CHARACTERIZATION OF URINARY ISOFLAVONE METABOLITES EXCRETED AFTER THE CONSUMPTION OF SOY FLOUR OR SOYBEAN PASTE USING LC-(ESI)MS/MS

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ABSTRACT

Isoflavones are thought to have an important role in the prevention of hormone-related diseases. This study was undertaken to identify the profile of urinary isoflavone metabolites collected within 24 h after the consumption of unfermented or fermented soy foods. The proportions of β-glycosides and aglycones to total isoflavone intake were much higher in soybean paste than soy flour. Twenty urinary isoflavone metabolites were identified using LC(ESI)-MS/MS. After enzymatic hydrolysis, glucuronide forms of daidzein and genistein accounted for 76 and 86% of total isoflavones, demonstrating that the majority of urinary isoflavone metabolites are glucuronide conjugates. When comparing the areas under curves of two foods, total urinary isoflavones excretion was higher in subjects consuming soybean paste than in subjects consuming soy flour. Genistein glucuronides are cleared more slowly than daidzein glucuronides after soy consumption. These findings indicate that the bioavailability of isoflavones may be influenced depending on the chemical composition of soy isoflavones.

PRACTICAL APPLICATIONS

Sensitive LC(ESI)-MS/MS methods characterizing the broad range of flavonoid metabolites formed in response to a "realistic" dietary exposure are critical for understanding what metabolites are circulating in plasma and have biological activity. Once identified these metabolites can be used to probe mechanisms of biological action, explore synergy between metabolites and better define their role in human health.

INTRODUCTION

Isoflavones, found primarily in soy foods, are plant-derived phenolic compounds that have weak estrogenic as well as antiestrogenic activities depending on the endogenous estrogen condition (Stob 1983; Setchell and Cassidy 1999). The two predominant isoflavones in soy foods are the β-glycosides genistin and daidzin. These are present in soy foods in different chemical forms including: the aglycones, β-glycosides, malonyl and acetyl glycosides (Wang and Murphy 1996; Koh and Mitchell 2007). Upon ingestion, the β-glycoside moiety is hydrolyzed by microbial β-glycosidase resulting in the formation of the aglycones genistein and daidzein. These compounds are subsequently absorbed in the gut and transported to the liver. During the absorptive processes, the isoflavones may be conjugated with glucuronic acid or sulfonic acid in hepatic and/or epithelial cell membranes (Figs. 1 and 2) and can be bound to plasma proteins such as albumin (Setchell and Cassidy 1999). The metabolites are more readily transported in the plasma and excreted into urine due to their hydrophilic characteristics as compared to the parent aglycones.

The bioavailability of isoflavones is influenced by multiple factors including: the composition of intestinal microflora, gender, age, background diet, duration of exposure and chemical–physical nature of the isoflavones and the food matrix (Setchell and Cassidy 1999; Rafii *et al*. 2003; Faughan *et al*. 2004; Cassidy *et al*. 2006; Koh and Mitchell 2007). It is now clear that certain species of intestinal microflora can convert daidzein into *O*-desmethylangolensin (*O*-DMA) or

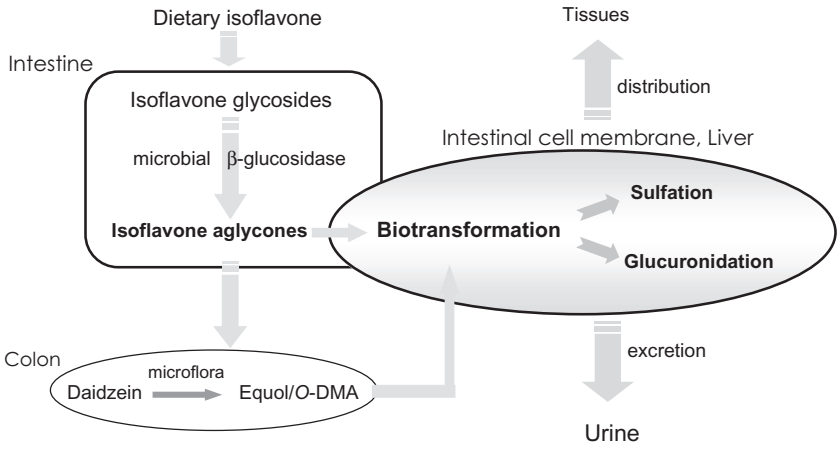

FIG. 1. GENERAL SCHEME FOR THE ABSORPTION AND METABOLISM OF ISOFLAVONES

equol (Atkinson *et al.* 2005), which demonstrates higher estrogenic activity than the parent daidzein (Markiewicz *et al.* 1993). The question of which form of isoflavones is more bioavailable remains unclear. Numerous studies report that the isoflavone aglycones are absorbed more rapidly in the intestine than the corresponding glycosides (Hutchins *et al.* 1995; Izumi *et al.* 2000; Cassidy *et al.* 2006; Koh and Mitchell 2007), whereas contrasting studies report no difference in overall bioavailability of the isoflavones aglycones or glycosides (Richelle *et al.* 2002; Zubik and Meydani 2003). However, these later studies, did not account for differences in the intake frequency of soy foods and intestinal microflora among individuals. For example, Lampe (2003) found that urinary isoflavones were higher in individuals who consumed soy foods more than once a month as compared with those consuming soy less than once a month or never. Numerous epidemiological studies show an association between soybean consumption and a reduced risk of breast cancer, prostate cancer, osteoporosis and cardiovascular disease (Setchell and Cassidy 1999; Dai *et al.* 2002; de Kleijn *et al.* 2002). Interestingly, the consumption of fermented soybean paste showed a significant inverse association with the risk of breast cancer in Korean women, whereas the consumption of total soy foods and unfermented soy foods such as soybeans, soybean curds and soy milk had no effects (Do *et al.* 2007).

Recently, Ismail and Hayes (2005) demonstrated that microbial β-glycosidase has higher activity toward β-glycosides as compared with malonyl and acetyl derivatives of isoflavones glycosides. This is significant as isoflavones in soybeans and unfermented soy products exist predominantly as malonyl–glycoside conjugates, whereas fermented soy products contain higher levels of the aglycones and β-glycoside forms (Coward *et al.* 1993; Wang and Murphy 1996; Koh and Mitchell 2007). Numerous ethnic diets incorporate fermented soy foods as part of a normal diet. For example, in the Korean population, total isoflavone intake per capita is estimated as 23.0 mg per day (Surh *et al.* 2006). However, fermented soy foods account for about 30% of this intake. This suggests that Koreans may consume relatively higher levels of the more bioavailable isoflavone aglycones as compared to a population consuming the same amount of isoflavone from unfermented soy foods. Additionally, more than 50% of Koreans are estimated to produce equol, whereas the level of equol conversion is estimated at 30% in the Western population (Akaza *et al.* 2004; Song *et al.* 2006).

The ratio and extent of isoflavone glucuronidation or sulfation may results in different physiological effects. Appreciating both qualitatively as well as quantitatively what types of isoflavones metabolites are formed and circulate in plasma will lead to an understanding of which forms are actually responsible for eliciting physiological effects. To date, a majority of studies investigating isoflavones bioavailability have relied on measuring the isoflavone aglycones, after they are liberated by enzymatic hydrolysis of the sugar moiety, in biological fluids (Doerge *et al.* 2000; Ritchie *et al.* 2004; Atkinson *et al.* 2005; Koh and Mitchell 2007). Measuring the agylcones simplifies the analysis and improves method sensitivity however information on metabolites is lost. This is critical as the metabolites are what circulate in the plasma and are likely responsible for the biological properties attributed to isoflavones. More recent studies have focused on understanding the chemical–physical properties that govern isoflavone bioavailability and metabolism once they are ingested. *In vitro* studies demonstrate that the aglycones, glucuronide and sulfated metabolites are all biologically active, although the response is dependent upon the chemical nature of the isoflavones and target protein involved (Wong and Keung 1997; Zhang *et al.* 1999). For example, isoflavone metabolites have been shown to be less estrogenic than the parent aglycones *in vitro* (Zhang *et al.* 1999; Kinjo *et al.* 2004). The rat microsomal uridine 5′-diphospho-glucuronosyltransferase has been shown to have greater affinity for genistein than daidzein, and

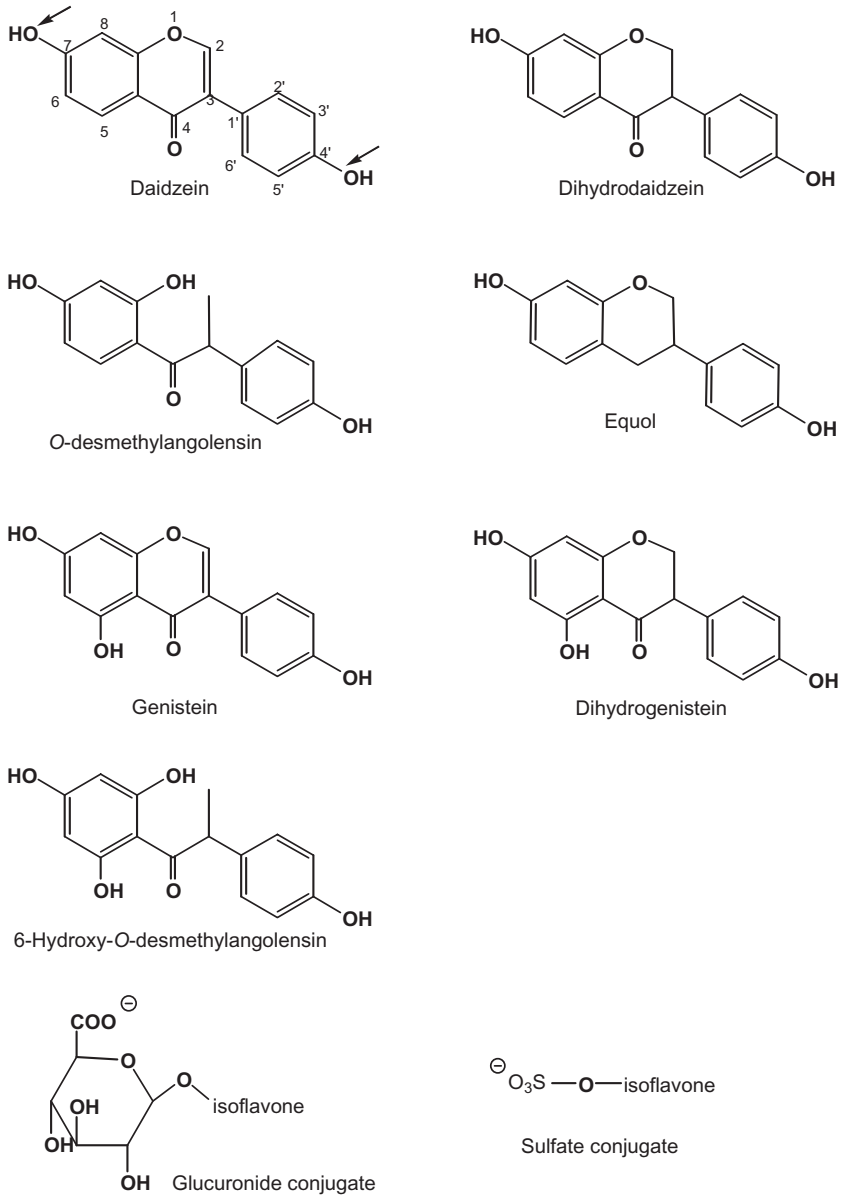

FIG. 2. CHEMICAL STRUCTURES OF GENISTEIN, DAIDZEIN AND THEIR PREDOMINANT METABOLITES
Arrows represent the major positions of conjugation during the metabolism.

genistein glucuronides have a twofold greater estrogen receptor binding affinity than daidzein glucuronides (Zhang *et al.* 1999). Rufer and Kulling (2006) found that reduced isoflavones such as dihydrodaidzein, dihydrogenistein, *O*-DMA and 6′-OH-*O*-DMA have the same or slightly lower antioxidant activities as compared to the parent isoflavones, whereas equol exhibits higher antioxidant capacity. Daidzein 4′-sulfates, daidzein 4′,7-disulfate and *O*-DMA have no estrogenic effects in endoplasmic reticulum β-transfected HeLa cells, whereas daidzein and daidzein 7-sulfate do (Totta *et al.* 2005). It has also been pointed out that sulfated and glucuronidated conjugates would not passively diffuse through cell membranes due to their increased hydrophilic properties. Therefore, isoflavone metabolites formed during absorptive processes would need to be rehydrolyzed to the aglycones in order to pass through cell membranes and induce estrogenic effects in target cells.

Accordingly, the current study investigates the formation and excretion kinetics of intact isoflavones metabolites after the consumption of either fermented soybean paste or unfermented soy flour in a population of healthy Koreans. An equivalent amount (20 mg) of total isoflavone was consumed as either 52 g of soybean paste or 12.2 g soy flour in a randomized, crossover trial consisting of two single-time point feedings and having 3-day run-in and washout periods between feeding. This corresponds to 0.2 and 16.5 mg of the sum of

malonyl-glycosides and acetyl-glycosides from fermented foods and unfermented soy products (Koh and Mitchell 2007). Urinary isoflavones metabolites were identified using high-performance liquid chromatography (HPLC) coupled with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS).

SUBJECTS AND METHODS

Subjects and Study Design

This study was approved by the Institutional Review Board Human Subjects Committee of University of California at Davis. This was a randomized, crossover trial consisting of two single-time point feedings. Ten healthy Koreans, five males and five females, were aged from 24 to 36 years and weighed between 49 and 95 kg (Koh and Mitchell 2007). Subjects were asked to avoid any isoflavones-containing foods, e.g., soybeans, soybean oil, soy-based products such as tofu, soy milk, soybean paste, soy sauce, soybean or mung bean sprouts, natto, beer, peanuts, snacks containing soy flour or cereals with soy ingredients, 3 days prior to the start of the first experiment and abstain until the completion of this study. We requested the subject to collect morning urine the day prior to each feeding. A portion of frozen soy product, either 52 g soybean paste or 12.2 g soy flour accounting for 20 mg isoflavone, was provided. After an overnight fast, the subject was instructed to eat one of the soy products and collect urine output into containers containing ascorbic acid and sodium azide for a period of 24 h. After the washout period of 3 days, the subject was requested to consume the other soy product after overnight fasting, and collect urine for 24 h post consumption. Urine samples were stored at −80C until analysis.

Chemicals

Daidzin (3-(4-hydroxyphenyl)-7-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-chromen-4-one, 96%) and equol (3,4-dihydro-3-(4-hydroxyphenyl)-(S)-2H-1-benzopyran-7-ol, 99%) were purchased from Indofine Chemical Co. Inc (Belle Mead, NJ). Genistin (4-hydroxy-3-(4-hydroxyphenyl)-7-[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]oxy-chromen-5-one,95%), genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, 98%), daidzein (7-hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, 98%) and HP-2 β-glucuronidae from Helix pomatia with sulfatase activity were purchased from Sigma Chemicals Co (St. Louis, MO).

Determination of Isoflavones in Soy Foods

The compositional analysis of soy isoflavones in fermented and unfermented soy foods was described previously (Koh and Mitchell 2007).

Analysis of Urinary Isoflavone Metabolites

Isoflavone metabolites were determined based on the method of Coldham *et al.* (1999) with a slight modification. Three milliliters of urine was diluted with an equal volume of 0.2% formic acid and applied to a C_{18} cartridge (500 mg, Fisher Scientific, Fair Lawn, NJ) conditioned with 6 mL of methanol and 6 mL of 0.2% formic acid. The column was washed with 6 mL of 0.2% formic acid three times and isoflavone metabolites were eluted with 3 mL of methanol. The elute was dried at 45C under a stream of nitrogen and redissolved in 300 μL of initial mobile phase and filtered through 0.2 μm filter prior to HPLC analysis. All samples were analyzed in duplicate. The mobile phase consisted of 1% formic acid in water (solvent A) and 25% acetonitrile in methanol (solvent B): 10% B for 5 min, 10–22% B from 5 to 20 min, 22–30% from 20 to 35 min, 30–46% from 35 to 36 min, 46–80% from 36 to 56 min and 80–85% from 56 to 60 min. A Phenomenex Prodigy ODS column (5 μm, 250 mm × 2.00 mm, Phenomenex, Torrance, CA) was equilibrated for 10 min between runs. The triple quadrupole ESI source parameters were the following: capillary 3.25 kV in the positive mode and extractor 2 V. The cone voltages varied from 15 to 30 V in order to optimize the intensity of the parent ion. The source block and desolvation temperature were set at 140 and 340C. The total ion chromatogram (TIC) was monitored at a mass range of m/z 100–800. The identification of isoflavone metabolites in urine was carried out with HPLC-electrospray ionization LC (ESI)-MS/MS in the positive mode on the basis of parent and daughter ion mass spectra.

Analysis of Urinary Isoflavone Aglycones

A 2 mL aliquot of the supernatant was incubated with 2 mL of 0.2 M acetate buffer (pH 5) and 20 μL β-glucuronidase and sulfatase (92,500 unit/mL β-glucuronidase and 7,500 units/mL sulfatase from Helix pomatia) for 16 h at 37C in the presence or absence of the β-glucuronidase inhibitor (*d*-saccharic, 1–4 lactone, 100 mmol/L). Isoflavone aglycones liberated after enzymatic hydrolysis were extracted according to the method of our previous study (Koh and Mitchell 2007). The aglycones released after digestion with enzyme mixture in the presence of *d*-saccharic, 1–4 lactone were used to calculate isoflavone sulfate concentration. The percentages of genistein or daidzein in urine as the glucuronide conjugates were obtained from the deduction of sulfate conjugates from total aglycones.

RESULTS AND DISCUSSION

Isoflavones in Soy Foods

Compositional analysis of the soy foods used in this study indicated that the malonyl derivatives were the most abun-

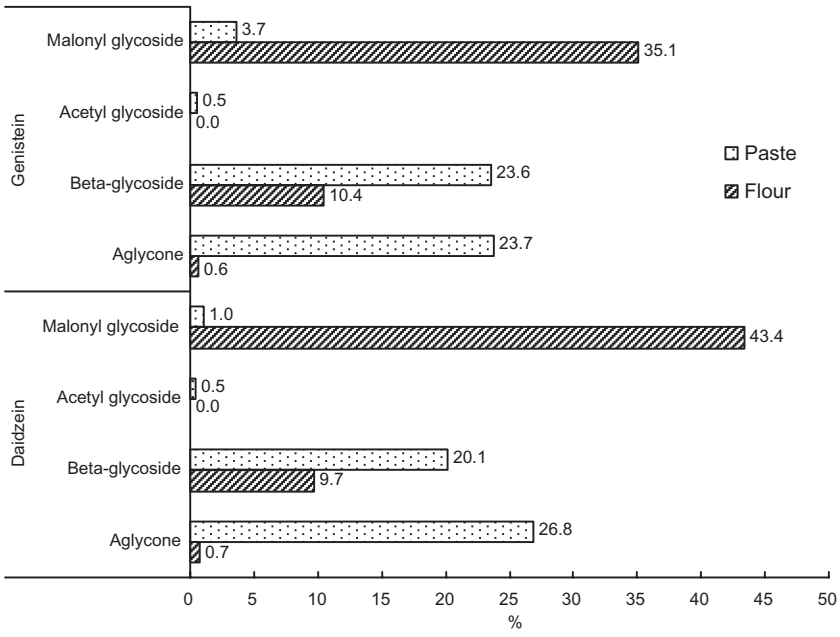

FIG. 3. RELATIVE COMPOSITION OF ISOFLAVONES IN SOY FLOUR AND SOYBEAN PASTE CORRESPONDING TO A TOTAL ISOFLAVONE AMOUNT OF 20 MG IN THE GIVEN FOOD MATERIAL

dant forms of isoflavones in unfermented soy flour, whereas the fermented soybean paste contained a higher proportion of aglycones and β-glycosides (Koh and Mitchell 2007). Such differences may arise from the conversion of acetyl and malonyl derivatives into β-glycosides or aglycones during soybean paste production, e.g., heat treatment and fermentation. Consequently, the proportions of β-glycosides and aglycones to total isoflavone intake were much higher in the fermented soybean paste as compared with soy flour (Fig. 3). Isoflavone glycosides are typically hydrolyzed into their corresponding aglycones prior to absorption (Setchell *et al.* 2002). Some bacteria, including *Escherichia coli* and *Clostridia*, have β-glycosidase activity with the ability to hydrolyze soy isoflavone glycosides (Slavin *et al.* 1998; Hur *et al.* 2000). Ismail and Hayes (2005) demonstrated that a β-glycosidase originating from *E. coli* was not effective at hydrolyzing the malonyl and acetyl glycosides of isoflavones and had much higher affinity toward daidzin and genistin, β-glycosides of daidzein and genistein, respectively. This suggests that the absorption of soy isoflavones is influenced by the composition and relative proportion of β-glycoside derivatives in the soy foods.

Identification of Urinary Isoflavone Metabolites

Isoflavone metabolites and profiles were identified in 24 h urine after the consumption of either the soy flour or soybean paste, and a representative HPLC chromatogram of the isoflavone separations monitoring 262 nm is shown in Fig. 4. HPLC eluents were monitored by LC (ESI)-MS/MS and the combined chromatographic trace and mass spectra suggest the presence of more than 20 possible isoflavone metabolites. Further identification of these potential isoflavone metabolites was achieved by first full scanning ions over a mass range of m/z 100–800 and identifying peaks that corresponded to mass values of all possible isoflavone metabolites. These peaks were then selected for MS/MS fragmentation and daughter ion monitoring. The results of the ESI-MS/MS identification of isoflavones metabolites are given in Table 1.

MS/MS fragmentation of peaks corresponding to compounds 2, 5, 8, 12 and 20 resulted in the production of a common fragment at m/z 255, indicating that these compounds are all derivatives of daidzein (Table 1). Mass spectra of peak 2 indicated that it was daidzein diglucuronide with a $[M+H]^+$ at m/z 607 and predominant fragment ions at m/z 431 and 255 resulting from the consecutive losses of two glucuronide moieties (176 Da). Mass spectra of peak 5 demonstrate that it was daidzein sulfoglucuronide with a $[M+H]^+$ at m/z 511 and predominant fragment ions at m/z 335, formed by the loss of a glucuronyl moiety (176 Da), and at m/z 255 derived from the consecutive losses a sulfonic acid moiety (80 Da). Peaks 8 and 12 (Table 1) displayed different retention behaviors on the HPLC column yet had identical molecular ions at m/z 431 and produce the same fragment ion at m/z 255 (Fig. 5). There are two conjugation sites on daidzein at C-7 and at the 4′ site (Fig. 2). It is established that the 4′-glucuronides of isoflavones are retained longer on reverse-phased sorbents as compared to their C-7-isomers (Clarke *et al.* 2002; Fang *et al.* 2002). Furthermore, Clarke *et al.* (2002) demonstrated that the proportion of daidzein 7-glucuronide is about twofold higher than daidzein 4′-glucuronide in

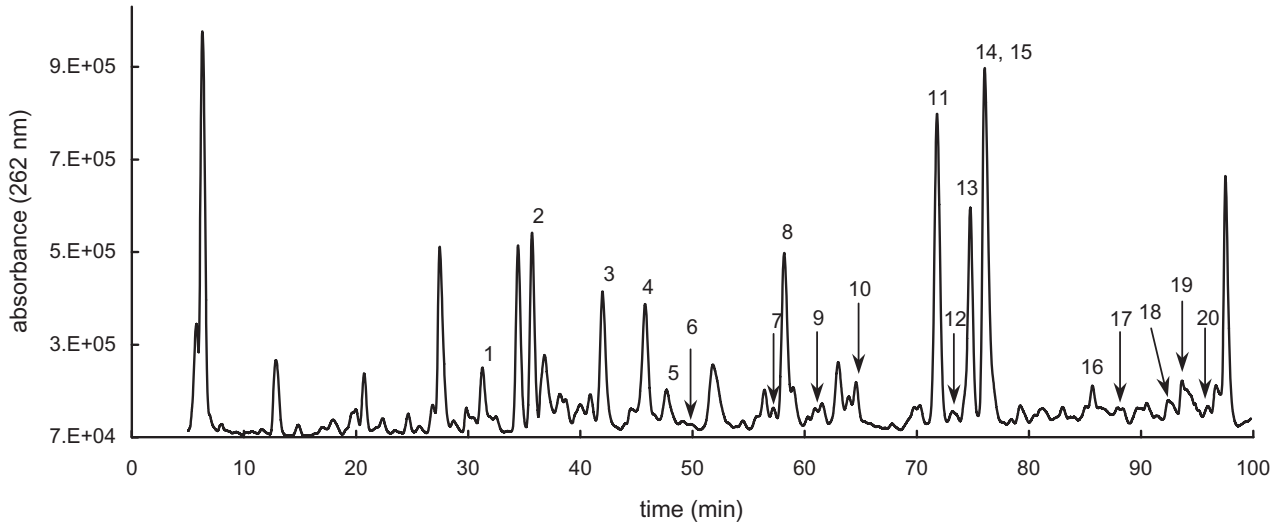

FIG. 4. HPLC CHROMATOGRAM (UV 262 NM) OF URINARY ISOFLAVONE METABOLITES EXCRETED AFTER THE INGESTION OF 52 G OF SOYBEAN PASTE (20 MG OF TOTAL ISOFLAVONE)

human urine. Based on ultraviolet (UV) chromatograms and peak intensities observed in the MS/MS chromatogram (Fig. 5), peak 8 was identified as daidzein 7-glucuronide and peak 12 was identified as the 4′-glucuronide. Peak 20 was identified as daidzein sulfate with a $[M+H]^+$ at m/z 335 and a fragment ion at m/z 255 resulting from the loss of the sulfonic acid moiety.

MS/MS fragmentation of peaks corresponding to compounds 3, 10, 15 and 17 resulted in the production of a common fragment at m/z 271, indicating that these were all derivatives of genistein (Table 1). Mass spectra of peak 3 indicated that this was genistein diglucuronide with a $[M+H]^+$ at m/z 623 and predominant fragment ions at m/z 447 and 271 resulting from the consecutive losses of two glucuronide

TABLE 1. ISOFLAVONE METABOLITES IDENTIFIED IN HUMAN URINE AFTER THE CONSUMPTION OF 20 MG ISOFLAVONES FROM SOY PASTE USING LC-ESI/MS/MS

Peak	t_R (min)	Isoflavone metabolite	$[M+H]^+$ (m/z)	MS^2 fragments ions (m/z)
1	31.26	Dihydrodaidzein diglucuronide	609	433 ($[M+H]^+$ – GlcUA), 257 ($[M+H]^+$ – GlcUA – GlcUA)
2	35.69	Daidzein diglucuronide	607	431 ($[M+H]^+$ – GlcUA), 255 ($[M+H]^+$ – GlcUA – GlcUA)
3	41.96	Genistein diglucuronide	623	447 ($[M+H]^+$ – GlcUA), 271 ($[M+H]^+$ – GlcUA – GlcUA)
4	45.76	Dihydrogenistein diglucuronide	625	449 ($[M+H]^+$ – GlcUA), 273 ($[M+H]^+$ – GlcUA – GlcUA)
5	47.67	Daidzein sulfoglucuronide	511	335 ($[M+H]^+$ – SO_3), 255 ($[M+H]^+$ – SO_3 – GlcUA)
6	50.02	O-DMA sulfoglucuronide	515	339 ($[M+H]^+$ – SO_3), 259 ($[M+H]^+$ – SO_3 – GlcUA)
7	57.50	6-OH-O-DMA glucuronide	451	275 ($[M+H]^+$ – GlcUA)
8	58.16	Daidzein glucuronide	431	255 ($[M+H]^+$ – GlcUA)
9	61.01	Dihydrodaidzein glucuronide	433	257 ($[M+H]^+$ – GlcUA)
10	64.58	Genistein sulfoglucuronide	527	351 ($[M+H]^+$ – SO_3), 271 ($[M+H]^+$ – SO_3 – GlcUA)
11	71.79	Dihydrogenistein glucuronide	449	273 ($[M+H]^+$ – GlcUA)
12	73.10	Daidzein glucuronide	431	255 ($[M+H]^+$ – GlcUA)
13	73.14	Dihydrogenistein glucuronide	449	273 ($[M+H]^+$ – GlcUA)
14	74.76	6-OH-O-DMA glucuronide	451	275 ($[M+H]^+$ – GlcUA)
15	76.06	Genistein glucuronide	447	271 ($[M+H]^+$ – GlcUA)
16	85.65	Dihydrogenistein glucuronide	449	273 ($[M+H]^+$ – GlcUA)
17	88.02	Genistein glucuronide	447	271 ($[M+H]^+$ – GlcUA)
18	92.41	O-DMA glucuronide	435	259 ($[M+H]^+$ – GlcUA)
19	95.90	Equol glucuronide	419	243 ($[M+H]^+$ – GlcUA)
20	96.80	Daidzein sulfate	335	255 ($[M+H]^+$ – GlcUA)

Peak numbers and retention times refer to HPLC trace in Fig. 3.

t_R, retention time; $[M+H]^+$, positively charged molecular ion; GlcUA,: glucuronyl unit; SO_3, sulfonyl unit.

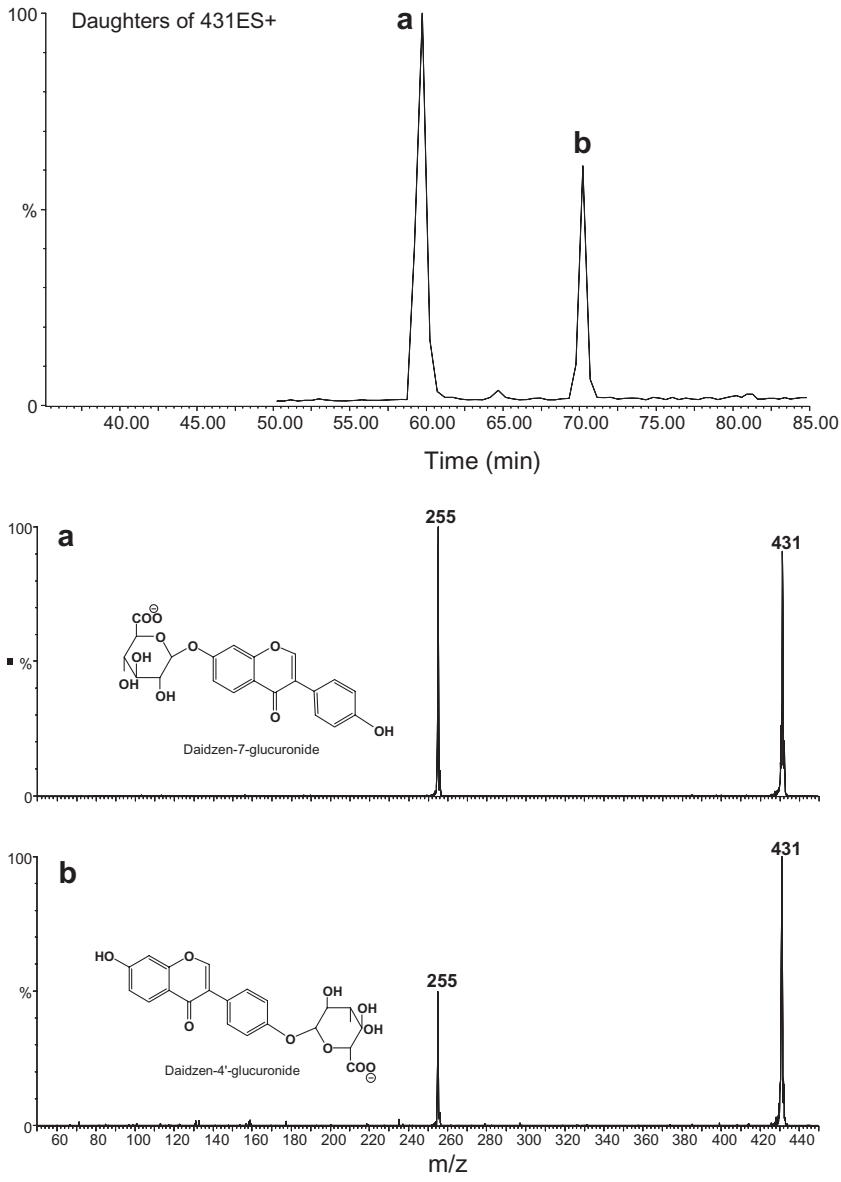

FIG. 5. SELECTED ION CHROMATOGRAM AT M/Z 431 AND MS/MS SPECTRA OF DAIDZEIN GLUCURONIDES

moieties. Mass spectra of peak 10 indicated it was genistein sulfoglucuronide with a $[M+H]^+$ at m/z 527 and fragment ions at m/z 351, formed by the loss of a glucuronyl moiety, and at m/z 271 derived from the consecutive losses a sulfonic acid moiety. Peaks 15 and 17 (Table 1) display different retention behaviors on the HPLC column yet had identical molecular ion masses at m/z 447 and produced a predominant fragment ion at m/z 271, indicating that they are isomers of genistein glucuronide (Fig. 6). Genistein has three conjugation sites at C-5, C-7 and at the 4′ position (Fig. 2). In general, the hydroxyl group at C-5 is not conjugated as it forms the hydrogen bond with the adjacent carbonyl group at C-4 (Coldham *et al.* 2002). Taking peak intensity and HPLC retention time into consideration, peaks 15 and 17 are tentatively identified as the 7-glucuronide and 4′-glucuronide, respectively.

MS/MS fragmentation of peaks corresponding to compounds 4, 11, 13 and 16 resulted in the production of a common fragment at m/z 273, indicating that these compounds are all derivatives of dihydrogenistein (Table 1). Mass spectra of peak 4 indicated that this was a dihydrogenistein diglucuronide with a $[M+H]^+$ at m/z 625 and predominant fragment ions at m/z 449 and 273 resulting from the consecutive loss of twio glucuronide moieties. Mass spectra of peaks 11, 13 and 16 indicated that these are isomers of dihydrogenistein glucuronide each having a $[M+H]^+$ at m/z 449 and

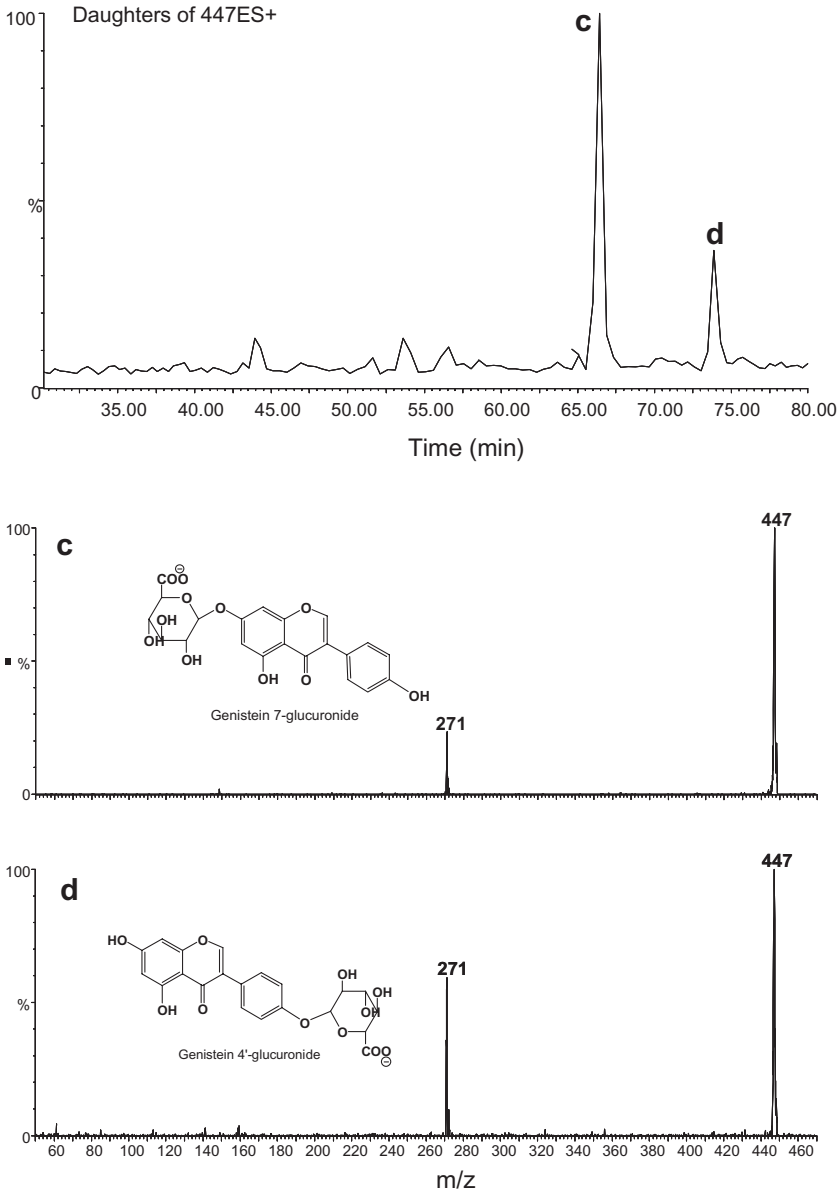

FIG. 6. SELECTED ION CHROMATOGRAM AT M/Z 447 AND MS/MS SPECTRA OF GENISTEIN GLUCURONIDES

predominant fragment ion at m/z 273 from the loss of the glucuronide moiety. Mass spectra of peak 9 indicated it was dihydrodaidzein with a $[M+H]^+$ at m/z 433 and a fragment ions at m/z 257, formed by the loss of a glucuronide moiety. Mass spectra of peaks 7 and 14 indicated that these are geometric isomers of 6-OH-*O*-DMA glucuronide, a product of microbial metabolism, with a $[M+H]^+$ at m/z 451 and a predominant fragment ion at m/z 275, formed by the loss of the glucuronide moiety. Mass spectrum of peaks 6 indicated that this is *O*-DMA sulfoglucuronide, again products of microbial metabolism, with a $[M+H]^+$ at m/z 515 and a fragment ions at m/z 339 and 259, formed by the consecutive loss of the glucuronide and sulfonic acid moieties. Peak 18 was identified as *O*-DMA glucuronide with a $[M+H]^+$ at m/z 435 and a fragment ion at m/z 259 formed by the loss of the glucuronide moiety. Mass spectra of peak 19 indicate that this is equol glucuronide with a molecular ion at m/z 419 and predominant fragment ion at m/z 243.

Urinary isoflavone levels are established as appropriate biomarker of soy consumption (Atkinson *et al*. 2002; Setchell *et al*. 2003; Ritchie *et al*. 2004). Qualitative assessment of urinary isoflavones metabolites can provide important information on the biotransformation pathways involved in isoflavone metabolism. Table 2 summarizes the profile of urinary isoflavone metabolites excreted within 24 h after the consumption of either unfermented soy flour or fermented

TABLE 2. ISOFLAVONE METABOLITES IDENTIFIED IN URINE AFTER THE CONSUMPTION OF SOY FLOUR AND SOYBEAN PASTE

Identified metabolites	Soy flour	Soybean paste
Daidzein diglucuronide	1	1
Dihydrodaidzein diglucuronide	1	1
Genistein diglucuronide	1	1
Dihydrogenistein diglucuronide	1	–[a]
Daidzein sulfoglucuronide	1	1
Genistein sulfoglucuronide	1	1
Daidzein glucuronide	2	2
O-DMA sulfoglucuronide	1	–[a]
Dihydrodaidzein glucuronide	1	1
Genistein glucuronide	2	2
Dihydrogenistein glucuronide	3	3
6-OH-*O*-DMA glucuronide	2	2
O-DMA glucuronide	1	1
Equol glucuronide	1	1
Daidzein sulfate	1	1
Total	20	18

[a] Not detected.

soybean paste. Twenty isoflavones metabolites were identified (Table 2). The glucuronide forms of both daidzein and genistein predominate, which is in agreement with other studies (Adlercreutz *et al.* 1995; Clarke *et al.* 2002; Shelnutt *et al.* 2002; Lampe 2003). Sulfoglucuronides were found to a lesser extent, with daidzein sulfate being the only isoflavone sulfate identified in this study. This can be explained by the observations of Shelnutt *et al.* (2002) who demonstrated that the concentration of daidzein sulfate in the plasma was 167% higher than of genistein sulfate and that one-fifth to one-half of the sulfate conjugates in plasma are excreted into urine. Thus, the levels of sulfate conjugates are either at or below the limit of detection herein. The number of urinary isoflavone metabolites identified was 20 and 18 after the ingestion of soy flour or soybean paste, respectively, and was due to differences in the dihydrogenistein diglucuronide and *O*-DMA sulfoglucuronide formed. To our knowledge, this is the first study to identify the intact urinary isoflavone metabolites after the consumption of fermented and unfermented soy products. It is well established that daidzein and genistein are reduced by the gut microflora, followed by C-ring cleavage to form *O*-DMA and 6′-OH-*O*-DMA, respectively. *O*-DMA was detected in all the subjects' urines; however, equol was detected in only one subject. When comparing the areas of isoflavone metabolites and intensities of mass spectra, only a minor part of the daidzein ingested appears to be further metabolized to *O*-DMA or equol via dihydrodaidzein. Watanabe *et al.* (1998) demonstrated that the individual urinary concentration of equol and *O*-DMA were quite variable in subjects and the percentages of recovery of daidzein ingested were 7.0 and 4.0%, respectively.

Composition of Urinary Isoflavone Metabolites

Accordingly, we determined the relative proportions of isoflavone aglycones and their metabolites in urine after the consumption of soy foods using three different conditions including: no enzymatic hydrolysis, incubation with glucuronidase and sulfatase in the presence of a glucuronidase inhibitor, and incubation with a glucuronidase and sulfatase and no inhibitor. The completion of hydrolysis was ensured by comparing the UV chromatograms of intact and hydrolyzed urinary metabolites. Isoflavone glucuronides, the sum of monoglucuronides and diglucuronides, were calculated by subtracting isoflavone levels after hydrolyzing with the mixture of glucuronidase and sulfatase in the presence of glucuronidase inhibitor from total isoflavone levels hydrolyzed without enzyme inhibition. The proportions of daidzein glucuronide and genistein glucuronide to total amount of daidzein and genistein were 76 and 86%, respectively, demonstrating that urinary isoflavones are predominantly conjugated with glucuronic acid and to a lesser extent sulfonic acid. It is not surprising that aglycones were not detected in urine in this study as hydrophobic aglycones are likely to accumulate in lipophilic tissues, e.g., breast, prostate via partitioning from the blood. These results are in agreement with the values of Zhang *et al.* (2003) showing that the glucuronides of daidzein and genistein were 73 $\pm$ 4% and 71 $\pm$ 5% of total daidzein and genistein in human urine excreted after soy consumption. Glucuronides may still be a good source of cellular isoflavone aglycones as the deglucuronidation of a flavonoid metabolite, luteolin monoglucuronide, was demonstrated in human serum (Shimoi *et al.* 2001).

Excretion Kinetics of Urinary Isoflavone Metabolites

The areas under curves (AUC) for genistein and daidzein after consumption of soy flour or soybean paste are given for one individual in Fig. 7. Levels of daidzein and genistein in urine peaked at 6 h after the ingestion of soy flour, returning to baseline 14 h. In contrast, the time to reach maximum urinary excretion was 10 h after the consumption of the fermented soybean paste. This difference in the time to reach the maximum excretion is likely linked to the higher concentration of readily bioavailable isoflavones in soybean paste. This is similar to a study by King and Bursill (1998) who demonstrated that the mean excretion rates for daidzein and genistein increased progressively reaching a peak at 6–12 h after a single soy meal. As expected, the concentrations of isoflavones increased more slowly for the first 3 h after ingestion of unfermented soy flour as compared to fermented soybean paste due to glycosidic forms. When comparing the AUC of the two soy foods, total urinary isoflavones excretion

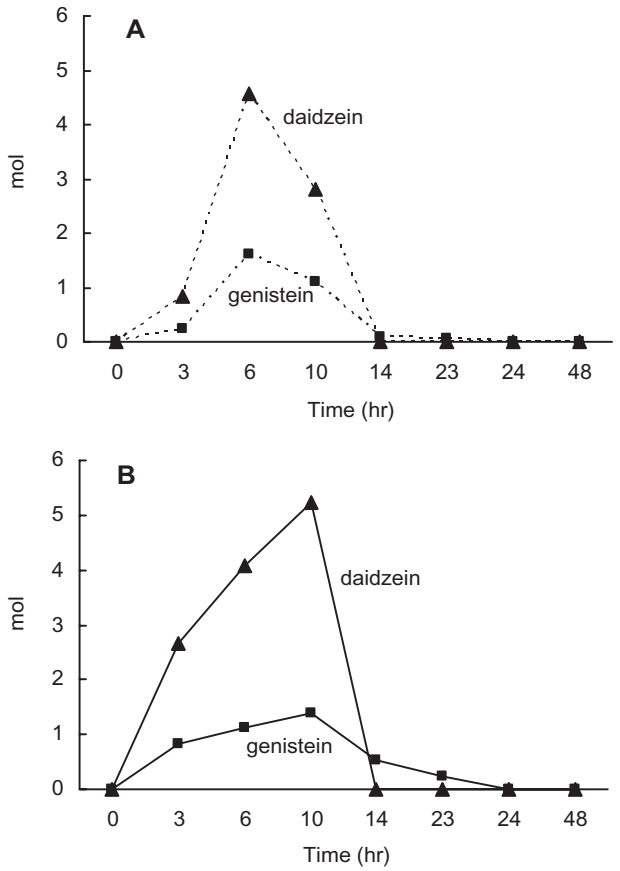

FIG. 7. URINARY KINETICS OF THE GLUCURONIDES OF DAIDZEIN AND GENISTEIN WITHIN 24 H AFTER THE CONSUMPTION OF EITHER UNFERMENTED SOY FLOUR (A) OR FERMENTED SOYBEAN PASTE (B)

was higher in soybean paste than soy flour. These results are in accordance with our hypothesis that the consumption of soybean paste consisting of over 50% isoflavone aglycones and higher levels of β-glycosides results in higher bioavailability of both daidzein and genistein as compared to soy flour, which contains predominantly malonyl glycosides that undergo less specific hydrolysis in the intestine prior to absorption. In contrast, Zubik and Meydani (2003) demonstrated that there was no difference in the apparent bioavailability of pure daidzein and genistein tablets when consumed as either aglycones or glycosides; however, malonylated forms were not considered in this study.

In both soy foods, the genistein glucuronides are cleared more slowly than the daidzein glucuronides (Fig. 7). This is in agreement with the result of Shelnutt *et al.* (2000). The difference in the urinary disposition of these two compounds may be due to the greater hydrophobicity of genistein, which results in the longer retention in tissues, or an enhanced excretion of genistein via bile as compared to daidzein (Birt *et al.* 2001). Interestingly, Xu *et al.* (1995) demonstrated that genistein is also likely to be more degraded by gut microflora in human intestine than daidzein, which again could result in lower urinary recovery.

CONCLUSION

Our results demonstrate that daidzein and genistein are absorbed in the intestine and are rapidly conjugated predominantly with glucuronic acid and excreted into urine within 24 h after the consumption of soy foods. Urinary excretion of genistein and daidzein was higher after the consumption of fermented soybean paste as compared to unfermented soy flour, indicating that isoflavones in fermented soy foods are more bioavailable than those in unfermented soy foods. These results should be considered when assessing the potential health effects arising from the consumption of different soy foods in a given population.

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