-2R (carboxylate-reduced) and AMP-2EE (enzyme and alkaline-degraded).

Sugar linkage	AMP-2	AMP-2R	AMP-2EE
Araf (1→	1.52	1.56	0.53
→5) Araf (1→	1.30	1.17	0.93
Man (1→	1.16	1.26	1.00
→2,4) Rha (1→	1.00	1.00	n/a
Gal (1→	n/a	1.00	0.42
→2) Gal (1→	1.78	1.74	2.2
→4) Gal (1→	2.28	2.65	n/a
→6) Glc (1→	1.39	1.43	1.01
→2) Glc (1→	1.25	1.29	0.98

Table 2 Effect of AMP-A, B, C on blood glucose levels in alloxan-induced diabetic rats.

Groups	Blood glucose concentration (mmol/dL)		
	Days after induction of diabetes		
	0 day	5 days	10 days
Normal	4.18 ± 0.45	4.54 ± 0.60	3.91 ± 0.54
Diabetic	22.53 ± 2.49	21.39 ± 2.86	19.62 ± 2.33
Diabetic+AMP-A	21.89 ± 2.71	22.12 ± 3.70	20.69 ± 4.18
Diabetic+AMP-B	22.01 ± 3.48	14.51 ± 5.54 *	13.18 ± 5.83 *
Diabetic+AMP-C	21.36 ± 2.45	18.03 ± 6.46	17.60 ± 4.98

After induction of diabetes, AMP-A, B, C were given daily at an oral dose of 100 mg/ kg body weight. The untreated-diabetic group received 2 ml water orally daily. Values are means ± S.E, n=10. * p<0.05, compared with the untreated-diabetic group.

Table 3 Effect of AMP-2 on blood glucose levels in alloxan-induced diabetic rats.

Groups	Blood glucose concentration (mmol/dL)		
	Days after induction of diabetes		
	0 day	7 days	11 days
Normal	3.94 ± 0.54	3.51 ± 0.32	4.34 ± 0.70
Diaaibetic	19.03 ± 3.43	19.42 ± 7.78	19.07 ± 7.21
Diabetic+AMP-2	18.77 ± 3.29	7.76 ± 3.23***	8.12 ± 4.15***
Diabetic+ Glibenclamide	19.21 ± 3.12	11.38 ± 5.31**	10.46 ± 5.64**

After induction of diabetes, AMP-2 was given daily at an oral dose of 50 mg/ kg body weight. The untreated-diabetic control group received 2 ml water orally daily. Glibenclamide was given daily at an oral dose of 2 mg/kg. Values are means ± SEM, N=9.
** p<0.05, *** p<0.01, compared with untreated-diabetic group.

betic group animals showed no significant change in blood glucose levels over a period of 11 days, but the rats treated with AMP-2 demonstrated significantly lowered blood glucose after 7 days at dose of 50 mg/kg (p<0.01). The effect of AMP-2 was better than glibenclamide (2.0 mg/kg) at 7d, but AMP-2 had no further improvement from 7 to 11 d.

DISCUSSION

At present, more than 300 polysaccharides have been isolated from natural sources, including plants, animals and microbes. Among these, many neutral and acid polysaccharides exhibited significant antidiabetic activities, such as reducing blood glucose and lipid levels, improving insulin resistance, increasing liver glycogen content and protecting pancreatic β-cells (Cheng *et al*. 2007; Du *et al*. 2007). However, there are only a few papers reporting the antidiabetic effects of protein-bound polysaccharides. For example, a protein-polysaccharide compound (PBPP) isolated from pumpkin containing 41.21% polysaccharide and 10.13% protein reduced blood glucose levels and increased serum insulin content in alloxan-induced diabetic rats (Li *et al*. 2005). In another example, a glyco-peptide glycoconjugates (GPS) isolated from the leaves of *Morus alba* included 86% carbohydrate and 11% protein. Four hours after administering this GPS, fasting blood sugar and random blood glucose levels decreased by 31.48 and 54.29%, respectively (Xue *et al*. 2005). The antidiabetic mechanism of PBPP and GPS are still unknown, but Jin *et al*. reported an acid proteoglycan (APFM) from *M. alba* exhibited significant antioxidant activity in a diabetic animal model, where it efficiently scavenged ·OH and O_2· in the organs of alloxan-induced diabetic mice, inhibited the production and accumulation of malondialdehyde, and increased the superoxide dismutase activity (Jin *et al*. 2007).

Alloxan is widely used in studies of experimental diabetes because this agent destroys pancreatic β-cells with specific selectivity. The alloxan molecule is structurally so similar to glucose that the GLUT2 glucose transporter in the β-cell plasma membrane accepts this glucomimetic and transports it into the cytosol (Gorus *et al*. 1982). Alloxan can generate reactive oxygen species (ROS) in the cyclic reaction with its reduction product, dialuric acid. In the β-cells the toxic action of alloxan is initiated by free radicals formed in a redox reaction (Munday *et al*. 1988). Autoxidation of dialuric acid generates superoxide radicals and hydrogen peroxide (Winterbourn *et al*. 1989). Paradoxically the thiols cysteine and GSH have long been reported to protect rats against the development of alloxan diabetes when injected together with alloxan (Lazarow *et al*. 1948). This observation can now be explained in light of the established molecular mechanism of alloxan action (Lenzen 2008).

We previously reported that the complex-polysaccharide fraction AMP-B from *A. macrocephalae* showed potent activity in normalizing the blood glucose level in alloxan-induced diabetic rats after oral administration. AMP-B also improved the characteristic diabetic symptoms of polyuria, polydipis, polyphagia and weight loss, decreased water and food consumption, and inhibited the atrophy of thymus and pancreas (Shan *et al*. 2003b). AMP-B was further purified to a glycoconjugate AMP-2. AMP-2 is a highly branched carbohydrate-protein conjugate. AMP-2 exhibited more significant hypoglycemic activity (50 mg/kg) than AMP-B (100 mg/kg) within 11 days. Although the exact hypoglycemic mechanism of AMP-2 is not clearly understood yet, we suggest that AMP-2 might protect β-cells of the pancreas against free radicals generated by alloxan and dialuric acid or increase the release of insulin. The precise structure and the anti-diabetic mechanisms of AMP-2 will be studied further in our laboratory.

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