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Note

Production of Hydroxlated Flavonoids with Cytochrome P450 BM3 Variant F87V and Their Antioxidative Activities

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A variant of P450 BM3 with an F87V substitution [P450 BM3 (F87V)] is a substrate-promiscuous cytochrome P450 monooxygenase. We investigated the bioconversion of various flavonoids (favanones, chalcone, and isoflavone) by using recombinant *Escherichia coli* cells, which expressed the gene coding for P450 BM3 (F87V), to give their corresponding hydroxylated products. Potent antioxidative activities were observed in some of the products.

Key words: flavonoid; P450 BM3; peptidyl-prolyl *cis-trans* isomerase; *Escherichia coli*; antioxidative activity

Cytochrome P450 monooxygenases are versatile biocatalysts that introduce oxygen into a vast range of molecules. A variant (mutant) of P450 BM3 with a Phe87Val (F87V) substitution [P450 BM3 (F87V)] has been shown to have promiscuous substrate specificity (affinity) for various small molecules, including aromatic compounds and sesquiterpenes, in the preparation of hydrogenated product(s) in previous studies.[1–6] The fusion protein, P450 BM3 (F87V) N-terminally fused to the PPIase, was much more soluble in *E. coli* cells when compared with the intact P450 BM3 (F87V) protein.[1] To the best of our knowledge, this P450 has not previously been shown to biotransform flavonoids, one of the two major families of plant pigments, flavonoids and carotenoids. Flavonoids have recently attracted considerable attention due to their beneficial effects on health, *e.g.*, their antioxidative activity,[7] antimicrobial activity,[8] and estrogenic activity,[9] and are considered to be of medicinal and nutritional importance. It may therefore be a promising approach to produce rare or novel flavonoids by enzymatic or biotechnological conversion. We aimed in this study to produce rare hydroxylated flavonoids with potent antioxidative activities by applying biotranformation with P450 BM3.

E. coli BL21 (DE3) cells carrying plasmid pFusionF87V in an LB medium containing ampicillin (final concentration of 100 μg/mL), 5-aminolevulinic acid hydrochloride (80 μg/mL), ammonium iron (II) sulfate (0.1 mM), and isopropyl β-D-thiogalactopyranoside (0.1 mM) were cultured at 20 °C for 19 h with rotary shaking (100 rpm). The cells were collected by centrifugation, and then re-suspended in a sodium phosphate buffer (50 mM, pH 7.2) containing glycerol (5%), proline (5 mM), EDTA (1 mM), and DTT (0.2 mM) as described.[10] Bioconversion reactions were carried out with various flavonoids (final concentration of 1 mM) containing flavones (flavone and 4′-hydroxyflavone), flavanones [flavanone, 2′-hydroxyflavanone, 3′-hydroxyflavanone, 4′-hydroxyflavanone, and naringenin (4′,5,7-trihydroxyflavanone)], chalcone, isoflavone, and isoflavanone in 96-deep-well plates with 0.5 mL each at 28 °C for 24 h with vortex shaking. The substrates used were purchased from Sigma-Aldrich Co. (Missouri, USA), Wako Pure Chemical Industries Co. (Osaka, Japan) or Tokyo Chemical Industry Co. (Tokyo, Japan).

The products were analyzed by HPLC as described.[10] The bioconversion reactions consequently proceeded, except for the flavones (flavone and 4′-hydroxyflavone) and isoflavone. The products from flavanone, 2′-hydroxyflavanone, 3′-hydroxyflavanone, 4′-hydroxyflavanone, naringenin, chalcone, and isoflavanone were then purified and identified.

The reaction mixture (100 mL from 2 plates) was extracted with EtOAc (100 mL × 2 times) to purify each product. The organic layer was concentrated *in vacuo* and analyzed by thin-layer chromatography (TLC) on silica gel [0.25 mm E. Merk silicagel plates (60F-254)]. The converted compounds were purified by silica gel column chromatography [12 mm i.d. × 250 mm, Silica Gel 60 (Kanto Chemicals, Tokyo, Japan)] and subsequent preparative ODS HPLC [10 mm i.d. × 250 mm, Pegasil ODS column (Senshu Scientific, Tokyo, Japan) at a flow rate of 3.0 mL/min] if necessary. The solvent conditions to purify each product and the yield are shown in Fig. 1.

The structures of the purified products were analyzed by mass spectrometry (MS) [HR-APCI-MS (Jeol JMS-T100LP) or HR-EI-MS (Jeol DX505W)] and nuclear magnetic resonance (NMR; 400 MHz, Bruker Avance 400), and the proposed structures were verified by comparing the MS and ^{1}H- and/or ^{13}C-NMR data with those reported. The product from flavanone was identified as 3-hydroxy-2-phenyl-chroman-4-one (3-hydroxyflavanone;[11] compound **1**) (Fig. 1). The relative

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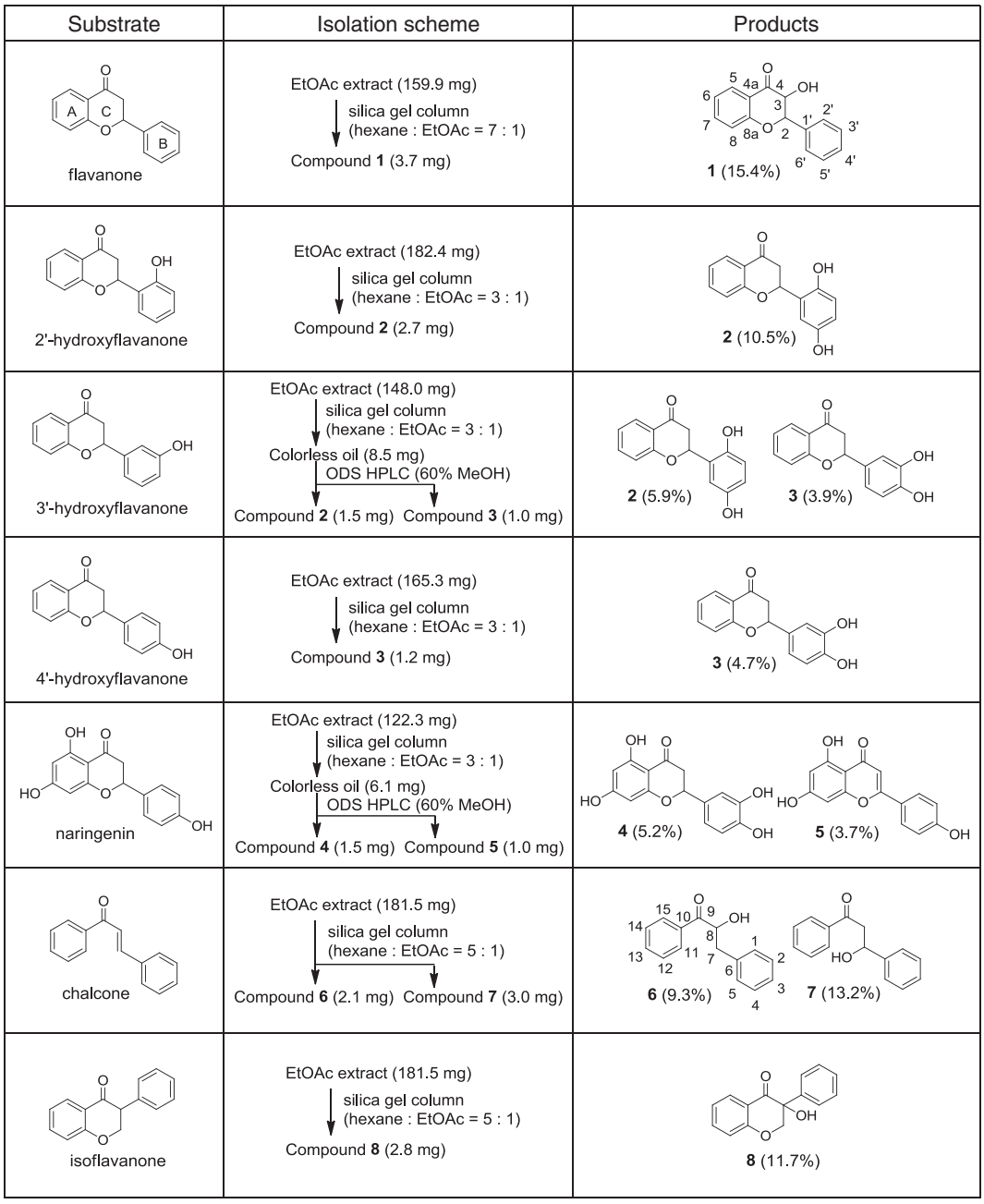

Fig. 1. Bioconversion of Various Flavonoids by Using Cells of *E. coli* BL21 (DE3) Carrying Plasmid pFusionF87V for Expression of the FKBP-P450 BM3 (F87V) Gene.

The mg values in parentheses in the isolation scheme column and the percentage values in parentheses in the product column represent the yield of each purified product.

stereochemistry of H-2 and H-3 was judged to be *trans* due to the large ^{1}H-^{1}H vicinal coupling constant. [^{13}C-NMR ($CDCl_3$) δ: 73.6 (C-3), 83.9 (C-2), 118.1 (C-8), 118.5 (C-4a), 122.1 (C-6), 126.1 (C-5), 127.5 (C-2′ and C-6′), 128.7 (C-3′ and C-5′), 129.3 (C-4′), 136.3 (C-1′), 136.9 (C-7), 161.7 (C-8a), 194.2 (C-4)]. The product from 2′-hydroxyflavanone was identified as 2-(2,5-dihydroxy-phenyl)-chroman-4-one (2′,5′-dihydroxyflavanone;[12] compound **2**) (Fig. 1). [^{1}H-NMR (CD_3OD) δ: 2.88–2.95 (2H, H-3), 5.73 (m, 1H, H-2), 6.62 (dd, J = 2.9, 8.6 Hz, 1H, H-4′), 6.67 (d, J = 8.6 Hz, 1H, H-3′), 6.98 (d, J = 2.9 Hz, 1H, H-6′), 7.06 (dd, J = 7.4, 8.1 Hz, 1H, H-6), 7.08 (d, J = 7.8 Hz, 1H, H-8), 7.55 (dd, J = 7.4, 7.8 Hz, 1H, H-7), 7.85 (d, J = 8.1 Hz, 1H, H-5). ^{13}C-NMR (CD_3OD) δ: 43.9 (C-3), 76.3 (C-2), 114.1 (C-6′), 116.7 (C-4′), 117.2 (C-3′), 119.2 (C-8), 122.1 (C-4a), 122.4 (C-6), 127.7 (C-5), 127.7 (C-1′), 137.4 (C-7), 148.0 (C-2′), 151.4 (C-5′), 163.6 (C-8a), 194.8 (C-3)]. The products from 3′-hydroxyflavanones were identified as compound **2** and 2-(3,4-dihydroxy-phenyl)-chroman-4-one (3′,4′-dihydroxyflavanone;[13] compound **3**) (Fig. 1). The product from 4′-hydroxy-flavanone was identified as compound **3**. The products from naringenin were identified as 2-(3,4-dihydroxy-phenyl)-5,7-dihydroxy-chroman-4-one (3′,4′,5,7-tetra-hydroxyflavanone;[14] compound **4**) (Fig. 1) and 5,7-dihydroxy-2-phenyl-chromen-4-one (apigenin; compound **5**) (Fig. 1) by directly comparing with an authentic sample by an HPLC analysis. We have already reported that apigenin was predominantly converted from naringenin with *E. coli* cells expressing the cyanobacterial P450 *CYP110E1* gene.[6] The products

Table 1. Inhibitory Effects of the Converted Products on Lipid Peroxidation in a Rat Brain Homogenate

Compound	IC_{50} (μM)
flavanone	81
1	8.4
2′-hydroxyflavanone	52
3′-hydroxyflavanone	>100
2	0.78
3	0.77
4′,5,7-trihydroxyflavanone (naringenin)	>100
4	1.2
5 (apigenin)	>100
chalcone	>100
6	>100
7	>100
isoflavanone	88
8	15
quercetin	0.78

from chalcone were identified as 2-hydroxy-1,3-diphenyl-propane-1-one (compound **6**)[15] (Fig. 1) and 3-hydroxy-1,3-diphenyl-propane-1-one (compound **7**)[16] (Fig. 1). The product from isoflavanone was identified as 3-hydroxy-3-phenyl-chroman-4-one (compound **8**)[17] (Fig. 1).

Since each substrate used in this study (excepting chalcone) was a racemic mixture at C-2, we examined the absolute configurations of the products by chiral HPLC analyses {Daicel Chiralcel OD-H column, 10 mm i.d. × 250 mm, Daicel Corporation, Tokyo, Japan; *n*-hexane/2-propanol solvent [9:1 (for **1** and **7**) or 3:1 (for **2**)]; 3.0 mL/min flow rate; detection by monitoring the maximum absorbance in the range of 200–500 nm}, using high-yield compounds **1**, **2** (the product from 2′-hydroxyflavone), and **7** as representatives. All the tested compounds gave two peaks derived from the two enantiomers [**1** (t_R 15.6 min and t_R 21.6 min), **2** (t_R 10.5 min and t_R 11.6 min), **7** (t_R 20.8 min and t_R 23.1 min)], the ratio of the two peaks being approximately 1:1. We judged from these results that all the products (**1**, **2**, **3**, **4**, **6**, **7**, and **8**) were racemic mixtures.

Since hydrogenated products may exhibit anti-oxidative activity due to their structures, we evaluated their *in vitro* inhibitory effects on free radical-induced lipid peroxidation in a rat brain homogenate.[18] The results are shown in Table 1. Compounds **1**, **2**, **3**, and **4** showed superior antioxidative activity when compared to the corresponding substrates. In particular, compounds **2**, **3**, and **4**, which possessed a *para*- or *ortho*-hydroquinone structure in the B ring, showed potent antioxidative activities, being almost identical to that of quercetin (a natural potent antioxidative flavone).

Considering the structures of the products produced in this study, P450 BM3 (F87V) possessed the characteristic of inserting oxygen at C-3 (C ring) or at the *ortho* or *para* position of phenolic OH (B ring) in the (iso)flavanone structure. The latter may be a feasible characteristic for the preparation of antioxidative flavanones, because the *para*- or *ortho*-diol structure was constructed in the converted products. P450s that showed low substrate specificity may have possessed a non-stereospecific character, resulting in the generation of the racemic products from the racemic substrates in this study. In respect of the optical resolution of these products, similar results have been obtained in the case of the epoxidation of 1-phenyl-4-butanol to synthesize 2-phenethyl-oxirane by CYP153A13a (data not shown).[19] The conversion of the flavones (flavone and 4′-hydroxyflavone) was not apparent in this study, while the flavanones were oxygenated. Our previous studies have shown such a result in some cases.[6,20,21] On the other hand, such a characteristic of inserting oxygen at C-3 (C ring) is likely to have been unique to P450 BM3 (F87V) among our enzymes that could oxygenate flavonoids.[6,20,21]

Among products **1**–**8**, compounds **1**, **3** and **4** have been reported to be generated by conventional biotransformation using microbial cells.[11,13,14] The bioconversion of naringenin to apigenin (**5**) has recently been reported with *E. coli* cells expressing the cyanobacterial P450 *CYP110E1* gene.[6] This is therefore the first report of the preparation of compounds **2**, **6**, **7**, and **8** by microbial bioconversion achieved by using recombinant *E. coli* cells that expressed the substrate-promiscuous P450 gene. Moreover, the natural occurrence of compounds **2**, **6**, **7**, and **8** has never before been reported, while many chemical reactions to prepare **6** and **7** have been described.[22,23] Compounds **2** and **8** were rare flavonoids whose preparation by organic synthesis has rarely been reported.[12,18]

We consider that our bioconversion method using recombinant *E. coli* would be superior to conventional biotransformation using microbial cells in respect of product foreseeability. The products produced in this study can be candidates for biologically active chemicals, including medicines, and further biological studies on these products are in progress.

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