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Cleavage of Methyl Ethers by O-Desmethylangolensin-Producing Clostridium Strain SY8519

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Abstract

We previously isolated an *O*-desmethylangolensin (*O*-DMA)-producing bacterium, Clostridium rRNA cluster XIVa strain SY8519. We studied the metabolism of soy isoflavonoids by strain SY8519 and found that the bacterium attenuated the activity of isoflavonoids by "decomposition". In this study, the *O*-methyl derivatives of isoflavones formononetin and biochanin A were fed to the bacterium. Formononetin and biochanin A were metabolized to *O*-DMA and 2-(4-hydroxyphenyl) propionic acid, respectively, which are the products of the original isoflavones. We could detect daidzein as an intermediate in the production of *O*-DMA from formononetin by time course analysis of the culture medium. Therefore, strain SY8519 can cleave the *O*-methyl ethers of isoflavones. We then examined the demethylation of *O*-methyl derivatives of gallic acid methyl ester by strain SY8519. The metabolite was almost 3-*O*-methylgallic acid methyl ester but not gallic acid ester. These results suggest that strain SY8519 also cleaves the *O*-methyl ethers of certain phenolic compounds by demethyl enzyme. We also compared the activities of the *O*-methyl derivatives of gallic acid methyl ester. As the number of the methyl ether decreased, the activities in tyrosinase inhibition and antioxidant assay increased. Therefore, compared with humans who have different microflora, humans who have strain SY8519 or who have similar microbial activity in their intestine would experience different effects after the intake of phytochemicals.

Keywords: *O*-desmethylangolensin-producing bacteria; Human; Metabolism; Microflora; Soy isoflavonoids

Introduction

In 1962, Westlake et al. reported the microbial degradation of flavonoids [1]. Nowadays, more than half century later, the microbial metabolism of soy isoflavones is interesting because of the unique activity of equol, an intestinal metabolite of daidzein [2]. However, soy isoflavones such as daidzein and genistein are also metabolized to other metabolites, such as *O*-desmethylangolensin (*O*-DMA) and 2-(4-hydroxyphenyl) propionic acid (2-HPPA) by intestinal bacteria in certain populations [3]. We have also isolated a Clostridium rRNA cluster XIVa strain SY8519 [4] that metabolizes daidzein and genistein to *O*-DMA and 2-HPPA, respectively (Figure 1a). We studied the metabolism of strain SY8519 and determined the absolute stereochemistry of 2-HPPA and *O*-DMA it produces [5,6]. In our previous study, we found that optically active (*R*)-*O*-DMA was obtained from racemic dihydrodaidzein and suggested the presence of a dihydrodaidzein racemase in the bacterium. Therefore, *O*-DMA may be obtained by cleavage of the C-O bond in the C-ring of dihydrodaidzein, which is an ether cleavage reaction. In the present study, we examined the cleavage of ether bonds by strain SY8519.

Materials and Methods

Materials

Formononetin and biochanin A were purchased from LKT Laboratories, Inc. (St. Paul, MN). 3,4,5-Tri-*O*-methylgallic acid methyl ester was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Methyl syringate (3,5-di-*O*-methylgallic acid methyl ester) was purchased from Alfa Aesar (Heysham, UK). Gallic acid methyl ester and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Mushroom tyrosinase and trimethylsilyldiazomethane were obtained from Sigma (St. Louis, MO).

Metabolism of soy isoflavonoids by strain SY8519

The fermentation of strain SY8519 and extraction of its metabolites were performed using procedures similar to those previously described [4]. In brief, the bacterium was grown in 200 mL Gifu Anaerobic Medium (GAM; Nissui Pharmaceuticals, Tokyo, Japan). Aliquots of this culture medium (10 mL) were distributed to sterilized vials, the isoflavonoid samples were added to the vials to a final concentration of 50 μ, respectively, and then the vials were incubated at 37°C under anaerobic conditions. After the incubation, the medium was acidified with 6 M HCl (0.5 mL), and the product was extracted using three, 3-mL aliquots of ethyl acetate. The solvent was removed under reduced pressure, the resulting residue was dissolved in 1.0 mL methanol, and a sample was subjected to high-performance liquid chromatography (HPLC) analysis after centrifugation.

HPLC analysis

The samples were analyzed using an HPLC apparatus equipped with a 250 × 4.6 mm i.d. Wakosil- II 5C18HG column (Wako). A solvent mixture containing water/methanol (55/45) at a flow rate of 1.0 mL/min at 40°C was used to elute *O*-DMA, daidzein, and formononetin. The peaks were determined by monitoring the absorbance at 254 nm.

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For 2-HPPA analysis, a water/methanol/trifluoroacetic acid (75/25/0.1) mixture was used as the eluent, and the UV detector was set to 235 nm. After each analysis, the column was washed with methanol, and then conditioned for the next analysis. The eluted peaks were identified by the co-injection with authentic samples.

Isolation of the methyl syringate metabolite produced by strain SY8519

The metabolite of methyl syringate by the bacterium was isolated using a procedure similar to that described previously [4,5]. In brief, the bacterium was grown in two bottles containing 500 mL GAM and 25 mg methyl syringate for 4 days. An ethyl acetate extract was applied to a Merck preparative TLC plate (1.0 mm thickness) and eluted with a solvent mixture containing hexane/ethyl acetate (1:1). Finally, the methyl syringate metabolite was purified by semi-preparative HPLC on a system equipped with 250 × 8.0 mm i.d. Develosil ODS-5-HG column (Nomura chemical, Aichi, Japan). The compound was eluted at ambient temperature using water/methanol/trifluoroacetic acid (80/20/0.1) at a flow rate of 2.4 mL/min. Finally, 3.5 mg of methyl 3-*O*-methylgallic acid methyl ester **(3)** was obtained by monitoring the absorbance at 254 nm.

Antioxidative assay using DPPH

A 0.25 mM ethanolic solution of DPPH (180 μM) was added to samples (0.1, 1.0 mM; 20 μM) dissolved in ethanol in a 96-well plate using a procedure similar to one previously reported [7]. After 5 min at ambient temperature, the absorbance at 520 nm was measured by using a Bio-Rad iMark microplate reader (Hercules, CA).

Mushroom tyrosinase inhibition

Reactions were performed in 96-well plates using the method described by Chen et al. with minor modifications [8]. In brief, solvent mixtures containing 1.0 mM L-tyrosine (170 μM) dissolved in 50 mM phosphate buffer (pH 6.8), ethanolic samples (10 μM), and 1,000 U/mL mushroom tyrosinase (20 μM) were placed in a plate. After brief gentle shaking, the reaction was allowed to proceed at ambient temperature for 30 min. The dopaquinone produced in each reaction was measured by determining the absorbance at 490 nm using a Bio-Rad iMark microplate reader.

Statistical analysis

The *p* values were calculated with the unpaired Student's *t* test. The significance level was set to 1% for each analysis.

Results and Discussion

In the initial experiments, formononetin '4'-*O*-methyl daidzein' was fed to strain SY8519. *O*-DMA was produced from formononetin as it had been from daidzein although the final concentration of the *O*-DMA produced from formononetin was lower than that produced from daidzein (Figure 1b). We then analyzed the time course of the metabolism to confirm the production of daidzein as a demethylated intermediate. As shown in Figure 1c, the concentration of *O*-DMA increased in a time dependent manner but a small amount daidzein was detected at an early stage. Figure 1c suggests that daidzein was indeed produced by demethylation as an intermediate. The daidzein produced was easily metabolized to *O*-DMA, whereas the rate of formononetin demethylation was slower than that of the consumption of daidzein. Since the concentration of formononetin decreased by the reaction processed. The production of daidzein would also decrease due to the reduction of the substrate formononetin. Thus, we think that the difference illustrated in figure 1b might have occurred even though we did not yet studied other pathways such as through *O*-methyl ether of *O*-DMA.

Figure 1c illustrates that strain SY8519 can cleave methyl ethers in the formononetin metabolism. This further suggests that the intestinal bacterium may produce 2-HPPA from biochanin A '4'-*O*-methyl genistein'. Therefore, we examined the production of 2-HPPA from biochanin A. We also compared the rate of metabolism using formononetin and biochanin A as the sbstrates. For this purpose, the two substrates were added to the SY8519-containing culture media, divided from one bottle, to achieve a final concentration 50 μ. After 2 days of incubation, the products were analyzed by HPLC. Figure 1d shows that 2-HPPA was produced from biochanin A and that the rate of this reaction was faster than that of the production of *O*-DMA from formononetin. Comparing the metabolism of daidzein and genistein, 2-HPPA was easily produced yielding data similar to those in figure 1d (data not shown). Therefore, we think that the difference in reaction rates between formononetin and biochanin A (Figure 1d) was mainly due to the subsequent steps, which involve cleavage of the C-ring of the dihydro-isoflavones, but not demethylation.

We were interested whether the demethylation performed by strain SY8519 depends on the substrate or the position of the ether. However, Figure 1 suggested that formononetin and biochanin A would not be good substrates for the study because the demethylation products were further metabolized. In subsequent experiments, we

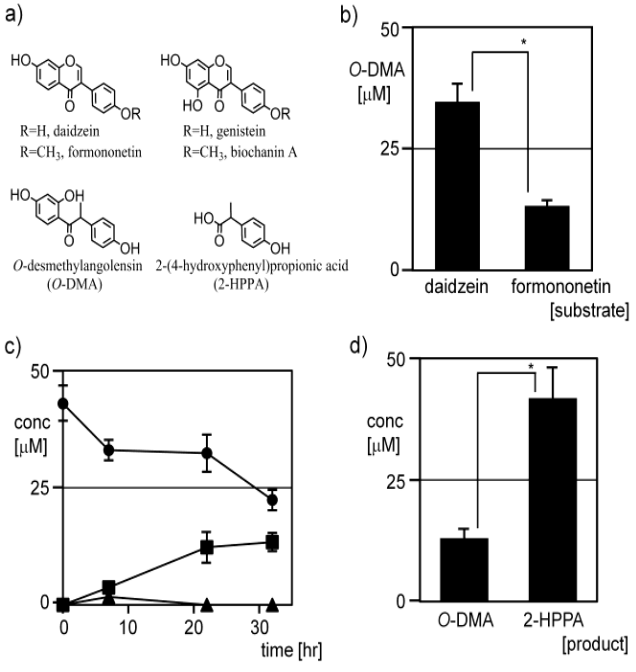

Figure 1: The metabolism on formononetin and biochanin A by strain SY8519:
a) Structures of soy isoflavonoids and metabolites studied.
b) Comparing the production of *O*-DMA from formononetin and daidzein. Samples (50 μM) were metabolized for 2 days in the presence of strain SY8519.
c) Time course of the metabolism of formononetin.
●: formononetin, ▲: daidzein, ■: O-DMA
d) Comparing the production of *O*-DMA and 2-HPPA from formononetin and biochanin A using 50 μM samples.
Figures are expressed as the mean ± SD of the three experiments. Bars represent standard deviations. **p*<0.01.

used methyl syringate (Figure 2a) as the substrate because we recently studied leptosperin, a gentiobiose derivative of **2**, obtained from Manuka honey [9]. Leptosperin have antibacterial activity and would be helpful for human beings. However, the metabolism of leptosperin in our body is not studied. Then, we first examined the metabolism by strain SY8519 using the aglycone. Metabolism of **2** by strain SY8519 produced a novel spot on TLC (hexane/ethyl acetate; 1/1). The spot had stronger reducing activity than **2**, as evidenced by its reaction with $FeCl_3$. At first, we thought that this product was methyl gallate (**4**) obtained by the demethylation, but commercially available methyl gallate displayed a different *Rf* value in this TLC analysis. The product was detected as a spot between **2** and **4** on the TLC plate. From its TLC profile and its potent reducing activity, we then thought the metabolite was a monomethyl ether of **4**, which had a catechol moiety (**3**). This structure was supported by the fact that the metabolite was produced as a minor product of the treatment of methanolic solution of **4** with trimethylsilyldiazomethane in the presence of Et_3N. We also isolated the metabolite from the culture media and compared its ^1H NMR spectrum with those reported [10]. With these data, we identified the metabolite to be 3-*O*-methylgallic acid methyl ester (**3**).

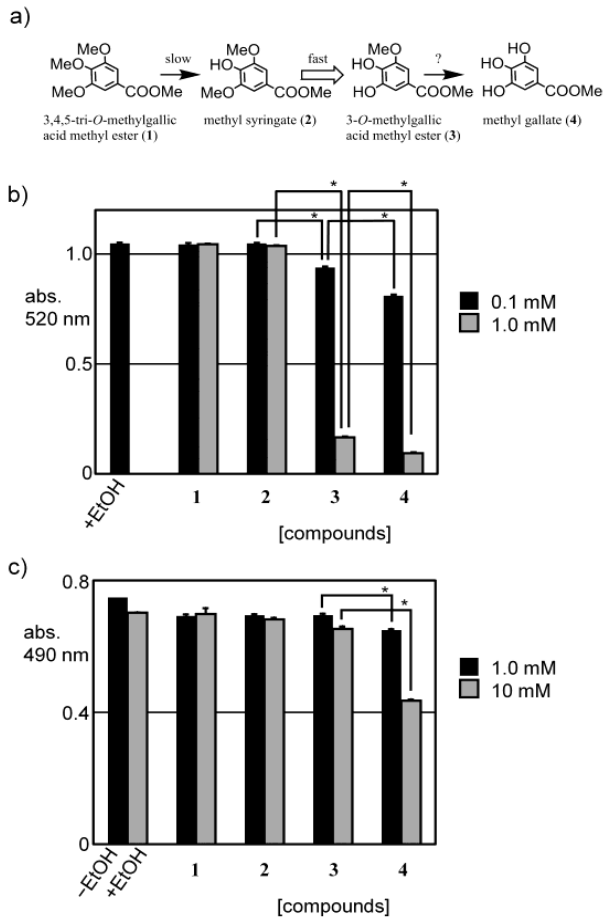

Figure 2: Demethylation of methyl gallate derivatives by strain SY8519:
a) Proposed mechanism for the production of 3-*O*-methylgallic acid methyl ester (**3**) from 3,4,5-tri-*O*-methygallic acid methyl ester (**1**).
b) Antioxidant activity of methyl gallate derivatives using DPPH.
c) Tyrosinase inhibition by methyl gallate derivatives.
Figures are expressed as the mean ± SD of three experiments. Concentration means that of the added sample and not the final concentration in the reaction mixture. Bars represent standard deviations although some bars are difficult to see because of the precision of the data. *p<0.01.

We then used 3,4,5-tri-*O*-methylgallic acid methyl ester (**1**) as an substrate. However, we did not observe major products other than **3** by TLC. The reactivity was similar to that reported for *Pseudomonas putida* [11]. Therefore, we did not observe selectivity for demethylation between the 3-*O*- and 4-*O*-methyl moieties of **1**. From the results, however, we conclude that strain SY8519 has selectively for the demethylation as like reported enzymes [11,12]. The different rate of demethylation produced **3** as a metabolite from **1** and **2** (Figure 2a).

We have reported that strain SY8519 produce (*R*)-*O*-DMA from racemic dihydrodaidzein [6]. However, we could not determine whether the mechanism of C-ring cleavage in dihydrodaidzein was because of the demethylase enzyme described herein. Some *O*-demethylases have been isolated [12,13]; however, their activity on the chroman skeleton such as dihydrodaidzein have not yet been determined. Therefore, we could not elucidate the mechanism by which the C-ring of dihydrodaidzein is cleaved to produce *O*-DMA. However, strain SY8519 is a unique strain that has both dihydrodaidzein racemase and demethylase. Moreover, the bacterium possesses an unidentified mechanism that produces 2-HPPA from genistein.

We began our study of soy isoflavonoid metabolism by human intestinal bacteria because the bacterial product equol has activity different from that of the substrate daidzein [14]. We have also compared the activities of the soy isoflavonoid metabolites with those of daidzein and genistein [5,15]. Therefore, we were interested in the activity of methyl 3-*O*-methylgallate (**3**), which is a metabolite produced by strain SY8519. Then, we examined the four *O*-methyl derivatives of methyl gallate (**1**-**4**) in an antioxidative activity assay and an enzyme inhibition assay. Figure 2b illustrates that the demethylation of methyl syringate enhanced the antioxidant activity, which might due to the presence of the catechol moiety of **3**. On the other hand, this catechol formation was not sufficient for tyrosinase inhibition (Figure 2c). From this two results as illustrated in Figures 2b and 2c, one demethylation does not always affect the activity. However, the demethylation might alter the activity of polyphenols in some cases.

In general, plant polyphenols are not present as the aglycones, but are present as derivatives, such as esters and glucosides. These polyphenols are thought to be liberated to the corresponding aglycones in the small intestine [16,17]. However, the metabolism of these methylated compounds is not fully understood. Some methyl ethers of polyphenols have different activity than that of the native form [18-20]. Our results suggest that certain populations alter the activity of polyphenol methyl ethers through intestinal demethylation.

In our previous reports [5,15], we suggested that the metabolism of daidzein and genistein by strain SY8519 attenuated their activities. In the present study, however, the same bacterium enhanced the activities of **1** and **2** (Figure 2). Recently, intestinal metabolism by bacteria is interested especially due to the production of potent phytoestrogen, equol [2]. However, several intestinal bacteria could not grow *in vitro* and still much intestinal bacteria are unidentified. Moreover, the bacterial metabolism of chemical compounds in the intestine is hardly elucidated. Therefore, animal studies including those using humans involving the consumption of phytochemicals will have to consider the metabolism of phytochemicals *in vivo*, including that caused by intestinal bacteria, which varies from individual to individual.

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