Isoflavone Aglycone Content and the Thermal, Functional, and Structural Properties of Soy Protein Isolates Prepared from Hydrothermally Treated Soybeans

Ana Paula Wally-Vallim, Nathan Levien Vanier, Elessandra da Rosa Zavareze, Rui Carlos Zambiazi, Luis Antônio Suita de Castro, Manoel Artigas Schirmer, and Moacir Cardoso Elias

Abstract: Soybeans were hydrothermally treated at 2 different temperatures (40 °C and 60 °C) and for 4 different hydration times (4, 8, 12, and 16 h) to (i) increase the isoflavone aglycone content in a soy protein isolate and (ii) evaluate the changes in thermal, functional, and structural properties of a soy protein isolate as a function of hydrothermal treatment conditions. Our study is the first to evaluate aglycone content, extraction yield, β-glucosidase activity, differential scanning calorimetry, protein digestibility, scanning electron microscopy, water absorption capacity (WAC), foaming capacity (FC), and foaming stability of soy protein isolates prepared from hydrothermally treated soybeans. For aglycone enhancement and the extraction yield maintenance of soy protein isolates, the condition of 40 °C for 12 h was the best soybean hydrothermal treatment. The structural rearrangement of proteins that occurred with the hydrothermal treatment most likely promoted the capacity of proteins to bind to aglycone. Moreover, the structure shape and size of soy protein isolates verified by scanning electron microscopy appears to be related to the formation of hydrophobic surfaces and hydrophobic zones at 40 °C and 60 °C, respectively, affecting the protein digestibility, WAC, and FC of soy protein isolates.

Keywords: aglycone, functional properties, isoflavone, soy protein

Practical Application: The aglycone content in the soy protein isolate can be improved with the hydrothermal treatment of soybeans. The temperature and time used for hydrothermal treatment must be selected in order to achieve a soy protein isolate with high aglycone content, extraction yield, and functionality. This technology is suitable for providing healthier soy protein isolates for food industry with improved functional and structural properties.

Introduction

Traditionally known as a good source of protein and lipids, soybeans have been widely used in Asian countries as the raw material for several soy-based foods, such as tofu, tempeh, and soymilk. Recently, the knowledge of health benefits promoted by soybean proteins and phytochemicals has increased interest in soy-based foods by Western populations. Many nutritional health benefits of soybean consumption have been attributed to isoflavones, mainly related to women's health. These health benefits include protection against uterine carcinoma (Eason and others 2005) and the relief of menopausal symptoms (Nahas and others 2007). Isoflavones are also used to treat airway inflammatory disease (Bao and others 2011), and their antioxidant activities can prevent oxidative damage (Dixit and others 2012).

Isoflavones can occur in the form of aglycones (daidzein, genistein, and glycitein) and β-glucoside conjugates, which include glucosides, malonylglucosides, and acetylglucosides (Tipkanon and others 2010). According to Izumi and others (2000), the β-glucosidase produced in the gastrointestinal tract hydrolyzes glucosides to aglycones, which are absorbed more rapidly and in higher amounts than their glucosides by humans due to the lower hydrophilicity of aglycones and their lower molecular weights.

Among the soy products used for human consumption, soy protein isolate is widely used in the food industry because of its functional properties. Wang and Murphy (1996) studied the mass balance of isoflavones during soybean processing and verified that only 39.9% of the isoflavones in soy protein isolate are aglycones. Similarly, Shao and others (2009) studied the isoflavone profile of soy protein isolates prepared from low-, medium-, and high-isoflavone content soybeans and reported that aglycones comprise 46.7%, 47.7%, and 38.5% of the total isoflavones, respectively. Thus, alternative processing technologies must be developed to improve isoflavone aglycones in soy protein isolates as a method for providing healthier food products to the world's population. Some of this processing includes soaking, fermentation, coagulation, and protein precipitation.

When soybeans are soaked in water, the endogenous β-glucosidase present in the grain is activated, hydrolyzing the isoflavone glucoside forms to their aglycone forms (Ha and others 1992). The β-glucosidase activity varies as a function of hydration time and temperature (Tipkanon and others 2010). The

MS 20131697 Submitted 11/17/2013, Accepted 4/27/2014. *Authors Wally-Vallim, Vanier, Zavareze, Zambiazi, Schirmer, and Elias are with Depto. de Ciência e Tecnologia Agroindustrial, Univ. Federal de Pelotas, 96010-900, Capão do Leão, Brazil. Author Wally-Vallim is with Inst. Federal Sul-Riograndense, Campus Pelotas – Visconde da Graça, 96060-290, Pelotas, Brazil. Author Castro is with Embrapa Clima Temperado, Rodovia BR 392, km 78, Caixa Postal 403, 96010-971, Pelotas, Brazil. Direct inquiries to author Vanier (E-mail: nathanvanier@hotmail.com).*

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hydrothermal treatment of soybeans has been used to improve the aglycone levels in soybean grains (Carrão-Panizzi and others 2004; Góes-Favoni and others 2010) and in soy germ flour (Tipkanon and others 2010); however, the effects of time and temperature used during soybean hydrothermal treatment with a focus on the soy protein isolate have not been studied.

The objective of this study was to evaluate the aglycone content and the thermal, functional, and structural properties of soy protein isolates prepared from hydrothermally treated soybeans at 2 different temperatures (40 °C and 60 °C) and 4 different hydration times (4, 8, 12, and 16 h).

Materials and Methods

Material

Soybeans (*Glycine max* L. Merril) were cultivated on a farm at Capão do Leão in the State of Rio Grande do Sul, Brazil, and were harvested when the moisture content was approximately 13%. The grains were subsequently submitted to a cleaning process. Subsequently, the grains were treated hydrothermally. All the chemicals used in this study were of analytical grade or better.

Hydrothermal treatment

The soybeans (100 g) were distributed in perforated bags within a beaker, covered with distilled water 1:5 (w/v), and submitted to 2 different temperatures (40 °C and 60 °C) and 4 different hydration times (4, 8, 12, and 16 h), in triplicate. The beakers containing the samples were distributed in a water bath (Bubnoff, Quimis, Diadema, Brazil) under a controlled temperature of 40 °C or 60 °C. The grains were dried in an oven (at the end of each hydration time) at 40 °C under forced air flow until the moisture content reached 13%. Next, the grains were ground in a laboratory mill (Perten 3100, Perten Instruments, Sweden). Nonhydrothermally treated soybeans were used as the control treatment.

The temperatures used in this study were intended to cover a temperature range above and below the critical temperature of soybean protein denaturation to detect a possible change in aglycone formation and functional properties of the proteins as a function of protein interaction with isoflavones.

Protein isolates preparation and extraction yield

The soybean flour was de-fatted using petroleum ether (LabSynth, Brazil). The soybean protein isolates were prepared according to the method described by Lui and others (2003). The de-fatted flour (50 g) was suspended in 1 L of distilled water and the pH was adjusted to 9.0 using NaOH (4 N). The slurry was heated for 45 min at 55 °C under continuous stirring. The slurry was then centrifuged (Excelsa^{TM} II, Mod. 206 BL, Fanem, São Paulo, Brazil) at 13000 × *g* for 20 min at 25 °C. The residue was collected and lyophilized (Micromodulyo E2 M2, Edwards). This fraction was referred to as the insoluble residue. The supernatant was also collected and its pH adjusted to 4.1 with HCl (6 N). It was mixed for 1 h for protein precipitation according to its isoelectric point. After acid precipitation, the slurry was centrifuged (Excelsa II) at 13000 × *g* for 20 min at 5 °C, and the precipitated material (soybean protein isolate) and the supernatant were collected and lyophilized.

The extraction yield of soy protein isolates was measured according to the formula: Extraction yield (%) = {[amount of soy protein isolate (g)/amount of de-fatted flour (g)] × 100}.

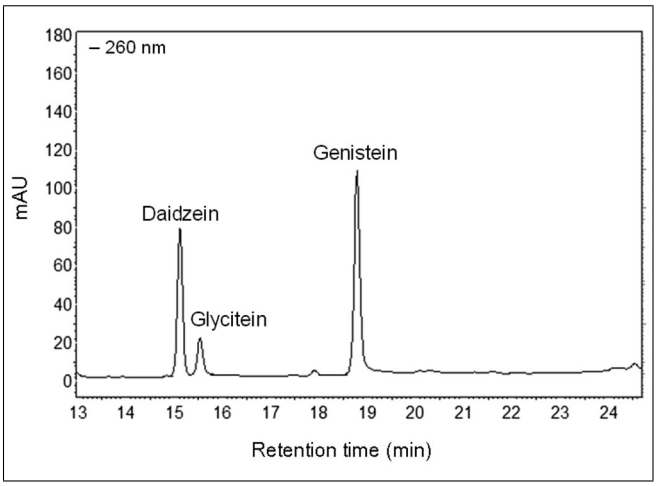

Figure 1–Mixture of isoflavones standard analyzed by high pressure liquid chromatography (HPLC).

Isoflavone extraction

The isoflavone extraction was performed according to the method described by Fukutake and others (1996), with modifications. Each sample (1 g) was transferred to a volumetric flask (10 mL). Methanol (80%) (LabSynth) was added to the flask, and the extraction proceeded for 2 h. Next, the slurry was centrifuged (Microcentrífuga – NT800, Nova Técnica, São Paulo, Brazil) at 9000 rpm for 6 min. The supernatant was transferred to a 1.5 mL vial and used for aglycone quantification.

Isoflavone quantification using high-performance liquid chromatography (HPLC)

The quantification of the aglycones was performed by HPLC according to the method described by Carrão-Panizzi and others (2004).

The extract was purified prior to the HPLC analysis; the first step utilized common filter paper, and the 2nd step was accomplished with a 0.45-μm syringe filter (FHLP 1300 PVDF, Millipore, Billerica, Mass., U.S.A.). Next, 10 μL of extract was injected in the HPLC system equipped with a single pump (model LC-10ATVP, Shimadzu, Japan), a solvent delivery module (FCV-10ALVP, Shimadzu, Japan), a degassing pump (DGU-14A, Shimadzu, Japan), a system controller (SCL-10ATVP, Shimadzu, Japan), a block heater oven (CTO-10ASVP, Shimadzu, Japan), and an auto sampler (SIL-10AF, Shimadzu, Japan). The HPLC separation of all of the compounds was performed using a chromatographic octadecyl reverse-phase Supelcosil^{TM} LC-18 column (25 cm × 4.6 mm × 5 μm). A UV/VIS detector was used for detection at 260 nm (UV/VIS SPD-10AVVP, Shimadzu, Japan).

A gradient elution was performed at a flow rate of 0.9 mL/min, initially using an 80% aqueous mobile phase (0.1% acetic acid in Milli-Q water) and a 20% organic mobile phase (consisting of 0.1% acetic acid in acetonitrile). The mobile phase proportion was changed over 15 min until a ratio of 50% aqueous mobile phase to 50% organic mobile phase was achieved. After 25 min, the mobile phase proportion was changed to 100% organic mobile phase (0.1% acetic acid in acetonitrile), which was maintained until the 35 min mark. Next, the mobile phase linearly returned to the initial condition, totaling 40 min of analysis. The column temperature ranged from 25 °C to 35 °C. The data were analyzed using Class-VP software.

The aglycone quantification was based on an external standard using an analytical calibration curve constructed with standards solutions of daidzein (98% purity, Sigma-Aldrich, St. Louis,

Table 1–Aglycone content (μg aglycone/g dry weight) of soy protein isolates prepared with hydrothermally treated soybeans at different time and temperature conditions.

			Time (h)			
Aglycones	Temperature (°C)	Control	4	8	12	16
Daidzein	40	93.03 aD[a]	99.74 bD	161.84 bC	223.93 bA	207.82 bB
	60	93.03 aE	112.95 aD	170.77 aC	243.09 aA	219.72 aB
Glycitein	40	29.40 aD	33.90 aD	66.40 bC	98.00 bB	117.60 aA
	60	29.40 aE	48.30 bD	82.70 aC	114.10 aB	122.80 aA
Genistein	40	97.84 aE	144.80 bD	220.00 bC	295.10 bA	230.90 bB
	60	97.84 aE	153.40 aD	235.43 aB	312.40 aA	242.90 aC
Total	40	220.27 aE	278.52 bD	447.40 bC	626.98 bA	556.27 aB
	60	220.27 aE	314.69 aD	488.87 aC	669.55 aA	585.38 aB

[a]The results are the means of 3 determinations with a standard deviation <10%. Values with different lowercase letters in the same column and uppercase letters in the same row, for each aglycone compound, are significantly different ($P < 0.05$).

Mo., U.S.A.), glycitein (98% purity, Sigma-Aldrich), and genistein (98% purity, Sigma-Aldrich). The retention times for daidzein, glycitein, and genistein were 15.3, 15.6, and 18.9 min, respectively (Figure 1).

Protein content

The nitrogen content was determined using the AACC method 46–13 (AACC 1995), and the protein content was obtained using a conversion factor of nitrogen to protein of 6.25.

β-glucosidase activity

The β-glucosidase activity in soybeans after hydrothermal treatment was determined according to the method described by Góes-Favoni and others (2010). One hundred milligrams sample of soy flour, and 1.5 mL of 0.05 M citrate buffer (pH 4.5) containing 0.1 M NaCl was maintained for 1 h at room temperature. The samples were centrifuged and the supernatant was kept for enzyme activity analysis. Two milliliters of the substrate 1 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*-NPG) (Sigma Chemical Co., St. Louis, EUA) in phosphate–citrate buffer (0.1 M pH 5.0) were transferred to a test tube and kept in a water bath at 30 °C for 10 min; then 0.5 mL of the supernatant was added and the tube left in the water bath at 30 °C for 30 min. The reaction was stopped with 2.5 mL of 0.05 M sodium carbonate and the contents were immediately measured in a spectrophotometer at 420 nm. The blank solution was composed of 2.5 mL of 0.05 M sodium carbonate solution, 2.0 mL of substrate solution, and 0.5 mL of 0.05 M citrate buffer (pH 4.5) containing 0.1 M NaCl. The para-nitrophenol (*p*-NP) released by the action of the enzyme was determined by referring to a calibration curve prepared from the *p*-NP (Sigma-Aldrich Co., St. Louis, Miss., U.S.A.) in concentrations that varied from 5 to 300 μM, according to Matsuura and Obata (1993). One activity unit (UA) was defined as the quantity of enzyme necessary to release 1 μmol of *p*-NP per minute under the experimental conditions. Results were expressed as β-glucosidase activity level (UA/g of sample on dry weight).

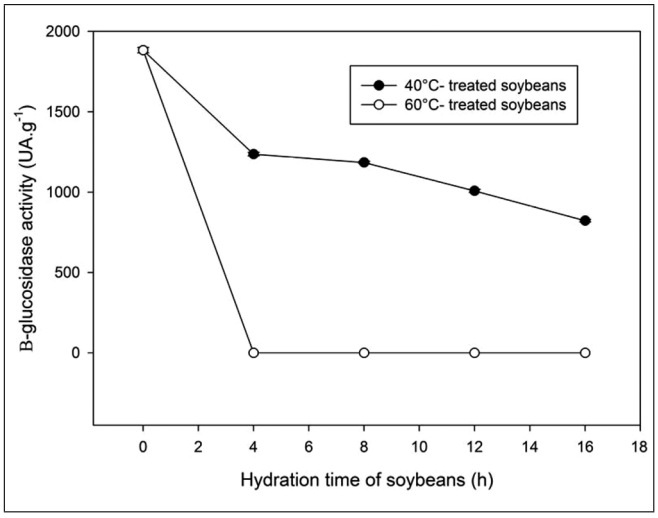

Figure 2–Residual β-glucosidase activity of soybeans after the hydrothermal treatment at 40 °C and 60 °C for 4, 8, 12, and 16 h.

Differential scanning calorimetry (DSC)

Thermal characteristics of the soybean protein isolates were determined using DSC (TA-60 WS, Shimadzu, Kyoto, Japan). Protein isolate samples (approximately 2.5 mg on a dry basis) were weighed directly in an aluminum pan (Mettler, ME-27331), and distilled water was added to obtain an aqueous suspension containing 75% water. The pan was hermetically sealed and allowed to equilibrate for 1 h before analysis. An empty pan was used as a reference. The sample pans were then heated from 20 °C to 110 °C at a rate of 10 °C/min. The onset temperature of denaturation (T_o), the peak temperature of denaturation (T_p), and the denaturation enthalpy (ΔH) were determined.

In vitro protein digestibility

The *in vitro* protein digestibility was determined in accordance with the method described by Pinto and others (2005). Aqueous protein solutions in 0.1 N HCl (10 mg/mL) were hydrolyzed with pepsin for 3 h at 37 °C under mild agitation, with a ratio of enzyme to substrate of 1:25. The hydrolysis was interrupted by adding trichloroacetic acid to a final concentration of 5%. After centrifugation (10000 × *g* for 20 min), the supernatant aliquots were used for nitrogen determination according to the Kjeldahl method (AACC 1995). Two blanks were prepared, a sample blank containing the protein sample under study and an enzyme blank, containing only the protease used. Casein was used as a control in each determination. The correlation between total nitrogen and nitrogen of the hydrolysate (supernatant) allows for the estimation of protein digestibility via the following formula: Protein digestibility (%) = [(*N* digested × 100)/*N* total].

Scanning electron microscopy

The structure of the lyophilized soy protein isolates was examined using a scanning electron microscope (Shimadzu, SSX-550, Japan). The material was initially suspended in acetone to obtain a 1% (w/v) suspension, and the samples were maintained in an ultrasound for 15 min. A small quantity of each sample was spread

Table 2–Aglycone and protein retention in soy protein isolate, insoluble residue and supernatant, and extraction yield (% of the initial de-fatted flour) of soy protein isolates as affected by time and temperature of soybean hydrothermal treatment.

		Soy protein isolate		Insoluble residue		Supernatant		
Temperature (°C)	Time (h)	Protein retention (%)	Aglycones retention (%)	Protein retention (%)	Aglycones retention (%)	Protein retention (%)	Aglycones retention (%)	Extraction yield (%)
Control		82.7 ± 2.7 a [a]	28.8 ± 0.9 e [a]	13.2 ± 0.4 e	7.1 ± 0.5 g	4.1 ± 0.1 c	64.1 ± 0.5 a	40.01 ± 1.26 a [a]
40	4	83.9 ± 3.1 a	32.1 ± 1.8 d	13.4 ± 0.9 e	4.4 ± 0.3 h	2.7 ± 0.2 e	63.5 ± 1.0 a	41.46 ± 1.90 a
	8	82.8 ± 3,5 a	45.2 ± 1.6 b	14.0 ± 0.5 e	12.7 ± 0.3 e	3.2 ± 0.2 d	42.0 ± 0.4 b	38.75 ± 1.30 a
	12	77.1 ± 1.4 b	52.5 ± 1.4 a	19.0 ± 1.7 d	14.4 ± 0.5 d	3.9 ± 0.2 c	33.1 ± 0.5 d	36.02 ± 1.05 b
	16	68.4 ± 3.4 c	46.2 ± 2.3 b	27.7 ± 1.9 c	19.5 ± 0.7 c	3.9 ± 0.1 c	34.4 ± 3.3 d	32.05 ± 1.79 c
60	4	53.4 ± 1.2 d	27.9 ± 0.2 e	42.0 ± 1.4 b	8.9 ± 0.7 f	4.6 ± 0.1 a	63.1 ± 0.3 a	28.27 ± 0.72 de
	8	44.5 ± 2.4 e	36.6 ± 1.7 c	50.7 ± 2.2 a	26.1 ± 0.8 a	4.8 ± 0.1 a	37.2 ± 0.2 c	22.44 ± 1.23 f
	12	54.2 ± 2.2 d	54.2 ± 2.6 a	43.1 ± 0.6 b	21.4 ± 0.4 b	2.6 ± 0.2 e	24.4 ± 0.4 e	26.95 ± 1.76 e
	16	54.5 ± 0.7 d	54.7 ± 1.1 a	42.2 ± 0.2 b	25.0 ± 0.6 a	3.3 ± 0.1 d	20.3 ± 0.2 f	29.29 ± 0.34 d

[a]The values are the means of 3 determinations ± standard deviation and represent the percentage of de-fatted flour proteins and aglycones in each fraction obtained during the soy protein isolation process. Values with different lowercase letters in the same column are significantly different ($P < 0.05$).

Table 3–Differential scanning calorimetry of soy protein isolates prepared with hydrothermally treated soybeans at different time and temperature conditions.

		β-Conglycinin (7S)			Glycinin (11 S)			
Temperature (°C)	Time (h)	T_o (°C)	T_p (°C)	ΔH (J/g)	T_o (°C)	T_p (°C)	ΔH (j/g)	Total enthalpy ΔH (j/g)
Control		72.54 [a]	76.20	0.66	83.60	90.69	2.19	2.85
40	4	73.19	77.71	0.43	84.73	91.90	1.57	2.00
	8	74.38	78.48	0.10	83.62	90.64	1.37	1.47
	12	74.98	77.23	0.84	84.53	90.96	1.73	2.57
	16	73.22	77.74	0.58	84.97	92.27	2.26	2.84
60	4	74.52	78.78	0.19	85.19	92.42	0.84	1.03
	8	75.17	77.97	0.17	84.61	90.79	1.77	1.94
	12	70.94	71.54	0.15	85.37	90.62	2.49	2.64
	16	nd	nd	nd	84.54	91.34	1.66	1.66

[a]The results are the means of 2 determinations.
T_o, onset temperature of denaturation; T_p, peak temperature of denaturation; ΔH, enthalpy of denaturation.

Table 4–*In vitro* protein digestibility (%), water absorption capacity (g/g), foaming capacity (FC; %), and foaming stability (FS; %) of soy protein isolates prepared with hydrothermally treated soybeans at different time and temperature conditions.

			Time (h)			
	Temperature (°C)	Control	4	8	12	16
In vitro protein digestibility (%)	40	72.0 ± 1.2 aC [d]	71.4 ± 1.9 aC	85.5 ± 1.5 aB	86.2 ± 1.0 aB	88.7 ± 1.3 aA
	60	72.0 ± 1.2 aB	66.5 ± 1.5 bC	70.5 ± 1.7 bB	72.7 ± 1.2 bAB	74.6 ± 2.0 bA
WAC (g/g) [a]	40	3.35 ± 0.1 aA [d]	2.96 ± 0.2 bB	3.09 ± 0.1 bB	3.12 ± 0.1 bB	2.97 ± 0.2 bB
	60	3.35 ± 0.1 aB	3.07 ± 0.2 aC	3.43 ± 0.1 aB	4.09 ± 0.2 aA	3.58 ± 0.2 aB
FC (%) [b]	40	66.67 ± 0.9 aE [d]	70.27 ± 2.2 bD	80.24 ± 1.9 bC	84.26 ± 1.4 bB	106.33 ± 2.5 aA
	60	66.67 ± 0.9 aB	90.33 ± 1.4 aA	90.33 ± 2.1 aA	92.27 ± 1.5 aA	90.10 ± 1.9 bA
FS (%) [c]	40	76.16 ± 0.2 aB [d]	76.42 ± 0.3 bB	90.04 ± 0.3 bA	89.29 ± 1.1 bA	90.66 ± 0.9 aA
	60	76.16 ± 0.2 aD	93.27 ± 0.2 aA	93.27 ± 0.5 aA	91.32 ± 0.7 aB	83.60 ± 0.2 bC

[a]Water absorption capacity.
[b]Foaming capacity.
[c]Foaming stability.
[d]The results are the means of 3 determinations ± standard deviation. Values with different lowercase letters in the same column and uppercase letters in the same row, for each analysis, are significantly different ($P < 0.05$).

directly onto the surface of the stub and dried in an oven at 32 °C for 1 h. Subsequently, all of the samples were coated with gold and examined in the scanning electron microscope under an acceleration voltage of 15 kV and magnifications of 20× and 100×.

WAC, foaming capacity (FC), and foaming stability (FS)

The WAC was determined according to the method described by Sosulski and others (1976). A 500 mg sample was homogenized in 5 mL distilled water for 1 min and allowed to stand for 30 min at 25 °C. Next, the material was centrifuged (Excelsa II) at 1200 × *g* for 30 min. The supernatant was then removed, and the precipitated material was weighed. The WAC was measured according to the following equation: WAC (g/g) = {[weight of precipitate (g)/weight of initial sample (g)] × 100}.

The FC and FS were recorded according to the methods described by Phillips and others (1987) and Dipak and Kumar (1986), respectively. For each protein isolate, 3.0 g was suspended in 100 mL of distilled water. The slurry was allowed to stand for 10 min at 25 °C. Next, the slurry was vortexed for 1 min and transferred to a 250 mL beaker, where the volume was measured before and after the mixing of the slurry. The FC was calculated according to the following formula: FC (%) = {[(vol. after mixing − vol. before mixing)/vol. before mixing] × 100}. The FS was analyzed by allowing the slurry to stand for 15 min after the mixing step.

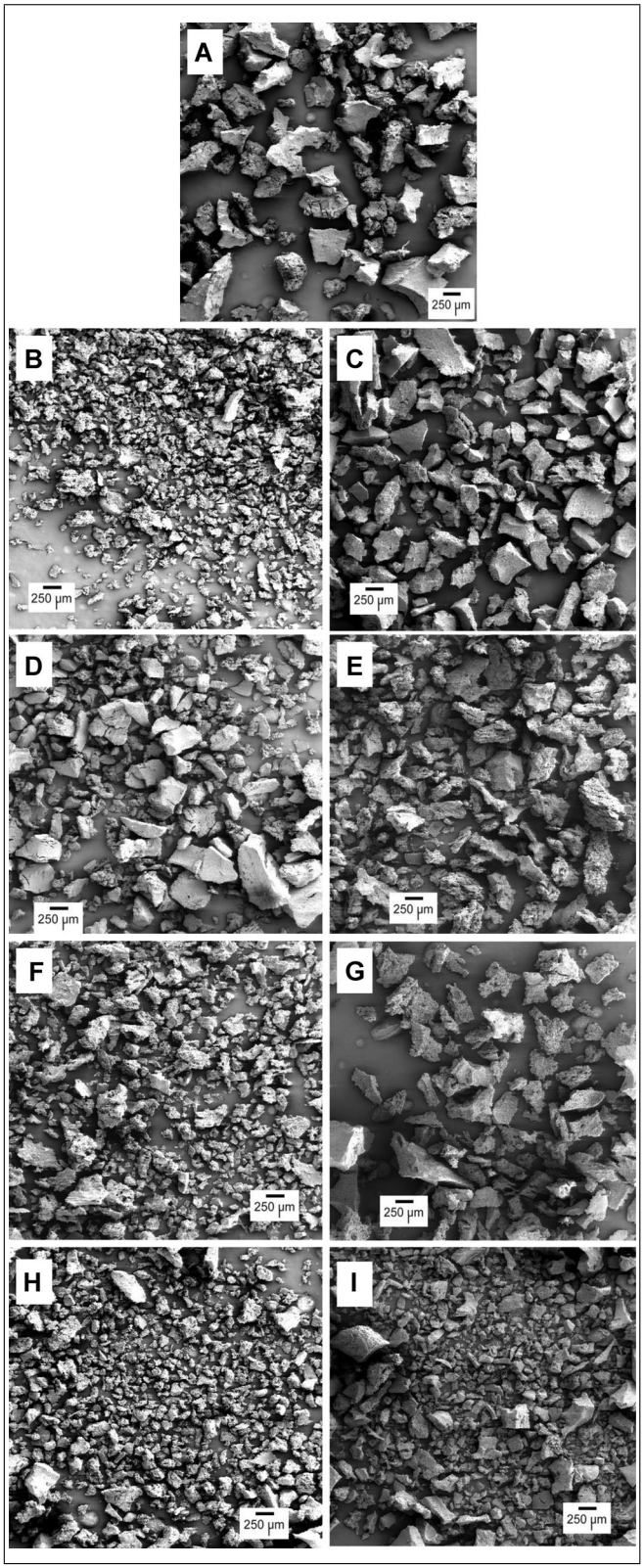

Figure 3–Scanning electron micrographs of soy protein isolates (SPI) prepared from hydrothermally treated soybeans at 20× magnification: (A) control treatment; (B) SPI from 40 °C, 4 h-treated soybeans; (C) SPI from 60 °C, 4 h-treated soybeans; (D) SPI from 40 °C, 8 h-treated soybeans; (E) SPI from 60 °C, 8 h-treated soybeans; (F) SPI from 40 °C, 12 h-treated soybeans; (G) SPI from 60 °C, 12 h-treated soybeans; (H) SPI from 40 °C, 16 h-treated soybeans; (I) SPI from 60 °C, 16 h-treated soybeans.

The FS was calculated according to the following formula: FS (%) = [(volume after 15 min/volume after mixing) × 100].

Statistical analysis

Analytical determinations for the samples were performed in triplicate, except for DSC, which was performed twice. A comparison of the means was ascertained by Tukey's test to a 5% level of significance using analysis of variance (ANOVA).

Results and Discussion

Aglycone content of soy protein isolates as affected by hydrothermal treatment of soybeans

The aglycone contents of the soy protein isolates are presented in Table 1. The hydrothermal treatment of soybeans increased the aglycone content in the protein isolates (Table 1). The aglycone distribution profile remained unchanged with the hydrothermal treatments, with genistein composing the majority of the aglycones, followed by daidzein and glycitein (Table 1). Daidzein and genistein increased with an increased time of hydration up to 12 h at both 40 °C and 60 °C. There was a reduction in the total aglycones of the soy protein isolate in the samples treated for 16 h at both 40 °C and 60 °C compared to the soy protein isolate prepared from 12 h hydrothermally treated grains (Table 1). According to Carrão-Panizzi and others (2004), the hydrothermal treatment of soybean promotes an increase in its β-glucosidase activity. We did not verify β-glucosidase activity in soybeans treated at 60 °C; however, it was verified in soybeans treated at 40 °C (Figure 2). Similar results were verified by Lima and Ida (2014), who reported no enzyme activity of soybeans hydrated at temperatures around 60 °C. The authors explained that β-glucosidase losses stability after its peak activity (Lima and Ida 2014). This fact can justify the decrease in β-glucosidase activity of soybeans treated at 40 °C as a function of hydration time, as well as justify the absence of β-glucosidase activity in soybeans hydrated at 60 °C. The reduction in total aglycones when 16 h of hydrothermal treatment was used compared to 12 h most likely occurred as a function of the thermal instability of isoflavones and/or aglycone leaching to the soaking water. According to Malaypally and Ismail (2010), isoflavones become unstable when proteins are denatured given that proteins act to protect isoflavones.

Protein and aglycone retention in soy protein isolates as affected by hydrothermal treatment of soybeans

The protein and aglycone retention in the soy protein isolate, the insoluble residue, and the supernatant during the protein isolation process are presented in Table 2. Initially, the de-fatted soy flour was composed of 55.3% of crude protein and 321.98 μg/g of aglycones (data not shown). After the analysis of each fraction obtained during the protein isolation process, the soy protein isolate fraction with the highest retention of proteins was from de-fatted soybean flour (82.7%), followed by the insoluble residue (13.2%) and the supernatant (4.1%). The hydrothermal treatment of soybeans before protein isolation changed the protein retention percentage in the 3 fractions (soy protein isolate, insoluble residue, and supernatant). The reduction and the increase in protein retention in the soy protein isolate and the insoluble residue, respectively, are related to the decrease in the extraction yield of the soy protein isolate (Table 2). This conclusion can be drawn given that the protein content of soy protein isolates was similar in the control and in the hydrothermally treated samples, varying

between 92.6% and 96.4% (data not shown). There was an increase in protein retention in the insoluble residue (Table 2) and a consequent decrease in the extraction yield of soy protein isolate (Table 2) from soybeans treated for 12 and 16 h at 40 °C and for all the hydration times at 60 °C compared to the control (protein isolate from nonthermally treated soybeans). The protein isolates from soybeans treated at 60 °C showed a lower extraction yield than the protein isolates from soybeans treated at 40 °C (Table 2). The structural rearrangement of proteins and denaturation can insolubilise proteins, preventing their extraction during the protein isolation process and increasing their retention in the insoluble residue, as verified in Table 2, where the insoluble residue from 60 °C-treated soybeans showed a higher protein retention than the 40 °C-treated soybeans.

The aglycones of the control treatment were distributed mainly in the supernatant, which retained 64.1% of the aglycones initially present in the de-fatted flour used for the protein isolation process (Table 2). A similar result was observed by Lui and others (2003), who reported an isoflavone retention of 52.0% in the supernatant. The hydrothermal treatment promotes an increase in aglycone retention in soy protein isolates, except for the 4 h and 60 °C treatment (Table 2). The highest aglycone retention percentage in a soy protein isolate was found when the soybeans were hydrothermally treated at 40 °C for 12 h (52.5%), at 60 °C for 12 h (54.2%), and at 60 °C for 16 h (54.7%; Table 2). The changes in the structural arrangement of proteins that occurs with hydrothermal treatment most likely promote the capacity of proteins to bind to aglycones. According to Wolf (1979), the thermal denaturation and dissociation of proteins promotes structural unfolding and the exposure of hydrophobic groups. These hydrophobic groups have affinity to phenolic compounds, such as aglycones (Naczk and others 2006). Accordingly, there was also an increase in aglycone retention in the insoluble residue fraction that contain fibers and proteins (data not shown), from 7.1% in the control treatment to 12.7%, 14.4%, and 19.5% in the insoluble residue obtained from the 8, 12, and 16 h-treated soybeans at 40 °C, respectively, and to 8.9%, 26.1%, 21.4%, and 25.0% in the insoluble residue from the 4, 8, 12, and 16 h-treated soybeans at 60 °C (Table 2). This makes the insoluble residue more attractive to industrial applications.

Differential scanning calorimetry

The protein isolates were evaluated for thermally induced changes using DSC. The DSC thermograms of protein isolates showed 2 peaks. According to Liu and others (2008), the 1st peak represents the 7S fraction (β-Conglycinin), while the 2nd one represents the 11S fraction (Glycinin).

The onset temperature of denaturation (T_o), the peak temperature of denaturation (T_p), and the denaturation enthalpy (ΔH) from DSC analysis of the soy protein isolates are presented in Table 3. The T_o and T_p of the β-Conglycinin fraction varied from 70.94 °C to 75.17 °C and 71.54 °C to 78.78 °C, respectively. The T_o and T_p of the Glycinin fraction varied from 83.60 °C to 85.37 °C and 90.62 °C to 92.42 °C, respectively. Similar results were verified by Sorgentini and others (1995), who reported 74 °C and 83 °C for the onset and peak temperatures of β-Conglycinin, respectively.

The ΔH represents the energy required to change the structure of the protein and/or to promote protein denaturation. When ΔH is reduced, it can be due to the structural rearrangement and/or partial denaturation of proteins. When ΔH disappears, it indicates that the denaturation was total. The soy protein isolate prepared from hydrothermally treated soybeans at 60 °C and 16 h did not yield denaturation temperatures (T_o and T_p) and, consequently, did not present enthalpy (ΔH) of the β-Conglycinin protein fraction (Table 3). At 60 °C and 16 h of hydrothermal treatment, only the Glycinin fraction showed T_o, T_p, and ΔH. The higher thermal stability of Glycinin compared to β-Conglycinin is due to its 21 disulfide bonds, of which 15 are intrasubunit and 6 are intersubunit (Damodaran 1988). Given that β-Conglycinin does not contain disulfide bonds, it shows lower stability against thermal treatment and, thus, lower T_o and lower ΔH than Glycinin. In comparing hydrothermally treated and nontreated soy protein isolates, hydrothermal treatment did not induce major changes in the protein electrophoretic patterns (data not shown), suggesting that hydrothermal treatment at 40 °C and 60 °C for as long as 16 h did not modify the protein profiles of soy protein isolate.

In vitro protein digestibility

The *in vitro* protein digestibility of soy protein isolates is presented in Table 4. The soy protein isolates prepared at 40 °C over 8, 12, and 16 h showed higher protein digestibility than the control treatment (Table 4). The increase in protein digestibility is most likely due to a structural rearrangement and/or a partial denaturation that occurred in the proteins, according to the decrease in the total ΔH from the DSC analysis (Table 4). Protein denaturation changes the protein conformation, maintaining its primary structure intact, implying a better protein digestibility by exposing new sites for enzymatic attack. When 60 °C was used for the hydrothermal treatment, there was an intense structural rearrangement, which was confirmed by the decrease in ΔH from DSC analysis. This rearrangement was particularly indicated by the absence of T_o, T_p, and ΔH of β-Conglycinin when the soybeans

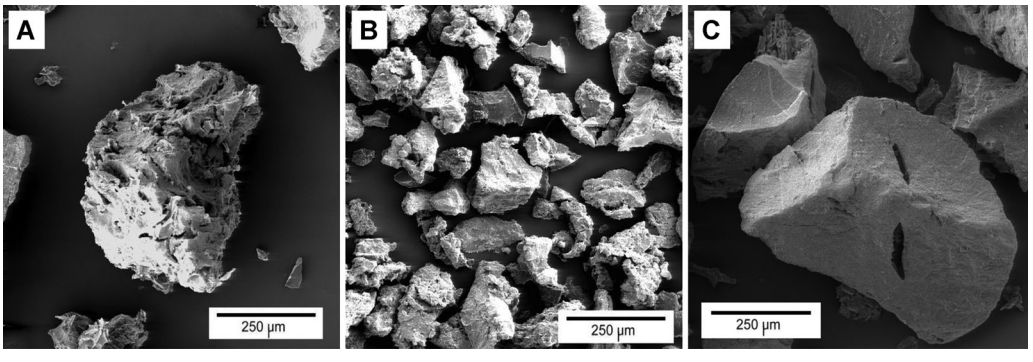

Figure 4–Scanning electron micrographs of soy protein isolates (SPI) prepared from hydrothermally treated soybeans at 100× magnification: (A) control treatment; (B) SPI from 40 °C, 16 h-treated soybeans; (C) SPI from 60 °C, 16 h-treated soybeans.

were treated at 60 °C over 16 h, indicating total denaturation of the 7S fraction (Table 3). Thus, enzyme attack becomes more difficult. These data are in agreement with Privalov (1979), who proposed a 2-step denaturation of globular proteins. The conversion of a native protein to a denatured state involves first the dissociation of the protein subunit followed by the disruption of hydrophobic interactions. However, at higher temperatures, denaturation occurs more intensively and is irreversible, characterized by reaggregation through the exposed hydrophobic regions and disulfide bonds. In addition, the increase in aglycone content most likely contributes to decrease the protein digestibility of the isolates from the 60 °C-treated soybeans. According to Stanley and Aguilera (1985), phenolics can form complexes with proteins, making proteins unavailable to enzymatic reactions.

Scanning electron microscopy

Scanning electron microscopy of the soy protein isolates at a magnification of 20× was performed to understand the effect of the temperature and time used in the hydrothermal treatment of soybeans on the particle size distribution of the protein isolates (Figure 3). The lyophilized soy protein isolates from 40 °C-treated soybeans (clearly observed in Figure 3B, 3F, and 3H) in general had smaller and more heterogeneous structures than the control treatment (Figure 3A). Moreover, the soy protein isolate from 60 °C-treated soybeans for 12 h of hydration time (Figure 3C, 3E, and 3G) did not differ with respect to particle size compared to the control treatment (Figure 3A). In addition, the structures were more homogeneous and were larger than for the 40 °C-treated soybean.

Figure 4 compares the particle surface of soy protein isolates prepared from 40 °C for 16 h-treated soybeans and 60 °C for 16 h-treated soybeans compared to control at a magnification of 100-fold. The soy protein isolate from the control treatment (nonhydrated soybeans before protein isolation) showed an irregular surface, as observed in Figure 4(A). When 40 °C was used during hydrothermal treatment for the most severe hydration time of our study (Figure 4B), there was a reduction in the structure size, but the structure appears to continue with an irregular surface. However, when 60 °C was used as the hydrothermal treatment for the same 16 h (Figure 4C), the structure size was similar to the control, but the surface was smoother. These results may be due to the changes in temperature leading to an unfolding of the soy protein molecules at 40 °C, with an exposure of hydrophobic groups on the surface of the molecules. A similar phenomenon was reported by Hu and others (2013), who studied the effects of ultrasound treatment on the structural properties of soy protein isolate dispersions. The hydrothermal treatment of soybeans at 60 °C most likely promoted an interaction of the hydrophobic surfaces, allowing for the formation of larger aggregates during freeze-drying than those from 40 °C-treated soybeans.

WAC, FC, and FS

The WAC, the FC, and the FS of the protein isolates prepared with hydrothermally treated soybean grains are presented in Table 4.

There was a decrease in the WAC of the protein isolates from 40 °C-treated soybeans and for the soy protein isolate prepared from 60 °C and 4 h-treated soybeans. However, in soy protein isolates from 60 °C-treated soybeans for 8 and 16 h, the WAC was in the same range as the control (Table 4). There was an increase in the WAC in the protein isolate prepared from the 60 °C and 12 h-treated soybeans compared to the control. According to Wagner and others (2000), protein denaturation increases its superficial hydrophobicity, promoting water repulsion. This explains the reduction in the WAC of isolates from 40 °C-treated soybeans. Takeiti and others (2004) reported that mild denaturation conditions cause the exposure of protein hydrophobic regions, reducing their WAC. When 60 °C was used for the hydrothermal treatment of soybeans, there was a partial denaturation of proteins (as verified in the DSC analysis) that possibly promoted the interaction of hydrophobic surfaces, allowing for the formation of new hydrophobic zones. The formation of hydrophobic zones promotes the reduction in water repulsion of the soy protein isolates, explaining why the soy protein isolates from the 60 °C-treated soybeans reacquired the WAC to the same level or higher than the control treatment.

The FC increased in the protein isolates prepared from hydrothermally treated soybeans compared to control (Table 4). There was a continuous increase in the FC of the protein isolates prepared from 40 °C-treated soybeans as a function of hydration time (4, 8, 12, and 16 h). However, the FC of the protein isolates prepared from 60 °C-treated soybeans increased when 4 h of hydration was used and remained unchanged for as long as 16 h of hydration at 60 °C (Table 4). The FS increased in protein isolates prepared from 40 °C-treated soybeans during 8, 12, and 16 h of hydration and increased in protein isolates prepared from 60 °C-treated soybeans for 4, 8, 12, and 16 h of hydration compared to control (Table 4). The highest FS was verified in isolates prepared from 60 °C-treated soybeans when 4, 8, and 12 h of hydration were used. However, when 16 h of hydration was used, the lowest temperature (40 °C) promoted a higher FS than the 60 °C treatment (Table 4).

In addition to the WAC, the protein unfolding that occurred in soybeans treated at 40 °C most likely exposed the hydrophobic surfaces of the proteins, allowing for an increase in the FC with an increase in hydration time. The highest extent of hydrophobic surfaces in soy protein isolates provides a great ability for the material to form bubbles by air occlusion in the nonpolar spaces from molecules. When soybeans were submitted to 60 °C during hydrothermal treatment, there was a strong structural rearrangement and/or denaturation of proteins (as reported in sections "Differential scanning calorimetry" and "Scanning electron microscopy") that increased the FC to a maximum degree during the first 4 h, remaining unchanged until 16 h of hydrothermal treatment at 60 °C (Table 4).

Conclusion

Our study focused on aglycone content and thermal, functional, and structural properties of soy protein isolate, being the 1st one to evaluate soy protein isolates prepared from hydrothermally treated soybeans under different time and temperature conditions. It was possible to increase the aglycone content of soy protein isolates by hydrothermal treatment of soybeans. Considering the observed aglycone enhancement and the extraction yield maintenance of soy protein isolates, we conclude that 40 °C for 12 h is the best soybean hydrothermal treatment used, providing a 184.6% increase in aglycone content with only a 10.0% decrease in extraction yield. The changes in structural arrangement of proteins that occurred with the hydrothermal treatment most likely promoted the capacity of the proteins to bind to aglycones.

The structural and functional characteristics of soy protein isolates were also affected by the hydrothermal treatment of soybeans. The soy protein isolates from soybeans that were treated at 60 °C for as long as 12 h showed a smooth surface, while those isolates

from 40 °C-treated soybeans showed an irregular surface and smaller structures than the control treatment. This structure shape and size appears to be related to the formation of hydrophobic surfaces and hydrophobic zones at 40 °C and 60 °C, respectively, affecting the protein digestibility, WAC, and FC of soy protein isolates.

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