Metabolism of Glycitein (7,4\[^D\]-Dihydroxy-6-methoxy-isoflavone) by Human Gut Microflora

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Abstract
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Keywords
Glycitein; isoflavone; microbial metabolism; dihydroglycitein; dihydro-6, 7, 4'-trihydroxyisoflavone

Disciplines
Food Science | Human and Clinical Nutrition | Other Nutrition

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Gut microbial disappearance and metabolism of the soy isoflavone glycitein, 7,4′-dihydroxy-6-methoxyisoflavone, were investigated by incubating glycitein anaerobically with feces from 12 human subjects. The subjects’ ages ranged from 24 to 53 years with a body mass index (BMI) of 20.9–25.8 kg/m² (mean BMI = 24.0 ± 1.1 kg/m²). Glycitein disappearance followed an apparent first-order rate loss. Fecal glycitein disappearance rates for the subjects segregated into three different groups described as high (k = 0.67 ± 0.14/h), moderate (k = 0.34 ± 0.04/h), and low (k = 0.15 ± 0.07/h) glycitein degraders (p < 0.0001). There was no dose effect on the disappearance rates for each subject from 10 to 250 µM glycitein (average k = 0.32 ± 0.03/h, p > 0.05). Four putative glycitein metabolites, characterized by liquid chromatography–mass spectrometry (electrospray ionization using positive ionization mode), were dihydroglycitein, dihydro-6,7,4′-trihydroxyisoflavone, and 5′-O-methyl-O-desmethylangolensin. Two subjects produced a metabolite tentatively identified as 6-O-methyl-equol, and one subject produced daidzein as an additional metabolite of glycitein. These results show that glycitein is metabolized by human gut microorganisms and may follow metabolic pathways similar to other soy isoflavones.

KEYWORDS: Glycitein; isoflavone; microbial metabolism; dihydroglycitein; dihydro-6,7,4′-trihydroxyisoflavone

INTRODUCTION

Isoflavones are a subclass belonging to a larger group of polyphenolic compounds called flavonoids. Isoflavones mainly occur in plants of the Leguminosae family (1). Soybeans and soybean-derived foods are the main sources of the isoflavones genistein (5,7,4′-trihydroxyisoflavone), daidzein (7,4′-dihydroxy-isoflavone), and glycitein (7,4′-dihydroxy-6-methoxyisoflavone) where the total isoflavone concentration can reach up to 2 g per kg fresh weight (2). Glycitein comprises less than 10% of the total isoflavone amount in soybeans and soybean foods but comprises about 50% of the isoflavone mass in soy germ (3). The three soy isoflavones are hydroxylated in the 7- and 4′-positions of the isoflavone skeleton (Figure 1), which makes them structurally similar to β-estradiol (4). Apparently, because of this similarity, isoflavones are weakly estrogenic and bind to estrogen receptors (4). Glycitein was more estrogenic in a mouse uterine growth assay than was genistein, when fed in equal amounts (5). Epidemiological studies have reported beneficial relationships between isoflavone intake and decreased risk of chronic diseases, although more recent studies have not observed these relationships (6–9). Isoflavones have been proposed to decrease the risk of coronary heart disease, certain types of cancers (10, 11), and osteoporosis (12) by a variety of biological functions and mechanisms.

Isoflavones are found as both glucosides and aglucons in soy foods (13). Oral ingestion of isoflavone glucosides leads to their hydrolysis to aglucons in the small intestine by bacterial β-glucosidase activity and β-glucosidases in gastrointestinal mucosal cells (14). The free aglucons resulting from hydrolysis can be absorbed, rapidly glucuronidated, and undergo first pass hepatic metabolism (15) or be further metabolized by the gut microflora. Antibiotic administration was shown to decrease the production of isoflavone metabolites in humans (16). Experiments with germ-free rats revealed that isoflavone metabolites were not excreted in the urine (17). Understanding the metabolism of isoflavones in the gut is important because this process...
may affect their bioavailability and their overall absorption and biological activities in vivo (18).

The anaerobic bacterial metabolism of genistein and daidzein by gut microflora has been studied extensively. Genistein metabolites resulting from anaerobic metabolism in the human gut have been identified as dihydrogenistein (19–23), 6′-hydroxy-O-desmethyldaidzein (6-OH-ODMA) (20–23), 4-hydroxyphenyl-2-propionic acid (22, 23), and phloroglucinol (22). Anaerobic bacterial metabolism of daidzein resulted in dihydrodaidzein (19–21), O-desmethyldaidzein (24, 25), equol (19, 25, 26), and cis-4-hydroxy-equol (20, 21). In contrast, glycitein microbial metabolites have not been well-characterized in vitro. Glycitein comprises less than 10% of the total isoflavone amount in soybeans and soybean foods and may be the reason that its metabolism has not been well-studied. However, glycitein comprises about 50% of the isoflavone mass in soy germ (3). Glycitein was demethoxylated in vitro by Eubacterium limosum to 6,7,4′-trihydroxyisoflavone (27). Recently, glycitein metabolites, dihydroglycitein, 5′-O-methyl-O-desmethyldaidzein, and 6-O-methyl-equol have been isolated and characterized in human urine (21).

Earlier studies by our group showed that the rate of disappearance of glycitein was significantly slower than that of genistein (p < 0.0001) in an in vitro fecal fermentation system (18), and the human bioavailability of glycitein was significantly greater than genistein (28). The aim of this study was to further characterize glycitein metabolism in humans by investigating the kinetics and variability of the apparent fecal degradation reaction and the metabolites of glycitein using an in vitro fecal fermentation system.

**MATERIALS AND METHODS**

**Chemicals.** Glycitein was synthesized according to Lang’at-Thoruwa et al. (29). Daidzein and 2,4,4′-trihydroxydeoxybenzoin were synthesized using the method of Song et al. (3). Genistein was synthesized according to a modification of Chang et al. (30). The purities of glycitein, genistein, daidzein, and 2,4,4′-trihydroxydeoxybenzoin were 99±%. 6,7,4′-Trihydroxyisoflavone was purchased from Indofine Chemical Co., Inc. (Hillsborough, NJ). Dihydrodaidzein, dihydrogenistein, equol, and O-desmethyldaidzein were purchased from Plantech U.K. (Reading, England). All other chemicals including high-performance liquid chromatography (HPLC) grade acetonitrile, methanol, acetic acid, and dimethyl sulfoxide were purchased from Fisher Scientific (Fairlawn, NJ). All aqueous solutions were prepared using Milli-Q system (Millipore Co., Bedford, MA) HPLC grade water (MQ water).

**Subject Protocol.** Four men and eight women volunteered from Iowa State University and the surrounding Ames area. The subjects were in good health and not taking any medication. The subjects’ ages ranged from 24 to 53 years with a body mass index (BMI) of 20.9–25.8 kg/m² (mean BMI = 24.0 ± 1.1 kg/m²). The ethnicities of the subjects included six Caucasians, three African Americans, one Chinese immigrant, one Asian-Indian, and one Latino. Subjects were given written instructions about soy-containing foods to avoid for 1 week before collecting one fresh fecal sample in sealed sterile containers (Sage Products Inc., Crystal Lake, IL). Approval of the study design was obtained from the Iowa State University Human Subjects Research Committee in 2003.

**Isoflavone Fermentation and Extraction.** In vitro fecal fermentation systems and the isoflavone extraction from the fermentation systems were carried out according to Simons et al. (31) with the following changes. Daidzein, glycitein, and genistein were added to a fecal suspension at 100 μM. Glycitein dose response was investigated by adding glycitein to fecal suspensions for final concentrations of 10, 50, 75, 100, and 250 μM glycitein. The negative control consisted of the fecal suspension without isoflavones. The positive control consisted of brain heart infusion media and 250 μM daidzein, genistein, and glycitein without the fecal suspension. All isoflavone fermentations were carried out in duplicate.

**HPLC Analysis.** The HPLC system consisted of a Hewlett-Packard 1050 Series. Twenty microliters of isoflavone extract was injected onto a reversed phase 250 mm × 4.6 mm, 5 μm, C18 AM 303 column (YMC Co. Ltd., Wilmington, NC). The mobile phase consisted of 0.1% glacial acetic acid in water (A) and acetonitrile (B). Solvent B was increased from 30% to 50% in 14 min, increased to 100% in 5 min, and recycled back to 30% in 1 min. The flow rate was 1 mL/min. The wavelengths used for the detection of isoflavone and metabolite peaks and for the preparation of isoflavone standard curves were 254 and 280 nm. Chem station 3.0 software (Hewlett-Packard Co., Scientific Instruments Division, Palo Alto, CA) was used to integrate the peak area responses and to evaluate the ultraviolet absorbance spectra.

**Mass Spectrometry Analysis.** Analyses of glycitein metabolites were carried out using a Shimadzu 2010 LC-MS system (Kyoto, Japan) consisting of a Shimadzu 2010 liquid chromatograph with a dual wavelength photodiode array detector in series between the chromatograph and a Q-array-Octapole-Quadrupole mass analyzer. Detection of metabolites was performed using electrospray ionization (ESI) in the positive ion mode. The mobile phase for sample separation was performed under the same conditions as used for HPLC analysis except that the flow rate was 0.2 mL/min. The injection volume was 20 μL. Samples were introduced into the electrospray interface through an untreated fused silica capillary. A nitrogen gas flow of 4.5 L/min was used as the nebulizing and auxiliary gas for the mass spectrometer. The parameters applied to MS were as follows: block temperature, 200 °C; desolvation temperature, 400 °C; capillary voltage, 3.8 kV; and cone voltage, 20 V. The mass spectrometer was tuned and calibrated for the range of m/z 100–300. Daidzein, glycitein, 6,7,4′-trihydroxyflavone, and O-desmethyldaidzein were dissolved in 100% methanol at 25 μmol/L and analyzed to obtain authentic mass spectra prior to sample analysis.

**Data Analysis and Statistics.** The rate of disappearance of isoflavones in fecal suspensions was estimated by plotting In(% remaining isoflavone) vs time, and the negative of the slope was the apparent first-order degradation rate constant. An internal standard curve employing 2,4,4′-trihydroxydeoxybenzoin was used to estimate the concentration of isoflavones in the fecal suspensions. Zero time concentrations were corrected for recovery for each subject. Statistical evaluation of degradation rate differences was performed using the SAS system (version 8.1, SAS Institute, Cary, NC). Differences between the overall and the individual degradation rates of isoflavones were estimated using one-way analysis of variance. Isoflavone degradation phenotypes were identified using average linkage cluster analysis (32). The statistical significance of all analyses was set at α = 0.05.

**RESULTS AND DISCUSSION**

The glycitein concentrations studied in our fermentation systems represented probable doses of isoflavones in the human gut after ingestion of soy-containing foods (Figure 2). A plot
of the natural log of the percentage remaining for each isoflavone against time resulted in a straight line for all subjects, with correlation coefficients ranging from 0.75 to 0.99. These findings suggest that isoflavone degradation by gut microflora follows apparent first-order kinetics, which supports other investigations reporting isoflavone degradation kinetic data (18, 33). There were no significant differences in the average degradation rate for each glycitein dose across all subjects with an average \( k = 0.30 \pm 0.21/h, p > 0.05 \) (Figure 2).

Cluster analysis allowed us to determine if any groupings of similar isoflavone degradation rates occurred in these subjects. Three different degradation rate groupings were discerned for glycitein: high \((k = 0.67 \pm 0.14/h)\), moderate \((k = 0.34 \pm 0.04/h)\), and low \((k = 0.15 \pm 0.07/h)\) glycitein degraders \((p < 0.0001; \text{Figure 3})\). These phenotypes were observed for genistein and daidzein degradation rates as well (Table 1). These data were similar to that reported by Hendrich et al. (34), who found three isoflavone degradation phenotypes (high, moderate, and low) from nine men and 11 women.

The glycitein degradation rate, with an average \( k = 0.30 \pm 0.21/h, p < 0.0001 \), was significantly lower than genistein with an average \( k = 0.43 \pm 0.44/h, p = 0.02 \), but not different from daidzein with an average \( k = 0.16 \pm 0.17/h, p = 0.07 \). We believe that this difference is due to structural differences between the three isoflavones. It has been proposed that the hydroxy group in the 5th position on the A ring of the flavonoid structure is responsible for the rapid degradation of genistein by gut microflora (18, 35). We have shown in related studies that flavonoids with hydroxyl groups in the 5-, 7-, and 4′-positions were significantly more rapidly degraded in human fecal incubations than were flavonoids not possessing all of these hydroxyls (31).

Phenotypic trends were observed in all of the subjects (Table 1). Subjects 9, 13, and 26 had moderate and high degradation phenotypes and subjects 5, 8, 17, and 18 had low degradation phenotypes for all three isoflavones. We speculated that these differences in degradation phenotypes were because of distinct differences in individual gut bacterial populations. Our preliminary studies have shown different bacterial DNA profiles of rapid isoflavone degraders as compared with subjects of slow degradation phenotype using polymerase chain reaction—denaturing gradient gel electrophoresis (36). Different fecal bacterial species were identified in high glycitein degraders as compared with high genistein degraders. Subjects with low in vitro isoflavone degradation phenotypes may experience higher isoflavone bioavailability in vivo, as compared to subjects with high isoflavone degradation phenotypes. Zheng et al. (37) have shown that Asian women with low genistein degradation phenotypes experienced greater genistein bioavailability as compared with Asian subjects with high genistein degradation phenotypes. The overall average glycitein degradation rate (average \( k = 0.30 \pm 0.21/h)\) did not change when fermented with genistein and daidzein (average \( k = 0.23 \pm 0.19/h, p = 0.72 \)). These data suggest that the isoflavones may not compete with each other for metabolism by the gut microflora.

We hypothesized that the main glycitein metabolite should be dihydroglycitein based on reports identifying dihydrodaidzein and dihydrogenistein as the main metabolites of daidzein and genistein, respectively (19, 20). In vitro metabolism studies by Hur et al. (27) reported that glycitein was metabolized to 6,7,4′-trihydroxyisoflavone by E. limosum. E. limosum is able to O-demethylate methoxyl derivatives of benzoic acid (38) and is found in the human digestive tract (39). On the basis of the data in these studies, we predicted that 6,7,4′-trihydroxyisoflavone would be one of the metabolites of glycitein.

HPLC chromatograms were analyzed for the formation of new peaks in the fecal fermentation suspensions over the 24 h period. In two subjects, intact glycitein levels remained the same over the 24 h period and there was no evidence of new chromatographic peaks. For the other 10 subjects, two new peaks (peaks 1 and 4) appeared after 6 h of incubation at retention times 14.5 and 19.9 min, respectively (Figure 4A). Two additional new peaks (peaks 2 and 3) appeared after 24 h of incubation with retention times of 17.5 and 17.7 min, respectively (Figure 4A). Peak 2 was observed in only two of the 10 subjects producing metabolites. One subject showed an additional new peak at 15.9 min (peak 5) (Figure 4B). None of the other chromatogram peaks were associated with glycitein metabolism because they appeared in negative controls.

The UV spectra and retention time of peak 1 from HPLC analysis were identical to the chemically synthesized reference standard, 6,7,4′-trihydroxyisoflavone. However, mass spectrometric analysis using positive ESI (M + H) revealed that the molecular weights of peak 1 and 6,7,4′-trihydroxyisoflavone were different \( m/z \) 273 and 271, respectively. On the basis of these data, the identity of peak 1 was tentatively given as dihydro-6,7,4′-trihydroxyisoflavone. The UV spectra and reten-

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**Table 1. Cluster Analysis of Subjects’ Isoflavone Degradation Rates**

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>high</th>
<th>moderate</th>
<th>low</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>26</td>
<td>3, 11, 2</td>
<td>6, 8, 18, 17, 4, 5</td>
</tr>
<tr>
<td>9</td>
<td>26</td>
<td>13, 2, 3, 11, 4</td>
<td>8, 5, 18, 17</td>
</tr>
<tr>
<td>13</td>
<td>26</td>
<td>2, 3</td>
<td>8, 9, 5, 17, 11, 4, 18, 6</td>
</tr>
</tbody>
</table>

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a Degradation phenotypes of subjects. Subjects separated into three significantly different groups for each isoflavone, named high, moderate, and low degraders \((p < 0.0001)\). b Degradation rates expressed as averages ± standard deviation of the mean. c Whole numbers shown are subjects’ identification number. d Standard deviation could not be determined from a measurement of only one subject’s degradation rate.
tion time of peak 5 and daidzein were identical. MS analysis confirmed that peak 5 was daidzein with a molecular weight (M + H)$^+$ of 255.

Peaks 2–4 were more hydrophobic than glycitein (Figure 4A,B). The UV spectra of peaks 2–4 were similar to the daidzein microbial metabolites, equol, O-desmethylangolensin, and dihydrodaidzein, respectively. MS analysis for these three peaks showed molecular weights (M + H)$^+$ of m/z 271, 289, and 287, respectively, which would tentatively match 6-methoxy-equol, 5′-methoxy-O-desmethylangolensin, and dihydroglycitein, respectively, based on the MS and UV absorbance.

Glycitein is similar to daidzein structurally with exception of the methoxyl group at the 6-position. We expected to identify metabolites that were similar to the daidzein metabolites dihydrodaidzein, equol, and O-desmethylangolensin (19–21). We detected dihydroglycitein in the fecal incubations, but the major metabolite appearing in all metabolite-producing subjects was dihydro-6,7,4′-trihydroxyisoflavone at 6 h after incubation. These observations suggest that the first step in glycitein metabolism is reduction to dihydroglycitein and then O-demethylation to dihydro-6,7,4′-trihydroxyisoflavone. However, glycitein may first be O-demethylated to 6,7,4′-trihydroxyisoflavone and then further reduced to dihydro-6,7,4′-trihydroxyisoflavone. We did not detect 6,7,4′-trihydroxyisoflavone in our incubation mixtures suggesting that the step to dihydro-6,7,4′-trihydroxyisoflavone is either extremely rapid or that this route was not viable. The O-demethylation pathway of glycitein to 6,7,4′-trihydroxyisoflavone would be similar to the microbial metabolic pathways of formononetin (7-hydroxy-4′-methoxyisoflavone) and biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) (27).

Heinonen et al. (21) identified dihydrodaidzein, dihydrogenistein, and dihydroglycitein in the urine of six human subjects fed three soy bars, equivalent to a daily intake of 48.4, 40.2, and 4.1 mg of daidzein, genistein, and glycitein, respectively, for 2 weeks. These data imply that daidzein, genistein, and glycitein were metabolized to their respective dihydroisoflavones and absorbed across the intestinal wall. However, Bowey et al. (17) observed that dihydrogenistein and dihydrodaidzein were produced in the urine of germ-free rats. They concluded that dihydrogenistein, dihydrodaidzein, and dihydroglycitein are not products of microbial metabolism, which conflicts our observation of dihydroglycitein as a microbial metabolite of glycitein. Because previous studies have reported the microbial conversion of daidzein and genistein to dihydrodaidzein and dihydrogenistein, respectively (19–23), we propose that dihydroglycitein and other dihydroisoflavones are produced by both intestinal bacteria and gut mucosal enzymes.

The metabolite, 5′-OMe-O-desmethylangolensin, most likely results from C ring cleavage of dihydroglycitein, consistent with bacterial C ring cleavage observations of daidzein and genistein (16, 17). Heinonen et al. (21) identified 5′-OH-O-desmethylangolensin in addition to 5′-OMe-O-desmethylangolensin in human urine, which suggested direct C ring cleavage of dihydro-
that were not supported by data from fermentation systems. Dashed arrows indicate hypothesized pathways that were not supported by data from fermentation systems. 6,7,4′-trihydroxyisoflavone or demethylation of 5′-OMe-O-desmethylangolensin. We did not detect 5′-OH-O-desmethylangolensin in our fecal fermentation mixtures over the 24 h incubation period.

Production of daidzein from glycitein would result from direct demethoxylation of glycitein at the 6-position. However, only one subject produced daidzein in this study. Additionally, bioavailability studies in our laboratory have shown that daidzein was produced in only one out of 10 hamsters that were fed purified glycitein (unpublished data). Setchell et al. (40) reported that humans fed glycitin (glycitein-7-O-β-D-glucopyranoside) showed only minute concentrations of daidzein in plasma. Taken together, these results suggest that demethoxylation of glycitein may not be a major pathway of metabolism in humans and hamsters.

We observed the in vitro degradation of daidzein for each subject and did not observe any equol production. However, our in vitro fecal fermentation models are poor indicators of equol-producer phenotypes, because apparently, equol is only produced in response to chronic soy ingestion over a period of at least 3 days (41, 42). Therefore, we may have had equol producers among the 12 subjects. We proposed that 6-methoxyequol and 6-hydroxy-equol would be possible metabolites of glycitein, and we were able to identify 6-methoxy-equol in two subjects.

We realize that there were some limitations in our glycitein metabolite identification process. The metabolites were identified based on interpretation of the HPLC, MS, and UV data since we did not have authentic reference standards for direct comparisons. Nevertheless, the metabolites identified in these studies have allowed us to propose several microbial pathways for glycitein shown in Figure 5. The major pathways of glycitein microbial metabolism are reduction to dihydroglycitein (7,4′-dihydroxy-6-methoxy-isoflavone), followed by demethylation to produce dihydro-6,7,4′-trihydroxyisoflavone (6,7,4′-trihydroxyisoflavone) or, alternatively, C ring cleavage of dihydroglycitein to produce 5′-methoxy-O-desmethylangolensin. Minor pathways include direct demethylation of glycitein to daidzein (7,4′-dihydroxyisoflavone) and reduction of dihydroglycitein to 6-OMe-equol (7,4′-dihydroxy-6-methoxy-isoflavone).

The estrogenic activity of isoflavones may be affected by gut microbial metabolism. For example, equol, a metabolite of daidzein, has been reported to be more estrogenic than daidzein (43, 44). p-Ethylphenol, a metabolite of genistein, has no estrogenic activity even though genistein possesses estrogenic activity and other biological effects (45). Song et al. (5) have shown that glycitein possesses a lower in vitro estrogen receptor-binding affinity as compared to genistein but gave a higher estrogenic response in an in vivo mouse uterine enlargement assay. This higher estrogenic response probably resulted from the higher bioavailability of glycitein as compared to genistein or the formation of glycitein metabolites in the in vivo assays that possess higher estrogenic potencies than the parent compound. 6,7,4′-Trihydroxyisoflavone has been shown to exhibit weak estrogenic activity and binds the estrogen receptor β with little or no affinity (45, 46). It would be interesting to determine the estrogenic properties of the other glycitein metabolites. The gut microflora play a significant role in the bioavailability of isoflavones (18, 28, 37, 47). Plasma daidzein and genistein concentrations were negatively correlated with the daidzein and genistein microbial degradation rate constants, respectively (37), suggesting that increased isoflavone intestinal microbial degradation reduced the amount of intact isoflavones appearing in plasma after absorption. Additionally, daidzein and glycitein were more bioavailable than genistein, as reflected in urinary excretion as a percentage of ingested dose, because daidzein and glycitein were degraded at a much slower rate as compared to genistein (28).

In this study, we have found that glycitein was metabolized according to first order kinetics by human gut microflora but at a slower rate as compared to genistein. We found that there was significant interindividual variation in glycitein degradation, but the degradation rates were segregated into three different groups (p < 0.0001). We tentatively identified three major glycitein metabolites (dihydroglycitein, dihydro-6,7,4′-trihydroxyisoflavone, and 5′-OMe-O-desmethylangolensin) in 10 of 12 subjects, while two subjects failed to produce any metabolites. Of the 10 metabolite producers, two subjects apparently produced 6-OMe-equol and one subject produced daidzein. The biological significance and exact identity of the glycitein metabolites have yet to be determined.

**LITERATURE CITED**


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