Exploiting Designed Oxidase-Peroxygenase Mutual Benefit System for Asymmetric Cascade Reactions

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**Bacterial stains, enzymes, chemicals and instruments**

*E. coli* C41 strain was used to express OleT<sub>je</sub>, and BL21 (DE3) was used to express P450-BM3 and ANEH, while Rosetta (DE3) was used to express FDH. The KOD hot start DNA polymerase was obtained from Novagen.

All chemicals were purchased from 9ding chemistry, Bide Pharmtech, Tokyo Chemical Industry, Macklin Biochemical, Thermo Fisher, Sangon, or Aladdin.

Shimadzu LC-20AT high-performance liquid chromatography, Shimadzu UV-2450 UV-visible spectrophotometer were used for detection.

**Gene cloning and directed evolution**

| Library | Primer mixture | Primer | sequence | ratio |
|---------|---------------|--------|----------|-------|
| Phe     | F4            | F4     | 5'-GATAAAAAACTTAAAGTCAGCAGGKTAAAAATTGTACGTAITTTGCA GGAAGACGGGTTAKTTTACAGCTTGGACCATGAAAAA -3' | 4     |
|         | F5            | F5-1   | -CTATCAAATTATTACATTCTTA <br> KTTGCGGGACAGAAACAAACAGT GTC -3' | 1     |
|         |               | F5-2   | CTATCAAATTATTACATTCTTA <br> KTTTACAGCTTGGACCATGAAAAA <br> GTC | 1     |
|         | F6            | F6     | 5'-CTATCAAATTATTACATTCTTA <br> WTTKTTGGACACAGAAACAAACAGT GTC -3' | 1     |
|         | F7            | F7-1   | GCGCTGCGCTTATGGCAACTGTCGTTTCTCTATATGCA | 1     |
|         |               | F7-2   | CGAAGCGCTGCGCTTATGGCAACTTTCGCTGCTTTCCTATATGCA <br> CAAAAG | 1     |
|         | F8            | F8-1   | CTATCAAATTATTACATTCTTA <br> ATGTCGACGACCAACAAACAGT GTC | 1     |
|         |               | F8-2   | CTATCAAATTATTACATTCTTA <br> ATGTCGACGACCAACAAACAGT GTC | 1     |
|         |               | F8-3   | CTATCAAATTATTACATTCTTA <br> ATGTCGACGACCAACAAACAGT GTC | 1     |
|         |               | F8-4   | CTATCAAATTATTACATTCTTA <br> ATGTCGACGACCAACAAACAGT GTC | 1     |
|         | F9            | F9     | 5'-GCGCTGCGCTTATGGCAACTGTCGTTTCTCTATATGCA | 1     |
|         |               | F7-1   | GCGCTGCGCTTATGGCAACTGTCGTTTCTCTATATGCA | 1     |
|         |               | F7-2   | CGAAGCGCTGCGCTTATGGCAACTTTCGCTGCTTTCCTATATGCA <br> CAAAAG | 1     |
|         | R4            | R4-1   | 5'-GACCAGTTGGGTTCGTTACGGCAAMTAGAATGTAATAATTGGA <br> TAG -3' | 1     |
|         |               | R4-2   | GACCAGTTGGGTTCGTTACGGCAAMTAGAATGTAATAATTGGA <br> TAG | 1     |
|   |   |   |   |
|---|---|---|---|
| **R5** | **R5-1** | 5'-CACAAAGCCTTCAGGTTTTAAGCGTAAAGTTTCTTTAATAATCCAGCTCG-3' | 1 |
|   | **R5-2** | 5'-CACAAAGCCTTCAGGTTTTAAGCTAAAGTTTCTTTAATAATCCAGCTCG-3' | 1 |
|   | **R5-3** | CACAAAGCCTTCAGGTTTTAAATAAAGTTTCTTTAATAATCCAGCTCG | 1 |
|   | **R5-4** | CACAAAGCCTTCAGGTTTTAAAATAAAGTTTCTTTAATAATCCAGCTCG | 1 |
| **R6** | **R6** | 5'-GACCACCTTTGTTTTTCGTGCTCAAAMAAWCTAAGAAATGTAATAATTGAT | 4 |
| **R7** | **R7-1** | 5'-TGCAATAGGAAAAACGAGGAGGTGGCCATAAGCCAGCGC | 1 |
|   | **R7-2** | 5'-CTTTTGCAATAGGAAAAACGAGGAGTTGGCCATAAGCCAGCGCCTTCG-3' | 1 |
| **R8** | **R8** | 5'-CACAAAGCCTTCAGGTTTTAAAAMARAAGTTTCTTTAATAATCCAGCTCG-3' | 4 |
| **R9** | **R9-1** | GACCACCTTTGTTTTTCGTGCTCAAATAGAAATGTAATAATTGATAG | 1 |
|   | **R9-2** | GACCACCTTTGTTTTTCGTGCTCAAATAGAAATGTAATAATTGATAG | 1 |
|   | **R9-3** | GACCACCTTTGTTTTTCGTGCTCAAATAGAAATGTAATAATTGATAG | 1 |
|   | **R9-4** | GACCACCTTTGTTTTTCGTGCTCAAATAGAAATGTAATAATTGATAG | 1 |
| **R10** | **R10-1** | TGCAATAGGAAAAACGCAGCGCAGTTGGCCATAAGCCAGCGC | 1 |
|   | **R7-1** | 5'-TGCAATAGGAAAAACGCAGCGCAGTTGGCCATAAGCCAGCGC-3' | 1 |
|   | **R7-2** | 5'-CTTTTGCAATAGGAAAAACGCAGCGCAGTTGGCCATAAGCCAGCGCCTTCG-3' | 1 |
| **R11** | **R11-1** | CACAAAGCCTTCAGGTTTTAAAAAAAAGTTTCTTTAATAATCCAGCTCG | 2 |
|   | **R11-2** | CACAAAGCCTTCAGGTTTTAAAGTAAMAGTTTCTTTAATAATCCAGCTCG | 2 |
General procedure of directed evolution

To increase the enantioselectivity of P450-BM3 toward the epoxidation of styrene, the same V library was screened as in past work. Then we got three mutants which showed enhanced (R)-selectivity (M1) and reversed (S)-selectivity (M2 and M3) compared to WT. Then these mutants were used as templates to do the second cycle of directed evolution; the same sites were selected to be scanned by phenylalanine, when M1 used as template; F4-R4, F5-R7 and F7-R5 were used to amplify the fragments 75–82-263-264, 263-264-328 and 328-437-438. To amplify the mega-primer, these fragments were mixed as templates, F4-R5 were used as primers to amplify the fragments containing all the mutations, and the last step used the mega-primer to amplify the whole express plasmid. Same strategies have been applied to construct the F library when M2 and M3 were used as templates. For M2, F4-R6, F6-R7, F7-R8 were used to amplify the three fragments and F4-R8 were used to amplify the mega-primer. For M3, F4-R9, F8-R10, F9-R11 were used to amplify the three fragments, and F4-R11 were used to amplify the mega-primer. All libraries were transformed into BL21 (DE3), expression and library screening was performed as before; each library was screened considering 368 colonies. Two mutants from the library which used M2 as templates showed reversed (R)-selectivity (S01 and S02), and two mutants from the library which used M3 as template showed enhanced (S)-selectivity (S03 and S04), but better mutants were not achieved from the library which used M1 as template. To further increase the (R)-selectivity, S02 was selected as template to do the next step directed evolution due to its high activity. Accordingly, 4 more sites were selected to do the V and F scan stimulatory, F10-R12 and F11-R13 were used to amplify 78-82-181 and 181-262 fragments, then F10-R13 were used to amplify the mega-primer. After screening 184 colonies, the best (R)-selective mutant S05 was obtained. All genotypes of the mutants are list in Table S2.

Expression and lysis of BM3-ANEH, S05, OleTJE, and FDH

Single-clone bacteria were carefully picked by toothpicks and placed into a 10 mL culture tubes with 3-6 mL LB media, and the corresponding antibiotic (kanamycin 50 µg mL\(^{-1}\))
chloramphenicol 35 µg mL⁻¹ or ampicillin 100 µg mL⁻¹) was added. After 10 h, 37 °C incubation, 2 mL turbid bacteria fluid was transferred into a 2 L flask with 200 mL TB media and the corresponding antibiotic added. Expression was induced at an optical density (OD₆₀₀) of 0.6 by adding of 20 µL (OleTJE) 40 µL (SO5 and BM3-ANEH) or 100 µL (FDH) 1 M IPTG (isopropyl β-D-1-thiogalactopyranoside) and 100 µL of 1 M δ-aminolevulinic acid (ALA). Expression in the shaker occurred at 25 °C (OleTJE, SO5, BM3-ANEH), or 18 °C (FDH) 16-20 h, 160 rpm. After expression, cells were harvested by centrifugation (8000 rpm 2 min), washed by the PBS buffer (100 mM, pH 8.0), and concentrated to about 10-15 mL. Lysozyme was added, shaken in the ice-water-bath for 30 minutes, and stored at -80°C until lysis. Before lysis, cells were thawed in ice-water at 4 °C. Cells were disrupted by sonication for 15 minutes (400 w, 2 s pulse and 4s pause; SCIENTZ-IID). The cell debris was removed by ultracentrifugation (12000 rpm, 1 h, 4 °C). The crude protein was quick-frozen by liquid nitrogen and then freeze-dried (SCIENTZ-10ND) to obtain enzyme powder.

**Purification of OleTJE and SO5**

After expression as described before, washing by the PBS buffer (100 mM, pH 8.0) was performed, followed by concentration to about 10-15 mL with 10% glycerin, and 300 mM NaCl (OleTJE). The lysis and ultracentrifugation procedure was the same as described before. After ultracentrifugation, the supernatant was mixed with 20 µL hemin (10 mM) in ice-water for 30 minutes, then 5 mL Ni-NTA was added and the mixture was stirred for 30 minutes. The mixture was washed by buffer A (0.1 M KPi, 10% glycerin, 50 mM imidazole, pH 7.4) with more than 5 times the volume of Ni-NTA, until the protein concentration (detected by nano-300 spectrophotometer UV-Visible Allsheng) of outflow liquid was less than 1µM. Then the Ni-NTA with 6-Histag protein was combined and washed by buffer B (0.1M KPi, 10% glycerin, 500 mM imidazole pH 7.4). All the red liquid was collected in a corresponding ultrafiltration tube (OleTJE 30000 Da, SO5 50000 Da), and concentrated (5000 rpm 10 min) to no more than 2 mL. The liquid was then passed through PD-10 Columns (GE Healthcare), and eluted by buffer C (0.1 M KPi, 10% glycerol, pH 7.4) to remove the imidazole. The concentration was determined by Cheng’s protocol².

**Lysate cascade reaction**

Reactions were performed in 1.5 mL polypropylene centrifuge tubes with a final volume of 500 µL. The reaction mixtures were prepared in KPi buffer (0.1 M, pH 8.0) containing 300 mM NaCl, 100 mM ammonium formate, 5 mM phenylpropionic acids la-j as substrates, 800 µM NADP+, 2 g/L FDH, 10 g/L BM3-ANEH, 20 g/L OleT, 30 °C, 800 rpm, 24 h on a mini shaker (MS-100, Allsheng). The reactions were quenched by adding 50 µL concentrated hydrochloric acid. Substrates and products were extracted by 500 µL ethyl acetate. Organic phases were blown to dryness with nitrogen, and dissolved with the same volume of isopropanol for HPLC detection. All products had the same characteristic properties as reported by Zhi Li et al³.

**Catalase activation and inhibition experiments**

Reactions were performed in 2 mL polypropylene centrifuge tubes with a final volume of 500 µL. The reaction mixtures were prepared in KPi buffer (0.1 M, pH 8.0) containing 100 mM ammonium formate, 5 mM phenylpropionic acid as substrate, 800 µM NADP+, 2 g/L FDH, 5 µM
SO5; 5 μM OleTJE were involved when reaction B was performed. All mixtures were incubated at 30 °C, 800 rpm for 12h. To evaluate the H$_2$O$_2$ influence, an excessive amount (>1200 U) of catalase was added to the system. Extraction and analysis of products were performed as described above.

**Concentration of OleTJE and SO5-mutant detection**
Freeze-dried lysate powder was dissolved in KPi buffer (0.1 M, pH 8.0) to 100 mg/mL respectively, then followed Cheng’s protocol to determine the concentration of both P450s.

**Detecting uncoupling of SO5 experiments**
Reactions were performed in 2 mL vials with a sealed lid. Freeze-dried lysate SO5 power was dissolved in 485 mL KPi buffer (0.1 M, pH 8.0) to 1 mM, and a blank was scanned by UV-vis (Allsheng, nano-300). Then 10 μL NADPH solution (100 mM) was added, and gentle shaking was continued. Absorbance A1 was scanned at 340 nm. Then 5 mL of substrate styrene was added (1 M, dissolved by DMSO). Vials were sealed and shaken at 30 °C for 5 minutes. Absorbance A2 was scanned after shaking. Considering spontaneous degradation of NADPH, control groups were set without substrate. All reactions were performed in parallel three times. NADPH consumption was calculated by the reduced proportion of A2 to A1. Production of styrene oxide was measured by GC.

**Detection methods**
Conversion and enantioselectivity were determined by HPLC with an AS-H column. When some of the graphics (products 2b, 2c, 2d, 2f, 2h, 2i, 2j ) failed to separate the (R)- and (S)-peaks clearly, we changed the proportion of mobile phase to 94:6 (n-hexane:IPA; v:v) for these reactions. All chiral diols have been described previously in the literature, their analytical data matching ours.
| code   | template P450-BM3 total mutations | ee(%) product |
|--------|-----------------------------------|--------------|
| Val    | WT                  | 20 (R)       |
|        | WT                  |              |
|        | L75V-F87V-I263V-A328F | 65 (R)       |
|        | L75V-F87V-A264V-A328V-T438V | 56 (S)       |
|        | L75V-F87V-L437V      | 63 (S)       |
| Phe    | M2                  | 90 (R)       |
|        | SO1                 |              |
|        | L75V-F87V-A328F-L437F-T438F | 90 (R)       |
|        | SO2                 |              |
|        | L75V-F87V-A328F-L437V-T438F | 90 (R)       |
| Val+Phe| SO2                | 96 (R)       |
|        | SO5                 |              |
|        | L75V-V78F-F87V-L181F-L262V-A328F-L437V-T438F | 92 (R)       |
|        | SO7                 |              |
|        | L75V-F87V-L181F-A328F-L437V-T438F | 92 (R)       |
|        | SO6                 |              |
|        | L75V-V78F-A82V-F87V-L181V-A328F-L437V-T438F | 95 (R)       |
| Phe    | M3                  | 80 (S)       |
|        | SO3                 |              |
|        | L75F-F87V-I263F-A264V-A328V-L437F-T438V | 80 (S)       |
|        | SO4                 |              |
|        | L75F-F87V-A264F-A328V-T438F | 80 (S)       |
HPLC graphs of reactions

Figure S1: HPLC graph of reaction of substrate 1a

Figure S2: HPLC graph of reaction of substrate 1b
Figure S3: HPLC graph of reaction of substrate 1c

Figure S4: HPLC graph of reaction of substrate 1d
Figure S5: HPLC graph of reaction of substrate 1e

Figure S6: HPLC graph of reaction of substrate 1f
Figure S7: HPLC graph of reaction of substrate 1g

Figure S8: HPLC graph of reaction of substrate 1h
Figure S9: HPLC graph of reaction of substrate 1i

Figure S10: HPLC graph of reaction of substrate 1j
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(3) Wu, S.; Chen, Y.; Xu, Y.; Li, A.; Xu, Q.; Glieder, A.; Li, Z., Enantioselective trans-Dihydroxylation of Aryl Olefins by Cascade Biocatalysis with Recombinant *Escherichia coli* Coexpressing Monooxygenase and Epoxide Hydrolase. *ACS Catal.* **2014**, *4*(2), 409-420.