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Hor-Gil Hur · Jackson O. Lay Jr. · Richard D. Beger
James P. Freeman · Fatemeh Rafii

Isolation of human intestinal bacteria metabolizing the natural isoflavone glycosides daidzin and genistin

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Abstract Fecal bacteria from a healthy individual were screened for the specific bacteria involved in the metabolism of dietary isoflavonoids. Two strains of bacteria capable of producing primary and secondary metabolites from the natural isoflavone glycosides daidzin and genistin were detected. The metabolites were identified by comparison of their HPLC/mass, ^{1}H NMR and UV spectra with those of standard and synthetic compounds. Both *Escherichia coli* HGH21 and the gram-positive strain HGH6 converted daidzin and genistin to the their respective aglycones daidzein and genistein. Under anoxic conditions, strain HGH6 further metabolized the isoflavones daidzein and genistein to dihydrodaidzein and dihydrogenistein, respectively. The reduction of a double bond between C-2 and C-3 to a single bond was isoflavonoid-specific by strain HGH6, which did not reduce a similar bond in the flavonoids apigenin and chrysin. Strain HGH6 did not further metabolize dihydrodaidzein and dihydrogenistein. This is the first study in which specific colonic bacteria that are involved in the metabolism of daidzin and genistin have been detected.

Keywords Isoflavonoid · Phytoestrogen · Intestinal bacteria · Biotransformation · Reduction

H. G. Hur · F. Rafii (✉)
Division of Microbiology,
National Center for Toxicological Research,
U.S. FDA, Jefferson, AR, 72079-9502, USA
e-mail: frafii@nctr.fda.gov,
Tel.: +1-870-5437342, Fax: +1-870-5437307

J. O. Lay Jr. · R. D. Beger · J. P. Freeman
Division of Chemistry,
National Center for Toxicological Research,
U.S. FDA, Jefferson, AR, USA

Present address:
H. G. Hur
School of Agricultural Biotechnology, College of Agriculture and Life Science, Seoul National University,
103 Seodoon-Dong, Suwon 441-744, Korea

Introduction

Isoflavonoid phytoestrogens are found in various plants, specifically in the legume family, and induce estrogen-like effects in mammalian systems. The two major isoflavonoids found in soybeans are daidzin and genistin (the glycoside conjugates of daidzein and genistein, respectively), and they account for more than 0.1% of the dry weight of soybeans (Adlercreutz 1995). In plants, daidzin and genistin act as signal molecules to symbiotic nitrogen-fixing bacteria (Cooper et al. 1999; Phillips 1992). In mammalian systems, soy isoflavonoids exhibit a number of biological activities, including inhibition of cell proliferation (Coward et al. 1993; Fotsis et al. 1993), anti-oxidative effects (Arora et al. 1998), and enzyme-inhibitory effects (Keung and Vallee 1993). They also show anti-estrogenic activity by competing with endogenous estrogens for receptor binding (Shutt and Cox 1972; Verdeal and Ryan 1979; Martin et al. 1978). Dietary phytoestrogens have been considered for the treatment and prevention of hormone-dependent diseases (Messina et al. 1994).

Colonic bacteria play an important role in the metabolism of isoflavonoids and their conjugates following consumption (Kelly et al. 1993; Rafii 1999). This results in the formation of compounds with estrogenic activities higher or lower than those of natural isoflavonoids. In vitro studies of germ-free or antibiotic-treated experimental animals and in vivo incubation of isoflavonoid glycosides with fecal material have shown that the isoflavonoid glycosides undergo hydrolysis by glucosidases from fecal bacteria, leading to the production of isoflavonoid aglycones, which can be further metabolized by the anaerobic intestinal bacteria (Chang and Nair 1995; Joannou et al. 1995; Kelly et al. 1993, 1995; Shutt et al. 1967).

In vitro fermentation of daidzein and genistein with human fecal samples under anoxic conditions resulted in the production of dihydrodaidzein, benzopyran-4,7-diol, 3-(4-hydroxyphenyl), and equol from daidzein, and dihydrogenistein from genistein (Chang and Nair 1995). However, the specific bacteria from the human intestinal tract

that are involved in the metabolism of these compounds are not known.

From a human fecal sample, we have isolated a gram-positive bacterial species that can reduce the double bond between C-2 and C-3 of daidzein and genistein to a single bond, leading to the production of dihydrodaidzein and dihydrogenistein. In addition, this strain, and another strain identified as *Escherichia coli*, have glucosidase activities, converting daidzin and genistin to the aglycones daidzein and genistein, respectively.

Materials and methods

Bacterial isolation and culture conditions

A human fecal sample from a healthy individual was collected and used to inoculate 10 ml water covered with 2 ml of sterilized mineral oil in a test tube. The sample was serially diluted in water and aliquots were plated on brain-heart infusion (BHI) agar plates under anoxic conditions. The plates were incubated in an anaerobic glove box (Forma Scientific, Marietta, Ohio) under a 5% CO_2, 10% H_2, and 95% N_2 atmosphere at 37 °C for 2 days. Two hundred different bacterial colonies were isolated and grown in 10 ml BHI medium under anoxic conditions (5% CO_2, 10% H_2, and 95% N_2).

Glucosidase activity in bacteria from the human intestinal tract

The bacterial isolates from the human intestinal tract were tested for glucosidase activity using 400 μg *p*-nitrophenyl-β-D-glucopyranoside (ml^{-1}) in 4 ml of BHI medium.

The strains were incubated overnight in the presence or absence of the substrate at 37 °C. For controls, BHI medium was incubated with 400 μM substrate for the same duration. Aliquots (500 μl) of the cultures were centrifuged at 20,000×*g* for 5 min using a Brinkmann centrifuge (Brinkmann Instruments, Westbury, N.Y.). The amount of *p*-nitrophenol released in the supernatants was measured at 405 nm using a model EL 312e spectrophotometer (Bio-Tek Instruments, Winooski, Vt.). The bacterial strain with the highest β-glucosidase activity, designated HGH21, was selected for further study of the metabolism of natural isoflavone glycosides.

Screening bacteria from the human intestinal tract for the metabolism of isoflavonoids

Strains isolated from the human intestinal tract were assayed for their ability to metabolize the isoflavonoid daidzein by combining eight different bacterial strains for each assay. Fifteen sets of bacterial cultures were grown in BHI medium containing daidzein for 2 weeks under anoxic conditions (5% CO_2, 10% H_2, and 95% N_2) at 37 °C. The bacterial cultures were extracted and the extracts were analyzed by HPLC (see below). Individual strains from the set that metabolized daidzein were further screened in the presence of daidzein as described above. One gram-positive bacterium, designated strain HGH6 (ATCC number BAA-96), was found to metabolize daidzein. This strain also had β-glucosidase activity as high as that of strain HGH21 (ATCC number BAA-97) and could deconjugate daidzin to daidzein.

Metabolism of isoflavonoids and their glycosides

Strains HGH6 and HGH21 were grown anaerobically in the presence or absence of 400 μM of the isoflavone glycosides daidzin and genistin at 37 °C for 7 days. This concentration did not have any effect on cell growth. Strain HGH6 was also incubated with 400 μM of the isoflavonoids daidzein and genistein, and with the flavonoids apigenin and chrysin under anaerobic conditions for 7 days. For controls, BHI medium was also incubated with 400 μM of the isoflavonoids or flavonoids. The experiments were done in duplicate.

Sample preparation for HPLC analysis

One ml of each bacterial culture grown in the presence or absence of isoflavonoids and of the control was extracted with 3 ml ethyl acetate. Two ml of each extract was dried and dissolved in 0.5 ml of 90% acetonitrile solution. The samples were filtered through Phenomenex 0.4-μm nylon filters (Torrance, Calif.) and 20 μl of each sample was used for HPLC analysis.

HPLC analysis of metabolites

The Star HPLC system from Varian (Walnut Creek, Calif.) and a Spherisorb C18 reversed-phase column (4.6×250 mm, S5, ODS 2, Deeside Ind Est, Queensferry, Clwyd, Wales, U.K.) were used. Solvent A was 10% acetonitrile in 0.1% acetic acid and solvent B was 90% acetonitrile in 0.1% acetic acid. For the detection of the metabolites of daidzin, daidzein, apigenin and chrysin, the mobile phase consisted of an isocratic elution for 10 min with 10% B, a linear gradient for 40 min to 30% B, a linear gradient for 10 min to 60% B, followed by isocratic elution for 5 min with 60% B at a flow rate of 1 ml/min. For the detection of metabolites of genistin and genistein, the mobile phase consisted of an isocratic elution for 15 min with 10% B, a linear gradient for 40 min to 40% B, a linear gradient for 5 min to 60% B, and an isocratic elution for 5 min with 60% B. All of the samples were monitored at 260 nm and the spectra of the peaks were determined from 220 nm to 450 nm.

Synthesis of dihydrodaidzein and dihydrogenistein

Catalytic transfer hydrogenation was performed according to Wähälä et al. (1998) with some modifications. Daidzein or genistein (5 mg) was dissolved in 25 ml ethanol containing 5 mg of ammonium formate and 5 mg of 10% palladium on activated charcoal (Pd/C) in 75-ml crimp-sealed septum vials. The air in the septum vials was evacuated and replaced with hydrogen gas. The solutions were stirred at room temperature and monitored by HPLC for the production of dihydrodaidzein and dihydrogenistein. The reaction solutions were centrifuged at 20,000×*g*, followed by evaporation of the supernatant to dryness. The dried crystals were dissolved in ethyl ether, dried and dissolved in methanol before purification by HPLC.

Purification of synthetic dihydrodaidzein and dihydrogenistein

The HPLC system consisted of a Model 660 quaternary pump and gradient controller from Waters (Milford, Mass.), a Rheodyne 7125 injector with a 100-μl loop (Rheodyne, Cotati, Calif.), and an Ultrasphere C18 reversed-phase column (10×250 mm, ODS, 5 μm, P.J. Cobert, St. Louis, Mo.). For the purification of dihydrodaidzein, the mobile phase consisted of a linear gradient of 50% methanol to 90% methanol at a flow rate of 1 ml/min for 40 min. For the purification of dihydrogenistein, the mobile phase consisted of a linear gradient of 70% methanol to 95% methanol for 20 min. All samples were monitored with a Waters 486 UV detector at 225 nm.

Liquid chromatography/mass spectrometry

The samples were analyzed by atmospheric pressure chemical ionization (APCI) using a TSQ-7000 liquid chromatograph-mass spectrometer (LC/MS/MS) (Finnigan, San Jose, Calif.). The vaporization was set to 400 °C and the capillary at 250 °C. The MS (Q1) was set to the mass for the presumed protonated molecules. The second MS (Q2) was set to scan from low mass (~20 Da) to the mass of Q1. Fragments were obtained using Ar (~0.7 mT) at

50 eV collisions. Otherwise the mass spectrometer settings were the default values for APCI. The HPLC column was a Phenomenex Prodigy ODS-2, 3×250 mm, with a flow rate of 250 µl/min. Mobile phase A was 95% methanol in 0.1% formic acid and B was 5% methanol in 0.1% formic acid. The gradient was as follows: 50% A for 5 min, followed by a linear gradient to 90% A at 10 min and then isocratic until the end of the run. The injection of 10 µl of each sample and other operations of MS were performed manually.

Nuclear magnetic resonance for identification of synthesized dihydrodaidzein and dihydrogenistein

Daidzein, dihydrodaidzein, genistein, and dihydrogenistein were analyzed by ^{1}H NMR spectroscopy at 500 MHz on a Bruker 500 MHz NMR spectrometer operating at 301 °K. Daidzein and genistein were dissolved in $CDCl_3$ and MeOD. Dihydrodaidzein and dihydrogenistein were dissolved in MeOD. The chemical shifts were defined by assigning the MeOD peak to 3.31 ppm. The spectral width was 7500 Hz with a 1.0-s delay time. Nuclear Overhauser effect (NOE) difference and proton decoupling NMR experiments were performed at every proton frequency for all four compounds.

Chemicals

Daidzin (daidzein-7-*O*-glucoside) was from Indofine (Somerville, N.J.). Apigenin (4′,5,7-trihydroxyflavone), chrysin (5, 7-dihydroxyflavone), daidzein (4′,7-dihydroxyisoflavone), genistein (4′,5,7-trihydroxyisoflavone), genistin (genistein-7-*O*-glucoside), and *p*-nitrophenyl-β-D-glucopyranoside were from Sigma (St. Louis, Mo.). HPLC-grade acetonitrile and methanol were from J.T. Baker (Phillipsburg, N.J.).

Results

β-Glucosidase activity of strains HGH6 and HGH21

Several strains of bacteria with β-glucosidase activities were isolated using the substrate *p*-nitrophenyl-β-D-glucopyranoside. One strain, designated HGH21, had the highest amount of activity. This strain was identified by the MIDI system (MIDI, Newark, Del.) as *E. coli*. It produced 540 µM of *p*-nitrophenol from 1,327 µM (400 µg/ml) of *p*-nitrophenyl-β-D-glucopyranoside in 24 h. This strain, along with another strain of a gram-positive anaerobic bacterium, designated HGH6, which also had a similar amount of β-glucosidase activity and could not be identified by conventional methods, were tested for the metabolism of daidzin and genistin.

Metabolism of isoflavonoids by strain HGH6 and *E. coli* HGH21

Strain HGH6 was incubated with daidzin (daidzein-7-*O*-glucoside) or genistin (genistein-7-*O*-glucoside) under anoxic conditions for 7 days. HPLC analysis detected metabolites eluting at 23.8 and 25.8 min from the cultures incubated with daidzin and metabolites eluting at 33.7 and 38.9 min from the cultures incubated with genistin. These metabolites were absent in the BHI medium incubated with daidzin and genistin and in control bacterial cultures incubated without isoflavonoids for the same duration (Figs. 1, 2). The retention times and UV spectra of the metabolites eluting at 23.8 and 25.8 min in daidzin cultures corresponded with those of standard dihydrodaidzein and daidzein. Similarly, the retention times and UV spectra of the metabolites eluting at 33.7 and 38.9 min in genistin cultures corresponded with those of standard dihydrogenistein and genistein. ^{1}H NMR spectra of synthetic dihydrodaidzein and dihydrogenistein were identical with those previously reported by Wähälä et al (1998). These metabolites were further identified as dihydrodaidzein and daidzein, and dihydrogenistein and genistein by mass spectral analysis (Figs. 3, 4). Dihydrodaidzein

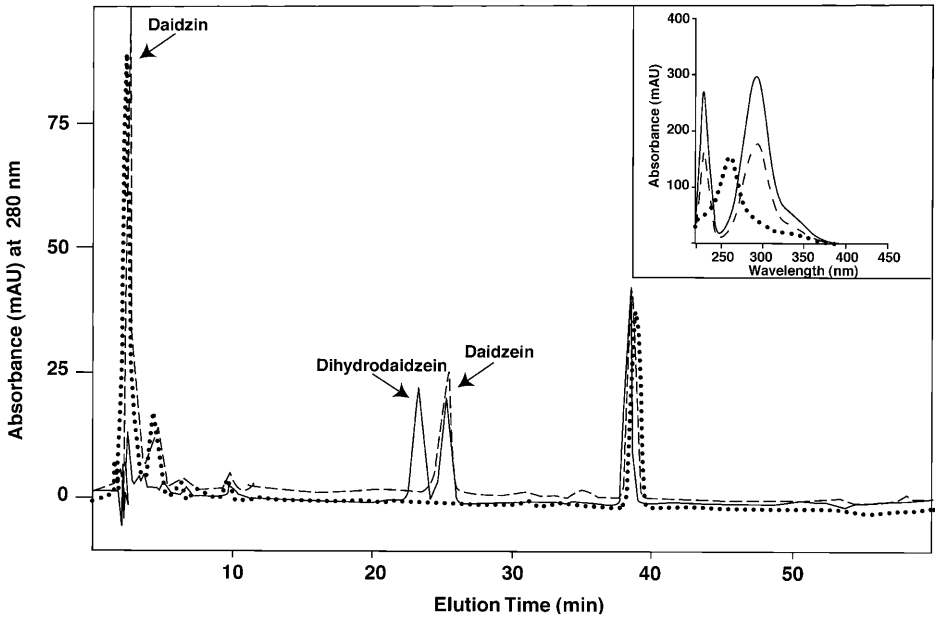

Fig. 1 HPLC elution profile of daidzin after 7 day anaerobic incubation with BHI (*dotted line*) strains HGH6 (*smooth line*) and HGH21(*hatched line*). *Inset* UV spectrum of daidzein (*dotted line*), dihydrodaidzein produced by strain HGH6 (*smooth line*) and synthetic dihydrodaidzein (*hatched line*)

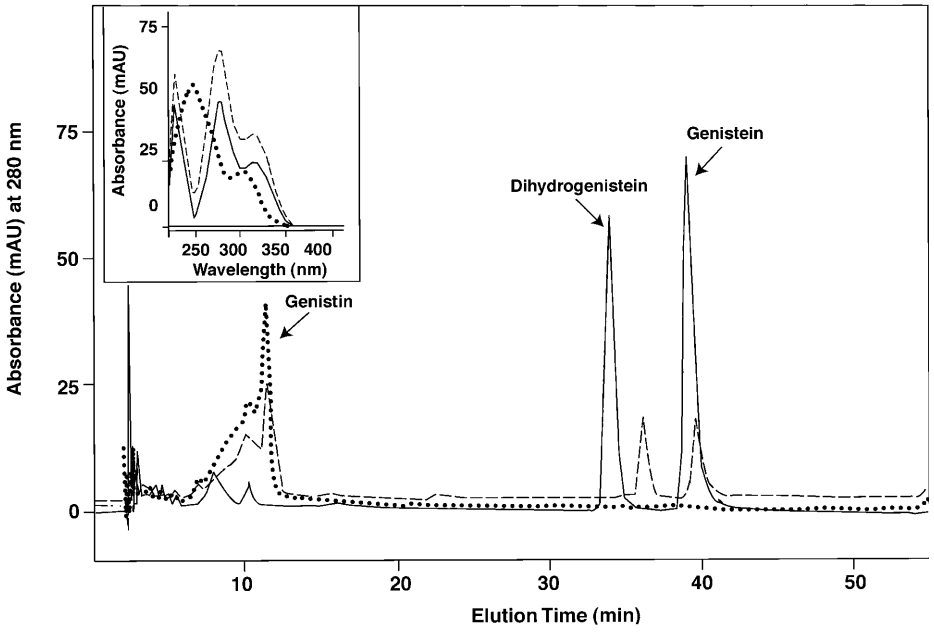

Fig. 2 HPLC elution profile of genistin after 7-day anaerobic incubation with BHI (*dotted line*), strains HGH6 (*smooth line*) and HGH21(*hatched line*). *Inset* UV spectrum of genistein (*dotted line*), dihydrogenistein produced by strain HGH6 (*smooth line*) and synthetic dihydrogenistein (*hatched line*)

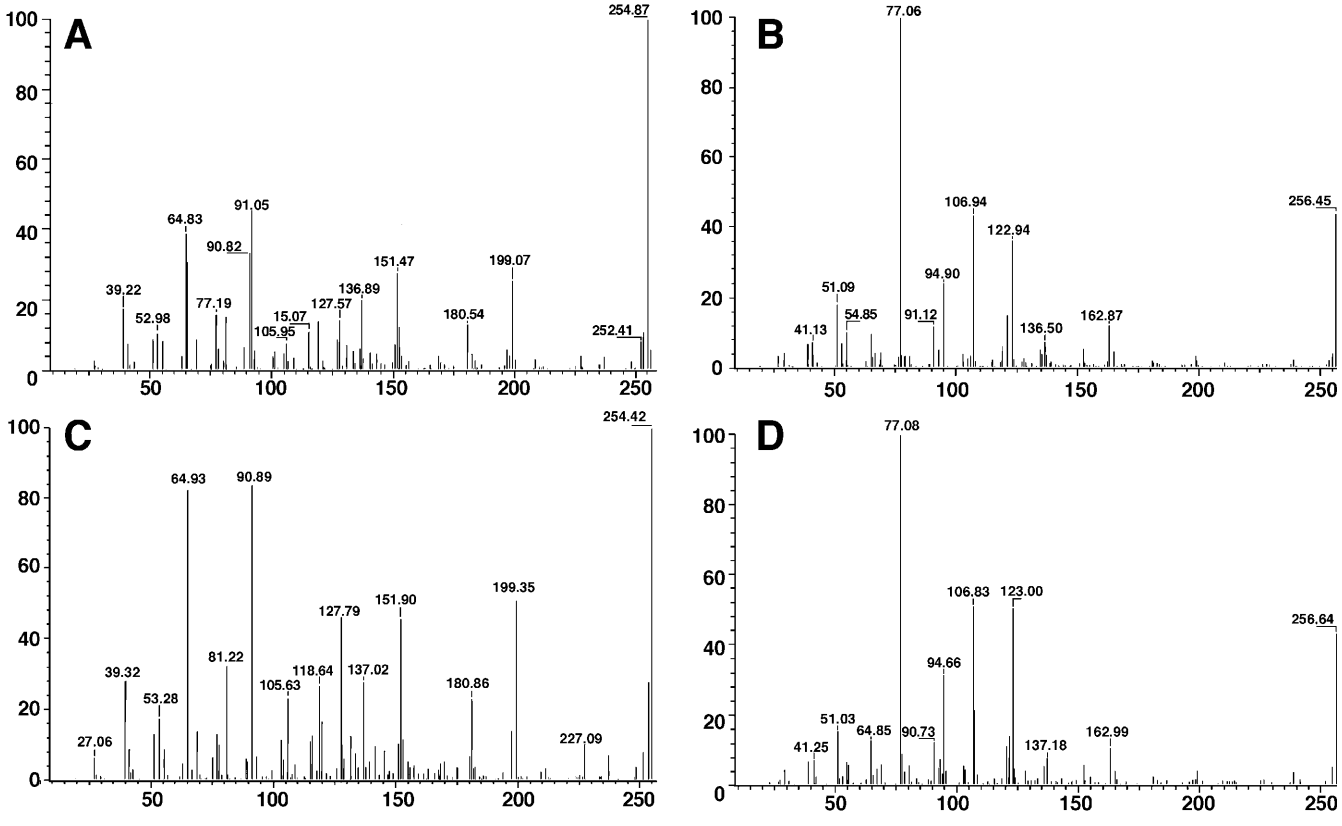

Fig. 3 EI Mass spectra of the standard daidzein (**A**), chemically synthesized dihydrodaidzein (**B**), daidzein produced by strain HGH6 and *E. coli* 21 (**C**) and dihydrodaidzein produced by strain HGH6 (**D**)

and dihydrogenistein were also produced after incubation of daidzein and genistein with strain HGH6. The 7-day incubation of 400 μM of the aglycones daidzein and genistein with strain HGH6 under anoxic conditions resulted in production of 37.2 μM of dihydrodaidzein and 361.7 μM of dihydrogenistein from daidzein and genistein, respectively. Strain HGH6 was also incubated with the flavonoids apigenin (4′,5,7-trihydroxyflavone) and chrysin (5,7-dihydroxyflavone) under the same conditions. However, strain HGH6 did not metabolize these compounds. Incubation of *E. coli* HGH21 with genistin or daidzin for 7 days resulted in production of the aglycones daidzein and genistein, respectively (Fig. 1). Dihydrodaidzein and dihydro-

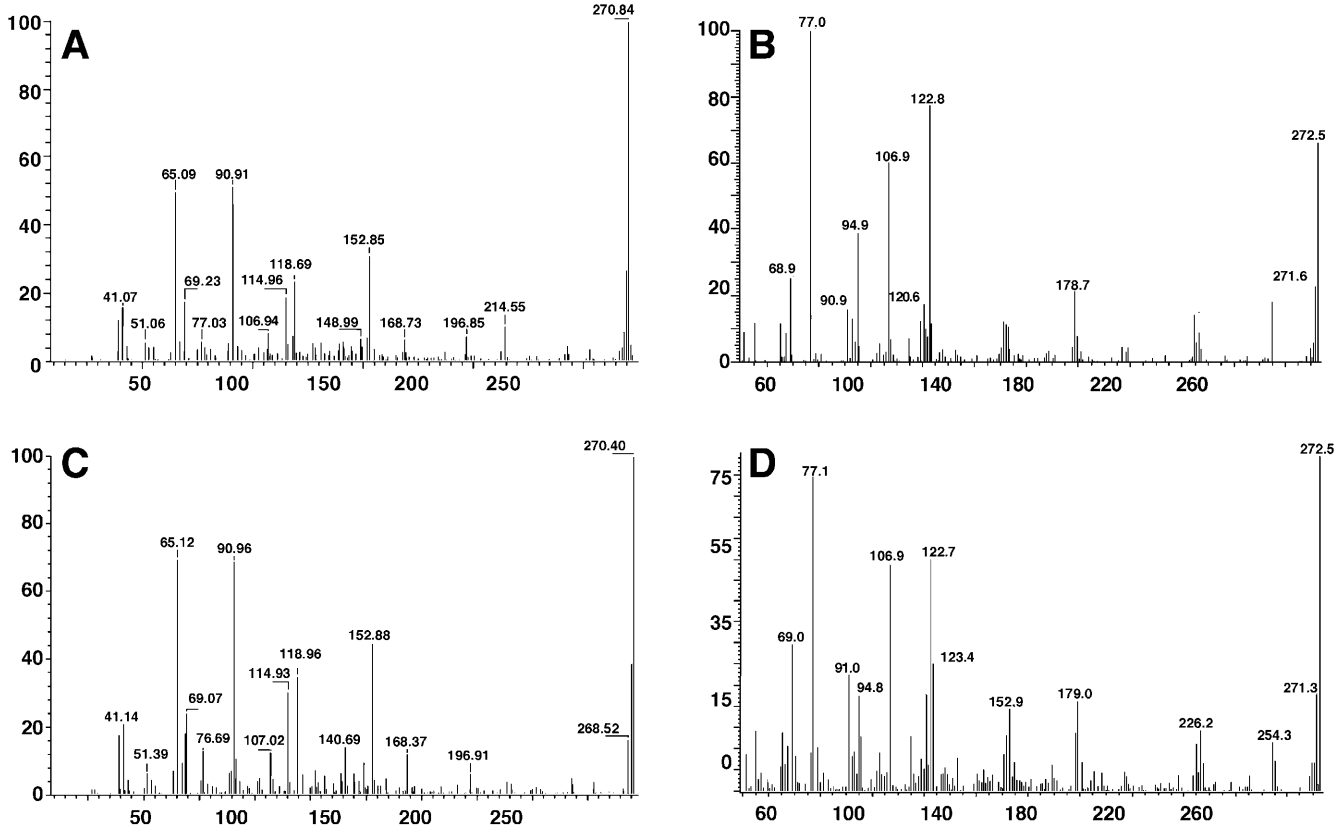

Fig. 4 EI Mass spectra of the standard genistein (**A**), chemically synthesized dihydrogenistein (**B**), genistein produced by strain HGH6 and *E. coli* 21(**C**) and dihydrogenistein produced by strain HGH6 (**D**)

genistein were not detected in *E. coli* HGH21 cultures grown anaerobically.

Discussion

Epidemiological and migrant studies support the hypothesis that western diet has a role in high incidence of hormone-dependent cancers, colon cancer and coronary heart disease (Adlercreutz et al. 1995). Similar studies have shown the beneficial effects of soybean phytoestrogens in Asian and vegetarian populations. Increased soy consumption is associated with a reduced risk of breast, colon and prostate cancer. In vitro studies also indicate the significant beneficial effects of these compounds on biological functions, such as dose-dependent inhibition of cancer cells (Adlercreutz 1993, 1995; Anderson et al. 1998; Arora et al. 1998; Divi et al. 1997; Fotsis et al. 1993; Kirk et al. 1998; Su et al. 2000; Zhou et al. 1998). Therefore, the availability and consumption of soybean isoflavonoids have recently increased in the United States. However, previous reports have shown variation in the metabolic response to dietary isoflavonoids in humans (Kelly et al. 1995). In vivo studies of metabolites in the plasma and urine of individuals consuming soy isoflavonoids have shown differences in the quantity and type of estrogenic and non-estrogenic metabolites (Joannou et al. 1995; Kelly et al. 1995). In vitro studies of metabolites produced from the total microflora of these individuals showed that the bioavailability of soybean isoflavonoids depends upon the ability of the gut microflora to metabolize these compounds (Joannou et al. 1995; Kelly et al. 1995). However, the specific bacteria involved in the metabolic process are not known. To understand the reasons for the metabolic variation, the human intestinal microflora were screened for the specific bacteria that metabolize two of the most potent natural phytoestrogenic isoflavone glycosides, genistin and daidzin. Two species of bacteria, *E. coli* HGH21 and an unidentified strain HGH6, converting genistin and daidzin to the more estrogenic genistein and daidzein, were found. Bokkenheuser et al. (1987) showed the hydrolysis of the flavonoid glycosides rutin and quercitrin by *Bacteroides* species from human intestinal flora. The isoflavone glycosides are less estrogenic than their respective aglycones and are very poorly absorbed compared with their corresponding aglycones because of their higher hydrophilicity and molecular weights (Brown 1988; Xu et al. 1995). Therefore, these bacteria with glucosidase activity are potentially important in the production of compounds with higher estrogenicity and better absorption, affecting the bioavailability and pharmacokinetics of natural phytoestrogens.

In addition, strain HGH6 was involved in the further modification of the isoflavonoid C-ring. This strain reduced a double bond between C-2 and C-3 of the C-ring

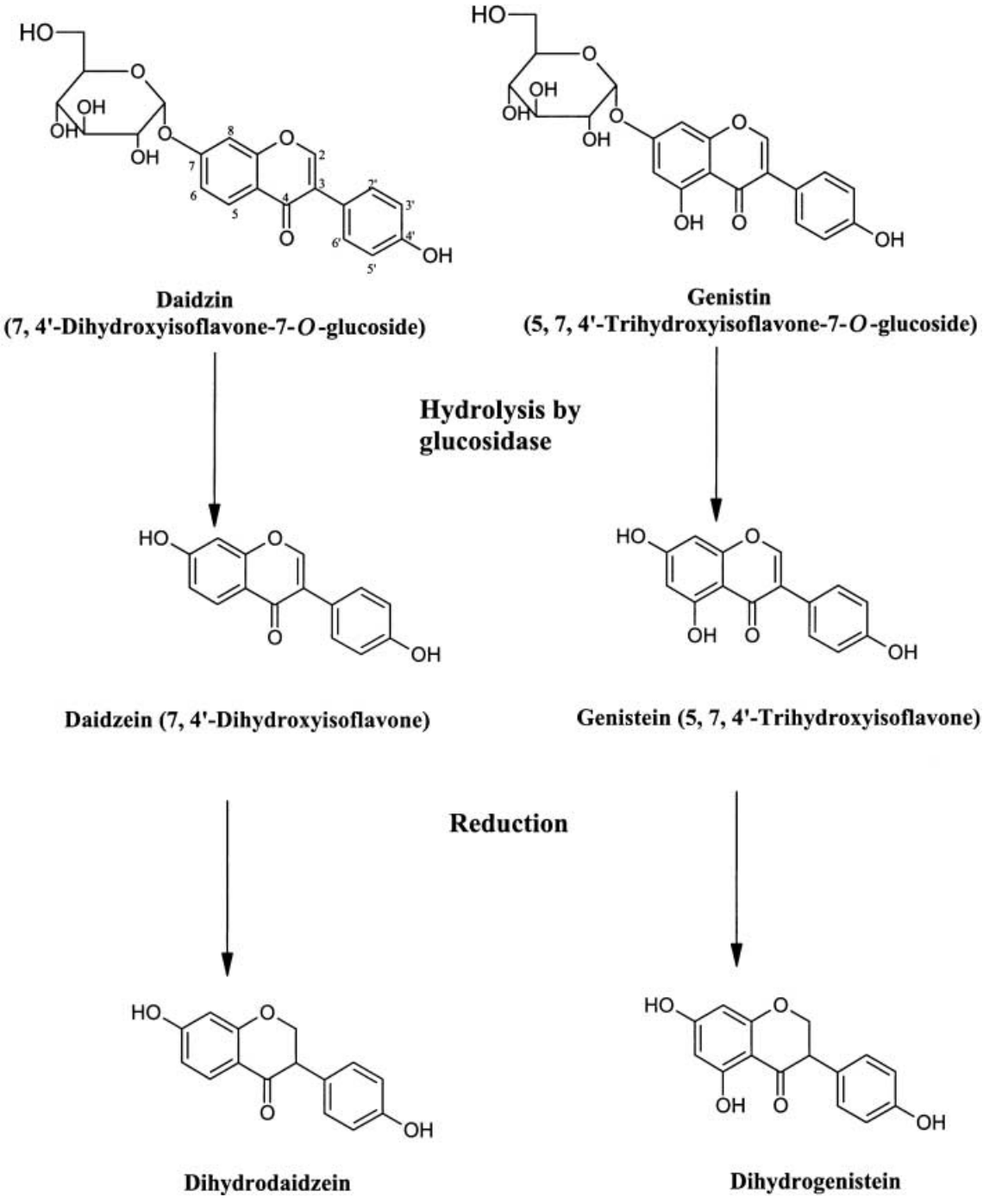

Fig. 5 Proposed metabolic pathways of 7-*O*-glucoside-conjugated isoflavonoids by strain HGH6

of both daidzein and genistein to a single bond, resulting in the formation of dihydrodaidzein and dihydrogenistein, respectively (Fig. 5). A similar reduction reaction was previously reported for *Clostridium paraputrificum*, which reduced deoxycorticosterone to tetrahydrodeoxycorticosterone (Bokkenheuser et al. 1975). In our experiments, however, *C. paraputrificum* did not reduce daidzein to dihydrodaidzein or tetrahydrodaidzein (data not shown).

These reduction products appear to be the first secondary metabolites produced as the result of ring modification of isoflavonoids by the colonic microbial flora. The metabolites were found after incubation of daidzein and genistein with total colonic microflora and also in the urine of individuals consuming soy isoflavonoids (Chang and Nair 1995; Joannou et al. 1995). Joannou et al. (1995) postulated that in some individuals dihydrodaidzein is converted to the estrogenic metabolite equol via tetrahydrodaidzein by the intestinal microflora. In our experiments, strain HGH6 did not reduce dihydrodaidzein to tetrahydrodaidzein. Furthermore, strain HGH6 did not re-

duce the similar bond in the flavonoids apigenin and chrysin. Therefore, the reduction of a double bond between C-2 and C-3 of the C-ring to a single bond appeared to be isoflavonoid-specific in strain HGH6.

The total colonic flora of the individual who participated in this study converted daidzein to equol. Since dihydrodaidzein and dihydrogenistein were the final products of the incubation of daidzein and genistein with strain HGH6, it appears that more than one bacterial species could be involved in the completion of the metabolic cycle of daidzein to equol and genistein to 6′-hydroxy-*O*-demethylangolensin.

Daidzein and genistein in legumes act as a signal to trigger the establishment of symbiosis with bacteria in the family Rhizobiaceae (Phillips 1992; Steele et al. 1999; Cooper et al 1999). Although the metabolism of daidzein and genistein by rhizobia associated with plants (which results in the production of aromatic products that can influence *nod* gene-inducing activity) has been shown previously (Cooper et al. 1999; Rao and Cooper 1994, 1995; Steele et al. 1999), this is the first study illustrating the metabolism of the isoflavonoids daidzin and genistin to dihydrodaidzein and dihydrogenistein by specific human intestinal bacteria.

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