Production of Bioactive 3'-Hydroxystilbene Compounds Using the Flavin-Dependent Monooxygenase Sam5^S

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The flavin-dependent monooxygenase Sam5 was previously reported to be a bifunctional hydroxylase with coumarate 3-hydroxylase and resveratrol 3'-hydroxylase activities. In this article, we showed the Sam5 enzyme has 3'-hydroxylation activities for methylated resveratrols (pinostilbene and pterostilbene), hydroxylated resveratrol (oxyresveratrol), and glycosylated resveratrol (piceid) as substrates. However, piceid, a glycone-type stilbene used as a substrate for bioconversion experiments with the Sam5 enzyme expressed in *Escherichia coli*, did not convert to the hydroxylated compound astringin, but it was converted by in vitro enzyme reactions. Finally, we report a novel catalytic activity of Sam5 monooxygenase for the synthesis of piceatannol derivatives, 3'-hydroxylated stilbene compounds. Development of this bioproduction method for the hydroxylation of stilbenes is challenging because of the difficulty in expressing P450-type hydroxylase in *E. coli* and regiospecific chemical synthesis.

Keywords: Monooxygenase, Sam5, hydroxystilbene, piceatannol

The generation of diverse hydroxyl moieties of natural products in microorganisms and plants is largely achieved through the modified reactions catalyzed by monooxygenases and cytochrome P450 enzymes (P450s). Understanding the natural evolution of the function of monooxygenases and P450 hydroxylases should accelerate the production of diverse structures for these natural products. Several microbial monooxygenases and P450 hydroxylases were identified to catalyze the *ortho*-hydroxylation of stilbenes and flavonoids [1–4]. For instance, monooxygenase HpaBC was reported to act on a series of simple 4-hydroxyphenylacetates analogous to tyrosine, *p*-cresol, and phenol as well as simple phenylpropanoid compounds such as *p*-coumaric acid, umbelliferone, naringenin, and resveratrol [3–7]. Recently, we found that a flavin-dependent monooxygenase, Sam5 from actinomycetes, not only converts *p*-coumaric acid to caffeic acid but also possesses resveratrol *ortho*-hydroxylation activity, producing piceatannol [8]. In addition, the Sam5 monooxygenase has a 5.7-fold higher resveratrol *ortho*-

hydroxylation activity for producing piceatannol compared with its coumarate 3-hydroxylase activity. Stilbenes represented by resveratrol are dibenzyl polyphenolic compounds produced in various plant families that protect against external environmental stresses. Although the basic chemical structure of stilbene is widespread throughout various plants, diverse species-specific substitution patterns exist. Among the stilbenes, resveratrol (3,4',5-trihydroxystilbene) is the most popular and widely studied. Resveratrol has been shown to have useful pharmacological activities against inflammatory, chronic, neurodegenerative, and cardiovascular diseases [9]. Indeed, the resveratrol taken by humans is eventually converted to piceatannol (3,3',4',5-tetrahydroxystilbene) in the human liver [10]. Piceatannol is known to have advanced functions compared with the parent compound resveratrol; for example, tyrosine kinase inhibition and anti-adipogenic activities [11, 12]. Thus, the additional introduction of hydroxyl groups is a commonly used strategy by nature to increase the stability, solubility, and biological activities of

Fig. 1. Schematic representation of the regioselective hydroxylation of stilbene compounds by Sam5 monooxygenase.

these compounds [13]. A variety of 3'-hydroxylated stilbene have been found to possess attractive properties for potential pharmaceutical uses [12, 14, 15]. However, these compounds exist at low abundance in nature, which limits their cost-effective isolation and broad applications [16]. Meanwhile, chemical synthesis is a major approach for these compounds, but it often requires relatively long synthetic sequences and uses expensive catalysts and reagents. Therefore, we speculated that Sam5 monooxygenase might also possess the capability of hydroxylating even more various stilbene compounds and not just resveratrol. Thus far, there have been no reports on monooxygenases that catalyze the hydroxylation of stilbene derivatives, such as pinostilbene, pterostilbene, oxyresveratrol, and piceid, other than resveratrol (Fig. 1).

In this study, we first selected methylated resveratrols

(pinostilbene and pterostilbene), hydroxylated resveratrol (oxyresveratrol), and glycosylated resveratrol (piceid) as substrates to survey the plasticity of the Sam5 active site because compared with its substrate, resveratrol shares a phenol moiety. For this purpose, we used a recombinant *Escherichia coli* containing a *sam5* gene expression plasmid (pET-Sam5), which was previously reported [8]. The recombinant *E. coli* strain harboring pET-Sam5 was cultured in synthetic medium (3 g/l KH_2PO_4 , 7.3 g/l K_2HPO_4 , 8.4 g/l MOPS, 2 g/l NH_4Cl , 0.5 g/l NaCl, 0.1 ml/l trace elements, 5 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g/l $(\text{NH}_4)_2\text{SO}_4$, and supplemented with 1 mM IPTG, 15 g/l glucose and 50 mg/l kanamycin) [17]. Upon induction with 1 mM IPTG, the resulting strain was cultivated in the presence of 6.5 mg/l of the substrates pinostilbene (25.2 μM), pterostilbene (25.4 μM), oxyresveratrol (26.6 μM), and piceid (16.7 μM). After a 24 h incubation, we

Fig. 2. HPLC profiles of the bioconversion experiments with pET-Sam5-harboring *Escherichia coli* fed with the stilbene compounds pinostilbene (A), pterostilbene (B), oxyresveratrol (C), and piceid (D).

Standard stilbene (lower panel) and stilbene-supplemented *E. coli* (upper panel). The absorbance was monitored at 320 nm.

analyzed the extract by HPLC (Fig. 2). As shown in Figs. 2A, 2B, and 2C, the substrates pinostilbene, pterostilbene, and oxyresveratrol were completely consumed, and three noticeable new peaks appeared in the HPLC chromatograms. The major peaks in Figs. 2A, 2B, and 2C exhibited parent mass ion peaks at m/z 259.15, 273.12, and 261.09 $[M+H]^+$, respectively, which corresponded to one hydroxylation of pinostilbene, pterostilbene, and oxyresveratrol (an addition of 15 Da; Fig. 3). It was expected that these hydroxylated compounds could have a hydroxy group located in the 3' position of the B-ring in each parent compound (Fig. 1). When the bioconversion rate was calculated through a quantitative comparison of the feeding standard substrates (6.5 mg/l), the conversion ratios for pinostilbene, pterostilbene, and oxyresveratrol in *E. coli* with the *sam5* gene were roughly 54%, 37%, and 51%, respectively. The bioconversion ratios, which were lower than the HPLC profile (Fig. 2), were assumed to have derived from the instability of the

compound. This phenomenon might be because the resveratrol and piceatannol derivatives were broken down in *E. coli* [18]. The MS/MS analysis of these new peaks had different fragments in the ion pattern compared with the pinostilbene, pterostilbene, and oxyresveratrol standards, with peaks of m/z 241.03, 255.05, and 243.00 $[M+H]^+$, which means that two hydroxyl groups located in the B-ring were also identified (Fig. S1). Thus, the new peaks at 9.2 and 12.1 min were confirmed as 3-methyl-piceatannol and 3,5-dimethyl-piceatannol respectively. However, we could not confirm the hydroxylation position of the oxyresveratrol products because it was not possible to establish the exact position of hydroxylation for either the C2' or C6' position by analyzing the MS/MS fragmentation pattern analysis. To confirm the hydroxylation position, we cultured the *E. coli* harboring the pET-Sam5 in 4 L with 10 mg of oxyresveratrol and purified the hydroxylated oxyresveratrol (5.4 mg) for structural investigation by NMR analysis. The

Fig. 3. Selected mass ion chromatogram of new peaks produced by bioconversion experiments with pET-Sam5-harboring *E. coli* fed with pinostilbene (A), pterostilbene (B), and oxyresveratrol (C).

structural elucidation of the purified compounds was done with ¹H and ¹³C NMR spectroscopy (Table S1). The NMR experiments were done on a Bruker AVANCE spectrometer (700 MHz; Bruker Inc., USA). The purified compounds exhibited the characteristic paired signals including those of oxyresveratrol suggesting that the purified compound is a modified oxyresveratrol and contains a C-3' hydroxylated of B-ring resveratrol skeleton (Table S1 & Fig. S3). Interestingly, the glycosylated substrate piceid did not form a new peak in the same HPLC analysis (Fig. 2D). The reason could be that Sam5 may not have substrate specificity for a glycosylated compound or that piceid may not pass through the membrane of the *E. coli*.

To confirm the function of the Sam5 enzyme whether the bioconversion activities match enzymatic *in vitro* reactions, we expressed and purified the His-tagged Sam5 enzyme from *E. coli*. The expression and purification methods

followed a previously reported method [8]. Flavin-dependent monooxygenases use NAD(P)H and O₂ as co-substrates. NAD(P)H reduces FAD, and the reduced FAD reacts with oxygen, and the activated oxygen is used for further reactions. Additionally, the *in vitro* reaction was done with a flavin reductase (the Fre enzyme from *E. coli* [19]; see the Supporting Information) that supplies FADH₂ to a FADH₂-utilizing monooxygenase. The Sam5 monooxygenase activity for each stilbene was investigated by measuring the conversion of substrate (100 μM pinostilbene, pterostilbene, oxyresveratrol, and piceid) to each hydroxylated product during 60 min. In the enzymatic reaction, all of the substrates were observed as a new peak on the HPLC profile (Figs. S2 and 4). In that case of using pinostilbene, pterostilbene, and oxyresveratrol as substrates, each new peak at 9.2, 12.1, and 6.0 min exactly matched those of the bioconversion experiment (Fig. S2). Interestingly, piceid

Fig. 4. HPLC profiles (A) and selected mass ion chromatogram (B) of the in vitro enzyme reaction of the purified His-tagged Sam5 enzyme with piceid as the substrate.

(A) The peak at 4.9 min was found to correspond to hydroxylated piceid (astringin). (B) Selected mass ion chromatogram of hydroxylated piceid (m/z 407.1 $[M+H]^+$).

formed a new peak at 4.9 min in the same HPLC analysis (Fig. 4). Additionally, the new peak in Fig. 4 had a parent mass ion peak at m/z 407.10 $[M+H]^+$ under a positive ESI mode, which corresponds to one hydroxylation of piceid (an addition of 15 Da; Fig. 4B). Its identity was confirmed as a hydroxylated piceid, astringin (MW 406), from the MS/MS analysis (Fig. S1D) of the m/z 407.10 $[M+H]^+$ ion, with a prominent product ion at m/z 245.06 $[M+H]^+$ (protonated piceatannol), which indicates the loss of the glucose moiety. The presence of m/z 245.06 $[M+H]^+$ indicates that the two hydroxyl groups are located in the B-ring of resveratrol (Fig. S1D). These data strongly mean that the peak at 4.9 min is a glucoside on the A-ring as well as a hydroxylate on the B-ring of resveratrol [20]. The in vitro conversion ratios for pinostilbene, pterostilbene,

oxyresveratrol, and piceid in enzyme reactions with the Sam5 and Fre proteins were roughly 50%, 38%, 23%, and 31%, respectively. As a result, piceid could not have passed well through the membrane of *E. coli* in this bioconversion condition like as through a human intestinal cell line [21]. Thus, glycosylated piceatannol, astringin, produced by a β -glucosidase can occur in the human liver and small intestine, which would enhance the quantity of piceatannol available from oral administration or a diet [22]. Moreover, several reports have shown that the methylation of resveratrol, pinostilbene, and pterostilbene, results in the enhancement of its bioavailability and bioactivity compared with resveratrol [23]. In particular, pterostilbene had increased bioactivity on aging and longevity in *Caenorhabditis elegans* [24]. Moreover, pterostilbene showed 2–5-fold

lower IC₅₀ values than those of resveratrol by cell viability tests in human colon cancer cells [25]. Pterostilbene effectively scavenges peroxy radicals and reduces singlet-oxygen-induced peroxidation at levels similar to those of resveratrol [26].

Herein, we found that the Sam5 enzyme can selectively hydroxylate bioactive stilbene compounds, for which the regiospecific hydroxylation of complex aromatic compounds is still quite challenging for chemical synthesis. This led to the exploration of more appropriate methods for their production by biotechnological production in microbial hosts. Recently, the biotechnological production can be pursued by reconstructing biosynthetic pathways in controllable microbial systems [16]. However, this method also needs more diverse enzymatic resources for the production of various compounds. This biohydroxylation approach with high efficiency is highly desirable and of great practical value, and can meet the demands for catalytic enzymes.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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