Food & Function

PAPER

# α-Glucosidase inhibitory activity of protein-rich extracts from extruded adzuki bean in diabetic KK-A$^y$ mice

Cite this: *Food Funct.*, 2014, **5**, 966

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Received 24th October 2013
Accepted 27th January 2014

DOI: 10.1039/c3fo60521c

www.rsc.org/foodfunction

An extrusion process has been widely used for the development of many functional foods. The aim of this study was to assess the effect of the extrusion process on the α-glucosidase inhibitory properties of adzuki bean protein in type 2 diabetes model KK-A$^y$ mice. The extruded adzuki bean protein (EA) was prepared by adding 1% and 2% into the diet for 42 days. It was found that the fasting blood glucose concentration was significantly decreased with the EA-2 group compared with the control diabetic mice group. In addition, there was a significant decrease in serum triglyceride, blood urea nitrogen levels and increased high density lipoprotein (HDL)-cholesterol. Meanwhile, hepatic lipids were improved and the content of α-dicarbonyl compounds in the kidney were reduced in mice fed with EA. These results suggest that the intake of EA could moderate type 2 diabetes and diabetic complications.

## Introduction

Diabetes, especially type 2 diabetes, is rapidly becoming an enormous health burden by decreasing the quality of life and causing death and disability across the whole world, all at a huge economic cost.[1] Diabetes is, in part, related to the amount of carbohydrates in the diet. Acting as a key enzyme for carbohydrate digestion, intestinal α-glucosidase is one of the glucosidases located at the epithelium of the small intestine.[2] α-Glucosidase has been recognized as a therapeutic target for modulation of postprandial hyperglycemia, which is the earliest metabolic abnormality to occur in type 2 diabetes mellitus.[3] The inhibition of intestinal α-glucosidases would delay the digestion and absorption of carbohydrates and consequently suppress the postprandial hyperglycemia.[4,5]

Extrusion cooking has been applied to modify the structure, improve the solubility, and increase the soluble fiber content of fibrous materials, such as plant cell-wall rich materials, brans and hulls of various cereals and legumes.[6] During extrusion cooking, a variety of steps, including feed transport, mixing, heating, forming, and drying, occur within a short time. The food components in the extruder barrel experience high temperature, shear, and pressure during extrusion cooking.[7,8] Extrusion cooking can affect and change the nature of many food constituents, like proteins and polyphenols, by changing the physical and chemical properties.

Adzuki beans have been a subject of extensive investigation due to their biological activities. Recently, they have been recommended as suitable foods for diabetic patients due to their proteins and phenolic compounds, which may offer extra benefits for the amelioration of diabetes.[9,10] Itoh *et al.*[11] reported that adzuki beans possess inhibition activity against α-glucosidase in streptozotocin (STZ)-induced diabetic rats. We also previously observed that extruded adzuki bean protein (EA) suppressed the elevation of blood glucose and plasma insulin levels after oral administration in normal and streptozocin-treated rats. Diabetic KK-A$^y$ mice have been frequently used as an animal model for noninsulin-dependent diabetes.[12,13] The symptoms of this animal model are similar to those of diabetic patients and the mice exhibit metabolism abnormalities, such as an absolute or relative lack of insulin, hyperglycemia and glucose intolerance, higher lipids and so on. We are interested in the antidiabetic effect of EA in KK-A$^y$ mice. Here, we report our investigations on the effect of EA on diabetic KK-A$^y$ mice.

## Materials and methods

### Preparation of the extruded adzuki bean protein (EA)

The extrusion was carried out in a laboratory twin screw extruder type DS56 (Saixin machinery, Shandong, China) with the following operation parameters: feed speed 20 g min$^{-1}$, screw speed 160 rpm, moisture content 16% (wet base), temperature settings for the feeding zone to die zone 80-110-150-135-80 °C. A five percent (w/v) bean flour slurry was adjusted to pH 9.5 with 1 M NaOH at room temperature, mixed for 1 h and centrifuged for 15 min at 2000× *g*. Then, the pH was adjusted to 4.5 with 1 M HCl to precipitate the protein. The proteins were recovered by centrifugation at 2000× *g* for 15 min

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followed by removal of the supernatant. The protein curd was washed with distilled water and the curd was re-dispersed in distilled water. The washed precipitate was collected and immediately put into a freeze dryer. The protein composition of the extruded adzuki beans was 86.04%, determined according to the methods of AOAC 14.108.[14] The amino acid composition of EA is shown in Table 1.

### Diets

The diets were prepared as previously described.[15] The control diet was prepared by mixing the following ingredients in proportion (% diet): cornstarch, 28.21; soybean meal, 11.62; soybean powder, 4.13; soybean, 0.28; wheat flour, 14.00; wheat bran, 7.42; fish powder, 0.84; $CaCO_3$, 1.33; $CaPO_4$, 1.54; mineral, 0.56; vitamin, 0.07; sucrose, 10.00; lard, 10.00; yolk powder, 10.00; The other two experimental diets were prepared by adding 1% (EA-1) and 2% (EA-2) protein powder (provided by the Chinese Academy of Agricultural Science, Beijing, China). The powered diets were mixed with a gelatin solution (20 g $L^{-1}$) in a ratio of 200 g diet powder per liter of solution. Once the gelatin had set, the diets were cut into pieces of approximately 10 g cubes and stored at −20 °C.

### Design of the animal experiment

The experiments were carried out according to the method of Yao *et al.*[16] Fourty male diabetic KK-$A^y$ mice were obtained from the Department of Laboratory Animal Science Center (Beijing, China). The diabetic mice were divided into four groups according to their weight and blood glucose levels to make the average weight and blood glucose levels similar among the groups. During the following 6 weeks, two groups were continued to be fed with the control diet and the two EA groups were fed with the experimental diets. The acarbose group animals were given 25 mg $kg^{-1}$ acarbose per day. They were allowed free access to the diet and water. The level of food intake and body weight were measured during the treatment. All the mice were housed individually in stainless steel wire-bottom cages in an air-conditioned room kept at a controlled ambient temperature (22 ± 1 °C), humidity (50 ± 10%), and a 12 h light/dark cycle. Blood samples were taken weekly from the tail vein after overnight fasting. Glucose was determined using a glucose analyzer (ACCU-CHEK Active, Roche, Shanghai, China). On the morning of the oral glucose tolerance test (OGTT), the fasting animals were given glucose orally (2 g $kg^{-1}$). The blood glucose levels were measured at 0 (before oral glucose), 30, 60, 90, and 120 min after glucose administration. Feces were collected for 2 days using a metabolic cage from day 40 to day 42. On day 42, after 12 h of fasting, each mouse was sacrificed under anesthesia with diethyl ether, and blood was obtained from the inferior vena cava. The liver and kidney were collected, and serum was immediately prepared from the blood. All the samples were frozen in liquid $N_2$ and stored at −80 °C until analysis. The experiment was carried out according to the European Community Guidelines for the Use of Experimental Animals and approved by the Peking University Committee on Animal Care and Use.

Table 1 Amino acid composition (%) of EA

| | EA |
|---|---|
| Asp | 6.84 ± 0.03 |
| Thr | 2.24 ± 0.01 |
| Ser | 3.08 ± 0.05 |
| Glu | 11.80 ± 0.01 |
| Gly | 2.48 ± 0.01 |
| Ala | 2.96 ± 0.21 |
| Cys | 0.04 ± 0.01 |
| Val | 3.16 ± 0.02 |
| Met | 0.76 ± 0.06 |
| Iie | 2.52 ± 0.01 |
| Leu | 4.64 ± 0.02 |
| Tyr | 1.80 ± 0.02 |
| Phe | 3.44 ± 0.06 |
| Lys | 4.52 ± 0.01 |
| His | 2.80 ± 0.01 |
| Arg | 3.88 ± 0.03 |
| Pro | 2.28 ± 0.02 |

### Biochemical analysis of the serum and liver

The plasma insulin (DSL-1600 Insulin RIA kit, Diagnostic Systems Laboratories, USA) level was measured using ELISA kits (Nanjing Jiancheng, Nanjing, China). C-peptide (ADL, San Diego, CA) and glucagon (RapidBio Laboratory, Calabasas, CA) were determined using commercial enzyme-linked immunosorbent assay kits. The plasma total cholesterol, triglycerides, and blood urea nitrogen (BUN) were measured using an auto-biochemical analyzer (Hitachi 7600, Japan).[17,18] Liver lipids were measured using commercial kits (Nanjing Jiancheng, Nanjing, China).

### Fecal component analysis

The collected feces were lyophilized and weighed (dry weight). The powdered dry feces were extracted and purified using the method described by Nagahora *et al.*[19] The cholesterol and TG levels in the feces were measured using the same enzymatic kit used in the plasma analysis.

### Determination of α-dicarbonyl compounds in the kidney

α-Dicarbonyl compounds were analyzed for 2,3-diaminonaphthalene (DAN) adducts, identified as fluorescing derivatives according to the method of Nagahora *et al.*[19] The kidney sample (100 mg) was homogenized with 1 mL of ice-cold 10 mM sodium phosphate buffer at pH 7.4 and was centrifuged at 14 000*g* for 15 min. The supernatant was transferred into a new tube, and the proteins were precipitated by adding 50 μL of 0.005% 2,3-pentanedione and 1 mL of 6% perchloric acid, and the sample was centrifuged at 3350*g* and 4 °C for 20 min. The supernatant was neutralized with 2 mL of a saturated sodium bicarbonate solution and was reacted with 0.1% (w/v) 2,3-diaminonaphthalene overnight at 4 °C. The derivatized compounds were extracted with 4 mL of ethyl acetate three times, and the solvent was dried under reduced pressure. The dried extract was

dissolved with 200 μL of methanol for HPLC analysis. Standards (3-DG, GO, and MG) were derived with 2,3-diaminonaphthalene, as described above.

HPLC analysis was carried out on a Syncronis-C18 column (4.6 × 250 mm, Thermo Fisher Scientific, West Palm Beach, USA) at 40 °C using a PU-2089 Plus pump (JASCO Co.; Tokyo, Japan). Chromatographic separation was performed with isocratic elution using 10 mM sodium phosphate–acetonitrile (70 : 30, v/v). The flow rate was 1 mL $min^{-1}$, and the injected volume was 5 μL. The wavelength used for the detection was 503 nm (excitation, 271 nm) on an FP-920 fluorescence spectrophotometer (JASCO Co.).

### Statistical analysis

All the values were expressed as the mean ± SD. The data were analyzed using one-way analysis of the variance (ANOVA). Differences with $p < 0.05$ were considered to be significant.

## Results and discussion

### Food intake and body weight

The present study found that EA and acarbose had no effect on the final body weight of KK-$A^y$ mice during the 6 week period of treatment (Table 2).

### Fasting blood glucose levels and oral glucose tolerance

KK-$A^y$ mice have high fasting and nonfasting blood glucose levels, similar to human type 2 diabetes. Oral administration of EA and acarbose for 6 weeks caused a dose dependent decrease in the blood glucose level compared with the control diabetic group (Fig. 1). EA and acarbose appeared to improve the glucose tolerance in KK-$A^y$ mice (Fig. 2). The KK-$A^y$ control mice showed a sharply increased blood glucose concentration at 30 min after glucose loading and maintained this high level over an additional 30 min. All the EA-treated KK-$A^y$ mice showed decreases in the blood glucose levels at 30, 60, 90 and 120 min compared with the control KK-$A^y$ mice (Fig. 2). Itoh *et al.*[20] reported that adzuki beans possess inhibition activity against α-glucosidase in streptozotocin (STZ)-induced diabetic rats due to anthocyanins and phenolic compounds. We have found that extruded adzuki bean protein (10 mg $mL^{-1}$) inhibited rat intestinal α-glucosidases (60.44%) *in vitro*. Further animal studies revealed that the oral intake of extruded adzuki bean extract reduced postprandial blood glucose by 15.6% and 30.9% following a sucrose challenge in normal and streptozocin-treated rats, respectively.[12]

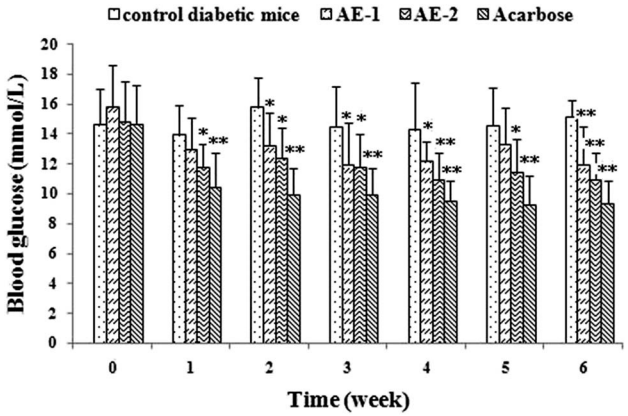


Fig. 1 Changes in blood glucose levels in the different experimental groups. Each column represents the mean ± S.D. of 10 animals. *$p < 0.05$, **$p < 0.01$, compared with the control diabetic mice.

### Serum analyses

EA-2 and acarbose significantly lowered the levels of plasma triglycerides (TG), and increased the content of high-density lipoprotein cholesterol (HDL-C). The serum total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), C-peptide, glucagons, and insulin levels were unchanged after the consumption of the diets containing EA. The results reported here are in agreement with a previous study in which the serum TG levels were reduced in obese rats that were fed water extracts of adzuki beans.[21] Bayraktar and Thiel have observed that acarbose lowered the TG levels,[22] but the insulin content was unchanged.[23] Blood TC and LDL-C correlate directly with the risk of heart diseases, whereas HDL-C correlates inversely with the risk.

Diabetic hyperglycemia induces the elevation of plasma urea nitrogen, which is considered to be a marker of renal dysfunction.[24] As shown in Table 3, the plasma urea in the EA-2 group reduced significantly the plasma urea nitrogen by 19.9% compared with the value of the diabetic control group, indicating that EA may be capable of ameliorating impaired diabetic kidney function in addition to its hypoglycemic control.

### Hepatic lipids

The hepatic total cholesterol and triglycerides in EA mice were significantly lower than those in the control mice. However, HDL-C, and LDL-C were unchanged after the consumption of the diets containing EA.

Several components of grains have the ability to reverse some alterations in lipid metabolism. It has been suggested that the

Table 2 Effect of EA on body weight and food intake (week) in diabetic KK-$A^y$ mice

| | Initial body wt (g) | Final body wt (g) | Food intake (g) |
|---|---|---|---|
| Control diabetic mice | 35.59 ± 1.54 | 37.41 ± 1.23 | 10.71 ± 0.89 |
| EA-1 | 36.26 ± 1.43 | 38.75 ± 1.55 | 10.25 ± 0.91 |
| EA-2 | 35.99 ± 1.48 | 38.93 ± 1.78 | 11.53 ± 1.42 |
| Acarbose | 35.74 ± 1.68 | 38.14 ± 1.44 | 10.67 ± 1.21 |


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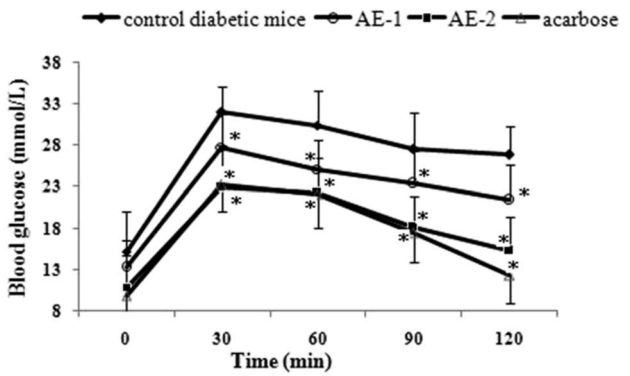


Fig. 2 Oral glucose tolerance test at the end of the 6 week treatment. *$p < 0.05$ compared with the control diabetic mice.

vegetal protein induces modifications in cholesterol metabolism by the action of bioactive peptides, or by the differences in the amino acid pattern.[25] Similar results were reported by Kitano–Okada *et al.*,[21] who observed that adzuki bean extract treatment in a high-fat group resulted in reductions in the total hepatic lipid accumulation and lipid secretion into the feces. A previous study reported that lupin protein affects the expression of the hepatic genes involved in lipid metabolism in hypercholesterolemic rats.[24] Sirtori *et al.*[26] also observed an increase in the mRNA abundance of the sterol regulatory element-binding protein-2 and LDL receptor along with a decrease in mRNA concentrations of 3-hydroxy-3-methylglutaryl-CoA reductase in mononuclear blood cells from hypercholesterolemic subjects after a 6 week intervention with lupin protein. Apart from the specific amino acid profile of the lupin protein, several bioactive peptides as well as entire proteins are equally capable of demonstrating favorable properties.[27] The liver showed inflammation in STZ-induced diabetic rats. It is well known that the liver is a central organ in lipogenesis, gluconeogenesis, and cholesterol metabolism. Hepatic lipid metabolism is influenced by the balance between the degradation and synthesis and/or import and export of TG and fatty acids. Hepatic TG accumulates, finally resulting in hepatic steatosis.[28]

### α-Dicarbonyl compounds in the kidney

The content of 3-DG in the kidney was not significantly altered among the groups (Table 3). GO and MG were lower than that of the control diabetic mice (Table 3), suggesting that the EA used in the present study reduced carbonyl stress.

Hyperglycemia accelerates nonenzymatic glycation, leading to the accumulation of advanced glycation end products (AGEs), which are associated with the development of diabetic complications such as diabetic nephropathy.[29] Therefore, AGEs are key metabolites that can be used to evaluate the degree of complications of diabetic nephropathy.[30] The α-dicarbonyl

Table 3 Serum, liver, kidney, and feces parameters in diabetic KK-$A^y$ mice

| | Control diabetic mice | EA-1 | EA-2 | Acarbose |
|---|---|---|---|---|
| **Serum** | | | | |
| TC[b] (mmol $L^{-1}$) | 5.77 ± 0.96 | 5.83 ± 0.05 | 5.73 ± 1.12 | 5.74 ± 0.07 |
| TG[c] (mmol $L^{-1}$) | 1.81 ± 0.60 | 1.56 ± 0.35[a] | 1.42 ± 0.28[a] | 1.44 ± 0.25[a] |
| HDL-C (mmol $L^{-1}$) | 4.12 ± 1.06 | 4.69 ± 0.28[a] | 5.46 ± 0.74[a] | 4.61 ± 0.25[a] |
| LDL-C (mmol $L^{-1}$) | 1.79 ± 0.17 | 1.42 ± 0.28 | 1.56 ± 0.26 | 1.63 ± 0.19 |
| C-peptide (μg $mL^{-1}$) | 37.80 ± 1.92 | 42.96 ± 6.53 | 39.96 ± 3.81 | 41.62 ± 2.46 |
| Glucagons (μg $mL^{-1}$) | 30.40 ± 0.59 | 31.80 ± 4.77 | 30.09 ± 2.61 | 33.53 ± 1.51 |
| Insulin (μg $mL^{-1}$) | 27.97 ± 5.76 | 31.57 ± 3.79 | 30.52 ± 4.05 | 31.27 ± 3.42 |
| BUN[d] (mmol $L^{-1}$) | 10.56 ± 0.86 | 10.04 ± 0.80 | 8.46 ± 1.28[a] | 10.34 ± 0.67 |
| **Liver** | | | | |
| TG (mg $dL^{-1}$) | 195.27 ± 4.84 | 172.39 ± 4.62[a] | 143.11 ± 3.69[a] | 147.84 ± 5.83[a] |
| TC (mg $dL^{-1}$) | 12.41 ± 1.52 | 7.54 ± 2.24[a] | 4.91 ± 1.31[a] | 6.24 ± 0.81[a] |
| HDL-C (mg $dL^{-1}$) | 1.34 ± 0.17 | 1.22 ± 0.13 | 1.31 ± 0.11 | 2.27 ± 0.23[a] |
| LDL-C (mg $dL^{-1}$) | 2.42 ± 0.30 | 2.16 ± 0.35 | 2.02 ± 0.28 | 2.07 ± 0.35 |
| **Kidney** | | | | |
| 3-Deoxyglucosone (nmol $g^{-1}$) | 0.07 ± 0.01 | 0.07 ± 0.01 | 0.06 ± 0.01 | 0.07 ± 0.01 |
| Glyoxal (nmol $g^{-1}$) | 13.21 ± 0.63 | 7.25 ± 0.21[a] | 6.42 ± 0.38[a] | 13.10 ± 0.27 |
| Methylglyoxal (nmol $g^{-1}$) | 2.67 ± 0.46 | 1.25 ± 0.28[a] | 1.13 ± 0.24[a] | 2.54 ± 0.31 |
| **Feces** | | | | |
| TC (mmol $L^{-1}$) | 3.31 ± 0.12 | 3.53 ± 0.81 | 3.60 ± 1.15 | 3.49 ± 0.95 |
| TG (mmol $L^{-1}$) | 2.03 ± 0.21 | 1.83 ± 0.16 | 1.97 ± 0.28 | 2.12 ± 0.21 |
| HDL-C (mmol $L^{-1}$) | 0.16 ± 0.06 | 0.11 ± 0.04 | 0.17 ± 0.09 | 0.10 ± 0.03 |
| LDL-C (mmol $L^{-1}$) | 0.93 ± 0.62 | 0.73 ± 0.12 | 1.09 ± 0.36 | 0.82 ± 0.14 |
| Total bile acid (μmol $L^{-1}$) | 113.00 ± 15.16 | 153.67 ± 26.49[a] | 173.00 ± 13.09[a] | 142.33 ± 14.28[a] |

[a] $p < 0.05$, compared with the diabetic control mice. [b] TC, Total cholesterol. [c] TG, Triglycerides. [d] BUN, blood urea nitrogen.

compounds, 3-deoxyglucosone (3-DG), glyoxal (GO), and methylglyoxal (MG), are known to be intermediate products of glycation. These compounds have higher reactivity with proteins than the reactivity of reducing sugars, and they damage proteins (the products are AGEs) and lipids. Therefore, the reaction is called carbonyl stress, which has a vital role in the pathogenesis of diabetic nephropathy and the progression of renal failure.

AGEs have been regarded as an important mediator of the untoward effects of hyperglycemia because the progression of diabetic complications could be prohibited by inhibitors of glycoxidation products that do not change glycemia. AGEs stimulate a variety of cellular responses, including matrix production, pro-fibrotic responses, and pro-inflammatory responses, *via* AGEs and the protein and mRNA expression of their receptor (RAGE), a specific cell-surface receptor on several cell types, including glomerular mesangial cell. In addition, the increased level of circulating RAGE associated with immune complexes in glomeruli may play a role in albuminuria and tissue injury.[31]

### Fecal lipids

The excretion of bile acid in the feces of the EA-1 and EA-2 mice were significantly greater than that of the control group (Table 3). However, the excretion of the total cholesterol, triglycerides, HDL-C, and LDL-C in the feces was not increased by the consumption of EA (Table 3). It is known that excessive cholesterol is mainly eliminated by mammals in the form of fecal bile neutral and bile acids. The present study found that the total bile acid was the lowest in the control followed by the EA-1, and EA-2 groups in a decreasing order, suggesting that EA may be an inhibitor of bile acid absorption.

Soybean protein has been reported to lower the serum cholesterol level *via* the inhibition of cholesterol absorption by the binding of cholesterol in the digestive organ, promotion of the metabolism of cholesterol to bile acid due to the lowering enterohepatic circulation of bile acid.[32] In general, the inhibition of cholesterol absorption in the jejunum,[33] enhancement of the excretion of neutral sterols and bile acid in feces by the inhibition of bile acid reabsorption in the ileum,[34] lowering cholesterol synthesis in the liver, slower cholesterol secretion from the liver to the blood, and promotion of blood low-density lipoprotein to the liver, are considered mechanisms of lowering serum cholesterol.

## Conclusions

In conclusion, the present study indicates that the intake of EA in KK-A$^y$ mice attenuated both the increase of blood glucose and the accumulation of hepatic lipids. These effects may be due to EA regulating the absorption and metabolism of sugars and lipids. Furthermore, the study demonstrated for the first time that EA could suppress the generation of α-dicarbonyl compounds in the kidney. Therefore, the present study suggests that EA may be capable of the attenuation of the pathology of type 2 diabetes and inhibition of the progress of diabetic complications.

## Acknowledgements

This work was supported by the earmarked fund for Modern Agro-Industry Technology Research System (nycytx-018) and the Sci & Tech Innovation Program of CAAS.

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