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C-Ring Cleavage of Isoflavones Daidzein and Genistein by a Newly-Isolated Human Intestinal Bacterium *Eubacterium ramulus* Julong 601

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Abstract Julong 601, a Gram-positive anaerobic bacterium strain capable of cleaving the C-ring of isoflavones daidzein and genistein, was isolated from human feces. BLAST search revealed that its complete 16S rDNA gene sequence has 99% similarity to *Eubacterium ramulus*. Metabolites of daidzein and genistein were determined as *O*-desmethylangolensin (*O*-Dma) and 2-(4-hydroxyphenyl) propionic acid (2-HPPA), respectively, based on UV, EI-MS, and ^{1}H and ^{13}C NMR spectral analyses. Enantiomers of *O*-Dma and 2-HPPA were isolated by chiral stationary-phase HPLC (CSP HPLC). Cleavage of the C-ring of daidzein and genistein by strain Julong 601 was highly enantioselective. Specific rotation ($[\alpha]_D$) and circular dichroism (CD) spectra of the enantiomers are reported here for the first time. Biotransformation kinetics of daidzein and genistein indicated that the C-ring of genistein has a higher susceptibility to bacterial degradation than that of daidzein.

Key words: C-ring cleavage, daidzein, genistein, enantiomer, isolation, human intestinal bacteria

Much attention has been given to the isoflavones of soybeans, particularly daidzein and genistein, due to their significant physiological activities, including anticarcinogenic [1, 8], antimutagenic [6], antioxidant [10], and antiproliferative effects on tumor cells [7]. Human intestinal microflora plays a pivotal role in the metabolism of isoflavones prior to absorption. The metabolic fate of isoflavones greatly influences our health, because some of their metabolites show biologically more active properties [16]. The intestinal anaerobic metabolites of daidzein, such as dihydrodaidzein, equol, and *O*-desmethylangolensin (*O*-Dma), and those of genistein, such as dihydrogenistein, 6-OH-*O*-Dma, and 4-*p*-ethylphenol, have been detected in various tissues and organs of man and animals that once consumed soybean diet [2, 4, 11].

However, isolation of a single bacterium specifically involved in the metabolism of isoflavones has only been carried out recently [9]. To date, two single bacterial strains capable of cleaving the C-ring of isoflavones were isolated from human feces. One single bacterial strain, HGH 136, was reported to cleave the C-ring of isoflavone daidzein to form *O*-desmethylangolensin (*O*-Dma) [9]. The other strain, which was identified as *Eubacterium ramulus*, was reported to cleave the C-ring of (iso)flavones, including rutin, quercetin, luteolin, daidzein, and genistein [13, 14]. Based on the metabolites detected in the urine of man and other animals, dihydrodaidzein and 2-dehydro-*O*-Dma were two intermediates for the formation of *O*-Dma from daidzein [11]. Similarly, genistein was biodegraded to 2-HPPA through the intermediates of dihydrogenistein and 6-hydroxy-*O*-Dma [4].

In this study, an anaerobic bacterium involved in the C-ring cleavage of daidzein and genistein to form *O*-Dma and 2-HPPA was isolated from human feces. In addition, the enantiomers of *O*-Dma and 2-HPPA were separated and characterized.

Materials and Methods

Chemicals

Daidzein and genistein were purchased from Indofine (Somerville, NJ, U.S.A.), and brain heart infusion (BHI) powder from Difco Co. (Detroit, MI, U.S.A.). Acetonitrile and ethyl acetate used were of HPLC-grade. Previously synthesized *O*-Dma by Hor-Gil Hur at Kwangju Institute of Science and Technology, Gwangju, Korea, was used as a standard compound [9].

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Isolation and Identification of Bacteria

Fresh human fecal sample from a healthy individual, who has consumed soy products as one of major food sources, was serially diluted and spreaded on BHI agar plates in an anaerobic chamber containing 5% CO_2, 10% H_2, and 85% N_2 at 37°C. Random combinations of 10 isolated single bacterial colonies were used to isolate the metabolically active bacterial colony toward daidzein, which were incubated for 3 days in BHI liquid medium containing 0.1 mM daidzein. Each colony from 10 isolated single bacterial colony combination, which showed the positive activity to daidzein, was individually incubated with daidzein to isolate specific bacterial colony(ies) responsible for the conversion of daidzein. Strain Julong 601, which showed the highest metabolic activity to daidzein, was also tested for its ability to transform genistein under the condition described above.

Amplification and Sequencing of 16S rDNA

Chromosomal DNA was extracted from the strain Julong601 using 0.05 N NaOH. One milliliter of the bacterial culture was sampled in an Eppendorf tube and centrifuged at 15,000 ×*g* for 5 min. The supernatant was discarded and 30 µl of 0.05 N NaOH was added in the tube. Duplicates were prepared. The tubes were incubated in boiling water for 15 min for extraction of the genomic DNA. The 16S rDNA sequence of Julong 601 was amplified by employing two universal primers, p27F (5'-AGAGTTTGATCMTGGCTCAG-3'), p1492R (5'-GGYT-ACCTTGTTACGACTT-3') [12]. The polymerase chain reaction (PCR) program used for amplification was as follows: 94°C for 5 min, followed by 29 cycles consisting of 94°C for 1 min, 55°C for 30 sec, and 72°C for 1 min and 30 sec, and a single final extension step consisting of 72°C for 10 min. The PCR product was purified from the electrophoresis gel using an Accu Prep™ gel purification kit (Accu. Chem. Sci. Corp. Westbury, NY, U.S.A.), and a ToPo-cloning kit (Invitrogen™, Carlsbad, CA, U.S.A.) was used for the ligation and transformation of the 16S rDNA PCR-product. Partial nucleotide sequences were obtained by using an ABI 377 XL upgrade DNA Sequencer (Perkin Elmer, Boston, MA, U.S.A.) and software provided by the manufacturer. The internet similarity BLAST search was performed on the full-length sequence of 16S rDNA against database entries. The full-length gene sequence of 16S rDNA for Julong 601 was submitted to NCBI GenBank with an accession number AY269188.

Isolation and Purification of Metabolites

Two-hundred µl of the bacterial culture containing daidzein was extracted with 1 ml ethyl acetate and evaporated to dryness using an Automated Environmental Speed Vac® AES1010 (ThermoSavant, New York, NY, U.S.A.). The residue was redissolved in 200 µl of acetonitrile, and 10 µl of the solution was injected to a Varian HPLC instrument equipped with a photodiode array detector (PDA) and a Waters ODS2 C_{18} column (5 µm particle size, 4.6×250 mm, Fullerton, CA, U.S.A.). The eluting solution consisted of 10% acetonitrile in 0.1% acetic acid (A) and 90% acetonitrile in 0.1% acetic acid (B). Elution was carried out as follows: A:B at 70:30 (v/v) for 15 min; linearly to 50:50 (v/v) for 10 min; linearly to 70:30 for 10 min. The flow rate was 1 ml min^{-1}. Enantiomeric metabolites, *O*-Dma and 2-HPPA, were separated on a Sumi-chiral column, OA-7000 (8×250 mm, Sumica Chem., Osaka, Japan) using a Thermo HPLC P2000 (ThermoSeparation Products, Riviera Beach, FL, U.S.A.). The isocratic mobile phase was 40% acetonitrile in 20 mM potassium buffer (pH 3.0) at a flow rate of 1.0 ml min^{-1}.

For bulk biosynthesis of the metabolites, strain Julong 601 was grown in two 500-ml flasks containing 200 ml BHI liquid medium overnight. Daidzein or genistein (40 mM stock solution in *N,N*-dimethyl formamide) was added to the overnight culture media in the final concentration of 800 µM. The bacterial culture was then incubated in the anaerobic chamber for one week. The bacterial culture was then extracted three times with an equal volume of ethyl acetate, and evaporated to dryness using an Eyela New Rotary Vacuum Evaporator NE (Rikakikai Co., Tokyo, Japan). The condensed metabolites were redissolved in 4 ml methanol and purified on an LC-918 Recycling Preparative HPLC system equipped with a polymeric gel filtration column JAIGEL-W251 (500 mm×ϕ20O.D. and an RI-50 detector (Anal. Ind. Co., Tokyo, Japan). The isocratic mobile phase was 100% methanol at a flow rate of 3 ml min^{-1}.

Identification and Characterization of Metabolites

The purified metabolites of daidzein and genistein were analyzed by EI-MS and NMR spectrometer. EI-MS was obtained on a JMS-AX50510A mass spectrometer (JEOL, Co. Ltd., Japan) in a positive mode (EI^+). The source temperature was 250°C, and the ionization voltage was 70 eV. An electron multiplier of 1.2 kV was used. ^1H and ^{13}C NMR spectra in acetone-d_6 were obtained from a 400 MHz Jnm-La NMR spectrometer (JEOL, Co. Ltd., Japan) at a temperature of 296 K. For ^1H NMR analysis, 16 transients were acquired with a 3 sec relaxation delay using 32 K data points. The 45°C pulse was 5.6 µsec with a special width of 1,000 MHz. ^{13}C NMR was obtained with a special width of 8,000 MHz, collecting 64 K data points. Specific rotation ($[\alpha]_D$) in ethanol was obtained from a Polarimeter Autopol (Rudolph, NJ, U.S.A.). CD measurement in ethanol was recorded on a Jasco J-715 spectrometer (Jasco Co. Tokyo, Japan).

Biotransformation Kinetics of Daidzein and Genistein

Strain Julong 601 was inoculated into 100 ml BHI medium in a 250-ml flask and grown under the anaerobic condition at 37°C overnight. As the optical density of the bacterial culture approached to 1.0 at 600 nm, daidzein or genistein was added to the culture in a final concentration of 0.5 mM. Two-hundred μl of the culture was sampled with time and extracted with 1 ml ethyl acetate. The metabolites were analyzed by HPLC using the methods described previously. All experiments were performed in triplicate.

Results and Discussion

Metabolism of Daidzein and Genistein by Julong 601

Julong 601, newly isolated from human feces, was found to be a Gram-positive anaerobic bacterium. Results of BLAST search of the bacterial nucleotide database revealed that the full-length 16S rDNA gene sequence for the bacterium has 99% similarity to that of *Eubacterium ramulus*.

The elution time and UV spectrum for the metabolite of daidzein, which gave absorbance maxima at 277 and 316 nm, were identical to those of standard *O*-Dma (Fig. 1a). On the other hand, the UV spectrum for the metabolite of genistein showed absorbance maximum at 275 nm (Fig. 1b). EI-MS spectra of the metabolites of daidzein and genistein

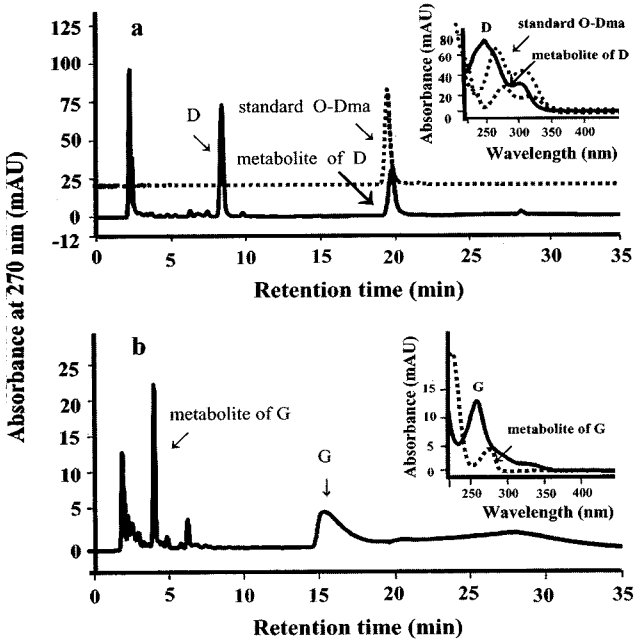

Fig. 1. HPLC elution profiles of daidzein (a) and genistein (b) after 3 days incubation with strain Julong 601 in BHI liquid medium.

Insert: UV spectra of daidzein and genistein (regular line); daidzein metabolite (thin dotted line), synthesized standard *O*-Dma (thick dotted line), and genistein metabolite (dotted line). D, daidzein; G, genistein.

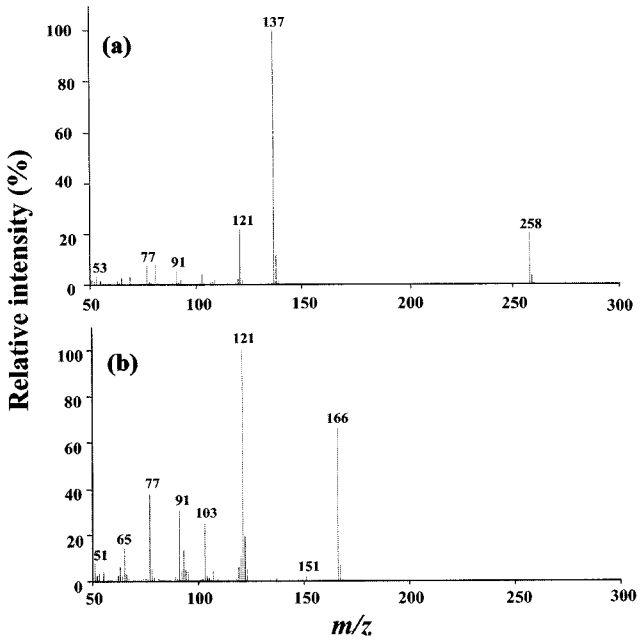

Fig. 2. EI-MS (*m/z*, rel. int.) spectra for the metabolites of daidzein (a) and genistein (b) produced by strain Julong 601.

gave molecular ion peaks of $[M+H]^+$ at *m/z* 258(20) (Fig. 2a) and *m/z* 166(65) (Fig. 2b), consistent with the molecular formulas of $C_{15}H_{14}O_4$ (*O*-Dma) and $C_9H_{10}O_3$ (2-HPPA), respectively (Fig. 3). Other daughter ion peaks of *O*-Dma and 2-HPPA are as follows. *O*-Dma (*m/z*): 137(100), 121(21), 91(5), 77(7); 2-HPPA (*m/z*): 121(100), 103(25), 91(30), 77(37), 65(14). For further structural identification, the metabolites were subjected to ^1H and ^{13}C NMR analyses. ^1H NMR data of *O*-Dma were identical to those of the previously synthesized one [9]. Here, we report for the first time ^{13}C NMR data for *O*-Dma, and ^1H and ^{13}C NMR data for 2-HPPA. NMR data for the biosynthesized *O*-Dma and 2-HPPA are as follows. *O*-Dma: ^1H-NMR ($(CD_3)_2CO$, 400 MHz): 7.88 (1H, d, J=9.0 Hz, H-6'), 7.18 (2H, d, J=8.5 Hz, H-2",6"), 6.76 (2H, d, J=8.5 Hz, H-3",5"), 6.33 (1H, dd, J=9.0 Hz, 2.5 Hz, H-5'), 6.27 (1H, d, J=2.5 Hz, H-3'), 4.75 (1H, q, J=7.0 Hz, H-2), 1.41 (3H, d, J=7.0 Hz, 3-CH_3). ^{13}C-NMR ($(CD_3)_2CO$, 100 MHz): 166.9 (C-4'), 165.5 (C-2'), 157.1 (C-4"), 134.0 (C-1"), 133.7 (C-6'), 129.4 (C-2",6"), 116.4 (C-3",5"), 112.9 (C-1'), 108.7 (C-5'), 103.6 (C-3'), 46.1 (C-2), 19.5 (C-3). 2-HPPA: ^1H-NMR ($(CD_3)_2CO$, 400 MHz): 7.15 (2H, d, J=8.5 Hz, H-2',6'), 6.77 (2H, d, J=8.5 Hz, H-3',5'), 3.61 (1H, q, J=7.0 Hz, H-2), 1.36 (3H, d, J=7.0 Hz, 3-CH_3). ^{13}C-NMR ($(CD_3)_2CO$, 100 MHz): 176.5 (C-1), 157.0 (C-4'), 133.1 (C-1'), 129.3 (C-2',6'), 115.9 (C-3',5'), 45.1 (C-2), 19.2 (C-3).

A single bacterial species from human feces was found to cleave, without any intermediate, the C-ring of isoflavone daidzein [9, 14], which is consistent with the results of our

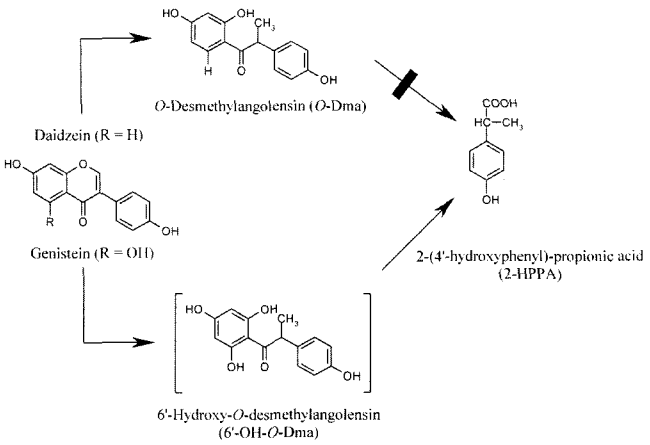

Fig. 3. Proposed metabolic pathways of daidzein and genistein by strain Julong 601 under anaerobic condition.

study. Furthermore, previous works [2, 15] proposed that the final metabolite of genistein cleaved by gut microflora was 4-*p*-ethylphenol because this compound was found in the plasma and urine of rat and man [2, 15]. On the other hand, Coldham *et al.* [4] detected 2-HPPA instead of 4-*p*-ethylphenol as the final metabolite of genistein in rat and human fecal cultures using a labeled ^{3}H-genistein. Based on the researches, Coldham *et al.* [4] proposed the metabolic pathway of genistein in rat as genistein→dihydrogenistein →6-OH-*O*-Dma→2-HPPA→4-*p*-ethylphenol. However, Scheofer *et al.* [14] proposed a different metabolic pathway as genistein→6-OH-*O*-Dma→2-HPPA. In our research, neither dihydrogenistein nor 6-OH-*O*-Dma was detected in the bacterial culture with strain Julong 601. The reason that our study did not show both dihydrogenistein and 6-OH-*O*-Dma could be explained by Coldham *et al.* [4], where they reported that 6-OH-*O*-Dma was transformed very quickly into 2-HPPA. Based on the current results with *O*-Dma production from daidzein and 2-HPPA production from genistein, strain Julong 601 metabolized genistein to 2-HPPA through 6-OH-*O*-Dma (Fig. 3). However, in this study dihydrogenistein is not involved in the metabolism of genistein by strain Julong 601. The reaction to produce 2-HPPA from daidzein was not identified by the strain (Fig. 3).

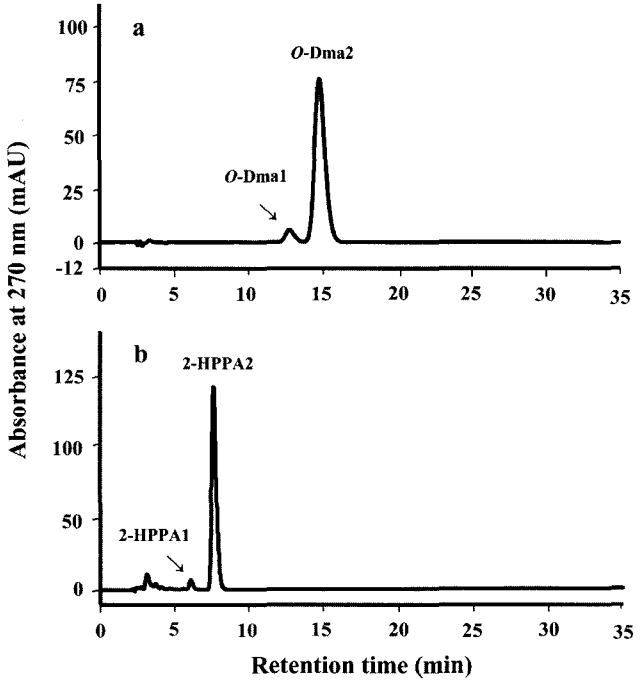

Fig. 4. HPLC elution profiles of *O*-Dma (a) and 2-HPPA (b) on a sumi chiral column, OA-7000.

Isolation and Characterization of Enantiomers

Enantiomers of *O*-Dma and 2-HPPA due to the chirality at C-2 were isolated by chiral stationary phase HPLC (CSP HPLC) (Fig. 4). Enantiomeric excess (% e.e) of *O*-Dma and 2-HPPA were 90 and 98%, respectively, indicating that the high enantioselective manner of the reaction was mediated by strain Julong 601. The $[\alpha]_D^{24}$ values of *O*-Dma2 and 2-HPPA2 were - 139.6° and - 38.6°, respectively. However, $[\alpha]_D^{24}$ values of *O*-Dma1 and 2-HPPA1 could not be measured due to their limited amounts. 2-HPPA2 was determined to have an *S*-form based on the finding that the *S*-form of 2-HPPA isolated from *Pterocarpus indicus* has a

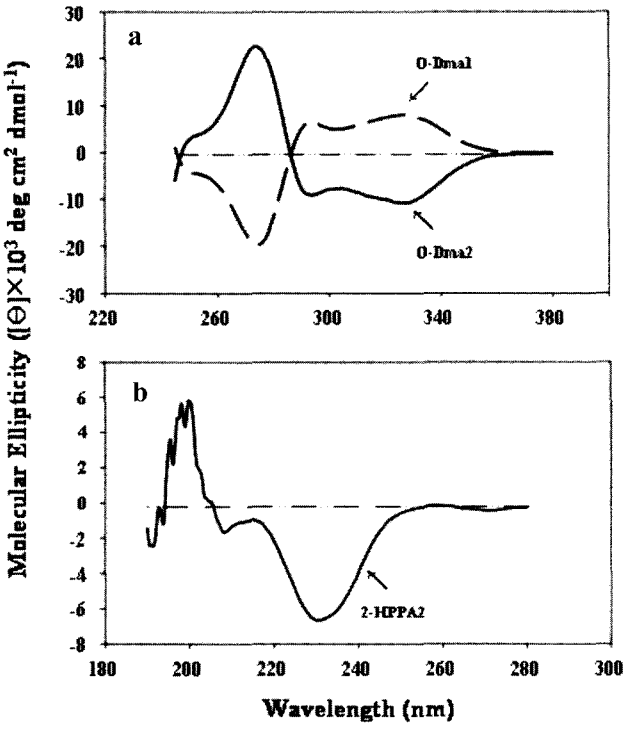

Fig. 5. CD spectra of enantiomers of *O*-Dma (a; *O*-Dma1 with dashed line and *O*-Dma2 with solid line) and 2-HPPA2 (b) in ethanol.

negative $[\alpha]_D^{24}$ value (-36° in $CHCl_3$) [3]. However, the absolute configuration of *O*-Dma2 could not be determined, because reference on the optical rotation of *O*-Dma is not currently available. CD spectra of the enantiomers of *O*-Dma in ethanol exhibited positive and negative cotton effects in the 240-360 nm region (Fig. 5a). Because of the lack of *R*-form of 2-HPPA (2-HPPA1), only the *S*-form of 2-HPPA (2-HPPA2) was measured (Fig. 5b).

Biotransformation Kinetics of Daidzein and Genistein

Kinetics of daidzein and genistein, biotransformed by human intestinal bacterium Julong 601, are shown in Fig. 6. Genistein was transformed into 2-HPPA very rapidly and completely within 6 h of incubation (Fig. 6b), whereas biotransformation of daidzein into *O*-Dma underwent slowly, as shown in Fig. 6a. On the other hand, such a significant difference in the biotransformation susceptibility of daidzein and genistein was not observed in the previous data obtained from the incubation of *Eubacterium ramulus* [14]. With regards the enzymatic susceptibility of genistein, Griffiths *et al.* [5] suggested that the 5-OH group in the A-ring in genistein may contribute to the enhanced C-ring cleavage Taken together, strain Julong 601 showed a high enantiomeric selectivity in terms of the metabolite production from isoflavones daidzein and genistein. The strain appeared to have a relative substrate specificity, in which genistein is more susceptible than daidzein. In addition, the strain produced 2-HPPA via a possible metabolite 6-OH-*O*-Dma from genistein to, which did not occur in the metabolism of daidzein.

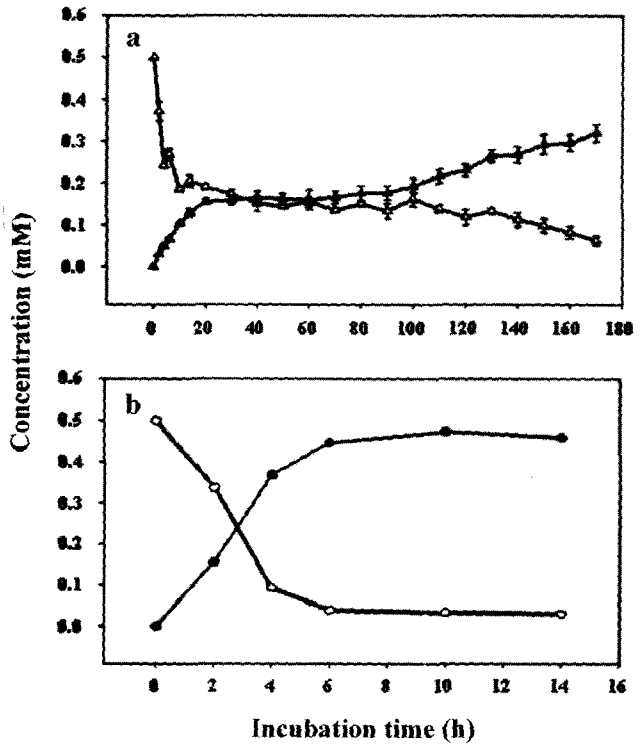

Fig. 6. Biotransformation of daidzein (a; concentrations of daidzein (△) and *O*-Dma (▲)) and genistein (b; concentrations of genistein (○) and 2-HPPA (●)) by strain Julong 601 in BHI medium.

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