JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Heat and pH Effects on the Conjugated Forms of Genistin and Daidzin Isoflavones

KEVIN MATHIAS, BARAEM ISMAIL, CARLOS M. CORVALAN, AND KIRBY D. HAYES*

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

Isoflavones occur primarily as glycosides (namely, malonyl-, acetyl-, and non-conjugated β-glycosides) and a small percentage as the bioactive aglycon. The different chemical structures of isoflavones can dictate their stability during processing. Therefore, our objective was to determine the effects of pH and thermal treatments on conjugated isoflavones with regard to interconversions and loss. Conjugated daidzin and genistin were heated at 25, 80, and 100 °C under neutral, acidic, and basic conditions. Changes in isoflavone derivatives were monitored using high-performance liquid chromatography. Along with interconversions, considerable loss in total known isoflavone derivatives was noted for each isoflavone, especially under elevated pH and temperature. The malonylglycosides showed more stability than acetylglycosides, especially under acidic conditions. Overall, loss in isoflavone derivatives was significantly higher for daidzin than for genistin glycoside forms. Our results highlighted the significance of chemical structure with regard to stability, which is a key factor in determining soy processing conditions.

KEYWORDS: Isoflavones; β-glycosides; malonylglycosides; acetylglycosides; stability; pH; processing conditions

INTRODUCTION

Epidemiological and clinical studies have shown that the consumption of soybeans decreases the risk of various diseases afflicting humans. A large number of researchers have intensified efforts to determine the active components of soy associated with health benefits, where many agreed that isoflavones are the most beneficial group (*1*−*3*). Three isoflavone types (daidzin, genistin, and glycitin) are known to exist in soybean. These isoflavones occur primarily as β-glycosides with a small percentage as the principal bioactive aglycon. The glycosides can exist in three forms: malonyl-, acetyl-, and non-conjugated β-glycosides. Essentially, a β-glycosidic form has a sugar moiety attached to the aglycon structure, and the conjugated forms have either a malonyl or an acetyl group esterified at the 6″-*O*- of the sugar moiety (**Figure 1**). In raw soybeans, malonylgenistin and malonyldiadzin are the most abundant forms followed by their respective non-conjugates and acetyl conjugates; glycitein derivatives, however, are found in marginal concentrations (*4*). Soybeans are commonly processed into a wide variety of soy or soy-based products. The difference in chemical structure among the known forms/types of isoflavones can affect their stability during various processing conditions and, consequently, their bioavailability. Research is still at an unprecedented high to investigate the health benefits, toxicity level, minimum requirements, and the best biologically available form of isoflavones in soybean. While all these issues are being studied, and until they are resolved, isoflavones stability is certainly a key factor in the determination of processing conditions of soy-based products.

The production of soy-based food products involves several types and intensities of processing conditions, which might have a substantial influence on the amount and profile of the isoflavones in soy products to a varying extent (*4*−*6*). In particular, the profile of isoflavones has been shown to depend on processing temperature and time (*7*, *8*). Malonylglycosides and acetylglycosides are thermally unstable and can readily be converted to their respective more heat-stable non-conjugated β-glycosides (*9*). In general, research has indicated that isoflavones were not destroyed under normal thermal processing conditions but rather were subject to interconversions between the different forms (*9*); however, additional reactions might have occurred, especially when interconversions were not equimolar (*4*). Murphy et al. (*10*) and Wang and Murphy (*11*) have shown significant variation in the total amount of isoflavones among several soy products that underwent different processing operations. Along with interconversions, a significant amount of loss/degradation was apparent under variable processing conditions. Researchers have seen up to 65% loss in total amount of isoflavones after processing in relation to their initial concentration in the raw soybeans (*12*). Measured losses of isoflavones were mainly related to leaching into discarded cooking water (*2*, *13*) or any other discarded fraction (*12*), efficiency of extraction procedures (*5*, *9*, *14*−*16*), aqueous-alcohol solutions

* Corresponding author. Telephone: 765-496-2864. Fax: 765-494-7953. E-mail: hayesk@purdue.edu.

10.1021/jf061322a CCC: $33.50
Published on Web 09/12/2006

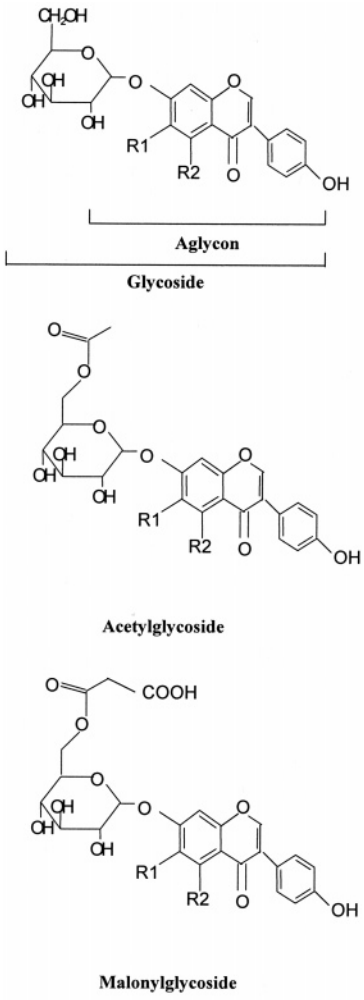

Figure 1. Aglycon, glycoside, acetylglycoside. and malonylglycoside where R1 can be H in the case of daidzin and genistin or CH_3O in the case of glycitin, while R2 can be H in the case of daidzin and glycitin or OH in the case of genistin.

used in the production of certain soy products like soy protein concentrates and isolates (*17*), and process temperature and pH with regard to soy matrices (*18*).

Jackson et al. (*12*) have measured isoflavone contents in the waste stream, and although the amounts represented a significant portion of the loss observed (65%), 20% loss still remained unexplained. Rostagno et al. (*19*) referred to the occurrence of isoflavone degradation with prolonged storage. Park et al. (*20*), on the other hand, measured the isoflavone profiles in soy flour and showed a decrease in total isoflavones (20.6% ± 7.9 SD) for all cultivars after heating at 121 °C for 40 min. A kinetic model was developed by Chien et al. (*8*) for the four known derivatives of genistin under dry and moist heat conditions in a closed model system. Their results not only showed conversions but also significant degradation of isoflavones that was not related to soy matrices, waste streams, or extraction procedures. The rates of degradation observed as compared to the rates of conversion were relatively high. However, their kinetic model did not include pH variation as a factor. Also, their model included only genistin derivatives; therefore, there was no indication to whether similar conversions and loss would be observed for diadzin, which is also present in significant amount in soy products.

Along with the interconversions between the known chemical forms of isoflavones resulting in a variable profile that might affect their biological activity, a significant amount of known isoflavone derivatives are being lost. Although several attempts have been made to study stability and conversions of isoflavones, the literature still lacks a complete understanding of the chemical modification of the isoflavone structure as affected directly by pH and heat. Total amount of biologically relevant isoflavones is what contributes to the nutritional value of soy products. It is hypothesized that the different chemical structure of each isoflavone form/type dictates its stability during processing under variable pH and temperature conditions. Since processing conditions can result in considerable interconversions and degradation, understanding the chemical modification of each known isoflavone compound as affected by pH and heat, factors that can change dynamically during processing, is a prerequisite to control degradation. Also, studying conditions that lead to interconversions might provide new insights for nutritional studies directed toward the effect of structural form on bioavailability.

The prevalence of malonyl- and acetylglycosides, mainly those of daidzin and genistin isoflavones, in processed soy products (*10*) warrants the investigation of parameters that will directly affect the chemical modifications of these glycosides occurring during processing, regardless of protein−isoflavone interactions. Therefore, our objective was to perform basic research to determine the direct effects of pH and thermal treatments on conjugated isoflavones with regard to interconversions and loss.

MATERIALS AND METHODS

Materials. High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Fisher Scientific (Hanover Park, IL). Isoflavone standards genistin, daidzin, genistein, daidzein, malonylgenistin, acetyldiadzin, and acetylgenistin were purchased from LC Laboratories (Woburn, MA). Malonyldiadzin was purchased from Fisher Scientific. Standard solutions of 500 ppm were prepared using 80% aqueous methanol.

HPLC Analysis. A Shimadzu HPLC system, equipped with SIL-10A VP autoinjector, 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 reversed-phase column was used for isoflavone analysis. Separation and quantification of isoflavones was achieved using the HPLC method outlined by Ismail and Hayes (*21*) with one modification regarding calibration. For the malonylgenistin and non-conjugated genistin determinations, a calibration scheme was followed with only the genistin series present in the calibration standard solutions, and that was to provide a better statistical fit. The % CV (relative standard deviation (std dev/mean × 100)) of the method, in all cases, was less than 5%.

Effects of Heat and pH Treatments on the Conjugated Forms of Genistin and Daidzin. A two-factor experimental design, performed in triplicate, was used to examine the effects of heat, pH, and their interaction on the conjugated forms of daidzin and genistin (malonyldaidzin, MDin; acetyldaidzin, ADin; malonylgenistin, MGin; and acetylgenistin, AGin). Diluted HCl (0.01 N HCl) solution (pH = 2) for acidic conditions, deionized distilled water (DDW) for neutral, or a diluted NaOH (0.0001 N) solution (pH = 10) for alkaline conditions were used. The extreme pH treatments were purposely chosen to magnify the pH effect if any. Since the pH range chosen was wide, the use of one type of buffering agent could not be achieved, and using different buffering agents might contribute to unaccounted for variations. Therefore, on the expense of risking some shifts in the pH throughout the treatment, no buffering agents were used. And even if shifts in pH were to occur, it would still be in acidic, neutral, or alkaline ranges for the starting pH 2, pH 7, or pH 10 solutions, respectively. An aliquot (20 μL) of each of the conjugated isoflavone solutions (500 ppm) was placed separately into three 2 mL screw-cap Eppendorf tubes, and then 980 μL of pH 2 solution, DDW, or pH 10 solution was added. The three Eppendorf tubes of each conjugated isoflavone then were placed into a 25 °C water bath (GCA model 260, Precision Scientific, Chicago,

Table 1. Mean Amounts (nmol) of MDin, ADin, Din, and Dein Present in Samples of Malonyldaidzin and Acetyldaidzin Subjected to 2 h of Various pH and Heat Treatments[a]

starting isoflavone	treatment	MDin	ADin	Din	Dein	total known Din derivatives[b]	total loss in known Din derivatives[c]
malonyldaidzin	control	4.25 a	0.00 b	0.15 h	0.00	4.39 a	0 e
	pH 7, 25 °C	4.02 a	0.00 b	0.26 h	0.00	4.29 a	0.11 e
	pH 2, 25 °C	4.11 a	0.00 b	0.20 h	0.00	4.31 a	0.08 e
	pH 10, 25 °C	3.73 b	0.00 b	0.50 g	0.00	4.23 ab	0.16 de
	pH 7, 80 °C	3.0 c	0.00 b	1.08 e	0.00	4.03 b	0.36 d
	pH 2, 80 °C	3.54 b	0.00 b	0.68 f	0.00	4.22 ab	0.17 de
	pH 10, 80 °C	1.62 e	0.00 b	2.00 c	0.00	3.62 c	0.77 c
	pH 7, 100 °C	0.91 f	0.00 b	2.39 b	0.00	3.30 de	1.09 b
	pH 2, 100 °C	1.97 d	0.00 b	1.51 d	0.00	3.48 cd	0.91 bc
	pH 10, 100 °C	0.18 g	0.14 a	2.78 a	0.00	3.10 e	1.13 a
acetyldaidzin	control	0.00	4.11 a	0.00 g	0.00	4.11 ab	0 e
	pH 7, 25 °C	0.00	4.18 a	0.00 g	0.00	4.18 a	0 e
	pH 2, 25 °C	0.00	4.12 a	0.00 g	0.00	4.12 ab	0 e
	pH 10, 25 °C	0.00	2.34 e	1.17 d	0.00	3.51 d	0.61 c
	pH 7, 80 °C	0.00	3.69 b	0.07 g	0.00	3.76 c	0.36 d
	pH 2, 80 °C	0.00	3.15 c	0.84 e	0.00	4.00b	0.10 e
	pH 10, 80 °C	0.00	0.06 g	3.19 a	0.00	3.24 e	0.88 b
	pH 7, 100 °C	0.00	2.96 d	0.49 f	0.00	3.45 d	0.68 c
	pH 2, 100 °C	0.00	1.42 f	2.04 c	0.00	3.46 d	0.66 c
	pH 10, 100 °C	0.00	0.00 g	2.93 b	0.00	2.93 f	1.19 a

[a] Mdin, malonyldaidzin; ADin, acetyldaidzin; Din, daidzin; Dein, daidzein. Means in each column per starting isoflavone, followed by the same letter, are not significantly different according to the Tukey–Kramer multiple means comparison test ($P \leq 0.05$). [b] The summation of all remaining known derivatives (MDin, ADin, Din, and Dein). [c] Amount of undetected isoflavones as compared to the control.

IL) for 2 h, with temperature fluctuation of ± 1 °C. The exact procedure was repeated using heat treatments of 80 °C and 100 °C. After heat treatment for 2 h, 4 mL of methanol (99.95%) was added to the content of each tube to result in 2 ppm isoflavone solutions. Each isoflavone solution then was filtered through 0.45 μm syringe filters into two vials and stored at −20 °C. A control was prepared, for each heat treatment, where 20 μL of each conjugated isoflavone solution (500 ppm) was mixed with 980 μL of DDW, then diluted to a 2 ppm isoflavone solution as stated above, and stored at −20 °C. All samples and controls were subjected to HPLC analysis, where isoflavone concentrations (ppm) were calculated based on peak areas using calibration curves plotted in Microsoft Excel (2003). Isoflavone concentrations (ppm) were converted to moles for better comparisons since each isoflavone has a different molecular weight. Percent interconversions between detected known isoflavone derivatives and percent loss in known isoflavone derivatives were calculated as follows:

Equation 1:

$$\% \text{ interconversions} = A + B + C$$

A = % conversion into respective conjugated form = [(conjugated form produced after treatment)]/[starting isoflavone in control (amount of specific isoflavone from prior to treatment)] × 100

B = % conversion into respective non-conjugated form = [(non-conjugated form in treated sample − non-conjugated form present in control (conjugated isoflavone controls sometimes contain minimal amounts of non-conjugated glycosides)]/[starting isoflavone in control] × 100

$$C = \% \text{ conversion into respective aglycon form} = \frac{(\text{amount of aglycon form produced ater treatment})}{\text{starting isoflavone in control}} \times 100$$

Equation 2:

% loss in total known isoflavone derivate 2 = [(total isoflavones in the control − total isoflavones in the treated sample)]/[total isoflavones in the control (starting isoflavone + other isoflavone form present in control, if any)] × 100

Effects of Heat and pH Treatments on Non-Conjugated Genistin and Daidzin. The effect of pH 2,pH 7, and pH 10, at 100 °C on non-conjugated daidzin (Din) and genistin (Gin) was studied following the exact procedure outlined in the previous section.

Statistical Analysis. Analysis of variance (ANOVA) was carried out utilizing SPSS 11.5 for Windows (*22*). Data were analyzed using two-factor factorial analysis in a completely randomized design with heat and pH as factors when studying changes within each isoflavone form. Percent interconversions between detected known isoflavone derivatives and percent loss in known isoflavone derivatives were compared among the four conjugated isoflavones, within each treatment (pH and heat), using one-way ANOVA with isoflavone type as factor. 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 were determined ($P \leq 0.05$) using the Tukey−Kramer multiple means comparison test.

RESULTS AND DISCUSSION

Effects of Various Heat and pH Treatments on Conjugated Daidzin Forms. The various heat and pH treatments exerted a significant ($P \leq 0.05$) effect on the conjugated forms of Din (**Table 1**). As compared to the control, MDin concentration decreased under alkaline conditions and as temperature increased, with a subsequent increase in the formation of Din (**Table 1**). It has been reported that alkaline conditions promote conversions of conjugated forms to their respective non-conjugates (*14*). Conversions of MDin into Din were observed under acidic conditions; however, they were less significant as compared to neutral and alkaline conditions, even with elevated temperatures. Wang et al. (*23*) reported that heating Din and Gin conjugated forms under acidic conditions released free isoflavones. In the present study, no conversions into daidzein (Dein) were noted in any of the treatments. This observation could be due to the low molarity of the acid used (0.01 M) in the present study as compared to a molarity of up to 3 M used by Wang et al. (*23*). Decarboxylation of MDin into ADin did not seem to occur in any of the treatments, except for pH 10 at 100 °C, where only about 3.3% of MDin was converted to ADin. Decarboxylation is normally favored under dry heating (>150 °C) conditions (*8*, *9*, *24*). Heating soymilk, however, at 80 °C caused malonlyglycosides to be converted almost exclusively

Table 2. Mean Amounts (nmol) of MGin, AGin, Gin, and Gein Present in Samples of Malonylgenistin and Acetylgenistin Subjected to 2 h of Various pH and Heat Treatments[a]

starting isoflavone	treatment	MGin	AGin	Gin	Gein	total known Gin derivatives[b]	total loss in known Gin derivatives[c]
malonylgenistin	control	4.01 a	0.00 d	0.12 f	0.00 c	4.13 a	0 d
	pH 7, 25 °C	3.81 a	0.00 d	0.18 f	0.00 c	3.99 ab	0.15 cd
	pH 2, 25 °C	4.04 a	0.00 d	0.13 f	0.08 b	4.25 a	0.02 d
	pH 10, 25 °C	3.81 a	0.00 d	0.24 f	0.00 c	4.05 a	0.14 d
	pH 7, 80 °C	2.48 c	0.04 c	1.12 d	0.00 c	3.64 bc	0.50 abc
	pH 2, 80 °C	3.17 b	0.00 d	0.63 e	0.12 ab	3.92 abc	0.21 bcd
	pH 10, 80 °C	2.00 d	0.03 c	1.60 c	0.00 c	3.63 bc	0.50 ab
	pH 7, 100 °C	0.82 e	0.17 a	2.63 b	0.00 c	3.62 bc	0.51 ab
	pH 2, 100 °C	2.02 d	0.04 c	1.60 c	0.18 a	3.84 abc	0.30 abcd
	pH 10, 100 °C	0.37 e	0.05 b	3.12 a	0.00 c	3.54 c	0.60 a
acetylgenistin	control	0.00	4.52 a	0.12 e	0.00 b	4.63 ab	0 d
	pH 7, 25 °C	0.00	4.67 a	0.13 e	0.00 b	4.80 a	0 d
	pH 2, 25 °C	0.00	4.60 a	0.16 e	0.00 b	4.76 a	0 d
	pH 10, 25 °C	0.00	3.10 c	1.34 c	0.00 b	4.44 abcd	0.19 bcd
	pH 7, 80 °C	0.00	3.80 b	0.24 e	0.00 b	4.04 e	0.59 a
	pH 2, 80 °C	0.00	3.40 c	1.10 cd	0.00 b	4.51 abc	0.13 cd
	pH 10, 80 °C	0.00	0.23 e	3.89 a	0.00 b	4.13 de	0.51 ab
	pH 7, 100 °C	0.00	3.20 c	1.0 d	0.00 b	4.19 cde	0.45 abc
	pH 2, 100 °C	0.00	1.61 d	2.55 b	0.22 a	4.39 bcde	0.25 bcd
	pH 10, 100 °C	0.00	0.12 e	3.95 a	0.06 b	4.13 de	0.51 ab

[a] MGin, malonylgenistin; AGin, acetylgenistin; Gin, genistin; Gein, genistein. Means in each column per starting isoflavone, followed by the same letter, are not significantly different according to the Tukey–Kramer multiple means comparison test ($P \leq 0.05$). [b] The summation of all remaining known derivatives (MGin, AGin, Gin, and Gein). [c] Amount of undetected isoflavones as compared the control.

to their respective non-conjugates (*9*). In that study, production of acetyl conjugates, monitored over a period of 3 h, was found to be minimal. Furthermore, the slight decarboxylation that might have occurred under the present moist conditions was most likely followed by a consequent de-esterification, as will be shown when discussing conversions of the acetylglycosides. Chien et al. (*8*) showed that, under both dry and moist heating of MGin, AGin formation increased then decreased with prolonged heating time (up to 3 h). The total amounts of known Din derivatives in treated MDin samples decreased mainly under elevated pH and temperature conditions. This observation indicated either complete degradation of the isoflavone or further conversions into other unknown isoflavone derivatives that are undetectable by the HPLC protocol used.

Overall results showed that MDin was most stable at pH 2 and least stable at pH 10. Ungar et al. (*25*) studied the thermal stabilities of the aglycon forms of Din and Gin at pH 7 and pH 9, where their kinetic analysis showed that the degradation rates for both isoflavones were higher at pH 9 than pH 7. Regardless of pH, stability of MDin significantly ($P \leq 0.05$) decreased as temperature increased. The highest percentage of loss in known Din derivatives was observed under pH 7 (25%) and pH 10 (30%) at 100 °C. However, the highest percent of interconversions (65%) was observed only under pH 10 at 100 °C.

Acetyldaidzin reaction to various pH and heat treatments seemed to be quite different from that of MDin (**Table 1**). After an alkaline treatment at 25 °C for 2 h, ADin dropped by 43%. The drop in ADin became even more dramatic under alkaline conditions as temperature increased, where a complete depletion was noted at 100 °C. Less conversions of ADin into Din were observed under neutral conditions as compared to acidic and alkaline conditions, even with elevated temperatures. No conversions into Dein were noted in any of the treatments. Under alkaline conditions, significantly higher Din was observed in ADin sample heated at 80 °C than that heated at 100 °C, which indicated degradation of Din at higher temperature. This observation was confirmed when Din was heated at 100 °C under alkaline conditions for 2 h, where a drop of 16% in total Din was noted (data not shown). The drop in total amounts of known Din derivatives in treated ADin samples was less significant under neutral and acidic conditions as compared to alkaline conditions at both 80 and 100 °C (**Table 1**).

Results indicated that ADin was most stable at pH 7 and least stable at pH 10, an observation that was slightly different from that of MDin. Overall, and regardless of pH, percent interconversions and loss in known Din derivatives increased as temperature increased. The best condition for ADin interconversions was specifically pH 10 at 80 °C treatment. Increasing the heat treatment to 100 °C, under alkaline conditions, led to a significant increase in percent loss of known Din derivatives (**Table 1**).

Effects of Various Heat and pH Treatments on Conjugated Genistin Forms. Under various pH and heat treatments, changes in the Gin conjugated forms (**Table 2**) followed a similar trend as that of the Din conjugated forms, with some exceptions. As was observed in treated MDin samples, MGin concentration decreased under alkaline conditions and as temperature increased, with a subsequent increase in the formation of Gin. Our findings were similar to the results reported by Chien et al. (*8*), who showed that MGin was readily converted to Gin under moist heat treatment at 100 °C. Our results showed that less conversions of MGin into Gin occurred under acidic conditions as compared to neutral and alkaline conditions, even with elevated temperatures. Unlike what was observed for treated Din conjugated forms, genistein (Gein) production was noted under acidic conditions, which increased slightly as temperature increased. It seemed like production of Gein occurs more readily than production of Dein under the tested acidic conditions. In the study carried out by Chien et al. (*8*), production of aglycons occurred only under dry heat (200 °C) and not under moist heat. However, their moist heat treatment was carried out under normal pH conditions. In the present work, slight decarboxylation of MGin into AGin seemed to occur as temperature increased, more so under neutral conditions. Decarboxylation under acidic conditions seemed to

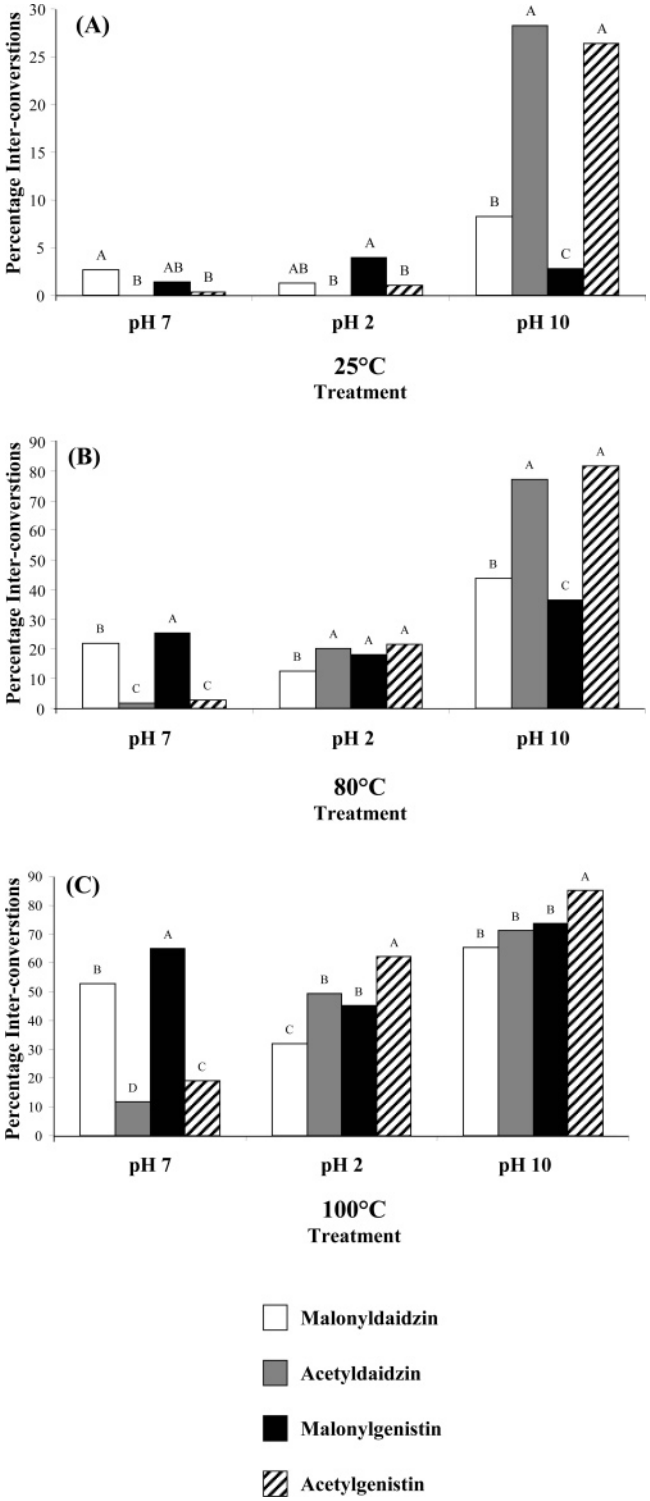

Figure 2. Interconversion percentages of the conjugated forms of daidzin and genistin after 2 h treatment under pH 7, pH 2, and pH 10 at 25 °C (**A**), 80 °C (**B**), and 100 °C (**C**). Interconversion percentages were obtained from the data presented in **Tables 1** and **2**. Different letters over the bars indicate significant differences between the four conjugated forms within each treatment and not across, according to the Tukey–Kramer multiple means comparison test ($P \leq 0.05$).

be the least favored, which is most probably due to the system being moist versus dry. As for decarboxylation that occurred under alkaline condition, it was most likely followed by de-esterification as observed in treated AGin samples under alkaline conditions (**Table 2**). The total amounts of known Gin derivatives in treated MGin samples decreased mainly under elevated pH and temperature conditions. However, the decrease noted here seemed to be less pronounced than the decrease in total amounts of known Din derivatives in treated MDin samples (**Table 1**).

Overall, and as was noted for MDin, MGin was most stable at pH 2 and least stable at pH 10, with the stability significantly ($P \leq 0.05$) decreasing as temperature increased. Treating MGin at 100 °C under acidic conditions showed considerably higher total interconversions with minimum loss. Treating MGin at 100 °C under alkaline conditions, however, showed the highest

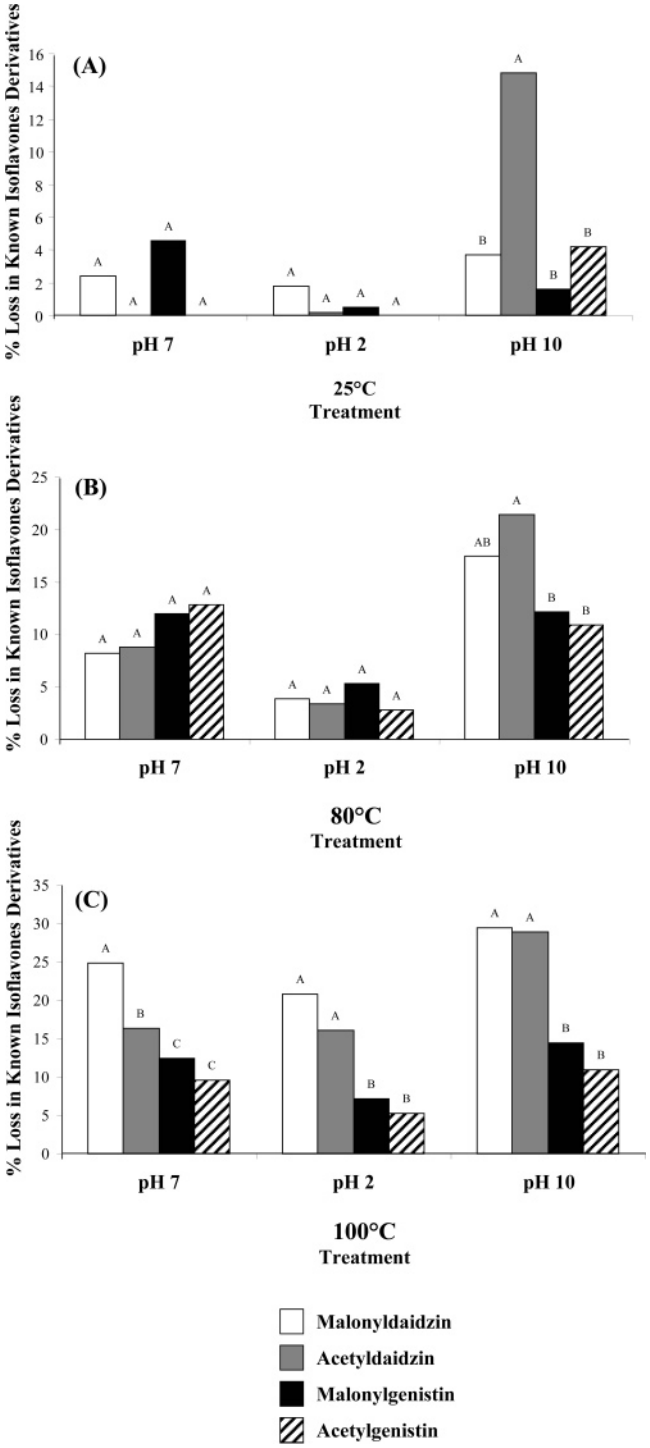

Figure 3. Percent loss in known isoflavone derivatives of the conjugated forms of daidzin and genistin after 2 h treatment under pH 7, pH 2, and pH 10 at 25 °C (**A**), 80 °C (**B**), and 100 °C (**C**). Loss percentages were obtained from the data presented in **Tables 1** and **2**. Different letters over the bars indicate significant differences between the four conjugated forms within each treatment and not across, according to the Tukey–Kramer multiple means comparison test ($P \leq 0.05$).

total interconversions (74%), without much increase in total loss (**Table 2**).

As was noted for Din conjugated forms, AGin reaction to various pH and heat treatments seemed to be quite different from that of MGin (**Table 2**). After an alkaline treatment at 25 °C for 2 h, AGin dropped by 32%. The drop in AGin was even more significant under alkaline conditions as temperature increased. Unlike Mgin, Agin seemed to be sensitive to not only alkaline conditions but also to acidic conditions and even more so as temperature increased. The non-conjugated glycoside (Gin) produced under alkaline conditions did not vary in AGin samples heated at 80 °C and at 100 °C, unlike what was noted for the non-conjugated glycoside (Din) produced from ADin under the same conditions. Genistin produced did not seem to undergo degradation as Din did, which was an observation confirmed after heating Gin at 100 °C under alkaline conditions (data not shown). It has been noted by other researchers that Din derivatives are more prone to degradation than Gin derivatives (*25*, *26*). As temperature increased, the total known Gin derivatives, of treated AGin samples, dropped more significantly

under alkaline conditions as compared to neutral and acidic conditions.

Although AGin was most stable under neutral conditions, overall minimal loss was observed under acidic conditions (**Table 2**). Regardless of pH, percent interconversions significantly increased as temperature increased; however, loss increased a little as temperature increased to 80 °C and was not significantly different from loss at 100 °C, mainly at pH 7 and pH 10 (**Table 2**). Maximal interconversions and loss in treated AGin samples were observed under alkaline conditions at both 80 and 100 °C; however, the loss was only 11% of the total AGin in the control. Considerable interconversions (62%) were also noted under acidic conditions at 100 °C, with loss being only about 5%.

Comparing the Reactions of Conjugated Din and Gin under Various Treatments. Results so far indicated differences in the reactions of the conjugated forms of Din and Gin to various pH and heat treatments. Therefore, data presented in **Tables 1** and **2** were used to compute percent total interconversions and percent loss in known isoflavone derivatives. And, to compare stabilities of the four treated conjugated forms, statistical comparisons of interconversions and loss were carried out. Under most of the treatments, significant ($P \leq 0.05$) differences were observed in percent interconversions (**Figure 2**) and percent loss (**Figure 3**) of treated conjugated forms (MDin, ADin, MGin, and AGin). Percent interconversion in treated samples did not seem to vary much under neutral and acidic conditions at both 25 and 80 °C (**Figure 2A,B**). However, under alkaline condition ADin and AGin showed significantly higher interconversions than MDin and MGin at both 25 and 80 °C. At 100 °C, under both acidic and alkaline conditions, AGin showed the highest interconversions (**Figure 2C**), while MGin under neutral condition showed maximal interconversions. Percent loss of known isoflavone derivatives did not significantly differ under neutral and acidic conditions at both 25 and 80 °C (**Figure 3A,B**). However, under alkaline condition ADin showed significantly higher loss than all other conjugated forms, and at 80 °C both ADin and MDin showed significantly higher loss than Gin conjugated forms. At 100 °C, under all pH conditions, Din conjugated forms showed significantly higher loss (almost double) than Gin conjugated forms (**Figure 3C**). Similar findings were reported by Grun et al. (*26*) when loss in total isolfavones of tofu heated in water at 80, 90, and 100 °C was mostly due to a decrease in the Din series.

Overall, our results showed that the chemical structure of isoflavones dictates their stability under variable pH and temperature conditions. Along with interconversions, considerable loss in total known isoflavones, was noted for each type/form of isoflavones, especially under elevated pH and temperature. This loss could be due to either a complete degradation or a transformation into isoflavone derivative(s) that could not be detected (i.e., showed no distinctive peaks) with the current HPLC method yet still comprise biological relevance. Further work is needed, using modified HPLC and mass spectroscopy techniques, to identify these derivatives and confirm whether or not they are of biological relevance through nutritional studies. Our results indicated that similar interconversions and loss in total known isoflavones are occurring during processing as a result of pH and heat fluctuation, irrespective of interference of the soy matrix. To complement and further investigate our findings, it is recommended to kinetically study conversions and loss of various types/forms of isoflavones under a narrower range of pH and at various temperatures similar to the current soy processing conditions, which includes dry and moist processing.

Our results highlighted that even a small difference in molecular structure as that between Din and Gin forms, which is only the presence of OH group at R2 position (**Figure 1**), can contribute to major differences mainly in percent loss. Also the malonyl moiety with the carboxyl group as compared to the acetyl moiety seemed to provide more stability to the isoflavone, especially under acidic conditions. These findings provide basic information to soy food producers aiming toward enhancing the nutritional value of their products, through minimizing loss and maximizing the isoflavone form/type of the most biological significance, without sacrificing the quality characteristics. This being said, it is worthwhile noting that the form/type of isoflavone that is most biologically significant is yet to be known. Therefore, while nutritionists investigate the biological significance of each type/form of isoflavone, it is a necessity to fully understand their distinct reaction to various processing conditions.

LITERATURE CITED

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

(2) Messina, M. Soy, soy phytoestrogens (isflavones), and breast cancer. *Am. J. Clin. Nutr.* **1999**, 70, 574−575.

(3) Kurzer, M. S. Hormonal effects of soy isoflavones: studies in premenopausal and postmeopausal women. *J. Nutr.* **2000**, *130*, 660S−661S.

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

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

(6) Coward, L.; Smith, M.; Kirk, M.; Barnes, S. Chemical modifications of isoflavones in soyfoods during cooking and processing. *Am. J. Clin. Nutr.* **1998**, *68*, 1486S−1491S.

(7) Kudou, S.; Fleury, Y.; Welti, D.; Magnolato, D.; Uchida, T.; Kitamura, K.; Okubo, K. Malonyl isoflavone glycosides in soybean seeds (*Glycine max* Merrill). *Agric. Biol. Chem.* **1991**, *55*, 2227−2233.

(8) 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.

(9) 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.

(10) 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.

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

(12) Jackson, C. J. C.; Dini, J. P.; Lavandier, C.; Rupasinghe, H. P. V.; Faulkner, H.; Poysa, V.; Buzzell, D.; DeGrandis, S. Effects of processing on the content and composition of Isoflavones during manufacturing of soy beverage and tofu. *Process. Biochem.* **2002**, *37*, 1117−1123.

(13) Hendrich, S.; Murphy, P. A. Isoflavones: source and metabolism. In *Handbook of Nutraceuticals and Functional Foods*; Wildman, R. E. C., Ed.; CRC Press: Boca Raton, FL, 2001; pp 55−75.

(14) Klump, S. P.; Allred, J. L.; MacDonald, J. M.; Ballam, J. Determination of isoflavones in soy and selected foods containing soy by extraction, saponification, and liquid chromatography:

collaborative sudy. *J. Assoc. Off. Anal. Chem. Int.* **2001**, *84*, 1865−1883.
(15) Lin, F.; Giusti, M. M. Effects of solvent polarity and acidity on the extraction efficiency of isoflavones from soybeans (Glycine max). *J. Agric. Food Chem.* **2005**, *53*, 3795−3800.
(16) Achouri, A.; Boye, J. I.; Belanger, D. Soybean isoflavones: efficacy conditions and effect of food type on extractability. *Food Res. Int.* **2005**, *38*, 1199−1204.
(17) Coward, L.; Barnes, N. C.; Setchell, K. D. R.; Barnes, S. Genistein, daidzein, and their β-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. *J. Agric. Food Chem.* **1993**, *41*, 1961−1967.
(18) 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.
(19) Rostagno, M. A.; Palma, M.; Barroso, C. G. Short-term stability of soy isoflavones extracts: sample conservation aspects. *Food Chem.* **2005**, *93*, 557−564.
(20) Park, Y. K.; Aguiar, C. L.; Alencar, S. M.; Mascrenhas, H. A. A.; Scamparini, A. R. P. Conversion of malonyl β-glycosides isofalvones into glycoside isoflavones found in some cultivars of Brazilian soybeans. *Cienc. Tecnol. Aliment.* **2002**, *22*, 130−135.
(21) Ismail, B.; Hayes, K. β-Glycosidase activity toward different glycosidic forms of isoflavones. *J. Agric. Food Chem.* **2005**, *53*, 4918−4924.
(22) Statistical Program for Social Sciences (SPSS). *SPSS for Windows*, Release 11.5.; SPSS Inc., Chicago, IL, 2002.
(23) Wang, G.; Kuan, S. S.; Francis, O. J.; Ware, G. M.; Carman, A. S. A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products. *J. Agric. Food Chem.* **1990**, *38*, 185−190.
(24) Farmakalidis, E.; Murphy, P. A. Isolation of 6-*O*-acetyldaidzin and 6-*O*-acetylgenistin from toasted defatted soy flakes. *J. Agric. Food Chem.* **1985**, *33*, 385−389.
(25) Ungar, Y.; osundahunsi, O. F.; shimoni, E. Thermal stability of genistin and daidzin and its effect on their antioxidant activity. *J. Agric. Food Chem.* **2003**, *51*, 4394−4399.
(26) Grun, I. U.; Adhikari, K.; Li, C.; Li, Y.; Lin, B.; Zhang, J.; Fernando, L. N. Changes in the profile of genistin, daidzin, and their conjugates during thermal processing of Tofu. *J. Agric. Food Chem.* **2001**, *49*, 2839−2843.

Received for review May 10, 2006. Revised manuscript received August 1, 2006. Accepted August 3, 2006.

JF061322A