Tohoku J. Exp. Med., 2015, **236**, 71-79

Hypoglycemic, Hypolipidemic and Antioxidant Effects of Peptides from Red Deer Antlers in Streptozotocin-Induced Diabetic Mice

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Diabetes mellitus is a serious chronic metabolic disorder. To develop novel anti-diabetic drugs from nature sources has always been the focus of research. Red deer (*Cervus elaphu* Linnaeus) antler is one of the most famous Chinese traditional medicines. We found that the peptides of 5-10 kDa from red deer antlers (PRDA) promoted the growth of cultured rat islet cells. The purpose of this study was to investigate the anti-diabetic actions of PRDA *in vivo* and purify a pure active peptide. We therefore investigated the hypoglycemic, hypolipidemic and antioxidant effects of PRDA in streptozotocin-induced diabetic mice and isolated a pure anti-diabetic peptide. PRDA, given intraperitoneally (75, 150, or 300 μg/kg), significantly decreased the blood glucose levels, significantly increased the insulin concentrations, and remarkably improved the lipid metabolism in the diabetic mice. PRDA significantly increased the superoxide dismutase activity, catalase activity and the total antioxidant capacity in the serum and liver, and simultaneously decreased the malondialdehyde levels. The activities of hexokinase and pyruvate kinase, two important enzymes involved in glucose utilization, were also significantly increased in the liver of the PRDA-treated diabetic mice. Moreover, a novel anti-diabetic peptide isolated from PRDA significantly promoted the viability of cultured rat insulinoma cells. The molecular mass of the purified peptide was 7064.8 Da under mass spectrometry, and its N-terminal amino acid sequence was identified as LSPFTTKTYFPHFDLSHGSA. Thus, PRDA may be useful in managing the hyperglycemia, hyperlipidemia, and oxidative stress in diabetes, and the anti-diabetic peptide is a promising drug for the treatment of diabetes.

Keywords: anti-diabetic; diabetic; pancreatic cell; peptide; red deer antler
Tohoku J. Exp. Med., 2015 May, **236** (1), 71-79.

Introduction

Diabetes mellitus (DM) is a group of chronic metabolic disorders characterized by hyperglycemia caused by defects in insulin secretion, action, or both; chronic hyperglycemia seriously damages systems such as blood vessels and nerves (O'Connell et al. 2008). In 2011, an estimated 366 million people were diagnosed with diabetes, and the incidence is predicted to increase to 552 million by 2030 (Whiting et al. 2011).

Numerous hypoglycemic agents have been clinically used to control blood glucose level, including insulin injection and oral drugs such as sulfonylureas, biguanides, α-glucosidase inhibitors, and thiazolidinediones. Some unexpected adverse effects have been experienced in the use of these agents. For instance, thiazolidinediones such as rosiglitazone, may induce edema (Bailey et al. 2010), heart failure (DREAM Trail Investigators 2008), and bone loss (Mancini et al. 2009). An increasing number of products with anti-diabetic properties have recently been extracted from natural sources (Bae et al. 2013; Yuan et al. 2013).

Diabetes is often accompanied by hyperlipidemia and oxidative stress, which will trigger diabetes complications and aggravate disease progression. It is, therefore, important to develop novel hypoglycemic, hypolipidemic and antioxidant drugs especially from nature sources. Deer antler has been used in the East as a traditional animal-based medicine for over 2000 years to prevent or treat various diseases, including bone-resorption diseases (Lee et al. 2011), rheumatoid arthritis (Kim et al. 2008), and vascular necrosis (Shi et al. 2010), as well as for improving hematopoiesis (Lee et al. 2012). Deer antlers are not traditionally used to treat DM, but peptides from sika (*Cervus nippon* Temminck) antlers have been shown to reduce the blood glucose levels of diabetic KK-Ay mice and promote glucose consumption in HepG2 human hepatoma cells (Huang et al. 2010). These results suggest that antler peptides from red deer, one of the two major deer species in China, could be a potential source of hypoglycemic agents. In our previous

Received December 19, 2014; revised and accepted April 24, 2015. Published online May 16, 2015; doi: 10.1620/tjem.236.71.
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study, we found that the 5-10-kDa peptides promoted the proliferation of mouse islet cells *in vitro* (data not shown). Therefore, the present study investigated the effects of 5-10-kDa peptides from dried red deer (*Cervus elaphu* Linnaeus) antlers on the blood glucose levels and other indices related to diabetes in streptozotocin-induced diabetic mice, as well as purified and characterized a novel anti-diabetic peptide from the fraction.

Materials and Methods

Materials and chemicals

The dried red deer antlers were provided by the Hua Shidan Pharmaceutical Co., Ltd in Xinjiang, China. Ultra filtration packages were purchased from Sartorius. All of the reagent kits for the determination of biochemical parameters were obtained from Jiancheng Bioengineering Institute, Nanjing, China. All chemicals were of the highest purity available.

Preparation of 5-10-kDa peptides from dried red deer antler (PRDA)

PRDA were prepared as follows: Dried red deer antlers from some red deer were sawn into segments of about 5 cm length and soaked in 50% ethanol for 1 week, and then were cut into sections of about 1 mm thickness. After dried at 37°C, the sections were pulverized. Dried red deer antlers (50 g) ground to pass 60 meshes were soaked twice in 5 mM acetate buffer (pH 3.5) (1:10, 1:10, w/v) for 8 hours at 4°C. The extract was centrifuged at 10,000 rpm. The supernatants were filtered and pooled. Ethanol was then added to a final concentration of 50% (v/v) and the mixture was stored for 3 hours at 4°C. The mixture was then filtered to remove precipitates and was evaporated under reduced pressure in a rotary evaporator at 37°C to obtain total peptides from red deer antlers. Ultra-filtration was used to fractionate and obtain the 5-10-kDa peptides from the total peptides, which were dialyzed against distilled water and lyophilized finally. The peptides were analyzed via Tricine-SDS-PAGE based on the method by Schägger (2006). The molecular mass of the peptides was determined by comparing its electrophoretic mobility with those of low-molecular-weight marker proteins (SANGON, China). Peptide content was estimated using bicinchoninic acid (BCA) reagent (Beyotime, China), with bovine serum albumin as the standard. PRDA (about 50 mg) were obtained from 50 g of antler based on the amount of proteins and peptides. The PRDA lyophilized powder contained 95.4% proteins and peptides and 2.9% moisture without any polysaccharide or nucleic acid.

Purification of an anti-diabetic peptide

A cation exchange CM-Sepharose column (5 ml) was employed to separation the peptides further from the PRDA. The column was equilibrated and initially eluted with 20 mM acetate buffer (pH 4.5). The anti-diabetic fraction (CMP) was fractionated using 150 mM NaCl in the equilibration buffer at the flow rate of 1 ml/min. The CMP was lyophilized and dissolved in 20 mM acetate buffer (pH 4.5) containing 150 mM NaCl and injected into a Superdex 30 column (GE Healthcare). Chromatography was then performed at a flow rate of 0.8 ml/min. The main fraction (SP) was collected and lyophilized for further separation via reversed phase chromatography on a SOURCE 15RPC column (1 ml, GE Healthcare). The SP was dissolved in 95% buffer A (0.1% TFA/H_2O) + 5% buffer B (0.065% TFA/acetonitrile). The peptides were eluted using sequential linear gradients from 95% buffer A + 5% buffer B to 72% buffer A + 28% buffer B in 5 min, followed by 72% buffer A + 28% buffer B for 30 min at a flow rate of 1 ml/min. The fraction R4 was separated on a Bio-Bond C18 column (250 mm × 4.6 mm, particle 3 μm, DIKMA). The fraction eluted using linear gradients from 70% buffer A (0.1% TFA/H_2O) + 30% buffer B (0.1% TFA/acetonitrile) to 65% buffer A (0.1% TFA/H_2O) + 35% buffer B (0.1% TFA/acetonitrile) for 45 min at a flow rate of 1 ml/min was lyophilized and stored at −20°C. The peptides were monitored by measuring the absorbance at 214 nm online.

Molecular mass of a pure anti-diabetic peptide

The pure peptide was analyzed using 16% Tricine-SDS-PAGE. The gel was stained with Coomassie Brilliant Blue after electrophoresis. The molecular mass of the isolated peptide was determined determine by comparing its electrophoretic mobility with those of low-molecular-weight marker proteins (SANGON, China).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) was also employed to determine the molecular weight of the purified peptide using an Applied Biosystems 4700 Proteomics Analyzer.

N-terminal amino acid sequence analysis

The pure peptide obtained from the Bio-Bond C18 column was subjected to amino acid sequence analysis. The freeze-dried powdered peptide was dissolved in 0.5 ml of phosphate buffered saline (2 mg/ml) and loaded onto the column supplied by Hewlett Packard for N terminal amino acid sequence analysis. The column was then analyzed using a Hewlett Packard 1000A protein sequencer equipped with an HPLC system (Wong and Ng 2003).

Animals and experimental design

The study was conducted on six-week-old C57BL/6J mice (Yangzhou University Laboratory Animal Center at Jiangsu, China). C57BL/6J mice are the most widely used inbred strain and also susceptible to streptozotocin-induced diabetes. The mice were housed in a room maintained at 24°C to 26°C with a normal day/night cycle. The mice were provided with a basal diet and free access to drinking water.

The animals were allowed to acclimatize to the laboratory environment for 1week and then randomly divided into six groups (n = 6 mice per group) as follows: untreated (control); streptozotocin-induced diabetic mice receiving physiologic saline via intraperitoneal injection (diabetic); streptozotocin-induced diabetic mice receiving metformin at 200 mg/kg body weight (diabetic + MET); and streptozotocin-induced diabetic mice treated with PRDA at 75, 150, and 300 μg/kg body weight, respectively, via intraperitoneal injection (diabetic + LD, diabetic + MD and diabetic + HD, respectively). LD, MD and HD indicated low dose, medium dose and high dose, respectively.

The doses of 75, 150, and 300 μg/kg body weight were chosen carefully after reviewing available literature and conducting preliminary experiments. There are very limited reports of anti-diabetic action of fractions from red deer antler. To choice appropriate doses, we could only reference the dose used by other hypoglycemic peptides from natural resources (Xue et al. 2010; Hou et al. 2013), 10 μg/kg body weight initially. But the fact was that PRDA failed to decrease the blood glucose of diabetic mice. Then, we increased the dosage progressively by 2-fold, 3.3-fold and 5-fold. A slightly hypoglycemic effect was observed only at the dose of 50 μg/kg body

weight. So, we enhanced the dose by 50%, 75 μg/kg body weight. The result in small pilot study indicated that PRDA decreased blood glucose significantly. We felt that a dose of 75 μg/kg body weight was the lowest effective dose. Then, 75, 150, and 300 μg/kg body weight provided in pre-test leaded to hypoglycemic effect significantly in diabetic groups in a dose-dependent manner.

The mice were fasted overnight and then injected intraperitoneally with streptozotocin (Sigma, USA) at 45 mg/kg body weight for five days. The streptozotocin was injected within 10 min after dissolving in 10 mM sodium citrate at pH 4.5. The control mice were injected with sodium citrate buffer as the vehicle control. Blood samples were collected from the retro-orbital plexus of each mouse under mild anesthesia at 1week after streptozotocin administration. Fasting blood glucose (FBG) was estimated using a glucose estimation kit. Mice with glucose levels exceeding 16.5 mM were included in the study as stable hyperglycemic animals. When stable hyperglycemia was achieved, the animals were treated once daily for 6 weeks. The PRDA were dissolved in saline and sterilized by filtration, and the dosages of PRDA at 75, 150, and 300 μg/kg body weight were determined by peptide content.

All procedures were performed in accordance with the animal welfare legislation of Jiangsu Province in China (Jiangsu Provincial People's Government Order No. 45) and were approved by the Experimental Animal Ethical Committee of China Pharmaceutical University.

Biochemical Parameters

An oral glucose tolerance test (OGTT) was performed to assess the insulin activity at 24 hours before the termination of the experiment. The fasted overnight mice were fed glucose (2 g/kg). Blood was collected from the orbital sinus of alternate eyes at 0, 15, 30, 60, and 120 min after glucose administration for glucose estimation.

Upon completion of the treatment, blood samples were collected for biochemical estimation. The fasting serum insulin content was obtained using an ELISA kit. The fasting blood glucose level was measured using a glucose estimation kit.

The total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and non-esterified fatty acids (NEFA) levels in the serum were determined using kits according to the instructions of the manufacturer.

Superoxide dismutase (SOD) activity and catalase (CAT) activity, the total antioxidant capacity (T-AOC), and malondialdehyde (MDA) content in the serum and liver were determined using commercially available kits.

Excised liver homogenate was prepared in normal saline. The homogenate was centrifuged at 2,500 rpm, and the resulting supernatant was collected to measure hexokinase (HK) activity and pyruvate kinase (PK) activity using commercially available kits. Enzyme activity was expressed as units per gram of protein in the homogenate.

Cell culture

RINm5f rat insulinoma cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 200 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified 5% CO_2 incubator. Upon reaching 80% confluence, the cells were digested with 0.25% trypsin and were passaged at a ratio of 1:4 every three days.

Promoting islet cell proliferation of the pure anti-diabetic peptide

The cells that grew well were mixed thoroughly to form a single cell suspension and were plated at a density of 2×10^4 cells per well on 96-well plates. The cells then were treated with or without the pure anti-diabetic peptide at a dose up to 200 nM for 48 hours. Exendin-4 (Ex-4), a peptide agonist of the glucagon-like peptide (GLP) receptor, at a dose of 20 nM was used as the positive control. Cell proliferation was measured using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as previously described (Shen et al. 2011).

Recovery effect on streptozotocin-damaged islet cells of the pure *anti-diabetic peptide*

Some of the cells were treated with 4 mM streptozotocin for 2 hours before anti-diabetic peptide treatment. The cells were then incubated in the presence or absence of the pure anti-diabetic peptide concentrations at a dose up to 200 nM for 48 hours. The positive control was the same as above and cell proliferation was measured by the MTT assay as previously mentioned.

Statistical analysis

The values are presented as means ± SEM. The statistical significance was evaluated by one-way ANOVA using the statistical software Origin 8.0 (Origin Lab Corporation, Northampton, MA, USA).

Results

Tricine-SDS-PAGE analysis of the PRDA

The Tricine-SDS-PAGE analysis of the PRDA is shown in Fig. 1. Adding an equal volume of ethanol precipitated most of the macromolecular proteins (Fig. 1, lane 6). The total peptides were successively ultra-filtered using membranes with nominal molecular weight cut-offs (MWCO) of 5-10 kDa to obtain peptides with molecular mass ranging from 5 kDa to10 kDa. The > 10-kDa fraction

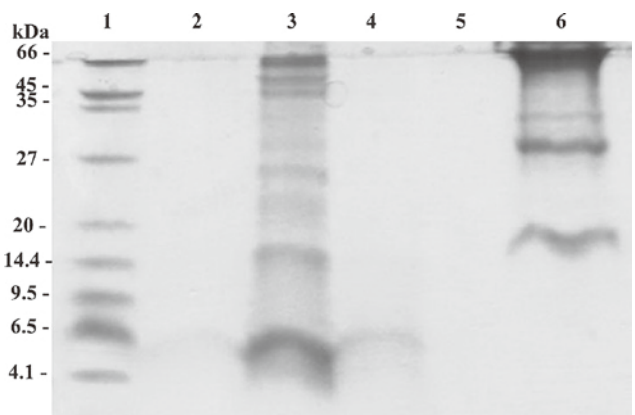

Fig. 1. Tricine-SDS-PAGE analysis of proteins from red deer antlers.
The proteins from red deer antlers were analyzed by Tricine-SDS-PAGE. The lanes are as follows: lane 1, low-range molecular weight standards; lane 2, samples of total peptides; lane 3, proteins or peptides of > 10 kDa; lane 4, peptides of 5-10 kDa; lane 5, peptides of < 5 kDa; and lane 6, peptides precipitated with ethanol. The gel was stained with Coomassie Brilliant Blue.

contained macromolecular bands, which also contained the band with a molecular mass of about 6.5 kDa (Fig. 1, lane 3). The 5-10-kDa peptides (Fig. 1, lane 4) showed a single band with a molecular mass of about 6.5 kDa, whereas the < 5-kDa peptides did not show any band (Fig. 1, lane 5). Accordingly, the specific active peptides in the 5-10-kDa fraction of the red deer antler were those with a molecular mass of about 6.5 kDa as shown in lane 4.

Purification

Four fractions were collected after CM-Sepharose chromatography, which were performed using 50, 100, 150, and 200 mM NaCl. The anti-diabetic activity was recovered in the 150 mM NaCl fraction (CMP). The CMP was subsequently purified via gel filtration on a Superdex 30 column. The anti-diabetic peptide was located in the main fraction, which was subsequently separated via RP-HPLC on a SOURCE 15RPC Column. The fourth of the five fractions (R4) with anti-diabetic activity was purified on a Bio-Bond C18 column. The purified anti-diabetic peptide was located in the main fraction.

Molecular weight of anti-diabetic peptide

The molecular weight of anti-diabetic peptide was determined via Tricine gel electrophoresis. The peptide showed a single band at about 6.5 kDa (Fig. 2, inset). Using mass spectrometry, the molecular weight of anti-diabetic peptide was determined to be 7,064.8 Da (Fig. 2).

N-terminal amino acid sequence

The N-terminal amino acid sequence of the peptide was LSPFTTKTYFPHFDLSHGSA. Comparison of this sequence with peptides in the database revealed no exact protein match.

Fasting blood glucose levels and OGTT

The fasting blood glucose levels diabetic mice were significantly elevated by 171.2 ± 11.1% ($p < 0.01$); however, after the 6-week PRDA (75-300 μg/kg BW) treatment, the increased blood glucose was significantly ($p < 0.05$) reduced by 6.6 ± 5.8, 18.2 ± 5.8 and 32.6 ± 7.3%, respectively (Fig. 3A, FBG data).

In the OGTT, the PRDA-treated groups (75 μg/kg, 150 μg/kg and 300 μg/kg BW) showed a significant increase in rate of clearance of glucose as compared to untreated-diabetic group (Fig. 3A). The area under the curve (AUC) was calculated (Fig. 3B). The result showed that the AUC significantly increased ($p < 0.01$) in the streptozotocin-induced diabetic mice compared with that in the control group. After 6 weeks of PRDA administration, the AUC of the three treatment groups (diabetic + LD, diabetic + MD, and diabetic + HD) significantly decreased compared with that of the diabetic group in a dose-dependent manner ($p < 0.01$).

Fasting serum insulin levels

Fig. 4 revealed the fasting serum insulin levels of all the groups. The insulin concentration of untreated diabetic mice fell significantly ($p < 0.01$) compared to the non-diabetic control. After the treatment of PRDA, the levels increased significantly in a dose-dependent manner ($p < 0.05$).

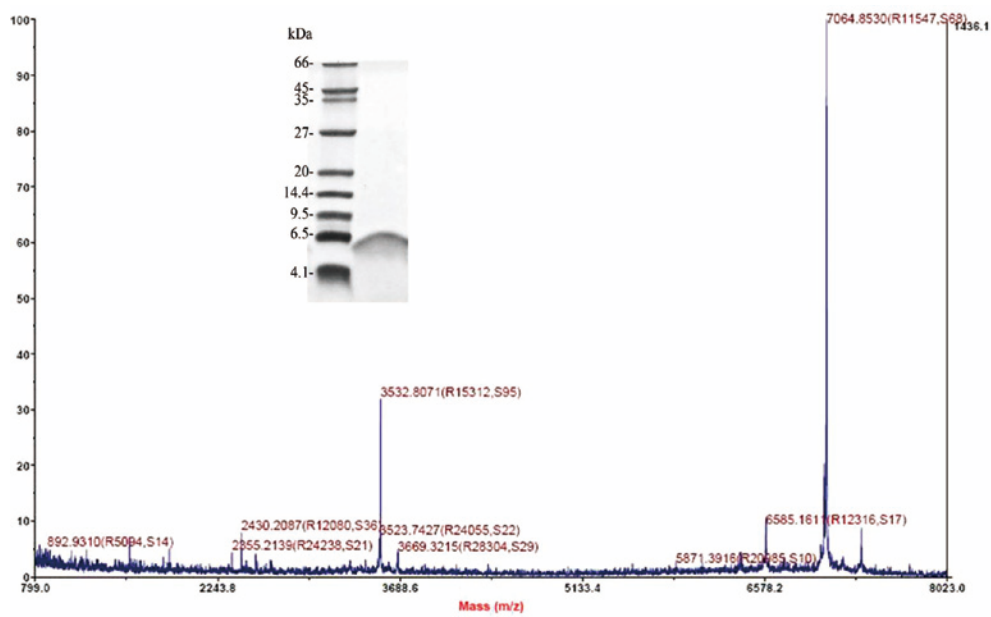

Fig. 2. Molecular mass determination of the pure anti-diabetic peptide.
Molecular mass determination of the anti-diabetic peptide via matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). Inset: SDS-Tricine gel electrophoresis for molecular weight determination of isolated peptide (stained with Coomassie Brilliant Blue).

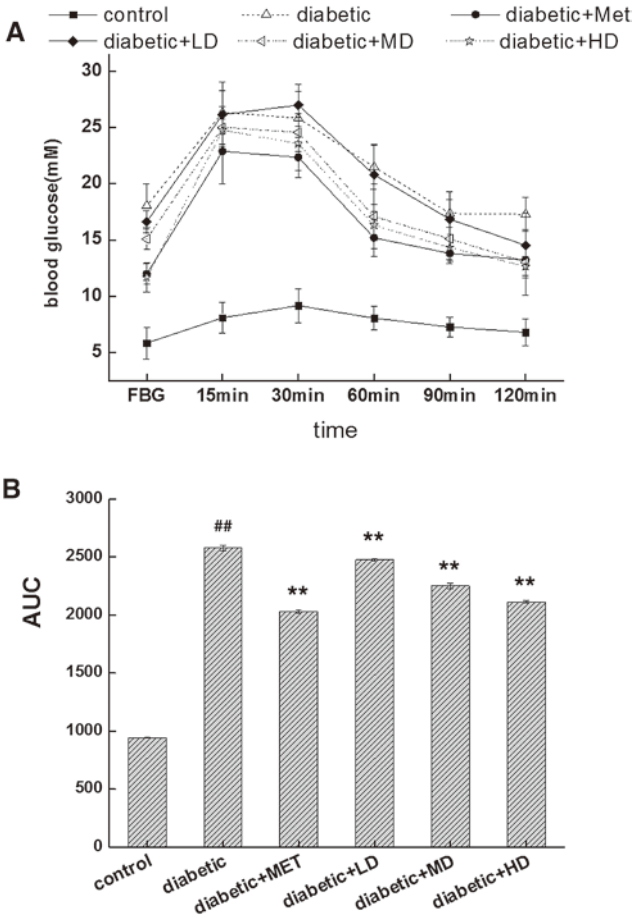

Fig. 3. Effects of PRDA on oral glucose tolerance test and area under the curve.
(A) Oral glucose tolerance test (OGTT) and (B) Area under the curve (AUC) of diabetic mice after treated with or without PRDA for 6 weeks and control mice. Data are expressed as means ± SEM; $n = 6$ (*$p < 0.05$ and **$p < 0.01$, compared with untreated diabetic mice; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, compared with the control). LD, MD and HD indicate low dose, medium dose and high dose of PRDA, respectively. FBG, fasting blood glucose.

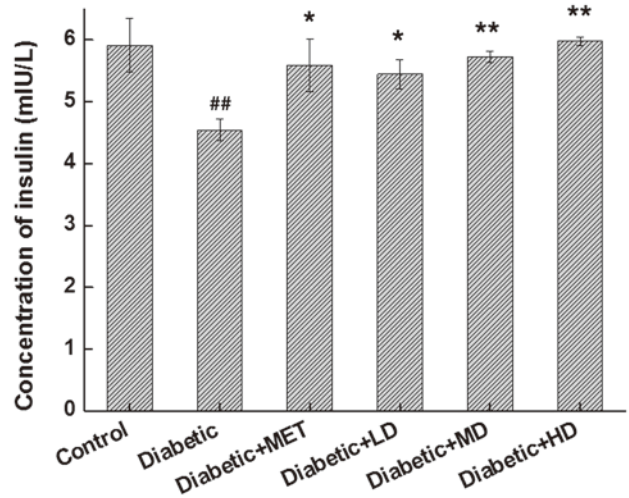

Fig. 4. Effects of PRDA on fasting serum insulin concentrations.
Fasting serum insulin concentrations of diabetic mice after treated with or without PRDA for 6 weeks and control mice. Data are expressed as means ± SEM; $n = 6$ (*$p < 0.05$ and **$p < 0.01$, compared with untreated diabetic mice; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, compared with the control). LD, MD and HD indicate low dose, medium dose and high dose of PRDA, respectively.

Estimation of lipid profile

The plasma TC, TG, HDL-C, and LDL-C levels of the mice are presented in Table 1. Compared to control mice, TC, LDL-C, and NEFA levels of untreated diabetic animal group were significantly increased ($p < 0.01$) (159.3 ± 14.8, 281.8 ± 14.4 and 69.4 ± 1.7%, respectively) and HDL-C levels decreased (64.8 ± 2.2%, $p < 0.01$). The low dose tested (75 μg/kg) caused a slight increase of by 12.9 ± 3.0% ($p > 0.05$) and PRDA at the doses of 150 and 300 μg/kg enhanced TC level by 20.0 ± 4.3 and 45.4 ± 1.0%, respectively ($p < 0.01$). The LDL and NEFA levels were also significantly decreased ($p < 0.01$), whereas the HDL-C levels were increased in the PRDA-treated animals in a dose-dependent manner. No change in TG was observed among the six groups.

Table 1. Effects of PRDA on lipid profiles in C57BL/6J mice.

group	Plasma lipid level (mg/dl)				
	TC	TG	LDL-C	HDL-C	NEFA
control	130.3 ± 8.0	77.6 ± 3.9	62.5 ± 1.1	91.6 ± 20.0	17.0 ± 2.5
diabetic	337.9 ± 19.3$^{\#\#}$	80.9 ± 4.1	238.6 ± 9.0$^{\#\#}$	32.2 ± 2.0$^{\#\#}$	28.8 ± 0.3$^{\#\#}$
diabetic + MET	172.7 ± 8.5**	79.3 ± 4.9	93.1 ± 1.4**	81.8 ± 6.8*	24.3 ± 3.9
diabetic + LD	294.2 ± 10.3	81.1 ± 5.6	196.8 ± 6.7**	32.0 ± 6.2	19.8 ± 2.0**
diabetic + MD	270.3 ± 14.6**	80.6 ± 7.9	173.3 ± 1.6**	66.0 ± 7.0**	18.6 ± 1.4**
diabetic + HD	184.6 ± 3.3**	72.2 ± 2.9	110.8 ± 2.2**	106.4 ± 13.9**	17.4 ± 1.2**

Each value represents the mean ± SEM, $n = 6$. * and ** represent $p < 0.05$ and $p < 0.01$ compared to untreated-diabetic mice, respectively. $^{\#\#}$ indicates $p < 0.01$ compared to control mice. LD, MD and HD indicate respectively low dose, medium dose and high dose of PRDA.

Table 2. Effects of PRDA on SOD, CAT, MDA and T-AOC levels in liver and serum of C57BL/6J mice.

group	control	diabetic	diabetic + MET	diabetic + LD	diabetic + MD	diabetic + HD
liver						
SOD (U/mg prot)	79.18 ± 4.94	58.38 ± 1.26$^{\#\#}$	64.36 ± 1.95	61.03 ± 0.47	71.57 ± 5.01*	80.79 ± 1.05**
CAT (U/mg prot)	40.85 ± 2.44	18.67 ± 0.76$^{\#\#}$	26.46 ± 1.06*	22.37 ± 1.18	28.06 ± 2.96*	33.83 ± 6.69**
MDA (nM/mg prot)	0.08 ± 0.002	0.15 ± 0.013$^{\#\#}$	0.07 ± 0.002**	0.11 ± 0.005*	0.09 ± 0.003**	0.07 ± 0.008**
T-AOC (U/mg prot)	1.51 ± 0.12	0.98 ± 0.09$^{\#}$	1.04 ± 0.08	0.84 ± 0.07	1.39 ± 0.25	1.63 ± 0.25*
serum						
SOD (U/ml)	95.02 ± 4.71	81.04 ± 1.03$^{\#\#}$	82.81 ± 5.38	86.70 ± 0.68	87.09 ± 0.87	92.03 ±1.90*
CAT (U/ml)	32.82 ± 0.67	28.45 ± 1.58	58.81 ± 3.81**	46.86 ± 1.42**	41.32 ± 0.20*	38.33 ± 0.45*
MDA (nM)	7.02 ± 0.22	8.62 ± 0.39$^{\#}$	6.83 ± 0.37*	8.07 ± 0.61	4.97 ± 1.02**	3.97 ± 0.38**
T-AOC (U/ml)	6.97 ± 1.46	4.48 ± 0.24$^{\#}$	5.77 ± 0.95	5.59 ± 0.96	6.08 ± 0.61**	6.39 ± 0.83*

Each value represents the mean ± SEM, $n = 6$. * and ** represent $p < 0.05$ and $p < 0.01$ compared to untreated-diabetic mice, respectively. $^{\#}$ and $^{\#\#}$ indicate $p < 0.05$ and $p < 0.01$ compared to control mice, respectively. LD, MD and HD indicate respectively low dose, medium dose and high dose of PRDA.

Antioxidant and lipid peroxidation parameter

The SOD activity, CAT activity, the T-AOC level, and the MDA content in the serum and liver of mice are illustrated in Table 2. The diabetic group had significantly increased MDA content ($p < 0.01$) and significantly decreased CAT activity ($p < 0.01$), SOD activity ($p < 0.01$) and T-AOC ($p < 0.05$) in liver compared to the control group. PRDA administration (75, 150, and 300 μg/kg BW) for 6 weeks increased the SOD activity, CAT activity, as well as T-AOC of the diabetic group and decreased their MDA level in a dose-dependent manner. The serum SOD activity and T-AOC level of the diabetic mice were significantly decreased, whereas the MDA concentration was increased compared with those of the control mice. However, PRDA treatment reversed the deteriorations in serum levels.

Hepatic hexokinase and pyruvate kinase activity

6 weeks after administrations of 150, 300 μg/kg, the hepatic HK activity was increased significantly by 134.7 ± 33.3% ($p < 0.05$), and 322.8 ± 51.7% ($p < 0.01$) compared to the untreated streptozotocin-induced diabetic mice (Fig. 5A), respectively. The liver PK activity of diabetic mice was significantly decreased by 79.7 ± 0.7% ($p < 0.01$) compared with that of the non-diabetic control. However, treatment with PRDA at 300 μg/kg BW significantly increased the PK activity compared to the diabetic group (101.9 ± 38.6%, $p < 0.05$) (Fig. 5B).

Promoting islet cells proliferation and recovery activities of pure anti-diabetic peptide

The islet cell proliferation activity of pure anti-diabetic peptide was determined (Fig. 6A). The anti-diabetic peptide promoted the cell proliferation significantly in a dose-related manner. In the three anti-diabetic peptide-treated groups, the islet cell viability increased by 4.3 ± 1.7, 5.9 ± 1.1%, and 9.6 ± 0.7% ($p < 0.01$), respectively compared with that of control cells (untreated cells). In islet cells recovery assay (Fig. 6B), the proliferation rate of streptozotocin-damaged cells reduced by 54.7 ± 0.3% ($p < 0.01$) compared to control cells. After treated with anti-diabetic peptide for 48 hours, the cells viability increased by 17.9 ± 8.0, 23.4 ± 7.1 and 76.2 ± 6.8%, respectively, compared to the model cells (streptozotocin-induced cells).

Discussion

A number of extracts from nature sources have exhibited hypoglycemic effects (Ansarullah et al. 2010; Ashok Kumar et al. 2012). The present study reports the hypoglycemic, hypolipidemic and antioxidant properties of PRDA, a 5-10 kDa fraction from red deer antler *in vivo*, as well as the abilities of the novel anti-diabetic peptide from PRDA to promote the proliferation and recovery of islet cells for the first time.

DM is defined as a group of metabolic diseases with elevated blood glucose levels (hyperglycemia) (Romero-Aroca et al. 2011). The fasting blood glucose level is an important basal parameter for monitoring diabetes (Makni et al. 2010). In the present study, the fasting blood glucose levels of streptozotocin-induced mice treated by PRDA were significantly lower than those of the diabetic mice. PRDA also significantly decreased the blood glucose level in glucose-loaded mice (OGTT) and AUC. The hypoglycemic activities of PRDA in streptozotocin-induced diabetic mice may partially be attributed to increased plasma insulin level; namely, the plasma insulin levels were elevated in PRDA-treated diabetic mice.

DM is commonly associated with dyslipidemia and characterized by increased TC, TG, and LDL-C, as well as decreased HDL-C, which represents a synergistic risk factor for cardiovascular disease (Kannel et al. 1971). The present results showed that PRDA decreased the serum TC and LDL-C levels and increased the HDL-C concentration in diabetic mice. Although PRDA did not affect the TG levels, the NEFA levels of the PRDA-treated mice decreased in a dose-dependent manner compared with that

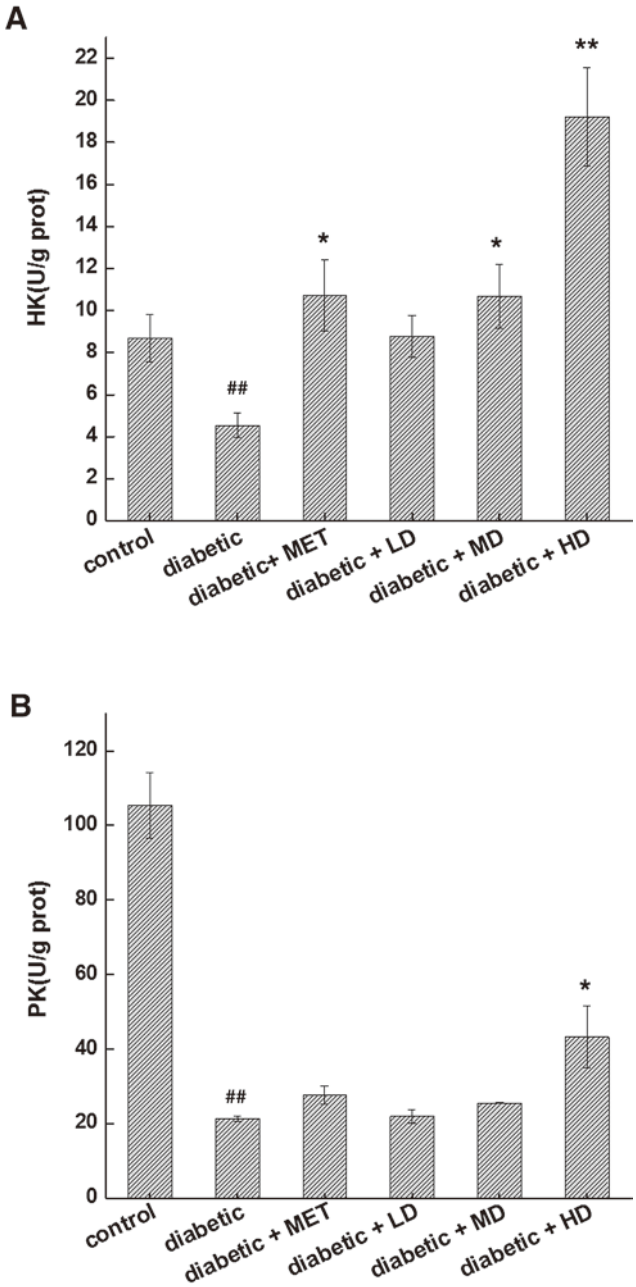

Fig. 5. Effects of PRDA on hepatic hexokinase activity and pyruvate kinase activity in streptozotocin-induced diabetic mice.
(A) Hepatic hexokinase activity and (B) hepatic pyruvate kinase activity of diabetic mice after treated with or without PRDA for 6 weeks and control mice. Data are expressed as means ± SEM; $n = 6$ ($^{*}p < 0.05$ and $^{**}p < 0.01$, compared with untreated diabetic mice; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, compared with the control). LD, MD and HD indicate low dose, medium dose and high dose of PRDA, respectively.

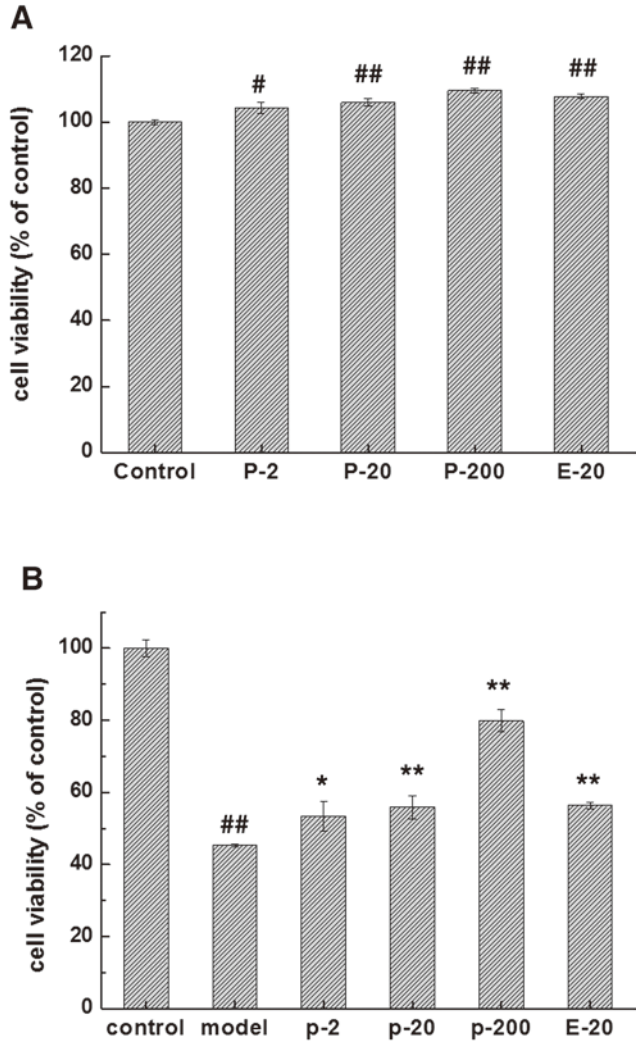

Fig. 6. Effects of the pure anti-diabetic peptide on RINm5f cell proliferation and recovery.
(A) RINm5f cell viability (% control) assay. RINm5f cells were treated with or without pour anti-diabetic peptide for 48 hours and cells viabilities (% control) were measured by MTT. (B) Streptozotocin-injured RINm5f cell viability (% control) assay. RINm5f cells were treated with or without pure anti-diabetic peptide for 48 hours after damaged by 4 mM streptozotocin for 2 hours, and cells viabilities (% control) were measured by MTT. Data are expressed as means ± SEM; $n = 5$ ($^{*}p < 0.05$ and $^{**}p < 0.01$, compared with the model; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, compared with the control). Control: β-cells treated without streptozotocin or anti-diabetic peptide; model: β-cells treated with streptozotocin; p-2, p-20, and p-200: β-cells injured by streptozotocin treated with anti-diabetic peptide at 2, 20, and 200 nM, respectively; E-20: β cells injured by streptozotocin treated with 20 nM exendin-4.

of the diabetic animals. Considering the increase in NEFA produced by TG breakdown from peripheral tissues provokes insulin resistance (Krebs and Roden 2005; Boden 2006), the PRDA probably improved fat storage in the peripheral tissue and alleviated insulin resistance.

Oxidative stress is an important factor in the etiology and pathogenesis of diabetes and it has been implicated in the development and progression of diabetic complications. Our data revealed a significant elevation in the MDA content in the liver and serum of diabetic mice. The increased

MDA content suggests decreased enzymatic and non-enzymatic antioxidants. Consistent with the MDA data, the untreated diabetic mice had decreased serum and liver SOD activity, CAT activity, and T-AOC. By contrast, the oxidation was reversed in the treatment groups compared with the diabetic mice. The results demonstrate that the PRDA may have antioxidant activity by acting on enzymatic and non-enzymatic antioxidants, which may be responsible for its hypoglycemic and hypolipidemic properties.

HK and PK play essential roles in the glucose metabolism of cells (Ritov and Kelley 2001; Wang et al. 2002). Impaired HK and PK activity contributes to the markedly decreased insulin levels in streptozotocin-induced diabetic animals (Sundaram et al. 2013) and the exaltation of HK and PK activities of diabetic mice treated with PRDA is due to increased insulin levels. Assuredly, the anti-diabetic activity of PRDA is found currently only in mice and further studies on clinical trials need to be implemented.

Peptides are attractive drug candidates because of their potent biological activities as well as high target specificities. Several peptides with hypoglycemic action from natural materials have been reported (Jang et al. 2010; Lu et al. 2012). Islet β cell dysfunction might be a key to the development of DM (Marchetti et al. 2008). Therefore, the therapeutic agents that can halt or prevent pancreatic β-cell failure will likely have a major impact on disease progression. Previous studies were successful on isolation of novel peptides with the activity of preserving pancreatic β-cell mass from nature materials. For example, exendin-4, isolated from *Heloderma suspectum* Venom (Eng et al. 1992), promotes the proliferation of β-cell and stimulates insulin secretion and has been used in DM2 clinical therapy. Antlers are rich in peptides, which are considered the main active ingredients. So, it is feasible to develop novel peptides from PRDA with the action of preserving pancreatic β-cell mass by stimulating β-cell proliferation. In present study, according to the protein and peptide content of PRDA lyophilized powder and Tricine-SDS-PAGE analysis, we hypothesized that the anti-diabetic ingredients of PRDA were peptides with molecular weight of about 6.5kDa, which was proved when a novel 7064.8Da peptide isolated from PRDA promoted islet cell proliferation and recovery. The difference between 6.5 kDa and 7064.8 Da lies in different measurement methods. The precision of electrophoretic measurement of molecular weight is kDa with an error of approximately ± 10%, while the accurate molecular weight can be obtained via MALDI-TOF MS, by which the error is less than ± 1 Dalton. In this article, the molecular mass of the peptide was shown as 6.5 kDa when it was detected with electrophoresis, and 7064.8Da was used to represent the molecular mass when it was determined by MALDI-TOF MS. Although the anti-diabetic effect of the peptide *in vivo* needs to be further confirmed, we established the β-cell model damaged by streptozotocin to simulate the experimental diabetic animal, and the peptide showed the activity of promoting damaged-cells recovery. All of these confirm the truth that the anti-diabetic peptide is one of the active ingredients of PRDA. Therefore, we consider the peptide as a potential treatment for diabetes. It is warranted to make a thorough research on the sequence and mechanism of anti-diabetic peptide.

Acknowledgments

Authors are thankful to Hua Shidan Pharmaceutical Co., Ltd in Xinjiang, China for providing dry red deer antler.

The work was supported by The Ministry of Science and Technology of the People's Republic of China [Science and Technology Supporting Xinjiang Foundation (No. 2008ZJ14), Scientific and Technical Personnel Servicing Enterprise Foundation (No. 2009GJG40009)].

Conflict of Interest

The authors declare no conflict of interest.

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