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METABOLISM OF DAIDZEIN AND GENISTEIN BY INTESTINAL BACTERIA

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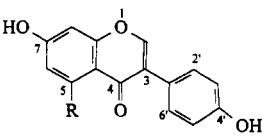
ABSTRACT.—The isoflavones daidzein [**1**] and genistein [**2**] were fermented with human fecal bacteria under anaerobic conditions. Dihydrodaidzein [**3**], benzopyran-4,7-diol, 3-(4-hydroxyphenyl) [**4**], and equol [**5**] were isolated from the fermentation broth of **1**. Only one metabolite, dihydrogenistein [**6**], was isolated and characterized from the fermentation broth of **2**. Metabolites **3–6** were identified by spectral methods.

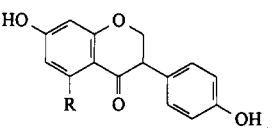
Endogenous and exogenous chemicals such as estrogens (1), androgens (2), and safflower yellow B (3), are metabolized by intestinal bacteria. The biological activities of these compounds can be altered dramatically through metabolism by intestinal bacteria, and these metabolites are often mutagenic (4,5). Soybean products are an integral part of the human diet in Asian countries. It has been postulated that soybeans, and especially their isoflavone components daidzein [**1**] and genistein [**2**], may provide protection against breast cancer and function as anticarcinogens (6). Also, **2** is reported as an inhibitor of tyrosine-specific protein kinases (7–9). The animal metabolism studies of **1** and **2** present in a soybean diet were carried out in sheep, rats, and human subjects. However, the metabolism of pure **1** and **2** in humans has not yet been reported.

Several isoflavone metabolites were detected in urine collected from human subjects on soy diets, namely, dihydrodaidzein, *O*-desmethylangolensin, glycitein, 6′-hydroxy-*O*-desmethylangolensin, equol, dihydrogenistein, and dehydro-*O*-desmethylangolensin (10–16), as glucuronide or sulfate conjugates (12,16). Equol [**5**] was the major metabolite identified in the urine of sheep, guinea pigs (17), and humans (15), and was also detected in the fermentation broth of soy protein incubated with human fecal bacteria (18). In another study, germ-free rats did not excrete **5** on a soy diet, whereas conventional rat urine contained **5** (11). This suggested that the intestinal bacteria metabolized **1** to **5**.

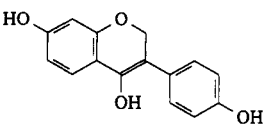
The flavonoids quercetin, kaempferol, and naringenin underwent C-ring cleavage at C-3 and C-4 upon incubation with *Clostridium* strains isolated from human intestinal bacteria (19). A similar fragmentation of isoflavonoids by intestinal bacteria is not known. *p*-Ethyl phenol was the major metabolite of **2** in the urine



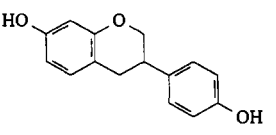
1 R=H
2 R=OH



3 R=H
6 R=OH



4 R=H



5 R=H

of ruminants (20) but not in human urine (15). Using gc-ms, Kelly *et al.* (15) confirmed the presence of dihydrodaidzein [**3**] and tetrahydrodaidzein in human urine. Compound **3** was detected in the urine of all human subjects studied, whereas tetrahydrodaidzein was found only in the urine of a single subject. Also, only one in twelve subjects excreted dihydrogenistein [**6**] in trace amounts. These data suggested that the metabolism of **1** and **2** varies in humans.

It is important to note that there are no published data on the intestinal metabolism of isoflavones, an important class of antioxidants present in many foods. Therefore, an in vitro metabolism study of **1** and **2** using human fecal bacteria was conducted.

Fermentation studies of endogenous and exogenous chemicals with fecal bacteria have provided valuable information to elucidate their metabolic pathways. The incubation of **1** with human feces afforded compounds **3–5**. After 72 h of incubation, the fermentation broth of **1** was purified by prep. tlc and hplc to afford **3**. ^{1}H-Nmr spectra of this compound showed the presence of two aromatic rings with an ABX substitution pattern in one ring and para-substitution in the other. The doublet of doublets signals which appeared at 4.57 and 4.53 ppm in **3** were assigned to the H-2 protons. A single proton (doublet of doublets) at 3.83 ppm confirmed the presence of H-3 in compound **3**. The absence of an olefinic proton in **3**, appearing at 8.02 ppm in **1**, indicated that the double bond between C-2 and C-3 in compound **1** was reduced. Therefore, the ^{1}H-nmr data of compound **3** indicated that it is dihydrodaidzein.

The ^{1}H-nmr spectra of compound **4** showed that the protons of rings A and B in **4** gave similar multiplicity and chemical shifts to the ring A and B protons in compound **1**. The H-2 singlet, which appeared at 8.02 ppm in **1** was absent in **4**. Therefore, the olefinic bond between C-2 and C-3 in **1** was reduced to yield **4**. A two-proton singlet at 4.10 ppm in compound **4** was assigned to the H-2 protons. This confirmed the presence of a double bond between C-3 and C-4 in **4** and, thus, the structure of this compound as benzopyran-4,7-diol, 3-(4-hydroxyphenyl). A third metabolite, isolated from the fermentation broth of **1** and purified by prep. recycle-hplc, was compound **5**. The ^{1}H-nmr spectral data of compound **5** was identical to the published values for equol (21).

The only metabolite isolated from

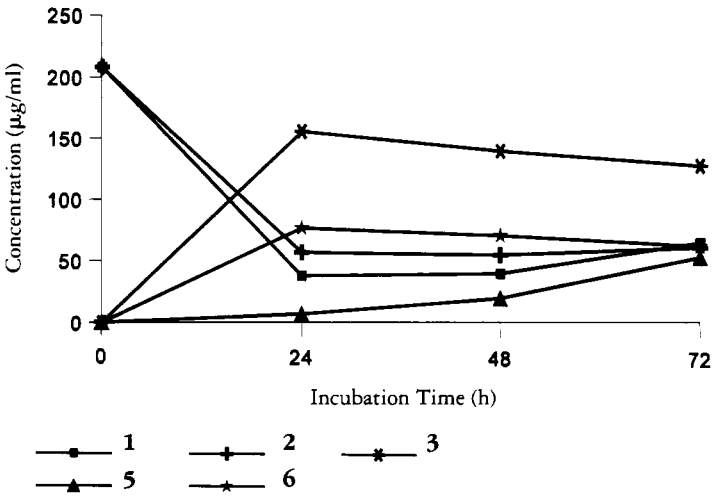


FIGURE 1. Rate of production of metabolites during the fermentation of **1** and **2** with human fecal bacteria for 72 h.

the fermentation broth of **2** was compound **6**. ^{1}H-nmr signals of **6** in the aromatic region were similar to those of **2**. The absence of a singlet at 8.30 ppm in **6** indicated that the olefinic bond between C-2 and C-3 in **2** was reduced. Three doublet of doublet signals appearing at 4.51, 4.44, and 3.83 ppm in **6** were assigned to the H-2a, H-2b, and H-3 protons, respectively.

The production of metabolites during the fermentation of isoflavones **1** and **2** with human fecal bacteria was monitored by hplc. Compound **3** was the major metabolite during the 72-h fermentation of compound **1** (Figure 1). Compound **5** was the major metabolite reported for **1** in urine from humans who consumed the soy diet. In our studies, the fecal bacterial metabolism of **1** afforded compound **3** in higher yield than **5**. This suggested that **3** was further metabolized to **5** prior to its excretion, a conclusion reached in previous in vitro studies (18). Because compound **4** was isolated in only a very small quantity, the quantification of this compound was not carried out.

The estrogenic activity of **2** in ruminants is considerably lower when given intraruminally compared with intramuscularly (10). This implies that the metabolism of **2** by rumen fluid may be responsible for the lack of estrogenic activity. *p*-Ethyl phenol, the reported endproduct of genistein [**2**] metabolism, was detected in the urine of soy-fed sheep. This compound was not detected in our fermentation studies. The hplc analysis of the fermentation products of **2** with fecal bacteria showed that the amount of **2** declined rapidly during 24 h. However, an equivalent increase in the amount of **6** was not observed (Figure 1). This suggests that **2** was metabolized to other compounds that were not detected in our study. We did not isolate any other metabolite from the fermentation broth of **2**.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H- and ^{13}C-nmr spectra were recorded on a Varian VXR 500 MHz spectrometer in CD_3OD solution at room temperature. Mass spectra were acquired on a JEOL HX-110 double focusing mass spectrometer (JEOL, Tokyo, Japan). Sep-Pak cartridges (C_{18}) were purchased from Waters (Milford, MA). Hplc analysis was performed with an hplc system equipped with an automatic gradient controller, an autosampler, and a photodiode-array detector (Waters, Milford, MA). Prep. recycle-hplc (LC-20) and C_{18} reversed-phase columns (Jaigel, S-343-15; 15 μm, 250×20 mm) were purchased from Japan Analytical Industrial Ltd. (Tokyo, Japan). C_{18} reversed-phase capcell pak columns (AG-120 S-5 μm, 30% carbon loading, 5 μm, 250×10 mm and AG-120 S-5 μm, 30% carbon loading, 5 μm, 250×4.6 mm) were bought from Shiseido Co., Ltd. (Tokyo, Japan).

CHEMICALS AND FERMENTATION MEDIA.—Daidzein [**1**] and genistein [**2**] were synthesized in our laboratory (22). Compounds **3**, **5**, and **6** were isolated from the fermentation of both **1** and **2** and used as standards for the quantification of these compounds in the fermentation media. BHI dehydrated media was purchased from Difco Lab (Detroit, MI) and vitamin K, heme, cystine hydrochloride, and resazurine were purchased from Aldrich Chemical Company (Milwaukee, WI).

IN VITRO ANAEROBIC FERMENTATION.—BHI medium (3.7 g/25 ml) was supplemented with vitamin K (20 μl/100 ml) and heme (0.5 mg/100 ml) and mixed with 5 mg of the isoflavones, daidzein [**1**] or genistein [**2**]. Cystine hydrochloride (50 mg/100ml) and resazurine (0.4 ml/100 ml medium from a 25 mg/100 ml stock solution) were used as reducing agent and O_2 indicator, respectively (23). The pH of the medium was adjusted to 7 with 1 N NaOH solution and autoclaved for 15 min under anaerobic conditions. Fresh human feces (1 g) were suspended in pre-reduced BHI medium (10 ml). Pre-reduced supplemented BHI medium (25 ml) was inoculated with the fecal suspension (0.5 ml) and incubated for 3 days at 37° under anaerobic conditions. The fermentation was carried out on pre-reduced supplemented medium with test compounds (5.2 mg/ml each of **1** and **2**, separately) and feces, medium with feces alone, and medium with test compounds alone under identical conditions. After 3 days of incubation, 1 ml of the fermentation broth was sampled and analyzed by hplc. The remaining fermentation broth was lyophilized for the isolation and purification of the respective metabolites.

The metabolisms of daidzein [**1**] and genestein [**2**] were investigated in separate fermentation runs. The percentage conversions of compounds **3**–**6** were calculated from the average yields of five consecutive fermentations.

PURIFICATION OF FERMENTATION PROD-

UCTS.—The fermentation broth of **1** (125 ml) was lyophilized and the resulting solid was extracted with hexane-$CHCl_3$ (1:1, 25 ml×2). The hexane/$CHCl_3$ extract was discarded. The residue was then extracted with MeOH (25 ml×2). The MeOH extract was evaporated to dryness *in vacuo* and the residue was purified via prep. recycle-hplc using a C_{18} reversed-phase column. The solvent system MeOH-H_2O (60:40) was used as the mobile phase under isocratic conditions at a flow rate of 3 ml/min. The metabolites were detected under uv at 210 nm. The fractions containing the isoflavone and its metabolites were further purified by hplc on a C_{18} reversed-phase capcell pak column using MeOH-H_2O (40:60) as the mobile phase at a flow rate of 1 ml/min. The compounds were monitored by a PDA detector at 210 nm.

The lyophilized fermentation broth of **2** (100 ml) was purified by vlc with $CHCl_3$-MeOH (4:1) as solvent system. Fractions containing isoflavones and their metabolites were combined and purified by prep. recycle-hplc on a C_{18} reversed-phase column using MeOH-H_2O (60:40) as the mobile phase at a flow rate of 3 ml/min. The metabolites were monitored under uv at 210 nm.

HPLC ANALYSIS OF ISOFLAVONES AND THEIR METABOLITES.—The fermentation broth (1 ml) was passed through a C_{18} Sep-Pak cartridge that was pre-conditioned with MeOH (5 ml) and H_2O (10 ml). The cartridge was then washed successively with H_2O (5 ml), MeCN-H_2O (30:70) (1 ml), and finally with MeCN-H_2O (90:10) (2 ml). The MeCN-H_2O (90:10) eluate was analyzed for isoflavones and their metabolites on a C_{18} reversed-phase capcell pak column. The mobile phase was MeCN and H_2O under a linear gradient of MeCN-H_2O (30:70) to 100% MeCN (final) in 15 min at a flow rate of 0.5 ml/min. The column was eluted with 100% MeCN for an additional 10 min. The compounds were monitored using a PDA detector and the data were collected at 200–360 nm and processed to obtain the results at 210 nm. The isoflavones have comparable absorption maxima at 210 and 262 nm. The hplc analysis of the isoflavones and their metabolites was carried out at 210 nm, because the uv spectra of the metabolites showed absorption maxima at 210 nm. The metabolites formed during the fermentation of **1** and **2** were monitored by withdrawing samples (1 ml) from the fermentation broth at 24, 48, and 72 h, respectively.

Calibration curves for compounds **1–3**, **5**, and **6** were created by analyzing the respective solution by hplc as mentioned above. The solutions were prepared by the serial dilution of the respective stock solutions to afford 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25.0 μg/ml concentrations, respectively. Calibration curves were generated by the Millenium 2010 chromatograph manager using the following equation: $y=A+Bx$, where y = response calculated for the standard peak at 210 nm; A = intercept of calibration curve; B = slope of calibration curve; x = the amount of standard, respectively.

Dihydrodaidzein [**3**].—20% conversion; ^{1}H- and ^{13}C-nmr spectral data were identical to the published results (15).

Benzopyran-4,7-diol, 3-(4-hydroxyphenyl) [**4**].—15.5% conversion; identification was made on the basis of comparison with literature reports (15).

Equol [**5**].—10.6% conversion; ^{1}H-nmr data were identical to those reported in the literature (21); ^{13}C nmr δ 155.70 (C-4′ or C-7), 155.42 (C-4′ or C-7), 154.39 (C-8a), 131.97 (C-1′), 129.25 (C-5), 127.41 (C-2′, C-6′), 114.52 (C-3′, C-5′), 112.69 (C-4a), 107.20 (C-6), 101.90 (C-8), 70.29 (C-2), 37.53 (C-3), 31.12 (C-4); eims *m/z* 242 (82), 120 (100).

Dihydrogenistein [**6**].—16% conversion; ^{1}H-nmr spectral data were identical to the reported values (15,16); ^{13}C nmr δ 196.50 (C-4), 166.71 (C-4′), 164.00 (C-5), 162.92 (C-8a), 156.16 (C-7), 128.84 (C-2′, C-6′), 125.88 (C-1′), 114.65 (C-3′, C-5′), 101.48 (C-4a), 95.33 (C-8), 94.10 (C-6), 70.77 (C-2), 49.74 (C-3); eims *m/z* 272 (25), 153 (100).

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