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Biotransformation of Plant Secondary Metabolite Decursin by *Mycobacterium* sp. PYR1001

Ki-Yeon Kim,[†] Sanghyun Lee,[‡] and Chang-Jun Cha*,[†]

[†]Department of Biotechnology (BK21 Program) and [‡]Department of Applied Plant Science, Chung-Ang University, Anseong 456-756, Republic of Korea

Decursin and its structural isomer decursinol angelate are major secondary metabolites in the root of *Angelica gigas* Nakai which possess several chemotherapeutic properties. We isolated bacteria capable of transforming decursin and determined metabolites and biotransformation kinetics. Decursinol angelate was not metabolized to any significant extent. Resting cells of *Mycobacterium* sp. PYR1001 were able to transform decursin. After 24 h incubation, 5 mM of decursin was completely transformed to a metabolite, the structure of which was determined by NMR and mass spectral analyses to be decursinol. This conversion was shown to be catalyzed by an esterase activity, and the activity was found to be specific for decursin. These results suggest that strain PYR1001 can be successfully used to transform decursin for the production of decursinol, a compound known to have cancer chemopreventive activity.

KEYWORDS: Biotransformation; *Angelica gigas*; decursin; decursinol; *Mycobacterium*

INTRODUCTION

The dried root of *Angelica gigas* Nakai (Umbelliferae), Cham-Dang-Gui (the Korean name), has been traditionally used in oriental herbal medicines for thousands of years to treat colds, hepatitis, arthritis, typhoid, and many other disorders and marketed as a functional food for women's health care in Europe and the United States (*1*). The curative potential of *A. gigas* has led to efforts to isolate bioactive secondary metabolites from this plant, and several coumarin derivatives have been isolated (*2*). Among these coumarins, decursin and decursinol angelate (**Figure 1**) are the most abundant components in the root of *A. gigas* (*3*) and have been shown to exhibit several pharmacological properties such as neuroprotective (*4*), antibacterial (*5*), antiangiogenic (*6*), and cancer chemotherapeutic activities (*7*−*10*).

Plant secondary metabolites are not only sources of drugs but also because of their chemical diversity and complexity they provide potential structural scaffolds for the synthesis of novel drug candidates (*11*, *12*). On the other hand, the chemical complexity often represents a major obstacle in the production of these pharmaceuticals by chemical synthesis. Isolation of some pharmaceutically active compounds from their natural sources is also typically low yielding (*11*). Biotransformation technology by using a wide variety of microbial metabolic activities could potentially be used to develop novel pharmaceuticals. Many pharmaceutical candidates were successfully produced by biotransformation processes (*11*−*13*). Moreover, large quantities of metabolites can be produced by microbial transformation more effectively than by experimental animals, cell cultures, or mammalian enzyme systems (*14*, *15*).

Since decursin and decursinol angelate have a variety of biological activities and their contents in the plant are much higher than any other coumarins, biotransformation studies were conducted to determine if bacteria are capable of metabolizing decursin and/or decursinol angelate to produce biologically active compounds.

*To whom correspondence should be addressed. Phone: +82 31 670 4840. Fax: +82 31 675 0432. E-mail: cjcha@cau.ac.kr.

MATERIALS AND METHODS

Chemicals and Reagents. Crystalline decursin and decursinol angelate were prepared as described by Lee et al (*5*). Media for microbial culture were purchased from Conda (Madrid, Spain). Dimethyl sulfoxide (DMSO), HPLC-grade acetonitrile, and ethyl acetate were obtained from Fisher Scientific (Somerville, NJ). NMR solvents were purchased from Isotec, Inc. (Miamisburg, OH).

Bacterial Strains and Culture Conditions. Two bacterial strains capable of degrading pyrene were tested for their ability to transform decursin and decursinol angelate. Pyrene-degrading bacteria were initially isolated by enrichment culture using pyrene as a sole source of carbon and energy. The strains were cultured in R2A medium at 30 °C by shaking at 200 rpm.

Identification of Bacterial Strains. Bacterial strains designated as PYR1001 and US6-1 were identified by analysis of 16S rRNA gene sequence and further confirmed by physiological and biochemical tests. The 16S rRNA gene was amplified by PCR using 27F and 1492R primers (*16*), and the nucleotide sequence was determined at the Macrogen DNA Sequencing Center (Seoul, Korea).

Biotransformation Conditions. Bacterial cells were harvested at the exponential growth phase by centrifugation, washed twice with 100 mM potassium phosphate buffer (pH 7.0), and resuspended in a reduced volume of the reaction mixture. These resting cells were used for biotransformation reactions. The reaction was initiated by adding decursin or decursinol angelate at a final concentration of 5 mM to the resting cell suspension. The reaction mixtures (a total volume of 2 mL) were incubated for 24 h at 30 °C on a rotary shaker operating at 200 rpm.

Extraction, Isolation, and Analysis of Metabolites. The reaction mixtures were extracted three times with 4 mL of ethyl acetate. The ethyl acetate phase was recovered and evaporated in vacuo. The resulting residue was dissolved in acetonitrile and filtered through a 0.2 μm nylon membrane.

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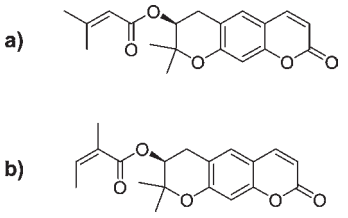


Figure 1. Chemical structures of (a) decursin and (b) decursinol angelate.

HPLC analysis was carried out using an OmniSpher C18 column (5 μm, 250 × 4.6 mm, Varian) attached to a ProStar 335 system (Varian) with a photodiode array detector monitored at 340 nm. Elution was performed using a linear gradient of 40−95% acetonitrile over 20 min at 1.0 mL/min. The metabolites were fractionated using a semipreparative scale HPLC system consisting of the same type of column (5 μm, 250 × 10.0 mm, Varian) and a flow rate of 5.0 mL/min. The metabolite was isolated and further analyzed for its structural identification by gas chromatography−mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) spectrometry.

Identification of Metabolite. The metabolite was dissolved in deuterated acetonitrile (99.96 atom % ^{2}H) with TMS as an internal standard for ^{1}H NMR analysis. The NMR measurements were made at 300 MHz on a Gemini 2000 spectrometer (Varian). The mass spectral analyses were performed on GC-MS (Micromass Autospec) equipped with a DB-5 column (30 m, 0.25 μm film thickness, 0.2 mm i.d.; Agilent Technologies). The mass spectrometer was operated in electron impact (EI) mode at 70 eV.

Preparation of Cell-Free Crude Extracts. Cells were harvested from 400 mL of R2A culture broth at the exponential growth phase and washed twice with 100 mM potassium phosphate buffer (pH 7.0). The cells were disrupted by an ultrasonicator (model 5501, Ohtake, Tokyo). The lysate was centrifuged at 13 000*g* for 1 h, and then the resulting supernatant was passed through a 0.2 μm sterilized syringe filter to remove cell debris and unbroken cells. The filtrate was used as a crude enzyme extract for biotransformation activity. The protein concentration of cell-free extracts was determined by the method of Bradford (*17*) using the Bio-Rad protein assay kit (Bio-Rad Laboratories).

Enzymatic Biotransformation. Biotransformation activity by cell-free extract was assayed in 100 mM potassium phosphate buffer (pH 7.0) by measuring the consumption of the substrate using HPLC. The cell-free extract was incubated with 1 mM decursin in a final reaction volume of 2.5 mL for 400 min at 30 °C. Samples (250 μL) were taken at intervals, and then the reaction was terminated by extracting with ethyl acetate. The resulting extracts were subjected to HPLC for analysis of decursin and decursinol. Boiled cell-free extract was used as a control.

RESULTS AND DISCUSSION

Screening for Bacteria Capable of Biotransformation. When resting cells of PYR1001 and US6-1 were incubated with 5 mM of decursin and decursinol angelate as substrates, respectively, only strain PYR1001 was able to metabolize decursin and a new metabolite was observed with an HPLC retention time of 16.1 min (**Figure 2**). No strains transformed decursinol angelate (data not shown). Control experiments containing the boiled cells of strain PYR1001 showed no metabolism, suggesting that the transformation of decursin was biologically mediated.

Identification of Strain PYR1001. Strain PYR1001 was strictly aerobic, Gram-positive, oxidase-positive, and catalase-positive. The 16S rRNA gene sequence analysis revealed that strain PYR1001 has the highest sequence similarity of 99.4% with *Mycobacterium gilvum* ATCC43909^T.

Biotransformation of Decursin by *Mycobacterium* sp. PYR1001. The kinetics of formation of decursin metabolite by resting cells of *Mycobacterium* sp. PYR1001 are shown in **Figure 3**. While the amount of decursin continued to decrease during the incubation time of 24 h, there was a corresponding increase in the amount of reaction product. After 16 h incubation, 95% of decursin was transformed. No changes were observed in control experiments. The final yield of the product was 52%.

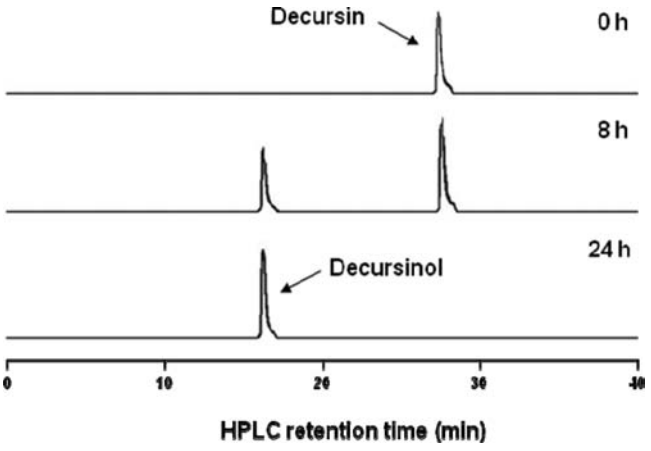


Figure 2. HPLC profiles of the biotransformation of decursin to decursinol by the resting cells of *Mycobacterium* sp. PYR1001.

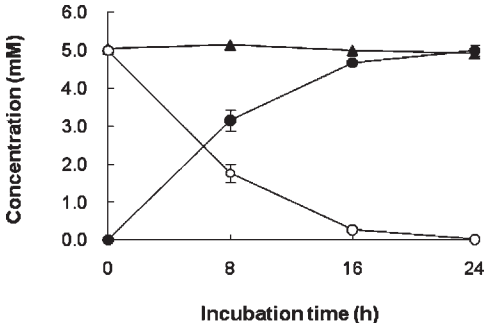
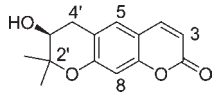


Figure 3. Biotransformation kinetics of decursin by the resting cells of *Mycobacterium* sp. PYR1001: (▲) decursin control; (○) decursin; (●) metabolite. Control experiments were performed using the boiled cells of strain PYR1001. The error bars correspond to the standard deviations of duplicate experiments.

Identification of Biotransformation Metabolite. The metabolite was purified by HPLC and subjected to mass spectrometry and ^{1}H NMR spectroscopy for its structural identification. ^{1}H NMR spectra of a coumarin moiety (δ 6.16 and 7.74, a doublet of 1H each due to H-3 and H-4) and a pair of aromatic protons para to each other (δ 6.68 and 7.31, a singlet of 1H each), along with a geminal dimethyl group (δ 1.31 and 1.29, a singlet of 3H each) and a $-CH_2CH$ system (δ 2.77 and 3.06, 1H each and δ 3.80 due to H-3′ proton) indicated its similarity to decursinol. ^{1}H NMR data and the chemical structure of the metabolite are shown in **Table 1**. The *m*/*z* ratio of the metabolite was determined to be 247 $[M + H]^+$ (calcd for $C_{14}H_{15}O_4$, 247). Both ^{1}H NMR spectra and molecular mass data were in accordance with published data for decursinol (*19*−*20*). Thus, the isolated metabolite was successfully identified as decursinol.

Transformation of Decursin to Decursinol by Cell-Free Extracts from *Mycobacterium* sp. PYR1001. Because we speculated that the reaction mechanism for the biotransformation of decursin to decursinol involved hydrolysis of decursin to decursinol and 3-methylbut-2-enoic acid, an esterase would have been the enzyme responsible for catalyzing the reaction. To confirm whether the reaction occurred enzymatically, cell-free extract was used for the biotransformation of decursin to decursinol. As shown in **Figure 4a**, decursin was also transformed to decursinol by cell-free extract. Boiled extract did not convert the substrate (data not shown). The activity increased linearly according to the different concentrations of protein used in the assay (**Figure 4b**), suggesting that the reaction

Table 1. Assignment of ^{1}H NMR Data and the Chemical Structure of the Metabolite Produced by *Mycobacterium* sp. PYR1001



position	^{1}H NMR for metabolite, ppm (*J*, Hz)
4	7.74 (1H, d, 9.5)
5	7.31 (1H, s)
8	6.68 (1H, s)
3	6.16 (1H, d, 9.5)
3′	3.80 (1H, t, 5.3)
4′a	3.06 (1H, dd, 17.0, 5.3)
4′b	2.77 (1H, dd, 17.0, 5.3)
gem $CH_3 \times 2$	1.31 (3H, s), 1.29 (3H, s)

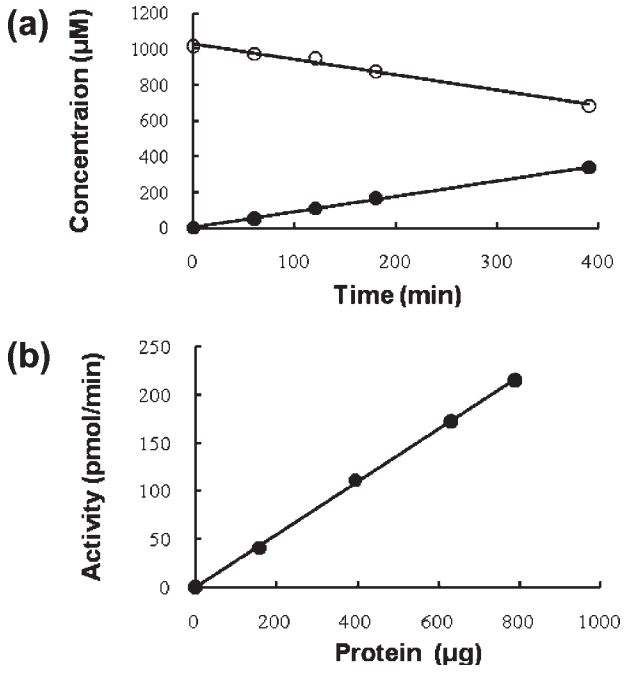


Figure 4. Enzymatic biotransformation of decursin to decursinol by the cell-free extracts of *Mycobacterium* sp. PYR1001: (**a**) enzymatic consumption of decursin (○) and the production of decursinol (●); (**b**) decursin biotransformantion activity by various quantities of protein of the cell-free extracts used in the assay.

occurred enzymatically and is most likely an esterase. The specific activity of decursin bioconversion was estimated to be 0.26 nmol/min/mg of protein. Interestingly, decursinol angelate, a structural isomer of decursin, was not converted to any metabolites by both cells and cell-free extracts, indicating that the esterase enzyme is regiospecific.

Numerous biological activities such as anticancer (*10*, *21*), antiangiogenic (*22*), acetylcholinesterase (AChE) inhibitory (*18*), neuroprotective (*23*), and anti *Helicobacter pylori* (*24*) activities have been reported for decursin, decursinol angelate, and decursinol. Interestingly, the results from several studies revealed that decursinol, where the 3-methylbut-2-enoic acid and (*Z*)-2-methylbut-2-enoic acid of decursin and decursinol angelate are replaced with a hydroxyl group, respectively, shows relatively higher or unique biological activities in comparison with those of the parent compounds. Decursin and decursinol angelate were found to be more potent for cancer inhibitory activities in cell-based experiments (*10*, *21*, *22*, *25*, *26*) but less effective than decursinol in in vivo experiments (*22*, *25*). These results indicate that decursinol, rather than decursin and decursinol angelate, plays an important role in anticancer activity in vivo. The AChE inhibitory activity of decursinol is also much higher than that of decursin (*18*). AChE hydrolyzes acetylcholine (ACh), and the resulting ACh deficiency causes memory impairments in Alzheimer's disease (*18*). Structure−activity studies suggested that a free hydroxyl group of decursinol at C-3′ is crucial for AChE inhibitory activity (*18*).

Isolation of decursinol from its natural source *A. gigas* is also typically low-yielding; the amount of decursinol extracted with methanol did not exceed 0.05% of the plant tissue mass, whereas the content of decursin and decursinol angelate can be as high as 4.6% and 3.7% of the dried root of *A. gigas*, respectively (*3*).

Decursinol has been prepared by organic synthesis (*20*, *27*), which started from the commercially available compounds umbelliferone and esculetin and resulted in the desired product but required 8 and 10 reaction steps, respectively (*20*). Synthesis of decursinol has been recently achieved more efficiently by using a microbial system (*19*). Herath et al. reported the production of decursin metabolites by microbial transformation. They used *Sepedonium chrysospermem* (ATCC 13378) and six other fungal strains to convert decursin to decursinol and decursidinol with a yield of 1.25%, respectively. However, the present study showed that the bacterial strain *Mycobacterium* sp. PYR1001 stoichiometrically transformed decursin exclusively to decursinol. To the best of our knowledge, this is the first report on the bacterial metabolism of decursin. Furthermore, the enzyme activity responsible for the hydrolysis of decursin was determined. Interestingly, the esterase enzyme was found to be specific for decursin. Decursinol angelate did not lead to decursinol production. Our study also suggested that this bacterial strain can be more useful than the previously studied fungal strains when a high yield of decursinol production is desired.

In summary, the present study demonstrated that the bacterial strain *Mycobacterium* sp. PYR1001 was able to transform decursin to decursinol enzymatically. Further studies on the esterase and the gene encoding the enzyme are currently in progress. Biotransformation technology by using this bacterial system can be applied for commercial production of decursinol as a pharmaceutical candidate for treating cancer and Alzheimer's disease.

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