JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

The Effects of Processing and Extraction Conditions on Content, Profile, and Stability of Isoflavones in a Soymilk System

KATHERINE R. NUFER, BARAEM ISMAIL,* AND KIRBY D. HAYES

Food Science Department, Purdue University, 745 Agriculture Mall Drive, West Lafayette, Indiana 47907

The effect of processing temperature and pH as well as enzyme-assisted extraction on the content and profile of isoflavones in a soymilk system was investigated. Isoflavone content in thermally treated soymilk at pH 7 and pH 9 was determined following a standard solvent extraction or an enzyme-assisted extraction protocol. Upon thermal processing, at both pH 7 and pH 9, significant interconversions were noted, indicated by the observed decrease in malonylglucosides with the concurrent increase in β-glucosides. Enzyme-assisted extraction resulted in enhanced isoflavone extraction efficiency and revealed significant loss in total isoflavone content upon processing. This observation suggested that protein−isoflavone interactions, which are dependent on the protein structure and isoflavone form, affect isoflavone extractability, leading to underestimation of any loss that might have occurred in previously reported thermal studies. Accurate isoflavone measurements are essential to determine the processing conditions that result in the least loss of the biologically relevant isoflavone content.

KEYWORDS: Isoflavones; soymilk; pH; processing conditions; isoflavone extraction; isoflavone conversions and loss

INTRODUCTION

Soy isoflavones were shown to be associated with the prevention of several diseases such as cancer, cardiovascular disorders, bone health problems, and postmenopausal symptoms (*1*−*4*). As a consequence, demand for soy-based food products increased substantially over the past couple of decades. Today, many soybean breeders and processers use isoflavone content as a marketing tool.

The bioavailability and the physiological contributions of isoflavones have been the subject of controversial research. Thus, the mechanisms by which isoflavones exert their beneficial effects are not fully understood. However, it is likely that these mechanisms and ultimately the health benefits depend on the isoflavone chemical structure as well as the total amount consumed. Soybeans have 6″-*O*-malonylgenistin as the major isoflavone, followed by genistin, 6″-*O*-malonyldaidzin and daidzin, all of them contributing to about 83−93% of the total isoflavones (*5*). Processing conditions have a substantial influence on the profile of isoflavones, mainly converting conjugates to their respective nonconjugates (*6*−*8*). However, processing may also cause "loss" in the form of undetectable degradation products (*9*−*11*), which may or may not be biologically valuable. While the issue of bioavailability gets solved, characterizing the effect of typical processing conditions on the fate of isoflavones, with respect to their structural modifications and total biologically relevant content, remains a fundamental scientific need.

Literature reports conflicting results with regard to isoflavone loss during processing and their content and profile in different matrices. The protein component of a soy matrix may influence the impact of processing on isoflavone stability as well as their extractability. Hydrogen bonding, ionic and covalent binding, and mainly hydrophobic interactions are involved in the formation of protein−polyphenol complexing (*12*). Having a polyphenolic nature, isoflavones are thought to associate with the interior moiety of the native form of the globular soy protein. Disregarding the effect of structural differences and the association with the protein moiety on the stability and extractability of isoflavones, will certainly lead to conflicting results. Protein may have a protective effect against the degradation of isoflavones upon processing. Also, protein content and the level of protein denaturation may affect isoflavone extractability such that the measured amount of isoflavones does not reflect the true amount in the sample. A greater percentage of total isoflavones was extracted from a low protein soy product (soymilk) than from a high protein soy product (soy protein isolate) after a single extraction, indicating that protein−isoflavone interactions may complicate extractability (*13*).

On the other hand, thermal processing causes denaturation and unfolding of the protein, which potentially can lead to an

* To whom correspondence should be addressed. E-mail: bismailm@umn.edu. Phone: 612-625-0147. Fax: 612-625-5272. Current Address: Food Science and Nutrition, University of Minnesota, 1334 Eckles Avenue, St. Paul, Minnesota 55108-1038.

10.1021/jf802876d CCC: $40.75
Published on Web 01/23/2009

easier extraction of the exposed protein-bound isoflavones. This would make comparison of unprocessed and processed samples inaccurate due to the differences in the state of the protein (native vs denatured) in the samples. Ferruzzi and Green (*14*) found that catechins in a high protein tea−milk system were more efficiently extracted when an enzyme-assisted extraction method was used. On the basis of this finding, it is hypothesized that interrupting protein−isoflavone interactions via proteolysis may improve extraction efficiency and thus allow for better comparison between different soy samples. To date, the effect of proteolysis on isoflavone analytical extraction efficiency has not been demonstrated. Isoflavone−protein interactions can be strongly influenced by not only thermal treatment but also by the pH of the system. Variation in pH will cause changes in the ionization of the proteins, affecting isoflavones interactions with proteins and thus their extraction rate. Increase in measured isoflavone concentrations was observed upon neutralization of soy protein isolate (SPI) samples at the time of analytical extraction (*15*). To date, the combined effect of pH and temperature on isoflavone stability and analytical extraction efficiency has not been examined in a complex aqueous system such as soymilk. The pH of processed soymilk can vary between 6.2 and 8.5, on average, depending on the pH of the plant water, the amount of calcium carbonate or calcium citrate added, protein percent, and the heat treatment (aseptically processed vs pasteurized).

Soymilk processing is not standardized across the industry. For instance, the intensity of thermal treatment varies; soymilk can be pasteurized at 95 °C for ∼15 min or ultrahigh temperature (UHT) processed at 150 °C for 1−2 s (*16*). As a result, a wide variation is noted in the content and profile of isoflavones in soymilk from various sources. Murphy et al. (*17*) reported significant variation in isoflavone content among different brands of soymilk and between lots of the same brand. The primary impact of thermal treatment on isoflavones observed by Murphy et al. (*18*), in a soymilk system, was conversion from malonyl and acetylglucosides to nonconjugated β-glucosides. The researchers (*18*) did not detect significant loss in total isoflavone content upon thermal processing of soymilk in a closed system at 80 °C for 3 h. However, in closed aqueous buffer systems, thermal processing caused significant conversion as well as degradation/loss of isoflavones (*9*−*11*). Differences in recorded loss between model systems and complex systems could be attributed to protein−isoflavone interactions. The enhanced isoflavone analytical extraction due to thermal denaturation of protein might have resulted in masking the loss in isoflavones upon processing of the soymilk.

Because of the high consumption of soymilk and the variety of conditions employed during its processing, there is a need to study the combined effect of temperature and pH on the isoflavone profile and content in soymilk. Processing may be optimized to promote interconversion between isoflavone forms while minimizing loss in total content. In addition, utilization of enzyme-assisted extraction may give insights into the nature of protein−isoflavone interactions and how these interactions may impact stability and accurate assessment of isoflavone content in soymilk. Therefore, the objective of this work was to investigate the effect of processing temperature and pH as well as enzyme-assisted extraction on content, profile, and stability of isoflavones in a soymilk system.

MATERIALS AND METHODS

Materials. Raw soymilk was obtained from Sunopta Inc. (Minnetonka, MN). High performance liquid chromatography (HPLC) grade methanol (no. BP1105-4) and acetonitrile (ACN) (no. A998-4) were obtained from Fisher Scientific (Hanover Park, IL). Lyophilized porcine trypsin (no. T0303-10G; 14300 U/mg) was obtained from Sigma Chemical Co. (St. Louis, MO), dissolved in deionized distilled water (DDW) or Tris buffer (0.05 M, pH 8) with/without NaCl (0.1 M) at various dilutions, and stored at −20 °C until use. Isoflavone standards: genistin, daidzin, malonylgenistin, acetyldiadzin, and acetylgenistin were purchased from LC Laboratories (Woburn, MA), malonyldiadzin and malonylglycitin were purchased from Wako Chemicals, USA, via Fisher Scientific, and genistein, daidzein, and glycitein were purchased from Indofine Chemical Co. (Somerville, NJ). A concentrated solution (500 ppm) of each standard was prepared in 80% methanol in water (v/v). Solutions were stored at −20 °C and were brought to room temperature (23 °C) and completely redissolved before use. Sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) reagents: laemmli buffer (no. 161-0737), Prestained, broad-range molecular weight standards (no. 161-0318), criterion tris-HCl 4−15% gradient precast gels (no. 345-0028), and 10X Tris/glycine/ SDS running buffer (no. 161-0732) were obtained from Bio-Rad Laboratories (Richmond, CA).

Effects of Processing pH and Temperature on Isoflavone Content in Soymilk. A two-factor experimental design, performed in triplicate, was used to examine the effects of temperature, pH, and their interaction on isoflavone content in soymilk. Raw soymilk, prepared by grinding lipoxygenase-null soybeans (specific variety not revealed) with water at 95 °C, was obtained from Sunopta, Inc. (Minnetonka, MN). The soymilk was divided into six 1 L samples. A triplicate of samples was adjusted to approximately pH 9 (range: 8.96−9.14) with 2 N NaOH, while the remaining triplicate was left at their initial pH of approximately 7 (range: 6.94−7.05). The pH values were chosen based on the observed pH range of pasteurized and aseptically processed soymilk from the current market. An aliquot (500 mL) of each sample was left with no further treatment and served as a control. Another aliquot (150 mL) of each sample was distributed into 2 mL glass ampules that were sealed and placed in a water bath at 95 °C (±1) for 1 h. The contents of the ampules for each sample were pooled and frozen at −20 °C until freeze-drying. Freeze-drying, moisture analysis, and isoflavone extractions were carried out according to Wang and Murphy (*5*), with the extractions carried out in duplicate. Extracts were analyzed for isoflavone content by HPLC. Isoflavone concentration was calculated based on peak areas whereby area responses were integrated by Shimadzu software (version 5). Final concentration of each isoflavone was calculated as mol/gram dry sample.

HPLC Analysis. To separate and quantify isoflavones, a Shimadzu HPLC system was used, equipped with SIL-10A VP auto injector, SPD-10A VP UV detector set at a wavelength of 256 nm, and a 250 mm × 4.6 mm, S-5 μm, YMC pack ODS AM-303 C18 reverse phase column (YMC Inc., Wilmington, NC). The HPLC analysis method used was as described by Ismail and Hayes (*19*) with modification only in the calibration approach. A seven-point HPLC calibration was carried out using seven diluted standard solutions (0.1, 0.5, 1.0, 2.0, 4.0, 8.0, 10.0 ppm) containing all 12 isoflavone standards, prepared in 80:20 methanol to water and filtered through 0.45 μm syringe filters.

Development of an Enzyme-Assisted Extraction Method. Several combinations of time, reaction media, and trypsin (14300 U/mg) level, as presented in **Table 1**, were used to determine the optimum conditions for hydrolysis of the protein in soymilk. Partial hydrolysis of soy protein has been achieved with trypsin (*20*−*22*), thus this enzyme was chosen for the enzyme-assisted extraction method. In 50 mL plastic screw-cap containers, 1 g of finely ground, freeze-dried nontreated soymilk was combined with trypsin, which was dissolved in DDW or Tris buffer (0.05 M, pH 8) with/without NaCl (0.1 M), and the volume was brought to 7 mL using DDW or Tris buffer (0.05 M, pH 8) with/without NaCl (0.1 M). The mixture was incubated at 37 °C for the indicated time (**Table 1**), while stirring. After incubation, aliquots (50 μL) of each sample were diluted to 1 mL with DDW in Eppendorf tubes, except in one set of samples (the first six listed in **Table 1**) where 10 μL aliquots were diluted to 1 mL. An aliquot (50 μL) of each diluted sample was mixed with 50 μL DDW and 200 μL Laemmli buffer in screw-cap Eppendorf tubes and boiled for 5 min. Samples were placed on ice and then frozen at −20 °C until electrophoresis.

Table 1. Experimental Conditions for the Hydrolysis of Protein in Soymilk by Trypsin

level of trypsin[a] (mg/g sample)	reaction media	incubation times (h)
0.025	DDW	0.5, 1
0.05	DDW	0.5, 1
0.20	DDW	0.5, 1
0.25	DDW	0.5, 1
0.50	DDW	0.5, 1
2.0	DDW	0.5, 1
20	buffer[b]	2, 4, 6
20	buffer + NaCl[c]	2, 4, 6
200	buffer	2, 4, 6
200	buffer + NaCl	2, 4, 6
100	DDW	0.5, 1, 2
100	buffer	0.5, 1, 2
200	DDW	0.5, 1, 2
200	buffer	0.5, 1, 2

[a] (14300 U/mg). [b] Tris buffer, 0.05 M, pH 8. [c] NaCl, 0.1 M.

SDS-PAGE. SDS-PAGE was carried out to visualize the extent of hydrolysis of soy protein following the various enzyme treatments. Molecular weight standards and samples (20 μL) were loaded onto 18-well 4−15% gradient tris-HCl gels and electrophoresed in 10X tris/glycine running buffer (diluted 1:10 (v/v) with DDW) at 200 V for 1 h. The gel was stained for 1 h with Coomassie blue (50% methanol, 10% glacial acetic acid, 0.25% Coomassie brilliant blue G250) and destained overnight in 10% glacial acetic acid and 5% methanol.

Testing Isoflavone Extraction Efficiency Using the Enzyme-Assisted Protocol. To assess the effect of soy protein hydrolysis on isoflavones extraction efficiency, freeze-dried nontreated soymilk samples, in triplicate, were subjected to either extraction following the method outlined by Murphy et al. (*18*), while using only 1 g of sample instead of 2 g, or enzyme-assisted extraction, using the conditions established for enzymatic hydrolysis. The extraction method outlined by Murphy et al. (*18*) is a modified method based on the one outlined by Wang and Murphy (*5*), used in the earlier experiment, with addition of 7 mL of water to the extracting solution, which consisted of 10 mL acetonitrile and 2 mL HCl (0.1 N). The addition of water seemed to enhance the extraction rate of isoflavones (*18*). This modified extraction was chosen for this experiment to accommodate the addition of an aqueous enzyme solution instead of just water to carry out the enzyme-assisted extraction. For the enzyme-assisted extraction, finely ground, freeze-dried soymilk (1 g) was combined with 2 mL of trypsin in DDW (50 mg/mL) and 5 mL of DDW in a 50 mL screw-cap plastic container and stirred at 37 °C for 1 h. Following incubation, 10 mL of ACN and 2 mL of HCl (0.1 N) were added and the sample was stirred at 400 rpm at room temperature (23 °C) for 2 h. The extract was filtered through Whatman no. 42 filter paper and dried on a rotary evaporator at <40 °C for 1 h. Dried extracts were redissolved in 80% methanol and filtered through a 0.45 μm syringe filter into HPLC vials. A control was prepared by following the same outlined extraction protocol; however, the sample was incubated at 37 °C for 1 h with no enzyme. Extracts from both extraction protocols, and the control, were analyzed for isoflavone content by HPLC as described above.

Monitoring the Effect of Processing Temperature and pH on Isoflavone Chemical Modification Using Various Extraction Protocols. Extraction of isoflavones from the heated (95 °C for 1 h) soymilk samples at various pH (pH 7 and pH 9) and from their respective controls was carried out, in triplicate, using the extraction protocols described in the previous section. Extracts from both extraction protocols, and the control, were analyzed for isoflavone content by HPLC as described above. Percent interconversions between detected known isoflavone derivatives and percent loss in total known isoflavone derivatives were calculated as follows:

$$\%\ \text{interconversion from total conjugate forms into respective } \beta\text{-glucoside form} = \frac{\text{final}[\beta\text{-glycoside}] - \text{initial}[\beta\text{-glycoside}]}{\text{initial}[\text{derivatized conjugates}]} \times 100 \quad (1)$$

$$\%\ \text{loss in total known isoflavone derivatives} = \frac{\text{initial}[\text{known isoflavone derivatives}] - \text{final}[\text{known isoflavone derivatives}]}{\text{initial}[\text{known isoflavone derivatives}]} \times 100 \quad (2)$$

"Initial" concentrations refer to the amount present in the pH 7 raw sample, which served as the control. "Derivatized conjugates" refer to the sum of acetylglucosides and malonylglucosides in a particular sample. "Known isoflavone derivatives" refer to the sum of β-glucosides and/or derivatized conjugates in a particular sample.

Statistical Analysis. Analysis of variance (ANOVA) was carried out utilizing SPSS 15 for Windows (*23*). Total content of isoflavones as well as content of each isoflavone type/form, obtained from following a specific extraction protocol, were compared among the four different pH/heat treatments, using two-factor factorial analysis, with pH and heat as factors. Also, total content of isoflavones as well as content of each isoflavone type/form, extracted using different extraction protocols, were compared within each pH/heat treatment using one-way ANOVA with extraction protocol as the factor. Following the enzyme-assisted extraction, percent interconversion between, and percent loss of, known isoflavone derivatives were compared using two-factor factorial analysis with treatment and isoflavone type as factors. Also, comparisons of percentages were made between isoflavone types within each treatment using one-way ANOVA. When a factor effect or an interaction was found significant, indicated by a significant F test ($P \leq 0.05$), differences between the respective means (if more than 2 means) were determined ($P \leq 0.05$) using Tukey−Kramer multiple means comparison test.

RESULTS AND DISCUSSION

Effects of Processing pH and Temperature on Isoflavone Content in Soymilk. Upon thermal processing, at both pH 7 and pH 9, significant interconversions were noted, indicated by the observed decrease in malonylglucosides with the concurrent increase in β-glucosides (**Table 2**). Accurate calculations of percent interconversion and loss in total isoflavone content, which occurred upon processing, could not be obtained because of the unexpected observed increase in the total measured isoflavone content upon processing at both pH 7 and pH 9 (**Table 2**). The data presented by Murphy et al. (*18*) also showed an increase in total molar concentration of isoflavones after processing at 80 °C for three hours; however, the data were not statistically analyzed. It is not plausible that isoflavones were created during thermal processing. Instead, interactions with protein may have caused the content of isoflavones to be underestimated in the nonheated samples. In the thermally processed samples, heating most likely caused denaturation and unfolding of the protein exposing hydrophobic groups and the isoflavones associated with them, thus potentially enhancing extraction efficiency. Therefore, a greater proportion of the true isoflavone content have been extracted from processed samples, resulting in a measured isoflavone content greater than that of the nonheated samples.

Development of an Enzyme-Assisted Extraction Method. An enzyme-assisted extraction method was developed in order to accurately measure isoflavone content, regardless of the protein denaturation state. Development of the method required: (a) establishing conditions for soy protein hydrolysis and (b) evaluating whether the established conditions improved isoflavone extraction efficiency. The extent of hydrolysis of soy protein following the various enzyme treatments listed in **Table 1** was visualized using SDS-PAGE (**Figure 1**). Conditions listed in **Table 1** but not included in **Figure 1** did not give any significant information relative to the final incubation conditions chosen (gels not shown). Proteolysis of soy protein was achieved after incubation with ≥100 mg enzyme/g sample in both buffer

Table 2. Isoflavone Content in Soymilk Processed under Various Temperature and pH Conditions

	isoflavone content[a]		
	glucosides		
treatment	daidzin	genistin	glycitin
pH 7, raw	407.0 c[b]	554.1 c	33.2 b
pH 7, processed	1015.5 b	1408.5 b	66.3 a
pH 9, raw	396.4 c	549.5 c	28.7 b
pH 9, processed	1230.8 a	1724.8 a	72.1 a
	malonylglucosides		
	malonyldaidzin	malonylgenistin	malonylglycitin
pH 7, raw	600.5 a	1318.9 a	42.6 a
pH 7, processed	279.7 c	675.4 c	21.5 bc
pH 9, raw	442.1 b	1055.3 b	26.1 b
pH 9, processed	135.7 c	366.0 d	18.2 c
	acetylglucosides		
	acetyldaidzin	acetylgenistin	acetylglycitin
pH 7, raw	53.7 b	51.0 b	3.7 a
pH 7, processed	96.0 a	101.7 a	50.8 a
pH 9, raw	48.8 c	48.2 b	40.6 a
pH 9, processed	39.1 d	48.7 b	23.6 a
	aglycons		
	daidzein	genistein	glycitein
pH 7, raw	27.9 a	23.8 a	2.0 a
pH 7, processed	30.1 a	27.9 a	1.4 a
pH 9, raw	28.2 a	32.7 a	1.4 a
pH 9, processed	28.2 a	25.1 a	1.4 a
	total isoflavones[c]		
pH 7, raw	3118.3 b		
pH 7, processed	3774.6 a		
pH 9, raw	2697.8 c		
pH 9, processed	3713.7 a		

[a] Isoflavone content expressed as nmol/g dry weight, taking into account the molecular weight of each isoflavone as follows: daidzin = 416, genistin = 432, glycitin = 446, malonyldaidzin = 502, malonylgenistin = 518, malonylglycitin = 532, acetyldaidzin = 458, acetylgenistin = 474, acetylglycitin = 488, daidzein = 254, genistein = 270, glycitein = 284. [b] Means in each column of each subsection followed by the same letter are not significantly different according to Tukey–Kramer multiple means comparison test ($P \leq 0.05$). [c] Total detected isoflavones.

and DDW for ≥60 min at 37 °C, as indicated by the migration of lower molecular weight hydrolyzed protein peptides down the SDS-PAGE gel (**Figure 1**). Thus, the incubation conditions established for the enzyme-assisted extraction method were: 100 mg trypsin/g sample for 60 min at 37 °C in DDW, which are the same conditions visualized in lane 15 of **Figure 1**. Trypsin exhibited optimum activity at pH 8. However, for simplification of the isoflavone extraction protocol to be used, DDW was chosen for the reaction medium because it was found to be suitable for proteolysis despite the lower pH.

To evaluate the effect of the established soy protein hydrolysis conditions on the efficiency of isoflavone extraction, total extracted isoflavones from soymilk subjected to trypsin hydrolysis, which was the treatment, were compared to the total extracted isoflavones from soymilk subjected to regular solvent extraction protocol (*18*), which served as the control. The total measured isoflavones (7796 nmol/g dry sample) extracted from the sample subjected to the enzyme-assisted extraction protocol was significantly ($P = 0.0143$) greater than that of the samples subjected to the control extraction protocol (6906 nmol/g dry sample). This observation is complemented by the findings of

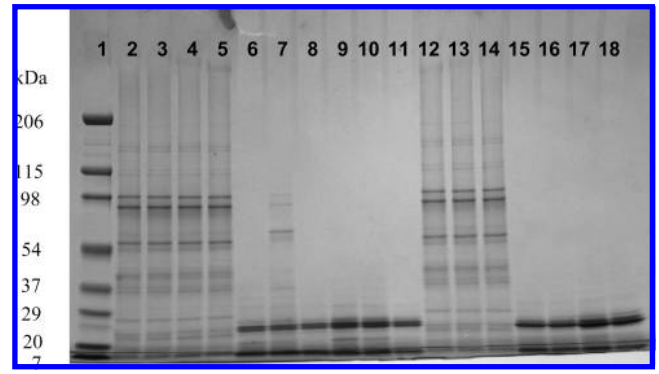

Figure 1. SDS-PAGE visualization of soy protein hydrolysis by trypsin (14300 U/mg) at 37 °C in Tris buffer, 0.05 M, pH 8 (lanes 2–11) and in DDW (lanes 12–18). Lane 1 is MW standards; lanes 2, 3, 4, and 5 are samples incubated with 0 mg trypsin/gram sample for 0, 30, 60, and 120 min, respectively; lanes 6, 7, and 8 are samples incubated with 100 mg trypsin/gram sample for 30, 60, and 120 min, respectively; lanes 9, 10, and 11 are samples incubated with 200 mg trypsin/gram sample for 30, 60, and 120 min, respectively; lanes 12, 13, and 14 are samples incubated with 0 mg trypsin/gram sample for 0, 60, and 120 min, respectively; lanes 15 and 16 are samples incubated with 100 mg trypsin/gram sample for 60 and 120 min, respectively; lanes 17 and 18 are samples incubated with 200 mg trypsin/gram sample for 60 and 120 min, respectively.

Ferruzzi and Green (*14*), where catechins were more efficiently extracted from a high protein tea–milk system following an enzyme-assisted extraction protocol. Trypsin-catalyzed hydrolysis caused the soy protein to be broken down into smaller peptides as evidenced by SDS-PAGE, thus the protein lost some of its primary, secondary, and tertiary structure. The hydrolysis most likely disrupted protein–isoflavone interactions, including hydrogen bonds, hydrophobic interactions, and electrostatic interactions, releasing isoflavones into the extraction solvent more readily and resulting in a more accurate measurement of the true isoflavone content.

Overall it was noted that the total isoflavone content obtained using the extraction method outlined by Murphy et al. (*18*) was significantly higher than the total isoflavone content (**Table 2**) obtained using the extraction method outlined by Wang and Murphy (*5*), with an without enzyme-assistance. This observation is attributed to the presence of water in the extracting solution, as explained by Murphy et al. (*18*). However, even with the enhanced extraction observed with the modified solvent method, enzyme-assisted extraction resulted in significantly higher total measured isoflavones (7796 vs 6906 nmol/g dry sample).

Effect of Processing Temperature and pH on Isoflavone Chemical Modification Using Various Extraction Protocols. Among the samples subjected to the control-extraction protocol, an increase in β-glucosides and a concurrent decrease in malonylglucosides within both the daidzin and genistin series was observed, indicating that conversion occurred at both pH 7 and pH 9 (**Table 3**). This is the primary conversion of interest as malonylglucosides and nonconjugated β-glucosides are the major contributors to the total isoflavone content. Similar conversions due to heat (*9*) and heat combined with pH (*9*) have been observed in model systems but have not been reported in soymilk. The glycitin series was not evaluated separately due to its minimal contribution (<2%) to the total isoflavone content, although detected glycitin derivatives are included in the total content.

The changes in isoflavone profile and content of the samples subjected to the enzyme-assisted extraction protocol followed

Table 3. Isoflavone Content in Soymilk Processed under Various Temperature and pH Conditions, Extracted Following Trypsin-Assisted Extraction or Regular Solvent Extraction Protocol

		isoflavone content[b]						
		daidzin series			genistin series			
treatment	extraction protocol[a]	Din*	Mdin*	Adin*	Gin*	Mgin*	Agin*	total[c]
pH 7, raw	control-extraction	757 c[d]	2286 b	53.8 b	746 c	2908 b	43.7 b	6911 b
	enzyme-assisted extraction	836 c	2461 a	65.8 b	835 c	3178 a	46.9 b	7552 a
pH 7, processed	control-extraction	1783 b	1197 c	97.5 a	1828 b	1648 c	99.9 a	6777 b
	enzyme-assisted extraction	1818 b	1164 c	97.9 a	1880 b	1607 c	97.6 a	6766 b
pH 9, raw	control-extraction	814 c	2360 ab	47.2 bc	839 c	3007 ab	44.2 b	7226 ab
	enzyme-assisted extraction	866 c	2338 ab	61.5 b	899 c	3038 ab	44.3 b	7383 ab
pH 9, processed	control-extraction	2184 a	673 d	37.1 c	2290 a	945 d	42.4 b	6247 c
	enzyme-assisted extraction	2355 a	694 d	39.0 c	2465 a	993 d	44.1 b	6672 bc

[a] Control extraction: extraction following the method outlined by Murphy et al. (2002); enzyme-assisted extraction: extraction following incubation with 100 mg trypsin (14300 U/mg)/gram sample for 60 min at 37 °C. [b] Expressed as nmol/gram dry weight. [c] Total detected isoflavones, including glycitin series (data not shown). [d] Means in each column followed by the same letter are not significantly different according to Tukey−Kramer multiple means comparison test ($P \leq 0.05$). *Din = daidzin; Mdin = malonyldaidzin; Adin = acetyldaidzin; Gin = genistin; Mgin = malonylgenistin; Agin = acetylgenistin.

the same trend as that of the samples subjected to the control-extraction protocol. However, samples subjected to the enzyme-assisted extraction protocol showed significant loss in total detectable isoflavones upon processing at pH 7 and pH 9 when compared to the nonheated sample at pH 7. Whereas in samples subjected to control extraction, loss due to processing was not evident at pH 7. The enhanced extraction of isoflavones upon denaturation of the protein, most likely has masked the loss that occurred due to processing at pH 7, when control-extraction was employed. On the other hand, loss was apparent at pH 9. It seems that there is a combined effect of pH on both extractability as well as stability of isoflavones. Malonyl carboxyl is ionized at pH > 8, and so are some of the hydroxyl groups of the isoflavone itself. Increased ionization increases reactivity and reduces stability (*11*). Ionization of the hydroxyl groups on the isoflavones might promote electrostatic interactions or repulsion with the protein moiety, thus affecting extractibility and stability of the isoflavone. Further studies are needed to better understand the effect of high pH on the association of isoflavones with the protein.

Isoflavone content of the raw samples at pH 7, determined using the enzyme-assisted extraction protocol, was significantly greater than that determined using the control extraction protocol (**Table 3**), indicating that protein−isoflavone interactions were disrupted upon enzymatic proteolysis. This enhanced extraction was not observed in the processed samples at pH 7, where thermal processing might have already weakened or disrupted these interactions due to protein denaturation. Therefore, proteolysis in the processed samples at pH 7 did not further enhance the extraction. Within both the daidzin and genistin series, the enzyme-assisted extraction method primarily increased the extraction of malonylglucosides in raw samples at pH 7 (**Table 3**), suggesting that malonyl-forms exert stronger interaction with the protein than the other isoflavone forms. Malonylglucosides have an additional ionizable group compared to the other isoflavones, which may allow for increased interactions with the protein. The precise nature and strength of isoflavone−protein interactions has not been characterized among the different isoflavone structures, thus it is an area that requires further investigation.

Extraction of isoflavones from samples at pH 9 did not seem to be enhanced by enzyme-assisted extraction (**Table 3**). This is most likely due to the change in charge distribution on the protein, which might affect the strength of isoflavone−protein interactions. For better understanding, this also requires further investigation.

Isoflavone content of all samples subjected to the enzyme-assisted extraction protocol was consistently greater than or equal to that of the samples subjected to control-extraction protocol (**Table 3**). Therefore, percent interconversion and percent loss of isoflavones were determined using the data obtained from the enzyme-assisted extraction protocol. Percent interconversions from conjugated to nonconjugated β-glucosides upon thermal treatment were 32% and 38% at pH 7 and 50% and 60% at pH 9 for genistin and daidzin forms, respectively. The significantly ($P \leq 0.05$) greater percentages of conversion due to processing at alkaline pH are consistent with reported observations in SPI (*15*) and model systems (*10*). Additionally, 10.4% and 11.6% loss in total detectable isoflavones were observed upon processing at pH 7 and pH 9, respectively. The observed loss is mainly attributed to the conversion of conjugated glucosides to undetected products that may or may not be complete degradation products. The nonconjugated β-glucosides are relatively stable under these conditions (*10*). There was no significant difference in percent loss due to processing at pH 7 compared to pH 9, unlike what was observed in a model system (*10*). Moreover, the observed percent conversion and percent loss in soymilk was lower than those observed by Mathias et al. (*10*) in a buffer system subjected to similar processing conditions. The noted difference between buffer and soymilk systems could be attributed to the protein moiety, which may exert a protective effect against conversion and degradation of the associated isoflavones.

Overall results confirmed that isoflavone content and profile in soymilk systems are affected by processing temperature and pH and that interactions with protein can affect the accuracy of evaluating these effects. Results highlighted the inappropriate comparison of isoflavone content in processed vs unprocessed soymilk systems following standard extraction protocols. Enzyme-assisted extraction resulted in enhanced isoflavone extraction efficiency, at pH 7, thus enabling a more accurate comparison between treated soymilk samples. Following an enzyme-assisted extraction protocol revealed significant loss in total isoflavone content upon processing, contrary to what has been reported previously (*18*). This observation suggested that protein−isoflavone interactions affect isoflavone extractability, which may have masked any loss that might have occurred in previously reported thermal studies. Accurate isoflavone measurements are

essential to determine the processing conditions that result in the least loss of the biologically relevant isoflavone content. Therefore, more work is needed to (1) optimize an enzyme-assisted extraction taking into account the protein level and denaturation state in a specific matrix and (2) determine the effect of the protein content and structure on the stability of the associated isoflavones.

LITERATURE CITED

(1) Cohen, L. A.; Zhou, Z.; Pittman, B.; Scimeca, J. A. Effect of intact and isoflavones-depleted soy protein on NMU-induced rat mammary tumorigenesis. *Carcinogenesis* **2000**, *21*, 929–935.

(2) Kwon, T. W.; Song, Y. S.; Kim, J. S.; Moon, G. S.; Kim, J. I.; Honh, J. H. Current research on the bioactive functions of soyfoods in Korea. *J. Korean Soybean Dig.* **1998**, *15*, 1–12.

(3) Lamartiniere, C. A.; Moore, J. B.; Brown, N. M.; Thomson, R.; Harden, M. J.; Barnes, S. Genistein suppresses mammary cancer in rats. *Carcinogenesis* **1995**, *16*, 2833–2840.

(4) Song, T. T.; Hendrich, S.; Murphy, P. A. Estrogenic activity of glycitein, a soy isoflavone. *J. Agric. Food Chem.* **1999**, *47*, 1607–1610.

(5) Wang, H.; Murphy, P. A. Isoflavone content in commercial soybean foods. *J. Agric. Food Chem.* **1994**, *42*, 1666–1673.

(6) Barnes, S.; Kirk, M.; Coward, L. Isoflavones and their conjugates in soy foods: extraction conditions and analysis by HPLC-Mass spectrometry. *J. Agric. Food Chem.* **1994**, *42*, 2466–2474.

(7) Wang, H. J.; Murphy, P. A. Mass balance study of isoflavones during soybean processing. *J. Agric. Food Chem.* **1996**, *44*, 2377–2383.

(8) Coward, L.; Smith, M.; Kirk, M.; Barnes, S. Chemical modification of isoflavones in soy foods during cooking and processing. *Am. J. Clin. Nutr.* **1998**, *68*, 1486S–91S.

(9) Chien, J. T.; Hsieh, H. C.; Kao, T. H.; Chen, B. H. Kinetic model for studying the conversion and degradation of isoflavones during heating. *Food Chem.* **2005**, *91*, 425–434.

(10) Mathias, K.; Ismail, B.; Corvalan, C.; Hayes, K. D. Temperature and pH effects on the conjugated forms of genistin and daidzin isoflavones. *J. Agric. Food Chem.* **2006**, *53*, 7495–7502.

(11) Vaidya, N. A.; Mathias, K.; Ismail, B.; Corvalan, C. M.; Hayes, K. D. Kinetic modeling of malonylgenistin and malonyldaidzin conversions under alkaline conditions and elevated temperatures. *J. Agric. Food Chem.* **2007**, *55*, 3408–3413.

(12) Boye, J. I. Protein-polyphenol interactions in fruit juices. *Recent Res. Dev. Agric. Food Chem.* **1999**, *3*, 85–107.

(13) Achouri, A.; Boye, J. I.; Belanger, D. Soybean isoflavones: efficacy of extraction conditions and effect of food type on extractability. *Food Res. Int.* **2005**, *38*, 1199–1204.

(14) Ferruzzi, M. G.; Green, R. J. Analysis of catechins from tea−milk beverages by enzyme-assisted extraction followed by high performance liquid chromatography. *Food Chem.* **2006**, *99*, 484–491.

(15) Rickert, D. A.; Meyer, M. A.; Hu, J.; Murphy, P. A. Effect of extraction pH and temperature on isoflavone and saponin partitioning and profile during soy protein isolate production. *J. Food Sci.* **2004**, *69*, C623−C631.

(16) Gandhi, N. R.; Hackbarth, H. R., Chen, M. Soy milk compositions and methods of preparation. U.S. Patent 6,663,912, December 16, 2003.

(17) Murphy, P. A.; Song, T.; Buseman, G.; Barua, K.; Beecher, G. R.; Trainer, D.; Holden, J. Isoflavones in retail and institutional soy foods. *J. Agric. Food Chem.* **1999**, *47*, 2697–2704.

(18) Murphy, P. A.; Barua, K.; Hauck, C. C. Solvent extraction selection in the determination of isoflavones in soy foods. *J. Chromatogr., B* **2002**, *777*, 129–138.

(19) Ismail, B.; Hayes, K. β-Glycosidase activity toward different glycosidic forms of isoflavones. *J. Agric. Food Chem.* **2005**, *53*, 4918–4924.

(20) Kamata, Y.; Shibasaki, K. Degradation sequence of glycinin by tryptic hydrolysis. *Agric. Biol. Chem.* **1978**, *42*, 2103–2109.

(21) Kim, S. Y.; Park, P. S. W.; Rhee, K. C. Functional Properties of Proteolytic Enzyme Modified Soy Protein Isolate. *J. Agric. Food Chem.* **1990**, *38*, 651–656.

(22) Shutov, A. D.; Kakhovskaya, I. A.; Bastrygina, A. S.; Bulgama, V. P.; Horstmann, C.; Müntz, K. Limited proteolysis of β-conglycinin and glycinin, the 7S and 11S storage globulins from soybean (*Glycine max* (L.) Merr.): structural and evolutionary implications. *Eur. J. Biochem.* **1996**, *241*, 221–228.

(23) *Statistical Program for Social Sciences (SPSS). SPSS for Windows*, release 15; SPSS Inc.: Chicago, IL, 2006.

Received for review September 15, 2008. Revised manuscript received December 3, 2008. Accepted December 17, 2008.

JF802876D