Altering the Regioselectivity of Cytochrome P450 BM3 Variant M13 toward Genistein through Protein Engineering and Variation of Reaction Conditions

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ABSTRACT: The biocatalysts responsible for the enzymatic synthesis of hydroxygenisteins, derivatives of genistein with multiple activities, usually show regioselective promiscuity, hydroxylating genistein to form a mixture of multiple products, which, in turn, results in a cumbersome separation and purification. Hence, it is highly desired to explore the underlying mechanism regulating the regioselectivity of hydroxylases. M13 is a variant of cytochrome P450 BM3 with oxidant activity toward genistein. Herein, genistein was demonstrated to be hydroxylated by M13 to form a mixture of 3′-hydroxygenistein (3′-OHG) and 8-hydroxygenistein (8-OHG), each giving 4% conversion with a ratio of 1:1. Protein engineering toward M13 was thus performed to improve its regioselectivity. When isoleucine at position 86 was mutated into cysteine, the resultant variant M13I86C displayed improved regioselectivity toward 3′-OHG with an increased conversion of 8.5%. The double mutation M13I86CP18W further boosted the conversion of 3′-OHG to 9.6%, and the ratio of 3′-OHG to 8-OHG increased to 12:1. Conversely, both CoCl2 and glucose 6-phosphate (G6P) could lead to more 8-OHG. When Co2+ reached 37.5 mM, M13I86CP18W could give an 8-OHG conversion of 22.4%. The maximal ratio of 8-OHG to 3′-OHG reached 130 when 62.5 mM Co2+ was included in the reaction mixture. With the increase of G6P from 10 to 40 mM, the conversion of M13I86CP18W to 8-OHG gradually increased to 22.6%, while the conversion to 3′-OHG decreased to 6%. Thus, both intrinsic residues and external reaction conditions can affect the regiospecificity of M13, which laid the foundation for the selection of suitable biocatalysts for the hydroxylation of genistein.

INTRODUCTION

Genistein is a naturally occurring isoflavone primarily found in soybeans and soy food (Figure 1). Moreover, it has been reported that genistein displays multiple activities, such as anticancer, anti-inflammatory, and hypotensive effects. Various structural modifications in genistein had thus been carried out with the aim to improve the efficacy or the solubility. Among these structural modifications, hydroxylation, which is achieved mainly by enzyme-mediated biocatalysis, is an important modification because hydroxylated derivatives of genistein exhibit improved pharmaceutical activities. 3′-Hydroxygenistein (3′-OHG; Figure 1), for example, shows multiple biological effects such as anticancer, anti-inflammatory, neuroprotective, anti-obesity, antioxidant, and antiviral activities. 8-Hydroxygenistein (8-OHG; Figure 1) displays antioxidant, anticancer, and antimitogenic effects. A rare hydroxylated derivative of genistein, was demonstrated to have hepatoprotective activity. These data collectively indicate that hydroxylated derivatives may be one of the important sources of active lead compounds.

Hydroxylation of genistein is achieved mainly through enzyme-mediated biocatalysis. The biocatalytic enzymes responsible for the hydroxylation of genistein are generally cytochrome P450 proteins, like BM3 (CYP102A1) from Bacillus megaterium,19 CYP57B3 from Aspergillus oryzae,20−23 and CYP105D724 and CYP107Y1 from Streptomyces avermitilis MA4680.25 Moreover, other monoxygenases such as tyrosinase,6,11,12,26,27 and a flavin-dependent monoxygenase Sam5 from Saccharothrix espanaensis28 have also been applied to hydroxylate genistein to form hydroxylated derivatives. Besides the enzymes, whole cells including Streptomyces sp. 060524,29 A. oryzae,30−31 Streptomyces griseus, and Streptomyces cattaneae3 could be used as biocatalysts to hydroxylate genistein.

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Of these biocatalysts, many enzymes are catalytically promiscuous, hydroxylating genistein to form multiple products. For example, CYP57B3 was demonstrated to be capable of hydroxylating genistein at C6, C8, and C3’ positions, respectively. 20,21 Sam5 was able to hydroxylate genistein to yield two products.28 Also, both BM3 mutants M13 (R47L/L86I/F87V/L188Q) and M15 (R47L/E64G/F87V/E143G/L188Q/E267V) can hydroxylate genistein to form two products, respectively.19 The catalytic flexibility undoubtedly enriches the product diversity, thereby increasing the possibility to obtain lead compounds. However, promiscuous enzymes usually result in a mixture of multiple products, which makes it difficult to separate and purify the target products. In this way, the promiscuity of enzymes is a double-edged sword in the enzymatic synthesis of hydroxylated genistein. How to effectively regulate this double-edged sword and balance its role in the diversity and specificity of desired products are very important for the enzymatic synthesis of hydroxylated derivatives of genistein.

Cytochrome P450 BM3 is a soluble polypeptide in which the P450 hydroxylase is naturally fused to its redox partner to afford BM3, which has the highest catalytic activity due to its self-sufficient electron transfer.32 Moreover, a high degree of structural plasticity of BM3 directs the generation of varied mutants with varied substrate specificity.33,34 Taken together, BM3 is a promising biocatalyst for the hydroxylation of active compounds. Hence, it is highly desired to explore the underlying mechanism of BM3 regulating catalytic promiscuity and substrate specificity. M13, a variant of BM3, was a promiscuous hydroxylase converting genistein to form two metabolites.35 M13 may be developed into a biocatalyst for the hydroxylation of genistein due to its self-sufficient catalysis and extreme plasticity. The underlying mechanism of M13 regulating promiscuity and specificity was thus explored in this research. First, we demonstrated that M13 displayed promiscuous regioselectivity toward genistein, hydroxylating genistein to form 3’-OHG, 8-OHG, and an unknown dihydroxygenistein (Figure 1). Subsequently, docking-directed protein engineering toward M13 was performed with the aim to balance the product promiscuity and specificity. M13 variants, namely, M13I86C and M13I86CP18W, displayed improved regiospecificity toward 3’-OHG. On the contrary, both Co2+ and glucose 6-phosphate (G6P) could lead to more 8-OHG. These data collectively indicate that both the intrinsic protein structure and the external reaction conditions can affect the regiospecificity of M13 toward genistein.

RESULTS AND DISCUSSION

Substrate Docking. Genistein was docked into the catalytic site of M13, using the MOE algorithm. The retrieved hits were ranked according to their MOE docking scores (in kcal/mol). The four highest-ranking poses exhibited the docking score of −9.63, −9.47, −9.38, and −9.08 kcal/mol, respectively, suggesting that they were tightly fitted into the active sites of M13 (Figure 2). At least two orientations among the four highest-ranking poses were generated (Figure 2). In the first orientation, the B ring of genistein was close to the heme of M13 (Figure 2A,B,D), while in the other orientation, the A ring of genistein was in close proximity to the heme (Figure 2C). Three best-ranking docking conformations of genistein having the B ring oriented toward heme were identified (Figure 1). The C3’ position of genistein was found to be the closest substrate atom to the heme in these three conformations. The distance of C3’ to heme was 5.49, 5.66, and 4.23 Å, suggesting this atom would be attacked in the hydroxylation reaction (Figure 2A,B,D). Likewise, when the A ring was close to the heme, C8 is the closest atom to the heme, and this atom is thus prone to hydroxylation (Figure 2C).
Taking together, C3′ and C8 are the most vulnerable positions for M13-catalyzed hydroxylations. Under the action of M13, genistein is most likely to be hydroxylated to form 3′-OHG and 8-OHG.

**Protein Expression and Purification of M13.** An expression plasmid pET28aM13 was introduced into *Escherichia coli* Transetta (DE3) (TransGen Biotech, Beijing, China) to generate a recombinant strain Transetta (DE3) [pET28aM13] for heterologous expression. After isopropyl-β-d-thiogalactopyranoside (IPTG) induction, the crude extract of Transetta (DE3) [pET28aM13] was subjected to SDS-PAGE analysis. As shown in Supporting Information Figure S1A, an intense band with a molecular mass of 120 kDa, consistent with the theoretical mass of the recombinant M13 protein, was present in the crude extract expressing the M13 gene. On the contrary, no corresponding band was detected in the control crude extract. These data collectively suggested a soluble expression of M13 in *E. coli*. The recombinant protein was purified to near homogeneity, and the concentrations of purified M13 were determined to be 68.6 mg/mL (Figure S1B). The purified protein was used as the biocatalyst for hydroxylation of genistein unless otherwise specified.

**M13-Mediated Hydroxylation toward Genistein.** After being incubated with purified M13 protein at 37 °C for 2 h, genistein was hydroxylated into two new products with respective retention times of 24.8 min (P1) and 27.5 min (P2) (Figure 3A). The UV spectra of the two newly formed metabolites are consistent with that of genistein, indicating that they have a similar skeleton with genistein (Figure 3B). Genistein has a maximum absorption at 260 nm due to the absorption of the A-ring benzoyl system. The maximum absorption of hydroxyl metabolite P1 shifts toward a longer wavelength (267 nm), suggesting that a hydroxyl modification occurs on the A ring. On the contrary, P2 exhibits the same maximum absorption at 260 nm as genistein, indicating that a hydroxyl group is located on the B ring.

Both gave [M − H]- ion at m/z 285.0373 in the full-scan mass spectrum, corresponding to mono-hydroxylated metabolites of genistein (Figure 3C,D).

**Regioselectivity Improvement toward 3′-OHG.** M13-catalyzed hydroxylation toward genistein generated almost equal amounts of 8-OHG and 3′-OHG with a conversion rate of 96.2% for 3′-OHG. After incubation with 10 mg/mL purified M13 protein for 2 h, approximately 50% of the genistein was converted to 3′-OHG (Table 1).

| position | δC | δH |
|----------|----|----|
| 2        | 154.46, CH | 8.11, 1H, s |
| 3        | 123.20, C   |             |
| 4        | 182.10, C   |             |
| 5        | 155.43, C   |             |
| 6        | 99.59, CH   | 6.30, 1H, s |
| 7        | 154.24, C   |             |
| 8        | 125.98, C   |             |
| 9        | 147.11, C   |             |
| 10       | 105.99, C   |             |
| 1′       | 124.13, C   |             |
| 2′,6′    | 131.24, CH  | 7.37, 2H, d (8.7 Hz) |
| 3′,5′    | 116.07, CH  | 6.85, 2H, d (8.6 Hz) |
| 4′       | 158.59, C   |             |

These two metabolites were collected for NMR measurements. Based on 1H- and 13C-NMR data (Figures S2–S5 and Tables 1 and 2), together with previously reported values, 2P1 and P2 were assigned to be 8-OHG (Table 1) and 3′-OHG, respectively (Table 2). The hydroxylated behavior of M13 toward genistein is consistent with the docking analysis.

**Table 1. NMR Spectroscopy Data for 8-OHG in CD3OD (δ in ppm, J in Hz)**
Table 2. NMR Spectroscopy Data for 3′-OHG in CD3OD (δ in ppm, J in Hz)

| position | δC       | δH       |
|----------|----------|----------|
| 2        | 154.53, CH | 8.03, 1H, s |
| 3        | 123.67, C  |          |
| 4        | 181.96, C  |          |
| 5        | 163.65, C  |          |
| 6        | 100.15, CH | 6.18, 1H, d(2.1 Hz) |
| 7        | 166.47, C  |          |
| 8        | 94.72, CH  | 6.29, 1H, d(2.1 Hz) |
| 9        | 159.54, C  |          |
| 10       | 105.89, C  |          |
| 1′       | 124.59, C  |          |
| 2′       | 121.48, CH | 6.99, 1H, d(2.0 Hz) |
| 3′       | 146.04, CH | 6.79, 1H, d(8.1 Hz) |
| 4′       | 146.60, C  |          |
| 5′       | 116.12, CH | 6.82, 1H, dd (8.0 Hz, 2.0 Hz) |
| 6′       | 117.24, C  |          |

of 4%, making it difficult to separate these compounds (Figure 4 and Table 3). Protein engineering toward M13 was thus performed using site-directed mutagenesis. The purified variants were used as biocatalysts for genistein hydroxylation. Of these variants, M13I86CP18W was able to further improve the regioselectivity toward 3′-OHG (Figure 4). Under the action of M13I86CP18W, more 3′-OHG was generated from genistein with a conversion rate of 9.6%. On the other hand, the conversion rate of 8-OHG declined to 0.8%, which increased the ratio of 3′-OHG to 8-OHG in the reaction mixture to 12:1 (Figure 4, Table 3). These data revealed that the two residues I86 and P18 had effects on the regioselectivity of M13 toward 3′-OHG.

Regioselectivity Improvement toward 8-OHG. The accumulated evidence indicated that divalent metal ions had effects on P450 activity.35,36 Hence, the effect of Co2+ on the regioselectivity of M13 was also investigated. When Mg2+ in the reaction mixture was changed to Co2+, the regioselectivity of M13I86CP18W toward genistein varied (Figure 6A). 8-OHG became the major metabolite in the reaction mixture with Co2+. The conversion rate of 8-OHG increased with the rise of Co2+ concentration in the reaction mixture. When the final concentration of Co2+ in the reaction mixture increased to

![Figure 4. HPLC profiles of hydroxygenisteins catalyzed by M13 and its variants.](https://dx.doi.org/10.1021/acsomega.0c05088)
37.5 mM, the conversion of genistein to 8-OHG was the highest, reaching 22.4% (Figure 6A,B). In addition, a third metabolite with a retention time of 21.0 min was obtained. This newly formed metabolite was identified as a dihydroxylated genistein with a mass of m/z 301.03436 (Figure 6C). This dihydroxylated metabolite displayed a UV spectrum with maximum absorption of 267 nm, similar to that of 8-OHG (Figure 6D). This evidence, together with the catalytic behavior of M13 toward genistein, suggested that this dihydroxylated derivative was 3′,8-dihydroxygenistein (3′,8-DOHG).

With the continuous increase in Co²⁺ concentration, the conversion of 8-OHG began to decrease. When Co²⁺ increased to 62.5 mm, the conversion of 8-OHG decreased to 2.6%. On
the contrary, Co²⁺ was able to inhibit the generation of 3’-OHG. As exhibited in Figure 6, the conversion rate of 3’-OHG declined with the increase of Co²⁺ in the reaction mixture. When Co²⁺ in the reaction mixture was 62.5 mM, 3’-OHG was present in trace amount and the conversion rate was only 0.02%. Thus, with the increase of Co²⁺ from 25 to 62.5 mM, the ratio of 8-OHG to 3’-OHG rose significantly (Figure 6 and Table 3).

G6P, a substrate of glucose 6-phosphate dehydrogenase (G6PD) in the pentose phosphate pathway, was believed to be critical to NADPH regeneration. Hence, the effect of G6P on the regioselectivity was also investigated. Results indicated that besides Co²⁺, G6P also had an effect on the regioselectivity of M13I86CP18W (Figure 7). When G6P was present in low concentration (5 mM), M13I86CP18W yielded 3’-OHG as the major product. The conversion rate of 3’-OHG was more than 10 times that of 8-OHG (Figures 4 and 7A and Table 3). With the increase in G6P concentrations from 10 to 40 mM, the conversion of 8-OHG gradually increased, while that of 3’-OHG decreased (Figure 7). The ratio of 8-OHG to 3’-OHG was thus positively correlated with the concentration of G6P. Moreover, the presence of 3’8-OHDOH was observable when the final concentration of G6P was above 5 mM (Figure 7A). These data clearly showed that 3’8-OHDOH began to appear with an increase in the yield of 8-OHG. On the contrary, the content improvement of 3’-OHG did not lead to the formation of 3’,8-DOH. These facts suggested that genistein was first hydroxylated by M13I86CP18W to form 8-OHG and 3’-OHG. Next, 8-OHG was further hydroxylated to yield 3’8,DOH under the action of M13I86CP18W (Figure 1).

Taking together, the product regioselectivity of M13 toward genistein can be altered by protein engineering or the reaction conditions. However, M13I86CP18W could yield a third metabolite besides 8-OHG and 3’-OHG, which was not conducive to the separation and purification of reaction products. Hence, more works should be carried out to further improve the regioselectivity of M13 and its variants.

### MATERIALS AND METHODS

#### Plasmids, Strains, and Chemicals.
A plasmid pET28a-M01A82W containing the M01A82W variant (R47L/A82W/F87V/L188Q/E267V) was used as a template for the mutation. Specifically, under the action of Fast Mutagenesis System (TransGen Biotech, Beijing, China), M01A82W was mutated into another BM3 variant M13 (R47L/L86I/F87V/L188Q), thereby generating another plasmid pET28aM13.19 Next, pET28aM13 was used as the template to yield diverse pET-28a (+) derived plasmids containing M13 variants using Fast Mutagenesis System. The primers used for the mutation are listed in Table S1. The presence of the desired mutations in M13 was confirmed by DNA sequencing.

*E. coli* strains Trans1-T1 and Transetta (DE3) (TransGen Biotech, Beijing, China) served as hosts for recombinant plasmid amplification and enzyme expression, respectively.

Genistein was purchased from Yuanye (Shanghai yuanye Bio-Technology Co., Ltd., Shanghai, China).

#### Structure Preparation of M13 Protein and Substrates.
The crystal structure of a P450 BM3 variant (A82F/F87V) in a complex with omeprazole was downloaded from the Protein Data Bank (PDB entry:4KEY) and the ligand (omeprazole) and water molecules were removed from BM3 protein using MOE software (Molecular Operating Environment, Chemical Computing Group, Montreal, Canada). Using the crystal structure of the wild-type P450 BM3 as the template, the three-dimensional (3D) structure of M13 was generated by amino acid substitution through the MOE Protein Builder tool. The 3D structure of the M13 protein was further refined by MOE QuickPrep functionality with default settings, including corrections of structural errors, addition of hydrogens, and calculation of partial charges. The refined M13 model was subject to energy minization, and the parameters were set to AMBER10: EHT force field and RMS gradient of 0.05. The structure of genistein was prepared using the same procedure as that of M13 protein, including structural refinement and energy minimization.

#### Molecular Docking.
The active sites of M13 protein were predicted using the “Site Finder” feature, and the site located closest to the heme was filled with dummy atoms for specification. Genistein was docked into the active site of M13 protein using the DOCK module of the MOE algorithm. In these dock simulations, “Triangle Matcher/London dg” and “Induced Fit/GBVI-WSA dg” parameters were chosen as the placement and refinement methods, respectively.

#### Protein Expression and Purification.
The pET-28a (+)-derived expression plasmids were transformed into *E. coli* strain Transetta (DE3) for heterologous expression. Specifically, a single colony harboring pET-28a (+)-derived plasmid was inoculated into LB medium with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol and incubated at 37 °C overnight. Next, one milliliter overnight culture was transferred into 50 mL M9 medium with appropriate antibiotics (50 µg/mL kanamycin and 34 µg/mL chloramphenicol), CaCl₂ (0.1 mM), MgCl₂ (1 mM), and FeSO₄ (0.1 mM) and then continued to stirred until OD₆₀₀ reached 0.6–0.7. At this point, induction expression was performed at 28 °C for 36 h by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-
aminolevulinic acid to the final concentrations of 0.4 and 1 mM, respectively. Cells were harvested by centrifugation (10,625g, 5 min) and the resulting pellet was resuspended in phosphate-buffered saline (PBS) buffer (50 mM, pH 7.4). The suspension was disrupted by sonification, and the resultant supernatant was subject to purification by metal affinity chromatography. The concentration of purified proteins was quantified by Super-Bradford Protein Assay Kit (CoWin Bio, Beijing, China).

**Hydroxylation Assays.** Hydroxylation assays were performed in 200 μL of mixtures containing 1 μL of substrate (100 mM), 1 μL of NADPH (100 mM), 1.5 μL of NADP (100 mM), 2 μL of G6P (500 mM), 1 μL of MgCl2 (2.5M), and 1.5 μL of purified protein (68.6 mg/mL).

The final concentrations of G6P were varied to explore the effect of G6P on the regioselectivity of M13 toward genistein. Moreover, MgCl2 was replaced by CoCl2 in the hydroxylation assays to probe the catalytic effect of Co2+ on the regioselectivity of M13.

The reaction mixtures were incubated at 37 °C for 2 h and then 200 μL of ethyl acetate was added to stop the hydroxylation reaction. The reaction mixtures were extracted using ethyl acetate and the collected solvent was dried using vacuum evaporation. The resultant residue was dissolved in 100 μL of methanol and then filtered. Fifty microliters of the filtrate was removed for analysis by high-performance liquid chromatography (HPLC). The conversion rates of M13 and its variants toward genistein were determined according to the following eluting program: 0−35 min, linear gradient from 50 to 100% B (v/v); 35−36 min, linear gradient from 50 to 100% B (v/v); 36−40 min, held at 100% B (v/v); 40−42, 100 to 5% B (v/v); and 42−45 min, kept at 5% B (v/v). C18 column was kept at ambient temperature, and the peaks were detected at a wavelength of 265 nm.

Hydroxylated products of genistein collected from the C18 column were injected into a Thermo Exactive Plus Orbitrap mass spectrometer for high-resolution mass spectrometry (HRMS) measurement. An ESI source was employed in a positive ionization mode. Full MS scans were acquired over the range of m/z 100−1500.

NMR spectra were recorded on a Bruker 600 MHz AVANCE III system, as introduced in our previous reports. Briefly, the samples were run in deuterated methanol (CD3OD) at 25 °C. Chemical shifts were recorded in δ (ppm) with the residual methyl signals in CD3OD as the references. The NMR assignments of hydroxygenisteins were based on 1H and 13C chemical shifts.

**ASSOCIATED CONTENT**

1. Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05088.

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**Notes**

The authors declare no competing financial interest.

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