



Kunitz trypsin Inhibitor and phytic acid levels in conventional and genetically modified soybean seeds from Londrina and Ponta Grossa, South Brazil

Olívio Fernandes Galão^{1*}, Mercedes Concórdia Carrão-Panizzi², José Marcos Gontijo Mandarino³, Rodrigo Santos Leite³, Thiago Claus⁴ and Jesuí Vergílio Visentainer⁴

¹Departamento de Química, Universidade Estadual de Londrina, Rod. Celso Garcia Cid, Km 380, 86051-990, Londrina, Paraná, Brazil. ²Departamento de Trigo, Empresa Brasileira de Pesquisa Agropecuária, Passo Fundo, Rio Grande do Sul, Brazil. ³Empresa Brasileira de Pesquisa Agropecuária, Centro Nacional de Pesquisa de soja, Londrina, Paraná, Brazil. ⁴Departamento de Química, Universidade Estadual de Maringá, Maringá, Paraná, Brazil. *Author for correspondence. E-mail: thiagoclaus@hotmail.com

ABSTRACT. Differences in the levels of antinutritional components - Kunitz trypsin inhibitor and phytic acid - were determined for conventional and genetically modified soybean cultivated in Londrina and Ponta Grossa, South Brazil. Trypsin inhibitor levels in the conventional cultivars of Londrina and Ponta Grossa varied from 14.56 mg g⁻¹ (BRS 267) to 20.40 mg g⁻¹ (BRS 261) and from 13.51 mg g⁻¹ (BRS 232) to 19.65 mg g⁻¹ (BRS 268), respectively, whereas in the genetically modified (GM) cultivars, they were found to be 14.16 mg g⁻¹ (BRS 242 RR) to 18.21 mg g⁻¹ (BRS 255 RR) for Londrina, and 13.68 mg g⁻¹ (BRS 244 RR) to 18.73 mg g⁻¹ (BRS 256 RR) for Ponta Grossa. Average levels of phytic acid in the Londrina's conventional and GM samples were estimated as 2.05 and 1.78 mg g⁻¹, respectively, and in the Ponta Grossa conventional and GM samples, the respective average values were lower, 1.60 and 1.51 mg g⁻¹.

Keywords: enzymes, genetic improvement, antinutritional factors.

Teor de inibidor de tripsina Kunitz e ácido fítico em sojas convencionais e transgênicas de Londrina e Ponta Grossa do Sul do Brasil

RESUMO. Diferenças em níveis de inibidor de tripsina Kunitz e ácido fítico foram determinadas para cultivares de soja convencionais e transgênicas cultivadas em Londrina e Ponta Grossa, Sul do Brasil. Os níveis de inibidor de tripsina na região de Londrina e Ponta Grossa para variedades convencionais variaram de 14,56 mg g⁻¹ (BRS 267) a 20,40 mg g⁻¹ (BRS 261) e de 13,51 mg g⁻¹ (BRS 232) a 19,65 mg g⁻¹ (BRS 268), respectivamente, enquanto para as variedades transgênicas foram encontrados valores de 14,16 mg g⁻¹ (BRS 242 RR) a 18,21 mg g⁻¹ (BRS 255 RR) para Londrina e 13,68 mg g⁻¹ (BRS 244 RR) a 18,73 mg g⁻¹ (BRS 256 RR) para Ponta Grossa. A média de teor de ácido fítico em variedades convencionais e transgênicas de Londrina teve variação de 2,05 a 1,78 mg g⁻¹, respectivamente, enquanto para as mesmas variedades de Ponta Grossa, a média variou de 1,60 a 1,51 mg g⁻¹.

Palavras-chave: enzimas, melhoramento genético, fatores antinutricionais.

Introduction

Trypsin is an enzyme produced by the pancreas that enables the digestion of proteins present in animal and human feeding (LEBENTHAL; LEE, 1980; MANDARINO, 2010). Protease inhibitors, constituting one of the soybean protein fractions, represent approximately 6.0% of all existing proteins (BRANDON et al., 1993; MANDARINO, 2010). The presence of these inhibitors in soy-based foods and dietetic products may cause pancreatic nodular hyperplasia in monogastric animals, as shown by studies with guinea pigs (BARROS et al., 2008). In soybeans, two different protease inhibitors can be found - chymotrypsin, also called the Bowman-Birk

inhibitor (BBI), and trypsin, known as the Kunitz trypsin inhibitor (KTI) (STAHLHUT; HYMOWITZ, 1983; MANDARINO, 2010). About 80% of trypsin activity is due to the KTI (BRANDON et al., 1989; MANDARINO, 2010). This feature is essential for genetic improvement of soybean cultivars, concerning their quality, since they are used as a food source for humans and animals. The identification of genetic and environmental factors appears to be important in defining gene sources or culture conditions in order to improve the characteristics of soybeans with reduced levels of the above-mentioned compounds. Thermal processing of grains has made it possible to

achieve about 80 - 90% reduction in the KTI activity (JIAO et al., 1992). Although the protease inhibitors are considered to be one of the antinutritional factors observed in soybeans and other legumes, several *in vitro* studies have exhibited their inhibitory action on the expression of some genes in breast, skin, bladder, colorectal, lung, pancreatic, mouth and esophageal cancer, in addition to their antioxidant properties (CÁRDENAS et al., 2010; BRANDON et al., 1993).

Phytic acid is a naturally occurring organic compound formed during maturation of most legume seeds and cereal grains, and it exists in the form of several isomers (DAI et al., 2010). The effect of these isomers salt forms is crucial for storing phosphorus in seeds (RIBEIRO et al., 1999).

Among the bioactive compounds in soybeans, phytic acid has characteristics that are both harmful and beneficial to the human body. The harmful properties refer to the chelating effect on divalent minerals, since six anionic phosphate groups that contain oxygen atoms with free electrons are present in their structure, thus facilitating the complexation of phytic acid with divalent metals, such as copper, zinc, iron and calcium. Such complexes can lead to decreased bioavailability of these minerals in the body, altering their solubility, functionality, digestibility and nutrient absorption (HARLAND; NARULA, 1999; WOYENGO et al., 2010). This phenomenon is known as the antinutritional effect. This effect is more severe in animals, since lower bioavailability of the minerals in the presence of phytic acid has been experimentally confirmed for their diet, whereas the results in humans have shown little or no changes in the quantities of the minerals normally found in the body, except in children, malnourished adults, pregnant women and elderly persons (LAJOLO et al., 2004; MANDARINO, 2010).

Beneficial properties of phytic acid have received much attention in the last decade. Phytic acid can act as a potent antioxidant, once it binds to mineral ions, and lipid oxidation in the body is impaired by these ions. It is considered that at low concentrations the antioxidant properties may be responsible for both the anticarcinogenic effects and the reduction of kidney and cardiovascular disorders in the body; therefore, it is important to analyze the phytic acid impact on human health (SZKUDELSKI, 1997; SHAMSUDDIN, 2002; LAJOLO et al., 2004; WOYENGO et al., 2010).

Thus, the objective of the study was to determine the differences in levels of antinutritional components - Kunitz trypsin inhibitor and phytic acid - for conventional and genetically modified soybean cultivated in Londrina and Ponta Grossa, South Brazil.

Material and methods

Kunitz Trypsin Inhibitor (KTI)

The KTI levels were quantified in 14 conventional (Embrapa-48, BRS 184, BRS 213, BRS 232, BRS 233, BRS 257, BRS 258, BRS 259, BRS 260, BRS 261, BRS 262, BRS 267, BRS 268, BRS 282) and 6 genetically modified (BRS 242 RR, BRS 244 RR, BRS 245 RR, BRS 246 RR., BRS 255 RR and BRS 256 RR) soybean cultivars according to the methodology developed by Kakade et al. (1974) and modified by Hammerstrand et al. (1981).

Sample preparation

Five grams of samples ground in a cold mill were placed in 250 mL Erlenmeyer flasks. In order to remove grease, approximately 50 mL of hexane were added, and the mixtures were constantly agitated with a magnetic stirrer for 12 hours at room temperature. After this procedure, the hexane was drained off, and the samples were transferred to Petri dishes and placed into an evaporation chamber to completely evaporate the solvent. Then, the degreased samples were sieved through a 100 mesh sieve, and the "flour" obtained was subjected to the thin-layer extraction procedure for the KTI (HAMMERSTRAND et al., 1981).

KTI extraction

The KTI extraction experiments were performed in triplicate. 1.0 g of the degreased fine powder samples and 50 mL of 0.01 N NaOH were placed into 250 mL Erlenmeyer flasks. The mixtures were agitated with a magnetic stirrer for 3 hours at room temperature. The pH of extracting solutions was adjusted to 9.2. Then, 2.0 mL aliquots of the solutions were pipetted into a 100 mL volumetric flask, and the volume was completed with distilled water. These diluted extracts were further used for the KTI quantification.

KTI determination

KTI quantification procedures were carried out in triplicate, according to Hammerstrand et al. (1981). After the extraction experiments, three aliquots of the diluted extracts were taken for each extraction replication. Five test tubes were prepared: three of them were used for the KTI quantification in the extract samples (Tubes 1, 2, and 3), one tube was "blank" (Tube 4), and the other one contained the KTI standard (Tube 5). 2.0 mL of the diluted extracts were added to Tubes 1, 2, 3, and 4, and 2.0 mL of distilled water were added to Tube 5. Then, 2.0 mL of KTI solution (0.0020 g KSTI 100 mL⁻¹ 0.001 N HCl) were added to Tubes 1, 2, 3, and 5.

Then, the tubes were agitated and placed in a water bath at 37°C for 10 min. After that, 5.0 mL of BAPA (benzoyl-DL-arginine-p-nitroanilide) preheated at 37°C were added to the five tubes.

The BAPA solution was prepared by dissolving 0.080 g of BAPA in 2.0 mL of dimethyl sulfoxide; the mixture was transferred to a 200 mL volumetric flask, and the volume was completed with the Trizma buffer preheated at 37°C. The solution was stable for 4h.

The Trizma buffer solution was prepared by dissolving 1.21 g of Trizma and 0.59 g of calcium chloride dihydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) in 180 mL of distilled water, and the pH was adjusted to 8.2 using 1 M HCl; the mixture was transferred to a 200 mL volumetric flask, and the volume was completed with distilled water. The solution was stable for 8 hours.

After the addition of the BAPA reagent, each tube was agitated and then immediately returned to the water bath at 37°C for 10 minutes. Afterwards, 1.0 mL 30% acetic acid was added to all the tubes, which were agitated again. After this step, 2.0 mL of the KTI were added to Tube 4 (“blank”). All the solutions were filtered through Whatman No. 3 filter paper, and their absorbances were determined using a spectrophotometer at a wavelength of 410 nm. It has been recommended to set the time interval between the introduction of the solutions into the wake of the tubes at 30 seconds, which would be enough to homogenize the solutions and mark the exact reaction time. The KTI levels (contents) were calculated as follows:

$$KTI \text{ (mg.g}^{-1}\text{)} = \frac{\text{Absorbance standard} - \text{Absorbance of the sample} \times 2500}{9.5 \times \text{sample weight(g)}}$$

Phytic acid

Phytic acid levels in the studied soybean seeds were determined according to the method proposed by Latta and Eskin (1980) and modified by Bordignon et al. (2000).

Phytic acid extraction

To perform the phytic acid extraction, 1.0 g of the “flour” samples was transferred to 125 mL Erlenmeyer flasks, mixed with 10 mL of 0.8M HCl, and subjected to constant agitation at 2-250 rpm. Then, the extraction solutions were centrifuged at 2000 rpm for 10 minutes. The supernatants were used for the separation of the phytic acid on an ion-exchange column (HONIG et al., 1984).

Phytic acid isolation

For the procedures of isolation of the phytic acid, an ion exchange column was used, containing 0.5 g

of a Dowex resin (1 x 40-50) loaded on an inch-thick “bed” of glass wool. The column was washed with distilled water and a balanced 0.7 M NaCl solution. Then, 2.0 mL of the extracting solutions (supernatants) were injected into the column. Undesirable cations were eluted from the column using 0.1 M NaCl, and the phytic acid was eluted with 10 mL of 0.7 M NaCl. The entire eluate was collected for the phytic acid spectrophotometric determination.

Phytic acid spectrophotometric determination

To carry out the phytic acid spectrophotometric analyses, a standard curve was constructed for the dodeca-sodium phytate water solution. To prepare a phytic acid working solution, 15 mL of the phytic acid stock solution, previously obtained by dissolving 35.29 mg of 85% phytic acid in 1000 mL of distilled water, were diluted in distilled water in a 100 mL volumetric flask. Then, to construct the curve, 0.1-0.5 mL aliquots of this working solution were transferred to the test tubes and diluted to 3.0 mL with distilled water. Afterwards, 1.0 mL of Wade reagent was added, and the mixtures were measured spectrophotometrically at 500 nm. The Wade reagent was prepared as follows: 0.03 g of ferric chloride monohydrate ($\text{FeCl}_3 \cdot \text{H}_2\text{O}$) and 0.30 g of sulfosalicylic acid were dissolved in distilled water, and the final volume of this solution was completed to 100 mL. The ideal absorbance of the Wade reagent should range between 0.490 and 0.530.

Results and discussion

Figure 1 summarizes the levels of KTI obtained. On average, they did not differ significantly for the conventional cultivars of Londrina and Ponta Grossa (17.19 and 17.20 mg g^{-1} , respectively). The transgenic cultivars presented a slight variation, with average values of 16.75 and 15.77 mg g^{-1} for Londrina and Ponta Grossa, respectively. The phytic acid levels in the conventional cultivars of Londrina and Ponta Grossa (Figure 2) had average values of 1.81 and 1.41 mg g^{-1} , respectively. As to the GM crops, the average values were 1.57 mg g^{-1} (Londrina) and 1.33 mg g^{-1} (Ponta Grossa). The phytic acid levels determined for the cultivars grown in Ponta Grossa were lower than for those sown in Londrina, probably due to the lower phosphorus contents in Ponta Grossa soils, with the subsequent synthesis of smaller banded grains. Table 1 shows the variation of KTI levels by region and genetic varieties.

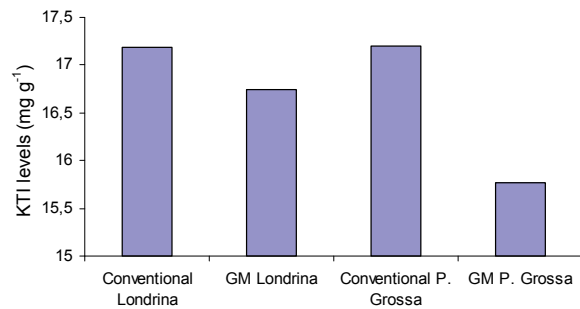


Figure 1. KTI levels in 20 soybean cultivars (average values).

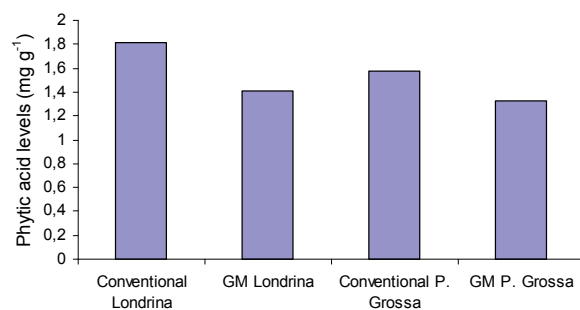


Figure 2. Phytic acid levels in 20 soybean cultivars (average values).

Table 1. Mean values (standard deviations), mg g⁻¹, for the KTI levels in soybean cultivars of Londrina and Ponta Grossa.

Conventional cultivars	Londrina	Ponta Grossa
Embrapa 48	18.54 ± 0.10 ^{abcd}	18.82 ± 0.03 ^{abc1}
BRS 184	18.69 ± 0.08 ^{ab}	15.13 ± 0.04 ^{de}
BRS 213	17.91 ± 0.17 ^{bcd}	15.91 ± 0.03 ^{cde}
BRS 232	17.12 ± 0.12 ^{cdefg}	13.51 ± 0.19 ^e
BRS 233	17.27 ± 0.14 ^{cdefg2}	16.30 ± 0.10 ^{bcd2}
BRS 257	17.19 ± 0.12 ^{cdefg}	18.89 ± 1.70 ^{ab}
BRS 258	15.27 ± 0.07 ^{ghi}	17.17 ± 1.71 ^{abcd}
BRS 259	17.48 ± 0.08 ^{bcddefg}	18.15 ± 0.13 ^{abc}
BRS 260	19.37 ± 0.04 ^{abc3}	18.43 ± 0.02 ^{abc3}
BRS 261	20.40 ± 0.07 ^a	16.38 ± 0.02 ^{bcd}
BRS 262	16.59 ± 0.11 ^{defgh4}	16.75 ± 2.17 ^{bcd4}
BRS 267	14.56 ± 0.01 ^{hi}	16.97 ± 0.81 ^{abcd}
BRS 268	15.73 ± 0.07 ^{efghi}	19.65 ± 0.47 ^a
BRS 282	15.70 ± 0.02 ^{ghi}	18.75 ± 0.08 ^{abc}
Transgenic cultivars (GM)	Londrina	Ponta Grossa
BRS 242 RR	14.16 ± 0.02 ⁵	14.48 ± 0.28 ^{de5}
BRS 244 RR	14.30 ± 3.17 ⁶	13.68 ± 0.10 ⁶
BRS 245 RR	17.62 ± 0.22 ^{bcddef}	14.73 ± 0.04 ^{de}
BRS 246 RR	17.96 ± 0.13 ^{bcddef6}	16.68 ± 0.14 ^{bcd6}
BRS 255 RR	18.21 ± 0.81 ^{abcd}	16.32 ± 0.02 ^{bcd}
BRS 256 RR	18.17 ± 0.07 ^{abcd7}	18.73 ± 0.08 ^{abc7}

Means followed by the same characters are not different as regards the planting sites according to the Tukey's test (at 5%).

Germination may increase the phytase activity by mobilizing phytate, which becomes more available for the divalent minerals, such as copper, zinc, cobalt, manganese, iron and calcium, at lower levels, thus improving the nutritional quality of germinated soybeans or sprouts (BAU; DEBRY, 1979). Table 2 shows the variation of the content of phytic acid by region and genetic varieties.

Table 2. Mean values (standard deviations), mg g⁻¹, for the phytic acid levels in soybean cultivars of Londrina and Ponta Grossa.

Conventional cultivars	Londrina	Ponta Grossa
Embrapa 48	1.56 ± 0.01 ^{hi}	1.79 ± 0.04 ^{abcd}
BRS 184	1.55 ± 0.08 ¹	1.45 ± 0.04 ^{ghj1}
BRS 213	2.16 ± 0.03 ^{bcddef}	1.86 ± 0.03 ^{ab}
BRS 232	2.16 ± 0.15 ^{cdef}	1.65 ± 0.11 ^{cdef}
BRS 233	1.79 ± 0.10 ^{ghi}	1.53 ± 0.14 ^{fgh}
BRS 257	2.21 ± 0.06 ^{bcd}	1.73 ± 0.09 ^{abcde}
BRS 258	2.11 ± 0.08 ^{cdef}	1.59 ± 0.01 ^{efg}
BRS 259	2.07 ± 0.18 ^{def}	1.61 ± 0.11 ^{defg}
BRS 260	1.74 ± 0.04 ^{ghi}	1.17 ± 0.06 ^k
BRS 261	2.41 ± 0.19 ^{ab}	1.53 ± 0.08 ^{fghi}
BRS 262	2.30 ± 0.08 ^{bcd}	1.33 ± 0.05 ^{jk}
BRS 267	2.36 ± 0.05 ^{abc}	1.68 ± 0.02 ^{cdef}
BRS 268	2.39 ± 0.40 ¹	1.60 ± 0.03 ^{defg}
BRS 282	1.96 ± 0.12 ^{efg2}	1.92 ± 0.04 ²
Transgenic cultivars (GM)	Londrina	Ponta Grossa
BRS 242 RR	1.81 ± 0.05 ^{gh3}	1.73 ± 0.04 ^{abcde3}
BRS 244 RR	1.21 ± 0.07 ⁷	1.39 ± 0.10 ^{bij}
BRS 245 RR	1.77 ± 0.09 ^{ghi}	1.25 ± 0.09 ^{jk}
BRS 246 RR	1.92 ± 0.07 ⁸	1.28 ± 0.08 ^{jk}
BRS 255 RR	1.80 ± 0.12 ^{ghi4}	1.82 ± 0.02 ^{abc4}
BRS 256 RR	2.19 ± 0.06 ^{bcd}	1.63 ± 0.03 ^{defg}

Means followed by the same characters are not different as regards the planting sites according to the Tukey test (at 5%).

Conclusion

The KTI elimination or reduction through genetic improvement techniques can significantly enhance the digestibility and nutritional value of soy-based products. Heat treatment may inactivate the largest portion of KTI. However, the BBI, which presents a great number of disulfide bonds (-SS-) in its molecule, appears to be quite resistant to the denaturing action of either heat or pepsin. KTI activity may be completely inhibited by heating grains with water at 100°C for 30 min. Phytic acid in many plant species is completely degraded after the heat treatment at 50°C and pH 5.0 for 200 min.

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Received on June 28, 2012.

Accepted on February 4, 2013.

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