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Hydroxylation of flavanones by cytochrome P450 105D7 from *Streptomyces avermitilis*

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ABSTRACT

Flavanones have a wide range of pharmacological activities. Previously, we showed that CYP105D7, a cytochrome P450, from *Streptomyces avermitilis* can catalyze hydroxylation of diclofenac at the C4′ position. Here, we demonstrated that CYP105D7 also catalyzes hydroxylation of two flavanones, naringenin and pinocembrin. Naringenin was hydroxylated at the 3′-position in a regiospecific manner to yield eriodictyol. Spectroscopic analyses showed that CYP105D7 binds to naringenin and pinocembrin in a weakly cooperative manner with an affinity of 103 μM and 52 μM, and a Hill coefficient of 1.25 and 1.47, respectively. A possible binding model of naringenin was investigated by molecular-docking analyses. The substrate-binding pocket of CYP105D7 is sufficiently wide to accommodate two naringenin molecules simultaneously, and the C3′ atom of the proximal molecule is in the appropriate location for aromatic hydroxylation.

1. Introduction

Cytochrome P450s (abbreviated to CYP or P450) constitute a superfamily of heme proteins and catalyze remarkably diverse oxygenation reactions that are found throughout nature, from Archaea to humans [1]. This family of enzymes can catalyze a wide range of synthetically challenging oxidation reactions: hydroxylation, epoxidation, *O*-demethylation etc. [2]. In recent years, P450s have gained considerable attention as biocatalysts in biotransformation because they can introduce an oxygen atom into a non-activated C—H bond in a regiospecific and stereospecific manner [3,4].

Streptomyces avermitilis MA4680 is one of the most important industrial microorganism used for production of the anthelmintic agent avermectin (an antiparasitic agent used widely for human/veterinary medicine and agricultural pesticides) [5]. The genome contains 33 genes encoding P450 related to the antibiotic biosynthesis and xenobiotic degradation. Among them, functional and structural characterization of CYP105P1, CYP105D6, and CYP105D7 has been reported by our previous studies [6–9]. In particular, CYP105D7 has been investigated extensively, and shows broad substrate specificity. For instance, CYP105D7 hydroxylates 1-deoxypentalenic acid at the 1-position to produce pentalenic acid (a shunt product in the biosynthesis of the pentalenolactone family of metabolites) [8]. CYP105D7 also catalyzes hydroxylation of the isoflavone daidzein at the 3′-position with redox partners of putidaredoxin and putidaredoxin reductase in *Escherichia coli* [10].

Recently, we reported bioconversion of a non-steroidal anti-inflammatory drug diclofenac to 4′-hydroxydiclofenac by CYP105D7 and elucidated the crystal structure in complex with diclofenac [9]. Structural and spectroscopic analyses showed that CYP105D7 binds two diclofenac molecules in the active-site pocket with slight (but significant) cooperativity.

Flavonoids are ubiquitous in photosynthesizing cells. They are widely found in the plant kingdom and exhibit a wide range of biologic activities. Flavanones, a type of flavonoids, are found in citrus fruits and have many beneficial pharmacologic properties, including antioxidant, anti-inflammatory, anticarcinogenic activities [11]. For example, naringenin has been reported to be a good inhibitor of aromatase (a major strategy in the treatment of breast cancer) [12,13]. Moreover, it has been shown that naringenin contained in grapefruit juice inhibits drug-metabolizing P450s in microsomes from the human liver potently [14,15]. Recently, *Streptomyces clavuligerus* has been reported to produce naringenin [16]. This is the first report that naringenin is naturally produced by

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a prokaryote. Several studies have focused on hydroxylation of flavonoids by P450s from mammals, plants, and fungi [17–23]. However, bioconversion of flavonoids by bacterial P450s is not well understood.

We examined the conversion of several flavonoids by CYP105D7. We found that CYP105D7 can hydroxylate two flavanones (naringenin and pinocembrin; Fig. 1). Their affinities and modes of ligand binding were measured by spectroscopic analyses. A possible binding model for naringenin was demonstrated by molecular-docking analyses.

2. Materials and methods

2.1. Materials

(±)-Naringenin (5,7,4′-trihydroxyflavanone), (±)-eriodictyol (5,7,3′,4′-tetrahydroxyflavanone), pinocembrin, pinobanksin, and apigenin were obtained from Sigma-Aldrich (St Louis, MO, USA). Chrysin and kaempferol were purchased from Shanghai Yinggong (Shanghai, China). All other chemicals were from commercial sources and of the highest grade available.

2.2. Co-expression of CYP105D7 with an electron transport system for a bioconversion assay

Transformants of *E. coli* BL21 (DE3) harboring pET11:*sav7469*:*camA-camB* (which encodes the genes for putidaredoxin reductase and putidaredoxin of *Pseudomonas putida*) were constructed according to our previous work [9]. Transformants were cultured in 10 mL of LB medium with 100 μg/mL of ampicillin with shaking at 200 rpm for 18 h at 37 °C. A portion of the culture was inoculated into 10 mL of M9 medium (M9 salts, 1% casamino acid, 0.4% glucose, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM $FeCl_3$, 100 μg/mL of ampicillin) at a ratio of 1:100, and then incubated with shaking at 150 rpm at 37 °C until absorbance at 600 nm reached 0.8 (typically for 2.75 h). Then, protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside and 5-aminolevulinic acid, the first compound in the porphyrin synthesis pathway, with final concentrations of 0.1 and 0.5 mM, respectively. Then, further incubation was conducted for 20 h at 22 °C. *E. coli* cells were harvested by centrifugation at 3000 rpm for 10 min at 4 °C, and washed twice with 5 mL of cold solution containing 50 mM sodium phosphate buffer, pH 7.2, 1 mM ethylenediamine tetra-acetic acid (EDTA), 2 mM dithiothreitol, and 10% (*v*/*v*) glycerol. Washed cells were suspended in 1 mL of the same buffer and used for the bioconversion assay.

2.3. Bioconversion of flavonoids and high-performance liquid chromatography/liquid chromatography–mass spectrometry (HPLC/LC–MS) analyses

For whole-cell conversion, an aliquot (500 μL) of cell suspension of recombinant *E. coli* harboring pET11:*sav7469*:*camA-camB* was used, and substrates (flavonoids) dissolved in methanol were added to a final concentration of 0.15 mM. The reaction mixture was incubated with shaking at 150 rpm for 6 h at 30 °C on a rotary shaker. An aliquot (0.5 mL) of ethyl acetate was added to stop the reaction at the end of incubation. Then, the mixture was shaken for 2 min, and the supernatant collected by centrifugation at 12,000 rpm for 5 min. After centrifugation, extracted samples were dried in a centrifugal vacuum concentrator (IKA, Guangzhou, China) and dissolved in methanol (200 μL). During the experiment, the isolated compound from *E. coli* recombinant cells without a substrate was used as the reference. An LC-20AT HPLC system (Shimadzu, Kyoto, Japan) equipped with an ultraviolet (UV)-visible absorption detector, a DGU-20A5 degasser, and a COSMOSIL packed column ($5C_{18}$-MS-II; 4.6 i.d. × 250 mm; particle size, 5 μm; Nacalai Tesque, Kyoto, Japan), and an LC-20AD HPLC system (Shimadzu, Kyoto, Japan) equipped with an SPD-M20A ultraviolet (UV)-visible absorption detector, and an InertSustain C18 column (4.6 × 250 mm; 5 μm), were used to separate bioconversion products. Conditions for HPLC were: mobile phase, linear gradient of 30–80% methanol for 60 min; flow rate, 0.8 mL/min; detection at 290 nm. A tandem system of HPLC-Chip/MS 1260 Infinity, combined with 6230 time-of-flight (TOF) LC/MS (Agilent Technologies, Santa Clara, CA, USA) with atmospheric-pressure soft electrical ionization in negative-ion mode was used for analyses of metabolites produced by CYP105D7 and an authentic standard (eriodictyol). LC conditions were: column, reverse-phase ODS (HC-C18(2) 150 × 4.6 mm; Agilent Technologies); 30–80% methanol; flow rate, 0.6 mL/min; temperature, 40 °C; UV detection wavelength, 290 nm.

2.4. Expression and purification of CYP105D7 for spectroscopic analyses

CYP105D7 protein with a 4-His tag at the C-terminus was expressed, as described previously [8]. Proteins were purified on a HiTrap Chelating HP 5-mL column (GE Healthcare, Piscataway, NJ, USA) with a linear gradient of 10–500 mM imidazole and concentrated by ultrafiltration. The P450 concentration was measured by CO difference spectroscopy [24]. Quality of protein purification was verified by the ratio of absorbance at 280 nm per Heme peak.

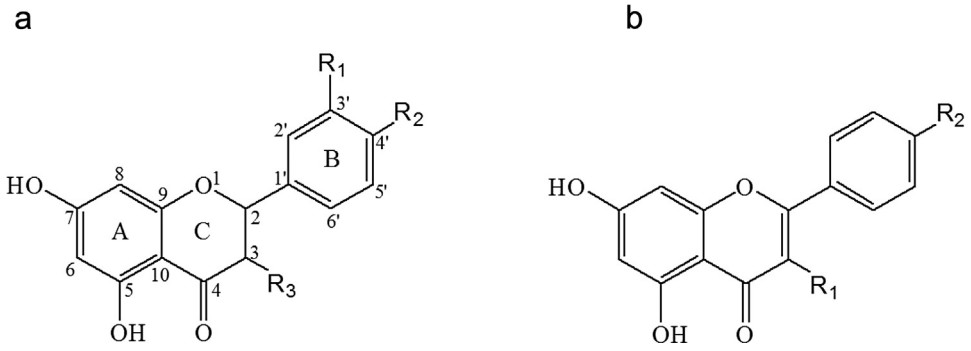

Fig. 1. Flavonoids and hydroxylated products in the present study. (a) Flavanones: naringenin (R_1 = −H, R_2 = −OH, R_3 = −H), pinocembrin (R_1 = −H, R_2 = −H, R_3 = −H), eriodictyol (R_1 = −OH, R_2 = −OH, R_3 = −H), 3′-hydroxypinocembrin (R_1 = −OH, R_2 = −H, R_3 = −H), and pinobanksin (R_1 = −H, R_2 = −H, R_3 = −OH). (b) Flavones and flavonol: apigenin (R_1 = −H, R_2 = −OH), chrysin (R_1 = −H, R_2 = −H), and kaempferol (R_1 = −OH, R_2 = −OH). Naringenin was hydroxylated in a regioselective manner by CYP105D7 at the 3′-position to produce eriodictyol. Hydroxylation of pinocembrin at the same position produces 3′-hydroxypinocembrin, but the hydroxylated product of pinocembrin by CYP105D7 was not determined in this study.