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Effects of heating on protein quality of soybean flour devoid of Kunitz inhibitor and lectin

F.P.P. Machado [a], J.H. Queiróz [a,*], M.G.A. Oliveira [a], N.D. Piovesan [b], M.C.G. Peluzio [c], N.M.B. Costa [c], M.A. Moreira [a]

[a] Department of Biochemistry and Molecular Biology, Federal University of Viçosa, Viçosa, Brazil
[b] Institute of Biotechnology Applied to Agriculture (BIOAGRO), Federal University of Viçosa, Viçosa, Brazil
[c] Department of Nutrition and Health, Federal University of Viçosa, Viçosa, Brazil

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Abstract

The effects of autoclaving on protein quality of soybean flours prepared from a conventional soybean (CSB) and an isoline lacking Kunitz inhibitor and lectin (KFLF) were studied. The heating was efficient in the urease, trypsin inhibitors and lectin inactivation, being 15 min sufficient to reduce more than 90% of these compounds and provide protein solubility over 80%. The results of PER, NPR and weight gain showed that heating equally improved the nutritional quality of both soybean flours, although higher autoclaving time was required for KFLF. No significant improvement was observed on NPU and *in vivo* digestibility of the diets containing KFLF at any heating time. As these later results were similar to those obtained with diets containing CSB, they show the importance of the heating to improve the nutritional value and show that other components rather than trypsin inhibitors and lectins impair the nutritive value of raw soybean.

Keywords: *Glycine max*; Kunitz inhibitor; Lectin; Protein quality; Heating

1. Introduction

Soybean has been widely used in human and animal nutrition because of its favorable agronomic characteristics, relatively low price, high quantity and quality of its protein and oil (Liu, 2000), and important functional properties for the development of different types of foods for humans (Traina & Breene, 1994). Moreover, the consumers' awareness of the beneficial effects reported on health has increased its consumption (Albertazzi, 2002; Bus & Worsley, 2003).

However, the presence of several antinutrients, mainly Kunitz and Bowman–Birk proteases inhibitors and lectins reduces the nutritional value of soybean (Liener, 1994). They may cause unfavorable physiological effects (Buttle et al., 2001; Vasconcelos et al., 2001) and decrease weight gain in animals (Palacios et al., 2004). Processing based on wet heat or biochemical treatments are able to inactivate these components (Bajpai, Sharma, & Gupta, 2005; Liener, 1994; Osman, Reid, & Weber, 2002), and heat is the most efficient. Another way to improve the nutritive value of soybean is the development of varieties lacking Kunitz inhibitor and lectins (Douglas, Parsons, & Hymowitz, 1999; Monteiro, Moreira, Costa, Oliveira, & Pires, 2003; Vasconcelos et al., 2001). Even better results are obtained when genetically improved soybeans are processed under wet heat (Friedman, Brandon, Bates, & Hymowitz, 1991; Palacios et al., 2004).

The aims of this work were to assess the result of the genetic elimination of Kunitz inhibitor and lectin from

* Corresponding author. Tel.: +55 31 3899 3045.
E-mail address: jqueiroz@ufv.br (J.H. Queiróz).

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soybean as well as the effects of heating on protein quality.

2. Materials and methods

2.1. Chemicals

Bovine pancreatic trypsin (type III), *N*α-benzoyl-DL-arginine-*p*-nitroanilide (D,L-BApNA), dimethyl sulfoxide and urease were purchased from Sigma Chemical Company (St. Louis, MO). Mineral and vitamin mixtures were purchased from Rhoster (Vargem Grande Paulista, São Paulo, Brazil). All the other chemicals used were of analytical grade.

2.2. Soybean samples and processing

The two high-protein soybean lines used in this work were originated from the Monarca variety through backcross and developed by the Soybean Quality Breeding Program of the Institute of Biotechnology Applied to Agriculture at Federal University of Viçosa (BIOAGRO/UFV). One line contained Kunitz inhibitor and lectins, and the other lacked these antinutrients. The donor lines of high protein content, lacking KTI and lectin genes were BARC-8, BRM 925 262 and Columbia, respectively. Flours were prepared from conventional (CSB) and improved (KFLF) soybean lines according to the procedure described by Monteiro et al. (2003) and sifted through a 0.5 mm opening sieve. Then, they were placed in a 1-cm deep layer in erlenmeyers, closed and autoclaved (1.7 atm, 121 °C) for 5, 10, 15 and 25 min.

2.3. Analytical methods

2.3.1. Proximate composition and urease activity

Proteins (method 36/IV), lipids (method 32/IV), moisture (method 12/IV) and ash (method 18/IV) were determined according to the methods described by Brasil (2005). The factor 6.25 was used for conversion from nitrogen to crude protein. The determination of urease activity was conducted as described by AOCS (1978).

2.3.2. Protein solubility

Protein solubility measurement was based on the soluble nitrogen determination in 0.2% sodium hydroxide solution and the results were expressed as percent of soluble nitrogen in relation to total nitrogen in sample (Brasil, 1991, with modifications). Soybean flour (2 g) was stirred with 100 mL of 0.2% sodium hydroxide solution at 150 rpm for 20 min at 25 °C and centrifuged at 3840*g* for 15 min. Supernatant (15 mL) was taken for protein determination using the Kjedhal method described by Brasil (2005). The soluble nitrogen percentage (SN%) was determined according to the formulas:

$$\text{Soluble nitrogen\% (SN\%)} = \left[\frac{(V_S - V_B) \times 0.05 \times f \times 1.4}{SW}\right] \times 100$$

$$\text{Nitrogen solubility index\% (NSI\%)} = \frac{SN\%}{TN\%} \times 100$$

where V_S is the 0.05 M HCl volume (mL) used for sample titration, V_B the 0.05 M HCl volume (mL) used for blank titration, f the HCl standardization factor, SW the sample weight and TN is the sample total nitrogen.

2.3.3. Electrophoresis

The one-dimensional SDS–PAGE was carried out according to Laemmli (1970) with modifications. The stacking gel contained 6% of acrylamide–bisacrylamide in 0.5 M Tris–HCl buffer (pH 6.8) and 0.4% of SDS. The separation gel contained 14% of acrylamide–bisacrylamide in 0.5 M Tris–HCl buffer (pH 8.8) and 0.4% of SDS. The running buffer was prepared with 0.5 M of Tris–HCl buffer (pH 8.8), 1.92 M of glycine and 1% of SDS. Soybean flour (15 mg) was dissolved in 500μL of buffer (92 mM of Tris–HCl, pH 8.1, 23 mM of $CaCl_2 \cdot 2H_2O$, 20% of sucrose). The extract was centrifuged at 15,700*g* for 15 min. The supernatant (60 μL) was added to 30 μL of sample buffer (0.188 M of Tris–HCl, pH 6.8, 30% of glycerol, 6.9% of SDS, 2% of mercaptoethanol, bromophenol blue) and boiled at 100 °C for 3 min. Electrophoresis started at 70 V and was tuned up to 120 V after a tracer dye entered the resolving gel. The gels were stained in solution containing 0.15% of Coomassie Brilliant Blue R-250, 9% of acetic acid and 45% of methanol solution. The unstaining solution contained 7.5% of acetic acid and 25% of methanol.

2.3.4. Trypsin inhibitor activity

Trypsin inhibition was performed by modifying the method originally described by Erlanger, Kokowosky, and Cohen (1961) and the results were expressed as milligram of inhibited trypsin/gram of extract protein. Defatted soybean flour (100 mg) was stirred with 15 mL buffer (0.1 M of Tris–HCl, pH 8.2, containing 20 mM of $CaCl_2$) at room temperature for 3 h. After, the extract was centrifuged at 35,600*g* for 30 min. Then, 100 μL of the extract suspension, 50 μL of trypsin solution (0.1% in 1 mM HCl) and 450 μL of buffer (0.1 M of Tris–HCl, pH 8.2, containing 20 mM of $CaCl_2$) were mixed in tube and incubated at room temperature for 5 min. Aliquot of 500 μL of this reaction was added into another tube containing 500 μL of buffer (0.1 M Tris–HCl, pH 8.2, containing 20 mM $CaCl_2$) and 500 μL of D,L-BApNA solution (200 μL from stock solution, prepared with 130.47 mg D,L-BApNA in 5 mL dimethyl sulfoxide, diluted with 10 mL buffer). After 5 min, the reaction was terminated by adding 300 μL of acetic acid (60%), mixed thoroughly, and absorbance was measured at 410 nm against reagent blank (enzyme and extract were substituted by the buffer).

The measurement of extract crude protein was carried out according to the methodology outlined by Smith et al. (1985) using bovine serum albumin (0.2%) as standard. Reagents *A* (1% of bicinchoninic acid, 0.16% of potassium tartrate, 1.8% of sodium carbonate, 0.4% of sodium hydroxide and 0.95% of sodium bicarbonate, pH 11.3) and *B* (1% of copper sulfate) were mixed in ratio 50:1 and to 1 mL of this solution 50 μL of extract suspension was added. After 30 min at 37 °C, the reaction was cooled for 20 min and the absorbance was measured at 562 nm. The trypsin inhibitor activity was calculated as follows:

$$\text{Milligram of inhibited trypsin/gram of extract protein} = \frac{(A \times B)}{C \times 1000 \times P}$$

where A is the difference between the enzyme control and sample absorbance, B the sample dilution factor, C the trypsin factor [0.019, Kakade, Simons, Liener, and Lambert (1972)], and P is the extract protein concentration in g/mL.

2.3.5. Lectin haemagglutinating activity

Haemagglutinating activity was estimated according to the method of Oliveira et al. (2003) using rabbit erythrocytes. The haemagglutinating activity was expressed as the reciprocal of the minimum concentration of the sample (expressed as mg/mL) that causes agglutination of rabbit erythrocytes (Friedman et al., 1991).

2.4. Protein quality evaluation

Male weaning 23-day-old Wistar rats weighing 50 ± 5 g were randomly divided into 12 groups of six rats. They were housed individually in wired-bottom cages in a temperature controlled room (25 °C) with 50% relative humidity and 12 h photoperiod. Food and water were given *ad libitum* during fourteen days of the experiment. A basal diet (nitrogen-free) was prepared based on the formulation described by Reeves, Nielsen, and Fahey (1993) for growing rodents (AIN-93G) (Table 1). The control diet was prepared incorporating casein to the basal diet. The experimental diets contained CSB or KFLF heated for 0, 5, 10, 15 or 25 min. Control and experimental diets contained 10% crude protein. Protein Efficiency Ratio (PER), Net Protein Utilization (NPU) and Net Protein Ratio (NPR) were determined as described by De Paula et al. (2004). Feces were collected between the 6th and 9th days and the true digestibility was evaluated according to Monteiro et al. (2003). Body weight gain, PER, NPR, NPU and true digestibility were measured after a 14-day experiment.

Table 1
Composition of basal diet[a]

Components	(g/100 g)
Soybean oil	7.00
Sucrose	10.00
Cellulose	5.00
Salt mixture	3.50
Vitamin mixture	1.00
Dextrinized cornstarch	13.20
L-Cystine	0.30
Choline bitartrate	0.25
Cornstarch	To make up 100

[a] The basal diet was prepared based on the formulation (AIN-93G) described by Reeves et al. (1993) for growing rodents.

2.5. Statistical analysis

Results were expressed as mean ± standard deviation of triplicate obtained by a mean of three determinations. The data were analyzed using the software SAEG 9.0 (Ribeiro, 2001). Significant differences were determined at the $p < 0.05$ level. The averages of the treatments were used for regression analyses.

3. Results and discussion

3.1. Proximate chemical composition

The breeding did not promote great differences on the evaluated components (Table 2). Both CSB and KFLF showed high protein content, although the oil content was moderate. The values obtained in the present work were similar to those previously reported to other soybean varieties (Monteiro et al., 2003; Yamada, Barcelos, Sousa, & de Lima, 2003).

3.2. Urease activity (UA)

The heating treatment applied in this study was efficient to inactivate urease (Fig. 1). The exponential model was better adjusted for CSB ($UA = 2.39 \times 0.82 \times t$, $r^2 = 0.97$, $p < 0.05$) and KFLF ($UA = 2.89 \times 0.86 \times t$, $r^2 = 0.73$, $p < 0.05$). The values obtained in the current experiment were in the range reported in other works (Grieshop et al., 2003; White, Campbell, & Mcdowell, 2000).

Different soybean samples with equal or similar urease activity may contain different quantity of trypsin inhibitors. In the current study, CSB and KFLF showed urease activities of 0.10 and 0.12 ΔpH, respectively, after 15 min heating while trypsin inhibition for the same samples was 3.1 and 1.4 mg trypsin inhibited/g protein (Fig. 2).

Values of urease activity lower than 0.3 ΔpH indicate adequate inactivation of trypsin inhibitors and lectins

Table 2
Proximate chemical composition of soybean flours

Samples[a]	Proteins[b]	Lipid[b]	Moisture[b]	Ash[b]
CSB (%)	43.96 ± 0.52	15.83 ± 0.08	9.12 ± 0.04	5.22 ± 0.06
KFLF (%)	45.55 ± .06	15.84 ± 0.06	9.40 ± 0.08	5.23 ± 0.03

[a] Conventional (CSB); Kunitz and lectin free (KFLF) soybean flours.
[b] Values are average ± standard deviation of triplicates analysis.

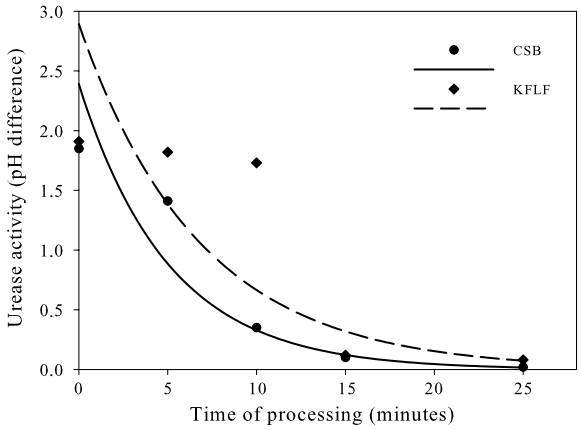

Fig. 1. Urease activity of conventional (CSB) and improved (KFLF) soybean flours submitted to different heating times.

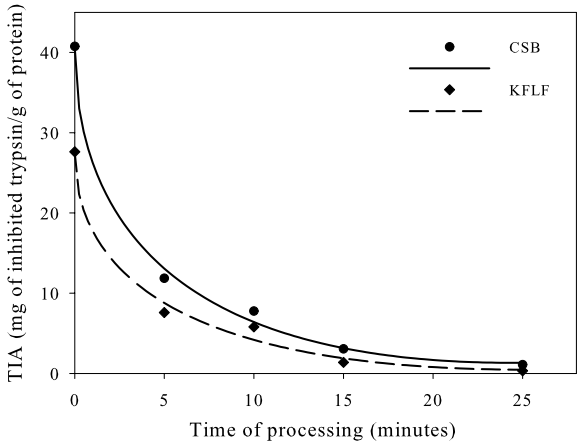

Fig. 2. Trypsin inhibitory activity of conventional (CSB) and improved (KFLF) soybean flours submitted to different heating times.

(Anderson-Hafermann, Zhang, & Parsons, 1992; Olguin et al., 2003; White et al., 2000; Wiriyaumpaiwong, Soponronnarit, & Prachayawarakorn, 2004). According to the results of the current study, adequate inactivation was achieved by autoclaving both CSB and KFLF for 15 min.

3.3. Trypsin inhibitor activity (TIA)

The genetic elimination of Kunitz inhibitor reduced the trypsin inhibition (Fig. 2). The square root model was better adjusted to the experimental data of CSB ($TIA = 40.56 - 15.88 \times t^{1/2} + 1.6 \times t$, $r^2 = 0.99$, $p < 0.05$) and KFLF ($TIA = 27.45 - 10.74 \times t^{1/2} + 1.06 \times t$, $r^2 = 0.98$, $p < 0.05$).

Raw soybean genetically improved was expected to have an even lower trypsin inhibition values because Kunitz inhibitor is the major antitryptic component in soybean seeds (Friedman et al., 1991). Similar results were reported in other studies (Carvalho, Oliveira, Barros, & Moreira, 1999; Miura, Binotti, Camargo, Mizubuti, & Ida, 2001; Monteiro et al., 2003). Soybean varieties lacking Kunitz inhibitor may have different inhibition trypsin levels due to growing location (Kumar, Rani, Tindwani, & Jain, 2003), variation of lipoxygenases level (Carvalho et al., 1999), presence of other types of trypsin inhibitor (Tan-Wilson et al., 1987; Wang, Kaizuma, Takahata, & Hatakeyama, 1996, 2005) and antinutrients such as phytates and tannins (Liener, 1994).

The elimination efficiency of trypsin inhibitors in soybean depends on the moisture level before processing (White et al., 2000), type of processing (Arndt, Hardy, Sugiura, & Dong, 1999; Olguin et al., 2003; Wiriyaumpaiwong et al., 2004) and on the binomial time–temperature used (Qin, Elst, Bosch, & Van Der Poel, 1996). The heating also promotes a partial denaturation of soy proteins, increasing their hydrolysis by digestive enzymes (Trugo, Donangelo, Trugo, & Knudsen, 2000). Conversely, the excessive heating may result in marked decrease of protein solubility and nutritional value (Arndt et al., 1999; Iwe, Van Zuilichem, Ngoddy, & Lammers, 2001).

The trypsin inhibition of an adequately processed soybean product should be below 4 mg g^{-1} (Qin, Verstegen, & Van Der Poel, 1998). According to the fitted equations to TIA in this study, heating times of approximately 15 and 10 min were necessary to process CSB and KFLF, respectively. Friedman et al. (1991) also found that low trypsin inhibitor soybeans needed shorter heating time than conventional soybeans to reduce their TIA to a safe level.

3.4. Protein solubility (PS)

The square root model was better adjusted for CSB ($PS = 86.81 - 0.014 \times t^2$, $r^2 = 0.98$, $p < 0.05$) and KFLF ($PS = 89.5 - 0.2 \times t - 0.01 \times t^2$, $r^2 = 0.99$, $p < 0.05$). The values obtained in the current experiment (Fig. 3) were in the range reported in other works (Grieshop et al., 2003; White et al., 2000).

Heating for 25 min reduced protein solubility of both CSB and KFLF, although the values were higher than those observed by Arndt et al. (1999). These authors found reduction of approximately 28% in protein solubility after autoclaving soybean flour for 20 min, whereas in the current

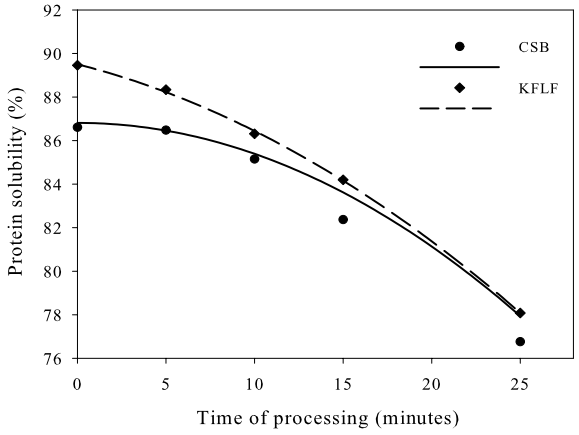

Fig. 3. Protein solubility of conventional (CSB) and improved (KFLF) soybean flours submitted to different heating times.

study it was about 11%. Differences on the level of reduction of protein solubility can be due to the type and conditions of heating process (Grieshop et al., 2003; Wiriyaumpaiwong et al., 2004).

Severe heating treatments denature proteins better than inactivate inhibitors (Qin et al., 1998, 1996). In the present work, even at 25 min the values of protein solubility were in the acceptable range (70–85%) as indicated by Araba and Dale (1990), the haemagglutinating activity reduced entirely and the trypsin inhibition decayed almost completely. Conversely, Arndt et al. (1999) noticed that autoclaving soybean flour during a period beyond 20 min provoked no significant change on the trypsin inhibition while protein solubility decreased markedly.

3.5. Haemagglutinating activity (HA)

The haemagglutinating activity values of the conventional soybean flour (CSB) (Fig. 4) were in the acceptable range of values previously reported (Friedman et al., 1991) and they were adjusted to the non-linear model ($HA = 1.2939 \times 0.85613 \times -t$, $r^2 = 0.96$, $p < 0.05$). The improved soybean raw flour did not present haemagglutinating activity and its analysis through SDS–PAGE confirmed the absence of lectins (datum not shown). In the current work the haemagglutinating activity for CSB after heating for 25 min was not observed, while Friedman et al. (1991) observed haemagglutinating activity after heating for 60 min. It can be explained by the variation of the lectin levels in different soybean varieties (Armour, Perera, Buchan, & Grant, 1998; Friedman et al., 1991).

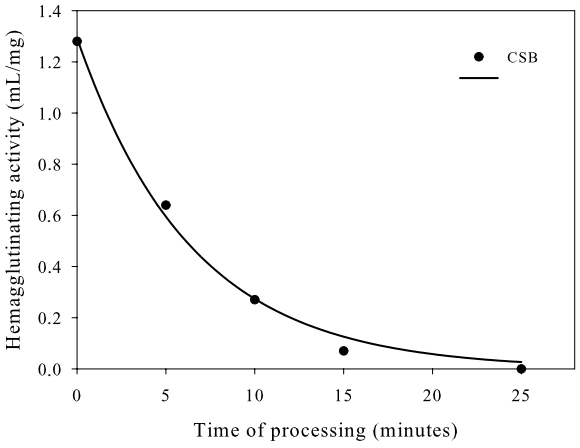

Fig. 4. Haemagglutinating activity of conventional soybean flour (CSB) submitted to different heating times.

Even completely inactivating lectins after heating for 25 min, activities of trypsin inhibition and urease were detected, similarly to the results obtained by Qin et al. (1998). This confirms that lectins were not adequate parameters to evaluate the heating level in soybean flour, as they demonstrated larger sensibility to heating than trypsin inhibitors and urease. Although high temperatures reduce markedly the lectin levels in soybean, the severe heating may be more effective in denaturing proteins than inactivating trypsin inhibitors as well as lectins.

3.6. Protein quality

Rats fed with a diet containing raw CSB showed similar weight gain ($p > 0.05$) to those fed with diet containing raw KFLF (Table 3). Autoclaving CSB for 5 min and KFLF for 15 min improved significantly their nutritional value. The weight gain obtained in the current study was in agreement to other studies (Armour et al., 1998; Friedman et al., 1991; Sarwar, 1997; Turner & Liener, 1975).

PER and NPR increased significantly by heating CSB for 5 min and KFLF for 10 min. Further heating times did not promote any significant change in protein quality ($p > 0.05$) (Table 3). Similar results were described in other works (Friedman et al., 1991; Miura et al., 2001; Pires, Almeida, Rosa, & Costa, 2006; Turner & Liener, 1975). Autoclaving CSB for 15 min resulted in significant improvement of true digestibility, while autoclaving it for 5 min increased NPU. On the contrary, even heating KFLF for 25 min these parameters did not improve

Table 3
Protein quality parameters of experimental diets

Diets[a]	Processing time (min)	Weight gain (g)[*]	PER[*]	NPR[*]	TD (%)[*]	NPU[*]
Casein	–	71.33 ± 8.87^{A}	4.05 ± 0.15^{A}	4.65 ± 0.21^{A}	98.59 ± 0.77^{A}	59.83 ± 1.62^{A}
CSB	0	24.50 ± 8.50^{D}	1.79 ± 0.42^{E}	2.58 ± 0.51^{D}	82.79 ± 1.86^{D}	21.99 ± 6.73^{D}
KFLF	0	27.50 ± 7.77^{D}	2.15 ± 0.42^{DE}	3.00 ± 0.51^{D}	86.72 ± 5.49^{BCD}	31.55 ± 4.57^{C}
CSB	5	41.83 ± 3.13^{BC}	2.62 ± 0.19^{BCD}	3.28 ± 0.25^{BC}	83.99 ± 1.63^{CD}	32.09 ± 3.44^{BC}
KFLF	5	32.00 ± 5.59^{CD}	2.39 ± 0.27^{CD}	3.18 ± 0.32^{CD}	87.11 ± 0.81^{BCD}	33.51 ± 2.45^{BC}
CSB	10	42.00 ± 6.87^{BC}	2.77 ± 0.21^{BC}	3.46 ± 0.20^{BC}	87.51 ± 2.98^{BCD}	35.35 ± 4.10^{BC}
KFLF	10	36.00 ± 4.98^{BCD}	2.73 ± 0.33^{BC}	3.53 ± 0.33^{BC}	87.70 ± 1.52^{BCD}	37.29 ± 2.67^{BC}
CSB	15	42.67 ± 6.74^{BC}	2.90 ± 0.24^{BC}	3.62 ± 0.24^{BC}	88.32 ± 3.14^{BC}	36.90 ± 3.08^{BC}
KFLF	15	45.33 ± 10.80^{BC}	3.00 ± 0.17^{B}	3.72 ± 0.29^{B}	91.22 ± 3.38^{B}	39.53 ± 2.74^{BC}
CSB	25	46.67 ± 3.98^{B}	2.94 ± 0.13^{B}	3.61 ± 0.14^{B}	89.56 ± 2.08^{B}	40.78 ± 1.88^{B}
KFLF	25	44.17 ± 8.06^{BC}	2.99 ± 0.29^{B}	3.71 ± 0.34^{B}	90.91 ± 1.98^{B}	36.55 ± 2.18^{BC}

PER, protein efficiency ratio; NPR, net protein ratio; NPU, net protein utilization; TD, true digestibility.

[a] Prepared with conventional (CSB) or Kunitz and lectin free (KFLF) soybean flours; casein (control).

[*] Values are averages ± standard deviation after 14 days. Means followed by at least one different letter within a same column are significantly different ($p < 0.05$).

($p > 0.05$) (Table 3). However, comparing diets prepared with raw soybean, NPU was significantly higher for KFLF than CSB. The values of NPU obtained in the present experiment for raw and heated soybean flours were in the range reported in the literature (Monteiro et al., 2003; Vasconcelos et al., 2001).

The true digestibility was similar to that obtained by Miura et al. (2001). They attributed their results to the lower consumption and excretion during raw soybean flour diet than heated soybean flour diet. In addition, interactions between proteins and other compounds, the presence of non-digestible oligosaccharides and fiber-like compounds may promote reduction in apparent digestibility (Olguin et al., 2003).

Genetically improved soybean was expected to have protein quality better than the conventional soybean, as reported by Palacios et al. (2004). But weight gain, PER, NPR and true digestibility were not significantly different between them at each heating time, although NPU was significantly different between the raw soybean flours (Table 3).

Comparing this study with others, differences in evaluated protein quality were expected because of the variations on the quantity of different types of trypsin inhibitors (Carvalho et al., 1999; Tan-Wilson et al., 1987; Wang et al., 1996, Wang, Takahata, Kono, & Kaizuma, 2005) and other antinutrient components in different soybean varieties (Liener, 1994). The type of processing employed (Trugo et al., 2000; Wiriyaumpaiwong et al., 2004) and the moisture level before processing (White et al., 2000) also contributed to these differences.

4. Conclusion

The flour obtained from the genetically improved raw soybean presented a lower level of trypsin inhibition and did not present haemagglutinating activity, comparing with the flour produced from the conventional soybean. However, the biological assay did not confirm the improvement of the nutritional quality in the improved raw soy flour, with the exception of NPU, which was achieved only with the use of heating. The most appropriate processing time was 15 min. This treatment promoted a satisfactory inactivation of trypsin inhibitors and lectin, without causing great reduction of the protein solubility, allowing the improvement of the nutritional quality in both flours. Future studies are suggested to evaluate the nutritional value of soybean varieties devoid of Bowman–Birk, Kunitz inhibitors and lectins, as to study the effect of several heat and no heat treatments on them, as well as to characterize other components that impair the nutritive value of this protein source.

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