Journal of Industrial and Engineering Chemistry 18 (2012) 72–76

Contents lists available at SciVerse ScienceDirect

Journal of Industrial and Engineering Chemistry

journal homepage: www.elsevier.com/locate/jiec

# Multiple sequence alignment-inspired mutagenesis of marine epoxide hydrolase of *Mugil cephalus* for enhancing enantioselective hydrolytic activity

Sung Hee Choi [a], Hee Sook Kim [a], Eun Yeol Lee [b,*]

[a] Department of Food Science and Biotechnology, Kyungsung University, Busan 608-736, Republic of Korea
[b] Department of Chemical Engineering, Kyung Hee University, Gyeonggi-do 446-701, Republic of Korea

ARTICLE INFO

*Article history:*
Received 16 October 2010
Accepted 17 January 2011
Available online 9 November 2011

*Keywords:*
Epoxide hydrolase
Multiple sequence alignment
Mutagenesis
*Mugil cephalus*

ABSTRACT

The marine fish microsomal epoxide hydrolase (mEH) of *Mugil cephalus* was engineered to enhance the enantioselective hydrolytic activity by multiple sequence alignment-inspired mutagenesis. The amino acid sequences of *Aspergillus niger*, *Rhodotorula glutinis*, zebra fish and human mEH were aligned and analyzed for identifying target amino acids. Single-point mutants (Q170K, E186K, E378D) and double-point mutants (E378D-Q170K, E378D-Y348F, E378D-Y348H) were developed and their hydrolytic activities were compared. The double-point mutant, E378D-Q170K, exhibited an enhanced hydrolytic activity by 4.6-fold, compared to the wild-type *M. cephalus* mEH. Enantiopure (*S*)-styrene oxide could be readily prepared with high enantiopurity more than 99%*ee* by using the double-point mutant.


## 1. Introduction

Epoxide hydrolases (EHs) can be applied to enantioselective hydrolysis of racemic epoxides for the production of chiral epoxides and vicinal diols [1]. Chiral epoxides and vicinal diols are synthetic intermediates for the preparation of high-value-added chiral pharmaceuticals due to their excellent reactivity and chirality [2].

The availability of high performance enzymes is the very essential requirement for the implementation of biocatalytic process for the preparation of chiral intermediates [3]. To meet the needs of high performance enzymes, the engineered EHs with improved properties have been developed by rational design and directed evolution [4,5]. Various high-throughput-screening methods have been developed to screen library generated by directed evolution more efficiently [6]. Gene libraries of *Agrobacterium radiobacter* and *Aspergillus niger* were generated by error-prone polymerase chain reaction (epPCR) and DNA shuffling, and then the EH varients were screened based on a high-throughput assay such as electron spray ionization (ESI)-mass spectrometer (MS) analysis [7–9].

Recently, we cloned and characterized a marine fish microsomal EH (mEH) from *Mugil cephalus* [10]. The marine fish mEH proved to have some advantages such as less inhibition to diol product and broad substrate spectrum. However, the hydrolytic activity was rather low compared to microbial EH. For this reason, it is required to enhance the activity via directed evolution or rationale protein engineering for its desired application. We have successfully applied comparative homology modeling-based site-directed mutagenesis to improve the catalytic activity [11]. Only a small number of amino acids such as single, double and triple-point mutations needed to be modified to improve the activity.

Multiple sequence alignment analysis can be applied for the identification of mutation target points for enhancing the catalytic activity [12–14]. Multiple sequence alignment analysis can be an efficient way to search out the target amino acids when comparative homology models based on the knowledge of the three-dimensional structure of the relevant proteins are available. In this study, multiple sequence alignment analysis was employed for a semi-rational educated identification of target amino acids to enhance enantioselective hydrolytic activity of *M. cephalus* mEH. The characteristics of the engineered EH were analyzed for the preparation of enantiopure styrene oxide.

## 2. Materials and methods

### 2.1. Multiple sequence alignment, bioinformatics analysis and comparative modeling

The conserved domains and catalytic triads of *M. cephalus* mEH were compared with other mEHs by multiple sequence alignment using BioEdit program. The three-dimensional models of *M. cephalus* mEH were constructed by using SWISS-MODEL server based on X-ray crystallographic structure of *Aspergillus niger* EH

* Corresponding author. Fax: +82 31 204 8114.
*E-mail address:* eunylee@khu.ac.kr (E.Y. Lee).

1226-086X/$ – see front matter © 2011 The Korean Society of Industrial and Engineering Chemistry. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jiec.2011.11.085

**Table 1**
Oligonucleotide sequences used in site-directed mutation.

| Target | Gene sequence (5′ → 3′) |
|---|---|
| Q170K | GAAGCCCCTCATAAAAAAGGGTTCAACACCCTCG |
| Q170K | CGAGGGTGTTGAACCCTTTTTTATGAGGGGCTTC |
| E186K | GTTTTCCTGACACTGATGAAGCGTTTGGGGTTCTC |
| E186K | GAGAACCCCAAACGCTTCATCAGTGTCAGGAAAAC |
| E378D | GCTGCCTTCCCTGGAGACCTGATGCATTGCCCTAAATC |
| E378D | GATTTAGGGCAATGCATCAGGTCTCCAGGGAAGGCAGC |
| Y348F | GTCCTCCATGCGCTTCTTCAAAGAGAACTTTAAG |
| Y348F | CTTAAAGTTCTCTTTGAAGAAGCGCATGGAGGAC |
| Y348H | GTCCTCCATGCGCTTCCACAAAGAGAACTTTAAG |
| Y348H | CTTAAAGTTCTCTTTGTGGAAGCGCATGGAGGAC |

(pdb code: 1qo7). Superimposition of *M. cephalus* mEH mutants on that of *A. niger* mEH was conducted by using RasMol and DeepView programs. The folding energy was *in silico* analyzed for predicting the structural stability of *M. cephalus* mEH mutants by using Prosa 2003.

### 2.2. Site-directed mutagenesis

The site-directed point mutations of *M. cephalus* mEH gene were carried out by using Quikchange II site-directed mutagenesis kit (Stratagene Co, USA) with primers shown in Table 1. The recombinant pET-21b(+)/mMcEH plasmid was used as the template for site-directed mutagenesis [11]. The mutagenic PCR operation conditions were as follows: 30 s at 95 °C, followed by 12 cycles of 30 s at 95 °C, 1 min at 55 °C, 7 min 20 s at 68 °C, ending with 2 min on ice to cool the reaction to 37 °C. *Dpn*I restriction enzyme was added directly to the reaction tube and the reaction was immediately incubated at 37 °C for 1 h to degrade the methylated template. The purified PCR product was transformed into *Escherichia coli* DH5α and cultured on the LB agar plate containing 50 μg/ml of ampicillin. The mutations were confirmed by DNA sequencing using the T7 forward and T7 terminator reverse primer.

### 2.3. Recombinant cell culture and batch enantioselective hydrolysis of racemic epoxides

The recombinant *E. coli* BL21 (DE3) harboring recombinant pET-21b(+)/mutated mMcEH plasmid was cultured at 37 °C for 2 h to be an $OD_{600}$ of 0.4–0.6 in shaking incubator at 190 rpm. And then, the recombinant cells were cultured at 15 °C for 24 h to express the mutated mEH genes by addition of 1 mM IPTG.

To analyze the expressed protein, the suspension cells with or without IPTG induction were cooked at 95 °C for 10 min with Laemmli buffer, separated on 12% SDS-polyacrylamide gel, and blotted onto a nitrocellulose membrane. The membrane was incubated with polyclonal antibody against hexahistidine (H-15, Santa Cruz Biotechnology Inc., USA) and peroxidase-conjugated anti-rabbit IgG (Jackson Immunoresearch, USA), and then visualized with CN/DAB (4-chloronaphthol/3,3′-diaminobenzidine) Substrate Kit (Pierce, USA).

Enantioselective hydrolysis of racemic styrene oxide was conducted in 1 ml 100 mM $KH_2PO_4$ buffer (pH 7.4) in 10 ml screw-cap bottles sealed with a rubber septum. Enantioselective hydrolysis reaction was started with the addition of 20 mM racemic styrene oxide in a shaking incubator at 30 °C and 230 rpm. The reaction was stopped by extraction with equal volume of cyclohexane. The progression of hydrolysis reaction was periodically analyzed by chiral GC analysis of samples.

### 2.4. Analysis

Cell concentration was measured by a spectrophotometer at 600 nm (UV mini-1240, Shimadzu, Japan). Enantiomeric excess ($ee = 100 \times (S - R)/(S + R)$) and yield for enantiopure styrene oxide were determined by chiral GC analysis. The reaction mixture was extracted with equal volume of cyclohexane, and 1 μl of the organic layer was analyzed by GC with a fused silica capillary beta-DEX 120 column (0.25 mm ID × 30 m, 0.25 mm film thickness,

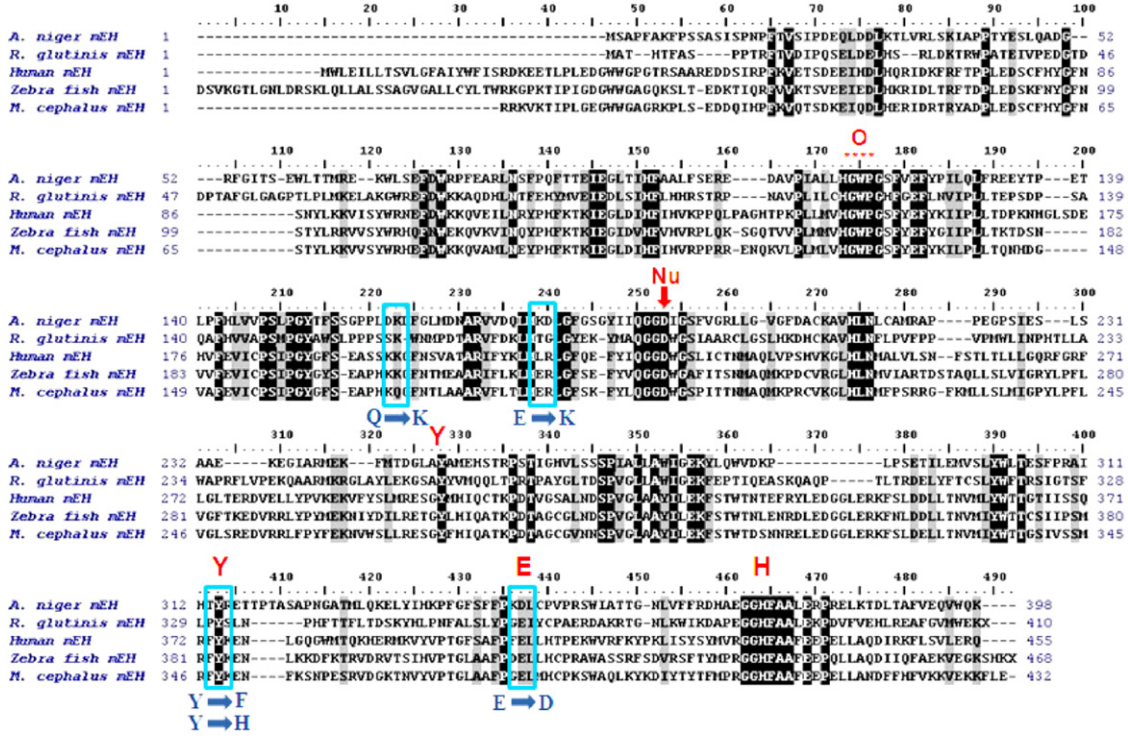


**Fig. 1.** Multiple sequence alignments of *M. cephalus* mEH with other EHs. Nu, E and Y are nucleophilic aspartic acid, acidic amino acid and two tyrosine residues for the hydrogen bonding with epoxide ring of substrates, respectively. O and H represent oxyanion hole and histidine, respectively.