Hypoglycaemic and anorexigenic activities of an α-amylase inhibitor from white kidney beans (Phaseolus vulgaris) in Wistar rats

M. A. Tormo*, I. Gil-Exojo, A. Romero de Tejada and J. E. Campillo

Department of Physiology, Faculty of Medicine, University of Extremadura, Apartado de Correos 108, 06071 Badajoz, Spain

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An inhibitor of α-amylase was isolated and purified from an extract of white kidney beans (Phaseolus vulgaris). The acute oral administration of the inhibitor (50 mg/kg body weight) to adult Wistar rats together with a starch load (2 g/kg body weight suspended in NaCl (9 g/l)) reduced the increase in glycaemia over the basal value (NaCl, 222 (SEM 49); inhibitor, 145 (SEM 16) mmol/l; P<0·01), a reduced weight gain (NaCl, 52 (SEM 3); inhibitor, 1·33 (SEM 8·9) g/21 d; P<0·01), a reduced food intake (NaCl, 23·07 (SEM 0·31); inhibitor, 19·50 (SEM 0·49) g/21 d; P<0·01), and some intestinal enzymes such as maltase (NaCl, 87 (SEM 7); inhibitor, 127 (SEM 11) U/g proteins; P<0·05). The present study has shown, for the first time, that the prolonged administration of an α-amylase inhibitor reduces blood glucose levels and body-weight gain in Wistar rats.

α-Amylase inhibitor; Glycaemia; Weight; Food intake; Disaccharidases

Inhibitors of α-amylase have been detected in many cereals and some pulses (Bowman, 1945; Jaffe & Lette, 1968; Marshall & Lauda, 1975; Mulimani & Rudrappa, 1994). In particular, the kidney bean (Phaseolus vulgaris) contains high levels of the inhibitor (Pusztai, 1966; Whitaker et al. 1982; Moreno et al. 1990). Elevated levels of ingestion of these raw foods lead to intestinal and metabolic alterations in man and in other animals (Rosenberg, 1982; Maranesi et al. 1984; Pusztai et al. 1995). The use in the early 1980s of crude extracts of kidney beans as starch blockers to control obesity and non-insulin-dependent diabetes mellitus was unsuccessful due to the low inhibitor content (Bo-Linn et al. 1982; Liener et al. 1984) and the presence of lectins and trypsin inhibitors in those extracts (Rosenberg, 1982). Subsequent human studies showed that a perfusion of the partially purified inhibitor in the duodenum significantly inhibited amylase activity during the ingestion of a starch diet (Layer et al. 1985). Ingestion of the purified inhibitor together with 50 g starch was found to reduce postprandial glycaemia levels in both healthy and diabetic subjects (Layer et al. 1986a). The long-term effect of the inhibition of intraluminal amylase activity in the gastrointestinal tract is, however, poorly understood.

The present study investigated the effect of the acute and chronic administration of an α-amylase inhibitor (α-AI) isolated and purified from white kidney beans on the levels of glycaemia and insulinemia, body weight, food intake, weight and length of the gastrointestinal tract, and disaccharidase activity of the brush-border membrane of enterocytes in rats.

Materials and methods

Purification of the α-amylase inhibitor

Purification of the α-AI was performed following the method described by Pusztai et al. (1995). Basically, bean meal (1 kg) was mixed in 10 litres acetic acid (20 mmol/l) containing 0·2 g ascorbic acid/l by stirring for 30 min, and, after adjusting to pH 5·0 with 1 M-NaOH, the slurry was stirred for another 2 h. After being left to stand in a cold room overnight, the extract was centrifuged (10 000 g for 15 min), and, after adjusting to pH 7·0 with 1 M-HCl, the supernatant was removed by centrifugation (3000 g for 10 min) and the supernatant fraction adjusted to pH 3·8 with 1 M-HCl. After another night in a cold room, the extract was cleared by centrifugation (10 000 g for 15 min) and diluted 2-fold.

Abbreviations: α-AI, α-amylase inhibitor; IRI, immunoreactive insulin.

* Corresponding author: Dr M. A. Tormo, fax +34 924 289437, email matormo@unex.es
with distilled water. The diluted supernatant fraction was further purified by ion-exchange chromatography on a Sulfopropyl Fast Flow (Amersham Pharmacia Biotech, Sant Cugat del Valles, Barcelona, Spain) column (50 mm × 75 mm, 150 ml bed volume) equilibrated with 25 mM-sodium formate buffer (pH 3.8). After the extract had passed through, the column was rinsed with formate buffer until the extinction value at 280 nm fell below 0.01, and then the α-AI was eluted with 0.15 M-NaCl in a formate buffer. The α-AI fractions from several chromatograms were combined and re-chromatographed through the Sulfopropyl Fast Flow column under the same conditions. To remove small-molecular-weight impurities, the concentrated eluates from the column were passed through a Sephacryl-100 column (Amersham Pharmacia Biotech, Barcelona, Spain) equilibrated with 50 mM-sodium phosphate buffer (pH 7.5), and the first peak containing α-AI was collected, dialysed against water, and freeze-dried. The yield was about 1.5–2.4 g α-AI/kg bean meal.

**Test of α-amylase inhibitor purity**

The haemagglutination activity of the α-AI preparations was measured according to a previously reported method (Le Berre-Anton et al. 1997). Briefly, in U-bottomed microtitration plates, 25 μl of 2-fold serial dilutions of α-AI (1 mg/ml) in 100 mM-Tris, 150 mM-NaCl buffer (pH 7.4) were mixed at room temperature with an equal volume of a 1% (v/v) suspension of thric-washed human O Rh+ erythrocytes in the same buffer. Haemagglutination was read 2 h later at room temperature and (as a control) after being left to stand at 4°C for 12 h.

A polyacrylamide gel electrophoresis was carried out using the Miniprotein II System (Bio-Rad Laboratories, Alcobendas, Madrid, Spain) with 15% acrylamide gel (Pusztaí et al. 1988).

**Animal experiments**

**Animals.** Adult (2-5 months) male Wistar rats (363 ± 11 g body weight) from our inbred colony were used. They had been maintained on a standard diet (maintenance diet; Panlab S.L., Barcelona, Spain; 61-41% (w/w) carbohydrate (100% starch), 3-96% fibre, 15-06% protein, and 2-66% fat) with free access to food and water, and housed in a room at 24°C with lighting from 08.00 to 20.00 hours. The animals were cared for in accordance with the principles of the Guide to the Care and Use of Experimental Animals (Real Decreto, 1988), and the protocol was approved by the animal ethics committee of the University of Extremadura.

**Acute effect.** Blood glucose and plasma insulin (immunoreactive insulin; IRI) were determined at 09.00 hours (time 0) after 18 h fasting. Then, without anaesthesia, using a gastric cannula attached to a syringe, starch (2 g/kg body weight, soluble potato starch; Sigma, Alcobendas, Madrid, Spain) was administered orally at 50% in NaCl (9 g/l) alone or with α-AI at the dose of 50 mg/kg body weight. Measurements of blood glucose and IRI were made at 15, 30, 60, 90, 120, 150, and 180 min. Blood was extracted from the tail of the animal and the blood glucose concentration was determined at the time of extraction from 2 μl blood with reactive strips read in a Glucocard Memory (A. Menarini Diagnostics, Barcelona, Spain). The rest of the blood was collected in pre-heparinised tubes and centrifuged. The plasma was stored at −70°C until the insulin assay.

**Chronic effect.** α-AI (50 mg/kg body weight) dissolved in NaCl (9 g/l) was administered through a gastric cannula in a single dose at 20.30 hours over a period of 21 d to the 2.5-month-old Wistar rats. Every day at 09.00 hours during this experimental period, food and water intake and body weight were determined, and blood glucose was measured immediately as described earlier. Also, on days 10 and 21 of the experimental period, blood samples were extracted and prepared as described earlier for the plasma IRI assay. After 21 d of treatment, the rats were killed in the morning by pentobarbitol overdose. The abdomen was cut open, and the small intestine, pancreas, liver, and large intestine were removed, rinsed with NaCl (9 g/l), blotted dry, and weighed. The small intestine was measured under 5 g tension. Epithelial cells of the small intestine were isolated according to the method of Watford et al. (1979). This method allows the isolation of metabolically competent enterocytes, and is based on the use of EDTA and gentle mechanical treatment of the small-intestine mucosa. The isolated cells were used to determine disaccharidases.

**Analytical methods**

To measure the in vitro inhibition of the amylase activity of the porcine amylase by the α-AI, the α-AI was dissolved in 0.02 M-sodium phosphate buffer (pH 6.9) containing NaCl (9 g/l), mixed with porcine pancreatic α-amylase (type I-A; Sigma-Aldrich Quı́mica S.A., Alcobendas, Madrid, Spain), and incubated for 30 min at 37°C to allow formation of the inhibitor–enzyme complex. The amount of uninhibited α-amylase was then determined using an amylase diagnostic kit (Dipal; Inquebor S.L., Granada, Spain) following the method of Wallenfels et al. (1978). The disaccharidase (maltase and sucrase) activity was determined in isolated epithelial cells, following the method of Dahlqvist (1964). The protein concentration was determined by the micro-Lowry method (Sigma-Aldrich Quı́mica, Alcobendas, Madrid, Spain). IRI was determined by RIA with a rat insulin kit which uses a specifically synthesized antibody against rat insulin (DRG’s Instrument GmbH, Marburg, Germany).

**Calculation, expression of results and statistical analysis**

In the studies of the acute effect, the increment of blood glucose and IRI concentration were calculated planimetrically. Values are expressed as the mean and standard error of the mean. Statistical analyses were performed using the program InStat for Macintosh version 1.12. Repeated-measures ANOVA was used to assess changes in the level of glycaemia and IRI during the treatment in the same experimental group. When P<0.05, the significance of the difference was estimated by the Bonferroni test. The Mann–Whitney U test was used to determine
Results

The in vitro $\alpha$-amylase activity was suppressed by 70% with 0.15 mg $\alpha$-AI/ml and by 94% with 1.5 mg $\alpha$-AI/ml (Fig. 1). Fig. 2 shows that the oral administration of the starch overload (2 g/kg body weight) led to an increase in glycaemia. The maximum value was attained at 50 min after administration. The level remained above the basal value throughout the study period. The simultaneous administration of starch and inhibitor (50 mg/kg body weight) led to a reduction in glycaemia values. The reduction was found to be statistically significant ($P<0.05$) in comparing the increase in glycaemia over the basal value (blood glucose area under the curve) with (145 (SEM 16)) or without (222 (SEM 49) mmol/l £ 180 min) the inhibitor. The differences in total insulin secretion response (IRI area under the curve) with (18-15 (SEM 1-33)) and without (16-20 (SEM 0-92) ng/ml £ 180 min) the inhibitor were not statistically significant.

The oral administration of the inhibitor (50 mg/kg body weight dissolved in NaCl (9 g/l)) over 21 d at 20.30 hours to rats allowed free access to a standard diet was followed by a slight reduction in glycaemia values with respect to day 0. This reduction was significant ($P<0.01$), except for days 8, 10, and 16 when the significance level was $P<0.05$ from day 4 of treatment until the end of the study (Fig. 3). No significant changes were observed in glycaemia levels in the group of rats who were administered NaCl (9 g/l) alone, for any of the days of the study. There were significant differences in the glycaemia of the treated rats on days 10 ($P<0.05$) and 21 ($P<0.01$) with respect to the untreated rats on those same days. There was no significant reduction in IRI (ng/ml) in the $\alpha$-AI-treated rats on days 10 (2.46 (SEM 0.27)) and 21 (2.23 (SEM 0.17)) with respect to day 0 (2.72 (SEM 0.58)). Also, there were no significant differences in these insulin levels with respect to those measured on days 0 (2.75 (SEM 0.48)), 10 (3.23 (SEM 0.38)), and 21 (3.38 (SEM 0.67)) of the untreated rats.

During the chronic administration of the inhibitor, there was no reduction in water intake (Table 1), but there was a significant reduction in daily food intake. This anorexigenic effect of the inhibitor was reflected in a smaller weight increase of the rats administered the inhibitor ($P<0.05$) relative to the controls ($P<0.01$).

The $\alpha$-AI treatment over 21 d significantly reduced the weight of the small intestine, the large intestine, liver, and pancreas (Table 2). There was a significant increase

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**Fig. 1. In vitro inhibition of porcine pancreatic $\alpha$-amylase activity by the $\alpha$-amylase inhibitor purified from white kidney beans (Phaseolus vulgaris). The values are means of four determinations.**

**Fig. 2. Blood glucose values (mmol/l) obtained after the oral administration to 2.5-month-old Wistar rats of starch (2 g/kg body weight) suspended at 50% in NaCl (9 g/l) alone (NaCl; □) or with 50 mg $\alpha$-amylase inhibitor/kg body weight (■). The values are means of six determinations for each experimental group, with standard errors of the mean represented by vertical bars. Mean value was significantly different from that for NaCl at the same time: *$P<0.05$, **$P<0.01$.**

**Fig. 3. Evolution of the blood glucose values (mmol/l) of Wistar rats over the 21 d following the oral administration of NaCl (9 g/l) alone (NaCl; □) or containing 50 mg $\alpha$-amylase inhibitor/kg body weight (■). The values are means of six determinations for each experimental group, with standard errors of the mean represented by vertical bars. Mean value was significantly different from that for NaCl on the same day: *$P<0.05$, **$P<0.01$. Mean value was significantly different from that for inhibitor on day 0: †$P<0.05$, ††$P<0.01$.**
Table 1. Intake of water and food, and body-weight gain over the time of the experimental period, of 2-5-month-old Wistar rats subjected for 21 d to a treatment with an inhibitor of pancreatic amylase isolated from white kidney beans (Phaseolus vulgaris) (50 mg/kg body weight) suspended in NaCl (9 g/l)†

(Mean values and standard errors of the mean)

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<th>NaCl (n 6)</th>
<th>Inhibitor (n 6)</th>
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<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
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<td>Water (ml/d)</td>
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<tr>
<td>Food (g/d)</td>
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<tr>
<td>Body-weight gain (g/21 d)</td>
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<td>3</td>
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** Mean value was significantly different from that for NaCl (P<0.01).
† For details of diets and procedures, see p. 786.

(P<0.05) in maltase activity, but not in sucrase activity measured in the enterocytes isolated from the small intestine at the end of the 21 d of treatment with the inhibitor (Table 3).

Discussion

Method and purification yield

The α-AI preparations contained four polypeptide bands of 32, 29, 17, and 16 kDa, similar to the results reported by other workers (Le Berre-Anton et al. 1997). The test for haemagglutination activity showed no evidence of contamination of the α-AI preparation with kidney bean lectin; again, results that were similar to previous reports (Maranesi et al. 1984; Pusztai et al. 1995).

Hypoglycaemic effect

The results show that the α-AI isolated and purified from white kidney beans significantly reduces glycaemia levels in rats following both acute (reduction of postprandial glycaemia) and chronic (reduction of basal glycaemia) administration. Similar results have been described by other workers for growing non-diabetic Wistar rats (Kotaru et al. 1989), and for healthy and type-2 diabetic subjects (Layer et al. 1986a,b; Boivin et al. 1987; Jain et al. 1989, 1991). These previously reported studies were all carried out under acute conditions, for which reason the present study was designed to investigate the effect of the prolonged daily administration of the α-AI.

The results showed no significant changes in plasma insulin levels after α-AI treatment, whether with acute or chronic administration, although the values were apparently lower. Other workers (Kotaru et al. 1989) have reported similar findings, with a decline in plasma insulin levels after the administration of α-AI purified from the cranberry bean variety of P. vulgaris together with an experimental diet in growing male Wistar rats. Healthy and diabetic subjects (Layer et al. 1986a,b), who were administered 50 g starch together with 10 g inhibitor, presented reduced levels of postprandial plasma insulin and C-peptide during the time that glucose levels were greater than the fasting levels.

Intake of water and food and body weight

The present results demonstrated that the chronic administration of α-AI reduces food intake and weight gain. As the α-AI was administered by a gastric cannula the anorexigenic effect observed could not be attributed to a lack of palatability of the product reducing the energy intake. A similar anorexigenic effect has been known for many years (Jaffe & Lette, 1968; Puls & Kneup, 1973; Pusztai et al. 1995). Indeed, different preparations based on the inhibitor isolated from P. vulgaris are commercially available in the USA for the treatment of obesity. One of them is manufactured under the supervision of J. J. Marshall who, with C. Lauda, was the first to purify and characterise α-AI (Marshall & Lauda, 1975), which they denominated phaselamin. It has also been difficult to explain how the chronic administration of α-AI reduces food intake. Studies on human subjects have shown that the inhibition of pancreatic amylase is associated with a delay in gastric emptying, and that the arrival of a greater amount of undigested carbohydrates in the ileum also slows gastric emptying (Jain et al. 1989, 1991). One must therefore agree with the suggestions of other workers who studied an α-AI purified from wheat (Kataoka & DiMaggio, 1999) that it is most likely that amylase inhibition produces satiety and decreases food intake by delaying gastric emptying, and that the weight loss caused by the inhibitor is due to that...
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References


