International Food Research Journal 22(4): 1550-1556 (2015)
Journal homepage: *http://www.ifrj.upm.edu.my*

Effects of phytase and tannase on *in vivo* nutruitive utilisation of faba bean (*Vicia faba* L.) flour

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Article history

Received: 28 June 2014
Received in revised form: 28 November 2014
Accepted: 17 December 2014

Keywords

Faba bean
Phytates
Tannins
Phytase
Tannase

Abstract

Faba bean is a legume used as a food source by humans and animals. However, it presents antinutritional factors such as tannins and phytic acid, compounds that form complexes with proteins and minerals, respectively, decreasing faba bean's digestive value. Faba bean was treated with tannase and phytase, the effect of this treatment was measured in a study with rats, a group received a diet with raw faba bean and the other one a diet with treated faba bean. The enzymatic treatment was effective in reducing tannins and promoting the increase of inorganic phosphorus. The biological assay showed that the enzymatically treated faba bean was better than raw faba bean in the apparent digestibility of phosphorus, in the level of glucose, cholesterol and triacylglycerol. The enzymatic treatment of faba bean could improve the nutritional value of this legume while also decreasing environmental pollution.

Introduction

Faba bean (*Vicia faba* L.) is widely consumed as a part of basic human and animal nutrition. However, one of the constrains on the utilisation of faba bean as food or feed is the occurrence of some antinutritional factors, such as phytic acid and tannins (Luo and Xie, 2013). Phytic acid and tannins have the ability to form complexes with proteins, carbohydrates and mineral nutrients, making them unavailable for digestion and absorption (Matuschek *et al*., 2001; Sandberg, 2002; Almeida *et al*., 2008; Schlemmer *et al.*, 2009). Diets containing tannins are associated with decreased intake, weight gain, feed conversion efficiency and protein digestibility in animal studies (Chung *et al*., 1998; Porres *et al*., 2002). Elimination or inactivation of such antinutritional compounds is absolutely necessary to improve the nutritional quality of faba bean and effectively utilise its full potential as food or feed (Luo *et al.*, 2009; Luo *et al*., 2010a).

Enzymatic supplementation is a technique with increasing applicability for improving the nutritional characteristics of food. Treatment with exogenous enzymes is crucial in animal feed to improve weight gain and the absorption of protein (Urbano *et al*., 2007), phosphorus and minerals (Towo *et al*., 2006; Guggenbuhl *et al*., 2007).

Phytases are enzymes that hydrolyse phytic acid (myo-inositol hexakisphosphate), releasing phosphoric acid, free inositol and⁄or intermediate compounds such as esters of mono-, bi-, tri-, tetrakis, and pentakisphosphateinositol (IP1-IP5), depending on the degree of dephosphorylation and the release of minerals that can be chelated (Lei and Porres, 2003; Kumar *et al*., 2010). Thus, the dietary effect of this enzyme is mainly related to improvements in weight gain and the absorption of phosphorus and minerals.

Tannin acy hydrolase, commonly referred to as tannase (EC: 3.1.1.20), is an enzyme that hydrolyses esters and depside bonds of hydrolysable and condensed tannins, like tannic acid, epicatechin gallate, epigallocatechin gallate and chlorogenic acid (Banerjee *et al*., 2001; Garcia-Conesa *et al*., 2001; Battestin *et al*., 2008). It is important to mention that there is little research on the use of tannase and no research examining the use of tannase and phytase together in legumes for application in animal feed. The purpose of this study was to evaluate the effect of tannase and phytase to faba bean to reduce the effect of antinutritional factors, such as tannins and phytate, in addition, observe the *in vivo* effect on Wistar rats.

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Materials and Methods

Materials and sample preparation

Tannase was obtained from Changhen (Wuhan, China), with activity of 500 U g^{-1}; A fungal phytase was obtained from SUNSON (Beijing, China), with activity of 6000 U g^{-1} and the suggested dose based on application in animal feeds was 100 U kg-^{1} feed dry matter.; all other reagents were analytical grade. Faba beans (Qidou 2, cultivated in Jiangsu Province and harvested in 2010) were collected from local market of the same batch in Nanjing, Jiangsu Province, P.R. China. The mean moisture, protein, carbohydrate, fat, ash content were 12.6%, 21.2%, 55.4%, 0.4%, 3.2% respectively. Flours of faba bean were prepared in a hammer-mill type grinder (HY-04B, Beijing Xinhuanya, China) and sieved through a 0.4 mm screen..

Chemical analysis

Phenolic compounds

The extraction of phenolic compounds from the faba bean was performed using a 2.0 g sample and 20 ml of 70% v/v acetone (analytical grade) by applying 20 min ultrasonic treatment at 4ºC followed by overnight mechanical tumbling. The extract obtained was centrifuged at 1500×g at 5ºC for 15 min. Extracts were analysed for total phenolics by spectrophotometrical methods using the Folin-Ciocalteu's Phenol Reagent. (Anonymous, 2000). Hydrolysable tannins were determined by using the ferric ammonium sulphate reagent (Brune *et al.,* 1991), and condensed tannins were determined through the vanillin method (Price *et al*., 1978). The quantification was carried out through a calibration curve using gallic acid (Sigma), tannic acid (Sigma) and catechin (Sigma) as the standard for total phenols and hydrolysable and condensed tannins, respectively. The results were expressed in mg 100 g^{-1} of faba bean.

Inorganic phosphorus

The extraction was performed using 500 mg of the sample and 10 mL of 0.5 M HCl solution for 2 h under agitation in a orbital shaker at 200 rpm and at room temperature (25ºC); after the extraction, the medium was centrifuged at 1500×g, 5ºC, for 15 min (Towo *et al.*, 2006). The extract obtained was used to determine levels of inorganic phosphorus (Shimizu, 1992). The quantification was done using a calibration curve with K_2HPO_4 and expressed in mg 100 g^{-1} of faba bean.

Faba bean treatment with tannase and phytase

Faba bean flour was mixed in a beaker with water in a faba bean:water ratio of 15:85, and phytase and tannase enzymes were added at a concentration of 100 U kg^{-1} of faba bean flour. The process was conducted during 24 h, with agitation in a thermostatised bath at 35ºC.

Biological assay

Animals

For the biological assay, twenty male 21-day-old Wistar rats were obtained from the College of Animal Science-Jinling Institute of Technology. The animals were maintained at a controlled temperature of 23 ºC±2ºC with relative humidity between 50% and 60%, with alternating 12 h periods of light and dark. The experimental animals were weighed (56 g±7 g) and randomly divided into two groups of ten rats each. All animals were kept in individual cages; from the 10th to the 17th day the rats were kept in a metabolic cage designed to collect feces and urine, both of which were stored frozen until the analysis period.

The study lasted 28 days, after which the animals were sedated with halothane to enable blood collection and then sacrificed through cervical dislocation. The procedure for the biological assay was approved by the Ethics Committee on Animal Experimentation of the Jinling Institute of Technology.

Diet

The animals'diets were prepared in accordance with the recommendations of the American Institute of Nutrition (AIN-93G) (Reeves *et al*., 1993). The groups were divided into a raw faba bean (RFB) group, which was given a diet prepared with milled and sized raw faba bean (<0.4 mm), and a treated faba bean (TFB) group, which was given faba bean that had been enzymatically treated as described above. The feed and water were supplied randomly.

Biological analysis

To determine the apparent digestibility of nitrogen (A.O.A.C., 1999) and phosphorus, the amounts of each were quantified in the feces and urine (Shimizu, 1992), respectively. The blood was collected without anticoagulant and centrifuged at 300×g for 15 min at room temperature. From the obtained serum, the animals'glucose, cholesterol, triacylglycerol, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and phosphorus levels were determined using SUNSON brand kits; calcium and serum iron levels were determined by using JIANCHENG

reaction kits, following the protocols specified by the companies. Left and right femurs were collected, weighed, packed in plastic and kept frozen at -18ºC until analysis. At that point, each femur was dried in an oven at 105ºC for 12 h and incinerated at 550ºC to obtain light ashes, which were then weighed (A.O.A.C., 1999).

Statistical analysis

Data were analysed with SPSS (Statistical Package for the Social Sciences) 13.0 for windows. The mean and standard deviation of means were calculated. The data were analysed by one-way analysis of variance (ANOVA). Tukey test was used to separate means. Significance was accepted at a probability $P < 0.05$.

Results and Discussion

Chemical analysis

The faba bean flour was treated with tannase and phytase enzymes; before and after the enzymatic treatment, total phenols, hydrolysable tannins, condensed tannins and inorganic phosphorus were determined (Table 1). In our perivous study, after the phytase treatment, the phytate in faba bean flour was degradated completely (Luo *et al*., 2010a; Luo *et al*., 2010b).

Table 1. Total phenols, hydrolysable tannins, condensed tannins and inorganic phosphorus in faba bean before and after enzyme treatment

Determination	Raw faba bean*	Faba bean treated with enzymes*
Total phenols	680±2	65±1
Hydrolysable tannins	58±2	30±1
Condensed tannins	2586±12	842±5
Inorganic phosphorus	375±10	814±13

Results obtained in triplicate followed by standard deviation.
*Results expressed in mg 100 g^{-1} of faba bean

The concentration of phenolic compounds in faba bean grains was similar to values found in other studies on total phenols (TF) and hydrolysable tannins (HT), but the concentration of condensed tannins (CT) was above the mean. The high concentration of condensed tannins in this variety of faba bean is probably related to its rusticity and resistance to pests. In a study conducted with 13 different samples of faba bean grown in Canada, the concentration of tannins was analysed both among distinct genotype varieties and among the same varieties cultivated in different locations. The authors found a large difference among the samples: the concentration of tannins ranged from 50 mg 100 g^{-1} to 5420 mg 100 g^{-1} of faba bean. This study showed that the concentration of phenolic compounds in faba bean is not a constant feature even in the same variety, but is influenced primarily by the cultivar, soil fertility and climatic conditions (Oomah *et al.*, 2011).

The enzymatic treatment decreased the content of total phenol and hydrolysable and condensed tannins by 90%, 48% and 67%, respectively. The concentration of phosphorus in the medium increased considerably, by 118%. In a similar study sorghum grain treated through natural fermentation and the application of polyphenoloxidase and phytase enzymes resulted in a reduction of 62% for TF, 72% for HT and 81.3% for CT. The decrease in phenolic compounds during fermentation is associated with the polyphenoloxidase and may also be related to the microbial production of organic acids in the medium (Towo *et al.*, 2006).

Tannins are highly reactive and unstable compounds and can be physically, chemically and biologically degraded (Rodríguez *et al.*, 2008; Chen *et al*., 2001).

The tannase enzyme acts specifically on the ester and depsidic bonds, which are present in hydrolysable and condensed tannins, to originate compounds with simpler structures, such as gallic acid, digallic, catechins and glucose, which can be metabolised more easily by microorganisms (Osawa *et al*., 2000). Rodríguez *et al.* (2008) suggested that the biochemical route of hydrolysis of tannic acid with Lactobacillus plantarum tannase was the following: tannic acid→gallic acid and glucose→gallic acid is decarboxylated to form pyrogallol. The related enzymes were tannase and, possibly, gallate decarboxylase. (Kumar *et al.*, 1999) reported that the following biochemical route of degradation of tannic acid by Citrobacter freundii tannase: tannic acid→glucose and gallic acid→pyrogallol→acid 2-hydroxymuconic→pyruvate. The authors associated the biodegradation of tannic acid by *Citrobacter freundii* with the enzymes tannase, gallic acid decarboxylase and pyrogallol 1,2-dioxygenase.

Towo *et al.* (2006) found an 88% reduction of phytate and a consequent increase of 3.1% in the bioavailability of iron after the treatment of sorghum with natural fermentation and polyphenoloxidase and phytase enzymes, similar result was desmonstrated by Matuschek *et al*. (2001).

Biological assay

Table 2 displays the results for total ingestion, apparent digestibility coefficient of nitrogen and phosphorus and daily excretion of phosphorus by

Table 2. Total ingestion, apparent digestibility coefficient of nitrogen and phosphorus, phosphorus daily animal excrement

Groups	Total Ingestion (g)	ADC nitrogen (%)	ADC phosphorus (%)	Daily P excrement (mg)
RFB	185 ± 24^{a}	37 ± 8^{a}	-56 ± 13^{b}	38 ± 8^{a}
TFB	184 ± 21^{a}	41 ± 12^{a}	81 ± 17^{a}	7 ± 2^{b}

Results obtained from the ten animals mean followed by standard deviation.
The results in the same column followed by different letters indicate significant difference ($P < 0.05$) .
ADC, apparent digestibility coefficient; P, phosphorus.

the animals. It is important to note that no rats died during the study. The total ingestion and the apparent digestibility coefficient (ADC) of nitrogen in animals receiving diets with treated faba bean (TFB) and raw faba bean (RFB) have not showed significant difference ($P < 0.05$). Similar results were reported for sorghum diets 65% (Lizardo *et al*., 1995), 71% (Aning *et al.*, 1998), 78% (Al-Mamary *et al.*, 2001) and sorghum diet added 3-phytase from Hansenula polymorpha 93.2% and 6-phytase from Peniophora lycii, 93.7%, in the same concentration (500 U kg $^{-1}$) (Vallet *et al.*, 1994). The observed elevated fecal nitrogen excretion in animals fed on diets with raw faba bean could have resulted from either higher levels of endogenous nitrogen in faeces, impaired digestion of dietary protein, or both.

The ADC of phosphorus for the TFB group was 81%, this result was significantly different ($P < 0.05$) compared to raw faba bean. Guggenbuhl *et al.* (2007) replaced that diets formulates with different phytase present the following ADC of phosphorous to pigs 41% (Quantum phytase - 250 U kg^{-1}), 46% (Quantum phytase - 500 U kg^{-1}), 46% (Natuphos phytase - 500 U kg^{-1}) and 45% (Ronozyme P phytase - 740 U kg^{-1}). Liebert and Portz (2007) reported an ADC of phosphorous of 61% and 50% to diet formulated by 3-phytase from Hansenula polymorpha and 6-phytase from Peniophora lycii, respectively, in the same level (500 U kg^{-1}).

The ADC of phosphorous verified in our study is higher than the levels demonstrated in most studies that used diets enriched with phytases (Guggenbuhl *et al.*, 2007; Liebert and Portz, 2007), this difference in apparent phosphorus digestibility between the studies can be related to the method of enzymatic application; in our study, the faba bean was submitted to treatment prior to the diet preparation. In the majority of studies, phytase was added to the feed at the exact moment that it was made available to the animals; consequently, it acted only when it reached the gastrointestinal tract, and could be hindered due to adverse conditions, such as low pH, low water concentration, less-than-optimal temperature conditions, or contact with other enzymes, such as proteases. Therefore, it is recommended to treat the feed prior to its intake; a good alternative could be for companies that manufacture animal feed to treat the feed before it is received by the rural producer.

We verified a negative ADC of phosphorous (-56%) for the diet with RFB. Similarly, Goncalves et al. (2005) reported a negative digestibility for zinc in standardised fish diets (without the addition of phytase), prepared with sorghum, rice bran, extruded soy bran and maize gluten, was evaluated at -390%, -106%, -21% and -98%, respectively. The authors suggest that part of this mineral is mobilised from organic and/or water reserves. Zinc is an important essential trace metal, it functions in the organism are immune response onset and regulation, antioxidant activity, enzymatic cofactor, spermatogenesis and steroidgenesis, vitamin A metabolism, insulin storage and release, energetic metabolism, proteins synthesis, stabilisation of macromolecules, regulation of the ADN transcription and cellular division (Salgueiro *et al.*, 2000).

We verified that the phosphorus (P) daily excrement content was significantly ($P<0.05$) lower for the TFB group than that observed in the RFB group. This results confirms the positive effect of enzymes on the reduction of phosphorus excretion. From an environmental point of view, this is important information: once the enzymatic effectiveness of hydrolysing phytate in releasing P and improving its digestibility can be proven, we can reduce the addition of mineral-based phosphorus to animal diets. This will probably reduce both the product's final price and the excretion of phosphorus into the environment.

In Porres *et al.* (2008) study, it was observed that after applying phytase to a lupine-based diet for rats a daily P excretion was 11, 10.6 and 14.4 (mg day^{-1}) for the control group (casein-based diet), raw lupine and lupine with added phytase, respectively. The results for glucose, cholesterol, triacylglycerol, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels are presented in Table 3. Regarding glucose, it was found that the group fed with enzymatically-treated faba bean showed a significant plasmatic concentration ($P < 0.05$), higher than that measured in animals fed with RFB. Cholesterol was significantly higher ($P < 0.05$) for the RFB group

Table 3. Biochemical determinations in the serum of the studied animals

Groups	Glucose	Cholesterol	Triacylglycerol	AST (U L^{-1})	ALT (U L^{-1})
RFB	21 ± 5^{b}	125 ± 17^{a}	51 ± 4^{a}	145 ± 12^{a}	72 ± 8^{a}
TFB	29 ± 6^{a}	74 ± 12^{b}	53 ± 5^{a}	76 ± 8^{b}	67 ± 11^{a}

Results obtained from the ten animal mean followed by the standard deviation.
The results in the same column followed by different letters indicate significant difference ($P < 0.05$) .
*Results expressed in mg 100 mL^{-1}

Table 4. Concentration of calcium, phosphorus and iron minerals in the plasma and ashes in the studied animals' femur

Groups	Calcium*	Phosphorus*	Iron†	Femur ashes (%)
RFB	14 ± 2^{a}	12 ± 2^{a}	85 ± 8^{a}	28 ± 2^{a}
TFB	14 ± 2^{a}	13 ± 1^{a}	82 ± 6^{a}	30 ± 2^{a}

Results obtained from ten animals mean followed by the standard deviation.
The results in the same column followed by different letters indicate significant difference ($P < 0.05$) .
*Results expressed in mg 100 mL^{-1}. †Results expressed in µg 100 mL^{-1}.

compared to the TFB group. Triacylglycerols (TAG) did not differ statistically ($P < 0.05$) between the groups. It is possible that higher plasmatic glucose concentration is related to a higher digestibility of nutrients in general. Plasmatic cholesterol increase could be the result of changes in metabolic regulation regarding the synthesis of cholesterol.

It is significant that the RFB group showed a statistically higher quantity ($P < 0.05$) of AST enzyme compared to the TFB group, which is probably related to a hepatic disturbance caused by anti-nutritional compounds in the non-treated faba bean. Aning *et al.* (1998) previously reported an increase in ALT enzyme in a diet prepared with progressive concentrations of a sub-product of malted sorghum for rats, this ALT increase is related to the increase in cyanide in the diet, originated from the sub-product malted sorghum.

Adebiyi *et al*. (2008) found that diets prepared with sorghum treated with an amylase from Rizophus sp. resulted in greater weight gain and improved hematological responses than that formulated with untreated sorghum. However, animals that received diets containing both untreated and treated sorghum showed adverse effect in the kidneys and liver cells.

In Table 4, the results for calcium, phosphorus and iron minerals existing in the serum and ashes from the femur are shown. The table shows that both the enzymatically-treated and raw faba bean groups have similar plasmatic levels of calcium, phosphorus and iron; the same occurs for femur ash content, with no significant difference ($P < 0.05$). Porres *et al*. (2008) study reported a reduction in mineral absorption (Mn, Fe, K and Zn) and an increase in phosphorus excretion for rats fed with phytasetreated lupine compared to the raw lupine-based diet. The authors explain this mineral reduction as a consequence of the increase in phosphate after phytase's action on the phytate, due to phosphate's ability to be linked to divalent cations, forming insoluble complexes, and to the presence of fibers.

This study indicated that the enzymatic treatment was effective in the reduction of tannins and phytate levels, and in the increase of free phosphorus concentration, which is very promising. The biological assay demonstrated that the enzymatic treatment of faba bean flour led to an apparent improvement in phosphorus digestibility, a reduction in phosphorus excretion, better biochemical indices for glucose and cholesterol and lower activity for the aspartate aminotransferase (AST) and alanine aminotransferase (ALT), compared to raw faba bean. However, enzymatic treatment had no significant effect on apparent protein digestibility or on concentrations of phosphorus, iron, or plasmatic calcium, as well as on the minerals existing in the femur.

Acknowledgement

This work was supported by National Science Foundation of China (31201318).

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