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Metabolism of Isoflavones Found in the *Pueraria thomsonii* Flower by Human Intestinal Microbiota

Kazuhiro HIRAYAMA[1]*, Yuki MATSUZUKA[2], Tomoyasu KAMIYA[2], Motoya IKEGUCHI[2], Kinya TAKAGAKI[2] and Kikuji ITOH[1]

[1]*Laboratory of Veterinary Public Health, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*

[2]*Research and Development Division, Toyo Shinyaku Co., Ltd., 7–28 Yayoigaoka, Tosu-shi, Saga 841-0005, Japan*

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Isoflavones contained in the root and flower of Kudzu (*Pueraria lobata* and related species) are suggested to be the critical component for its effects. Although metabolism of soy isoflavones has been well studied, the composition of isoflavones found in Kudzu is completely different from that of soy isoflavones. In the present study, we investigated whether isoflavones found in the flower of *Pueraria thomsonii*, a species of Kudzu, were metabolized by human fecal microbiota and murine small intestinal enzymes. Among 5 glycosidic isoflavones of the *Pueraria thomsonii* flower, tectorigenin 7-*O*-xylosylglucoside, tectoridin, genistin and glycitin were completely hydrolyzed by a homogenate of germfree mouse small intestine without contribution of bacteria. Released aglycones were not further metabolized, except that up to half of glycitein disappeared. Mouse small intestinal enzymes did not metabolize 6-hydroxygenistein 6,7-di-*O*-glucoside. Isoflavone aglycones as well as 6-hydroxygenistein 6,7-di-*O*-glucoside were highly metabolized by most of the human fecal suspensions. Metabolites were not detected with the present analytical methods in most cases. Although further investigations of the pharmacokinetics of *Pueraria thomsonii* flower isoflavones are needed, the results of the present study indicate active metabolism of *Pueraria thomsonii* flower isoflavones in the human intestine.

Key words: β-glucosidase; germfree; intestinal microbiota; isoflavones; metabolism; *Pueraria thomsonii*

INTRODUCTION

Both the root and flower of Kudzu (*Pueraria lobata* and related species in the genus *Pueraria*) have been traditionally used in Chinese medicine, and the effects of the flower of *Pueraria*, including the hepatoprotective effect and stimulation of recovery from alcohol intoxication, have been reported *(1-5)*. There are many species in the genus of *Pueraria*, and *Pueraria thomsonii* is one of those that have been used since ancient times. *P. thomsonii* flower contains a large amount of isoflavones and saponins as major bioactive components *(6)*.

Isoflavones have been linked with reductions in various disease risks. Soybean and soy foods have been identified as important dietary sources for isoflavones, and soy isoflavones have been widely studied *(7, 8)*. Isoflavones in foods are present predominantly as glycoside conjugates, except for in fermented foods *(9)*. They are hydrolyzed in the intestine as a first step of their metabolism and release their aglycones. It is well established that hydrolysis of the sugar moiety is required for their bioavailability and that isoflavone glycosides are not absorbed intact across the enterocyte in healthy adults *(10)*.

Although intestinal microfloral enzymes present in several groups of bacteria have been thought to be responsible for the hydrolysis of soy isoflavone glycosides, enzyme activity present in the small intestine is, at least partly, responsible for hydrolyzing isoflavonoid compounds in foods *(10)*. The isoflavonoid aglycones can undergo further metabolism to form more or less bioactive metabolites, and that metabolism may be important in relation to the biological efficacy of these compounds. In contrast to the hydrolysis of isoflavonoid glycosides, the crucial importance of the intestinal microbiota has been clearly demonstrated in soy isoflavone metabolism *(10)*.

Isoflavones present in *Pueraria* are also predominantly glycoside conjugates *(11)*, and the initial hydrolysis of sugar moiety and further metabolism of released aglycones appear to be important to the bioavailability and bioactivity of *Pueraria* isoflavones. However, the isoflavones found in the *P. thomsonii* flower are different from those in soy and are 6-hydroxygenistein 6,7-di-*O*-glucoside (6HGDG), tectorigenin 7-*O*-xylosylglucoside (TGXG), tectoridin, genistin, glycitin, tectorigenin, genistein and glycitein *(11)*. Among these isoflavones, 6HGDG, TGXG, tectoridin and tectorigenin, which

*Corresponding author. Mailing address: Laboratory of Veterinary Public Health, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan. Phone: +81-3-5841-3094. E-mail: akazu@mail.ecc.u-tokyo.ac.jp

amount to nearly 90% of the isoflavones, are present in the *P. thomsonii* flower and not present in soy foods. The metabolism of these *Pueraria* flower isoflavones in the intestine has not been elucidated. On the other hand, isoflavones found in soy, genistein, glycitein and their glycosides are only a small proportion of the isoflavones in the *P. thomsonii* flower.

In the present study, we investigated metabolism of isoflavones present in the *P. thomsonii* flower by human intestinal microbiota and mammalian small intestinal enzymes *in vitro*. Isoflavones were incubated with a homogenate of germfree mouse small intestine to investigate metabolism of these compounds, particularly hydrolysis of glycosides, without the activity of microbes. Then isoflavones were incubated with 10 human fecal suspensions anaerobically. In the incubation with human feces, isoflavone glycosides shown to be hydrolyzed to their aglycones without bacterial activity were excluded from the study because these glycosides would be rapidly metabolized in the human upper intestine, where very few bacteria harbor. Daidzein, which is a major isoflavone in soy but is not present in the *P. thomsonii* flower, was also included in this study because daidzein is metabolized by intestinal microbiota to a more estrogenic compound, equol *(10, 12)*.

MATERIALS AND METHODS

Chemicals

Tectorigenin 7-*O*-xylosylglucoside (TGXG), 6-hydroxygenistein 6,7-di-*O*-glucoside (6HGDG), tectoridin and tectorigenin were purchased from Tokiwa Phytochemical Co., Ltd. (Chiba, Japan). Genistin, glycitin and glycitein were purchased from Nagara Science Co., Ltd. (Gifu, Japan). The Faculty of Agriculture and Life Science, Hirosaki University supplied the 6-hydroxygenistein (6HG). Genistein, daidzein and equol were purchased from Funakoshi (Tokyo, Japan). Flavone was purchased from Sigma (St. Louis, MO, USA). Isoflavones were dissolved in DMSO at a concentration of 5 mmol/l, and equol was dissolved in DMSO at a concentration of 0.5 mmol/l.

Preparation of specimens

Germfree BALB/cA mice were bred and kept in the Laboratory of Veterinary Public Health, the University of Tokyo. Fourteen-week-old male mice were sacrificed, and the upper small intestine was removed. Small intestine was introduced into an anaerobic chamber and homogenized with 9 volumes of Brain Heart Infusion Broth (BHI; Difco, Detroit, MI, USA). The pH of the BHI was adjusted to 7.4 or 5.0. Ten human fecal samples were collected from healthy volunteers aged 20 to 65 years and stored at –80ºC until incubation with isoflavones. The subjects were comprehensively informed of the details of the clinical tests in accordance with the World Medical Association Declaration of Helsinki ethical principles for medical research involving human subjects, and written consent was obtained from the participants for their enrolment in the study. The experimental protocol was approved by the ethical committee of Toyo Shinyaku Co., Ltd. Fecal samples were introduced into the anaerobic chamber, and 100-fold fecal suspensions were prepared with BHI (pH 7.4). A mixture of these ten fecal suspensions was also prepared as an "average" human feces.

In vitro *metabolism of isoflavones by the mouse small intestine and human feces*

Each isoflavone solution was added to the mouse small intestinal homogenate or human fecal suspension at a concentration of 100 μmol/l and incubated anaerobically at 37ºC for 3 days. Among the isoflavones tested, TGXG, tectoridin, genistin and glycitin were omitted from the incubation with human fecal suspension because these glycoside-form isoflavones were totally transformed to aglycones by germ-free mouse small intestinal homogenate, i.e., without contribution of intestinal bacteria. To isolate both glycosidic and aglycone forms of isoflavones and their metabolites, incubated suspensions were extracted with the same volume of butanol 5 times. Flavone was added to each suspension for incubation as an inner standard prior to extraction. Butanol fractions were evaporated and redissolved with 50% ethanol.

HPLC analysis

The samples were analyzed by HPLC according to previous publications *(13, 14)* with modifications to analyze both aglycone and glycosidic isoflavones. HPLC analyses were carried out on an YMC-Pack ODS-AM (250 × 4.6 mm i.d.; 4 μm) reversed-phase column (YMC Co., Ltd., Kyoto, Japan). The temperature was set at 30ºC. Elution was performed at a flow rate of 0.8 ml/min with the following linear gradient: B in A (v/v) from 0%–20% in 20 min, from 20%–55% in 5 min, at 55% for 10 min, from 55%–100% in 10 min, at 100% for 5 min and at 0% for 5 min, where A was acetonitrile/water/ acetic acid (15:85:0.1; v/v/v) and B was acetonitrile/water/ acetic acid (50:50:0.1; v/v/v). Analytes were monitored by diode array detection at 260 nm (L-7455; Hitachi, Tokyo, Japan) for isoflavones and the fluorimetric detection at 280 nm excitation and 310 nm emission (L-7480; Hitachi) for equol simultaneously.

RESULTS

Metabolism of P. thomsonii *flower isoflavones and daidzein by mammalian small intestinal enzymes*

Three out of 5 glycosidic isoflavones found in the *P. thomsonii* flower, tectoridin, genistin and glycitin, were completely metabolized by incubation with germfree mouse small intestine for 3 days anaerobically under both pH 5.0 and 7.4 (Table 1). More than 95% of TGXG was also metabolized when the incubation was run under pH 5.0, while only 40% was metabolized under pH 7.4. These metabolized isoflavone glycosides were detected as their expected aglycones, tectorigenin from TGXG and tectoridin, genistein from genistin and glycitein from glycitin, although only about 65% of glycitin metabolized was detected as glycitein. Peaks that were not found before incubation were not detected under the present analytical conditions. On the other hand, 6HGDG was not hydrolyzed by a homogenate of germfree mouse small intestine regardless of pH.

When the aglycone-form isoflavones found in the *P. thomsonii* flower were incubated with germfree mouse small intestinal homogenate, the isoflavones were detected intact, except only about half of the glycitein was detected (Table 1).

Table 1. Metabolism of isoflavones found in the *Pueraria thomsonii* flower and daidzein by homogenates of small intestine of germfree mice at pH 7.4 and 5.0.

	pH 7.4		pH 5.0	
Substrate	Glycoside	Aglycone	Glycoside	Aglycone
6HGDG	106.2[a]	0.0	99.4	0.0
TGXG	60.7	43.7	4.9	97.1
Tectoridin	0.0	112.4	0.0	114.5
Genistin	0.0	105.7	0.0	103.0
Glycitin	0.0	62.1	0.0	65.5
Tectorigenin		107.1		104.5
Genistein		96.3		98.3
Glycitein		40.0		50.1
Daidzein		102.4		95.9

6HGDG, 6-hydroxygenistein 6,7-di-O-glucoside; TGXG, tectorigenin 7-O-xylosylglucoside

a: Values are percentages of compounds detected after incubation against compounds added.

Metabolism of P. thomsonii *flower isoflavones and daidzein by human fecal suspension*

6HGDG, which was not hydrolyzed by murine small intestinal enzymes, was completely metabolized by human fecal bacteria, and no glycoside-form isoflavones were detected after incubation for 3 days (Table 2). Four out of 10 fecal samples as well as the mixture of 10 fecal samples produced no detectable metabolite or isoflavone. Six out of 10 samples tested metabolized 9 to 56% of 6HGDG to its aglycone, 6HG, but the rest of the 6HGDG added to the suspensions was transformed to an undetectable compound.

Tectorigenin was metabolized completely by 8 of 10 fecal samples, and very small amount of tectorigenin was detected in 1 sample after incubation for 3days, while only about half of the tectorigenin was metabolized in 1 fecal sample (Table 2). In 5 fecal samples, 13 to 39% of the metabolized tectorigenin was detected as its metabolite, 6HG, and the rest of the tectorigenin was metabolized to an undetectable metabolite(s). No isoflavones or metabolites including equol except for 6HG were detected. Mixed human feces also metabolized tectorigenin completely, and no metabolite was detected.

Genistein was metabolized in 6 out of 10 fecal samples, and 94% of genistein was metabolized in 1 sample after

Table 2. Metabolism of 6HGDG, tectorigenin and genistein by human fecal suspensions.

Substrate	6HGDG		Tectorigenin		Genistein	
After incubation	6HGDG	6HG	Tectorigenin	6HG	Genistein	6HG
Sample no.						
1	0.0[a]	55.8	53.5	12.9	70.9	0.0
2	0.0	40.8	2.6	33.0	91.2	0.0
3	0.0	26.6	0.0	30.3	99.0	0.0
4	0.0	28.4	0.0	38.5	5.9	0.0
5	0.0	18.4	0.0	22.8	0.0	0.0
6	0.0	8.7	0.0	0.0	0.0	0.0
7	0.0	0.0	0.0	0.0	0.0	0.0
8	0.0	0.0	0.0	0.0	0.0	0.0
9	0.0	0.0	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.0	0.0	0.0
Mix	0.0	0.0	0.0	0.0	0.0	0.0

6HGDG, 6-hydroxygenistein 6,7-di-O-glucoside; 6HG, 6-hydroxygenistein; Mix, mixture of 10 samples

a: Values are percentages of compounds detected after incubation against compounds added.

Table 3. Metabolism of glycitein and daidzein by human fecal suspensions.

Substrate	Glycitein		Daidzein	
After incubation	Glycitein	Equol	Daidzein	Equol
Sample no.				
1	24.6[a]	0.0	84.5	2.4
2	17.5	0.0	90.8	0.0
3	2.7	0.0	60.3	0.0
4	10.7	0.0	96.4	0.0
5	7.1	9.9	8.0	58.8
6	0.0	9.3	0.0	60.3
7	12.2	0.0	62.6	0.0
8	3.2	1.9	13.9	15.1
9	0.0	0.0	81.4	0.0
10	5.6	0.3	8.4	32.7
Mix	8.0	0.2	30.1	19.6

6HGDG, 6-hydroxygenistein 6,7-di-O-glucoside; 6HG, 6-hydroxygenistein; Mix; mixture of 10 samples
a: Values are % of compounds detected after incubation against compounds added.

incubation for 3 days (Table 2). However, 70% of genistein was detected as intact genistein in 1 sample and more than 90% was found intact in 2 samples. Mixed samples metabolized genistein completely. No metabolites, including 6HG and equol, were detected from any incubates.

At least 75% of glycitein was metabolized by all fecal samples including the mixed sample (Table 3). Although most of the glycitein metabolized was converted to an unknown metabolite(s) that was not detected after incubation, a small amount of equol was detected in 4 of 10 fecal samples and the mixed sample. Half of the fecal samples produced equol from daidzein. The amount of equol detected varied and amounted to 2 to 60% of the daidzein (Table 3). The 4 samples that produced a small amount of equol from glycitein produced high amounts of equol from daidzein.

DISCUSSION

The root and flower of Kudzu contain high concentrations of isoflavones, and although they are not consumed as daily foods, they have been traditionally used in Chinese medicine *(1–5)*. Isoflavones often occur in nature as glycosides. The loss of sugar to release their aglycones is the first step for their metabolism in the intestine, and the bioavailability of isoflavones requires initial hydrolysis of the sugar moiety. Studies with soy isoflavones have revealed that glycosidic isoflavones have not been identified in plasma, and *in vitro* and *in vivo* studies have clearly demonstrated that isoflavone glycosides are not absorbed across the enterocyte of healthy adults. The absorption of intact isoflavone glycosides is unlikely to be of significance in the isolated small intestine *(10, 15, 16)*. Although it has been suggested that intestinal microbial enzymes are responsible for the hydrolysis *(8)*, some researchers refute the exclusive involvement of the gut microbiota. It has been reported that germfree animals also excrete a large amount of isoflavone aglycones after consumption of glycoside isoflavones in soya protein *(17)*. In addition, the main activity of bacterial activity is in the distal ileum and colon, and isoflavone absorption occurs extremely rapidly after ingestion, within minutes, suggesting that the absorption occurs in the upper intestine *(10)*. Thus, it appears that the major site of hydrolysis of isoflavone glycosides is likely the small intestine *(10)*. A number of membrane-bound β-glucosidases have been identified in the small intestine.

Therefore, we incubated a homogenate of small intestine from germfree mice, who have no intestinal bacteria, with isoflavones found in the *Pueraria thomsonii* flower. The results demonstrated that 4 out of 5 glycosidic isoflavones tested, TGXG, tectoridin, genistin and glycitin, can be highly hydrolyzed by mouse small intestinal homogenate, without bacterial activity, while 6HGDG was not hydrolyzed by mammalian enzymes. The whole amounts of hydrolyzed TGXG, tectoridin and genistin were detected as their expected aglycones, tectorigenin from TGXG and tectoridin and genistein from genistin, indicating that these isoflavones are not further metabolized without bacterial activity. On the other hand, only about 65% of glycitin was detected as its metabolite, glycitein. This result suggests that mammalian enzymes can, at least partly, metabolize glycitein. These results were confirmed when aglycone-form isoflavones were incubated with germfree mouse small intestinal homogenate. More than 95% of the tectorigenin and genistein, as well as the soy isoflavone daidzein, were detected intact after incubation, and about a half of the glycitein added was resolved.

Isoflavone aglycones can undergo further metabolism by intestinal microbiota and produce various metabolites prior to absorption or via enterohepatic circulation. Only a small portion of whole dietary phytoestrogens are excreted in urine, and the amounts of intact free aglycones in feces are low *(8)*. Thus, the majority of isoflavones ingested must be metabolized beyond deglycosylation. It is clearly demonstrated *in vivo* and *in vitro* that the intestinal microbiota, in contrast to the case of hydrolysis, has a crucial role in the metabolism of isoflavone aglycones. Administration of antibiotics reduced the plasma concentration of isoflavone metabolites *(18)*, and the metabolites were completely

absent in the urine of germfree rats fed soy *(19)*.

In the present study, isoflavones found in the *P. thomsonii* flower were incubated with fecal bacteria from 10 healthy volunteers. TGXG, tectoridin, genistin and glycitin were omitted from this experiment because our results demonstrated that these isoflavone glycosides would be rapidly deglycosilated to their aglycones and suggested that they would not reach the lower intestine, the main site of bacterial activity. Human fecal suspensions were highly capable of metabolizing *P. thomsonii* flower isoflavones. 6HGDG, which was not hydrolyzed by mouse small intestinal homogenate, was also deglycosylated completely and further metabolized.

Isoflavones have been linked with reduction of various disease risks *(7, 8, 10)*, and many of the effects are based on their estrogenic activity. The metabolites of the isoflavones often have different bioactivities from their parents, and so, the metabolism of the intestinal microbiota may be important in relation to the biological efficacy of isoflavones. For example, daidzein is mainly metabolized to equol, which is more estrogenic than its parent compound, or the less bioactive *O*-desmethylangolensin *(8, 10)*. It is suggested that the ability to produce equol is related to the biological efficacy of consumption of soy foods *(10, 12, 20)*. On the other hand, there is also some concern for detrimental human health effects of isoflavones due mainly to their estrogenic activities. In 2006, the Food Safety Commission of Japan announced recommendations concerning consumption of soy isoflavones, and the upper limit of safe daily consumption of soy isoflavones was set to 75 mg/day (as aglycones) in total and 30 mg/day as supplements.

In the present study, isoflavones found in the *P. thomsonii* flower were resolved with few exceptions, but, in most cases, their metabolites were not detected under the present analytical conditions. It is important to elucidate the fate of isoflavones after metabolism by intestinal microbiota to understand the kinetics of ingested *Pueraria* isoflavones. However, it is also very difficult to identify unknown metabolites. For example, Chen *et al. (21)* analyzed *in vivo* and *in vitro* metabolites of tectoridin using liquid chromatography-electrospray ionization tandem ion-trap mass spectrometry. Although they reported 6 metabolites from urine and feces of rats orally inoculated with tectoridin, two of them were glucuronide- and sulfate-conjugated tectorigenin and three were suggested to be host metabolites because they were also found in liver microsomal incubation mixtures. Only tectorigenin, the aglycone of tectoridin, was detected in the intestinal microbiota incubation mixture *(21)*. Interestingly, a small amount of equol was produced from glycitein in 4 samples and the mixed sample. To our knowledge, there is no publication reporting equol as a metabolite of glycitein, though 6-OH-equol has been reported *(22)*. Since Simons *et al*. *(23)* proposed direct demethoxylation of glycitein to daidzein as one of its minor pathways, equol might be produced from glycitein through production of daidzein. The production of equol was observed only in samples with high activity to produce equol from daidzein. Having said that, the amounts of glycitein and its glucoside, glycitin, in the *P. thomsonii* flower are quite limited *(11)*, and therefore, the amount of equol produced from the *P. thomsonii* flower by intestinal microbiota should be very low.

In conclusion, the results of the present study indicate that the isoflavones of the *P. thomsonii* flower, 6HGDG, TGXG, tectoridin and tectorigenin, which are particularly abundant in the *P. thomsonii* flower and not present in soy, as well as the isoflavones also found in soy, are actively metabolized in the human intestine. Although the formation of equol, a strong estrogenic metabolite, from *P. thomsonii* flower isoflavones is suggested to be limited, the metabolites were not detected in most cases, and the change in estrogenic activity after metabolism by intestinal microbiota was not investigated in the present study. The estrogenic activity of *P. thomsonii* flower isoflavones before and after metabolism by human intestinal microbiota, as well as the effects of the metabolism on the host, should be further investigated.

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