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Stereochemical determination of *O*-desmethylangolensin produced from daidzein

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ABSTRACT

We had isolated an *O*-desmethylangolensin (*O*-DMA)-producing bacterium, *Clostridium* rRNA cluster XIVa strain SY8519. According to chiral separation using HPLC, the SY8519-produced *O*-DMA exhibited high optical purity. To determine the absolute stereochemistry of *O*-DMA, we prepared 2-(4-hydroxyphenyl)propionic acid (2-HPPA) from the *O*-DMA using the Baeyer–Villiger reaction. From chiral analysis of the product, the major peak had the same stereochemistry to that of 2-HPPA produced from genistein by the same bacteria. As we have determined the stereochemistry of SY8519-produced 2-HPPA to have an *R* configuration, by the chemical synthesis of (*S*)-2-HPPA, the SY8519-produced *O*-DMA must also possess *R* stereochemistry at the 2-position. To study the stereoselective metabolism, we applied racemic dihydrodaidzein to SY8519. The *O*-DMA was isolated from the culture media and starting material was also recovered. The *O*-DMA produced was optically active in a similar manner to that produced from daidzein. However, the remaining dihydrodaidzein exhibited no difference between the enantiomers. These results suggested that SY8519 produces (*R*)-*O*-DMA from both enantiomers of dihydrodaidzein.

1. Introduction

Considerable attention has been focused on certain foods which can potentially prevent diseases or ageing. Soy isoflavones are amongst the most interesting food ingredients due to the variety of uses. However, previous studies have demonstrated that soy isoflavones, such as daidzein and genistein, are metabolized by intestinal bacteria in certain populations. The study of this metabolism is ongoing, but three metabolites have been identified in animal urine (Coldham et al., 1999; Yasuda, Ueda, & Ohsawa, 2001), human urine (Kelly, Nelson, Waring, Joannou, & Reeder, 1993) and bacterial fermentations (Schoefer, Mohan, Braune, Birringer, & Blaut, 2002; Wang, Kim, Lee, Hur, & Kim, 2004). Daidzein is converted to equol and *O*-desmethylangolensin (*O*-DMA) and genistein produces 2-(4-hydroxyphenyl)propionic acid (2-HPPA). Because all three metabolites have an asymmetric carbon, they have enantiomers. Enantiomers often show significantly different biological activity *in vivo*. Indeed, natural (*S*)-equol (Setchell et al., 2005) exhibits potent activity compared to that of the unnatural form (Muthyala et al., 2004). Therefore, the stereochemistry of the metabolites is important for determining the *in vivo* activity after soy intake.

In 2004, Wang et al. reported that an anaerobic bacteria enantioselectively produced 2-HPPA and *O*-DMA from genistein and daidzein, respectively. The results suggested that the metabolites produced in the human intestine would be produced enantioselectively. However, the stereochemistry of 2-HPPA and *O*-DMA were not determined in the previous study. Therefore, we are interested in the determination of the absolute stereochemistry of the metabolites.

We have isolated a *Clostridium* rRNA cluster XIVa strain SY8519 as an *O*-DMA-producing bacterium (Yokoyama, Niwa, Osawa, & Suzuki, 2010). SY8519 also decomposes genistein to 2-HPPA. We determined the absolute stereochemistry of the biosynthetic 2-HPPA by preparing (*S*)-2-HPPA from commercially available 2-(*S*)-phenylpropionic acid (Niwa, Yokoyama, & Osawa, 2013). We believe that this analytical method would be applicable for the determination of the stereochemistry of *O*-DMA if 2-HPPA was obtained from *O*-DMA. We then examined the preparation of 2-HPPA from *O*-DMA, which was produced by SY8519.

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2. Materials and methods

2.1. Materials

Racemic 2-HPPA, *m*-chloroperbenzoic acid (*m*CPBA) and K_2CO_3 were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Chemically synthesized *O*-DMA and biosynthesized (*R*)-2-HPPA were prepared according to previously published protocols (Niwa, Yokoyama, & Osawa, 2009; Niwa et al., 2013). Dihydrodaidzein was obtained from Toronto Research Chemicals Inc. (Ontario, Canada) or synthesized in our laboratory (Niwa et al., 2009). β-Glucuronidase was purchased from Sigma (St. Louis, MO). Sulfatase and $NaHCO_3$ were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation of O-DMA from daidzein by a SY8519 strain

The fermentation and isolation of *O*-DMA from the culture medium was performed according to a previously reported protocol (Yokoyama et al., 2010). For this study, *O*-DMA was purified by semi-preparative HPLC on a 250 × 8.0 mm i.d. Develosil ODS-5-HG column (Nomura chemical, Aichi, Japan). A solvent mixture containing H_2O/MeOH (55/45) at a flow rate of 2.4 ml/min at ambient temperature was used to elute the compound. *O*-DMA was collected by monitoring the absorbance at 254 nm.

2.3. Synthesis of 2-HPPA from O-DMA

To a reaction mixture of *O*-DMA (4.0 mg), K_2CO_3 (5.6 mg) and $NaHCO_3$ (34 mg) in $CHCl_3$ (3 ml), *m*CPBA (44 mg) dissolved in $CHCl_3$ (1.5 ml) was added (Kiyota, Nakashima, & Oritani, 1999). The reaction was performed by stirring at ambient temperature for 25 h. The reactant was mixed with 5% $NaHCO_3$ (50 ml), and the organic layer was discarded. The aqueous solution was acidified by the addition of 1 M HCl (50 ml) and the organic components extracted with EtOAc (80 ml). The EtOAc solution was washed with distilled water and saturated brine. The solvent was removed under reduced pressure after dried over anhydrous Na_2SO_4. The crude product was added to 35% MeOH (2 ml) and centrifuged. The supernatant was filtered and then subjected to semi-preparative HPLC as previously described (Niwa et al., 2013). Finally, 0.5 mg of 2-HPPA was obtained.

2.4. Chiral analysis using HPLC

Chiral analysis of dihydrodaidzein and 2-HPPA using HPLC was performed according to previously described methods (Wang, Shin, Hur, & Kim, 2005; Niwa et al., 2013). The chiral separation of *O*-DMA was performed with a slight modification of the reported conditions (Wang et al., 2004). Briefly, we used an eluent (20 mM phosphate buffer (pH 3.0):CH_3CN, 75:25) on a SUMICHIRAL OA-7000 column (i.d. 4.6 × 250 mm: Sumika Chemical Analysis Service, Osaka, Japan) at a flow rate of 1.0 ml/min at 40 °C. The UV detector was set to 254 nm.

2.5. Metabolism of dihydrodaidzein to O-DMA by SY8519

Commercially available dihydrodaidzein (20 mg) was metabolized by SY8519 in a similar manner to daidzein (Yokoyama et al., 2010). The culture medium was extracted with EtOAc similar to the method used in the preparation of the metabolites (Yokoyama et al., 2010; Niwa et al., 2013). The resulting residue was applied to a Merck preparative TLC plate (0.5 mm thickness) and eluted with a solvent mixture of *n*-hexane/EtOAc (1:1). Finally, 2.5 mg of *O*-DMA was obtained along with the recovery of 8.8 mg of dihydrodaidzein.

2.6. Isolation of O-DMA from human urine

Five female volunteers (21 and 22 years old) from our laboratory participated in our study with informed consent and approval from the Research ethical committee of our university (#13–007). In the first screening, they drank soy milk (200 ml; Kikkoman, Chiba, Japan) purchased from a local market prior to sleeping. A small amount of urine from each student was collected the next morning. The samples were treated with enzymes to liberate the polyphenols (Hall, O'Brien, & McCormack, 2007). Briefly, each 9 ml sample was mixed with 1 ml of a 0.5 M acetate buffer (pH 5.5). Then, the samples were treated with 250 μl of sulfatase (1000 U/ml) and 250 μl of β-glucuronidase (2500 U/ml) at 37 °C for 2 h. The enzyme-treated samples were extracted with EtOAc (3 × 3 ml), and the extracts were dried under reduced pressure. The residual samples were re-dissolved by the addition of 300 μl of MeOH and then 200 μl of H_2O. The samples were subjected to HPLC with an ODS column used to identify the producer of *O*-DMA.

2.7. Reversed-phase HPLC analysis

HPLC analysis of *O*-DMA was performed with an i.d. 4.6 × 250 mm column (Wakogel-II 5C18HG; Wako). An aqueous 40% MeOH solution was eluted at 1.0 ml/min at 40 °C with monitoring at 254 nm.

2.8. Chiral analysis of O-DMA obtained from human urine

An *O*-DMA producer drank soy milk and urine samples were collected. The sample (200 ml) was treated with the enzymes described above. *O*-DMA was purified in a similar manner to that used in the isolation from the bacterial fermentation. Finally, 0.2 mg of *O*-DMA was isolated and subjected to chiral separation as described in Section 2.4.

3. Results and discussion

Natural products often exhibit optical activity. Daidzein and genistein do not have enantiomers due to their structures. However, these compounds will have an asymmetric carbon after C-ring reduction. Therefore, some metabolites of soy isoflavonoids have enantiomers. In a previous study, Wang et al. (2004) reported that *O*-DMA produced by a bacterium is optically active. However, the stereochemistry has not yet been determined.

Strain SY8519 was identified as an *O*-DMA-producing bacterium by our group. We then analysed SY8519-produced *O*-DMA using a SUMICHIRAL OA-7000 column. The SY8519-produced *O*-DMA was also highly optically active (e.e. 90%). Next, we tried to determine the stereochemistry of the 2-position of *O*-DMA by derivatization to 2-HPPA. We achieved this by synthesising 2-HPPA, as shown in Fig. 1. The Baeyer–Villiger reaction would produce an ester of 2-HPPA from *O*-DMA. The resulting ester would be converted to 2-HPPA by alkaline hydrolysis. Then, we reacted *O*-DMA with *m*CPBA in the presence of K_2CO_3 and $NaHCO_3$. TLC analysis of the reaction product yielded a spot corresponding to the starting material, as well as an additional spot. In spite of our scheme, the product was 2-HPPA, which was identified by HPLC

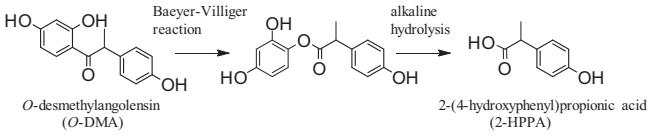

Fig. 1. Synthetic strategy for the preparation of 2-HPPA from *O*-DMA.

analysis and ^{1}H NMR spectroscopy. In a previous study, Corma, Fornés, Iborra, Mifsud, and Renz (2004) described a one-pot phenol production using the Baeyer–Villiger reaction. We have not identified the precise mechanism of this direct reaction. The optical activity of synthesized 2-HPPA was analysed by HPLC, and compared to that of commercially available racemic 2-HPPA and SY8519-produced (*R*)-2-HPPA (Fig. 2). Therefore, the predominant peak for the product was identified as (*R*)-2-HPPA. These results indicated that SY8519 produces (*R*)-*O*-DMA from daidzein, with a high selectivity. This result was reasonable as the same selectivity of the asymmetric reduction of the C-ring in the metabolism of daidzein and genistein is observed by the same bacterium.

Wang, Hur, Lee, Kim, and Kim (2005) reported that (*S*)-equol is produced from dihydrodaidzein by human intestinal bacterium. Recently, Shimada et al. (2012) identified a dihydrodaidzein racemase and described a metabolic pathway that produces optically active (*S*)-equol from racemic dihydrodaidzein. We also metabolized commercially available dihydrodaidzein by SY8519 and isolated the *O*-DMA produced and remaining dihydrodaidzein. Identification was performed by co-injection into an authentic sample for reverse-phase HPLC, and then, the isolated samples were subjected to HPLC equipped with a SUMICHIRAL OA-7000 column. The optical activity (e.e. 88%) of *O*-DMA (Fig. 3) was similar to that of the product from daidzein. The unreacted dihydrodaidzein exhibited no apparent difference between the enantiomers (Fig. 4). This result suggested that SY8519 produced (*R*)-*O*-DMA equally from racemic dihydrodaidzein. Our results were also consistent with previous results concerning bacterial production of equol from dihydrodaidzein racemate (Shimada et al., 2012). In addition, we proposed another mechanism that inverts the stereochemistry of (*S*)-*O*-DMA to the *R*-form, as shown in Fig. 5. Then, we applied commercially available racemic 2-HPPA to SY8519 and analysed the chirality. However, the recovered 2-HPPA still exhibited no optical purity (data not shown). These results suggested that very little (*S*)-*O*-DMA was produced from daidzein and racemic dihydrodaidzein. Therefore, we concluded that SY8519 has

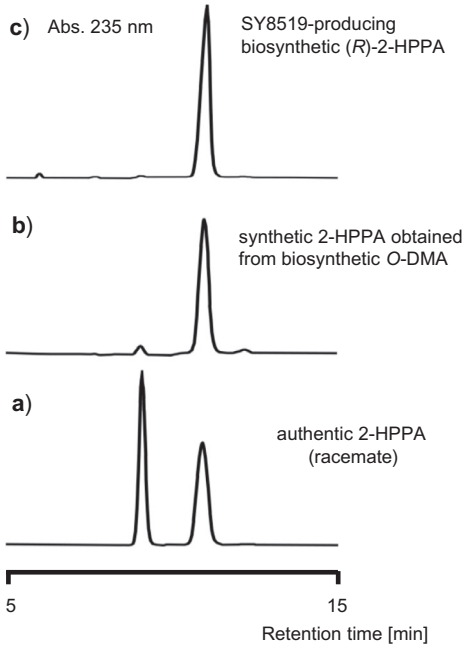

Fig. 2. Chiral separation of 2-HPPA obtained from SY8519-producing *O*-DMA. HPLC analysis was performed using a CHIRALPAK IB column as described previously (Niwa et al., 2013). (a) authentic racemic sample, (b) synthetic 2-HPPA from SY8519-produced *O*-DMA, and (c) strain SY8519 produced (*R*)-2-HPPA.

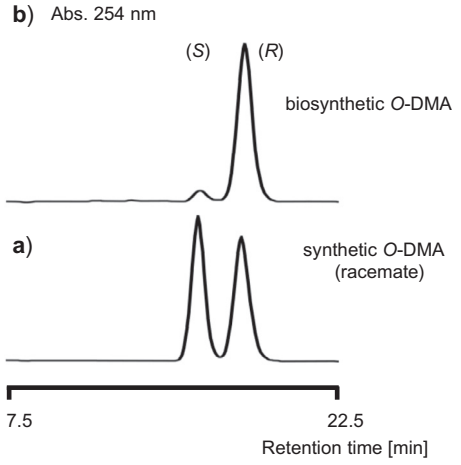

Fig. 3. Chiral separation of *O*-DMA produced from racemic dihydrodaidzein by a SY8519 strain. Dihydrodaidzein (20 mg) was metabolized by SY8519 for 2 days. *O*-DMA (2.5 mg) was isolated from the culture medium where 8.8 mg of dihydrodaidzein was recovered as described in Section 2. A solvent mixture (20 mM phosphate buffer (pH 3.0):CH_3CN, 75:25) was eluted on a SUMICHIRAL OA-7000 column at 1.0 ml/min at 40 °C. (a) Synthetic racemic sample and (b) isolated dihydrodaidzein purified from SY8519 strain product.

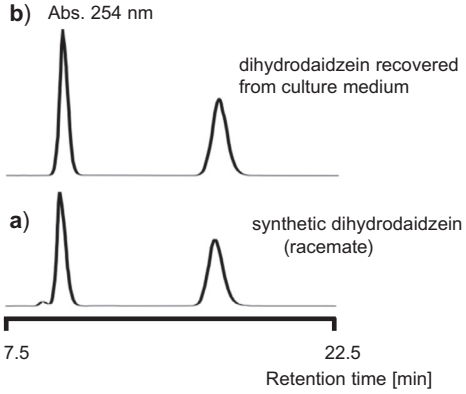

Fig. 4. Chiral separation of recovered dihydrodaidzein, which was obtained in Fig. 3. The samples were analysed by a previously published protocol (Wang et al., 2004). (a) Synthetic racemic sample and (b) an isolated sample recovered from the culture medium.

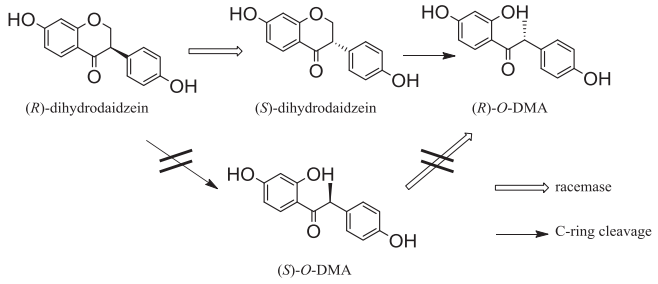

Fig. 5. Proposed mechanism for the production of (*R*)-*O*-DMA from raceme dihydrodaidzein by SY8519.

dihydrodaidzein racemase activity and produces (*R*)-*O*-DMA from racemic dihydrodaidzein, as shown in Fig. 5. The results in Figs. 3 and 4 suggested that the reaction of the racemase is faster than the cleavage of the C-ring of (*S*)-dihydrodaidzein.

Several *O*-DMA producing bacteria have been isolated (Hur et al., 2002; Schoefer et al., 2002; Yokoyama et al., 2010), and many

more unidentified *O*-DMA producing bacteria still exist, including a number in the human intestine. Next, we examined whether the SY8519-produced *O*-DMA structure is common. We analysed the stereochemistry of *O*-DMA obtained from human urine. Five female students in our laboratory drank soy milk prior to going to sleep and their urine was collected the next morning. Soy isoflavonoids are typically present as conjugates of glucuronide and sulfate in human urine (Clarke et al., 2002). Therefore, the five urine samples were treated with glucuronidase/sulfatase and subjected to reversed-phase HPLC. Although we did not perform a quantitative analysis, such as the recovery of daidzein or *O*-DMA concentration, only one sample had an apparent *O*-DMA signal on the chromatogram. Next, we isolated *O*-DMA from human urine and analysed the optical purity using HPLC. The isolated *O*-DMA also predominantly contained the *R*-enantiomer on the chromatogram. However, the optical purity (e.e. 80%) was less than that of the SY8519-produced one. We believe that the optical purity of the fragile α-position of the carbonyl was reduced by some process, such as glucuronidase/sulfatase treatment or circulation in the body. Because only one result was available for determination of the stereochemistry of *O*-DMA obtained from human urine, we were unable to determine if this was the case. However, our result indicated that some people selectively produce (*R*)-*O*-DMA from daidzein even though there is still the possibility of the presence of (*S*)-*O*-DMA-producing bacteria.

Recently, the importance of soy isoflavonoid metabolism has received considerable interest. However, studies are primarily focused on equol production due to its potent activity. However, studies involving *O*-DMA are limited (Kinjyo et al., 2004; Choi & Kim, 2013). Therefore, further investigation of soy isoflavone metabolites is required to understand the precise role of the activity of soy isoflavones *in vivo*. In these studies, we must also consider the stereochemistry of the soy metabolites, not only *O*-DMA but also 2-HPPA, to determine their precise activity, as has been performed for equol (Muthyala et al., 2004; Brown et al., 2010).

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