Biotransformation of Flavone by CYP105P2 from *Streptomyces peucetius*

Niraula, Narayan Prasad, Saurabh Bhattarai, Na-Rae Lee, Jae Kyung Sohng, and Tae-Jin Oh*

Institute of Biomolecule Reconstruction (iBR), Department of Pharmaceutical Engineering, SunMoon University, Asansi 336-708, Korea

Received: January 26, 2012 / Revised: April 9, 2012 / Accepted: April 10, 2012

Biocatalytic transfer of oxygen in isolated cytochrome P450 or whole microbial cells is an elegant and efficient way to achieve selective hydroxylation. Cytochrome P450 CYP105P2 was isolated from *Streptomyces peucetius* that showed a high degree of amino acid identity with hydroxylases. Previously performed homology modeling, and subsequent docking of the model with flavone, displayed a reasonable docked structure. Therefore, in this study, in a pursuit to hydroxylate the flavone ring, CYP105P2 was co-expressed in a two-vector system with putidaredoxin reductase (*camA*) and putidaredoxin (*camB*) from *Pseudomonas putida* for efficient electron transport. HPLC analysis of the isolated product, together with LC-MS analysis, showed a monohydroxylated flavone, which was further established by subsequent ESI/MS-MS. A successful 10.35% yield was achieved with the whole-cell bioconversion reaction in *Escherichia coli*. We verified that CYP105P2 is a potential bacterial hydroxylase.

Keywords: Cytochrome P450, flavone, redox partner, *Streptomyces peucetius*, whole-cell biotransformation

The reactions catalyzed by microorganisms involving flavones include oxidation, reduction, conjugation, and deglycosylation [5]. Numerous flavonoids biotransformed by microbes to improve their pharmaceutical activities have been studied. Recently, *Escherichia coli* has been identified as a suitable host for whole-cell bioconversion, although a few examples of the use of *Streptomyces* strains for biotransformation have also been reported [33]. Cytochrome P450s (CYP450s) are highly attractive biocatalysts, as they are responsible for selective hydroxylation of aromatic compounds, which is among the most challenging chemical reactions. These enzymes have gained steadily increasing attention during recent years, particularly because of the use of hydroxylated aromatics as precursors for pharmaceuticals. However, the low activity and the multicomponent nature of these enzymes often result in poor productivities. Therefore, co-expression is a powerful tool for the optimization of whole-cell CYP450 biocatalysis. The co-expression of genes encoding the redox partners of the CYP450 is necessary when the host cell lacks such electron transfer proteins or when the activity with an endogenous system is low [25]. The flavoprotein in this system, putidaredoxin reductase (CamA), contains FAD and is a strictly NADH-dependent ferredoxin reductase lacking the NADP-binding sequence [8, 23]. The iron sulfur protein, putidaredoxin (CamB), belongs to the [2Fe–2S] ferredoxin group and plays the role of an electron shuttle transferring the two electrons one at a time from putidaredoxin reductase to P450cam [7, 8].

Three classes of substrates, namely 7-ethoxycoumarin, fatty acids, and flavonoids [34], were selected to study the hydroxylating activity of CYP450s from *Streptomyces peucetius* by exploring their substrate specificity. Out of 23 CYP genes present in the genome of *S. peucetius*, four CYPs (CYP105F2, CYP105P2, CYP107N3, and CYP157C4) that were expressed as soluble proteins exhibited remarkable CO-difference spectra and were chosen as candidates for bioconversion of the substrates. CYP105F2 was previously verified for hydroxylation of oleandomycin [30]. Our initial attempt to identify possible substrates for CYP105P2 by binding studies and homology modeling turned out to be significant for the flavonoids (flavone, flavanone, quercitin, and naringenin) class of substrates [16]. Among other substrates such as macrolides, 7-ethoxycoumarin used for docking, flavone was best en suite in the constructed model with ring-B directed towards the reaction center; iron [16]. Intrinsically, P450 enzymes are not very active; they exhibit poor stability in their isolated form. Therefore, owing to this fact, we co-expressed CYP105P2 with the redox partner *camA/camB* in *E. coli* that readily generated the monohydroxylated flavone.

Furthermore, the structure-based pharmaceutical activation of flavones has led to extensive research of biotransformation...
by microorganisms [11, 13]. 5-Hydroxy-, 6-hydroxy-, and 7-hydroxyflavone can be hydroxylated to their corresponding 4'-hydroxylated products, and 5-hydroxyflavone can be transformed into dihydroxylated 5,3',4'-trihydroxyflavone by S. fulvissimus NRRL1453B [15]. Likewise, flavone can be converted to 2',3'-dihydroxyflavone and 3'-hydroxyflavone, and the 6-hydroxyflavone analog to 6,2'-dihydroxyflavone, by a recombinant S. lividans [29]. Hosny et al. [11] also found that S. griseus catalyzed hydroxylation and methylation of quercetin, fisetin, and catechin. These broad capacities for functional modification of flavones by microorganisms led us to use CYP450 to generate flavonoids with improved pharmacological potency.

Herein, we have experimentally verified the previously proposed docked structure of flavone into the CYP105P2 homology model. The whole-cell biotransformation of flavone into its monohydroxy derivative by co-expression of a putative CYP450 hydroxylase (CYP105P2) from S. peucetius ATCC27952 and heterologous redox partner (CamA/CamB) was successfully achieved with a 10.35% product yield (Fig. 1).

**Materials and Methods**

**Bacterial Strains, Plasmids, and Growth Conditions**

S. peucetius ATCC27952 was cultured in R2YE medium at 28°C for 2 to 3 days in baffled flasks for isolation of genomic DNA, and *E. coli* strains were cultured in Luria–Bertani (LB) broth or on LB agar plates at 37°C [26]. *E. coli* XL-1-Blue MRF’ (Stratagene, La Jolla, CA, USA) and *E. coli* ET12567 (John Innes Centre, UK), a demethylating (**dam dem hsdS On**’), host, were used for DNA manipulation and propagation of unmethylated plasmid DNA, respectively. For transformation, *E. coli* BL21(DE3) was used as the expression host throughout the study. Ampicillin (100 µg/ml) and streptomycin (35 µg/ml) were used for selection of recombinant strains in *E. coli* transformants. In some cloning experiments, 0.4 mM isopropyl-β-thiogalactopyranoside (IPTG) and 40 µl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were included in the LB agar plates for screening of transformants, pGEM-T Easy (Promega, USA) was used as the cloning vector, and pET32a+ (Novagen, USA), pCDFDuet-1 (Novagen, USA), and pIBR25 were used as expression vectors [31]. Reagent-grade chemicals were purchased from Sigma Co.

**DNA Manipulation and Sequence Analysis**

All general DNA manipulations, including ligations and enzymatic digestion of DNA, were carried out using standard methods [26]. Polymerase chain reaction (PCR) was performed in a thermocycler (Takara, Japan). The amplification conditions for PCR were 94°C for 7 min, followed by 30 cycles of 94°C for 1 min, 63°C for 1 min, and finally 72°C for 10 min. The PCR amplification was performed in a total volume of 20 µl containing 5 µl PCR Mix ([Buffer (10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl2, 50 mM KCl), 1 mM dNTP, Taq DNA polymerase]) (Genotech Corporation), 1 µl forward primer, 1 µl reverse primer, 1 µl template DNA, 1 µl dimethyl sulfoxide (DMSO), and 11 µl distilled water. A set of primers, 105P2F (5'-AGA GAA CTT GGG CTT TTC CCA GCC GCT TTC C-3') and 105P2R (5'-TCA CCA GGT GGG CAG C-3') (underlined letters indicate engineered restriction sites), was used to amplify CYP105P2 and then the PCR product (1,200 bp) was cloned into the EcoRI/HindIII sites of pET-32a (+) to construct pN105P2. Similarly, another set of primers, P2F (5'-AGA TTC AGT ATG TCC CAG CCC ACC G-3') and 105P2R (5'-TCA AAG CTT TCA CCA GGT GAG GGG C-3') was used to amplify the nucleotide sequences of CYP105P2 and then the PCR product (1,200 bp) was cloned into the EcoRI/HindIII site of pET-32a (+) to construct pN105P2. The PCR products were sequenced prior to cloning into the expression vector to verify that no mutations had been introduced during the PCR amplification.

The recombinant pN105P2 was introduced into *E. coli* BL21(DE3) by heat-pulse transformation. DNA fragments were isolated from the agarose gel and recovered in low-melting agarose dissolving buffer. Ethanol precipitation was carried out for further purification of genomic DNA and plasmid DNA. Similarly, two redox partners, *camA* and *camB* from *Pseudomonas putida*, which were previously cloned into the PETDuet-1 with the ampicillin resistance gene [24], were recloned into pCDFDuet-1, which contained the streptomycin resistance gene, to form pDCamAB. The recombinant pDCamAB was also introduced into *E. coli* BL21(DE3) by heat-pulse transformation. Computer-based analysis and comparison of nucleotide and protein sequences were performed with the programs BLAST, FASTA, CLUSTALW [32], and GENEDOC. Other CYPs involved in secondary metabolite production were searched at http://drnelson.utmem.edu/.

**Co-Expression of pN105P2 and pDCamAB in E. coli**

*E. coli* BL21(DE3) harboring pN105P2 and pDCamAB (Fig. S1) were cultured in 3 ml of LB medium with 100 µg/ml ampicillin and 35 µg/ml streptomycin at 37°C for 8 h and then transferred into 50 ml of fresh LB medium supplemented with appropriate antibiotics. Cells were allowed to grow in a shaking incubator at 37°C to an
OD$_{600}$ of about 0.6. The cultures were induced with 0.4 mM IPTG and further incubated at 20°C for 20 h. E. coli cells were harvested by centrifugation at 6,000 × g for 10 min, washed twice with cold 50 mM potassium phosphate buffer (pH 7.6), and then resuspended in 1 ml of the same buffer. The cells were disrupted ultrasonically and then centrifuged for 30 min at 12,000 × g. The molecular weight was determined after 12% SDS-PAGE based on the standard molecular weight marker from Novagen.

Reduced Carbon Monoxide Difference Spectra Measurement
Reduced CYP450 carbon monoxide (CO) difference spectra were measured as described by Omura and Sato [21]. A few crystals of sodium dithionite were added to the CYP450-rich fraction obtained from the heterologously expressed strains, and then mixed and divided into two cuvettes. The sample cuvette was saturated with 30 to 40 bubbles of CO at a rate of about 1 bubble per second, and the absorbance difference between reduced CYP450 against CYP450 bound to CO was measured using an extinction coefficient of 91 mM$^{-1}$ cm$^{-1}$.

Biotransformation of Flavone
E. coli BL21(DE3) harboring pN105P2 and pDCamAB was induced as described above. Twenty minutes after the addition of 0.4 mM IPTG, flavone was added to a final concentration of 0.5 mM, and the incubation was continued for 48 h at 20°C on a rotary shaker (200 rpm) to allow side-chain cleavage in the whole-cell biotransformation. E. coli harboring vectors alone was employed as the negative control. An equal amount of ethyl acetate was added to stop the reaction at the end of incubation, followed by centrifugation to obtain the supernatant.

The supernatants were analyzed by high-performance liquid chromatography (HPLC) and electron spray ionization-mass spectrometry (ESI-MS), followed by liquid chromatography mass spectrometry (LC-MS) analysis. The ESI-MS analysis was carried out at a probe temperature of 392°C with a source voltage of 31.2 V using Finnigan AQA (UK) mass spectrophotometer. Samples (2 µl) were injected via a 10 µl loop and were transferred at a flow rate of 1 ml/min using methanol as a solvent. The HPLC was performed under the following conditions: column, Mightysil RP-18 GP (150 × 4.6 mm ID; Kanto Chemical, Tokyo); gradient solvent system, 100% acetonitrile and water with 0.1% trifluoroacetic acid; flow rate, 1 ml/min; detection, absorbance at 260 nm. The LC-MS analysis was performed under similar conditions; the solvent was 100% acetonitrile and water with 0.1% trifluoroacetic acid; the flow rate was 1 ml/min. For GC-MS analysis, a 5 µl portion of the concentrated extract was analyzed (Shimadzu GC-17A, 70 eV, EI, positive-ion mode; 30 m × 0.25 mm bond capillary column, 5% phenylmethylsilicon) using a temperature program of 50 to 280°C and a temperature gradient of 20°C per minute. Further structural information was obtained with an electrospray ionization-mass/mass spectrometer (ESI-MS/MS).

RESULTS
Amino Acid Sequence Analysis
The amino acid sequence of CYP105P2 is deposited in GenBank under the accession number CAE53708.

CYP105P2 encodes a protein of 399 aa with an overall G+C content of 70.5%, which is characteristic for genes in the genus Streptomyces. Threonine in I-helix (oxygen-binding motif), glutamate and arginine in the EXXR motif of K-helix, and glycine and cysteine in the GXXXCXG signature of heme-binding domains are conserved in this enzyme. The deduced aa sequence of the putative CYP105P2 was aligned with other counterparts by ClustalW (Fig. 2A). Our sequence analysis revealed that CYP105P2 has 92% identity with CYP105P1 of S. avermitilis and 48% identity with CYP105S1 of S. tubercidicus. Similarly, it shared 47% identity with CYP105L of S. virginiae and 42% identity with CYP105F1 from S. lavendulae.
Co-Expression of pN105P2 and pDCamAB in E. coli
CYP450s receive the necessary electrons for oxygen cleavage and substrate hydroxylation from different redox partners [9]. Since cofactors and their regeneration are required for enzymatic oxidation reactions, biotransformation using the whole cell is favored as a method of biocatalysis. The putidaredoxin reductase (CamA) and putidaredoxin (CamB) from P. putida are the most widely studied type I electron transport system. The genome sequence of S. peucetius ATCC27952 available at our laboratory provided the entire coding region of CYP105P2. The 1,200 bp CYP105P2 was amplified and cloned at the EcoRI/HindIII site of pET32a(+) to yield pN105P2 (Fig. 1). Recombinants pN105P2 and pDCamAB were finally transferred into the host E. coli BL21(DE3) strain. Expressions were easily accomplished in the total cell by induction with 0.4 mM IPTG for 20 h at 20°C. The molecular masses of CYP105P2 (59 kDa, fused with thioredoxin protein and N-terminal His-Tag), CamA (45.6 kDa), and CamB (27 kDa) were confirmed by 12% SDS-PAGE (data not shown) and were consistent with the predicted sizes.

Reduced Carbon Monoxide Difference Spectra Measurement
For a reaction to occur between CYP450 and oxygen or carbon monoxide, the heme-iron must be reduced from the ferric to ferrous state so that these molecules may bind to the heme iron. The ferric to ferrous reduction of the heme protein was achieved by the addition of sodium dithionite to the sample and reference cuvettes, and carbon monoxide was gassed through the sample cuvettes only. CYP450 in the cytosolic fraction obtained from the heterologously expressed CYP105P2 and pDCamAB after sonication and centrifugation exhibited a CO-reduced difference maximum at 450 nm (Fig. 2B). The shift of about 30 nm observed in CYP105P2 means that the distribution of electron density at the heme is significantly perturbed.

Biotransformation of Flavone
Bacterial CYP450 systems usually require the presence of a ferredoxin reductase and a ferredoxin to couple electron flow from NAD(P)H to the terminal CYP450 component. Most bacterial CYP450s are one of two different classes: class I (or B-class) P450 receives electrons from a two-component reductase system, ferredoxin and NADH ferredoxin reductase, such as in P450CAM [8, 23]. The possible activity was detected by HPLC/LC-MS and GC-MS analysis of extracts from E. coli BL21(DE3) harboring pN105P2/pDCamAB in comparison with extract from the negative control. The isolated sample subjected to HPLC showed a possible hydroxylated peak at a retention time of 16.5 min. Subsequent LC-MS analysis of the peak clearly indicated a monohydroxylated product with a mass of 239 in positive mode (Fig. 3). Furthermore, flavones [M+H]+ ions exhibited m/z 221 [M+H-H2O]+, m/z 193 [M+H-H2O-CO]+, and other fragments, as shown in Fig. 4. Similar results have also been observed in the cases of luteolin, apigenin, and baicalein [1, 3, 18]. In this way, mass detection at 239 by MS-MS analysis also indicated the monohydroxylated flavone (Fig. 4) and the fragmentation pattern shows ring B-hydroxylation of flavone. Time-dependent biocconversion of flavone was maximal at 48 h. During the experiment, the compound isolated from the host with vector alone was taken as the reference for calculation of the percentage yield of our desired product, and the yield was 10.35%.

DISCUSSION
Whole-cell bioconversion from Streptomyces species has been well established [14]. More importantly, S. lividans is a suitable host for biotransformation [29]. We therefore carried out a comparative study of CYP105P2 in the in vivo bioconversion in S. lividans and S. peucetius using pIBR25 as an expression vector (Fig. S2). The parental strain of S. peucetius exhibited very low bioconversion of flavone into its monohydroxy derivative. Overexpression
of CYP105P2 in *S. peucetius* resulted in minimal detectable bioconversion of flavone. The CYP105P2 transformant of *S. lividans* TK24 also showed comparable bioconverted derivative. However, no conversion was achieved from the *S. lividans* TK24 strain. This may be due to oxidative activity by endogenous P450s present in the *Streptomyces* strain that metabolized the flavones into other derivatives. As the activity of the enzyme with the endogenous system was very low, *E. coli* BL21 (DE3) was chosen as a suitable host to express a single P450. To obtain novel derivatives of flavone, CYP105P2 was co-expressed with the redox partner camA/camB in *E. coli* BL21 (DE3) to allow hydroxylation of flavone.

There is considerable interest in the interactions of flavonoids with CYP450 isozymes, since this process has the potential to interfere with the metabolism of various drugs [10], although early studies indicated that this activity may be attributed to the competitive metabolism of flavonoids. Some flavonoids such as diosmin and flavanone have also been shown to be inducers of CYP450 enzymes [2, 4]. For instance, genistein, a substrate for *S. griseus*, induces soluble CYP450 SOY in the same organism [27]. By hydroxylation, presumably through CYP450, *S. griseus* produced a catechol that appeared to be a requisite for methylation either at carbon or at phenolic oxygens. Flavone was converted to 2',3'-dihydroxyflavone and 3'-hydroxyflavone in 13% and 2.4% yields, respectively, by a recombinant *S. lividans* strain [29]. Findings on the bioconversion of genistein by *S. griseus* and *S. catenulae* suggest that the pathways for its biotransformation require 3'-hydroxylation to form the catechol [12]. A recent study on P450s from white-rot fungus *Phanerochaete chrysosporium* also shows 3'-hydroxylation of flavone [17]. These discoveries on the hydroxylation of flavone serve as a suitable foundation for the specificity of monohydroxylation. Moreover, in this study, in the substrate-docked structure of the ferric enzyme, the closest C3, C4', and C5' atoms of the B-ring of the substrate flavone is located at a distance of approximately 4.7Å from the iron [16], which is a feasible distance for the reaction to occur. However, it is possible that the substrate could move close to the iron in other iron states, because NMR studies of P450BM3 revealed that the position of the substrate is different between the ferric and the ferrous iron states [19], which
would further facilitate the closest contact of substrate with iron. These facts substantiate the feasibility of hydroxylation of the target substrate, more precisely in the C3' position. Furthermore, the B-ring of flavone is bound and stabilized almost entirely via hydrophobic interactions with the aa residues side-chain facing towards the substrate-binding pocket as Ala183 (SRS3 in F-G loop), Pro81, Leu87, Leu88, Trp88 (SRS1 in B-C loop), Leu234 (SRS4 in I-J loop), Ile151 (E-helix), Phe389 (SRS6), Ala288, Arg290 (SRS5), Ile283, Gly284 (β 1–4), Ile236, Ala236, Ala238, Thr240, Thr242, and Met245 in the I-helix [16]. Notably, almost all the residues involved in flavone binding are located in the substrate recognition sites (SRs) [6]. In addition, alignment of CYP105P2 with flavonoid 3'-hydroxylase genes [28] also revealed significant similarity. In addition, biotransformation of unsubstituted flavone by many fungi species also yields 4'- and 3',4'-hydroxyflavone as the major products. Based on these findings, the most probable hydroxylation position is C3' on the B-ring of the flavone.

Furthermore, flavones are typically more potent antioxidants than flavanones. Therefore, desaturation of the C-ring to form aromatic flavones can alter the potency or produce new pharmacological activity. Moreover, a recent report on the biological activities of hydroxylated flavone based on numbers and/or positions of hydroxyl groups revealed highest activity for 6,7,3'-trihydroxyflavone [22], which can also be derived from 3'-hydroxyflavone. Therefore, isolation and purification of our target compound monohydroxyflavone (3'-hydroxyflavone) in sufficient quantities for structural and stabilization almost entirely via hydrophobic interactions with the aa residues side-chain facing towards the substrate-binding pocket as Ala183 (SRS3 in F–G loop), Pro81, Leu87, Leu88, Trp88 (SRS1 in B–C loop), Leu234 (SRS4 in I–J loop), Ile151 (E-helix), Phe389 (SRS6), Ala288, Arg290 (SRS5), Ile283, Gly284 (β 1–4), Ile236, Ala236, Ala238, Thr240, Thr242, and Met245 in the I-helix [16]. Notably, almost all the residues involved in flavone binding are located in the substrate recognition sites (SRs) [6]. In addition, alignment of CYP105P2 with flavonoid 3'-hydroxylase genes [28] also revealed significant similarity. In addition, biotransformation of unsubstituted flavone by many fungi species also yields 4'- and 3',4'-hydroxyflavone as the major products. Based on these findings, the most probable hydroxylation position is C3' on the B-ring of the flavone.

Furthermore, flavones are typically more potent antioxidants than flavanones. Therefore, desaturation of the C-ring to form aromatic flavones can alter the potency or produce new pharmacological activity. Moreover, a recent report on the biological activities of hydroxylated flavone based on numbers and/or positions of hydroxyl groups revealed highest activity for 6,7,3'-trihydroxyflavone [22], which can also be derived from 3'-hydroxyflavone. Therefore, isolation and purification of our target compound monohydroxyflavone (3'-hydroxyflavone) in sufficient quantities for structural elucidation would be a milestone in the study of flavonoids; this will require optimization of culture conditions and a purification protocol for flavonoids. In the same context, study of the electron transport system could also allow us to increase the CYP450 activities for hydroxylation of flavonoids. In addition, interconversion of flavanone into flavone and subsequent glycosylation of the hydroxylated derivative or glycosylation of hydroxylated flavonoids alone would certainly extend our understanding of the antioxidant potency of flavonoids.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0003461, 2011-0026856).

References