An *In Vitro* and *In Vivo* Study of the α -Amylase Activity of Phaseolamin

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ABSTRACT We evaluated the polypeptide profiles, inhibition of human salivary α -amylase activity, and hemagglutination properties of a commercial phaseolamin sample. We also performed an *in vivo* assay to investigate the effects of a commercial phaseolamin treatment (100, 500, or 1500 mg/kg) over 20 days on the glycemia, body weight, and serum biochemical parameters (total cholesterol, triglycerides, alanine aminotransferase, and aspartate aminotransferase) of nondiabetic and streptozotocin-induced diabetic rats. The *in vitro* evaluation showed defined protein profiles, low hemagglutination activity, and high α -amylase inhibition. None of the experimental groups treated with phaseolamin or acarbose showed decreases in body weight. Our data demonstrate that phaseolamin inhibits amylase activity *in vitro*, reduces blood glucose levels, decreases or attenuates some of the renal and hepatic effects of diabetes in streptozotocin-induced rats, and could therefore have therapeutic potential in the treatment or prevention of the complications of diabetes.

KEY WORDS: • carbohydrate blockers • diabetes • Phaseolus • serum biochemistry • streptozotocin

INTRODUCTION

DIABETES MELLITUS IS A CHRONIC DISEASE caused by hyperglycemia resulting from the underproduction or ineffective use of pancreatic insulin. According to the World Health Organization, more than 220 million people worldwide have diabetes.^{1,2} Studies on α -amylase inhibition (α -1,4-D-glucan-4-glucanohydrolases, EC 3.2.1.1) have suggested that new drugs can reduce postprandial hyperglycemia by delaying carbohydrate digestion (starch blockers) and thereby decreasing intestinal glucose absorption. This type of inhibitor can be used to treat type 2 diabetes mellitus and obesity.^{3–6} Phaseolamin is a glycoprotein that was characterized in 1975, is present in white beans and kidney beans (*Phaseolus vulgaris*), and contains an α -amylase inhibitor termed α -AI.⁷ Moreover, phaseolamin is used as a dietary supplement and is present in pharmaceutical products marketed as protein concentrates for weight management.^{5,8,9}

Several studies have shown that some commercially available amylase inhibitor extracts fail to influence starch digestion due to low α -amylase inhibition in humans.^{8,10–14} The ability of commercial amylase inhibitors to prevent starch metabolism is influenced by product extraction and manufacturing, which impacts anti-amylase activity. The aim of this study was to evaluate the polypeptide profile, human α -amylase inhibitory activity, and hemagglutination

activity of a commercial phaseolamin sample *in vitro*. Additionally, the subchronic effects of this sample were evaluated *in vivo* on the body weight, serum glucose level, lipid level, enzyme activity, and nitrogen metabolism of streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Preparation of the commercial phaseolamin sample

A commercial phaseolamin sample was acquired from a pharmacy (Uberlandia, MG, Brazil) as commercially prepared white bean extract. This extract was solubilized to a final concentration of 10 mg/mL in 0.15 M NaCl by stirring for 3 h at 20°C. The extract was then centrifuged (1000 g for 15 min) at 4°C, and the resulting supernatant was used in the *in vitro* assays. Protein content in the supernatant was estimated using a Bradford assay¹⁵ and analyzed on a 5–22% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).¹⁶

Hemagglutination activity

The hemagglutination assay was conducted in U-bottom microwell plates. The results were expressed in hemagglutination units (HU) per 50 μ L of sample using the previously described method.¹⁷

Inhibition of human α -amylase in vitro

The commercial phaseolamin extract was analyzed for inhibition of α -amylase activity using a 2-chloro-4-nitrophenyl-4- β -D-galactopyranosylmaltoside substrate (GalG2CNP;

Manuscript received 28 March 2013. Revision accepted 8 January 2014.

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Sorachim S.A., Lausanne, Switzerland) and salivary α -amylase enriched fraction. Human saliva samples were collected from five individuals and stored at -20°C for 48 h. The samples were thawed and centrifuged at 12,000 g for 10 min at 20°C . The supernatant was fractionated in a Q-Sepharose fast flow (Pharmacia Biotech, Uppsala, Sweden) column with a 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 10 mM EGTA buffer. The volume excluded from the Q-Sepharose column was dialyzed three times in ammonium bicarbonate buffer (pH 7.0), lyophilized, and solubilized in phosphate-buffered saline (PBS) at pH 7.2. This α -amylase enriched fraction was diluted 595-fold in 50 mM 2-(N-morpholino) ethanesulfonic acid buffer (Sigma-Aldrich, St. Louis, MO, USA) containing 5 mM CaCl_2 , 300 mM NaCl (pH 6.0). The commercial phaseolamin sample was then added (1:10 v/v) and incubated for 30 min. The reaction was initiated by adding 300 μL of 2 mM substrate. Increases in absorbance (i.e., CNP release) were measured every minute at 37°C and at a wavelength of 405 nm (Microplate reader, Molecular Devices, Sunnyvale, CA, USA). All assays were performed in triplicate.

Animals

Male Wistar rats (180–220 g) were used for the animal studies and housed under standard conditions ($22 \pm 1^{\circ}\text{C}$, humidity $60 \pm 5\%$, and a 12 h light:12 h dark cycle). The animals were fed a commercially available pellet diet (65.82% carbohydrate, 5.36% fiber, 21.0% protein, and 4.96% fat; Bio base, SC, Brazil) and received water *ad libitum*. All procedures for handling and euthanizing the animals were approved by the Ethics Committee on Animal Research of the Federal University of Uberlandia, Brazil (CEUA/UFU 051/08).

Induction of diabetes mellitus

The rats were allowed to acclimatize in their environment for one week and were then fasted for 24 h. The animals were anesthetized intraperitoneally with xylazine/ketamine (1:1 v/v) and then 40 mg/kg streptozotocin (Sigma-Aldrich) was freshly dissolved in 0.01 M citrate buffer (pH 4.5, injection volume of 2 mL/kg) and injected into the penile vein. The animals were fasted for another 90 min after the injection. Ten days after the streptozotocin injection, the rats with fasting blood glucose levels greater than 200 mg/dL were selected for subsequent experiments.

Experimental design

The animals were randomly divided into 10 groups (six rats each) with the following treatments: rats in an untreated nondiabetic control received 1 mL of distilled water (group 1: NDC); groups of nondiabetic rats treated with a commercial phaseolamin sample (100, 500, or 1500 mg/kg; groups 2–4: ND100, ND500, and ND1500 respectively); nondiabetic rats treated with acarbose (25 mg/kg) in an aqueous solution (group 5: NDA); rats in an untreated diabetic control received 1 mL of distilled water (group 6: DC);

groups of diabetic rats treated with a commercial phaseolamin sample (100, 500, or 1500 mg/kg; groups 7–9: D100, D500, and D1500 respectively); and diabetic rats treated with acarbose (25 mg/kg) in an aqueous solution (group 10: DA). All groups were treated daily for 20 days. The commercial phaseolamin sample was diluted in filtered water and administered by gavage each afternoon. The animals were observed continuously for 2 h on the first day and then every 24 h for up to 20 days. The examinations identified any physical signs of toxicity (writhing, hypnosis, dyspnea, and mortality) and measured body weight and blood glucose levels (Biocheck Glucose Test Strips; Bioeasy, MG, Brazil).

Serum biochemical parameters

After 20 days of treatment, the animals were fasted for 12 h and blood samples were collected from the portal vein via the laparotomy method. Total cholesterol, triglycerides, urea, creatinine, alkaline phosphatase, gamma-glutamyl-transferase, aspartate amino transferase, and alanine amino transferase levels were determined from serum samples. All of the parameters were measured at the Clinical Analysis Laboratory of the Faculty of Veterinary Medicine, Federal University of Uberlandia (ChemWell Automated Analyzer, Awareness Technology, Inc., Palm City, FL, USA) via colorimetric methods using commercial kits (Labtest Diagnostica, MG, Brazil).

Statistical analysis

The data were analyzed using Student's *t*-test ($n=6$) using SigmaStat v3.5 software (Systat Software, Inc., IL, USA) and expressed as means $\pm$ standard error of the mean (SEM). A *P*-value $< .05$ was considered significant.

RESULTS

Protein profile

An SDS-PAGE protein profile showed that the commercial phaseolamin sample contained a large number of polypeptides (Fig. 1). These polypeptides profile showed bands that may indicate the presence of proteins such as α -AI-1 (16–11 kDa), phytohemagglutinin (35–25 kDa), and phaseolin, a major vacuolar storage glycoprotein of the common bean (50–35 kDa).

Hemagglutination assay and *in vitro* inhibition of human α -amylase

The commercial phaseolamin sample exhibited low hemagglutination activity with 8 HU/50 μL for human type A+ erythrocytes and 4 HU/50 μL for human type B+, AB+, and O+ erythrocytes. The *in vitro* assay showed that the commercial phaseolamin sample inhibited α -amylase activity by 99%.

Toxicity

The rats treated with 100, 500, and 1500 mg/kg of the commercial phaseolamin sample did not show physical

FIG. 1. Protein profile of a commercial phaseolamin sample (phaseolamin, Ph). It is possible that polypeptides present between 50–35 kDa, 35–25 kDa, and 15–10 kDa correspond to phaseolin, phytohemagglutinin, and α AI-1 (α -amylase inhibitor-1) subunits respectively. A molecular weight marker (MW) was included (225–10 kDa).

signs of extract-induced toxicity, and there were no treatment-related deaths. Additionally, there were no symptoms of starch accumulation in the intestinal tract, such as diarrhea. None of the control group animals died.

Effect on glycemia and body weight

Tables 1 and 2 show the glucose levels and body weights of the nondiabetic and diabetic rats that received the com-

mercial phaseolamin sample. Relative to the control, glucose levels did not decrease ($P < .05$) in any of the nondiabetic treated groups (Table 1). However, glucose levels did decrease ($P < .05$) after the 20 day treatment for diabetic rats receiving 100, 500, or 1500 mg/kg of the commercial phaseolamin sample and the acarbose control (Table 2). Body weight did not decrease in any group after acute administration of the commercial phaseolamin sample.

Serum biochemical parameters after treatment

The effects of commercial phaseolamin treatment on total cholesterol, triglycerides, creatinine, and urea levels in nondiabetic and diabetic rats are listed in Tables 1 and 2 respectively. In nondiabetic rats, 500 and 1500 mg/kg doses decreased triglyceride levels, and the 500 mg/kg dose decreased creatinine levels relative to untreated nondiabetic rats. Additionally, the 100, 500, and 1500 mg/kg doses increased serum urea levels relative to the untreated nondiabetic rats (Table 1). In diabetic rats, the 500 and 1500 mg/kg doses decreased creatinine levels, and all doses reduced urea levels relative to untreated diabetic rats (Table 2). The 100 mg/kg treatment had no effect on creatinine levels in any group.

Tables 1 and 2 show the serum biochemical parameters for alkaline phosphatase (ALP), gamma-glutamyltransferase (γ GT), aspartate amino transferase (AST), and alanine amino transferase (ALT) levels in nondiabetic and diabetic rats. The 100, 500, and 1500 mg/kg doses and acarbose increased ALP, AST, and ALT in nondiabetic rats compared to untreated nondiabetic rats. Nevertheless, commercial phaseolamin treatment did not significantly alter γ GT, cholesterol, and triglycerides levels in diabetic and nondiabetic rats. Moreover, commercial phaseolamin treatment decreased ALT (1500 mg/kg dose) and ALP (all doses) in diabetic rats relative to untreated diabetics. However, the commercial phaseolamin treatment did not alter AST levels in diabetic rats.

DISCUSSION

The SDS-PAGE profiles showed that the commercial phaseolamin sample contained a qualitative similarity in

TABLE 1. BLOOD GLUCOSE LEVEL AND SERUM BIOCHEMICAL PARAMETERS OF NONDIABETIC RATS AFTER 20 DAYS OF TREATMENT

Parameters	NDC	ND100	ND500	ND1500	NDA
Glycemia (mg/dL)	104.12 $\pm$ 5.32	105.37 $\pm$ 4.57	113.00 $\pm$ 4.74	91.12 $\pm$ 6.10	113.12 $\pm$ 9.55
Total cholesterol (mg/dL)	65.28 $\pm$ 4.96	77.20 $\pm$ 3.62	75.55 $\pm$ 3.52	71.77 $\pm$ 3.57	71.90 $\pm$ 3.44
Triglycerides (mg/dL)	59.96 $\pm$ 9.57	45.66 $\pm$ 3.49	31.71 $\pm$ 3.99*	23.20 $\pm$ 2.24*	32.95 $\pm$ 4.72*
Creatinine (mg/dL)	0.62 $\pm$ 0.03	0.50 $\pm$ 0.05	0.50 $\pm$ 0.04*	0.56 $\pm$ 0.04	0.53 $\pm$ 0.04
Urea (mg/dL)	37.26 $\pm$ 1.68	59.22 $\pm$ 3.45*	51.15 $\pm$ 1.79*	51.15 $\pm$ 2.92*	52.28 $\pm$ 3.23*
ALP (U/L)	92.41 $\pm$ 7.45	149.06 $\pm$ 15.16*	124.17 $\pm$ 11.28*	124.17 $\pm$ 23.55*	134.81 $\pm$ 7.18*
γ GT (U/L)	5.38 $\pm$ 1.42	4.35 $\pm$ 0.97	3.11 $\pm$ 1.17	3.38 $\pm$ 0.91	9.13 $\pm$ 2.73
AST (U/L)	74.62 $\pm$ 3.52	105.25 $\pm$ 8.23*	88.00 $\pm$ 5.65	88.25 $\pm$ 4.54*	111.25 $\pm$ 8.07*
ALT (U/L)	36.37 $\pm$ 2.30	83.37 $\pm$ 7.2*	76.75 $\pm$ 3.39*	76.75 $\pm$ 6.06*	73.87 $\pm$ 4.92*

Each value represents mean $\pm$ standard error of the mean (SEM); $n=6$, each group; * $P < .05$ compared with NDC.

ALP, alkaline phosphatase; AST, aspartate amino transferase; ALT, alanine amino transferase; γ GT, gamma-glutamyltransferase; NDC, untreated nondiabetic control; ND100, nondiabetic treated 100 mg/kg dose; ND500, nondiabetic treated 500 mg/kg dose; ND1500, nondiabetic treated 1500 mg/kg dose; NDA, nondiabetic treated acarbose 25 mg/kg dose.

TABLE 2. BLOOD GLUCOSE AND SERUM BIOCHEMICAL PARAMETERS OF DIABETIC RATS AFTER 20 DAYS OF TREATMENT

Parameters	DC	D100	D500	D1500	DA
Glycemia (mg/dL)	568.57 ± 84.47	330.00 ± 85.68*	415.40 ± 53.48*	346.25 ± 69.18*	397.66 ± 39.33*
Total cholesterol (mg/dL)	70.45 ± 4.72	65.90 ± 2.09	65.60 ± 5.91	69.62 ± 3.30	82.68 ± 4.91
Triglycerides (mg/dL)	137.07 ± 40.63	114.53 ± 18.45	125.40 ± 13.42	95.92 ± 22.86	179.23 ± 43.33
Creatinine (mg/dL)	0.87 ± 0.05	0.66 ± 0.07	0.68 ± 0.06*	0.52 ± 0.17*	0.68 ± 0.07
Urea (mg/dL)	77.68 ± 5.59	57.03 ± 5.22*	54.54 ± 4.41*	48.88 ± 6.21*	61.13 ± 6.11
ALP (U/L)	559.32 ± 62.14	318.16 ± 79.77*	319.18 ± 77.68*	217.90 ± 62.05*	359.08 ± 55.53
γGT (U/L)	19.80 ± 9.35	10.11 ± 5.86	9.08 ± 3.42	10.62 ± 3.94	17.21 ± 10.73
AST (U/L)	116.85 ± 11.38	93.33 ± 10.84	120.80 ± 22.96	92.75 ± 13.71	92.00 ± 6.76
ALT (U/L)	93.71 ± 6.19	76.50 ± 10.59	64.40 ± 15.66	59.00 ± 9.24*	78.00 ± 10.94

Each value represents the mean ± SEM; *n* = 6, each group; **P* < .05 compared with DC.

four abundant polypeptides bands of approximately 45, 30, 16, and 11 kDa of previously published results.^{18–20} The polypeptide at 45 kDa may be phaseolin (45–50 kDa).²¹ This polypeptide is the most abundant, representing approximately 80% of total bean protein content.²² The 30 kDa polypeptide is characteristic of bean phytohemagglutinin, and the 16 and 11 kDa polypeptides are typical of α-AI inhibitors.²³

The low hemagglutination activity of the commercial phaseolamin sample in this study is low and consistent with previous reports.^{20,23–25} *In vitro* testing showed that this sample has a high inhibitory effect on amylase activity as also described by Sawada *et al.*²⁶ and Tormo *et al.*¹⁹

Acute treatments with a commercial phaseolamin sample decreased glucose levels in diabetic rats. This result is in agreement with previous studies where diabetic rats were chronically treated with phaseolamin.^{19,20,27} Administration of this commercial sample did not affect body weight. This agrees with previous studies where diabetic animals were given acute and subchronic phaseolamin treatments.^{28,29}

Diabetes is related to various defects in metabolism. Our data show that creatinine, urea, ALP, γGT, AST, and ALT levels increase in untreated diabetic rats compared to untreated nondiabetic rats. Elevated serum urea and creatinine are significant markers of renal dysfunction from diabetic hyperglycemia.³⁰ Serum enzymes such as ALP and ALT are used to evaluate hepatic disorders. An increase in activity of these enzymes reflects active liver damage.^{31,32} Moreover, elevated ALP and γGT levels may indicate cholestatic liver injury.^{33–35} On the other hand, Harikumar *et al.*²⁸ showed that acute and subchronic administration (90 days) of the Phase2® all-natural starch neutralizer (the first standardized product) to nondiabetic rats did not result in mortality or significant toxicity in AST, ALT, and ALP levels. Furthermore, Barrett and Udani³⁶ showed that Phase2 can induce weight loss and reduce spikes in blood sugar caused by carbohydrates through its α-amylase inhibiting activity.

Phaseolamin treatment did not affect total cholesterol and triglycerides levels in diabetic rats. This suggests that phaseolamin does not affect lipid metabolism in diabetic rats. Pereira *et al.*³⁷ found similar results when administering white bean flour to rats over 21 days.

The efficacy of commercial phaseolamin samples depends on the manufacturer's production and extraction

methods. Despite some uncertainty, phaseolamin with appropriate structural and physical-chemical properties has significant capacity *in vivo* to inhibit and mediate these conditions. Recent studies have shown that the α-amylase inhibitor is safe.⁸ There is a wide range of efficacy and safety for commercial “starch blockers” in both humans and animals.^{23,28,38}

It is important to consider the safe administration of phaseolamin because the pancreas is the organ most frequently affected by antinutritional factors present in raw white bean flour. However, the correlation between damage and pancreatic hyperamylasemia must be carefully considered.^{39,40} According to the study by Pereira *et al.*,³⁷ the decline of serum amylase suggests the occurrence of cell damage in the pancreas, although this was not confirmed by histological analysis. On the other hand, α-amylase inhibitors can reduce hyperglycemia and consequent oxidative stress in the pancreas by delaying carbohydrate digestion (starch blockers) and thereby decreasing intestinal glucose absorption. The phaseolamin sample evaluated in this study could be used to protect the pancreas by reducing hyperglycemia and the consequences of oxidative stress.

CONCLUSION

In conclusion, a commercial phaseolamin sample evaluated *in vitro* and *in vivo* exhibited defined protein profiles, low hemagglutination activity, and high α-amylase inhibitory activity. Moreover, phaseolamin treatment reduced blood glucose levels in diabetic rats without affecting body weight. This treatment also decreased or attenuated some renal and hepatic parameters in the serum of streptozotocin-induced diabetic rats. Consequently, phaseolamin could have therapeutic potential in the treatment or prevention of the complications of diabetes. Nevertheless, the effect of phaseolamin on distinct tissues requires further investigation.

ACKNOWLEDGMENTS

This study was funded by the Brazilian Governmental Agencies from the Conselho Nacional de Desenvolvimento Tecnológico (CNPq) and from the Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG) and Ministry of

Health of Brazil (grant number PPSUS- EDT3257/06 to FSE). Fellowships from the Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (PNPD/CAPES) to NMG and from FAPEMIG to FVA and FBF. FSE is a CNPq fellow.

AUTHOR DISCLOSURE STATEMENT

The authors declare that no conflict of interest exists.

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