Characterization of a Biflaviolin Synthase CYP158A3 from *Streptomyces avermitilis* and Its Role in the Biosynthesis of Secondary Metabolites

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**Abstract**

*Streptomyces avermitilis* produces clinically useful drugs such as avermectins and oligomycins. Its genome contains approximately 33 cytochrome P450 genes and they seem to play important roles in the biosynthesis of many secondary metabolites. The SAV_7130 gene from *S. avermitilis* encodes CYP158A3. The amino acid sequence of this enzyme has high similarity with that of CYP158A2, a biflaviolin synthase from *S. coelicolor* A3(2). Recombinant *S. avermitilis* CYP158A3 was heterologously expressed and purified. It exhibited the typical P450 Soret peak at 447 nm in the reduced CO-bound form. Type I binding spectral changes were observed when CYP158A3 was titrated with myristic acid; however, no oxidative product was formed. An analog of flaviolin, 2-hydroxynaphthoquinone (2-OH NQ) displayed similar type I binding upon titration with purified CYP158A3. It underwent an enzymatic reaction forming dimerized product. A homology model of CYP158A3 was superimposed with the structure of CYP158A2, and the majority of structural elements aligned. These results suggest that CYP158A3 might be an orthologue of biflaviolin synthase, catalyzing C-C coupling reactions during pigment biosynthesis in *S. avermitilis*.

**Key Words:** P450, *Streptomyces avermitilis*, CYP158A3, Flaviolin, 2-Hydroxynaphthoquinone

**INTRODUCTION**

*Streptomyces avermitilis* is a gram-positive soil-dwelling, filamentous actinobacterium. It has been of great pharmaceutical interest because it produces various useful drugs in human and veterinary medicine such as avermectins and oligomycins. The genome sequence of *S. avermitilis* was completed in 2003 and approximately 33 cytochrome P450 (P450) genes were found (Ikeda et al., 2003). These P450 enzymes seem to play important roles in the biosynthesis of many secondary metabolites in *S. avermitilis*.

P450 enzymes are the major heme-containing catalysts involved in the oxidation of various substrates (Ortiz de Montellano, 2015). They are found in nearly all organisms, from bacteria to plants and mammals (Guergerich, 2008). Genome sequencing projects continue to reveal new genes for P450 enzymes from microorganisms including bacteria, archaea, and fungi. To date, more than 21,000 P450 genes have been reported across all classes of organism (http://drnelson.uthsc.edu/cytochromeP450.html/) (Lee et al., 2015; McLean et al., 2015).

P450 enzymes from species in the genus *Streptomyces* are mainly integrated into biosynthetic operons for secondary metabolite pathways. These operons contain a series of enzymes for the production of an antibiotic, pigment, or some other secondary metabolite (McLean et al., 2015). *S. coelicolor* is a prototypic *Streptomyces* strain; its genome has 18 P450-encoding genes. Our previous study reported that CYP105N1 from *S. coelicolor* is a P450 enzyme involved in the biosynthesis of coelibactin, which is a siderophore implicated in zinc-dependent antibiotic regulation (Lim et al., 2012; McLean et al., 2015).

Two CYP158A enzymes (CYP158A1 and CYP158A2) have been found in *S. coelicolor*. Previously, Zhao et al. (2005a, 2005b, 2007, 2012) reported that CYP158A1 and CYP158A2 produce polymers of flaviolin (biflaviolin or triflaviolin), which
MATERIALS AND METHODS

Chemicals and enzymes

Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP+, and 2-OH NQ were purchased from Sigma (St. Louis, MO, USA). Isopropropyl-β-D-1-thiogalactopyranoside (IPTG) was purchased from Anatrace (Maumee, OH, USA). All the chemicals were of the highest grade commercially available.

Construction of expression plasmids

Our general approach has been described previously (Lim et al., 2012; Han et al., 2015). The open reading frame for CYP158A3 was isolated directly by PCR amplification from S. avermitilis. Substrate binding titrations and 2-hydroxynaphthoquinone (2-OH NQ) dimerization experiments were performed to determine the reaction catalyzed by CYP158A3.

Enzymes expression and purification

The expression and purification of CYP158A3 were carried out as previously described, with some modifications (Lim et al., 2012; Park et al., 2014). Briefly, Escherichia coli DH5α cells transformed with pCW (CYP158A3) and pGroEL/ES vectors were inoculated in Luria-Bertani (LB) broth containing 50 μg/mL ampicillin and 20 μg/mL kanamycin; cells were then pre-cultured overnight at 37°C. LB cultures were then seeded into 500 mL of Terrific broth (TB) expression medium containing threedimensional protein model was visualized in PyMOL (http://www.pymol.org/). The homology model was analyzed to identify possible substrate binding sites.

Homology modeling of CYP158A3 protein

Homology modeling was performed on the Swiss-Model server (https://swissmodel.expasy.org/). CYP158A2 (PDB entry: 1793) was used as a template for modeling. The resulting three-dimensional protein model was visualized in PyMOL.

Spectroscopic characterization

Sodium dithionite was added to reduce the ferric-form purified CYP158A3. CO-ferrous CYP158A3 complexes were generated by passing CO gas through solutions of ferrous CYP158A3. The UV-visible spectra were collected on a Cary Varian spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) in 100 mM potassium phosphate buffer (pH 7.4) at room temperature.

P450 enzyme catalytic activity assay

Hydroxylation of 2-OH NQ by CYP158A3 was analyzed by LC-mass spectrometry. The reaction mixture contained 2 nmol purified CYP158A3 enzyme, and 200 mM tert-butyl hydroperoxide (tBHP) in 0.50 mL of 100 mM potassium phosphate buffer (pH 7.4), along with 400 μM 2-OH NQ. Incubations were performed at 37°C for 1 h and terminated by adding 10 μL 2N HCl; this was followed by ethyl acetate extraction (three times). The reaction products were recovered from the organic phase after drying under N2. Oxidized products were analyzed by LC-mass spectrometry on a Shimadzu LCMS-2010 EV system (Shimadzu, Kyoto, Japan), using LC-MS software. Products were separated on a Shim-pack VP-ODS column (2.0 mm i.d. ×250 mm; Shimadzu) at a flow rate of 1.0 mL/min. The mobile phase contained 90% solvent A (0.5% formic acid and 0.01% trifluoroacetic acid in water, v/v) followed by a linear gradient of solvent B (0.5% formic acid and 0.01% trifluoroacetic acid in acetonitrile, v/v), increasing to 100% solvent B over 20 min, and finally holding for 10 min with solvent B at a flow rate of 0.8 mL/min (Zhao et al., 2005a). Mass spectra were recorded by electrospray ionization in negative mode to identify the metabolites. Interface and detector voltages were 4.4 kV and 1.5 kV, respectively. Nebulization gas flow was set to 1.5 mL/min. Interface, desolvation line, and heat block temperatures were 250°C, 230°C, and 200°C, respectively.

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RESULTS

Amino acid sequence alignments for CYP158A3

The SAV_7130 gene from S. avermitilis encodes a 404 amino acid enzyme, named CYP158A3 (http://drnelson.uthsc.edu/cytochromeP450.html/). Its sequence contains both a heme-binding domain (FXXGXXXFXG) and an EXXR motif in the K-helix, which are conserved in P450 enzymes (Fig. 1). Amino acid sequence alignment of CYP158A3 from S. avermitilis with that of CYP158A2 from S. coelicolor shows high sequence identity (81%; Fig. 1) (Notredame et al., 2000). The good sequence alignment to CYP158A2 suggests that the gene product of SAV_7130 may be a biflaviolin synthase from S. avermitilis possessing flaviolin dimerization catalytic activity.

Expression and purification of CYP158A3

Recombinant CYP158A3 protein was co-expressed with the molecular chaperones GroEL/ES in E. coli. The CO-reduced difference spectrum of E. coli cells transformed by pCW/158A3 expression vector (Fig. 2A) showed a typical P450 expression level of ~850 nmol P450 holoenzyme per liter of culture medium. CYP158A3 was purified by Ni²⁺-NTA affinity and size exclusion column chromatography. SDS-PAGE analysis showed a band with a size corresponding to that expected (45.1 kDa) from the open reading frame of the gene encoding CYP158A3, bearing a 6×His-tag (Fig. 2B). The absolute spectra of purified ferric-form CYP158A3 exhibited a Soret band at 416 nm, indicating a low-spin state; the distinctive α- and β-bands of ferric P450 were observed at 588 nm and 534 nm, respectively (Fig. 2B).

Binding analysis of CYP158A3

Binding titration analysis of CYP158A3 was performed to

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identify putative substrates. Titration of purified CYP158A3 with myristic acid produced a decrease at 416 nm and an increase at 378 nm, indicative of type I binding (Fig. 3A). The binding affinity ($K_d$) of myristic acid was calculated to be $98 \pm 19 \mu M$ (Fig. 3A). Since the substrate of CYP158A2, flavilolin, is not commercially available, its chemical analog, 2-hydroxynaphthoquinone (2-OH NQ), was used for the substrate binding analysis (Zhao et al., 2005a). Titration with 2-HNQ produced a decrease at 416 nm, however the spectral increase at 380 nm was not clear (Fig. 3B). The pseudo-type I binding titration results for CYP158A3 suggested that two molecules of 2-OH NQ might occupy the active site, consistent with a dimerization reaction. The calculated $K_d$ value for 2-HNQ was $18 \pm 2 \mu M$ (Fig. 3B), indicating tight substrate binding. Additionally, resveratrol, stilbenes, and lauric acid displayed similar spectral changes upon binding; however, their binding affinities were not high (data not shown).

**Catalytic activities of CYP158A3**

The catalytic activity of CYP158A3 towards 2-OH NQ was analyzed using LC-mass spectrometry. Purified CYP158A3 supported the catalytic turnover of 2-OH NQ, in the presence of tBHP, to yield the metabolic products displayed in the chromatogram (Fig. 4A). A total ion scan and selected ion monitoring mode scan for m/z=175 were consistent with the inference that the substrate peak at 7.8 min corresponded to 2-OH NQ (relative molecular mass: 174 Da) (Fig. 4B). The m/z of peak at 19.3 min indicated a mass of 361 Da, corresponding to the isomers of the dimeric form of 2-OH NQ after oxidation of the benzene ring (Fig. 4B). Dimeric isomers have not been definitively identified in the current analysis; further structural analysis will be required to define their detailed chemical structure.

**DISCUSSION**

*S. avermitilis* was first isolated in Japan in 1979, and was screened by Merck Sharp & Dohme, resulting in the discovery of the avermectins, which are famous anthelmintic and insecticidal agents (Burg et al., 1979; Demain, 1999). CYP171A1, a P450 enzyme encoded by the aveE gene from *S. avermitilis*, is involved in the biosynthesis of the avermectins, where it catalyzes the formation of the furan ring (Han et al., 2016; Lamb et al., 2003). Our previous effort to purify functional, recombinant CYP171A1 holoenzyme using an E. coli expres-
sion system was not successful. Presumably, the bacterial expression system may not be optimal for the correct folding of recombinant CYP171A1.

For further analysis of the molecular structure, we tried to obtain the three-dimensional structure of CYP158A3. However, we were unable to produce crystals of CYP158A3 that diffracted well. To improve protein crystallization, a clone of CYP158A3 was constructed in which the first seven amino acids were truncated from the N-terminus. The truncated clone was expressed in *E. coli*. The truncation resulted in reduced expression (up to 74 nmol P450 per liter of culture). Nonetheless, highly concentrated pure protein could be obtained. However, the second crystallization attempt using this truncated CYP158A3 was not successful either as suitable crystals did not form.

To provide alternative structural information, a homology model of CYP158A3 was constructed using the crystal structure of CYP158A2 (PDB entry: 1T93) as a template (Fig. 5). Overall, the helical structure of the CYP158A2 homology model matched that of previously reported P450 enzymes (Fig. 5). When the model was superimposed on the crystal structure of CYP158A2, most residues from both structures aligned, implying that CYP158A3 may be a biflaviolin synthase in *S. avermitilis*.

The spectra for myristic acid and 2-HO NQ binding to purified CYP158A3 showed standard type I binding (Fig. 3). Previously, CYP158A2 displayed a similar type I spectral titration to 2-OH NQ with a $K_d$ value of 43 μM (Zhao et al., 2005b). The calculated binding affinity of CYP158A3 in this study was a little stronger with a $K_d$ value of 18 μM (Fig. 3B). In our study, other aromatic compounds (resveratrol and stilbenes) also showed similar spectral changes associated with a type I binding mode (data not shown). Although the calculated binding affinities were not high, the type I binding patterns were clear, indicating that CYP158A3 may have an active site architecture suitable for compounds containing multiple aromatic rings. Meanwhile, an inhibitory type II binding mode was observed during the binding titration with theazole compound, econazole, which had a $K_d$ value of 0.2 μM towards CYP158A3 (data not shown). Zhao *et al.* (2007) showed different binding modes and dimer forms of flavilin produced by CYP158A1 biflaviolin synthase. In this study, the biflaviolin synthase activity of CYP158A3 also indicated the different dimer forms of 2-HO NQ (Fig. 4A). However, only a trace of enzyme activity was observed in the 2-OH NQ reaction of CYP158A3 (Fig. 4A) because 2-OH NQ lacking the 5- and 7-hydroxy groups create a more hydrophobic environment and disrupts the continuous water chain for proton delivery (Zhao *et al.*, 2005b).

In conclusion, recombinant *S. avermitilis* CYP158A3 was purified and its biochemical properties were characterized. The binding studies and enzymatic analyses suggested that CYP158A3 might be a biflaviolin synthase in *S. avermitilis*. The present findings will help elucidate the functional roles of P450 enzymes and provide biochemical insights into the complicated biosynthetic pathways of secondary metabolism in *Streptomyces* species.

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