International Journal of Biological Macromolecules 121 (2019) 326–332

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Multiple site-directed mutagenesis of a *Phaseolus vulgaris* epoxide hydrolase to improve its catalytic performance towards *p*-chlorostyrene oxide based on the computer-aided re-design

Chuang Li [a], Jun Zhao [a], Die Hu [b], Bo-Chun Hu [a], Rui Wang [a], Jia Zang [c,*], Min-Chen Wu [b,*]

[a] Key Laboratory of Carbohydrate Chemistry and Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, PR China
[b] Wuxi School of Medicine, Jiangnan University, Wuxi 214122, PR China
[c] The Affiliated Wuxi Matemity and Child Health Care Hospital of Nanjing Medical University, Wuxi 214002, PR China

ARTICLE INFO

Article history:
Received 12 April 2018
Received in revised form 25 August 2018
Accepted 8 October 2018
Available online 09 October 2018

Keywords:
Epoxide hydrolase
Computer-aided re-design
Site-directed mutagenesis

ABSTRACT

To improve the activity and regioselectivity of a *Phaseolus vulgaris* epoxide hydrolase (*Pv*EH3) towards *p*-chlorostyrene oxide (*p*CSO), the site-directed mutagenesis was conducted based on the computer-aided re-design. Firstly, seven single-site variants of a *Pv*EH3-encoding gene (*pveh3*) were constructed as designed theoretically and expressed in *E. coli* BL21(DE3), respectively. One transformant, *E. coli*/*pveh3*^{G170E}, had the higher EH activity towards racemic *p*CSO, while both *E. coli*/*pveh3*^{F187L} and /*pveh3*^{P237L} with enhanced regioselectivity coefficient α_S values. Secondly, to combine their respective merits, the double- and triple-site variants, *pveh3*$^{G170E/F187L}$, *pveh3*$^{G170E/P237L}$ and *pveh3*$^{G170E/F187L/P237L}$, were also constructed. Among all *E. coli* transformants, *E. coli*/*pveh3*$^{G170E/F187L/P237L}$ simultaneously had the highest EH activity of 20.3 U/g wet cell and α_S value of 95.2%, by which the hydrolysis of *rac*-*p*CSO enantioconvergently produced (*R*)-*p*-chlorophenylethane-1,2-diol with an enantiomeric excess of 93.2%. Furthermore, *Pv*EH3$^{G170E/F187L/P237L}$ expressed in *E. coli*/*pveh3*$^{G170E/F187L/P237L}$ was purified. Its specific activity and catalytic efficiency towards *rac*-*p*CSO were 4.1 U/mg protein and 1.81 mM^{-1} s^{-1}, which were 3.0- and 3.1-fold those of *Pv*EH3. Finally, the molecular docking simulation analysis indicated that *Pv*EH3$^{G170E/F187L/P237L}$ preferentially attacks the more hindered benzylic carbon of (*S*)-*p*CSO over *Pv*EH3, which was consistent with their α_S values measured experimentally.

1. Introduction

Epoxide hydrolases (EHs), existing widely in microorganisms, plants, invertebrates and mammals, can stereoselectively catalyze the opening of an active three-membered oxirane ring of racemic (*rac*-) epoxides, retaining epoxide enantiomers and/or affording enantiopure vicinal diols [1]. The vast majority of EHs (EC 3.3.2.9 and 10) belong to the α/β-hydrolase fold superfamily, sharing one α/β domain, that is, a β-sheet flanked by a cluster of α-helices, and one cap domain containing a variable cap-loop [2]. Reportedly, the EH-catalyzed hydrolysis of epoxides proceeded mainly in two steps. The nucleophilic Asp residue first attacks the carbon atom in an oxirane ring of epoxide, forming a hydroxyalkyl-EH intermediate. Then, a water molecule that is activated by His in the catalytic triad interacts with the intermediate, releasing a vicinal diol product [3,4]. According to the catalytic mechanisms of the given EH-epoxide pairs, the transformation of *rac*-epoxides can be divided into two pathways: kinetic resolution and enantioconvergent hydrolysis [5]. Compared with the kinetic resolution having an intrinsic limitation of 50% maximum yield of epoxide enantiomers, the enantioconvergent hydrolysis can produce chiral diols with up to 100% theoretical yield [6].

Recently, EHs have attracted much attention for the reason that enantiopure epoxides and vicinal diols can be prepared by EHs-catalyzed hydrolysis of epoxides in a highly stereoselective and environment-friendly manner [7]. As the highly value-added and versatile building blocks, they were used to synthesis of pharmaceuticals, agrochemicals and fine chemicals. For example, (*S*)-styrene oxide was applied to synthesize α1-, β2- and β3-adrenergic receptor agonists, anticancer agent — levamisole, anti-HIV agent — (−)-hyperolactone C and nematicide [8,9]. Another example is that a quite promising neuroprotective agent, Eliprodil, was applied in the treatment of ischemic stroke. Its (*R*)-(−)-enantiomer, having higher bioactivity *in vivo* than its (*S*)-(+)-antipode, can be synthesized from (*R*)-*p*-chlorophenylethane-1,2-diol (*p*CPED) [10].

The enantioconvergent hydrolysis of *rac*-epoxides by single EHs was an ideal bioprocess for preparing chiral diols, but few EHs had high and complementary regioselectivities that lead to high enantiomeric excess values of chiral diols (ee_p) [11]. Therefore, it was required to excavate novel EHs and to perfect the catalytic performance of existing EHs by

* Corresponding authors.
E-mail addresses: zangj1976@sina.com (J. Zang), biowmc@126.com (M.-C. Wu).

https://doi.org/10.1016/j.ijbiomac.2018.10.030
0141-8130/

protein engineering [12]. An *Aspergillus niger* M200 EH was subjected to saturation mutagenesis at nine amino acid sites lining in the substrate access tunnel and binding pocket. Through screening, the best mutant H:12-A1 was selected, whose regioselectivity towards (*R*)- or (*S*)-*p*-chlorostyrene oxide (*p*CSO) was higher than that of wild-type EH [13]. As to plant-derived EHs, the multiple site-directed mutagenesis of *Pv*EH1, one of *P. vulgaris* EHs, was carried out based on the rational re-design. The result indicated that the hydrolysis of *rac*-*p*CSO by *Pv*EH1$^{L105I/M160A/M175I}$, a triple-site mutant, produced (*R*)-*p*CPED with 70.9% ee_p, higher than that (51.4% ee_p) by *Pv*EH1 [14]. Furthermore, these directed modifications of EHs based on the computer-aided re-design also contributed to a better understanding about the catalytic mechanism of enantioconvergent hydrolysis [15].

In our previous studies, a *Pv*EH3-encoding gene (*pveh3*, GenBank accession no: MF420352) was amplified from *P. vulgaris* and expressed in *E. coli* BL21(DE3). The catalytic performance analysis indicated that *Pv*EH3, with an enantiopreference towards (*S*)-*p*CSO, can catalyze the hydrolysis of *rac*-*p*CSO in an enantioconvergent pathway. However, its unsatisfactory regioselectivity coefficient α_S (87.0%) led to low ee_p of (*R*)-*p*CPED, while poor specific activity (1.4 U/mg) to low catalytic efficiency towards *rac*-*p*CSO. In this work, to improve the regioselectivity and catalytic efficiency of *Pv*EH3, ten variants of *pveh3* were constructed by whole-plasmid PCR based on the computer-aided re-design, and expressed in *E. coli*. The EH activities and regioselectivity coefficients of *E. coli* transformants were determined and compared with those of *E. coli*/*pveh3*. Additionally, *Pv*EH3$^{G170E/F187L/P237L}$ expressed in *E. coli* was purified, and its specific activity and kinetic parameters were assayed. The source of *Pv*EH3$^{G170E/F187L/P237L}$ with enhanced α_S was primarily analyzed by molecular docking (MD) simulation.

2. Materials and methods

2.1. Materials

E. coli BL21(DE3) and pET-28a(+) (Novagen, Madison, WI) were used for the construction of recombinant plasmids and expression of EH genes. Both a recombinant plasmid (pET-28a-*pveh3*) and a *Pv*EH3-expressing *E. coli* transformant (*E. coli*/*pveh3*) were constructed and preserved in our lab. PrimeSTAR HS DNA polymerase and *Dpn* I endonuclease (TaKaRa, Dalian, China) were used for the single or multiple site-directed mutagenesis of *pveh3*. *E. coli* BL21(DE3) and its transformants were grown in LB medium, and induced by isopropyl-β-D-thiogalactoside (IPTG). *Rac*-*p*CSO, (*S*)-*p*CPED and (*R*)-*p*CPED (Energy, Shanghai, China) were used for the assays of activities and regioselectivity coefficients of *Pv*EH3 and its mutants. All other chemicals were of analytical grade.

2.2. Homology modeling and computer-aided re-design of PvEH3

Using *Pv*EH3 as a template, several plant EHs sharing more than 50% sequence identity with *Pv*EH3 were searched by a BLAST server at NCBI website (http://www.ncbi.nlm.nih.gov/), among which, five ones possessed superior catalytic performance (i.e., high activity and/or regioselectivity) in the enantioconvergent hydrolysis of *rac*-epoxides [11,16–18] were finally selected to conduct the multiple sequence alignment with *Pv*EH3. Based on the known crystal structure of *Solanum tuberosum* EH at 1.95 Å resolution (*St*EH, PDB: 2CJP), sharing 59.0% identity with *Pv*EH3, the three-dimensional (3-D) structure of *Pv*EH3 or its mutant was homologically modeled using the MODELLER 9.11 program (http://salilab.org/modeller/). The generated model was then subjected to molecular mechanics optimization using CHARMM27 force field supplied in GROMACS 4.5 package (http://www.gromacs.org/). Energy minimization (geometry optimization) was performed until the gradient of 0.01 kcal/Å/mol was reached. The geometry qualities of the models were validated by SAVES program (http://services.mbi.ucla.edu/SAVES/). Finally, a model with the best quality was chosen for further studies. Meanwhile, the 3-D structure of (*R*)- or (*S*)-*p*CSO was disposed by MM2 force field in a ChemBio3D Ultra 12.0 software (http://www.cambridgesoft.com/). Our previous study showed that *Pv*EH3 enantiopreferentially hydrolyzed (*S*)-*p*CSO over (*R*)-*p*CSO. To improve its catalytic activity towards (*R*)-*p*CSO (or *rac*-*p*CSO), the mutual action between the 3-D structures of *Pv*EH3 and (*R*)-*p*CSO was predicted by MD simulation using the AutoDock 4.2 program (http://autodock.scripps.edu/) to locate the most appropriate binding sites and orientation [19]. MD was conducted according to the protocol of Autodock 4.2. In general, all the rotatable bonds in (*R*)-*p*CSO automatically identified by the program were set as rotatable. The polar hydrogen was added to the substrate and all the catalytic residues were set as flexible residues. MD was allowed to run 50 times and up to 50 poses were generated. Finally, a representative pose with the lowest binding energy was selected. Then, the 3-D structure of the docked enzyme-substrate complex was also optimized by GROMACS 4.5 package and applied to present the binding conformation of the complex [16]. Based on the conformation of this optimized complex, the residues of *Pv*EH3 in proximity to (*R*)-*p*CSO within 12 Å were identified using a PyMol software (http://pymol.org/).

The multiple sequence alignment of *Pv*EH3 with other five plant EHs was carried out using the ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The above identified amino acids of *Pv*EH3 whose frequencies are below 60% among six EHs were considered to be non-conserved in this work. Considering the physicochemical properties of these non-conserved residues and their locations on the configuration of *Pv*EH3, several residues were selected to be substituted with the corresponding and frequently emerging ones among other five plant EHs, respectively, to construct a series of single-site mutants of *Pv*EH3.

2.3. Construction of variant genes and E. coli transformants

The site-directed mutagenesis of *pveh3* was performed using the one-step whole-plasmid PCR technique [20]. PCR primers were designed according to both the sequence of *pveh3* and the codons encoding mutation residues, and synthesized by Sangon (Shanghai, China) (Table 1). The seven single-site variant genes were PCR-amplified from pET-28a-*pveh3* as following conditions: an initial denaturation at 95 °C for 4 min, 30 cycles of at 98 °C for 10 s, 55 °C for 15 s and 72 °C for 8 min, and an extra elongation at 72 °C for 10 min. The target products were digested by *Dpn* I endonuclease at 37 °C for 6 h to decompose the original methylated template, and transformed into *E. coli* BL21(DE3), respectively, thereby constructing seven *E. coli* transformants, such as *E. coli*/*pveh3*^{G170E} and /*pveh3*^{F187L}. Analogously, two double-site variant genes, *pveh3*$^{G170E/F187L}$ and *pveh3*$^{G170E/P237L}$, were amplified from pET-28a-*pveh3*^{G170E} using two pairs of primers, F187L-F/F187L-R and P237L-F/P237L-R, and used to construct *E. coli*/

Table 1
PCR primers used for the single or multiple site-directed mutagenesis of *Pv*EH3.

Primer name	Primer sequence (5′ → 3′)[a]
T137P-F	AACCCCGAGATCAGA**CCA**GTCGATGCCA
T137P-R	TGGCATCGAC**TGG**TCTGATCTCGGGGTT
G170E-F	GCTGAAGTTGGGACT**GAA**TATGTGCTCAAA
G170E-R	TTTGAGCACATA**TTC**AGTCCCAACTTCAGC
Y171E-F	GAAGTTGGGACTGGG**GAG**GTGCTCAAAAAC
Y171E-R	GTTTTTGAGCAC**CTC**CCCAGTCCCAACTTC
F187L-F	CCTCCAATC**TTA**CCAAAGGGAGAGTACG
F187L-R	CGTACTCTCCCTTTGG**TAA**GATTGGAGG
D199S-F	GGATTCAACCCA**TCT**ATGACTAATTCC
D199S-R	GGAATTAGTCAT**AGA**TGGGTTGAATCC
P228G-F	ACGGGCTTCACTGGA**GGA**TTGAACTATTAC
P228G-R	GTAATAGTTCAA**TCC**TCCAGTGAAGCCCGT
P237L-F	TACAGAAATATGAAC**TTA**AATTGGGAGCTGAC
P237L-R	GTCAGCTCCCAATT**TAA**GTTCATATTTCTGTA

[a] The codons encoding mutation residues are underlined.