JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isoflavone Conjugates Are Underestimated in Tissues Using Enzymatic Hydrolysis

LIWEI GU,*,†,‡ MYRIAM LALY,† HEBRON C. CHANG,† RONALD L. PRIOR,† NIANBAI FANG,†,§ MARTIN J. J. RONIS,†,# AND THOMAS M. BADGER†,‡

Arkansas Children's Nutrition Center and Departments of Physiology/Biophysics, Pharmaceutical Sciences, and Pharmacology/Toxicology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72202

Many health effects of soy foods are attributed to isoflavones. Isoflavones upon absorption present as free form, glucuronide, and sulfate conjugates in blood, urine, and bile. Little is known about the molecular forms and the relative concentrations of soy isoflavones in target organs. Acid hydrolysis or enzymatic hydrolysis (glucuronidases and sulfatases) was used to study isoflavone contents in the heart, brain, epididymis, fat, lung, testis, liver, pituitary gland, prostate gland, mammary glands, uterus, and kidney from rats fed diets made with soy protein isolate. The heart had the lowest isoflavone contents (undetectable), and the kidney had the highest (1.8 ± 0.6 nmol/g total genistein; 3.0 ± 1.1 nmol/g total daidzein). Acid hydrolysis released 20−60% more aglycon in tissues than enzymatic digestion ($p < 0.05$), and both hydrolysis methods gave the same level of isoflavones in serum. Approximately 28−44% of the total isoflavone content within the liver was unconjugated aglycon, and the remainder was conjugated mainly as glucuronide. The subcellular distribution of total isoflavones was 55−60% cytosolic and 13−16% in each of the nuclear, mitochondrial, and microsomal fractions. These results demonstrated that (1) soy isoflavones distribute in a wide variety of tissues as aglycon and conjugates and (2) the concentrations of isoflavone aglycons, which are thought to be the bioactive molecules, are in the 0.2−0.25 nmol/g range, far below the concentrations required for most in vitro effects of genistein or daidzein.

KEYWORDS: Isoflavones; daidzein; genistein; tissues

INTRODUCTION

Reports of the potential health benefits of dietary soy include lowering plasma cholesterol concentrations, prevention of osteoporosis, and reduction of breast cancer incidence (*1*). Associated with soy protein are several phytochemicals, including isoflavones, which have been reported to modulate a wide variety of molecular, cellular, and physiological events (*2*, *3*). Genistein and daidzein are the most widely studied soy isoflavones in human diets, and soy foods are rich sources of these isoflavones (*4*).

Soy isoflavones circulate in several molecular forms, including glucuronide and sulfate conjugates, freely circulating aglycons, and protein-bound aglycons. A majority of absorbed isoflavones are excreted as conjugates into the urine, but a smaller percentage undergoes enterohepatic recycling (*5*). The conjugates of genistein and daidzein include monoglucuronides, monosulfates, diglucuronides, disulfates, and mixed conjugates with one site glucuronidated and one sulfated (*2*).

The lack of commercially available standards for conjugates has been a hindrance in their qualitative and quantitative measurement, and this has led to utilization of enzymatic hydrolysis of the isoflavone conjugates with subsequent detection and quantitation of the liberated aglycon (*6*, *7*). Currently, there are three commercially available enzyme preparations used by most investigators to study conjugates of genistein and daidzein: a mixture of sulfatase/glucuronidase prepared from *Helix pomatia*; an enriched β-glucuronidase; or an enriched sulfatase. Typically, the conjugated isoflavones in urine or serum are hydrolyzed with these enzymes; the aglycons liberated are separated and quantitated by HPLC or LC-MS; and the isoflavone concentrations are expressed as "aglycon equivalents". Commercially available extracts from *H. pomatia* contain glucuronidase and sulfatase activities toward the conjugates of genistein and daidzein in sufficient ratios to determine the "total aglycon" levels in serum and urine. The β-glucuronidase and sulfatase preparations have been used to determine the glucuronide and sulfate concentrations, respectively (*7*, *8*).

To assess the potential risks and benefits of dietary soy isoflavones and the mechanisms by which health effects occur,

* Author to whom correspondence should be addressed [telephone (501) 364-5985; fax (501) 364-3161; e-mail GuLiwei@uams.edu].
† Arkansas Children's Nutrition Center.
‡ Department of Physiology/Biophysics.
§ Department of Pharmaceutical Sciences.
# Department of Pharmacology/Toxicology.

10.1021/jf050802j CCC: $30.25
Published on Web 07/28/2005

Table 1. Diet Composition of Total Enteral Nutrition[a]

component	source	% of total calories
protein	soy protein isolate	14.2
carbohydrate	maltodextrin and dextrose	59.8
fat	corn oil	26.0

[a] Mineral and vitamin contents had been reported previously (*33*).

it is essential to have a more complete understanding of target tissue concentrations of the isoflavones and their metabolites. However, when we employed the above-described enzymes to assess tissue isoflavone concentrations, results indicated that these enzymes were not effectively hydrolyzing all of the conjugates in the tissues. These results were similar to data previously reported from our laboratory in which we speculated that mixed conjugates in plasma posed problems that resulted in less than all of the conjugated aglycons being enzymatically hydrolyzed (*7*). In the current study, we demonstrated that (1) enzyme hydrolysis may underestimate the isoflavone concentrations in several tissues and (2) acid hydrolysis releases more aglycons from conjugated isoflavones, possibly making this method a better approach in estimating the total isoflavone content of tissues.

MATERIALS AND METHODS

Materials. Daidzein (7,4′-dihydroxyisoflavone, ≥95%), genistein (5,7,4′-trihydroxyisoflavone, ≥95%), and biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) were purchased from Indofine, Inc. (Somerville, NJ). Equol (7,4′-isoflavandiol) was purchased from Plantech (Reading, U.K.). β-Glucuronidase (type B-3 from bovine liver, 4000 units/mg), sulfatase (type VIII, from *Abalone entrails*, reported sulfatase activity of 20−40 units/mg), and sulfatase type H-5 (aryl-sulfate sulfohydrolase from *H. pomatia*, reported sulfatase activity of 15−40 units/mg and glucuronidase activity of 400−600 units/mg, referred to as sulfatase/glucuronidase mixture in this paper) were purchased from Sigma (St. Louis, MO). Soy protein isolate was a gift from the Solae Co. (St. Louis, MO). Others solvents or chemicals of analytical grade were purchased from Fisher Scientific (Pittsburgh, PA).

Animals. Adult Sprague−Dawley female rats (220 g; $n = 6$) were fed an AIN-93G diet (*9*) formulated with soy protein isolate as the sole source of protein. Diet contained 0.27 mg/g of genistein and 0.21 mg/g of daidzein as aglycons. The daily intake of genistein and daidzein was estimated at 16.8 and 12.8 mg/kg of body weight, respectively. After 4 days of feeding, the rats were euthanized with 100 mg/kg Nembutal. Tissues (liver, uterus, mammary glands, and brain) were collected and stored at −70 °C. Blood was collected for serum, and 24-h urine samples were collected in the presence of ascorbic acid and sodium azide (0.1% w/w of each in urine) prior to the sacrifice day and stored at −70 °C. Tissues were pooled and pulverized under liquid nitrogen prior to analyses for isoflavones.

Male Sprague−Dawley rats (300 g; $n = 4$) were fed soy protein by total enteral nutrition. An intragastric cannula was surgically inserted into the stomach. The rats were allowed to recover for 14 days. Water was infused during the recovery period (*10*). Liquid diet containing soy protein isolate (**Table 1**) was infused into the stomach at 187 kcal/kg•75/day to meet all nutrient requirements for the rat established by the National Research Council (*11*). The daily intakes of genistein and daidzein were 17.3 and 13.6 mg/kg of body weight, respectively. After 21 days of feeding, rats were euthanized, and tissues were collected and kept frozen at −70 °C. The protocols were approved by the Animal Care and Use Committee of the University of Arkansas for Medical Sciences.

Subcellular Fractionation. Nuclear fractions, mitochondria, cytosol, and microsomes were prepared from rat liver using differential ultracentrifugation (*12*). Briefly, 2.5 g of pooled female rat liver was mixed with 15 mL of buffer (10 mM K_2HPO_4, 1.15% KCl, and 10 mM EDTA, pH 7.4) and homogenized on ice. The homogenate was subsequently centrifuged at 1000*g* for 5 min to obtain the nuclear fraction. The supernatant was centrifuged again at 10000*g* for 30 min to isolate the mitochondria. The remaining supernatant was centrifuged at 100000*g* for 1 h at 4 °C to collect the cytosol and microsomes. The pellets were reconstituted in 1 mL of ammonium acetate buffer (1 mol/L, pH 6.0), and 100 μL was used for enzymatic or acid hydrolysis.

Enzymatic Hydrolysis. Pooled female rat liver (250 mg) was homogenized in 1 mL of ice cold 1 mol/L ammonium acetate buffer (pH 6.0). Homogenates (100 μL; 20-mg equivalent of tissues) were brought to a final volume of 2 mL with buffer. One milliliter of enzyme solution in the same ammonium acetate buffer was mixed with the homogenate, and the mixture was incubated for 3 h at 37 °C. Enzyme used included sulfatase/glucuronidase (type H-5, 200 units of sulfatase), sulfatase (200 units), and glucuronidase (2000 units). For sequential digestion, the homogenates were incubated with glucuronidase at 37 °C for 3 h, and then sulfatase was added into the mixture and incubated for another 3 h. The sulfatase/glucuronidase was used for other samples (tissues, liver subcellular fractions, urine, and serum) for enzymatic hydrolysis.

Acid Hydrolysis. Homogenates of tissue (100 μL, 20-mg equivalent) in 2 mL of 1 mol/L ammonium acetate buffer (pH 6.0) were mixed with 1 mL of hydrochloric acid (6 mol/L). The tubes were capped and heated in boiling water at 100 °C for 30 min.

Isoflavone Extraction. Homogenates after enzymatic hydrolysis were acidified with 100 μL of glacial acetic acid and defatted with 5 mL of hexane twice. The hexane fraction was discarded, and the aqueous phase was dried under nitrogen flow for 15 min. Ethyl acetate (5 mL) was added to extract aglycons. This was repeated three times, and the ethyl acetate fractions were combined and dried under nitrogen at 50 °C. The dried extracts were reconstituted in 0.5 mL of 50% methanol/water containing a known amount of biochanin A as an internal standard. Tissue homogenate (20-mg equivalent) in 3 mL of ammonia acetate buffer was extracted in the same way to determine the unconjugated isoflavones. Addition of 100 μL of glacial acetic acid was omitted for acid-hydrolyzed tissues. Extraction of isoflavones from urine and serum samples has been described previously (*8*).

Isoflavone Analyses. Analyses were performed on liquid chromatography−mass spectrometry (LC-MS) (*7*). The extracts from samples containing internal standard (100 μL) were injected into a Discovery RP amide C16 high-performance LC column (25 cm × 4.6 mm, 5-μm particle size, Supelco, Bellefonte PA), and isoflavones were eluted with a mobile phase of solvent A (25% methanol containing 10 mM ammonium acetate and 71 mM triethylamine, pH 4.5) and solvent B (95% methanol containing 10 mM ammonium acetate and 71 mM triethylamine, pH 5.5) at a flow rate of 1.0 mL/min. Isoflavones were separated using a linear gradient from 45% solvent B to 65% solvent B over an 11-min period. The proportion of solvent B increased linearly from 65 to 95% over 5 min and was held for another 5 min before the return to initial conditions. The column was equilibrated for 6.5 min prior to any subsequent sample injections. Isoflavones were detected using a PE Sciex API 100 mass spectrometer by negative single-ion monitoring using a heated nebulizer atmospheric pressure−chemical ionization interface. Isoflavone concentrations in tissues were expressed as nanomoles per gram after normalization with biochanin A (*6*). The detection limit was 0.12 pmol of genistein or daidzein injected on column for this method.

Statistical Analyses. Data were analyzed by one-way ANOVA or Student's t test. A difference with $p < 0.05$ was considered to be significant.

RESULTS

Three enzyme preparations were used (alone or in combination) to evaluate the nature of conjugates in rat liver, and acid hydrolysis was employed to attain complete hydrolysis of isoflavone conjugates (**Figure 1**). The mean contents of unconjgated genistein and daidzein were ∼0.22 nmol/g, contributing to 28% of total genistein or 44% of total daidzein, respectively, on the basis of acid hydrolysis. It should be noted that total mean isoflavone values (bars) in **Figure 1** contain

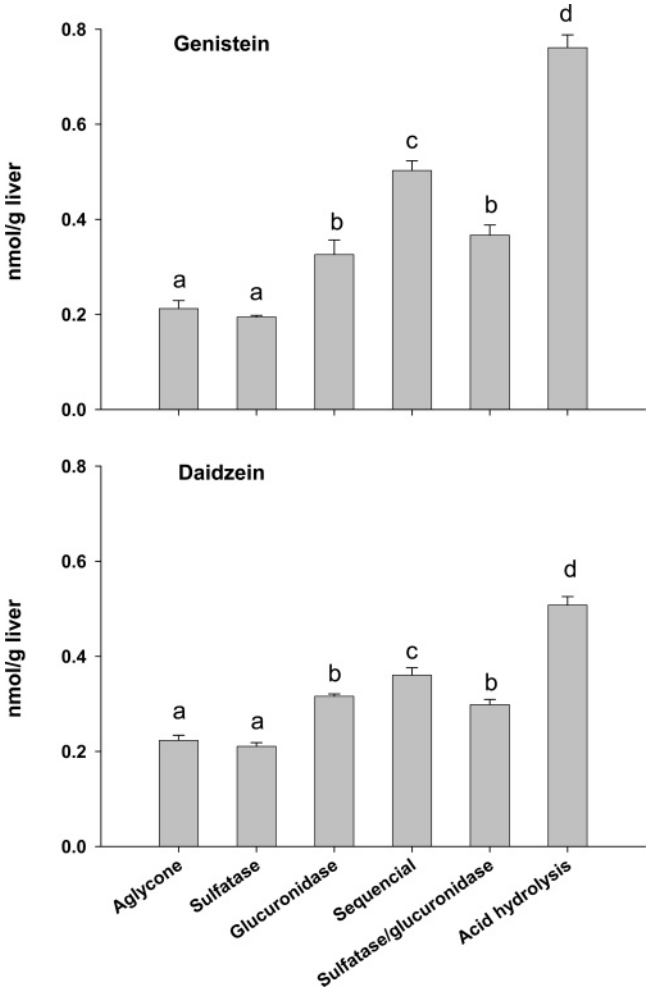

Figure 1. Isoflavone concentrations in pooled female rat liver after hydrolysis using various enzymes or hydrochloric acid. Aglycons were measured without hydrolysis. Data are mean ± SD of triplicate tests. Different letters on the bars indicate significant difference between treatments ($p < 0.05$).

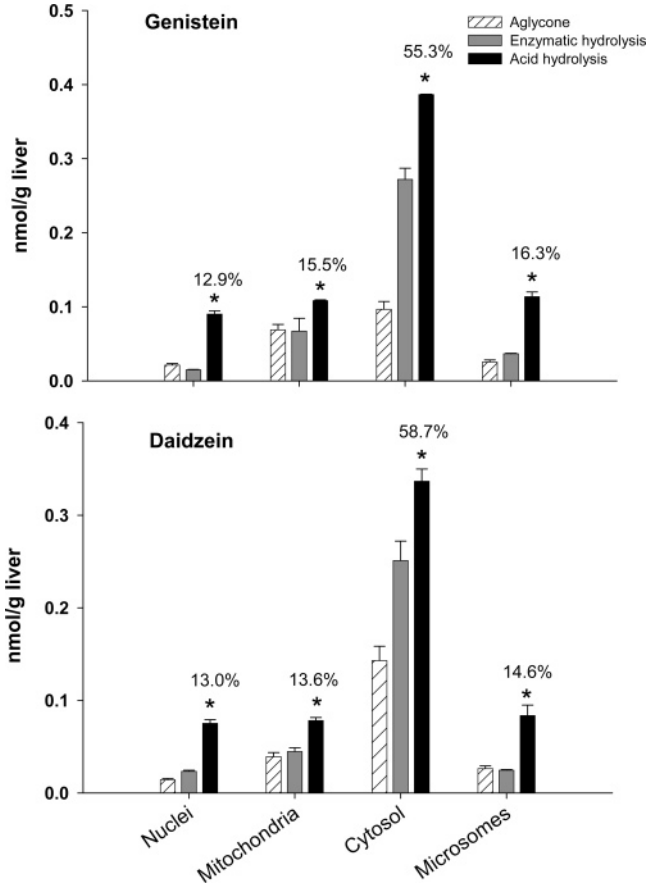

Figure 2. Distribution of isoflavone in the subcellular fractions of pooled female rat liver. Data are mean ± SD of duplicate tests. Asterisks indicate a significant difference between acid and enzymatic hydrolysis ($p < 0.05$). Proportion of isoflavones in the subcellular fractions on the basis of acid hydrolysis is labeled.

both the aglycons present prior to hydrolysis plus the aglycons released upon hydrolysis. The daidzein and genistein levels following sulfatase digestion were no greater than free aglycon levels (unconjugated levels), suggesting an absence of mono- or disulfate conjugates in liver. Glucuronidase released daidzein and genistein equal to ~42−54% of the aglycon level. The sulfatase/glucuronidase mixture did not yield greater levels of total aglycons than the glucuronidase alone, consistent with an absence of sulfates suggested by the sulfatase data. Sequential enzymatic hydrolysis released approximately 54% more genistein and 14% more daidzein than glucuronidase alone. Data in **Figure 1** also demonstrate that approximately 50% more genistein and 40% more daidzein were released by acid hydrolysis than the sequential enzymatic hydrolysis.

Figure 2 shows the subcellular distribution of isoflavones in nuclei, mitochondria, cytosol, and microsomes in rat liver. Greater isoflavone levels were found using acid hydrolysis than enzyme hydrolysis. In nuclei and microsomes, acid hydrolysis produced 3−5-fold higher isoflavone concentrations than were produced by enzymatic hydrolysis. Acid hydrolysis also produced more genistein and daidzein in mitochondria and cytosol ($p < 0.05$), whereas the differences were not as pronounced as in microsomes and nuclei. On the basis of acid hydrolysis, 55.3% and 12.9% of the total genistein levels were present in the cytosol and nuclei, respectively. Microsomes contained 16.3% of the total genistein. Daidzein had a subcellular distribution similar to that of genistein. Furthermore, the unconjugated genistein and daidzein contributed ~20−60% of total content of these isoflavones in each subcellular fraction.

The levels of genistein and daidzein in several tissues from female rats were compared using acid or enzymatic hydrolysis (**Table 2**). It was clear that more ($p < 0.05$) aglycons were recovered in kidney, uterus, liver, and mammary glands using acid hydrolysis than enzymatic hydrolysis. A difference of 3−10-fold was observed in the case of kidney. However, this did not hold true for genistein in the brain, where very low levels of isoflavones were detected. On the basis of acid hydrolysis, the isoflavone levels were the highest in kidney, followed by liver. Uterus and mammary glands are thought to be the target tissues of isoflavones. Uterus contained more isoflavones than mammary glands ($p < 0.05$). Enzymatic and acid hydrolyses of isoflavone conjugates produced the same level of daidzein in urine and serum and also the same level of genistein in serum. However, enzyme hydrolysis recovered more genistein in the urine than acid hydrolysis ($p < 0.05$).

Tissue distribution of isoflavones in male rats measured after acid hydrolysis is presented in **Table 3**. Tissue levels showed large variations among rats, although the isoflavone intakes were controlled to be the same on the basis of body weight. Highest levels of isoflavones were found in kidney. The concentrations of isoflavone in prostate and pituitary were also high. Testes and fat pad contained less genistein and daidzein than the liver. The isoflavone in the heart was below the detection limit.

Table 2. Isoflavone Contents in Pooled Female Rat Tissues, Serum, and Urine[a]

	nmol/g in wet tissues, nmol/mL in serum and urine			
	genistein		daidzein	
	acid hydrolysis	enzymatic hydrolysis	acid hydrolysis	enzymatic hydrolysis
brain	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01[b]	0.01 ± 0.01
mammary glands	0.11 ± 0.01[b]	0.03 ± 0.01	0.17 ± 0.01[b]	0.02 ± 0.01
uterus	0.20 ± 0.01[b]	0.04 ± 0.01	0.22 ± 0.04[b]	0.03 ± 0.01
liver	0.76 ± 0.03[b]	0.34 ± 0.02	0.51 ± 0.02[b]	0.30 ± 0.01
kidney	0.96 ± 0.09[b]	0.26 ± 0.03	1.02 ± 0.01[b]	0.10 ± 0.01
serum	0.24 ± 0.08	0.22 ± 0.04	0.17 ± 0.04	0.15 ± 0.03
urine	24.4 ± 2.5	31.9 ± 2.7[c]	40.2 ± 5.6	45.6 ± 6.2

[a] Data are mean ± SD of triplicate tests of pooled samples. [b] Values are significantly higher than those from enzymatic hydrolysis ($p < 0.05$). [c] Value is significantly higher than that from acid hydrolysis ($p < 0.05$).

Table 3. Isoflavone Contents in Male Rat Tissue Measured after Acid Hydrolysis[a]

	nmol/g of wet weight	
tissue	genistein	daidzein
heart	not detectable	not detectable
brain	0.03 ± 0.01	0.02 ± 0.01
epididymis	0.10 ± 0.05	0.33 ± 0.22
left fat pad	0.14 ± 0.04	0.15 ± 0.04
lung	0.16 ± 0.03	0.21 ± 0.07
testis	0.21 ± 0.06	0.12 ± 0.10
liver	0.26 ± 0.15	0.65 ± 0.32
pituitary gland	0.88 ± 0.34	0.92 ± 0.12
prostate gland	1.26 ± 0.67	1.69 ± 0.96
kidney	1.76 ± 0.65	3.05 ± 1.12

[a] Data are mean ± SEM of four rats.

The intra- and interassay coefficients of variation for total isoflavone quantitation in rat liver were 6.7 and 12.3%, respectively, using acid hydrolysis. Those variations were comparable to enzymatic methods (5.8 and 10.8%). The recovery rates of spiked daidzein and genistein standards in rat liver homogenate were 98 ± 2 and 94 ± 4% (mean ± SD, $n = 4$), respectively, after acid hydrolysis.

DISCUSSION

The UDP-glucuronosyltransferases in the endoplasmic reticulum and sulfotransferases in the cytosol of tissues actively convert the aglycons into glucuronides and sulfates, respectively. Because the known bioactivity of aglycon is greater than that of glucuronides and sulfates (*13*), conjugation would be expected to inactivate or reduce the bioactivity of soy isoflavones. We have been interested in determining if aglycons are the only bioactive soy isoflavones and what concentrations and molecular forms of isoflavones are found in target tissues of animals fed a diet containing a high level of soy protein isolate. The lack of isoflavone conjugates has hampered quantification of the isoflavone metabolites in serum, urine, bile, and tissues. The typical procedure employed is to measure the total aglycon equivalent of isoflavones, and this is accomplished by enzymatically hydrolyzing conjugates (deconjugation) and then using HPLC or LC-MS to assess the aglycons released (*6*−*8*). This can be used to measure the total genistein and daidzein equivalents. However, we have found that a complete hydrolysis is difficult to achieve in tissue homogenates by enzymes. One reason may be the limited access of enzymes to all of the conjugates within a cell. A portion of the isoflavones may exist in the organelles such as mitochondria, which remain partially undisrupted after homogenization (*12*). Furthermore, part of the isoflavones may be encapsulated in microsomes after homogenization, and those isoflavone conjugates could escape enzymatic hydrolysis. This would explain the observation that isoflavones in microsomes of liver after acid hydrolysis were higher than after enzymatic hydrolysis.

Isoflavone conjugates have been shown to bind with proteins, such as estrogen receptors α and β, but with lower affinity than aglycons (*13*, *14*), and protein-bound isoflavones may be resistant to enzymatic hydrolysis (*15*). This may explain in part the marked differences observed between the two methods of hydrolysis in the nuclei. The existence of other estrogen-binding proteins in the nuclei, cytosol, microsomes, and mitochondria could also reduce the level of isoflavones measured using enzyme hydrolysis (*16*).

Another reason is due to possible components in the tissues that inhibit β-glucuronidase or sulfatase activity, thus resulting in lower levels of aglycons than found with acid hydrolysis. One such component is D-glucaro-1,4-lactone, a potent inhibitor of β-glucuronidase. D-Glucaro-1,4-lactone equilibrates with D-glucaric acid in solution. D-Glucaric acid is the end metabolite of the D-glucuronic acid pathway and also a normal component of human blood (*17*). Although D-glucaro-1,4-lactone was not detectable in blood, a D-glucaro-1,4-lactone−glucuronidase complex had been detected in rat tissues (*17*). To our knowledge, no tissue component has been reported to inhibit sulfatases, although the existence of such inhibitors cannot be excluded. The higher isoflavone yield following acid hydrolysis of tissues may also be attributable to other acid liable conjugations between isoflavones and tissue components. Isoflavone 7-*O*-phosphates are suggested as such novel conjugates (*18*).

Recovery of the isoflavone aglycon from tissue after acid hydrolysis depends on hydrolysis of the conjugates and the stability of the aglycons. The acid hydrolysis conditions used here have been shown to have complete hydrolysis of isoflavone glycosides and high recovery of daidzein and genistein (*19*, *20*). We also found that acid hydrolysis of isoflavone conjugates in liver approached completion at 30 min. Daidzein and genistein standards were stable during acid hydrolysis, whereas equol dropped markedly over time (**Figure 3**). These observations agreed with previous reports that daidzein levels remained stable in acid hydrolysis conditions even with prolonged heating (*21*, *22*). Genistein had been shown to be less stable than daidzein in the acid (*21*, *23*). We did not observe the degradation of genistein standard due possibly to a shorter heating period. However, this may explain the higher total genistein in urine using enzymatic hydrolysis. Acid hydrolysis was too harsh and not applicable for the estimation of equol.

Estrogen-sensitive tissues have been considered to be the targets of isoflavones. Much attention has been paid to the mammary glands, uterus, pituitary gland, and prostate gland,

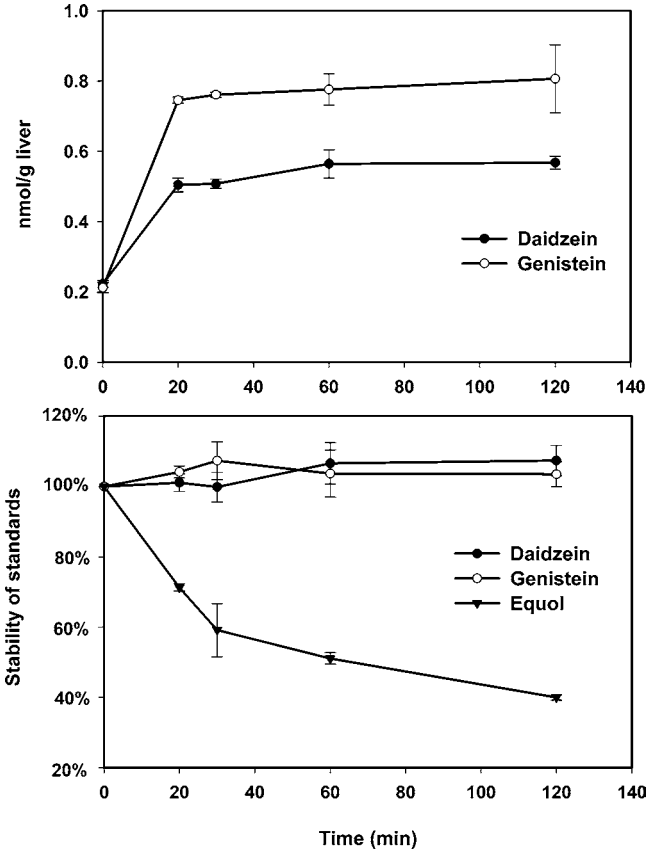

Figure 3. Time course of acid hydrolysis of pooled female rat liver (top) and stability of isoflavone standards during acid hydrolysis (bottom). Data are mean ± SD of duplicate tests.

where estrogen receptors are highly expressed and various physiological effects of isoflavones have been shown (*24*, *25*). Our results indicated substantial isoflavone levels in these target tissues. The presence of estrogen receptors does not seem to be an indicator of isoflavone content, because although the epididymis and the testis express levels of estrogen receptors similar to that of the pituitary gland (*26*), their isoflavone contents were severalfold lower than in the pituitary gland. We postulate that tissue isoflavones depend on the differential expression of uptake and efflux transporters, such as the organic anionic transporters and the *p*-glycoprotein, in organs (*27*). Tissue levels of isoflavones were found to be highest in the liver and kidney, where the isoflavones were metabolized and excreted and transporters were highly expressed (*28*). Lack of uptake transporters may explain the undetectable isoflavone content in heart.

In addition, isoflavones were found in all subcellular compartments, with the greatest concentrations in the cytosol (55−60%). Both aglycons and conjugates appear to be present in the nuclear, mitochondrial, and microsomal fractions, suggesting that isoflavones and their metabolites have access to virtually all cellular pathways.

It is important to point out that most in vitro studies of isoflavones have employed concentrations >10 μmol/L (*29*). However, the levels of total isoflavones found in serum and target tissues from the present study (0.02−3 nmol/g) were 3−5-fold lower than this value, and the free isoflavones in liver were far below that.

The tissue distribution of genistein measured using acid hydrolysis was in good agreement with that using ^{14}C-labeled genistein in Wistar rats (*30*). The genistein concentrations in female mammary glands, uterus, and liver in the present study were similar to those previously reported in Sprague−Dawley rats fed a diet fortified with genistein (0.1 mg/g of diet), but isoflavones in the brain were found to be markedly lower (*31*). We observed a much higher level of isoflavone in prostate than in testis, whereas much lower values for prostate had been reported previously using enzymatic hydrolysis (*31*, *32*). Although it is not clear as to why such discrepancies occurred, differences in the sources of isoflavones (soy protein versus pure aglycons added to the diet) and differences in the extent to which enzymatic hydrolysis underestimates the isoflavone levels may be partial explanations.

In summary, we demonstrated the existence of measurable levels of isoflavones in all organs and subcellular fractions studied, except for the heart. Isoflavone contents in tissue can be substantially underestimated using enzymatic hydrolysis in comparison to acid hydrolysis. However, the differences were not significant in serum. On the other hand, enzymatic hydrolysis remains preferable for urine. Equol, the major metabolite of daidzein in the rat, was found to be unstable in the acid hydrolysis conditions used. This study was the initial attempt in understanding genistein and daidzein contents in the target tissues. Further work is ongoing to more completely elucidate the levels of each molecular form of isoflavones and their microbial metabolites, including equol.

LITERATURE CITED

(1) Friedman, M.; Brandon, D. L. Nutritional and health benefits of soy proteins. *J. Agric. Food Chem.* **2001**, *49*, 1069−1086.
(2) Adlercreutz, H.; Mazur, W. Phyto-oestrogens and Western diseases. *Ann. Med.* **1997**, *29*, 95−120.
(3) Magee, P. J.; Rowland, I. R. Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. *Br. J. Nutr.* **2004**, *91*, 513−531.
(4) Setchell, K. D.; Cassidy, A. Dietary isoflavones: biological effects and relevance to human health. *J. Nutr.* **1999**, *129*, 758S−767S.
(5) Setchell, K. D. Absorption and metabolism of soy isoflavones—from food to dietary supplements and adults to infants. *J. Nutr.* **2000**, *130*, 654S−655S.
(6) Shelnutt, S. R.; Cimino, C. O.; Wiggins, P. A.; Badger, T. M. Urinary pharmacokinetics of the glucuronide and sulfate conjugates of genistein and daidzein. *Cancer Epidemiology, Biomarkers Prevention* **2000**, *9*, 413−419.
(7) Shelnutt, S. R.; Cimino, C. O.; Wiggins, P. A.; Ronis, M. J.; Badger, T. M. Pharmacokinetics of the glucuronide and sulfate conjugates of genistein and daidzein in men and women after consumption of a soy beverage. *Am. J. Clin. Nutr.* **2002**, *76*, 588−594.
(8) Cimino, C. O.; Shelnutt, S. R.; Ronis, M. J.; Badger, T. M. An LC-MS method to determine concentrations of isoflavones and their sulfate and glucuronide conjugates in urine. *Clin. Chim. Acta* **1999**, *287*, 69−82.
(9) Reeves, P. G.; Nielsen, F. H.; Fahey, G. C., Jr. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. *J. Nutr.* **1993**, *123*, 1939−1951.
(10) Ronis, M. J.; Lumpkin, C. K.; Ingelman-Sundberg, M.; Badger, T. M. Effects of short-term ethanol and nutrition on the hepatic microsomal monooxygenase system in a model utilizing total enteral nutrition in the rat. *Alcohol Clin. Exp. Res.* **1991**, *15*, 693−699.
(11) National Research Council. *Nutrient Requirements of the Laboratory Rat*, 4th ed.; National Academy Press: Washington, DC, 1995; pp 11−79.
(12) Hinton, R. H. *Subcellular Fractionation: A Practical Approch*; Oxford Universitiy Press: Oxford, U.K., 1997; pp 31−69.

(13) Zhang, Y.; Song, T. T.; Cunnick, J. E.; Murphy, P. A.; Hendrich, S. Daidzein and genistein glucuronides in vitro are weakly estrogenic and activate human natural killer cells at nutritionally relevant concentrations. *J. Nutr.* **1999**, *129*, 399−405.

(14) Kinjo, J.; Tsuchihashi, R.; Morito, K.; Hirose, T.; Aomori, T.; Nagao, T.; Okabe, H.; Nohara, T.; Masamune, Y. Interactions of phytoestrogens with estrogen receptors alpha and beta (III). Estrogenic activities of soy isoflavone aglycones and their metabolites isolated from human urine. *Biol. Pharm. Bull.* **2004**, *27*, 185−188.

(15) Press, M. F.; Xu, S. H.; Wang, J. D.; Greene, G. L. Subcellular distribution of estrogen receptor and progesterone receptor with and without specific ligand. *Am. J. Pathol.* **1989**, *135*, 857−864.

(16) Monje, P.; Boland, R. Subcellular distribution of native estrogen receptor alpha and beta isoforms in rabbit uterus and ovary. *J. Cell Biochem.* **2001**, *82*, 467−479.

(17) Walaszek, Z.; Szemraj, J.; Narog, M.; Adams, A. K.; Kilgore, J.; Sherman, U.; Hanausek, M. Metabolism, uptake, and excretion of a D-glucaric acid salt and its potential use in cancer prevention. *Cancer Detect. Prev.* **1997**, *21*, 178−190.

(18) Kanakubo, A.; Isobe, M. Differentiation of sulfate and phosphate by H/D exchange mass spectrometry: application to isoflavone. *J. Mass Spectrom.* **2004**, *39*, 1260−1267.

(19) Pettersson, H.; Kiessling, K. H. Liquid chromatographic determination of the plant estrogens coumestrol and isoflavones in animal feed. *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 503−506.

(20) King, R. A.; Broadbent, J. L.; Head, R. J. Absorption and excretion of the soy isoflavone genistein in rats. *J. Nutr.* **1996**, *126*, 176−182.

(21) Wang, G.; Kuan, S. S.; Francis, O. J.; Ware, G. M.; Carman, A. S. A simplified HPLC method for the determanation of phytoestrogens in soybean and its processed products. *J. Agric. Food Chem.* **1990**, *38*, 185−190.

(22) Mazur, W.; Fotsis, T.; Wahala, K.; Ojala, S.; Salakka, A.; Adlercreutz, H. Isotope dilution gas chromatographic-mass spectrometric method for the determination of isoflavonoids, coumestrol, and lignans in food samples. *Anal. Biochem.* **1996**, *233*, 169−180.

(23) Franke, A. A.; Custer, L. J.; Cerna, C. M.; Narala, K. Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 18−26.

(24) Adlercreutz, H.; Mazur, W.; Bartels, P.; Elomaa, V.; Watanabe, S.; Wahala, K.; Landstrom, M.; Lundin, E.; Bergh, A.; Damber, J. E.; Aman, P.; Widmark, A.; Johansson, A.; Zhang, J. X.; Hallmans, G. Phytoestrogens and prostate disease. *J. Nutr.* **2000**, *130*, 658S−659S.

(25) Setchell, K. D. Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. *Am. J. Clin. Nutr.* **1998**, *68*, 1333S−1346S.

(26) Kuiper, G. G.; Carlsson, B.; Grandien, K.; Enmark, E.; Haggblad, J.; Nilsson, S.; Gustafsson, J. A. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **1997**, *138*, 863−870.

(27) Chen, J.; Lin, H.; Hu, M. Absorption and metabolism of genistein and its five isoflavone analogs in the human intestinal Caco-2 model. *Cancer Chemother. Pharmacol.* **2005**, *55*, 159−169.

(28) Miyazaki, H.; Sekine, T.; Endou, H. The multispecific organic anion transporter family: properties and pharmacological significance. *Trends Pharmacol. Sci.* **2004**, *25*, 654−662.

(29) Barnes, S. Effect of genistein on in vitro and in vivo models of cancer. *J. Nutr.* **1995**, *125*, 777S−783S.

(30) Coldham, N. G.; Sauer, M. J. Pharmacokinetics of ^{14}C genistein in the rat: gender-related differences, potential mechanisms of biological action, and implications for human health. *Toxicol. Appl. Pharmacol.* **2000**, *164*, 206−215.

(31) Chang, H. C.; Churchwell, M. I.; Delclos, K. B.; Newbold, R. R.; Doerge, D. R. Mass spectrometric determination of genistein tissue distribution in diet-exposed Sprague−Dawley rats. *J. Nutr.* **2000**, *130*, 1963−1970.

(32) Dalu, A.; Haskell, J. F.; Coward, L.; Lamartiniere, C. A. Genistein, a component of soy, inhibits the expression of the EGF and ErbB2/Neu receptors in the rat dorsolateral prostate. *Prostate* **1998**, *37*, 36−43.

(33) Badger, T. M.; Ronis, M. J.; Lumpkin, C. K.; Valentine, C. R.; Shahare, M.; Irby, D.; Huang, J.; Mercado, C.; Thomas, P.; Ingelman-Sundberg, M. Effects of chronic ethanol on growth hormone secretion and hepatic cytochrome P450 isozymes of the rat. *J. Pharmacol. Exp. Ther.* **1993**, *264*, 438−447.

Received for review April 8, 2005. Revised manuscript received June 20, 2005. Accepted June 27, 2005. Funded by USDA-ARS 6251-51000-003-06S 002.

JF050802J