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ORIGINAL PAPER

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Isolation of an anaerobic intestinal bacterium capable of cleaving the C-ring of the isoflavonoid daidzein

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Abstract Colonic bacteria were screened for bacteria involved in the conversion of phytoestrogens. A gram-positive anaerobic bacterium, strain HGH 136, capable of conversion of the isoflavonoid daidzein, was isolated and identified as a *Clostridium* sp. The bacterium cleaved the C-ring of daidzein to produce *O*-demethylangolensin (*O*-Dma). This compound was identified by comparison of the HPLC retention time and UV spectrum of the metabolite with chemically synthesized *O*-Dma. The identity of the metabolite was confirmed by liquid chromatography-mass spectrometry and NMR using synthetic *O*-Dma as a standard. The bacterium incubated with synthetic dihydrodaidzein also produced *O*-Dma. After 3 days of incubation, 28% of added daidzein and 12% of added dihydrodaidzein were converted to *O*-Dma. This is the first study in which an anaerobic bacterium involved in the ring cleavage of daidzein to produce *O*-Dma has been identified.

Keywords Isoflavonoids · Phytoestrogens · Daidzein · Anaerobic bacteria · C-ring fission

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Introduction

Isoflavonoids are found in leguminous plants and have natural roles in plant defense and root nodulation (Peters et al. 1986; Phillips 1992). Daidzein (4′, 7-dihydroxyisoflavone) is one of the principal isoflavonoids found in soybeans and is present as the glycoside conjugate daidzin (Coward et al. 1993). It binds to estrogen receptors and elicits biological activities similar to those of natural estrogens. There is growing interest in the consumption of soy products because of the beneficial effects of isoflavonoids (Coward et al. 1993; Messina et al. 1994; Adlercreutz 1995; Anderson et al. 1998), which have been proposed for use in chemoprevention and therapy of hormone-dependent diseases.

Upon ingestion, isoflavonoids are converted by bacteria in the human intestinal microflora. This is essential for the absorption, bioavailability, and estrogenic activities of these compounds (Kelly et al. 1993, 1995; Xu et al. 1995). In vivo studies have shown variations in health benefits of phytoestrogens in different individuals, which have been attributed to dissimilarities in the populations of the colonic bacteria responsible for isoflavonoid conversion (Xu et al. 1995).

In vivo studies have shown that, after consumption of soy products rich in daidzein, the intestinal bacteria of some individuals produce the highly estrogenic compound equol, a reduction product of daidzein (Chang and Nair 1995; Joannou et al. 1995), or a nonestrogenic ring-cleavage product, *O*-demethylangolensin (1-(2′, 4′-dihydroxyphenyl)-2-(4″-hydroxyphenyl)-propan-1-one; *O*-Dma) (Kelly et al. 1995). The metabolites of daidzein, namely dihydrodaidzein, 3-(4-hydroxyphenyl)-benzopyran-4, 7-diol and equol, have been detected after incubation of daidzein with fecal bacteria under anoxic conditions (Chang and Nair 1995). In addition, dihydrodaidzein, tetrahydrodaidzein, 2-dehydro-*O*-demethylangolensin, and *O*-demethylangolensin have been found in the urine of various individuals after consumption of soy products (Joannou et al. 1995).

Studies of conversion of phytoestrogens by human intestinal flora have been limited to detection of products generated from incubation of these compounds with total fecal materials. We have previously detected colonic bacteria that produce daidzein and genistein from natural methylated isoflavonoids and a bacterium that reduces daidzein and genistein to dihydrodaidzein and dihydrogenistein, respectively (Hur and Rafii 2000; Hur et al. 2000). In this study, a single bacterial strain from the human intestinal tract that cleaves the C-ring of daidzein to produce a nonestrogenic metabolite was identified and characterized.

Materials and methods

Isolation and identification of a bacterium cleaving the daidzein C-ring

A human fecal sample was collected in 10 ml water and covered with 2 ml of sterilized mineral oil in a test tube. Serial dilutions were plated on brain heart infusion (BHI) agar plates under anoxic conditions and incubated in an anaerobic glove box under an atmosphere of $CO_2/H_2/N_2$ (5:10:85, v/v) at 37 °C for 2 days. About 200 bacterial colonies were isolated; each colony was inoculated into 10 ml BHI medium and incubated under anoxic conditions. Fifteen mixed bacterial cultures were made by combining several of these bacteria in BHI medium containing 100 µM daidzein and incubating the cultures for 2 weeks under anoxic conditions at 37 °C. The cultures were harvested and extracted with ethyl acetate. The ethyl acetate was evaporated and the residues were used for HPLC analysis. One of the bacterial culture combinations was found to cleave the C-ring of daidzein. The individual strains from this bacterial combination were grown in 5 ml of BHI medium in the presence or absence of 400 µM daidzein under anoxic conditions at 37 °C for 3 days until an OD of 0.5–1 OD_{600} was reached; the cultures were then harvested and extracted for HPLC analysis of the possible metabolites. As a control, BHI medium was incubated with 400 µM daidzein for the same duration. The experiments were done in duplicate. One anaerobic gram-positive rod, strain HGH 136, was found to convert daidzein. The BBL Crystal Anaerobe ID Kit from Becton-Dickinson (Cockeysville, Md., USA), fatty acid analysis, and 16S rRNA analysis were used to identify strain HGH 136.

Synthesis and isolation of dihydrodaidzein and *O*-demethylangolensin

Daidzein derivatives were synthesized according to the method described by Wähälä et al. (1998) with modifications (Hur et al. 2000). Daidzein (50 mg) was dissolved in 50 ml ethanol containing 100 mg ammonium formate and 200 mg 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 argon. The solutions were stirred at room temperature overnight. The reaction mixes were centrifuged at 17,000×*g*, and then the supernatant was decanted and evaporated to dryness. The dried crystals were dissolved in ethyl ether, evaporated to dryness and redissolved in 3 ml methanol. Preparative TLC plates were used to separate synthetic compounds using a solvent system consisting of toluene/methanol (19:1.2, v/v). When the TLC plates were exposed to UV light at 254 nm, four different bands with R_f values of 0.5, 1.2, 1.8 and 2.0 were observed. Each band was scraped from the TLC plates, dissolved in 20 ml methanol, evaporated to dryness and identified by GC-MS and NMR spectroscopy.

HPLC analysis

The bacterial cultures grown in the presence or absence of the isoflavonoids, and controls were extracted with ethyl acetate. The ethyl acetate layer was dried and dissolved in 0.5 ml 90% aqueous acetonitrile solution. The samples were filtered through 0.4-µm nylon filters (Phenomenex, Torrance, Calif.).

The Star HPLC system (Varian) consisted of a model 230 pump, a model 430 autosampler with a 100-µl loop, and a model 330 photo diode array spectrophotometer. A Spherisorb C18 reversed-phase column (4.6×250 mm, S5, ODS 2, Clwyd, Wales, UK) was used to separate the metabolites. The mobile phase consisted of 10% acetonitrile in 0.1% acetic acid (A) and 90% acetonitrile in 0.1% acetic acid (B) with a linear gradient of 10–20% B for 10 min, 20–40% B for 30 min, and 40–80% B for 15 min. The concentration of B was held at 80% for 5 min, then raised from 80% to 90% for 5 min. All of the samples were monitored at 280 nm; UV spectra of the peaks were scanned from 220 nm to 450 nm with the detector. The flow rate was 1 ml/min and the column was equilibrated after each injection.

Liquid chromatography-mass spectrometry

LC/MS analyses were carried out on an HP 5989B mass spectrometer equipped with an Hewlett-Packard 1090L/M HPLC. The mass spectrometer was operated in the negative electrospray ionization (ESI) mode. With the capillary exit voltage at –100 V, full scans were acquired from *m/z* 200 to 300. With the capillary exit voltage at –200 V, full scans were acquired from *m/z* 50 to 300. HPLC was carried out with a Prodigy ODS (3) 2.0×250-mm 5-µm 100Å HPLC column (Phenomenex). The mobile phase, delivered at 0.2 ml/min, was a linear gradient from 5% aqueous acetonitrile to 95% aqueous acetonitrile in 20 min with no buffer.

Nuclear magnetic resonance spectroscopy

The ^{1}H NMR spectra of daidzein, dihydrodaidzein and *O*-Dma were determined on a Bruker 500 MHz NMR spectrometer operating at 301 °K. Daidzein, dihydrodaidzein and *O*-Dma were dissolved in deuterated methanol. The chemical shifts were defined by assigning the deuterated methanol resonance peak to 3.31 ppm. The spectral width was 7,500 Hz with a 1.0-s delay time. For every proton resonance, nuclear Overhauser effect and homonuclear decoupling experiments were carried out.

Chemicals

Daidzein was from Indofine (Somerville, N.J., USA). HPLC-grade acetonitrile and methanol were from J.T. Baker (Phillipsburg, N.J., USA).

Results

Characterization of bacterium

Colonies of strain HGH 136 on BHI agar incubated anaerobically were clear and flat. The cells were gram-positive rods. Strain HGH 136 was catalase-negative and indole-positive. Using the BBLCrystal Identification System Anaerobic ID Kit, the bacterium utilized L-arginine, L-lysine, L-methionine, L-phenylalanine and escosyl. It did not utilize any other substrate in the kit. Fatty acid analysis identified the bacterium as *Clostridium sporosphaeroides* with low probability. However, analysis of the gene sequence of 16S rRNA did not confirm this identification (Fig. 1).

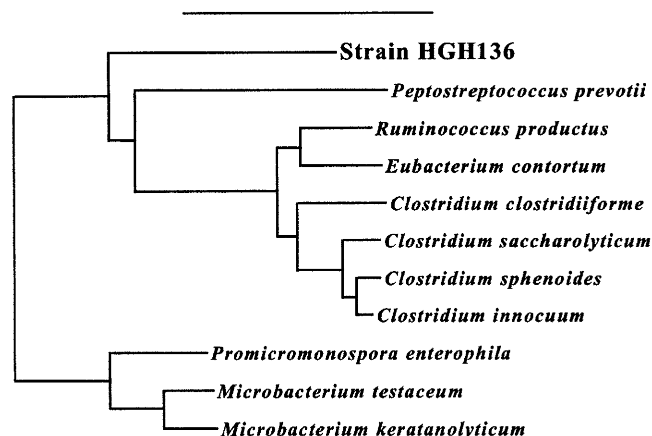

Fig. 1 Comparison of 16S rRNA from strain HGH 136 with the sequences of 16S rRNA of other bacteria in database. The 16S rRNA gene was analyzed by extracting the DNA, performing PCR with universal 16S primers, and sequencing with the same primers using standard methods. The resulting sequence of 520 bases was searched against MicroSeq database (Applied Biosystems) and Genbank (NCBI). *Bar* 9.521%

Therefore this strain is considered as *Clostridium* sp. strain HGH 136 based on the fatty acid analysis and 16S rRNA data (ATCC BAA-442).

Identification of synthetic *O*-Dma

Following chemical derivatization of daidzein and TLC analysis, four different compounds were detected with R_f values of 0.5, 1.2, 1.8 and 2. ^{1}H NMR spectral analysis of the compound with the R_f value of 2.0 showed a characteristic quadruplet peak at δ 4.66 ppm coupled to a doublet peak at δ 1.42 ppm for the C2 and C3 hydrogens of *O*-Dma, respectively. LC-MS showed an $[M-H]^-$ fragmentation pattern with *m/z* at 257, 239, 136 and 108. Based on the ^{1}H NMR and LC-MS analyses, the metabolite with R_f=1.2 was identified as dihydrodaidzein (Hur et al. 2000) and the material with R_f=2.0 as *O*-Dma (Table 1).

Table 1 ^{1}H-NMR analysis of *O*-demethylangolensin (*O*-Dma). All experiments were carried out at 500 MHz using a Bruker NMR spectrometer. The proton numbering scheme refers to Fig. 4. The solvent was deuterated methanol and the methanol peak was set to δ=3.31 ppm. Integration is the normalized volume under a chemical shift. Multiplicity is the peak splitting pattern and J(Hz) is the first-order coupling constant

Proton	δ (ppm)	Integration	Multiplicity	J (Hz)
C2-H	4.66	1	Quartet	6.8
C3-H	1.42	3	Doublet	6.8
C3′-H	6.32	1	Singlet	2.3
C5′-H	6.51	1	Doublet	8.8/2.3
C6′-H	7.74	1	Doublet	8.8
C2″-, C6″-H	7.11	2	Doublet	4.5

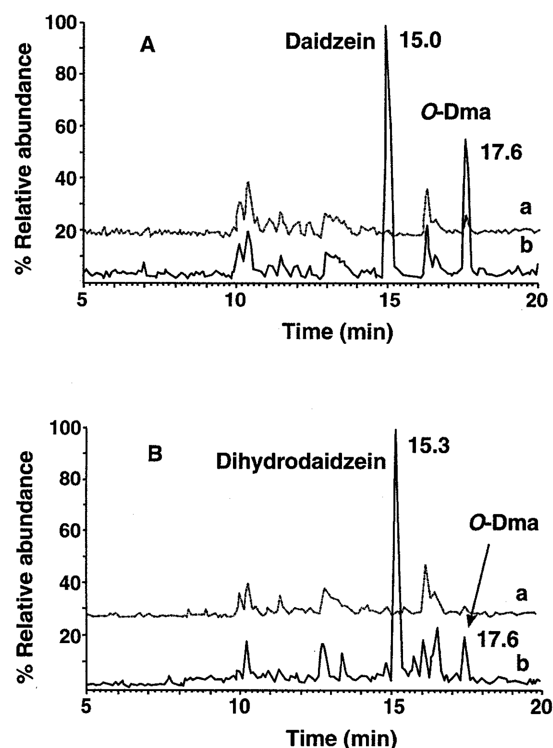

Fig. 2 HPLC elution profiles of daidzein (**A**) and dihydrodaidzein (**B**) incubated with *Clostridium* sp. strain HGH 136 in BHI for 3 days under anoxic conditions. *a* Controls without daidzein or dihydrodaidzein, *b* with daidzein or dihydrodaidzein

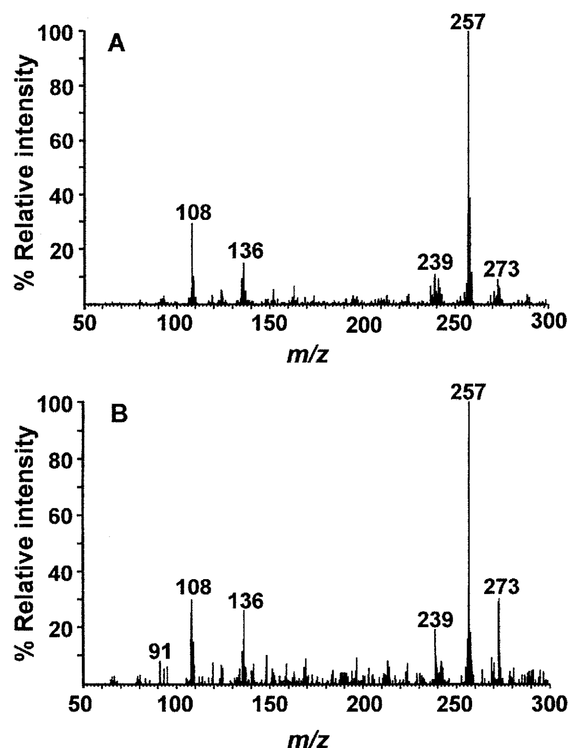

Fig. 3 Mass spectral analysis of **A** the chemically synthesized *O*-Dma and **B** the *O*-Dma metabolite produced by *Clostridium* sp. strain HGH 136

Conversion of daidzein and dihydrodaidzein

Strain HGH 136 was incubated with daidzein under anoxic conditions for 3 days. HPLC analysis detected a metabolite peak eluting at 17.6 min from the cultures incubated with daidzein. This metabolite was absent in sterile BHI medium incubated with daidzein and in bacterial cultures incubated without daidzein for the same duration (Fig. 2A). The retention time and UV spectrum of the metabolite eluting at 17.6 min in the cultures incubated with daidzein corresponded with those of synthetic *O*-Dma. The same metabolite was also observed in the culture of bacteria incubated with synthetic dihydrodaidzein (Fig. 2B). The metabolites produced from daidzein and dihydrodaidzein were further identified as *O*-Dma by mass spectral analysis (Fig. 3). The NMR values for the synthetic and metabolically produced *O*-Dma were identical. After 3 days incubation of strain HGH 136 with 400 μM daidzein, 112 μM of *O*-Dma was produced (28% conversion). Incubation with synthetic dihydrodaidzein resulted in 12% conversion to *O*-Dma for the same duration. However, no dihydrodaidzein metabolite peak was detected after incubation of daidzein with this bacterium.

Discussion

Soy isoflavonoids can bind to the human estrogen receptor and mimic estrogenic activities, including the benefits of estrogen for bones and the cardiovascular system, and the use of these compounds has therefore increased in recent years (Adlercreutz et al. 1993; Fotsis et al. 1993; Adlercreutz 1995; Divi et al. 1997; Anderson et al. 1998; Arora et al. 1998; Kirk et al. 1998). However, individual variations in the bioavailability and biological effects of isoflavonoids have been observed in subjects consuming soy phytoestrogens in clinical trials. These variations have been attributed to the effect of intestinal flora on isoflavonoid metabolism (Wilcox et al. 1990; Baird et al. 1995; Xu et al. 1995; Albertazzi et al. 1999). Joannou et al. (1995) postulated that the intestinal microflora act by two distinctive pathways to convert daidzein to the more estrogenic metabolite equol or the less estrogenic metabolite *O*-Dma. These investigators observed an inverse relationship between equol and *O*-Dma excretion in various individuals and attributed the metabolic pathway to variability in intestinal microflora (Kelly et al. 1993; Joannou et al. 1995).

Considering the importance of intestinal bacteria in the conversion of daidzein to either more or less estrogenic compounds, the paucity of information on the individual bacteria responsible for either of these pathways is surprising. In this study, we detected one strain of *Clostridium* from the human intestinal flora that renders daidzein less estrogenic. Our data show that strain *Clostridium* sp. HGH 136, isolated from human feces, cleaved the C-ring of daidzein to *O*-Dma under anoxic conditions. Following incubation of total fecal bacteria with daidzein for 7 days, dihydrodaidzein and equol were found as major peaks but no *O*-Dma was detected (data not shown). We previously identified another bacterial strain, strain HGH 6, that converts daidzein to dihydrodaidzein (Hur et al. 2000).

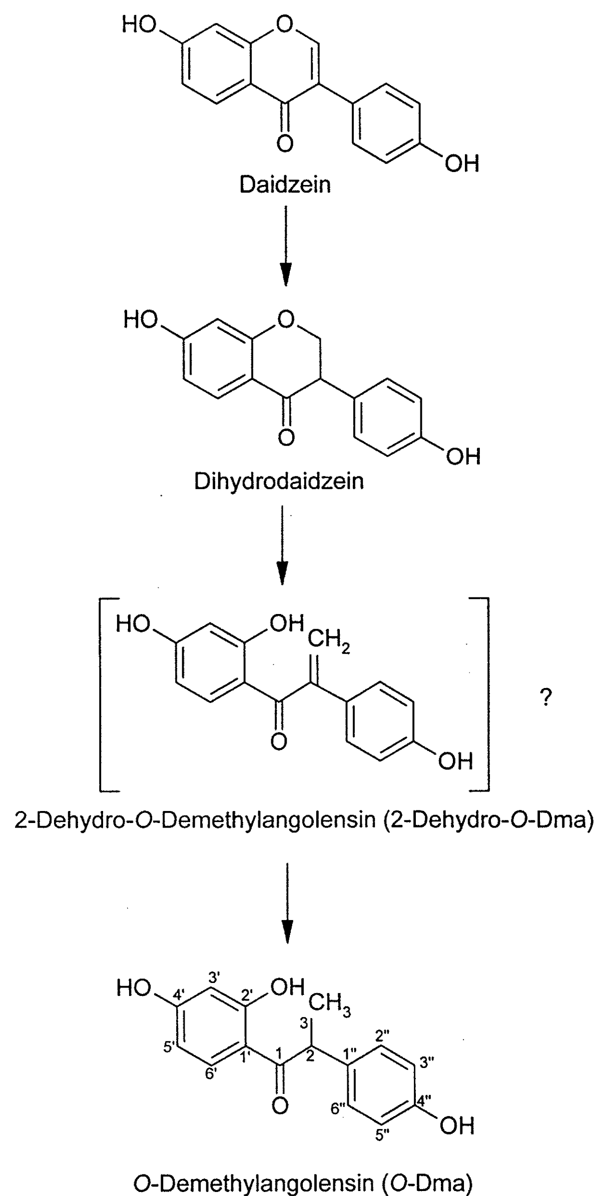

Fig. 4 Pathway of metabolism of daidzein to O-Dma as proposed by Joannou et al. (1995)

Joannou et al. (1995) found dihydrodaidzein, 2-dehydro-*O*-Dma, and *O*-Dma in the urine of some individuals following consumption of daidzein. They postulated that in these individuals, as the result of activities of the intestinal microflora, daidzein is first converted to dihydrodaidzein, which, in turn, is converted further to *O*-Dma through 2-dehydro-*O*-Dma. In our study, we found that strain HGH 136 carried out all of the steps necessary for the production of *O*-Dma from daidzein (Fig. 4). This strain, in addition to daidzein, converted synthetic dihydrodaidzein to *O*-Dma. The intermediates dihydrodaidzein and 2-dihydro-*O*-Dma were not detected in our

experiments (Fig. 2). If daidzein is converted to *O*-Dma via the pathway suggested by Joannou et al. (1995), it is possible that dihydrodaidzein and 2-dihydro-*O*-Dma, which could be produced by the bacterium, were transient intermediates and did not accumulate in the culture. It is not clear why synthetic dihydrodaidzein was not completely converted by the bacterium.

Previously, it has been shown that *Rhizobium* sp. strain NGR 234 produces resorcinol, *p*-coumaric acid and *p*-hydroxybenzoic acid by C-ring cleavage of flavonoids (Rao and Cooper 1994). Two human isolates, *Clostridium orbiscindens* (Winter et al. 1991) and *Eubacterium ramulus* (Schneider and Blaut 2000), and two rumen isolates, *Butyrivibrio* sp. (Cheng et al. 1969) and *Eubacterium oxidoreducens* (Krumholz et al. 1986), cleave the C-ring of flavonoids (not isoflavonoids) to produce hydroxyphenylacetic acids and phenylpropionic acids. The reduction products of daidzein also have been found as the result of the incubation of daidzein with fecal bacteria (Chang and Nair 1995) and with an anaerobic colonic bacterium (Hur et al. 2000). However, this is the first report of the isolation of an anaerobic bacterium capable of C-ring cleavage of phytoestrogenic daidzein to the much less estrogenic *O*-Dma. It is possible that these types of bacteria are more common in certain individuals and are more efficient for the conversion of phytoestrogens in the colon because of their higher numbers and the optimum growth conditions for high metabolic activities.

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