J. Microbiol. Biotechnol. (2018), 28(4), 561–565
https://doi.org/10.4014/jmb.1711.11047

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Hydroxylation of Resveratrol with DoxA In Vitro: An Enzyme with the Potential for the Bioconversion of a Bioactive Stilbene

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Received: November 27, 2017
Revised: January 9, 2018
Accepted: January 19, 2018

First published online
February 1, 2018

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pISSN 1017-7825, eISSN 1738-8872

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The late-stage doxorubicin biosynthesis pathway acting enzyme (DoxA) from *Streptomyces peucetius* CYP129A2 exhibited substrate promiscuity towards the stilbene group of compounds such as resveratrol. DoxA along with two accessory enzymes ferrdoxin reductase and ferredoxin from spinach hydroxylated resveratrol at the 3′-position in vitro to produce piceatannol. The product was identified by HPLC-PDA and high-resolution HR-qTOF-ESI/MS analyses in positive mode. The ESI/MS fragments resembled the hydroxylated product of resveratrol.

Keywords: DoxA, hydroxylation, monooxygenase, resveratrol, substrate flexibility

Natural stilbenes like resveratrol and piceatannol are plant secondary metabolites, and the hydroxylation of such secondary metabolites can enhance their solubility, stability, structural variations, and diverse pharmacological and biological activities [1, 2]. Furthermore, study has shown that resveratrol exerts a number of positive effects related to the heart, blood circulation, brain, and age-related diseases [3]. Resveratrol is hydroxylated in the human liver [4] into piceatannol, the latter of which exhibits numerous therapeutic effects beyond those of its parental compound, including anti-parasitic, antitumor, antioxidant, vasorelaxation, and anti-inflammatory effects [5]. Moreover, piceatannol is now associated with the prevention of hypercholesterolemia, arrhythmia, atherosclerosis, angiogenesis, and cardiovascular diseases [6], and because of these beneficial properties, its potential applications in the health, functional food, and cosmetics sectors have been identified. The main obstacles for the industrial use of these molecules are a low natural abundance and the difficulty and hazardousness of the chemical synthesis [7].

DoxA (CYP109A2) is a cytochrome P450 monooxygenase that plays a vital role in the production of the anticancer drug doxorubicin in *Streptomyces peucetius*, whereby it is engaged in the late biosynthetic stages of daunorubicin and doxorubicin [8]. It has been demonstrated that DoxA can catalyze three oxidation steps during the production of doxorubicin [9], and this proved the broad substrate specificity of DoxA with respect to the anthracycline group of substrates. We were curious whether DoxA can catalyze other substrates besides anthracyclines. We selected several flavonoids and stilbenoids randomly and performed an in silco docking study to validate the possible substrate for in vitro assay (Fig. 1). We predicted that DoxA could be applied for the hydroxylation of stilbenes such as resveratrol; therefore, the in vitro reaction of DoxA with resveratrol was performed in this study to expand the substrate flexibility of DoxA toward stilbenoids.

The expression and purification of DoxA were performed according to the previously described procedures [10]. The protein size (49 kDa) was similar to that in the previous reports (Fig. 2A) [10]. An extinction molecular coefficient of $\varepsilon_{450-490} = 91\ mM^{-1}\ cm^{-1}$ was used to calculate the protein concentration [11]. After the reduction of the DoxA with carbon monoxide (CO), two peaks were observed at 450 and 420 nm (Fig. 2B); here, the 30 nm difference between the peaks denotes a notable perturbation of the spreading

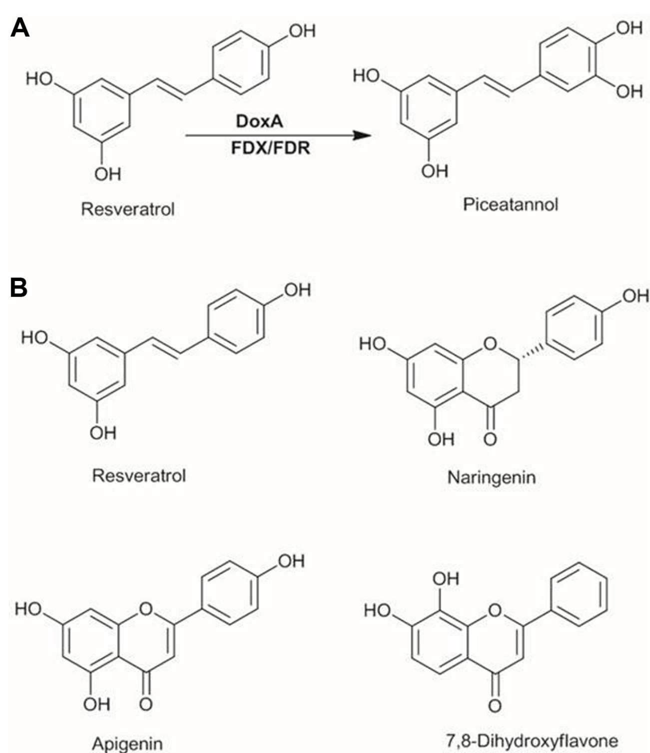

Fig. 1. Chemical structures of the substrates.

(**A**) The conversion of resveratrol to its hydroxylated product piceatannol is catalyzed by the *Streptomyces peucetius* DoxA. (**B**) The different substrates used in this study.

of the electron density at the heme. The in vitro DoxA reaction included the use of 1 μM of DoxA, 10 μM of spinach ferredoxin, and 3 μM of spinach ferredoxin reductase in 0.5 ml of 100 mM sodium-phosphate buffer (pH 7.6) that served as an NADPH-regeneration system, 10 mM of glucose-6-phosphate, 100 ng of catalase, 1 U of baker's yeast glucose-6-phsphate dehydrogenase, and 0.4 mM of NADPH. The final substrate concentration used was 150 μM. The reaction was incubated at 30°C for 3 h. The reaction was quenched by the addition of 0.5 ml of ethyl acetate, followed by a post-vortex centrifuging for 10 min. The supernatant was then collected and evaporated in nitrogen gas; the resultant dried sample was dissolved in acetonitrile, followed by the performances of HPLC (SPD-M20A; Shimadzu, Japan) and LC/MS analyses. The HPLC analysis of the extracted reaction mixture revealed a new peak P2 that was absent from the control, which comprised only the single peak, P1, at 308 nm. Peak P2 showed almost the same retention time (t_R) of 8.2 min with peak P of standard piceatannol (Fig. 3A). A successive analysis of the same reaction mixture by high-resolution quadruple time-of-flight electrospray ionization mass spectroscopy (HRQTOF ESI/MS) (ACQUITY UPLC; Waters Corp., USA) coupled with a SYNAPT G2-S (Water Corp., USA) showed a precisely monohydroxylated product mass of 245.08 in positive-ion mode (Fig. 3D). For identification of the compound, we compared the MS of both standard piceatannol (Fig. 3B) and the reaction product (Fig. 3D). The presence of *m/z* 245.08 $[M+H]^+$, *m/z* 171.09 $[M+H]^+$, and *m/z* 135.04 $[M+H]^+$ in both spectra gave the tentative identification of the compound as piceatannol. Similarly, MS/MS spectra of standard piceatannol (Fig. 3C) and the reaction product were compared (Fig. 3E). The existence of the molecular

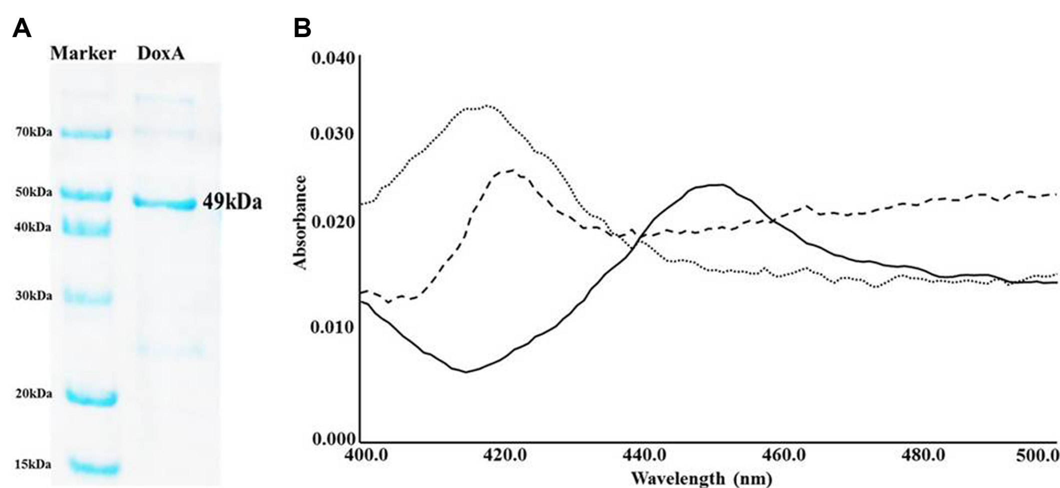

Fig. 2. Characterization of the purified DoxA from *S. peucetius*.

(**A**) SDS-PAGE analysis of DoxA that was used in the in vitro reaction, where the protein size is 49 kDa. (**B**) Carbon monoxide (CO)-reduced spectra of the overexpressed DoxA: Oxidized form, solid line; dithionate-reduced form, long-dash line; and CO-reduced complex, dotted line.

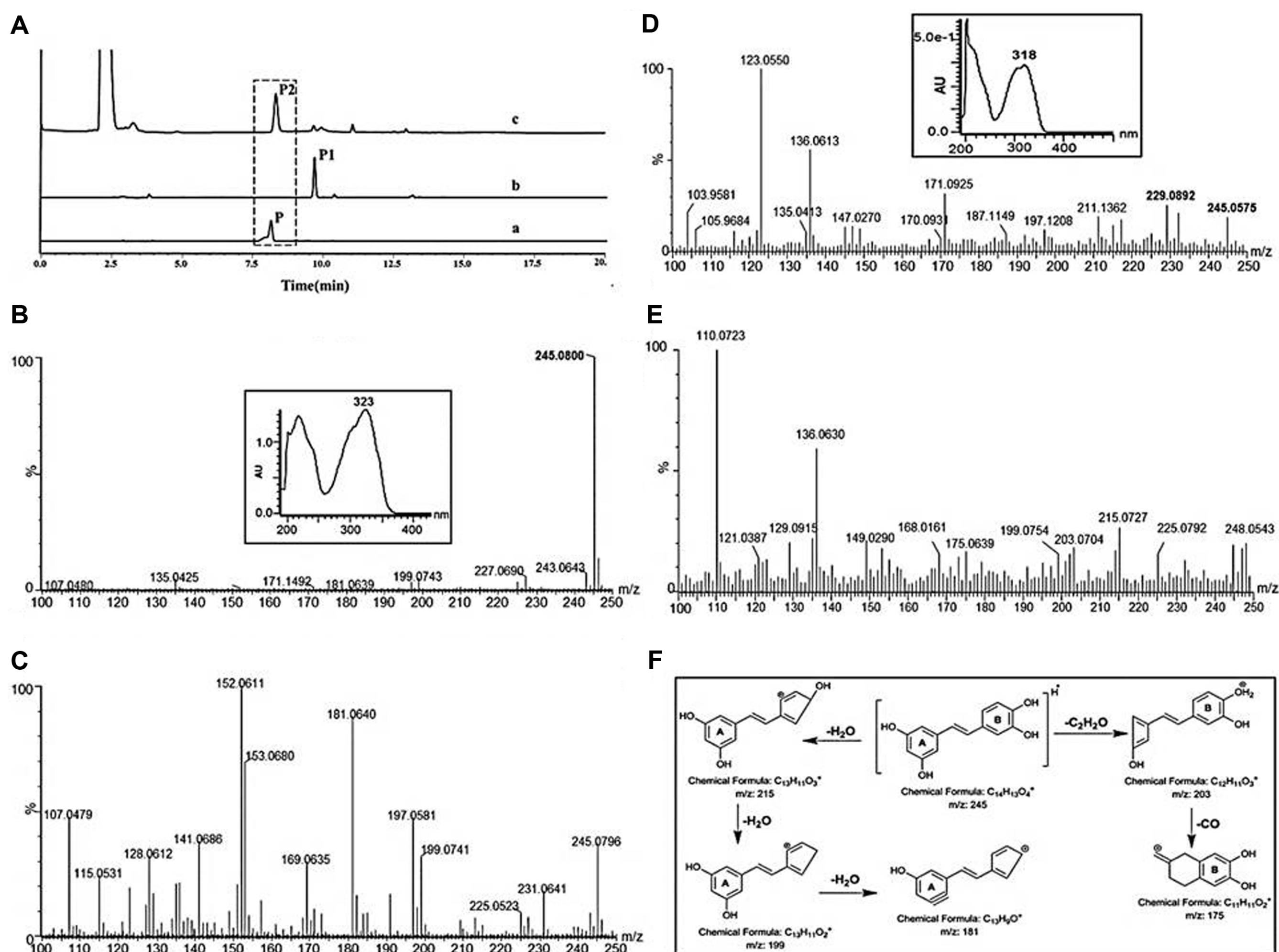

Fig. 3. HPLC and HRQTOF ESI/MS analyses of the reaction products and the standard piceatannol.

(**A**) HPLC chromatogram of the piceatannol (a), control (b), and reaction (c). (**B**) Full-scan positive (TOF) ion spray spectra of piceatannol (UV spectrum is shown in inset). (**C**) Full-scan positive MS/MS (TOF) ion spray spectra of piceatannol. (**D**) Positive-ion MS of DoxA reaction products (UV spectrum is shown in inset). (**E**) Positive-ion MS/MS spectra of the DoxA reaction products. (**F**) Probable fragmentation patterns of the compound are shown for selected fragments (structural difference from those shown may be possible).

ions and related fragments in both spectra, such as *m/z* 225.0792 $[M+H]^+$, *m/z* 215.0727 $[M+H]^+$, *m/z* 199.0754 $[M+H]^+$, *m/z* 203.0704 $[M+H]^+$, and *m/z* 175.0639 $[M+H]^+$, are typical for piceatannol (Fig. 3E) [12-14]. Moreover, after comparing the molecular ions and related fragments of piceatannol from the Human Metabolome Database (HMDB) [14], the fragments at *m/z* 203.070 $[M+H]^+$ and *m/z* 175.075 $[M+H]^+$, which indicate the ring-B hydroxylation position of resveratrol (Fig. 3F), confirmed that the product is piceatannol. Since we used commercially available redox partners that gave a trace amount of product, surprisingly no product was detected after using putidaredoxin and putidaredoxin reductase as auxiliary proteins. Although the in vitro reaction was also performed with other phenolic substrates such as naringenin, apigenin, and 7,8-dihydroxyflavone, no hydroxylated product was detected. The conversion of only the resveratrol could highlight the substrate selectivity of DoxA toward stilbene.

The in silico study consisted of the construction of the DoxA model and docking of four different substrates using Discovery Studio (ver. 3.5, 2012; Accelrys Inc., USA; http://www.accelrys.com). The templates for making the DoxA model were retrieved from the Protein Data Bank (PDB, http://www.pdb.org) and were aligned with the target and scanned for conserved sequences. MODELER, developed by Sali, was used for generating the DoxA model [10].

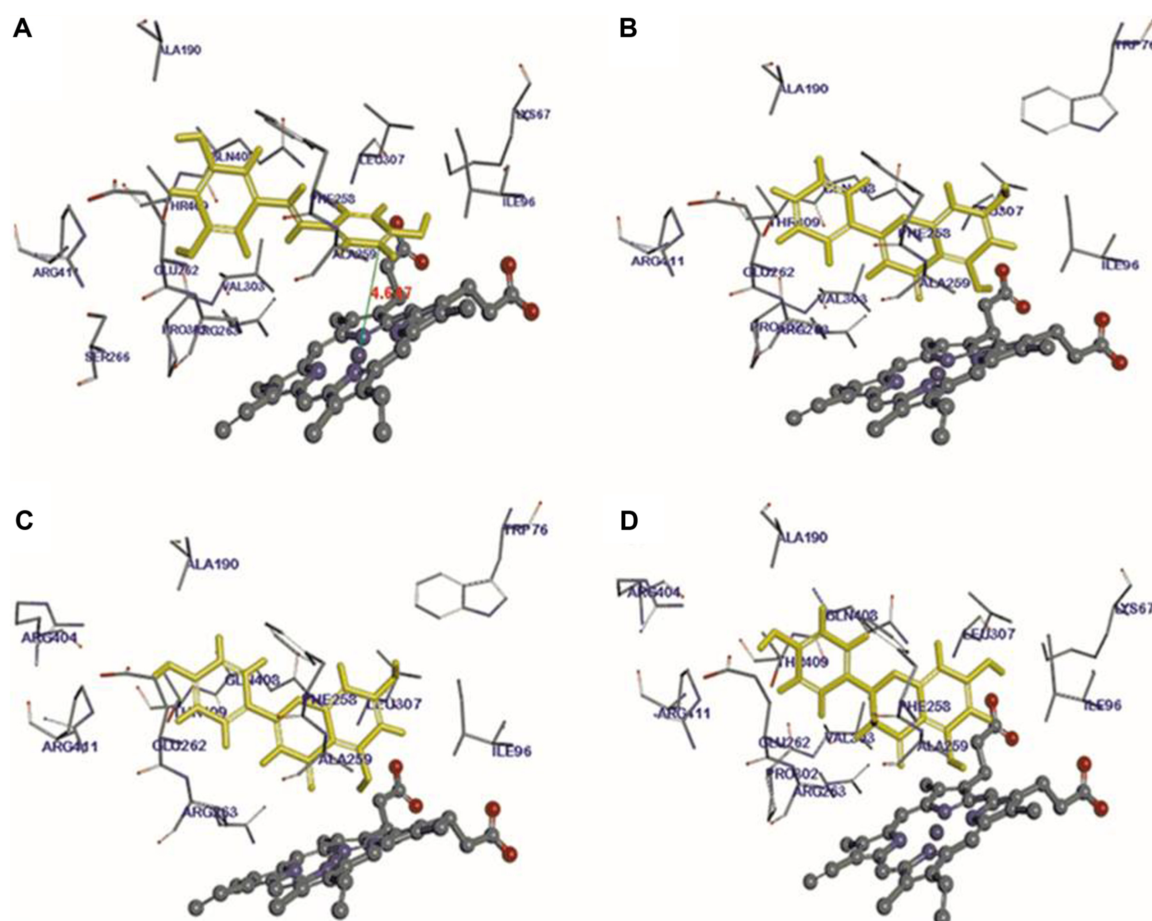

Fig. 4. The 3D structure of DoxA, showing a large pocket just above the heme-center that is believed to adjust to relatively large substrates.

Docking of resveratrol (**A**), 7,8-dihydroxyflavone (**B**), apigenin (**C**), and naringenin (**D**). Residues are denoted in dark blue color.

Orientation of the substrate and the binding energy were taken into consideration for the docking study. During the docking of substrates with the constructed DoxA model, we found that they were not located in the right position, except for the resveratrol C4′ that sits above the heme-prosthetic group and makes the shortest distance of 4.7 Å suitable for hydroxylation. That was an almost equal distance of 4.05 Å (data not shown) between DoxA and its native substrate daunorubicin. However, dihydroxyflavone, apigenin, and naringenin faced in a different orientation thus making it difficult to contact the heme-prosthetic group, and hence calculation of the binding energy was not possible (Fig. 4).

We have validated that in silico and experimental data combined can tell much about the substrate preference and flexibility of cytochrome P450. We also confirmed the idea that the conformation and distance from the heme-center also play a significant role in substrate binding and hydroxylation [15]. The monohydroxylated product of resveratrol proved that the bacterial P450 DoxA can be used for the production of the human metabolite piceatannol, which is more active and expensive than its parent compound. This study also demonstrated the flexibility of DoxA toward other plant phenolic substrates, besides its actual anthracycline substrates, and this knowledge could be useful for enhancement of the production of valuable natural products. An overall understanding of the actual structure (crystal structure), the development of the in vivo system, and the mutation on the specific site could enable the hydroxylation of resveratrol by the DoxA enzyme that achieves a considerable production of the human metabolite piceatannol.

Acknowledgments

This work was supported by the Sun Moon University Research Grant of 2016.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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