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Note

Hydroxylation of Oleanolic Acid to Queretaroic Acid by Cytochrome P450 from *Nonomuraea recticatena*

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A gene for cytochrome P450 (*moxA*) from *Nonomuraea recticatena*, coexpressed with *camAB* for pseudomonad redox partners in *Escherichia coli*, hydroxylated oleanolic acid to produce queretaroic acid. When we used the P450-induced whole-cell as a catalyst, only a small amount of queretaroic acid was produced, probably due to poor permeability of oleanolic acid into the *E. coli* cell. In an alternative approach with the cell-free reaction system, the conversion ratio increased up to 17%.

Key words: cytochrome P450; oleanolic acid; queretaroic acid; hydroxylation

Cytochromes P450 (P450s) are a superfamily of oxidative hemoproteins that catalyze an enormous variety of oxidative conversions of organic compounds, including hydroxylation, epoxidation, phenol coupling, heteroatom oxidation, dehalogenation, and C–C bond cleavage *via* successive hydroxylations. The ability of P450s to catalyze difficult oxidation with high regio- and stereoselectivity makes them attractive for numerous biotechnological applications.

We developed a useful bacterial P450-expression system with *Escherichia coli* that harbors a plasmid carrying genes for a P450 and *Pseudomonas* redox partners (*camAB*).[1,2] So far we have collected 213 bacterial P450 genes and constructed a P450 library to explore their ability to catalyze useful oxidative conversions.[3]

Oleanolic acid is a triterpene isolated from some plants that has various physiological activities such as anti-tumor,[4] anti-fungal,[5] insecticidal,[6] anti-HIV,[7,8] diuretic,[9] blood sugar level depression,[10,11] liver protection,[12] and anti-inflammatory activities.[13,14] Hence oleanolic acid can be a suitable model compound worth converting to its hydroxyl derivatives with superior physiological properties. We screened P450s of the P450 library for oleanolic acid hydroxylation activity and found that P450moxA, a P450 from rare actinomycete *Nonomuraea recticatena* IFO 14525, hydroxylated oleanolic acid at its C-30 of the methyl group to produce queretaroic acid as the main product (Fig. 1). Queretaroic acid is an ingredient in herb drugs and has been reported as to its physiological activity.[4] Here we report the novel hydroxylation of oleanolic acid to queretaroic acid by a bacterial P450, P450moxA, with a cell-free reaction system.

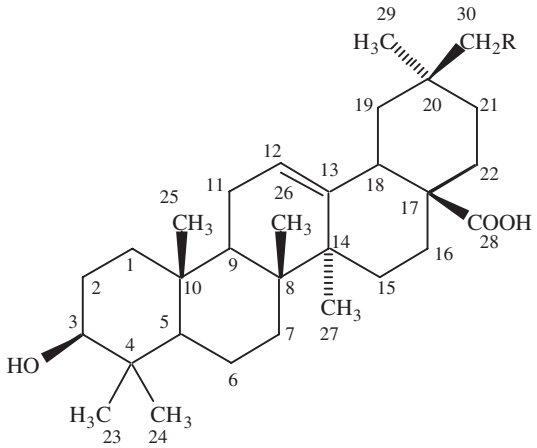

Fig. 1. Structure of Oleanolic Acid (R=H) and Queretaroic Acid (R=OH).

The gene coding P450moxA (*moxA*) and its downstream ferredoxin gene (*moxB*) were cloned together as genes responsible for compactin hydroxylation.[15,16] P450moxA is putatively classified into the CYP105 family from its amino acid sequence identity (49%) to P450sca-2 (CYP105A3).[17]

E. coli BL21 (DE3) was transformed with expression vector pET11a (Novagen, San Diego, CA) carrying *moxAB* and *camAB*, named pMoxAB-CamAB. P450-induced *E. coli* cells were prepared as described previously,[3] except for ethanol-glycerol treatment. It has been reported that ethanol and glycerol enhanced proper folding of the P450 protein.[18,19] After investigation of concentrations of ethanol and glycerol to optimize expression of P450moxA, we added 750 µl of

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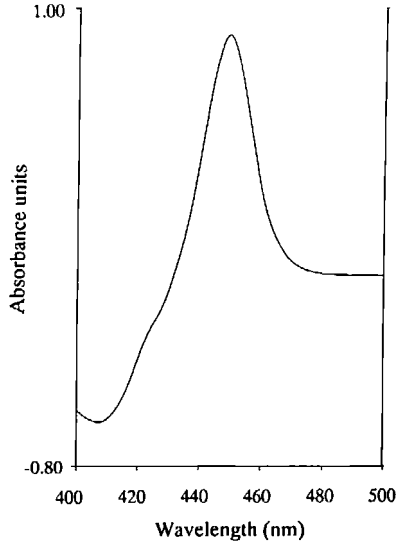

Fig. 2. Reduced CO Difference Spectrum of P450moxA Expressed in *E. coli* BL21 (DE3).

Cell extract of *E. coli* BL21 (DE3) expressing P450moxA was prepared. A typical reduced CO difference spectrum of P450moxA showed a Soret band at about 450 nm.

ethanol and 2.5 ml of 50% glycerol to 25 ml of the culture, which was further incubated for 20 min at 22 °C prior to the addition of isopropyl-thio-β-D-galactopyranoside (IPTG).

The expression of P450moxA in *E. coli* was verified by reduced CO difference spectral assay.[20] The CO-binding form of P450moxA in the reduced state with sodium dithionite showed the distinctive CO complex spectrum maximum at 450 nm that is characteristic of P450s (Fig. 2). The expression level of P450moxA was estimated at 2.5 μM in the culture of *E. coli* harboring pMoxAB-CamAB.

The P450moxA-induced *E. coli* cells were collected from 1.5 ml of culture and suspended in 1.5 ml of CV buffer (50 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol, 2 mM dithiothreitol, and 1 mM EDTA). Oleanolic acid dissolved in 30 μl of acetone was added to a final concentration of 200 μg/ml to the cell suspension. After incubation in a 24-well microplate at 28 °C on a shaker for 24 h, the product was extracted with 1.5 ml of ethyl acetate. The organic extract was evaporated under reduced pressure to dryness. The residue was reconstituted with 200 μl of methanol for HPLC analysis.

The HPLC analysis was performed with a Shimadzu LC-2010C HPLC system (Shimadzu, Tokyo) with a diode array ultraviolet detector set at 210 nm, fitted with a Develosil ODS-HG-5 column (150 × 4.6 mm i.d., Nomura Chemical, Aichi, Japan). The sample (10 μl) was chromatographed with a linear gradient of acetonitrile concentration in 5 mM phosphate buffer (pH 5.0) from 50% (v/v) to 85% (v/v) for 10 min, 85% (v/v) until 15 min, and 50% (v/v) until 20 min at a flow rate of 1.0 ml/min at 35 °C.

In the hydroxylation of oleanolic acid by P450moxA, only 3.3% of oleanolic acid was converted to queretaroic acid (Fig. 3A). In an alternative approach to improve the conversion reaction, we constructed a cell-free reaction system as follows: *E. coli* cells expressing P450moxA prepared from 1.5 ml of culture as described above were suspended in 300 μl of 50 mM Tris–HCl buffer (pH 7.4) containing 20% glycerol, and lysed using Bug-Buster reagent (Novagen, Darmstadt, Germany) with Benzonase nuclease (Novagen) and lysozyme (1.2 mg). To the cell lyzate, we added 300 μl of CamA-CamB solution prepared in advance from *E. coli* expressing *camAB* in the same manner as with the P450moxA solution, 2.6 mM NADH, 2.6 mM NADPH, 38 mM D-glucose, 6.25 U/ml glucose dehydrogenase, 0.1 mg/ml catalase, 0.5% randomly substituted methyl-β-cyclodextrin (Wacker Chemie, Munich, Germany) and 200 μg/ml oleanolic acid in a final volume of 800 μl. The reaction mixture was incubated at 30 °C for 20 h with shaking. With this cell-free reaction system, P450moxA successfully hydroxylated oleanolic acid to queretaroic acid with a conversion ratio of 17% (Fig. 3C). This increased conversion activity achieved by the cell-free reaction system perhaps indicates that oleanolic acid, due to its low penetration in the cell, cannot reach P450moxA before cell disruption. Another possibility is an improved electron transfer system supported by the NAD(P)H regeneration system. This possibility, however, can be excluded considering our preliminary result that conversion activity was decreased by the cell-free reaction system when we used diclofenac as a substrate that can penetrate into the cell for P450boxA-catalyzed 4′-C hydroxylation (data not shown).* No queretaroic acid production was detected in control cell lysate prepared from *E. coli* cells without *moxA* (Fig. 3B). In this bioconversion, we also observed three other products as minor components (Fig. 3C). The chemical structures and pharmacological significance of these products were not determined.

To obtain a larger amount of the product, large-scale conversion was carried out in the same manner. The product was extracted with 100 ml of ethyl acetate from the reaction mixture (330 ml) and purified by preparative HPLC on an Inertsil PREP-ODS column (250 × 20 mm i.d.; GL Sciences, Tokyo) at 220 nm at 40 °C with MeOH/water (70:30 [v/v]) as the mobile phase at a flow rate of 7.0 ml/min. A fraction containing queretaroic acid was evaporated under reduced pressure to give 3.0 mg of queretaroic acid as a white powder.

The identification of the product was based on analysis of NMR spectroscopy and electrospray ionization mass spectrometry (ESI-MS). The mass spectrum of the product showed 16 mass units more than that of oleanolic acid, indicating that the product was a

* P450boxA is a cytochrome P450 encoded by *boxA* (GenBank Accession no. AB180845) cloned from compactin hydroxylating *Streptomyces* sp. TM-7.

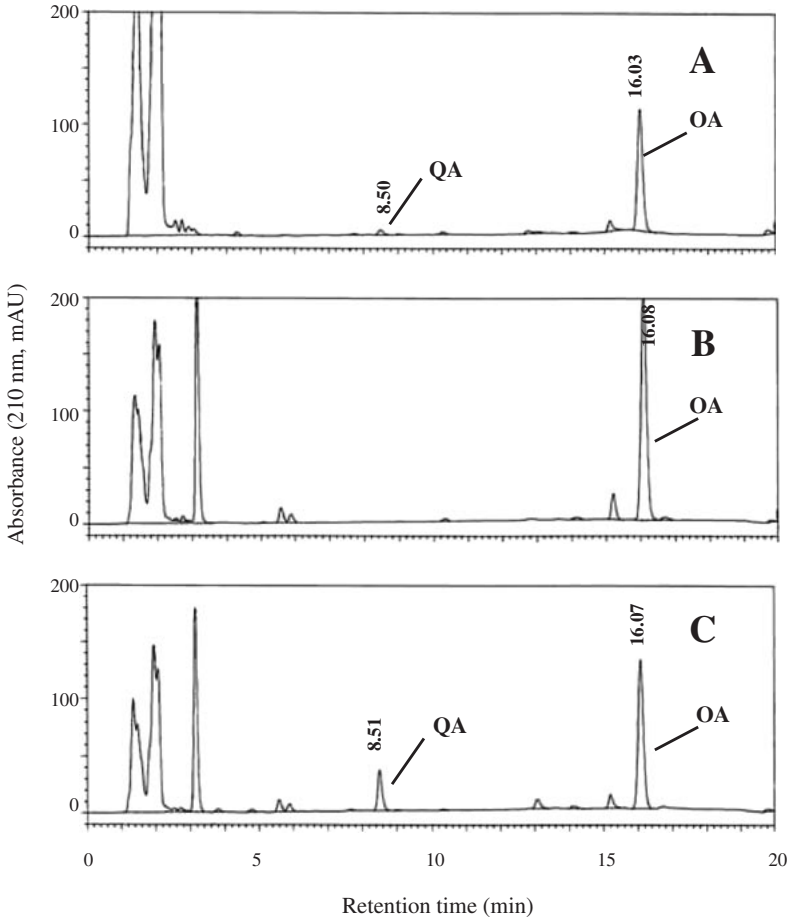

Fig. 3. Bioconversion of Oleanolic Acid (OA) to Queretaroic Acid (QA) by Whole Cells (A), a Cell-Free Reaction System without P450moxA (B), and a Cell-Free Reaction System with P450moxA (C).

Products detected by P450moxA reaction in the cell-free reaction system of oleanolic acid are indicated by arrow. Column, a Develosil ODS-HG-5 (150 × 4.6 mm i.d., Nomura Chemical, Aichi, Japan); flow rate, 1.0 ml/min; column temperature, 35 °C; detection, 210 nm; elution, an acetonitrile concentration linear-gradient in 5 mM phosphate buffer (pH 5.0) from 50% (v/v) to 85% (v/v) for 10 min, 85% (v/v) until 15 min, and 50% (v/v) until 20 min.

monohydroxylated derivative. The ^{1}H NMR spectrum (in methanol-d_6) of the product showed downfield methylene protons at δ 3.49, which corresponded to a carbon (δ 66.4) in the HMQC experiment. In the HMBC spectrum, this methylene carbon showed a correlation to methyl group protons (δ_H 0.88, δ_C 27.9). Two methyl groups at the 23-position (δ_H 0.97, δ_C 28.2) and the 24-position (δ_H 0.77, δ_C 16.4) were identified on the basis of HMBC correlations to C-3 (δ_H 3.14, δ_C 79.8). These results indicate that either the C-29 or the C-30 methyl group was hydroxylated. C-29-hydroxylation gives mesembryanthemoidigenic acid,[21] while C-30-hydroxylation gives queretaroic acid.[22] Comparing the oxygenated carbon signal (δ_C 66.4 in methanol-d_6) of the product with C-29 (δ_C 73.9 in pyridine-d_5) of mesembryanthemoidigenic acid or C-30 (δ_C 65.5 in pyridine-d_5) of queretaroic acid, whose data are reported in the literature, the product appeared to be queretaroic acid. Finally the product was identified as queretaroic acid (3β, 30-dihydroxy-12-oleanen-28-oic acid) by comparison of the ^{13}C NMR spectral data in pyridine-d_5 with the literature values.[22]

The whole-cell reaction using P450-expressed *E. coli* strains is useful in a search for their hydroxylation ability,[3] but it is a precondition that the compound to be hydroxylated permeates the *E. coli* cells. In this study, we found that even a compound with a poor permeability, like oleanolic acid, can be hydroxylated at higher efficiency by the cell-free reaction system. The cell-free reaction system requires some laborious handling to perform (*e.g.*, cell disruption and the addition of reagents to reconstitute the electron transport and NAD(P)H regeneration) as compared with the whole-cell reaction system. Still, it should be useful tool to detect products that cannot be detected by the whole cell reaction due to poor substrate permeability.

Queretaroic acid is likely to be produced from oleanolic acid by P450 hydroxylation in several plants, including *Clerodendron serratum Moon*. It is interesting that an actinomycete P450, P450moxA, catalyzes the same hydroxylation as botanical P450 does. This finding might indicate functional sharing of botanical P450s, at

least partly, with bacterial P450s. To the best of our knowledge, this is the first report that shows that an actinomycete P450 catalyzes the same hydroxylation as a botanical P450.

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