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Bowman-Birk and Kunitz Protease Inhibitors among Antinutrients and Bioactives Modified by Germination and Hydrolysis in Brazilian Soybean Cultivar BRS 133

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ABSTRACT: Soybean contains constituents that have antinutritional and bioactive properties. Enzymatic hydrolysis and germination can enhance the biological activity of these compounds in soybean. The objective of this study was to investigate the effect of germination, Alcalase (protease) hydrolysis, and their combination on the concentrations of antinutritional and bioactive compounds in Brazilian soybean cultivar BRS 133. A combination of germination and Alcalase hydrolysis resulted in the degradation of Bowman-Birk inhibitor (BBI), Kunitz trypsin inhibitor (KTI), and lunasin by 96.9, 97.8, and 38.4%. Lectin was not affected by any of the processing treatments when compared to nongerminated and nonhydrolyzed soy protein extract. Total isoflavones (ISF) and total saponins (SAP) increased by 16.2 and 28.7%, respectively, after 18 h of germination, while Alcalase hydrolysis led to the reduction of these compounds. A significant correlation was found between concentrations of BBI and KTI, BBI and lunasin, BBI and ISF, KTI and lunasin, KTI and ISF, KTI and SAP, lunasin and ISF, and ISF and SAP. Germination and Alcalase hydrolysis interacted in reducing BBI, ISF, and SAP. This study presents a process of preparing soy flour ingredients with lower concentrations of antinutritional factors and with biologically active constituents, important for the promotion of health associated with soybean consumption. In conclusion, 18 h of germination and 3 h of Alcalase hydrolysis is recommended for elimination of protease inhibitors, while bioactives are maintained by at least 50% of their original concentrations.

KEYWORDS: *soybean, germination, Alcalase hydrolysis, antinutritional factors, bioactive compounds*

INTRODUCTION

Soybean is classified as a legume and has been cultivated for more than 3000 years in Asia.[1] It is a major part of the Asian diet and gaining popularity in other parts of the world. Its popularity is associated with its health-promoting properties including promotion of cardiovascular health[2] and reduction of cancer risk.[3] These purported health benefits are due to the presence of biologically active compounds in soybean including isoflavones, saponins and bioactive peptides, and proteins. However, soybean also contains the so-called antinutritional factors such as trypsin inhibitors, flatulence-causing oligosaccharides, and lectins. Lectins are a group of proteins that bind specifically to sugars of glycoproteins and glycolipids on the surfaces of cells. The antinutritional factors limit the application of soybean in animal and human nutrition as they interfere with the utilization of proteins and minerals in the digestive tract.[4] For instance, Bowman-Birk trypsin/chymotrypsin and Kunitz trypsin inhibitors (KTIs) can cause an increase in the secretion of digestive enzymes by inducing hypertrophy and hyperplasia of the pancreas and reducing crude protein digestibility in monogastrics; this concept is still controversial in humans.[5] Lectins can bind to intestinal epithelium causing disruption of the brush border membrane and poorer growth of spleen and kidneys.[6] Hence, there is a need to find methods of removing these antinutritional factors, without affecting bioactive compounds, to further increase the acceptance and use of soybean as a part of the animal and human diet.

Different treatments such as heating, enzymatic hydrolysis, irradiation, use of chelating agents, and germination have been used to eliminate these antinutritional factors. For instance, a combination of heat denaturation and pepsin and pancreatin treatment showed great promise in completely reducing soybean lectin activity,[6,7] while γ-irradiation[8,9] and electron beam irradiation[10] resulted in reduced trypsin inhibitory activity, lipoxygenase activity, and phytic acid and oligosaccharide contents. Previous studies also showed that germination is an inexpensive and effective technology in reducing the concentration of antinutritional factors while enhancing protein digestibility.[11−13] However, to our knowledge, no previous study has shown the effect of combining germination and Alcalase hydrolysis on the concentration of antinutritional and bioactive compounds present in Brazilian soybean cultivar BRS 133. The Brazilian soybean cultivar BRS 133 is important in Brazil, because it can be grown in soils with low to medium fertility, allowing its production in different parts of Brazil. The protein concentration of the seed is approximately 38.5%, and the oil is 18%.[14]

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Hence, the present study was carried out with an objective of determining the effect of germination, Alcalase hydrolysis, and their combination on the antinutritional factors [Bowman-Birk inhibitors (BBIs), KTI, and lectin] and bioactive compounds (lunasin, saponins, and isoflavones) in Brazilian soybean cultivar BRS 133.

MATERIALS AND METHODS

Materials. Soybean of the Brazilian cultivar BRS 133 was provided by Embrapa Transferência de Tecnologia, Brazil.[14] Alcalase enzyme from *Bacillus licheniformis* (2.4 U/g of product) was purchased from Sigma (St. Louis, MO). Antibodies for soybean trypsin inhibitors were purchased from Abcam (Cambridge, MA), and both antibodies were of IgG isotype. BBI (mAb 238) was a mouse monoclonal antibody; its antibody−antigen binding region was determined to be the trypsin-reactive domain rather than the chymotrypsin-reactive domain. KTI was a rabbit polyclonal antibody; the immunogen used for the KTI antibody was the full-length protein from soybean. Lunasin antibody was a kind gift from Dr. Ben O. de Lumen, University of California at Berkeley, and lectin antibody was a kind gift from Dr. Lila Vodkin, University of Illinois at Urbana−Champaign.

Germination and Preparation of Alcalase Hydrolysates. Germination was carried out as previously reported.[15] Germination was performed during an equivalent time in the presence of light and in the dark for a total of 18 and 72 h at 25 °C. Germinated and non-germinated seeds were frozen, freeze-dried, and milled to obtain soybean flour. Soybean flour was defatted following the official method of AACC.[16] Alcalase hydrolysis was carried out using a previously reported protocol.[17] Briefly, 32 g of defatted soybean flour was suspended in 400 mL of distilled water and adjusted to pH 8.0 at 50 °C. Then, 2.5 mL of Alcalase enzyme was added to hydrolyze the proteins. Samples were treated for 0 (protein soluble extract), 1, 2, and 3 h (protein hydrolysate) at 50 °C, and the pH was maintained at 8.0 during the process. The reaction was stopped by adding 1.2 mL of 0.1 N HCl, pH 5.0. Hydrolysates were centrifuged at 14000*g* at 10 °C for 30 min to remove any precipitates, and the supernatants were ultrafiltered through a 0.8 kDa membrane (Microdyn-Nadir Industry, Wiesbaden, Germany) to remove salts. Soybean protein extracts and hydrolysates were frozen at −18 °C, freeze-dried, and kept at −20 °C. Samples were identified as GT-th where GT refers to the germination time either 0 (G0), 18 (G18), or 72 h (G72), and th refers to Alcalase hydrolysis time either 0 (0h), 1 (1h), 2 (2h), or 3 h (3h).

Soluble Protein. It was determined using Protein DC Microplate Assay (Biorad Laboratories, Hercules, CA). In a 96-well plate, 5 μL of diluted samples (1:50) was mixed with 25 μL of reagent A and 200 μL of reagent B, agitated, and incubated for 15 min at room temperature. The absorbance was read at 630 nm in an Ultra Microplate Reader (Biotek Instruments, Winooski, VT). The protein concentration (mg/mL) was calculated using a bovine serum albumin (BSA) standard curve [$y = 0.0002x - 0.0058$ ($r^2 = 0.997$), where y = absorbance and x = protein concentration (μg/mL)].

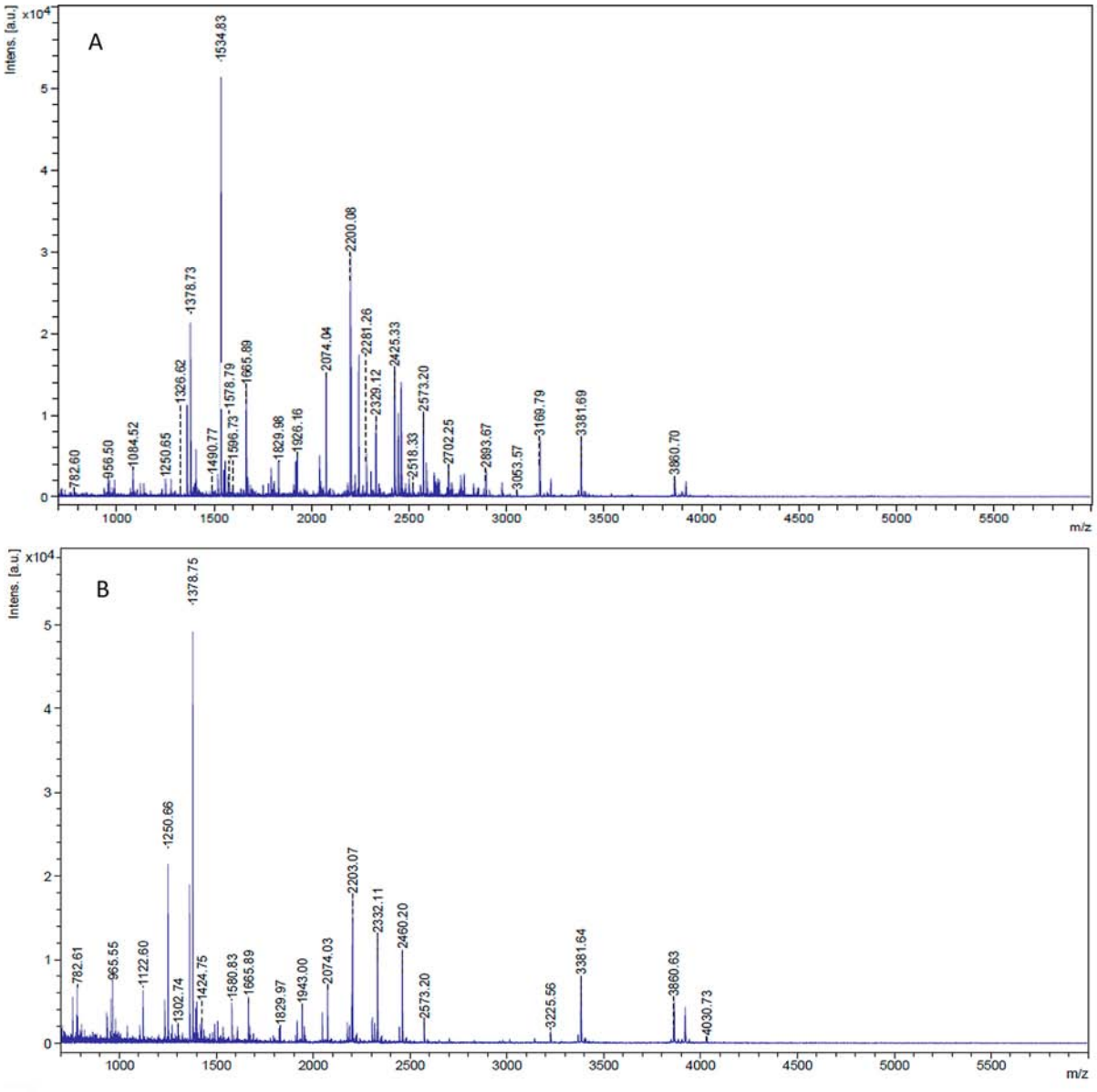
Peptide Mass Fingerprinting by Matrix-Assisted Laser Desorption/Ionization. Samples were dissolved in water and mixed with "Super-DHB" matrix (Sigma, St. Louis, MO) in 50% acetonitrile and 0.1% trifluoroacetic acid and deposited on a standard stainless steel target. On the basis of previous studies, the mass spectra were collected in positive ion mode with a scanning range of up to 20000 Da on an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a frequency tripled Nd:YAG solid state laser using the FlexControl 1.3 software package (Bruker Daltonics). Following external calibration, spectra were acquired at 500 Hz, summed, and saved for analysis. Analysis was performed using the FlexAnalysis 3.3 software package (Bruker Daltonics). Spectra were smoothed, and a baseline correction was applied using the software package.

Quantification of Isoflavones in Germinated and Hydrolyzed Soy Flour. Isoflavones quantification in germinated and hydrolyzed soy flour was performed as previously reported.[18] Dry hydrolysate (100 mg) was extracted with 1 mL of 80% methanol at 20 °C with vigorous shaking (80 rpm) in a G10 gyratory shaker (New Brunswick Sci., NJ) for 12 h. The pellet obtained after centrifugation at 5000*g* for 10 min was extracted again with 1 mL of 80% methanol by shaking for 2 h, and samples were centrifuged at 5,000*g* for 10 min. The combined supernatants (2 mL) were centrifuged at 12000*g* for 10 min and then used for HPLC analysis. Isoflavones were separated with a Waters HPLC system (Waters Corp., Milford, MA) using a 250 mm × 4.6 mm, 5 μm Prevail C_{18} column (Alltech Assoc., Deerfield, IL). A linear gradient composed of solvent A (water−acetonitrile−acetic acid, 95:5:0.1) and solvent B (acetonitrile−water−acetic acid, 95:5:0.1) was used at 1 mL/min. Following injection of 10 μL of sample, solvent B was increased from 5 to 20% over 10 min and 50% over 40 min. The column was then washed with 95% of solvent B for 3 min and equilibrated for 3 min at 5% B between runs. A Waters 996 photodiode array detector was used to measure UV absorbance at 260 nm. Isoflavone standards (daidzein, genistein, glycitein, and their glucosides) were purchased from LC Laboratories (Woburn, MA). Malonyl forms were identified based on the response factors of the corresponding β-glucosides and appropriate correction for the molecular mass differences. Each sample was analyzed in three replicates.

Quantification of Saponins in Germinated and Hydrolyzed Soy Flour. It was conducted as previously reported.[19] Briefly, saponins from the soybean flour, germinated soybean flour, and hydrolysates were extracted with dimethylsulfoxide/methanol (1:1) solution at room temperature for 4 h, followed by a 15 min of sonication at 50 °C and another 2 h extraction at room temperature. The extracts were then filtered through a 0.45 μm nylon filter. HPLC analysis was conducted on a Shimadzu LC-20 HPLC system (LC-20AT quaternary pump, DGU-20A5 degasser, SIL-20A HT autosampler, and a SPD M20A photodiode array detector, running under Shimadzu LCSolutions version 1.22 chromatography software, Columbia, MD). The column used was an Inertsil ODS-3 reverse phase C-18 column (5 μM, 250 mm × 4.6 mm, with a Metaguard column, from Varian). For saponin analysis, the initial conditions were 30% acetonitrile and 0.025% trifluoroacetic acid (TFA) in water, at a flow rate of 1 mL/min. The effluent was monitored at 210 nm on the VWD. After injection (typically 25 μL), the column was developed to 60% acetonitrile and 0.025% TFA in a linear gradient over 45 min. Standard curves based on nanomoles injected were prepared from purified soyasaponin I prepared in the laboratory, and the nanomolar extinction coefficient (5.78×10^{-6} nmol per mAbs unit) was used to quantify the saponins. Each sample was analyzed in three replicates.

Quantification of Lunasin, BBI, KTI, and Lectin in Germinated and Hydrolyzed Samples. Briefly,[20] germinated and hydrolyzed samples were suspended in TBS and diluted. One hundred microliters of diluted samples (1:5000) was plated in a 96-well plate and stored overnight at 4 °C. After blocking with 5% BSA in Tris-buffered saline with 1% Tween 20, lunasin rabbit polyclonal antibody (1:200 dilution) was used as the primary antibody, and antirabbit IgG alkaline phosphatase conjugate (1:1000) (Sigma-Aldrich, St. Louis, MO) was used as the secondary antibody. The reaction was stopped by adding 100 μL of 3 N NaOH at 25 min, and the absorbance (405 nm) was read at 35 min. A similar procedure was used for BBI, KTI, and lectin analyses. The primary antibody dilution for BBI was 1:1000 using BBI mouse monoclonal antibody (Agdia, Inc., Elkhart, IN), for KTI was 1:3000 using trypsin inhibitor rabbit polyclonal antibody (Abcam, Cambridge, MA), and for lectin was 1:200 using lectin rabbit polyclonal antibody. Antimouse IgG alkaline phosphatase conjugate (1:2000) (Sigma-Aldrich) was used as the secondary antibody for BBI. Standard curves were determined using purified lunasin (>90% purity, $y = 0.025x + 0.176$, $R^2 = 0.99$), purified BBI (70−90% purity, $y = 0.015x + 0.065$, $R^2 = 0.99$), purified KTI (>98% purity, $y = 0.079x + 0.523$, $R^2 = 0.98$), and purified lectin (>80% purity, $y = 0.008x + 0.181$, $R^2 = 0.99$). Each sample was analyzed in three replicates.

Statistical Analysis. Data were analyzed using the PROC GLM procedure of SAS v 9.2, and means were separated by Tukey test at $P < 0.05$ ($n \geq 3$). Interaction between germination and hydrolysis in affecting the concentration of each constituent was analyzed by Prism software using two-way analysis of variance (ANOVA) at $P < 0.05$. Correlation among concentrations of different antinutrients and



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Sample	Molecular Mass (Da)			Sample	Molecular Mass (Da)		
	<1000	1000 – 4000	>4000		<1000	1000 – 4000	>4000
G0-0h	1.52	98.48	0.00	**G18-2h**	5.41	93.90	0.66
G0-1h	3.06	94.78	2.17	**G18-3h**	7.94	91.68	0.38
G0-2h	0.73	99.17	0.01	**G72-0h**	2.39	97.61	0.00
G0-3h	7.70	91.96	0.34	**G72-1h**	2.05	97.66	0.29
G18-0h	5.36	94.18	0.45	**G72-2h**	2.45	96.83	0.72
G18-1h	4.36	95.30	0.34	**G72-3h**	2.60	96.87	0.54

Figure 1. Representative peptide mass fingerprints of germinated and Alcalase hydrolysates of Brazilian soybean cultivar BRS 133. G0-0h (A), G18-3h (B), and percentage distribution of peptide masses found in 12 germinated and Alcalase-hydrolyzed soybean flours (C). Samples are identified as follows: GT-th, where GT refers to germination time, either 0 (G0), 18 (G18), or 72 h (G72), and th refers to Alcalase hydrolysis time, either 0 (0h), 1 (1h), 2 (2h), or 3 h (3h).

bioactive compounds was analyzed by Prism software using Pearson's correlation and a two-tailed test at $P < 0.05$.

RESULTS

This study investigated for the first time the combined effect of germination and Alcalase hydrolysis on the concentration of protease inhibitors BBI and KTI, lectin, lunasin, isoflavones, and saponins in Brazilian cultivar BRS 133. Figure 1 presents representative matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) peptide mass fingerprints of the germinated and Alcalase-hydrolyzed samples. The use of Alcalase for 3 h of hydrolysis resulted in an increase on the percentage of

peptides of molecular mass <1000 Da when compared to nonhydrolyzed soy protein extract. On the other hand, germination for 18 h without Alcalase treatment gave the highest percentage of peptides with molecular mass of <1000 Da, very similar to samples with no germination and 3 h hydrolysis (G0-3h). Germination for 18 h resulted in increased susceptibility of the soy protein extract to Alcalase hydrolysis as evidenced by a higher percentage of peptides <1000 Da, while 72 h of germination only showed a minimal increase in the percentage of peptides with <1000 Da molecular mass. These masses mostly corresponded to those found in the Alcalase hydrolysates of purified glycinin and β-conglycinin as previously reported.[15,21]

Alcalase Hydrolysis but Not Germination Reduced the Concentration of Soy Protease Inhibitors. Figure 2 shows

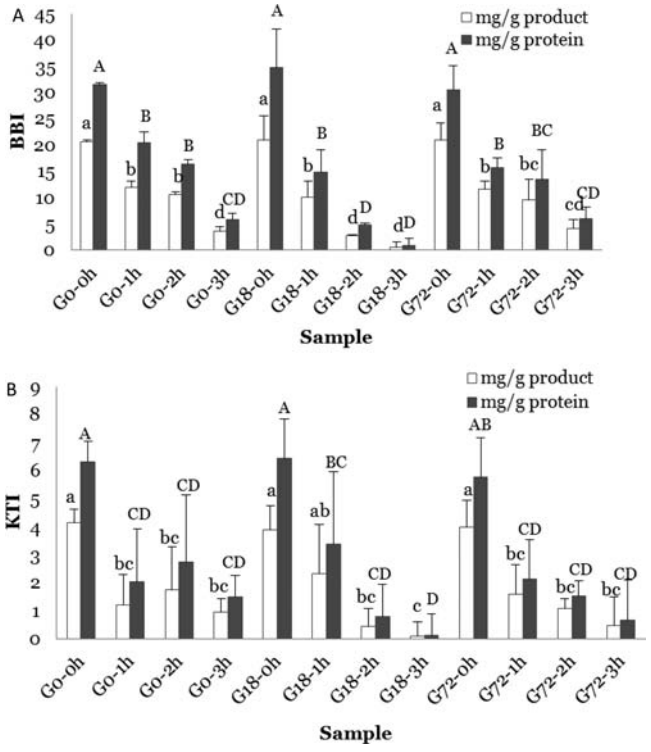


Figure 2. Effect of germination and Alcalase hydrolysis on the concentration of protease inhibitors in Brazilian soybean cultivar BRS 133. BBI (A) and Kunitz inhibitor (B). Soy protein extracts were obtained from soybean that was germinated and hydrolyzed by Alcalase as described in the Materials and Methods. Means followed by different letters are significantly different from each other ($n \geq 3$, $P < 0.0001$ for both BBI and KTI). Values are presented in noncapitalized letters for mg/g product and capitalized letters for mg/g protein.

BBI and KTI concentrations of germinated and Alcalase-hydrolyzed soybean protein extracts. The BBI concentration for nongerminated and nonhydrolyzed soy protein extract (G0-0h) was 20.7 ± 0.2 mg/g product. Alcalase hydrolysis of nongerminated soybean led to a significant time-dependent reduction of the concentration of BBI from 42.5 (1 h of hydrolysis) to 82.2% (3 h of hydrolysis). The same trend was observed on the effect of Alcalase hydrolysis after soy was germinated for 18 and 72 h. The highest reduction in BBI (96.9%) was after germination for 18 h and hydrolysis for 3 h. On the other hand, only germination for 18 and 72 h did not affect BBI concentration when compared to nongermination. Similar results were obtained on the effect of germination and Alcalase hydrolysis on KTI concentrations. Nongerminated and nonhydrolyzed soy protein extracts presented the highest concentration of KTI (4.2 ± 0.5 mg/g product). Alcalase hydrolysis for 3 h led to a significant reduction of KTI concentration (77.1%). Soy protein extract from germinated soy for 18 h and hydrolyzed for 3 h presented the highest reduction in KTI (97.8%).

Alcalase Hydrolysis Reduced Lunasin but Not Lectin in Soy Flour Hydrolysates. Figure 3A,B shows the effect of

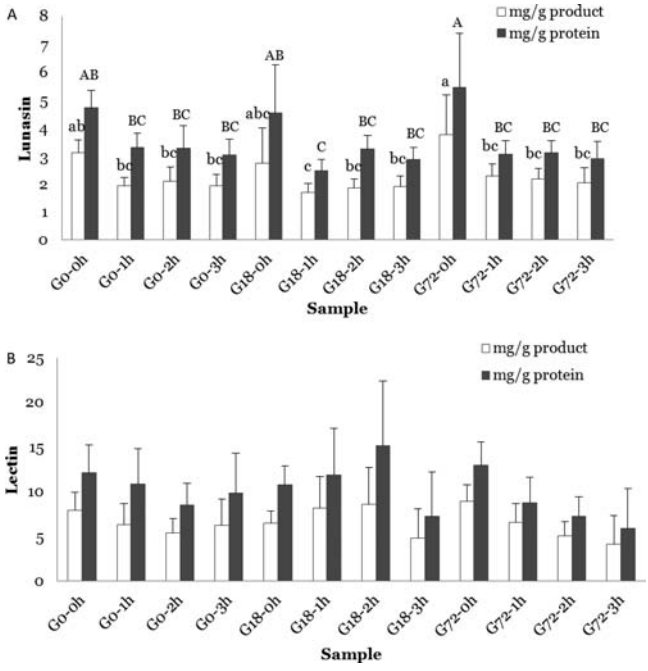


Figure 3. Effect of germination and Alcalase hydrolysis on the concentration of lunasin (A) and lectin (B) in Brazilian soybean cultivar BRS 133. Soy protein extracts were obtained from soybean that was germinated and hydrolyzed by Alcalase as described in the Materials and Methods. Means followed by different letters are significantly different from each other ($n \geq 3$, $P < 0.0001$ lunasin, and $P > 0.05$ for lectin).

germination and Alcalase hydrolysis on lunasin and lectin concentrations in Brazilian soybean cultivar, respectively. Lunasin concentrations from nonhydrolyzed soy protein extract ranged from 3.1 ± 0.5 to 3.8 ± 1.4 mg/g product. Lunasin showed resistance to Alcalase hydrolysis with a maximum of 45.1% reduction. The lectin concentration was not affected by either germination or Alcalase hydrolysis ($P > 0.05$).

Germination and Alcalase Hydrolysis Affected the Concentration of Soy Isoflavones. Table 1 presents the isoflavones concentration of the 12 germinated and Alcalase-hydrolyzed soy samples. The isoflavones concentration ranged from 2300 ± 96 to 4040 ± 63 μg/g product. Germination for 18 and 72 h led to a statistically significant increase in total isoflavone by 16.2 and 17.7%, respectively, when compared to nongerminated and nonhydrolyzed soy samples. In non-germinated samples, 2 h of Alcalase hydrolysis led to the highest reduction in isoflavone (33.2%). In 18 and 72 h germinated soy samples, 3 h of Alcalase hydrolysis resulted in the highest reduction in total isoflavones (23.3 and 24.5%, respectively). In nonhydrolyzed soy extract, germination for 18 and 72 h reduced isoflavone glycosides by 51.3 and 53.0%, respectively. Isoflavone glycosides refer to the isoflavone with sugar molecule attached to its structure including genistin, daidzin, and glycitin. In addition, germination for 18 and 72 h resulted in an increase in aglycone

Table 1. Isoflavone Concentrations (μg/g Dry Powder) of Germinated and Alcalase-Hydrolyzed Brazilian Soybean Cultivar BRS 133

	isoflavones											
sample	daidzin	glycitin	genistin	Mal-daidzin	Mal-glycitin	Mal-genistin	daidzein	glycitein	genistein	daidzein conjugate	glycitein conjugate	genistein conjugate	total[a]
G0-0h	668 ± 23	81 ± 3	414 ± 14	949 ± 31	70 ± 8	663 ± 40	288 ± 8	44 ± 2	264 ± 46	1905 ± 61	195 ± 10	1342 ± 61	3442 ± 57 b
G0-1h	525 ± 7	66 ± 8	354 ± 1	603 ± 6	40 ± 7	485 ± 1	320 ± 2	55 ± 3.6	344 ± 174	1448 ± 9	161 ± 14	1183 ± 173	2792 ± 178 de
G0-2h	326 ± 19	40 ± 10	237 ± 14	652 ± 41	47 ± 12	500 ± 27	288 ± 14	37 ± 2	173 ± 13	1266 ± 53	123 ± 4	911 ± 48	2300 ± 96 f
G0-3h	476 ± 13	77 ± 9	347 ± 6	583 ± 13	49 ± 2	452 ± 7	342 ± 9	43 ± 2	235 ± 13	1401 ± 34	168 ± 9	1034 ± 24	2604 ± 66 e
G18-0h	469 ± 15	63 ± 19	309 ± 7	1443. ± 35	108 ± 30	994 ± 4	336 ± 3	41 ± 2	235 ± 2	2249 ± 48	212 ± 36	1538 ± 10	3999 ± 93 a
G18-1h	576 ± 40	70 ± 17	353 ± 6	951 ± 9	60 ± 23	703 ± 14	234 ± 6	42 ± 3	178 ± 15	1762 ± 52	172 ± 38	1234 ± 32	3167 ± 47 c
G18-2h	723 ± 23	85 ± 11	467 ± 18	845 ± 31	68 ± 7	632 ± 27	178 ± 8	47 ± 18	120 ± 7	1746 ± 61	200 ± 16	1218 ± 52	3164 ± 99 c
G18-3h	778 ± 18	86 ± 13	477 ± 12	777 ± 18	48 ± 2	588 ± 11	167 ± 2	32 ± 0	113 ± 2	1723 ± 36	166 ± 13	1178 ± 24	3067 ± 53 c
G72-0h	453 ± 34	37 ± 10	319 ± 3	1446 ± 19	103 ± 15	1025 ± 15	330 ± 10	59 ± 6	277 ± 23	2229 ± 56	199 ± 17	1622 ± 31	4040 ± 63 a
G72-1h	479 ± 55	44 ± 7	347 ± 4	1000 ± 4	62 ± 8	786 ± 8	203 ± 4	48 ± 3	125 ± 2	1682 ± 59	155 ± 6	1257 ± 8	3094 ± 71 c
G72-2h	539 ± 25	70 ± 13	407 ± 20	985 ± 22	43 ± 7	809 ± 24	135 ± 5	40 ± 4	43 ± 2	1659 ± 33	153 ± 11	1260 ± 43	3072 ± 54 c
G72-3h	617 ± 21	33 ± 14	469 ± 10	851 ± 44	61 ± 2	734 ± 41	176 ± 7	48 ± 3	67 ± 4	1645 ± 61	142 ± 15	1270 ± 54	3056 ± 126 cd

[a]Means ± SDs followed by different letters are statistically different from each other ($P < 0.05$).

forms (corresponding to the isoflavone with no sugar moiety) by 2.8 and 11.9%, respectively.

Germination and Alcalase Hydrolysis Affected the Concentration of Soy Saponins. Saponins concentration of germinated and hydrolyzed soy samples ranged from 2.7 ± 0.2 to 7.4 ± 0.8 mg/g product (Table 2). Germination for 18 h resulted in a statistically significant increase in total saponin concentration, while 72 h of germination did not affect the concentration of total saponin when compared to nongerminated soy extract. Alcalase hydrolysis at each time point led to a significant reduction in total saponin concentrations when compared to their nonhydrolyzed counterparts. Germination for 18 h increased the total saponin A and total saponin B by 1.8- and 1.1-fold, respectively, while 72 h of germination increased the total saponin A by 11% and reduced total saponin B by 11%. Total saponin A includes soyasaponins A1 and A2, while the rest of the saponins were included in total saponin B. Alcalase hydrolysis for 3 h resulted in the total elimination of total saponin A in all soy hydrolysates, while total saponin B was reduced by 19.3 to 39.4%. Also, it can be seen that during hydrolysis the 3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one forms of saponins (soyasaponins βg, βA, and αg) were reduced, which might be attributed to partial conversion to soyasaponin I and then to soyasaponin II.

Correlations among Different Antinutrients and Bioactives in Germinated and Alcalase-Hydrolyzed Soy Flour. Figure 4 presents the relationship between concentrations of two antinutrients/bioactive compounds in germinated and Alcalase-hydrolyzed Brazilian soybean cultivar BRS 133. A strong significant positive association was found between the concentrations of BBI and KTI (r = 0.95), BBI and lunasin (r = 0.82), KTI and lunasin (r = 0.82), lunasin and total isoflavones (r = 0.73), and total isoflavones and total saponins (r = 0.81). There was a weak positive correlation between BBI and total saponins, KTI and total isoflavones, and KTI and total saponins. No statistically significant correlation was found among lectin and other compounds measured, BBI and total saponins, and lunasin and total saponins.

DISCUSSION

Soybean has played an increasing role in both human and animal nutrition over the past several decades in several parts of the world. This increase in popularity may be attributed to increasing evidence on the health-promoting effects of soy due mainly to the presence of biologically active components including isoflavones, saponins, proteins, and peptides. Several studies have shown that hydrolysis of soy proteins resulted in the production of peptides with biological activities including the capability to act as antioxidants and to inhibit adipogenesis and inflammation. For instance, Park et al.[22] showed that Alcalase hydrolysates, of commercially available soy protein, with a molecular mass of less than 3 kDa, possessed the highest antioxidant activity. In addition, Alcalase hydrolysis of nine soybean cultivars showed an improved antioxidant capacity of different soy protein hydrolysates.[23] Also, our previous study with germinated and hydrolyzed samples has shown that their antioxidant capacity ranged from 607.9 ± 32.1 to 740.6 ± 26.1 mM Trolox equiv/g hydrolysate and possessed anti-inflammatory properties against lipopolysaccharide-induced inflammation in RAW 264.7 macrophages.[15]

The presence of antinutritional factors in soybean including protease inhibitors and lectins has resulted in low protein efficiency ratio as well as low food intake in rats.[24] The concept of

Table 2. Saponin Concentrations (mg/g) of Germinated and Alcalase-Hydrolyzed Brazilian Soybean Cultivar BRS 133

	saponins								
sample	soyasaponin I (Bb)	soyasaponin II (Bc)	soyasaponin V (Ba)	4-acetyl-soyasaponin A1 (Ab)	4-acetyl-soyasaponin A2 (Af)	soyasaponin βg	soyasaponin βA	soyasaponin αg	total[a]
G0-0 h	1.7 ± 0.1	1.2 ± 0.03	0.2 ± 0.01	0.6 ± 0.03	0.0 ± 0.0	1.1 ± 0.04	0.6 ± 0.04	0.2 ± 0.01	5.7 ± 0.1 b
G0-1 h	2.1 ± 0.3	1.4 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.1	0.3 ± 0.04	0.1 ± 0.1	4.5 ± 0.7 bcd
G0-2 h	1.6 ± 0.2	0.9 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 0.2 e
G0-3 h	2.4 ± 0.3	1.5 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	3.9 ± 0.4 cde
G18-0 h	1.6 ± 0.2	1.0 ± 0.1	0.2 ± 0.03	0.8 ± 0.1	0.4 ± 0.1	2.0 ± 0.2	1.1 ± 0.1	0.2 ± 0.02	7.4 ± 0.8 a
G18-1 h	2.2 ± 0.3	1.3 ± 0.2	0.0 ± 0.0	0.3 ± 0.04	0.0 ± 0.0	0.6 ± 0.1	0.4 ± 0.03	0.1 ± 0.1	4.9 ± 0.7 bc
G18-2 h	2.8 ± 0.3	1.7 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	4.7 ± 0.7 bc
G18-3 h	2.6 ± 0.4	1.6 ± 0.3	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.3 ± 0.8 bcd
G72-0 h	1.1 ± 0.1	0.6 ± 0.03	0.3 ± 0.01	0.4 ± 0.03	0.3 ± 0.02	1.1 ± 0.1	0.6 ± 0.03	0.7 ± 0.03	5.2 ± 0.3 bc
G72-1 h	1.7 ± 0.03	1.0 ± 0.02	0.2 ± 0.01	0.3 ± 0.03	0.0 ± 0.0	0.6 ± 0.01	0.4 ± 0.0	0.6 ± 0.03	4.7 ± 0.1 bc
G72-2 h	1.5 ± 0.03	0.9 ± 0.01	0.1 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.02	3.0 ± 0.1 de
G72-3 h	2.5 ± 0.2	1.5 ± 0.1	0.2 ± 0.01	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.1	4.6 ± 0.4 bc

[a]Means ± SDs followed by different letters are statistically different from each other ($P < 0.05$). Soyasaponins III (Bb′), IV (Bc′), 4-acetyl-A7 (Ac), γg, and γa were not detected.

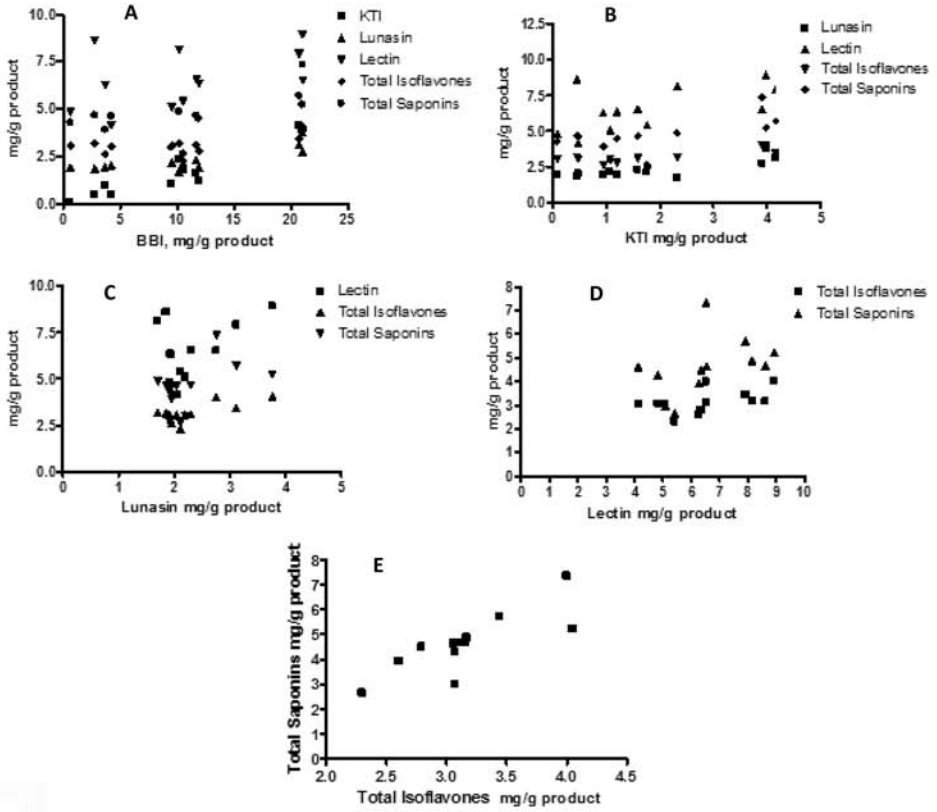
antinutritional properties of BBI is still controversial in humans. Human trials have confirmed no deleterious effects in human health; in addition, a number of relevant studies have evaluated their potential beneficial effects in human health.[25]

Our results showed that germination of Brazilian soybean cultivar BRS 133 for 18 and 72 h did not affect the concentration of protease inhibitors and lectin (G18-0h and G72-0h). It is important to mention that in this study, both trypsin inhibitory domains present in the BBI isoinhibitor were recognized by the antibody used, based on a previous study on the reactivity of the same BBI antibody (mAb 238).[26]

Previous studies have shown that germination alone had an effect on trypsin inhibitory activity in soybeans. Mostafa and Rahma[27] found that germination of soybean seeds for 6 days reduced trypsin inhibitory activity by 32%, while Bau et al.[28] showed that KTI and BBI degradation was enhanced if allowed to germinate for more than 4 days. Maximum reduction in trypsin inhibitory activity was obtained after 48−72 h of germination, and the activity increased when the germination was prolonged for 5 days.[29] These conflicting results on the effect of germination on the degradation of soybean protease inhibitors might be attributed to differences on the germination conditions. In this study, we germinated soybean for 18 and 72 h (dark-light conditions) at 25 °C, and we did not see degradation of BBI and KTI; Mohamed et al.[29] showed degradation of trypsin inhibitory activity after 48−72 h of germination under dark conditions at 25 °C. It is therefore important to determine an optimum germination condition that will result in the highest degradation of BBI and KTI.

On the other hand, Alcalase hydrolysis led to a time-dependent degradation of the protease inhibitors but not lectin. The reduction of protease inhibitors ranged from 40.5 to 97.8%, indicating the efficacy of Alcalase enzyme in degrading these proteins. Combined effects showed that germination and Alcalase hydrolysis interacted in reducing the concentration of BBI but not KTI and lectin (Table 3). The consumption of soybean with antinutritional factors such as trypsin inhibitors and lectin can cause growth depression.[30] A study of U.S. commercial soy foods showed that soymilk contained BBI (7.2−55.9 mg per 100 mL), while other soy products including tofu, tempeh, natto, soybean cake, and miso had BBI (0.7−19.2 mg/100 g product).[31] A recent study on the protease inhibitor activities of different soymilk-based infant formulas obtained in Ottawa, Canada, showed that six out of eight commercial products contained a residual soybean trypsin inhibitory activity (up to 55.6% trypsin inhibition) higher than the level being considered safe (10% residual).[32] Studies are needed on the long-term consumption of soymilk containing high residues of soybean trypsin inhibitory activity on human health.[33] The combination of germination and Alcalase hydrolysis can be used to produce ingredients that contain very low concentrations of BBI and KTI that can be used for soy-based products.

Germination for 72 h led to an increase in lunasin (21%), while germination for 18 h led to an increase in saponin concentration (28.7%). Previous studies on the effect of germination on isoflavones and saponins showed that an optimal increase was found at germination conditions of 63 h at 30 °C,[34] while lunasin was increased by 61.7% when germinated for 42 h at 25 °C. Germination affected the isoflavone profile of the soy flour.[20] Nongerminated soy protein extract showed a percentage distribution of 33.8, 48.9, and 17.3% for the glycosides, malonyl-glycosides, and aglycone forms of isoflavone, respectively. After germination, the malonyl-glycoside form of the isoflavones accounted for more than 50% of the total isoflavones and 63.7 and 63.6% after 18 and 72 h of germination, respectively. Germination resulted in the reduction of the glycosides (21% for 18 h and 20% for 72 h of germination) and aglycone (15.3% for 18 h and 16.5% for 72 h germination). These results are not in agreement with Shi et al.,[35] who showed that 77 and 30% of malonyl-daidzin and malonyl-genistin were converted to their corresponding glycosides and aglycone forms. Lee et al.[36] reported that concentrations of all isoflavones in soybean increased after 5 days of germination, while our results showed that only the malonyl and aglycone forms of the isoflavones increased after 18 and 72 h of germination. Jeong et al.[37] reported that total aglycone form of isoflavone increased after 48 h of germination at 20 and 25 °C depending on the soybean cultivar. Standardization for germination conditions that will result in the optimum concentration of isoflavone, especially the more biologically active aglycone forms, is needed. In addition, cultivar and growing conditions also affect the concentrations of isoflavones in soybeans. Our results showed that the aglycone form of isoflavone was increased after 72 h of germination, indicating that this germination condition increased



F

Comparison	P-value	r*	R^2**	95% Confidence Interval
A) BBI vs KTI	**< 0.0001**	**0.954**	**0.911**	**0.842 – 0.988**
BBI vs Lunasin	**0.001**	**0.816**	**0.667**	**0.456 – 0.947**
BBI vs Lectin	0.129	0.463	0.215	-0.151 – 0.819
BBI vs Total ISF	**0.023**	**0.647**	**0.419**	**0.116 – 0.890**
BBI vs Total SAP	0.052	0.573	0.328	-0.002 – 0.863
B) KTI vs Lunasin	**0.001**	**0.818**	**0.669**	**0.459 – 0.947**
KTI vs Lectin	0.056	0.563	0.318	-0.016 – 0.860
KTI vs Total ISF	**0.015**	**0.681**	**0.464**	**0.176 – 0.902**
KTI vs Total SAP	**0.028**	**0.629**	**0.396**	**0.087 – 0.884**
C) Lunasin vs Lectin	0.159	0.434	0.188	-0.187 – 0.807
Lunasin vs Total ISF	**0.007**	**0.733**	**0.537**	**0.274 – 0.920**
Lunasin vs Total SAP	0.125	0.468	0.219	-0.145 – 0.821
D) Lectin vs Total ISF	0.105	0.492	0.242	-0.115 – 0.831
Lectin vs Total SAP	0.172	0.422	0.178	-0.201 – 0.802
E) Total ISF vs Total SAP	**0.001**	**0.809**	**0.655**	**0.439 – 0.945**

*Pearson Correlation coefficient; **Coefficient of determination

Figure 4. Correlation of compounds affected by germination and Alcalase hydrolysis in Brazilian soybean cultivar BRS 133. Comparisons among BBI (A), KTI (B), lunasin (C), lectin (D), and total isoflavones (E). Statistical parameters for correlations as determined by the Prism program (F).

the amount of this more biologically active form of isoflavone. A previous study showed that germination of soybeans resulted in an increase in saponin concentration by 3.2-fold after 4 days of germination at 25 °C with a concomitant increase in the percentage of soysapogenol I from 1.8 to 7.3%.[38] We also found an increase in total saponin concentration only after 18 h of germination at 25 °C.

Lunasin concentrations in Alcalase-hydrolyzed samples did not differ among three hydrolysis times showing resistance of lunasin toward enzyme hydrolysis. A study on stability of lunasin against enzymatic hydrolysis showed that in vitro digestion by simulated intestinal fluid and simulated gastric fluid resulted in the digestion of synthetic and purified soy lunasin after 2 min, but in the presence of BBI, lunasin was protected from digestion.[39] Moreover, lunasin in soy protein was protected from digestion, a result that was attributed to the presence of protease inhibitors in the soy protein matrix. Our previous study also showed that 75% of the lunasin in lunasin-enriched soy flour was digested after 180 min of pepsin treatment, and only 3% remained after additional pancreatin exposure for 180 min.[40] The results of this study showed that at least 50% of the original lunasin remained in the soy hydrolysates even after 3 h of Alcalase digestion. The resistance of lunasin from digestion can be attributed to the protective effects of protease inhibitors present in the soy protein extract. The present study shows degradation of BBI only after Alcalase hydrolysis and resistance of lunasin only after germination. There seems to be some correlation in the protection of lunasin due to the presence of BBI.

The effect of enzymatic treatment on the isoflavone profile of soybean meal showed that treatment of Protease M resulted in the increased concentration of the aglycone form of isoflavone,

Table 3. Effect of Germination, Hydrolysis, and Their Interactions Affecting Components in Brazilian Soybean BRS 133[a]

	P values		
parameter	germination	hydrolysis	interaction
BBI	0.0005↓	<0.0001↓	0.0303↓
KTI	0.5049	<0.0001↓	0.1301
lunasin	0.0294↑	<0.0001↓	0.7738
lectin	0.6913	0.0715	0.3159
total isoflavones	<0.0001↑	<0.0001↓	0.0011↓
total saponins	<0.0001↑	<0.0001↓	0.0038↓

[a]Downward arrows indicate reduction in concentration, while upward arrows mean that the concentration was increased.

which was attributed to the presence of contaminating β-glucosidase in the enzyme preparation and found that the residue after hydrolysis contained a higher amount of isoflavones than the actual hydrolysates.[41] Our results showed that Alcalase hydrolysis led to a significant reduction in total isoflavones of the flour when compared to the nonhydrolyzed counterpart.

Interaction between germination and Alcalase hydrolysis showed a statistically relevant reduction in total isoflavones and total saponins, but the interaction had no effect on lunasin in soy protein extracts and soy protein hydrolysates (Table 3). Studies in vivo are needed to better understand the biological meaning of such correlations.

In summary, this study suggests that germination for 18 h and Alcalase hydrolysis for 3 h is optimum for the elimination of protease inhibitors, while the bioactive compounds are still present by at least 50% of their original concentrations. This is important in the preparation of soy flour ingredients for the promotion of health benefits associated with soybean consumption.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

BBI, Bowman-Birk inhibitor; KTI, Kunitz trypsin inhibitor; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; ANOVA, analysis of variance

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