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β-Glycosidase Activity toward Different Glycosidic Forms of Isoflavones

BARAEM ISMAIL AND KIRBY HAYES*

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

Isoflavones, a group of soybean components that significantly contribute to human health and disease prevention, exist in various chemical forms. The enzyme activity can be very sensitive to molecular structure; thus, the profile of the isoflavones can affect their rate of hydrolysis. The objective of this work was to study the β-glycosidase activities toward isoflavone β-glycosides and their conjugated forms. Hydrolysis experiments were conducted where β-glycosides and their conjugates were treated with β-glycosidase. Results confirmed that β-glycosidase can hydrolyze nonconjugated β-glycosides into aglycones. However, when the enzyme amount and/or activity were limited, significant differences in enzyme activity toward the β-glycosides were observed. On the other hand, β-glycosidase was not effective in hydrolyzing the conjugated glycosides to their respective aglycones, even with increased levels of the enzyme and with prolonged incubation. The transformation of conjugated glycosides into their respective β-glycosides will most likely result in increased hydrolysis rates and better absorption.

KEYWORDS: Isoflavones; β-glycosides; aglycones; malonylglycosides; acetylglycosides; β-glycosidase activity; hydrolysis

INTRODUCTION

The soybean [*Glycine max* (L.) Merrill], a popular food crop for its good quality protein and oil content, possesses significant health benefits that have been widely recognized for many years (*1*). In 1999, the Food and Drug Administration (*2*) approved a health claim that "25 g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease". As a consequence, consumer interest in the health benefits of soy foods is now at an unprecedented high. Accordingly, soy product sales in the United States have increased tremendously from $2.77 billion in 2000 to more than $3.5 billion in 2002 and are expected to grow 10−11% per year until 2005 (*3*). A large number of researchers have intensified efforts to determine the active components of soy associated with health benefits, with many agreed that isoflavones are the most beneficial group of soy components.

Many animal and human clinical studies as well as epidemiological studies have been conducted to prove the association of isoflavones with prevention of certain diseases, namely, cancer (*4*−*6*), cardiovascular disorders (*7*−*9*), bone health problems (*5*, *10*, *11*), and postmenopausal symptoms (*12*, *13*). These findings have generated a substantial interest in the food industry to market soy-based food products, such as tofu and soy milk, or soy-containing foods, such as yogurt and infant formulas, incorporating soy ingredients, namely, soy isolates, concentrates, or flours. Furthermore, soy isoflavone used in dietary supplements has grown to approximately $34 million at retail, with new products continuously being developed and launched (*14*).

The isoflavones in soy occur primarily as β-glycosides with a small percentage as the principal bioactive aglycone. The glycosides can exist in three forms, as malonyl, acetyl, and underivatized glucosides (**Figure 1**), and the percentage of each differs depending on product type and processing conditions (*15*−*17*). The chemical form is an important consideration since it influences biological activity, bioavailability, and therefore the physiological contributions of isoflavones (*18*). When consumed and during their passage through the intestinal tract, the glycosides require some form of active transport to be absorbed, which has not been proven to exist for isoflavones (*19*). Although Andluer et al. (*20*) observations indicated that glycosides genistine and daidzin were partly absorbed from the small intestine without previous cleavage, Setchell et al. (*21*) confirmed the absence of glycosides in the plasma and thus concluded that isoflavone glycosides are not absorbed intact across the enterocytes of healthy adults and agreed with Slavin et al. (*22*) that the glycosides are hydrolyzed by enzymes, mainly β-glucosidases of the intestinal microflora (*Escherichia coli* and *Clostridia*), into aglycones that will readily be absorbed across the enterocyte of the intestine. Hur et al. (*23*) isolated two strains of *E. coli* bacteria from human intestine and confirmed their capabilities of hydrolyzing isoflavone glycosides daidzin and genistin.

* To whom correspondence should be addressed. Tel: 765 496 2864. Fax: 765 494 7953. E-mail: hayesk@purdue.edu.

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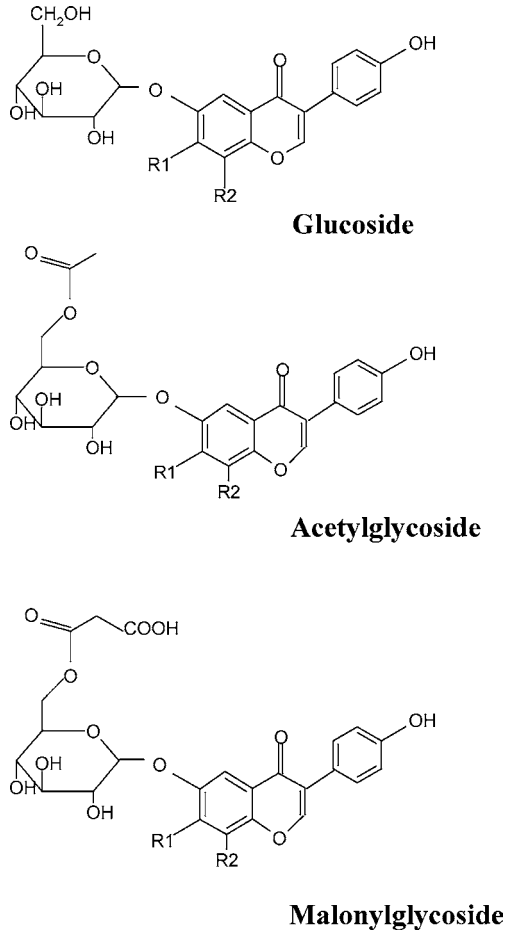

Figure 1. Glucoside, acetylglycoside, and malonylglycoside where R1 can be H in the case of daidzin and genistin or CH_3O in the case of glycitin, while R2 can be H in the case of daidzin and glycitin or OH in the case of genistin.

Conflicting opinions were advanced on whether the bioavailability of these isoflavones is greater when ingested as β-glycosides or as aglycones. Setchell et al. (*19*) confirmed that the bioavailability of isoflavones is greater when ingesting β-glycosides rather than aglycones, contradicting the findings of Izumi et al. (*24*) who confirmed the opposite. Zubik and Meydani (*25*), on the other hand, confirmed that the apparent bioavailability of genistein and daidzein is not different when consumed as either aglycone or glycoside. A variety of factors may contribute to the conflicting results, including ethnic background, dietary habits, intestinal microflora, food matrix, the administered dose, and the number of subjects in the study. All of those factors are now being taken into consideration by a number of researchers to eliminate irrelevant variability (*25*). One major factor that might explain a significant amount of the variability in the results is the actual glycoside profile. Setchell et al. (*19*) utilized pure glycosides of the underivatized form and compared them to the pure aglycone form. Izumi et al. (*24*) and Zubic and Meydani (*25*) used tablets of two different sources that contained all three forms of glycosides, whereby the profile of the glycosides differed from one source to the other, and compared them to tablets containing only the aglycone form. Obviously each group of researchers has used glycosides of different profile, and none of them gave any emphasis to that with regard to their results.

Research on isoflavone bioavailability has been limited to studying the extent of absorption of aglycone as compared to the glycosides as a whole. Considerable amounts of malonyl and acetyl groups are found to exist in many soy-based products, with the malonyl group being the most abundant among the three glycosidic forms, especially in less processed products. Thus, consumers of soy foods are ingesting glycosides with variable profiles. Knowing that enzyme activity can be very sensitive to molecular structure, it is hypothesized that the profile of the isoflavones in soy-based products will affect their rate of hydrolysis and absorption thereafter. On the basis of malonyl and acetyl glycosides being poorly hydrolyzed by β-glucosidases (*26*), Barnes et al. (*27*) assumed that these forms might be hydrolyzed in distant regions of the gut where bacterial concentrations are very high. However, neither of the two groups of researchers (*26*, *27*) has conducted distinctive experiments to prove this assumption and compared the hydrolysis rate among the three glycosidic forms.

The levels and profile of isoflavones incorporated into foods and supplements have been rather arbitrarily chosen, driven more by an attempt to maximize isoflavone levels in the product rather than by experimentally establishing the best content and profile of the isoflavones. The best isoflavone profile to be present in a dietary supplement for maximum hydrolysis and absorption is still not known. Thus, studying the enzymatic hydrolysis of each glycoside type will definitely form a basis for more convincing research on the bioavailability of isoflavones in soy. We hypothesized that the bacterial β-glucosidases present in the human gut have variable activity toward the different forms of glycosides, thus affecting their rate of hydrolysis. Therefore, the objective of this work was to study the activity of β-glucosidases from *E. coli* and almonds toward the different glycosides. Knowing the hydrolysis pattern of each glycoside will guide the industry, utilizing various processing techniques, to produce soybean products with specific isoflavone profiles that will enhance their absorption and thus their bioavailability.

MATERIALS AND METHODS

Materials. High-performance liquid chromatography (HPLC) grade acetonitrile and methanol were purchased from Fisher Scientific (Hanover Park, IL). Isoflavone standards daidzin, glycitin, genistin, malonylgenistin, acetyldaidzin, acetylglycitin, and acetylgenistin were purchased from LC Laboratories (Woburn, MA); malonyldaidzin and malonylglycitin were purchased from Fisher Scientific; and daidzein, glycitein, and genistein were obtained from Indofine Chemical Company (Somerville, NJ). A concentrated solution (500 ppm) of each standard was prepared using 80% aqueous methanol. Diluted isoflavone standard solutions were prepared with 80% aqueous methanol. The 12 isoflavone standards, each at a concentration of 4 ppm, were chromatographed individually, and retention times were recorded.

β-Glucosidase Enzymes. β-Glucosidase enzyme from *E. coli* (300 U/mg) was purchased from Indofine Chemical Company, and β-glucosidase enzyme from almonds (3542 U/mg) was obtained from Fisher Scientific. A 100 ppm solution of each enzyme was prepared.

HPLC Analysis. HPLC analysis used to separate and quantify isoflavones was based on a method described by Wang and Murphy (*28*), with modifications. 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. A linear HPLC binary gradient was used as follows: solvent A was 0.1% acetic acid in distilled water, and solvent B was 0.1% acetic acid in acetonitrile. Following the injection of 20 μL of a sample, solvent B was increased from 17 to 25% over 25 min, held at 25% for 5 min, increased to 30% over the next 10 min, and then held at 30% for 10 min; finally, solvent B was decreased back to 17% over the next 2 min and held at 17% for 8 min, to return the baseline to its original level. The flow rate was held at 1.2 mL/min throughout the run time, which was a total of 60 min. Both solvents were filtered through 0.45 μm Millipore nylon membrane filters (HNWP, 47 mm) and degassed before use.

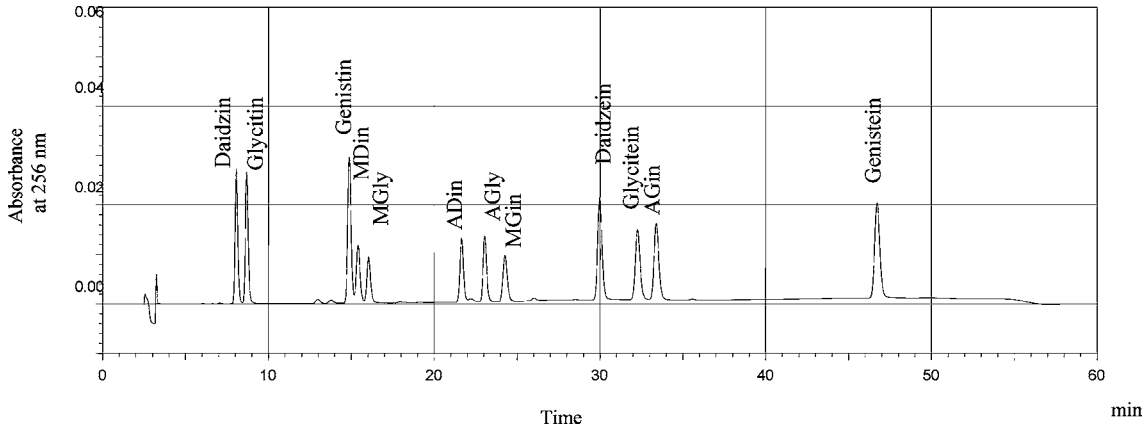

Figure 2. Reverse phase HPLC chromatography of the 12 isoflavones standards. MDin, malonyldaidzin; MGly, malonylglycitin; ADin, acetyldaidzin; AGly, acetylglycitin; MGin, malonylgenistin; and AGin, acetylgenistin.

Table 1. Enzyme–Substrate Reaction Parameters

experiment[a]	substrate type[b]	β-glucosidase origin	β-glucosidase units of activity[c]	maximum incubation time (h)
I	daidzin	*E. coli*	3	4
	glycitin	*E. coli*	3	4
	genistin	*E. coli*	3	4
	daidzin + glycitin + genistin	*E. coli*	9	4
II	daidzin + glycitin + genistin	*E. coli*	9	4
	daidzin + glycitin + genistin	almond	9	4
III	daidzin	*E. coli*	3	4
	malonyldiadzin	*E. coli*	3	4
	acetyldaidzin	*E. coli*	3	4
IV	glycitin	*E. coli*	3	4
	malonylglycitin	*E. coli*	3	4
	acetylglycitin	*E. coli*	3	4
V	genistin	*E. coli*	3	4
	malonylgenistin	*E. coli*	3	4
	acetylgenistin	*E. coli*	3	4
VI	genistin	*E. coli*	3	4
	malonylgenistin	*E. coli*	3	4
	acetylgenistin	*E. coli*	3	4
	genisitn	*E. coli*	6	4
	malonlygenistin	*E. coli*	6	4
	acetylgenistin	*E. coli*	6	4
VII	genisitn	*E. coli*	6	4
	malonlygenistin	*E. coli*	6	4
	acetylgenistin	*E. coli*	6	4
	genisitn	*E. coli*	6	24
	malonlygenistin	*E. coli*	6	24
	acetylgenistin	*E. coli*	6	24
VIII	genistin	almond	3	4
	malonlygenistin	almond	3	4
	genistin	almond	30	4
	malonlygenistin	almond	30	4

[a] Each experiment was carried out independently from the other. [b] The amount of each substrate incubated with the enzyme was 20 μg. [c] One unit releases 1 μmol of glucose per minute from salicin at 37 °C and a pH of 5.0.

Five diluted standard solutions containing all 12 isoflavone standards were prepared and filtered through 0.45 μm syringe filters. After the separation of the 12 isoflavone peaks as shown in **Figure 2** was confirmed, a five-point HPLC calibration was completed.

Enzyme Application and Hydrolysis Rates. Eight sets of independent experiments were carried out where daidzin, glycitin, and/or genistin isoflavones and/or their conjugated forms (malonyl and acetyl glycosides) were subjected to an enzyme treatment, each one separate and in certain combinations as illustrated in **Table 1**. Substrates were treated with enzyme in a phosphate buffer solution, pH 7.0, where 40 μL (containing 20 μg of substrate) of the concentrated standard solution (500 ppm) was placed in each of four 1 mL screw top conical tubes, β-glucosidase was added (**Table 1**), and then the volume was made up to 1 mL. All tubes were incubated at 37 °C in a water bath with a steady shaking where one tube was taken out every hour. Prior to HPLC analysis, the content of each tube was transferred quantitatively into a 10 mL Erlenmeyer flask and 4 mL of 99.7% methanol was added, which stopped the enzyme reaction. Each sample was filtered through 0.45 μm syringe filters and transferred into 2 mL vials. Samples then were subjected to HPLC analysis as described above where each sample was injected four times. Isoflavone concentration was calculated based on peak areas whereby area responses were integrated by Shimadzu software (version 5). An untreated blank solution for each enzyme-treated sample was analyzed, and all enzyme−substrate reactions were run in triplicate. Hydrolysis reactions were studied based on the depletion of the glycoside and the formation of its respective aglycone. Comparisons were made between the mean percentages of substrate hydrolysis of daidzin, genistin, and glycitin with *E. coli* β-glucosidase in separate solutions and in one mixture at a set level of the enzyme for a specific period of incubation (experiment I). The effect of β-glucosidase from different origins on the percent hydrolysis of the nonderivatized glycosides (daidzin, glycitin, and genistin all in one mixture) was studied (experiment II). Comparisons were made between *E. coli* β-glucosidase activities toward the three glycosidic forms of each isoflavone type, diadzin, glycitin, and genistin (experiments III, IV, and V). The effect of elevated level of *E. coli* β-glucosidase (experiment VI), as well as the effect of extended incubation time (experiment VII) on the hydrolysis reaction was studied for the genistin glycosides. Additionally, enzyme hydrolyses of genistin and malonylgenistin were studied when treated with two levels of almonds β-glucosidase (experiment VIII).

Stability of Conjugated Glycosides in Acidic Medium. Twenty microliters of concentrated (500 ppm) malonylgenistin, acetylgenistin, malonyldaidzin, and acetyldaidzin solutions was each placed in 1 mL of hydrochloric acid−potassium chloride buffer, pH 2.0, and incubated at 37 °C in a water bath for 2 h. Then, 4 mL of methanol was added, and samples were filtered through 0.45 μm syringe filter, placed in 2 mL vials, and analyzed for isoflavone content. A control was run for each sample, and the experiment was conducted in triplicate.

Statistical Analysis. Analysis of variance was carried out utilizing SPSS 11.5 for Windows (*29*). Percentage hydrolysis, aglycone produced upon hydrolysis, and glycoside-derived data were analyzed using factorial analysis in a completely randomized design with substrate type, substrate−mixture type, enzyme type, incubation time, and/or enzyme level as factors, depending on the experiment. 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 Duncan's multiple range test (DMRT) (*30*). The stability of malonyl and acetyl genistin under acidic conditions was tested using a two-tailed paired t-test ($p \leq 0.05$), in Microsoft Excel (2003).

RESULTS AND DISCUSSION

Percent Hydrolysis of the Nonconjugated Glycosides. Mean percentages of substrate hydrolysis of daidzin, genistin,

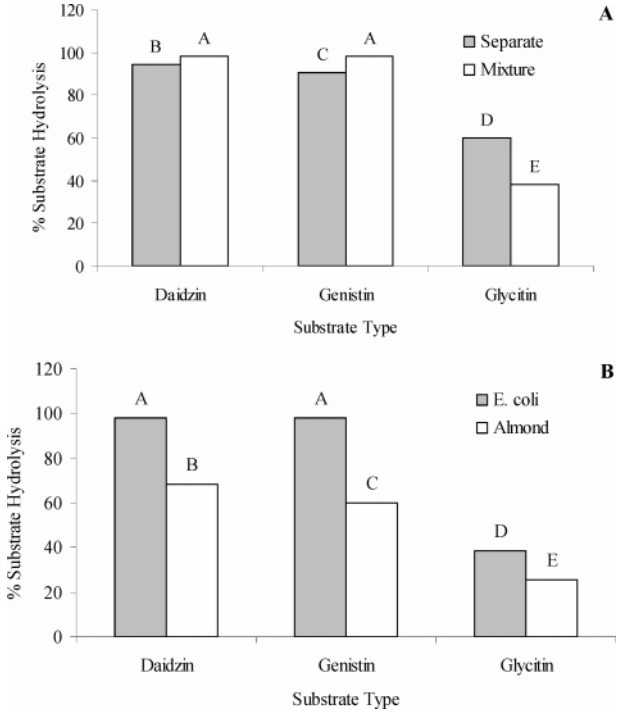

Figure 3. (**A**) Mean percentages of substrate hydrolysis of daidzin, genistin, and glycitin after 4 h of incubation at 37 °C with *E. coli* β-glucosidase in separate solutions and in one mixture (experiment I, **Table 1**). (**B**) Mean percentages of substrate hydrolysis of daidzin, genistin, and glycitin in one mixture after 4 h of incubation at 37 °C with *E. coli* β-glucosidase and with almonds β-glucosidase (experiment II, **Table 1**). Means with the same letter are not significantly different according to DMRT ($p \leq 0.05$).

and glycitin with *E. coli* β-glucosidase in separate solutions and in one mixture are presented in **Figure 3A**. Significant variations between the mean percent hydrolysis of the different substrates were observed when each was hydrolyzed separately by the enzyme and when all the substrates were hydrolyzed in a mixture. When treated with enzyme separately, daidzin had the highest percent hydrolysis followed by genistin. Glycitin was the least hydrolyzed by the enzyme, and its percent hydrolysis significantly dropped when all three glycosides were treated with the enzyme in a mixture. Apparently, the β-glucosidase had a higher affinity toward daidzin and genistin than glycitin, which explains the significant increase in the percent hydrolysis of both daidzin and genistin when all of the three glycosides were in one mixture and treated with 9 U of enzyme instead of 3 U for each when in separate solutions. This also can explain why no significant difference was observed between the percent hydrolysis of daidzin and that of genistin when treated with the enzyme in a mixture, since more enzyme was available to react with these two substrates because of the apparent lower affinity toward glycitin.

Percent hydrolyses of the three glycosides in a mixture treated with almond β-glucosidase were significantly lower than those of the three glycosides in a mixture treated with *E. coli* β-glucosidase (**Figure 3B**). Although a similar trend was observed, where the almond enzyme had more affinity toward daidzin and genistin than toward glycitin, overall it had lower activity than *E. coli* β-glucosidase toward all three glycosides.

***E. coli* β-Glucosidase Activity toward Each Isoflavone Glycoside and Its Respective Conjugates.** Aglycone produced after each glycosidic form was treated separately with *E. coli* β-glucosidase, and the glucoside derived from the conjugated forms after incubation without the enzyme is presented in **Table 2**. The concentration of daidzein produced after treating a solution of 0.01 μmol/mL daidzin with the enzyme was 0.0095 μmol/mL, which is equivalent to 95% of the original substrate amount (**Table 2**). On the other hand, the daidzein produced after treating solutions of malonyldaidzin and acetyldaidzin was only 10 and 2%, respectively, of the original substrate amount (0.01 μmol/mL). Furthermore, these small amounts of daidzein produced were not due to enzyme hydrolysis of the conjugated forms but were the products of hydrolysis of the daidzin derived nonenzymatically from the conjugated forms after 4 h of incubation at 37 °C (**Table 2**). Similar results were observed for glycitin and its conjugates and for genistin and its conjugates. A significant amount of aglycone was produced when the glucosides were treated with the enzyme, while only a low amount was produced when the conjugates were treated with enzyme. Again, this amount was the product of the enzyme hydrolysis that occurred after the nonenzymatic conversion of a small amount of the conjugated forms to their respective glycosides.

Table 2. *E. coli* β-Glucosidases Activity toward the Three Glycosidic Forms of Daidzin, Glycitin, and Genistin (Experiments III, IV, and V, **Table 1**)

experiment	glycoside	aglycone produced[a] (%)	nonconjugated glycoside derived[b] (%)
III	daidzin	95 a[c]	
	malonyldaidzin	11 b	14 a
	acetyldaidzin	2 b	3 b
IV	glycitin	61 a	
	malonylglycitin	1 b	8 a
	acetylglycitin	1 b	5 a
V	genistin	91 a	
	malonylgenistin	6 b	15 a
	acetylgenistin	14 b	5 b

[a] Percentage of aglycone produced on mole basis after 4 h of incubation of 0.01 μmol/mL with 3 U of *E. coli* β-glucosidase at 37 °C. [b] Percentage of nonconjugated glycoside derived on mole basis from the conjugated forms after 4 h of incubation without the enzyme. [c] Means in each column per experiment, followed by the same letter, are not significantly different according to DMRT ($p \leq 0.05$).

Effect of Elevated Levels of *E. coli* β-Glycosidase and Prolonged Incubation Time on the Hydrolysis of Genistin and Its Conjugates. After the solution was incubated with double the amount of enzyme, the amount of genistein produced increased significantly when genistin was the substrate; however, no significant changes occurred in the amounts of genistein produced from the conjugated forms (**Table 3**). The genistein produced when the conjugated form was the substrate did not show an increase since the amount of genistin derived during the incubation time is small and likely not enough to saturate the enzyme. Even prolonged incubation with double the amount of enzyme did not induce enzyme hydrolysis of the conjugated forms (**Table 4**). The only difference was that the genistin derived from acetylgenistin by chemical hydrolysis significantly increased after 24 h of incubation as compared to 4 h leading to a significant increase in the genistein produced.

Effect of Elevated Levels of Almonds β-Glycosidase on the Hydrolysis of Genistin and Malonylgenistin. Because of the availability of the almond β-glycosidase in concentrated form, we were able to treat the substrates with significantly increased enzyme activity (30 vs 3 U) to investigate whether such a high level of the enzyme would induce hydrolysis of the conjugated glycoside. Results obtained showed that with

Table 3. Effect of Elevated Levels of *E. coli* β-Glucosidase on the Hydrolysis of the Three Glycosidic Forms of Genistin (Experiment VI, **Table 1**)

enzyme level	genistin glycoside	aglycone (genistein) produced[a] (%)	genistin derived[b] (%)
3 U of activity	genistin	91 b[c]	
	malonylgenistin	6 c	16 a
	acetylgenistin	4 c	5 b
6 U of activity	genistin	98 a	
	malonylgenistin	4 c	17 a
	acetylgenistin	5 c	5 b

[a] Percentage genistein produced on mole basis after 4 h of incubation of 0.01 μmol/mL with *E. coli* β-glucosidase at 37 °C. [b] Percentage genistin (nonconjugated glycoside) derived on mole basis from the conjugated forms after 4 h of incubation without the enzyme. [c] Means in each column, followed by the same letter, are not significantly different according to DMRT ($p \leq 0.05$).

Table 4. Effect of Prolonged Incubation Time with 6 U of *E. coli* β-Glucosidase on the Hydrolysis of the Three Glycosidic Forms of Genistin (Experiment VII, **Table 1**)

time of incubation (h)	genistin glycoside	aglycone (genistein) produced[a] (%)	genistin derived[b] (%)
4 h	genistin	98 a[c]	
	malonylgenistin	5 c	12 ab
	acetylgenistin	5 c	6 c
24 h	genistin	96 a	
	malonylgenistin	8 c	10 b
	acetylgenistin	16 b	12 a

[a] Genistein produced after incubation of 0.01 μmol/mL with 6 U of *E. coli* β-glucosidase at 37 °C. [b] Genistin (nonconjugated glycoside) derived from the conjugated forms after incubation without the enzyme. [c] Means in each column, followed by the same letter, are not significantly different according to DMRT ($p \leq 0.05$).

Table 5. Effect of Elevated Levels of Almonds β-Glucosidase on the Hydrolysis of Genistin and Malonylgenistin (Experiment VIII, **Table 1**)

enzyme level	genistin glycoside	aglycone (genistein) produced[a] (%)	genistin derived[b] (%)
3 U of activity	genistin	72 b[c]	
	malonylgenistin	3 c	18 a
30 U of activity	genistin	99 a	
	malonylgenistin	6 c	20 a

[a] Percentage of genistein produced on mole basis after 4 h of incubation of 0.01 μmol/mL with almonds β-glucosidase at 37 °C. [b] Percentage of genistin (nonconjugated glycoside) derived on mole basis from the conjugated forms after 4 h of incubation without the enzyme. [c] Means in each column, followed by the same letter, are not significantly different according to DMRT ($p \leq 0.05$).

the increased level of the enzyme almost a complete hydrolysis of genistin into genistein occurred (**Table 5**), while no change was observed in the amount of genistein produced when malonylgenistin was the substrate.

Stability of Conjugated Glycosides in Acidic Medium. No significant change was observed in the concentration (μg/mL) of malonylgenistin or acetylgenistin, after 2 h of incubation at 37 °C in an acidic medium (pH 2). For both conjugated forms, no significant degradation or conversion occurred. Similar results were observed for malonydaidzin and acetyldaidzin.

Our results confirmed that β-glycosidase, regardless of origin (microbial or plant), can hydrolyze nonconjugated forms into aglycone where a complete hydrolysis could be achieved with higher levels of the enzyme. These findings are consistent with other research conclusions indicating that glycosides can be hydrolyzed into aglycone by gut β-glycosidase (*17*, *21*, *22*). However, our results show significant variations in the activity of the enzyme toward the three different types of nonconjugated glycosides, when the enzyme amount was limited and when the enzyme was from almond (a β-glycosidase with lower activity as compared to that of the *E. coli*). Daidzin was found to be the most hydrolyzed by β-glycosidase (from both origins), which might explain the findings of Xu et al. (*31*) and King (*32*) who, based on both plasma concentration and urinary excretion, concluded that daidzin is more bioavailable than genistin. On the other hand, our results showed that there was no significant difference in the percent hydrolysis between daidzin and genistin when both were subjected to higher enzyme activity. These findings might partially explain why Setchell et al. (*19*, *33*) were unable to confirm the findings of Xu et al. (*31*) and King (*32*). Each group of researchers conducted their studies on different groups of subjects that came from various ethnic backgrounds, had different dietary habits, and most likely differed in their intestinal microflora. These differences could cause variations in the amount as well as activity of the gut β-glycosidases. Lower levels of the enzyme as well as lower activity, as in the case of the almond enzyme, will cause variations in the hydrolysis rates of the nonconjugated glycosides. The glycoside profile used by each group of researchers could have been another factor that contributed to the variation in the results. Further research on the kinetics of the enzyme utilizing different substrates is needed for complete characterization of the enzyme toward the three nonconjugated glucosides (genistin, daidzin, and glycitin).

While β-glucosidases hydrolyzed the nonconjugated glycosides into aglycones, results confirmed that the enzyme from both origins was not effective in inducing any hydrolysis of the malonyl and acetyl glycosides to their respective aglycones, even with increased levels of the enzyme and with prolonged incubation. Structural differences among the three glycosidic forms were the cause of the variations in hydrolysis. The malonyl and acetyl groups on carbon number six of the glucose ring (**Figure 1**) probably cause ionic or steric hindrance of the enzyme, thus preventing it from detaching the glucose from the isoflavone.

Previous research indicated that the isoflavone profile is altered by normal thermal processing conditions, and conversions between the three glycosidic forms can occur (*34*). The malonyl and acetyl glycosides are considered to be heat sensitive and relatively unstable. Heat can cause deesterification of malonyl and acetyl glucosides, thereby leading to their respective β-glucosides (*35*). Although incubation temperature of our present work was only 37 °C, which is not a high temperature, conversions from the conjugated forms to their respective nonconjugated glycosides were observed, however, only in small amounts. Thus, the aglycones produced after incubating the conjugated forms with the enzymes were the products of the enzymatic hydrolysis of the nonconjugated glycosides derived during the incubation period through chemical hydrolysis.

Barnes et al. (*27*) suggested that the malonyl and acetyl glycosides might be hydrolyzed in distant regions of the gut where bacterial concentrations are very high. We increased the level of the enzyme significantly (30 vs 3 U) and still found no hydrolysis of the conjugated forms and almost complete hydrolysis of their respective glucoside. Even though our in vitro studies do not show any direct enzymatic hydrolysis of the conjugated forms, if any hydrolysis of these forms is to occur in vivo in the distant region of the gut, it will be very minimal and likely insignificant. Furthermore, we believe that the gut

microbes will not produce β-glucosidases in higher levels and activities than those used in our studies in vitro, especially when considering the substrate concentration. It is important to note that the longer it takes to hydrolyze and absorb a compound the more prone it becomes to degradation, especially under basic conditions (*36*) (the pH of the small intestine can reach up to 8.2). Our data show that the malonyl and acetyl conjugates of the two major isoflavones (genistin and daidzin) survived a simulated stomach pH condition, which indicates that they will make it almost intact to the intestine and might later on be degraded and lost.

The belief that aglycone is more bioavailable than its respective glycoside has led to the development of aglycone-enriched products; however, aglycones were found to have adverse effects on flavor (*37*). Furthermore, aglycones might be degraded before they get absorbed because the sugar moiety of the glycoside protects the isoflavone from being degraded early on in the small intestine, as argued by Setchell et al. (*19*). More research has been done to show that glycosides were not less bioavailable than aglycones (*19, 25*). The glycosides were found to be hydrolyzed by β-glucosidases of the intestinal microflora into aglycones that will readily be absorbed (*22*). However, none of the researchers studying the bioavailability of isoflavones gave any attention to the effect of the glycoside profile on hydrolysis rate. Our work confirmed that for the glycosides to be bioavailable they have to be in the nonconjugated forms. Because the data upheld our earlier hypothesis that isoflavone profiles affect their enzymatic hydrolysis, it is worthwhile to emphasize the need for bioavailability studies, whereby pure forms of malonyl and acetyl glycosides are ingested and then plasma concentrations are monitored.

Consumers of minimally processed soy foods are ingesting glycosides that are mainly of the conjugated forms. Intact soybeans, subjected to minimum processing conditions, have 6″-*O*-malonylgenistin as the major isoflavone (*28*). The glycosides might indeed be more bioavailable but only if they are in their nonconjugated forms. The production of soy-based food products involves several types and intensities of processing conditions. The health benefits of soy will therefore depend on the stability of isoflavones and the particular profile of their derivatives in the final product. The transformation of the conjugated forms into their respective nonconjugated glycosides will enhance the nutritional value of the soy products and thus ultimately increase consumption and economical value.

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