Characterization of P450 FcpC, the Enzyme Responsible for Bioconversion of Diosgenone to Isonuatigenone in *Streptomyces virginiae* IBL-14[†]

Wei Wang, Feng-Qing Wang, and Dong-Zhi Wei*

State Key Lab of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, China

Received 14 November 2008/Accepted 10 April 2009

A new cytochrome P450 monooxygenase, FcpC, from *Streptomyces virginiae* IBL-14 has been identified. This enzyme is found to be responsible for the bioconversion of a pyrano-spiro steroid (diosgenone) to a rare nuatigenin-type spiro steroid (isonuatigenone), which is a novel C-25-hydroxylated diosgenone derivative. A whole-cell P450 system was developed for the production of isonuatigenone via the expression of the complete three-component electron transfer chain in an *Escherichia coli* strain.

Nuatigenin-type steroids, such as nuatigenin and isonuatigenin (9, 13, 22), are rare natural steroidal sapogenins that are important pharmacological compounds. They are found in several healthy foods and traditional medicinal herbs. These compounds have been shown to have potential anticancer effects, antagonistic effects on rheumatoid arthritis, beneficial cardiovascular activities, and antimalarial activities. Examples include ophiopufosin in *Ophiopogon japonicus* (28), nuatigenoside in *Solanum sisymbriifolium* (13), avenacoside in oat (20), and glycosides in *Paris polyphylla* SM (7). Since the majority of these nuatigenin-type steroids are very rare, strategies for their isolation can lead to very high production costs. As a result, with a more economical production process in mind, it would be worthwhile to search for a suitable reagent capable of converting the abundant amounts of pyrano-spirostanol sapogenins found in nature, such as diosgenone, to rare nuatigenin-type steroids. At this time, we plan to focus on microbial transformation systems.

A previous article (25) described an actinomycete strain named *Streptomyces virginiae* IBL-14, isolated from soil, that can transform diosgenone to isonuatigenone by introducing a hydroxyl group to the tertiary C-25 atom of the F-ring (Fig. 1). To our knowledge, this was the first report of producing a rare nuatigenin-type spiro steroid from diosgenone by microbial biotransformation. The present study was conducted in order to identify the determinant enzyme from *S. virginiae* IBL-14 that catalyzes the biotransformation and to design a whole-cell cytochrome P450 system to produce isonuatigenone by using *Escherichia coli*.

Initial attempts to clone the genetic determinant for the biocatalytic activity. Based on preliminary biochemical results suggesting that the catalytic enzyme in the IBL-14 strain

is a cytochrome P450 monooxygenase (CYP), we attempted to isolate diverse CYP genes from *S. virginiae* IBL-14. The CYP-encoding genes *cyp10*, *cyp20*, *cyp24*, *cyp71*, *whyA*, and *yx1B* were isolated by PCR using degenerate primers (Table 1) designed to bind to the conserved oxygen- and heme-binding domains of CYPs (12, 14). Four additional CYPs (expressed by *cyp72*, *cyp73*, *cyp165*, and *fcpC*) in this strain were also identified using high-stringency primers (Table 1) designed specifically against genes for CYPs that are related to the bioconversion of steroids and tertiary hydroxylations (4, 21).

The *fcpC* gene product serves as the enzyme for the C-25 hydroxylation of diosgenone. To establish the *in vivo* function of the CYP gene products, we developed a genetic system in IBL-14 and disrupted each *cyp* gene by single-crossover homologous recombination. The inactivation of *fcpC* [to create the SCWF1 ($\Delta fcpC$) mutant] by the insertion of PKC1139 (5, 27), a temperature-sensitive plasmid containing an apramycin resistance gene, led to significant disruption in the production of isonuatigenone (Fig. 2, line b).

A 0.8-kb fragment of *fcpC* was used as a probe against an IBL-14 subgenomic library in order to identify the full-length gene (Fig. 3). *fcpC* encodes a peptide of 397 amino acids (with a molecular mass of 43.77 kDa) that belongs to the CYP105 family. The closest match in the databases to the deduced amino acid sequence is the sequence of a putative P450 monooxygenase encoded by a secondary-metabolite biosynthetic gene cluster in *Kitasatospora putterlickiae* (GenBank accession number DQ792514), sharing 65% identity with FcpC. The *fcpD* gene, downstream of *fcpC* in the genome, is considered to encode the native ferredoxin for FcpC. The start codon of *fcpD* is GTG instead of ATG, as in *fcpC*, and the first nucleotide of its initiation codon immediately precedes the stop codon of *fcpC*. In addition, a possible ribosome-binding site (GGGA) 6 nucleotides upstream of the initiation codon was found.

A complementation test for the *fcpC* disruption was conducted by introducing *fcpCD* (1.5 kb) under the control of a constitutively expressed *ermE** promoter (26). To this end, *fcpCD* was inserted into the plasmid pIB139 (creating pCFPC11, with a switch to kanamycin resistance) before conjugation into strain SCWF1. The diosgenone hydroxylation

* Corresponding author. Mailing address: State Key Lab of Bioreactor Engineering, New World Institute of Biotechnology, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China. Phone: 86-21-64252981. Fax: 86-21-64250068. E-mail: dzhwei@ecust.edu.cn.

[†] Supplemental material for this article may be found at <http://aem.asm.org/>.

[‡] Published ahead of print on 17 April 2009.

FIG. 1. (Bio)synthetic conversion of diosgenone (1) to isonuatigenone (2) and nuatigenone (3). Diosgenone can be transformed into isonuatigenone by cytochrome P450 FcpC from *S. virginiae* IBL-14. Nuatigenone is the rearrangement product of isonuatigenone during acidic work-up (8).

activity was restored in strain SCWF2 (SCWF1::pCFPCP11) (Fig. 2, line c). These results clearly show that the enzyme FcpC is responsible for isonuatigenone production.

Conversion of diosgenone to isonuatigenone in vitro. The *fcpC* gene was amplified from genomic DNA using PCR with the following primers: 5'-GCCCCcatatgAGTGAGTCCCTC CACACCGTC (where an NdeI recognition sequence is in lowercase and the start of the coding sequence of *fcpC* is underlined) and 5'-GGAGgaattcACTTCGCGTCCCAGG TGAC (where the sequence in lowercase is an EcoRI recognition site and the underlined sequence is the reverse complement of the *fcpC* translation stop codon and is followed by the 3'-end sequence of the *fcpC* gene). The resulting *fcpC* DNA fragment was digested with NdeI/EcoRI and ligated into a

FIG. 2. High-pressure liquid chromatography and ESI-MS analyses of the bioconversion of diosgenone catalyzed by wild-type IBL-14, mutants, and recombinant *E. coli* cells. The ESI-MS analyses of diosgenone (1) and isonuatigenone (2) show ion peaks at m/z 413.7 ($[M + H]^+$) and 429.6 ($[M + H]^+$), respectively. Lines correspond to the wild type (a), SCWF1 ($\Delta fcpC$) (b), SCWF2 (SCWF1::pCFPCP11) (c), and *E. coli* resting cells expressing *fcpC* and redox partners (d).

pET28 vector previously digested with NdeI/EcoRI (creating pFCP1). This strategy generated an N-terminally His₆-tagged FcpC construct (2, 24) for further biochemical characterization. After Ni-nitrilotriacetic acid chromatography (14, 29), the purified, red, recombinant P450 displayed (upon reduction) a signature peak at 450 nm in the CO difference spectrum (15, 19). Following desalting, FcpC was dissolved in 100 mM K₂HPO₄-KH₂PO₄, pH 7.3. A typical reaction mixture consisted of 1.90 ml of protein (containing 50 to 100 μ g of FcpC), 3.5 μ M spinach ferredoxin (Sigma), and 0.1 U of ferredoxin-NADPH reductase (Sigma) in a total assay volume of 2.0 ml (6). A total of 1 μ l of the diosgenone substrate (as a solution in isopropanol) was added to obtain final concentrations varying between 0.1 and 20 μ M. Following incubation at 30°C for 5 min, the reaction was started by the addition of NADPH to obtain a final concentration of 0.2 mM. The activity of the purified His-tagged FcpC was measured spectrophotometrically, following the consumption of NADPH at 340 nm and 30°C for 10 min (18). The data were used to fit the Lineweaver-Burk equation (in Microsoft Excel) in order to determine the

TABLE 1. PCR primers used for identifying CYP genes in this study

Primer	Primer sequence ^a (5' to 3')
O₂-binding domain primers	
OBD1	VTSGCSGGSCACGAGACSAC
OBD2	TSCTSCTSATCGCSGGSCACGAGAC
Heme-binding domain primers	
HBD1	GCSAGGTTTCGTSCCSAGGCACTGGTG
HBD2	GCACTGGTGSAYSCCGTGSCCGAA
HBD3	GCACTGGTGSACSCCGAASCAGAA
HBD4	GCAGAAGTGSACSCCGTGSCCGAA
High-stringency primers	
1F	CATGGACGACCCGSAGCAC
1R	CTGCCCCAGGCACCTGGTG
2F	CGCCGGGCACACAGACCAC
2R	TTCTGCCCGAGGCACTGGTG
3F	CTCATCGCAGGGCAGCAGAA
3R	TGTCCCGGTGGATGTCGAA
4F	CGGACCCACACAGGYTGCG
4R	GTCTCGTAGCCAGCATSAGCAG
5F	CGGAGATCTGCGGGATGTAC
5R	CCGATGCAGTAGTGGATGCC
6F	TACGACCCGCGGAGCACA
6R	TGGTGAACGCGGTGYCCGA

^a Ambiguity codes: S, C or G; Y, C or T; and V, G or A or C.

FIG. 3. Schematic diagram of the *fcpCD* operon of *S. virginiae* IBL-14. The size of each open reading frame in nucleotides (nt) and the predicted molecular mass in kilodaltons and the pI of each gene product are shown. In the sequence below, the start codon of *fcpD* is indicated by an arrow. The overlapping stop codon of *fcpC* and start codon of *fcpD* are underlined. The sequence of a possible ribosome-binding site (RBS) in *fcpD* is in italics.

enzymatic kinetic parameters. The production of isonuatigenone was detected by high-pressure liquid chromatography and electrospray ionization mass spectrometry (ESI-MS) analyses (data not shown).

Our results indicate that FcpC has a K_m of $2.4 \pm 0.8 \mu\text{M}$, a $V_{\max}$ of $0.024 \pm 0.011 \mu\text{mol/min/mg}$, and a turnover number of 1.06 min^{-1} for diosgenone hydroxylation. In comparison, the well-characterized bacterial CYP enzyme EryF, which is responsible for the hydroxylation of 6-deoxyerythronolide B to form erythronolide B in *Saccharopolyspora erythraea*, shows a turnover number of 52.8 min^{-1} for its natural substrate (1, 23). Since diosgenone is not the natural substrate for FcpC, this artificial substrate may not interact very well with the active site, leading to the observed lower turnover number. In addition, because the diosgenone substrate is not soluble in water (diosgenone concentration, $<20 \mu\text{M}$), it had to be dissolved in isopropanol in order to be added to the assay mixture. When the substrate solution was mixed with water, precipitation was observed immediately (diosgenone concentration, $>60 \mu\text{M}$). Given these observations, it is possible that the substrate may not have formed a proper solution under our assay conditions.

Bioconversion of diosgenone by *E. coli* resting cells expressing *fcpC* and redox partners. CYPs are highly attractive biocatalysts due to their abilities to catalyze a variety of regio- and stereospecific oxidation reactions of complex organic compounds under mild conditions. This remarkable feat is accomplished by taking advantage of the two-electron activated dioxygen, which is often challenging in organic synthesis (10). In order to activate molecular oxygen, a redox partner(s) is required to sequentially transfer two reducing equivalents from NAD(P)H to CYP (11). This inherent requirement of cytochrome P450 enzymes for protein partners significantly limits their application in biotechnology (16).

FcpC belongs to the class I P450 system, in which electrons are transferred from NADH or NADPH to the enzyme via a flavin adenine dinucleotide-containing reductase (ferredoxin reductase) and an iron-sulfur protein (ferredoxin). FcpD is considered to be the native ferredoxin for FcpC; however, the gene encoding the native *S. virginiae* IBL-14 ferredoxin reductase has not yet been cloned. Instead, preliminary CO-reduced spectrum results (see Fig. S11 in the supplemental material) indicate that SC4B10, one of three ferredoxin reductases from *Streptomyces coelicolor* A3(2) (3, 15, 17), helps increase the amount of active FcpC (11, 15, 16, 17). We constructed an operon in pFCP1 consisting of three genes and one expression plasmid by cloning *fcpD* and the ferredoxin reductase SC4B10 gene downstream of *fcpC* (Fig. 4). This new construct, pFCP2, was then expressed in *E. coli* BL21 Star(DE3)(pRARE).

After induction for 24 h with 0.2 mM IPTG (isopropyl- β -D-thiogalactopyranoside) at 20°C , transformed *E. coli* cells were harvested by centrifugation and resuspended in reaction buffer containing 100 mM K_2HPO_4 - KH_2PO_4 and 1% (vol/vol) glycerol, pH 7.5. Following the addition of a 0.15% (vol/vol) solution of diosgenone in isopropanol (30 mg/ml), the reaction was allowed to proceed at 20°C , with shaking at 160 rpm. The level of conversion achieved a product yield exceeding 5% after 24 h of incubation (Fig. 2, d).

The biotransformation of *E. coli* cells to express the complete electron transfer chain resulted in a 12-fold increase in the rate of isonuatigenone production compared to the rate

FIG. 4. Construction of an operon containing the *S. virginiae* IBL-14 cytochrome P450 gene *fcpC*, the corresponding ferredoxin gene *fcpD*, and the *S. coelicolor* SC4B10 ferredoxin reductase gene. The introduced gene fragments with restriction enzyme sites contained in pFCP2 are schematically presented.

obtained using an *E. coli* strain expressing only *fcpC* and *fcpD* (0.42%). As a control, all *E. coli* strains expressing only one of the three proteins of the electron transfer chain showed no substrate conversion. The increase in isonuatigenone production was mediated by the inclusion of ferredoxin and ferredoxin reductase, which together stabilize the P450 enzyme and provide an optimal electron transfer system (11, 15, 16, 17).

In conclusion, compared to the traditional isolation strategy (9, 13, 22), our whole-cell system using FcpC from *S. virginiae* IBL-14 expressed in *E. coli* can be a potentially valuable method for producing rare nusatigenin-type steroids. Our future work will focus on improving the present *fcpC* biotransformation system by using different hosts, promoters, vectors, and cloned redox partners for the extensive utilization of novel derivatives of steroidal drugs.

Nucleotide sequence accession number. The GenBank accession number for the *fcpC* and *fcpD* gene sequences identified in this study is EF646279.

We thank Hei-Qing Wang (Zhejiang University) for providing diosgenin and Mark Buttner (John Innes Institute, England) for providing plasmid PKC1139.

This research was financially supported by grants from the National Natural Science Foundation of China (no. 20777016) and the National Basic Research Program of China (no. 2009CB724703).

REFERENCES

- Andersen, J. F., and C. R. Hutchinson. 1992. Characterization of *Saccharopolyspora erythraea* cytochrome P450 genes and enzymes, including 6-deoxyerythronolide B hydroxylase. *J. Bacteriol.* **174**:725–735.
- Bell, S. G., and L. L. Wong. 2007. P450 enzymes from the bacterium *Novosphingobium aromaticivorans*. *Biochem. Biophys. Res. Commun.* **360**:666–672.
- Bentley, S. D., K. F. Chater, A. M. Cerdano-Tarraga, G. L. Challis, N. R. Thomson, K. D. James, D. E. Harris, M. A. Quail, H. Kieser, D. Harper, A. Bateman, S. Brown, G. Chandra, C. W. Chen, M. Collins, A. Cronin, A. Fraser, A. Goble, J. Hidalgo, T. Hornsby, S. Howarth, C. H. Huang, T. Kieser, L. Larke, L. Murphy, K. Oliver, S. O'Neil, E. Rabinowitz, M. A. Rajandream, K. Rutherford, S. Rutter, K. Seeger, D. Saunders, S. Sharp, R. Squares, S. Squares, K. Taylor, T. Warren, A. Wietzorrek, J. Woodward, B. G. Barrell, J. Parkhill, and D. A. Hopwood. 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature* **417**:141–147.
- Bernhardt, R. 2006. Cytochromes P450 as versatile biocatalysts. *J. Biotechnol.* **124**:128–145.
- Bierman, M., R. Logan, K. O'Brien, E. T. Seno, R. N. Rao, and B. E. Schoner. 1992. Plasmid cloning vectors for the conjugal transfer of DNA from *Escherichia coli* to *Streptomyces* spp. *Gene* **116**:43–49.
- Cane, D. E., and E. I. Graziani. 1998. Methymycin biosynthesis. Isolation of P450 monooxygenase activity in a cell-free system from *Streptomyces venezuelae*. *J. Am. Chem. Soc.* **120**:2682–2683.
- Chen, C. X., Y. T. Zhang, and J. Zhou. 1995. The glycosides of aerial parts of *Paris polyphylla* var. *yunnanensis*. *Acta Bot. Yunnan* **17**:473–478.

8. Faini, F., R. Torres, and M. Castillo. 1983. (25*R*)-Isonuatigenin, an unusual steroidal sapogenin from *Vestia lycioides*. *Phytochemistry* **23**:1301–1303.
9. Ferro, E. A., N. L. Alvarenga, D. A. Ibarrola, M. C. Helli6n-Ibarrola, and A. G. Ravelo. 2005. A new steroidal saponin from *Solanum sisymbriifolium* roots. *Fitoterapia* **76**:577–579.
10. Guengerich, F. P. 2001. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem. Res. Toxicol.* **14**: 611–650.
11. Hannemann, F., A. Bichet, K. M. Ewen, and R. Bernhardt. 2007. Cytochrome P450 systems—biological variations of electron transport chains. *Biochim. Biophys. Acta* **1770**:330–344.
12. Hyun, C.-G., J. M. Kim, S. K. Hong, and J. W. Suh. 1998. An efficient approach for cloning P450 hydroxylase genes from *Actinomyces*. *J. Microbiol. Biotechnol.* **8**:295–299.
13. Ibarrola, D. A., M. C. Helli6n-Ibarrola, N. L. Alvarenga, E. A. Ferro, N. Hatakeyama, N. Shibuya, M. Yamazaki, Y. Momose, S. Yamamura, and K. Tsuchida. 2006. Cardiovascular action of nuatigenosido from *Solanum sisymbriifolium*. *Pharm. Biol.* **44**:378–381.
14. Jungmann, V., I. Moln6r, P. E. Hammer, D. S. Hill, R. Zirkle, T. G. Buckel, D. Buckel, J. M. Ligon, and J. P. Pachlatko. 2005. Biocatalytic conversion of avermectin to 4"-oxo-avermectin: characterization of biocatalytically active bacterial strains and of cytochrome P450 monooxygenase enzymes and their genes. *Appl. Environ. Microbiol.* **71**:6968–6976.
15. Li, L., M. R. Waterman, A. J. Fulco, S. L. Kelly, and D. C. Lamb. 2003. Availability of specific reductases controls the temporal activity of the cytochrome P450 complement of *Streptomyces coelicolor* A3(2). *Proc. Natl. Acad. Sci. USA* **101**:494–499.
16. Li, S. Y., L. M. Podust, and D. H. Sherman. 2007. Engineering and analysis of a self-sufficient biosynthetic cytochrome P450 PikC fused to the RhFRED reductase domain. *J. Am. Chem. Soc.* **129**:12940–12941.
17. Moln6r, I., D. S. Hill, R. Zirkle, P. E. Hammer, F. Gross, T. G. Buckel, V. Jungmann, J. P. Pachlatko, and J. M. Ligon. 2005. Biocatalytic conversion of avermectin to 4"-oxo-avermectin: heterologous expression of the *ema1* cytochrome P450 monooxygenase. *Appl. Environ. Microbiol.* **71**:6977–6985.
18. Nebert, D. W., and H. V. Gelboin. 1968. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. I. Assay and properties of induced enzyme. *J. Biol. Chem.* **243**:6242–6249.
19. Omura, T., and R. Sato. 1964. The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**: 2370–2378.
20. Osbourn, A. E. 1996. Preformed antimicrobial compounds and plant defense against fungal attack. *Plant Cell* **8**:1821–1831.
21. Parajuli, N., D. B. Basnet, H. C. Lee, J. K. Sohng, and K. Liou. 2004. Genome analyses of *Streptomyces peuceitii* ATCC 27952 for the identification and comparison of cytochrome P450 complement with other *Streptomyces*. *Arch. Biochem. Biophys.* **425**:233–241.
22. Saijo, R., C. Fuke, K. Murakami, T. Nohara, and T. Tomimatsu. 1983. Two steroidal glycosides, aculeatiside A and B from *Solanum aculeatissimum*. *Phytochemistry* **22**:733–736.
23. Shafiee, A., and C. R. Hutchinson. 1987. Macrolide antibiotic biosynthesis: isolation and properties of two forms of 6-deoxyerythronolide B hydroxylase from *Saccharopolyspora erythraea* (*Streptomyces erythreus*). *Biochemistry* **26**: 6204–6210.
24. Taylor, M., D. C. Lamb, R. Cannell, M. Dawson, and S. L. Kelly. 1999. Cytochrome P450105D1 (CYP105D1) from *Streptomyces griseus*: heterologous expression, activity, and activation effects of multiple xenobiotics. *Biochem. Biophys. Res. Commun.* **263**:838–842.
25. Wang, F. Q., B. Li, W. Wang, C. G. Zhang, and D. Z. Wei. 2007. Biotransformation of diosgenin to nuatigenin-type steroid by a newly isolated strain, *Streptomyces virginiae* IBL-14. *Appl. Microbiol. Biotechnol.* **77**:771–777.
26. Wilkinson, C. J., Z. A. Hughes-Thomas, C. J. Martin, I. Bohm, T. Mironenko, M. Deacon, M. Wheatcroft, G. Wirtz, J. Staunton, and P. F. Leadlay. 2002. Increasing the efficiency of heterologous promoters in actinomycetes. *J. Mol. Microbiol. Biotechnol.* **4**:417–426.
27. Xiang, L. K., and B. S. Moore. 2002. Inactivation, complementation, and heterologous expression of *encP*, a novel bacterial phenylalanine ammonia-lyase gene. *J. Biol. Chem.* **277**:32505–32509.
28. Xu, T. H., Y. J. Xu, S. X. Xie, H. F. Zhao, D. Han, Y. Li, J. Z. Niu, and D. M. Xu. 2008. A novel steroidal glycoside, ophiopuroside A from *Ophiopogon japonicus* (Thunb.) Ker-Gawl. *J. Asian Nat. Prod. Res.* **10**:415–418.
29. Xue, Y., D. Wilson, L. Zhao, H. W. Liu, and D. H. Sherman. 1998. Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the pikC-encoded cytochrome P450 in *Streptomyces venezuelae*. *Chem. Biol.* **5**:661–667.