

Production of phytoestrogen *S*-equol from daidzein in mixed culture of two anaerobic bacteria

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Abstract An anaerobic incubation mixture of two bacterial strains *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16, which have been known to transform dihydrodaidzein to *S*-equol and daidzein to dihydrodaidzein respectively, produced *S*-equol from daidzein through dihydrodaidzein. The biotransformation kinetics of daidzein by the mixed cultures showed that the production of *S*-equol from daidzein was significantly enhanced, as compared to the production of *S*-equol from dihydrodaidzein by *Eggerthella* sp. Julong 732 alone. The substrate daidzein in the mixed culture was almost completely converted to *S*-equol in 24 h of anaerobic incubation. The increased production of *S*-equol from daidzein by the mixed culture is likely related to the increased bacterial numbers of *Eggerthella*

sp. Julong 732. In the mixture cultures, the growth of *Eggerthella* sp. Julong 732 was significantly increased while the growth of *Lactobacillus* sp. Niu-O16 was suppressed as compared to either the single culture of *Eggerthella* sp. Julong 732 or *Lactobacillus* sp. Niu-O16. This is the first report in which two metabolic pathways to produce *S*-equol from daidzein by a mixed culture of bacteria isolated from human and bovine intestinal environments were successfully linked under anaerobic conditions.

Keywords Isoflavone · Daidzein · *S*-equol · Intestinal · Anaerobic bacteria

Introduction

The dietary phytoestrogenic isoflavones daidzein and genistein are known to possess diverse physiological activities including anticarcinogenic (Adlercreutz et al. 1992; Hirano et al. 1994), antimutagenic (Hartman and Shankel 1990), and antioxidant (Jha et al. 1985) activities, as well as antiproliferative effects against tumor cells (Hirano et al. 1989). After ingestion of dietary isoflavones, they are subjected to biotransformation by gut microbiota to diverse metabolites including dihydrodaidzein, tetrahydrodaidzein, equol, and *O*-desmethylangolensin, which have been detected in human urine (Joannou et al. 1995; Lampe et al. 1998; Coldham et al. 1999; Rowland et al. 1999; Hur et al. 2000; Bowey et al. 2003; Heinonen et al. 2003; Zheng et al. 2003; Simons et al. 2005). In addition, isoflavone metabolites have been found in the urine of mares (Marrian and Haselwood 1932), domestic fowl (Common and Aims-worth 1961), pregnant macaques (Monfort et al. 1984),

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chimpanzees (Adlercreutz et al. 1986), and dogs (Juniwicz et al. 1988), and in the bovine rumen (Luk et al. 1983).

Equol, which is known to be the most effective in stimulating an estrogenic response among the isoflavone derivatives (Borriello et al. 1985; Sathyamoorthy and Wang 1997), was found in only 30–40% of the human population (Setchell and Adlercreutz 1988; Sathyamoorthy and Wang 1997). This is the result of individual differences in the gut microbiota responsible for equol production. Structural similarity of equol to human estrogen estradiol confers to compete for binding to the endocrine receptors against estradiol. In addition, equol with enantiomeric stereochemistry showed potentially different biological activities due to its different binding affinity toward the endocrine receptors ER α and ER β . *S*-equol, which is found in human urine, binds to ER β at a rate that is 13 times higher than that of *R*-equol and 2 times higher than that of (\pm) equol (Muthyala et al. 2004). *R*-equol, however, showed a binding preference to ER α with four times higher than that of *S*-equol and two times higher than that of (\pm) equol.

Although extensive research has been performed to search for a single bacterium capable of producing equol from isoflavones, there have been no reports to date, which describe a specific bacterium in relation to the production of equol produced from daidzein (Joannou et al. 1995). Wang et al. (2005a) reported isolation of human intestinal bacterium *Eggerthella* sp. Julong 732 that can produce *S*-equol from dihydrodaidzein, which is reduced at the C-2 and C-3 double bonds of daidzein. In addition, Wang et al. (2005b) and Hur et al. (2000) isolated bacterial strains gram-positive *Lactobacillus* sp. Niu-O16 and *Clostridium* sp. HGH6 from bovine rumen and human fecal contents, respectively, that can metabolize daidzein to dihydrodaidzein under anaerobic conditions.

In this study, we combined two reductive metabolic pathways, daidzein to dihydrodaidzein, and dihydrodaidzein to *S*-equol, to produce *S*-equol from daidzein under anaerobic conditions using two bacterial strains *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16, which were isolated from human fecal and bovine rumen contents, respectively.

Materials and methods

Chemicals

Daidzein was purchased from Indofine (Somerville, NJ, USA), and brain heart infusion (BHI) powder

was from Difco Co. (Detroit, MI, USA). Acetonitrile, methanol, and acetic acid were of HPLC-grade. Dihydrodaidzein and *S*-equol were previously synthesized and identified in our laboratories (Wang et al. 2005a, b).

Bacterial strains and incubation

Eggerthella sp. Julong 732, which was previously isolated from a human fecal sample (Wang et al. 2005a), is a gram-negative and straight short-rod bacterium responsible for *S*-equol production from dihydrodaidzein under anaerobic conditions. *Eggerthella* sp. Julong 732 cannot ferment glucose and lactose. *Lactobacillus* sp. Niu-O16, which was isolated from a bovine rumen sample (Wang et al. 2005b), is a gram-positive and straight to slightly curved bacterium responsible for the production of dihydrodaidzein from daidzein also under anaerobic conditions. *Lactobacillus* sp. Niu-O16 cannot hydrolyze gelatin, but starch and esculine. It can also produce a curd reaction with litmus milk. It can produce acid from glucose, lactose, sucrose, and maltose but not from mannitol, xylose, arabinose, and glycerol. Based upon cell morphology observations performed by transmission electron microscopy (TEM) (Model JEOL-1010 Electron Microscope, JEOL, Tokyo, Japan), the size of *Lactobacillus* sp. Niu-O16 was shown to be two to three times larger than that of *Eggerthella* sp. Julong 732 (pictures not shown).

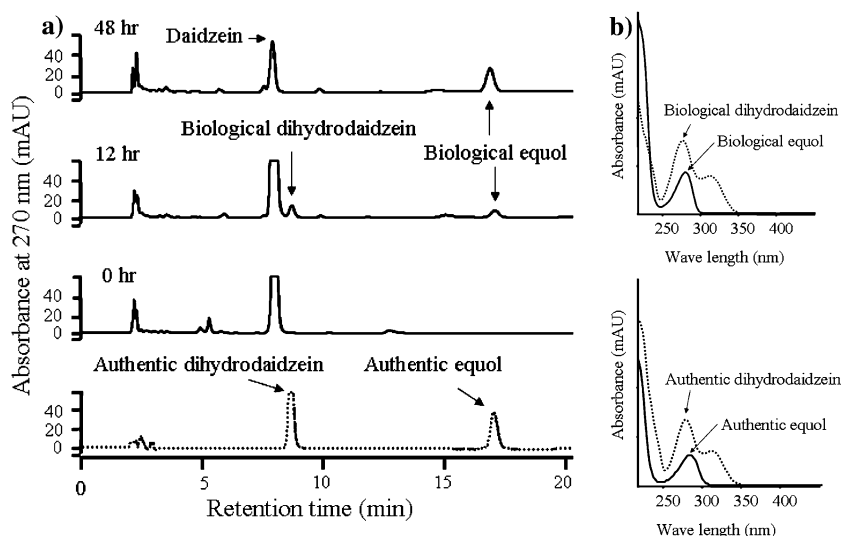
Prior to experiments, *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16 were maintained on BHI agar plates and inoculated into 20 ml of fresh BHI liquid medium in 45-ml tubes in an anaerobic chamber containing 5% CO₂, 10% H₂, and 85% N₂ at 37°C overnight. The cultures were washed three times with 20 mM phosphate buffer (pH 7.5), and adjusted to an optical density of 0.5 at 600 nm in BHI liquid medium. The bacterial cells were then dispensed into 6-ml cap tubes containing 3 ml of BHI medium. For the initial inoculation, bacterial numbers for *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16 were 0.6×10^7 /ml and 3.3×10^7 /ml, respectively. Daidzein in a 40 mM stock solution was added to the cultures for a final concentration of 1,200 μ M daidzein. Bacterial cultures were incubated anaerobically at 37°C and 200 μ l samples were taken at 0, 6, and 12 h, and continued every 12 h thereafter for a total of 72 h. Cell numbers were counted with a standard hemacytometer (Coulter Electronics, Krefeld, Germany) under a light microscope (Dongwon Precision Co. Ltd., Korea). Biotransformation kinetics of the growing bacterial cultures was performed under the same incubation time period as was performed for the bacterial counts. Gram-staining

of the strains was performed as described previously (Johnson et al. 1995). All experiments were performed in triplicate.

Identification of the metabolites

UV spectra and retention times of the metabolites produced from daidzein by the mixed culture of bacteria were compared with those of the standard compounds daidzein, dihydrodaidzein, and equol in HPLC profiles. Analytical HPLC profiles were obtained with a Varian ProStar HPLC (Varian, Walnut Creek, CA, USA) equipped with a photodiode array detector and a C18 column (5 μm particle size, 4.6 by 250 mm; Waters, Fullerton, CA, USA). The mobile phase was composed of 10% acetonitrile solution in water (solution A) and 90% acetonitrile solution in water (solution B), which were buffered with 0.1% acetic acid. The elution program was as follows: solution B was run at 30% for 15 min, linearly increased to 50% for 10 min, and then linearly increased to 70% for 5 min. The flow rate was 1 ml/min. All samples were monitored at 270 nm. UV spectra of the peaks were recorded from 200 to 400 nm. In order to separate racemates of the metabolites, a Sumi Chiral OA-7000 (5 μm particle size, 4.6 by 250 mm; Sumika Chemicals, Osaka, Japan) was used with a mobile phase composed of 40% acetonitrile in 20 mM potassium phosphate buffer (pH 3.0). The isocratic elution program with the mobile phase lasted for 35 min. The flow rate was 1 ml/min, and UV spectra of the peaks were recorded from 200 to 400 nm. EI-MS was obtained on a JMS-AX50510A mass spectrometer (JEOL, Co. Ltd. Tokyo, Japan) in positive mode (EI⁺). The source temperature was 250°C, and the ionization voltage was 70 eV. An electron multiplier of 1.2 kV, regular spectrum type, was used.

Fig. 1 HPLC elution profiles for the metabolism of the isoflavone daidzein by the mixed culture of *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16 (a). Inset UV spectra of both authentic and biological equol and dihydrodaidzein (b)



Results and discussion

Mixed cultures of *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16 completely reduced the heterocyclic C-ring of daidzein to produce *S*-equol under anaerobic conditions. HPLC chromatograms indicated that two metabolites of daidzein eluted at 8.5 and 17.1 min from the mixed cultures, which were incubated for 12 h (Fig. 1). Both retention time and UV spectral data for these two metabolites were identical to those of dihydrodaidzein and equol reported previously (Wang et al. 2005a, b). In addition, the EI-MS spectrum for the metabolite of daidzein shown at 17.1 min by co-culture of two isolated bacteria gave an (M + H)⁺ molecular ion peak at m/z 242 (relative intensity, 83%). The other daughter ion peaks (m/z) of the metabolite were 120(100), 123(72), 135(32), 107(24), and 91(11). The EI-MS spectrum for the metabolite shown at 8.5 min gave the (M + H)⁺ molecular ion peak at m/z 256 (relative intensity, 22%). The other daughter ion peaks (m/z) were 137(100), 120(52), 91(36), and 65(16). The EI-MS spectra obtained were well compared to the corresponding authentic compounds equol and dihydrodaidzein, respectively (Wang et al. 2005a, b).

Therefore, the metabolites of daidzein shown at 8.5 and 17.1 min were determined to be dihydrodaidzein and equol, respectively. Based on the retention time from the chiral column of the HPLC, biological equol production from daidzein by the mixed cultures was identical to authentic *S*-equol (Fig. 2) illustrating that the mixed cultures produced an enantiomeric pure *S*-equol. However, other intermediates such as tetrahydrodaidzein and/or dehydroequol were not detected during the metabolism of daidzein even though they were detected and proposed previously (Joannou et al.

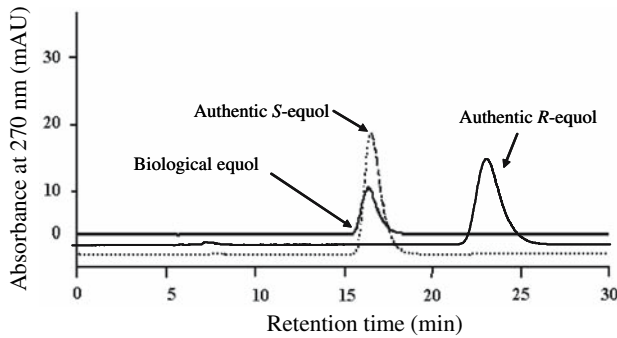


Fig. 2 HPLC elution profiles for biologically synthesized *S*-equal from daidzein by the mixed culture of *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16, and authentic *R*- and *S*-equal

1995). The biotransformation kinetics of daidzein by the mixed cultures of *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16 growing in BHI liquid medium with 1,200 μM daidzein is shown in Fig. 3. During bacterial growth, the mixed cultures produced *S*-equal and dihydrodaidzein in the amounts of 323 and 5 μM , respectively, in 12 h of incubation under anaerobic conditions. Production of *S*-equal gradually increased with time until 24 h, by which it reached its highest concentration in the medium at 754 μM . However, dihydrodaidzein was not detected after 24 h of incubation. The highest level of *S*-equal was observed during the stationary phase of the mixed cultures. As compared to our previous data (Wang et al. 2005a), the mixed cultures produced *S*-equal faster than did *Eggerthella* sp. Julong 732 alone, which did not produce *S*-equal from dihydrodaidzein until 48 h after the start of incubation. The reason for this is likely to be related with the significantly increased bacterial cell number of *Eggerthella* sp. Julong 732 in the mixed cultures (Fig. 4). Total bacterial cell numbers in the mixed

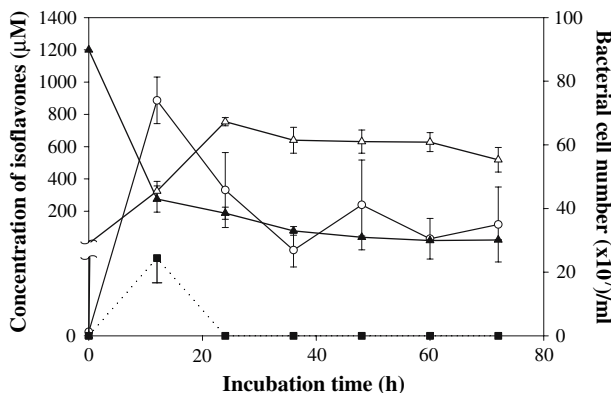


Fig. 3 Bacterial growth and biotransformation kinetics of daidzein by the mixed culture of *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16. Bacterial growth (open circle), substrate daidzein (filled triangle), and equal (open triangle) and dihydrodaidzein (filled square) produced from daidzein by the mixed culture

cultures reached its maximum of 75.6×10^7 cells after 12 h of incubation. The growth of human intestinal strain *Eggerthella* sp. Julong 732 in the mixed cultures was significantly enhanced as compared to the growth in the single culture of *Eggerthella* sp. Julong 732. The highest cell numbers of *Eggerthella* sp. Julong 732 in the mixed (Fig. 4a) and single (Fig. 4b) cultures were obtained at 12 h with 38.5×10^7 cells/ml and at 24 h with 2.5×10^7 cells/ml of incubation, respectively. Meanwhile, the growth of *Lactobacillus* sp. Niu-O16 in the mixed cultures was suppressed as compared to the growth of *Lactobacillus* sp. Niu-O16 alone. The highest cell numbers of *Lactobacillus* sp. Niu-O16 in the mixed (Fig. 4a) and single (Fig. 4b) cultures were obtained at 12 h with 35.4×10^7 cells/ml and 70.5×10^7 cells/ml, respectively. In the current study, we did not carry out experiments to elucidate physiological and biochemical interactions between the two bacteria that might affect

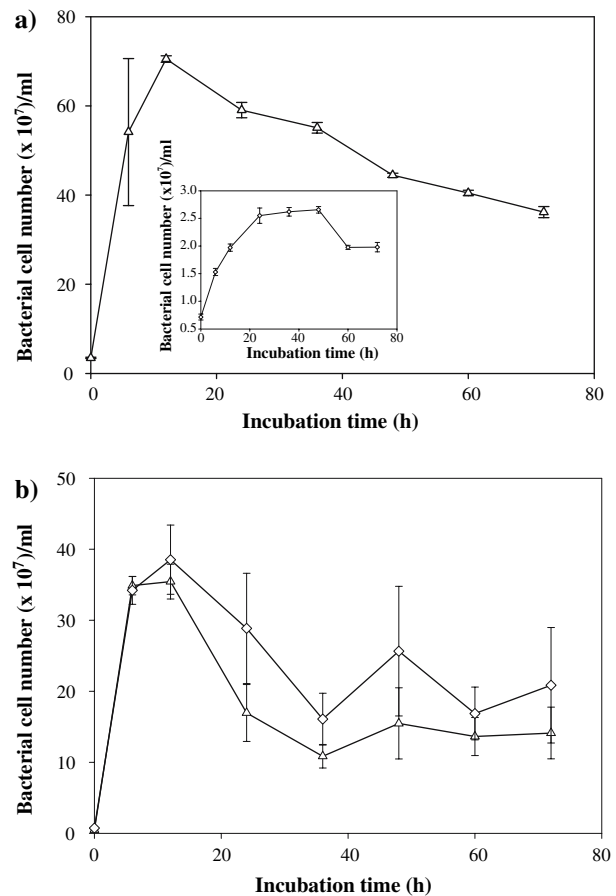
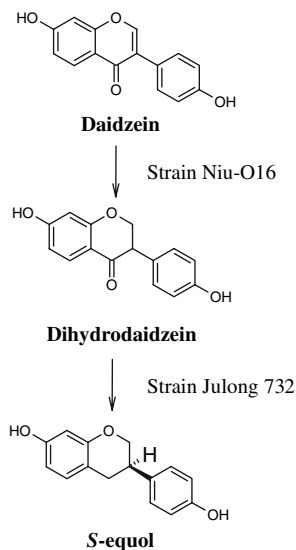


Fig. 4 Bacterial growth of *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16 in the medium containing daidzein. **a** Bacterial cell number of *Lactobacillus* sp. Niu-O16 from single culture (inset bacterial cell number of *Eggerthella* sp. Julong 732 from single culture); and **b** bacterial cell numbers of *Eggerthella* sp. Julong 732 (open diamond) and *Lactobacillus* sp. Niu-O16 (open triangle) from the mixture culture

Fig. 5 Proposed metabolic pathway from daidzein to *S*-equol through dihydrodaidzein by bovine rumen strain *Lactobacillus* sp. Niu-O16 and human intestinal strain *Eggerthella* sp. Julong 732 under anaerobic conditions



their growth in the mixed cultures. However, it will be necessary to accumulate more detailed knowledge in regard to the factors that might influence bacterial interactions before scale-up production of *S*-equol from daidzein. Equol, which is a urinary metabolite that originates from daidzein (Marrian and Haselwood 1932; Common and Aimsforth 1961; Axelson et al. 1982; Luk et al. 1983; Adlercreutz et al. 1986), has been shown to be the most potent phytoestrogenic compound among the derivatives of the natural isoflavone daidzein, lowering the incidence of hormone-dependent diseases such as breast cancer and prostate cancer (Sathyamoorthy and Wang 1997; Setchell et al. 2002; Akaza et al. 2004; Muthyala et al. 2004). Joannou et al. (1995) reported a urinary metabolic profile of isoflavones in which daidzein was biologically reduced to dihydrodaidzein to tetrahydrodaidzein to equol in sequential reactions, or was reduced to dehydroequol (7,4'-dihydroxyisoflav-3-ene). Recently, growing attention has been paid to the isolation of the intestinal bacteria capable of isoflavone metabolism. Hur et al. (2000) and Wang et al. (2005a) isolated anaerobic bacteria capable of metabolizing either daidzein to dihydrodaidzein or dihydrodaidzein to *S*-equol from human fecal or bovine rumen contents. A similar reaction for the reduction of daidzein to tetrahydrodaidzein, which is the metabolite most likely produced one step before equol, has been reported in bacterial metabolisms of steroids (French and Bruce 1995; Ren et al. 1996). For an example, morphinone reductase of *Pseudomonas putida* M10, which has been known as “old yellow enzyme” containing a yellow cofactor FMN, carries out reduction reaction of oxo-ene compound morphinone to oxo compound hydromorphone under the aerobic condition (French and Bruce 1995). Hydromorphone is

subject to further reduction reaction by reversible morphine dehydrogenase of *P. putida* M10 to dihydromorphone, which may correspond to tetrahydrodaidzein. Therefore, the biochemical reduction mechanisms by morphinone reductase and morphine dehydrogenase to the corresponding substrates will help elucidate further biochemical reactions of daidzein leading to *S*-equol by *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16. In the current study, a mixed culture consisting of human intestinal strain *Eggerthella* sp. Julong 732 and bovine rumen strain *Lactobacillus* sp. Niu-O16, successfully produced *S*-equol from daidzein through dihydrodaidzein under anaerobic conditions (Fig. 5).

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