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Cloning and Characterization of Zebrafish Microsomal Epoxide Hydrolase Based on Bioinformatics

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A gene encoding for a putative microsomal epoxide hydrolase (mEH) of a zebrafish, *Danio rerio*, was cloned and characterized. The putative mEH protein of *D. rerio* exhibited sequence similarity with mammalian mEH and some other bacterial EHs. A structural model for the putative mEH was constructed using homology modeling based on the crystallographic templates, 1qo7 and 1ehy. The catalytic triad consisting of Asp^{233}, Glu^{413}, and His^{440} was identified, and the characteristic features such as two tyrosine residues and oxyanion hole were found to be highly conserved. Based on bioinformatic analysis together with EH activity assay, the putative protein was annotated as mEH of *D. rerio*. Enantiopure styrene oxide with enantiopurity of 99 %*ee* and yield of 33.5 % was obtained from racemic styrene oxide by the enantioselective hydrolysis activity of recombinant mEH of *D. rerio* for 45 min.

Key words: Bioinformatics, *Danio rerio*, microsomal epoxide hydrolase, enantiopure styrene oxide, enantioselective hydrolysis

INTRODUCTION

Enantiopure epoxides are versatile synthetic building blocks for preparing more complex enantiopure pharmaceuticals and agrochemicals [11]. Various chemical approaches have been investigated to produce enantiopure epoxides *via* asymmetric epoxidation of olefins and enantioselective resolution of racemic epoxide substrates [3, 19]. Enantiopure epoxides can be prepared by kinetic resolution of racemic epoxides by epoxide hydrolase (EH) [5, 9, 21, 23]. EH catalyses an enantioselective hydrolysis reaction of an epoxide to the corresponding diol [2, 10]. EH is cofactor independent, easy to use, and relatively stable enzyme [6, 7].

Recently, genome sequencing is significant as the source of novel enzymes for industrial biocatalysis. Genome-wide identification of novel enzymes based on sequence similarity has been used to search useful biocatalysts from various genome data [8]. Although many efforts have been paid to interpret sequence information into the corresponding function using structural genomics and proteomics, the exact function of a putative protein cannot be readily identified by sequence information alone. Therefore, a putative protein should be cloned and expressed to annotate the exact function to date. Despite a generally low sequence similarity, EH belongs to the class of α/β hydrolase fold enzymes that possess a high conservation of catalytic triad composed of Asp, Asp(or Glu in microsomal EH) and His [16, 23].

EHs are ubiquitous and are found in mammalian cells, plants, bacteria, yeasts, fungi, and insects [4]. Up to date, EHs of fish have not investigated intensively yet [13]. The potential of fish EHs as biocatalysts for preparing enantiopure epoxides can be readily extended by recombinant gene expression. Therefore, it is of interest to identify and characterize EHs from fish bioresources for searching novel EH activity toward various epoxide substrates. In this paper, we mined genome data of zebrafish to discover a fish EH with the enantioselective activity towards various racemic epoxides substrates. The putative mEH was cloned and expressed to confirm the enantioselective EH activity, and the recombinant mEH was evaluated as biocatalysts for producing enantiopure epoxides.

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MATERIALS AND METHODS

Identification of a putative mEH in *D. rerio* and sequence homology analysis

Bioinformatic searches of nucleotide or protein sequence for fish EHs were performed using NCBI (http://www.ncbi.nlm.nih.gov/) and PredictProtein servers (http://www.predictprotein.org/). Comparison of the conserved features of mEH from *D. rerio* with other mammalian mEHs was performed by multiple sequence alignment in ClustalW (http://srs.ebi.ac.uk/). A model for *D. rerio* putative mEH was constructed using molecular modeling based on homology with crystallographic templates of *Aspergillus niger* mEH (pdb code: 1qo7) and *Agrobacterium radiobacter* sEH (pdb code: 1ehy). Molecular modeling was carried out using Modeller7v7 program [15] and Swiss-Model server, accessible *via* the EXPASY(http://www.expasy.org/).

Strains, plasmids and DNA manipulations

The tissue extract of *D. rerio* was used as the source of mRNA to clone the putative EH genes. The pGEM-T Easy vector (Promega, U.S.A.) was used for gene cloning and a pCold I vector (Takara, Japan) was used for the expression of EH in *Escherichia coli* BL21(DE3). Isolation of plasmid DNA from *E. coli* was conducted using Qiagen Miniprep Kit (Qiagen, Hilden, Germany). DNA from agarose gel was extracted and purified using Qiaquik Gel Extraction Kit (Qiagen, Hilden, Germany).

Isolation of total RNA from *D. rerio* and generation of cDNA library

Total RNA isolation and cDNA library generation was carried out as described previously [14]. Briefly, a tissue sample of a frozen zebrafish in liquid nitrogen was ground in a mortar and suspended in Zol B solution, and then the total RNA was extracted with of chloroform/isoamylalcohol. The RNA pellet was precipitated with isopropanol, dried and dissolved in diethylpyrocarbonate(DEPC)-treated water. The cDNA library of *D. rerio* was synthesized from the total RNA by RT-PCR using the First Strand cDNA Kit (Invitrogen, U.S.A.).

Cloning and expression of mEH of *D. rerio*

The putative mEH gene was amplified from cDNA library using two primers; forward, 5'-gacatatggatagcgtcaaaggacacttg-3' and backward, 5'-gagaattcttacttgtggcttttgccctcc-3'. Primers with *Nde*I and *Eco*RI restriction sites (underlined in the primers) were designed from the nucleotide sequence (GenBank ID: BC055594) of mEH protein of *D. rerio*. The PCR products were cloned into pGEM-T easy vector, subsequently inserted into pCold I expression vector, and the resulting recombinant plasmids were transformed into *E. coli* BL21(DE3) [17]. The recombinant *E. coli* was cultivated on LB medium supplemented with 50 μg/ml of ampicillin in a gyratory incubator with a shaking speed of 250 rpm. Initially, the cells were grown at 37°C for 2-3 h to be an OD_{600} of 0.4~0.6, and then cultivated at 15°C for 24 h to induce the production of putative mEH after adding of 1 mM IPTG.

To analyze the expressed gene products, the proteins were extracted from the recombinant cells, separated on 12% SDS-polyacrylamide gel, and blotted with polyclonal antibody against hexahistidine (H-15, Santa Cruz Biotechnology Inc., U.S.A.) and peroxidase-conjugated anti-rabbit IgG (Jackson Immunoresearch, U.S.A.), and then visualized with CN/DAB(4-chloronaphthol/3,3'-diaminobenzidine) solution (Pierce, U.S.A.).

Enantioselective hydrolysis by the recombinant *E. coli* possessing the mEH gene of *D. rerio*

The EH activity of the recombinant *E. coli* was confirmed by the analysis of hydrolysis reactions for enantiopure (*R*)- or (*S*)-styrene oxide (Aldrich Chemical Inc., U.S.A.). The reaction was conducted at 30°C in 10 ml 100 mM KH_2PO_4 buffer in 50 ml screw-cap bottles sealed with a rubber septum. A shaking water bath was used at 250 strokes min^{-1}. The reaction was started by adding 5 mM racemic styrene oxide, and stopped by adding an equal volume of diethyl ether for extraction of unreacted styrene oxide. The progression of reaction was followed by analyzing the samples withdrawn periodically from the reaction mixture.

Analyses

Cell concentration was measured by a spectrophotometer at 600 nm (UV1240, Shimazhu, Japan). Enantiomeric excess ($ee = 100 \times (S-R)/(S+R)$) and yield for enantiopure styrene oxide were determined by chiral GC analysis [12]. The reaction mixture was extracted with an equal volume of diethyl ether, and 1 μl of the organic layer was analyzed by chiral GC with a fused silica capillary beta-DEX 120 column (0.25 mm ID × 30 m, 0.25 mm film thickness, Sulpelco Inc., U.S.A.) fitted with a FID detector. The

temperatures of the column, injector, and detector were 100, 220, and 220°C, respectively.

RESULTS AND DISCUSSIONS

Gene mining for identification of mEH from genome data of *D. rerio*

EHs share a weak but significant sequence similarity to each other, which led to the conclusion that the EHs possess the same overall tertiary structure of catalytic activity site. All EHs have the catalytic triad consisting of an acidic nucleophile and a basic charge relay system. The characteristic oxyanion hole is also highly conserved. Therefore, the putative EHs can be identified *in silico* by data mining based on the characteristic conserved sequence information.

We mined the genome data of *D. rerio* to identify a putative mEH from fish bioresources. BLAST searched one putative mEH (Ephx1 protein, GenBank ID: AAH55594) and one putative mEH-like protein (GenBank ID: AAH45930) which we have expressed in *E. coli* and characterized [13]. The deduced amino acid sequence of the putative mEH was characterized by multiple sequence alignment with other mEHs using ClustalW (Fig. 1). The putative mEH of *D. rerio* have sequence identity with about 60% and 30% of mammalian mEHs and microbial EHs, respectively. In this molecular modeling study, we chose *A. niger* EH and *A. radiobacter* EH whose X-ray structure was reported. The identity between *A. niger* EH and *D. rerio* mEH is about 29% for the matched sequence, but many other amino acid residues exhibited close similarity. In the modeled catalytic active site, mEH of *D. rerio* possessed the catalytic triad consisting of Asp^{233}, Glu^{413}, and His^{440}. Two tyrosine residues (Tyr^{308} and Tyr^{383}) are conserved at the active site to form hydrogen bonds with epoxide substrates (Fig. 2).

Most of known EHs are α/β-hydrolase fold enzymes. The common feature of α/β-hydrolase fold enzymes is that they share a basic structure of α/β-hydrolase fold domain with a lid domain over an active site [1, 16, 23]. As shown in Fig. 3, the modeled structure for the putative mEH of *D. rerio* has a central domain consisting of a twisted eight-stranded parallel β-sheet except the second, which is antiparallel, flanked on both faces by α-helices like in mEH of *A. niger*. The putative mEH of *D. rerio* also has a similar lid domain consisting of six α-helices with a triangular structure. Therefore, the homology modeling suggested that the mEH of *D. rerio* has a similar topology of α/β-

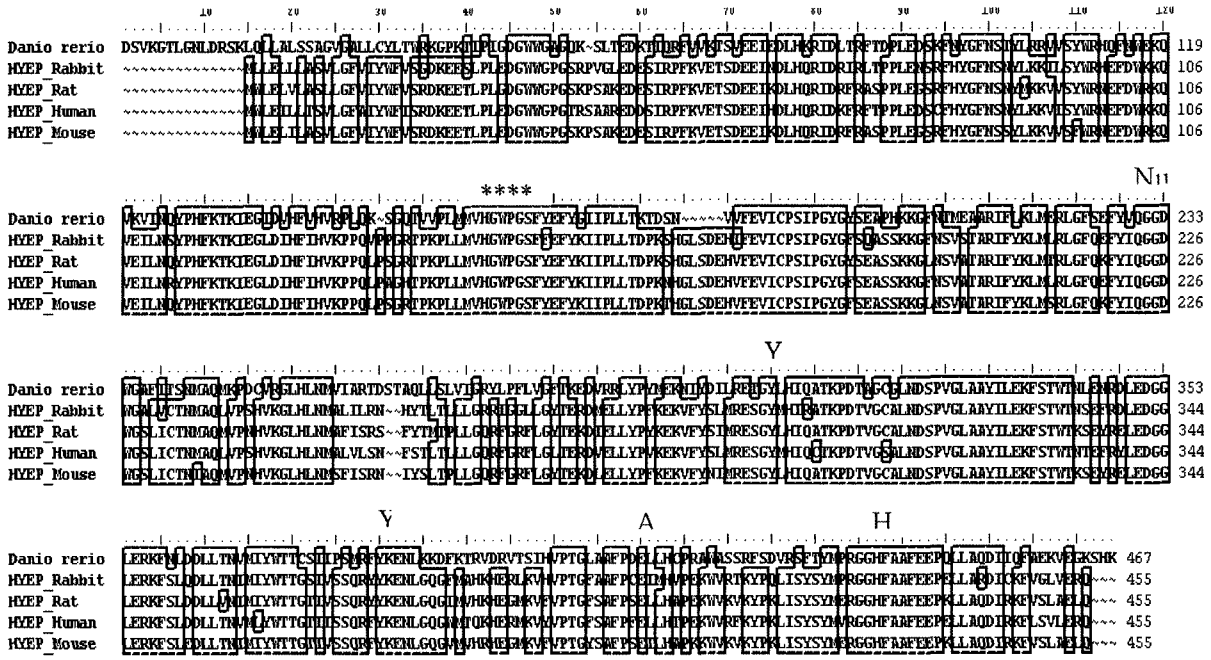

Fig. 1. Multiple alignments of amino acid sequences of the mEH of *D. rerio* with those of several organisms extracted from Swiss-Prot. The conserved domain regions of a catalytic triad and an oxyanion hole are represented with abbreviations. The residues that form the catalytic triad are indicated by Nu (nucleophile), A (acidic residue) and H (histidine). The amino sequences corresponding to the oxyanion hole are indicated by ****. Conserved identical amino acid residues are boxed with outline.

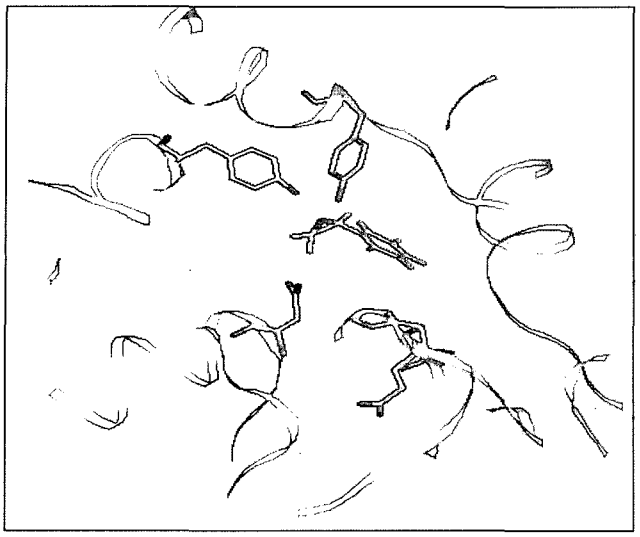

Fig. 2. The proposed active site of *D. rerio* mEH based on homology structural modeling. The ribbon representation was made using RASWIN [19]. The epoxide oxygen of the substrate (styrene oxide) is 'anchored' by two hydrogen bonds donated by tyrosine residues, Tyr308 and Tyr383. Asp233, His440 and Glu413 residues form a catalytic triad which is essential to the EH catalytic activity.

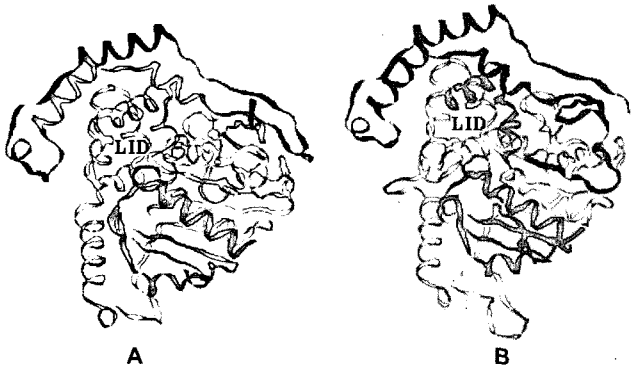

Fig. 3. The proposed α/β-fold structure of mEH of *D. rerio* based on homology modeling with the coordinates of two X-ray structures, 1qo7A.pdb (*Aspergillus niger*) and 1ehy.pdb (*Agrobacterium radiobacter* AD1). (A) *A. niger* EH (pdb code: 1qo7A) (B) mEH model of *D. rerio*. The position of the α-helical lid is highlighted with the label LID. N-terminal region and C-terminal region are represented by blue and red, respectively. The figure was drawn using RASWIN [19].

hydrolase fold EHs such as mEH of *A. niger* and sEH of *A. radiobacter*, which supports that the putative mEH of *D. rerio* would have an enantioselective EH activity.

Cloning and characterization of the putative mEH protein of *D. rerio*

While valuable enzyme can be readily discovered by gene mining of genome data, its function cannot be

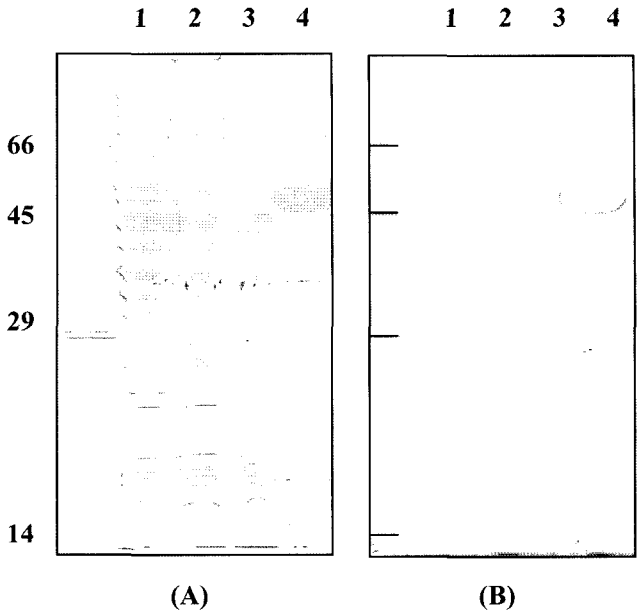

Fig. 4. SDS-PAGE (A) and immunoblot analysis (B) of the expressed mEH proteins in *E. coli*. The proteins of cell extracts were separated on 12% gel and electrotransferred on to nitrocellulose paper for immunoblotting. Primary Ab and secondary Ab were polyclonal anti-hexahistidine antibody and peroxidase conjugated polyclonal antibody, respectively. Lane 1 and 2: pColdI vector-transformed *E. coli* without or with induction, lane 3 and 4: pColdI-mEH without or with induction.

definitely predicted by sequence information alone. As a result, the putative gene has to be cloned and characterized to annotate the function. The gene of the putative mEH was cloned from a cDNA library generated by RT-PCR for the isolated total mRNA of *D. rerio*. A 1.4 kb DNA fragment of PCR product was cloned into pGEM-T easy vector and pCold I vector, sequentially. The cloned gene was sequenced and confirmed that all amino acid sequences were same with those of mEH protein of *D. rerio* in GenBank. The cloned gene encodes 468 amino acids and exhibited a band in SDS-PAGE with the corresponding mass of 5.6 kDa (Fig. 4). Most of the putative mEH protein was expressed as a soluble form in *E. coli*.

We also identified a putative MEST(mesoderm-specific transcript)/EH gene that is expressed particularly in mesodermal tissues in early embryonic stages. The putative MEST/EH gene shares about 74% amino acid sequence similarity with other MEST/EHs from mouse, rat, human and xenopus (data not shown). The putative MEST/EH gene possess the α/β-hydrolase fold that is common to a number of hydrolytic enzymes of widely differing catalytic function. A catalytic triad consisting of Asp^{155}, Asp^{292} and

His^{319} is conserved and the amino acid sequences corresponding to the oxyanion hole are also conserved. The generic sm-x-nu-x-sm-sm motif was conserved on the MEST/EH gene, where sm is a small residue, x is any residue and nu is the catalytic nucleophile. All these data mining indicate that MEST/EH probably exhibits hydrolytic activity toward epoxide substrates. To verify this reasoning, we cloned the MEST/EH gene and expressed the cloned gene in *E. coli*. Whereas western blot analysis clearly showed that the MEST/EH gene was highly expressed in *E. coli*, the recombinant *E. coli* exhibited no hydrolytic activity toward various epoxide substrates. One probable reason why MEST/EH does not have a hydrolytic activity is that only one probable Tyr^{262} is identified in the amino acid sequences of MEST/EH of *D. rerio*, whereas two tyrosine residues, $Tyr^{308}$and Tyr^{383}, are conserved in mEH. In general, the tyrosine residues play a key role in a general acid catalysis by forming hydrogen bonds with the oxide oxygen, facilitating a nucleophilic attack by the catalytic aspartic residue. It has been reported that the EH of *A. radiobacter* still exhibited a hydrolytic activity when one of two tyrosine residues, Tyr^{215}, was mutated to phenylalanine [18]. In contrast, one of two tyrosine residues of mEH of *D. rerio* and EH of *Rhodotorula glutinis* was mutated into phenylalanine, the mutated EHs showed no hydrolytic activity toward epoxide substrates (unpublished results), showing that it needs to be further investigated whether the requirement of two tyrosine residues is prerequisite for obtaining EH activity or not.

Kinetic resolution of racemic styrene oxide by the recombinant *E. coli* possessing mEH gene of *D. rerio*

Enantioselective hydrolysis of racemic styrene oxide was carried out at 5 mM to confirm the EH activity of the putative mEH of *D. rerio*. The recombinant *E. coli* possessing the putative mEH gene of *D. rerio* preferentially degraded the (*R*)-enantiomer, when incubated for 10 min at 30°C and pH 7. We conclude that the putative mEH identified using gene mining based on sequence information has an enantioselective hydrolysis activity. The fish gene of mEH was successfully expressed as a soluble form in *E. coli*, indicating that the recombinant *E. coli* with fish mEH can be used as a whole-cell biocatalyst to prepare enantiopure epoxides. The kinetic preference of the fish EH toward (*R*)-enantiomer is same with those of mammals.

Batch kinetic resolution by the recombinant *E. coli* was carried out at 30°C, pH 7.0 and initial concentration of 5 mM racemic styrene oxide. Cell weight was calculated as dry cell weight (dcw). The reaction was started by adding 40 mg dcw ml^{-1}. Samples were withdrawn periodically to monitor the progression of the enantioselective hydrolysis reaction. As shown in Fig. 5, (*R*)-styrene oxide was readily hydrolyzed into the corresponding diol and (*S*)-styrene oxide with the enantiopurity of 99 % *ee* was obtained after 45 min. Resolution yield was 33.5%. We concluded that the recombinant *E. coli* possessing the *D. rerio* mEH could be a good whole-cell biocatalyst for production of enantiopure (*S*)-styrene oxide.

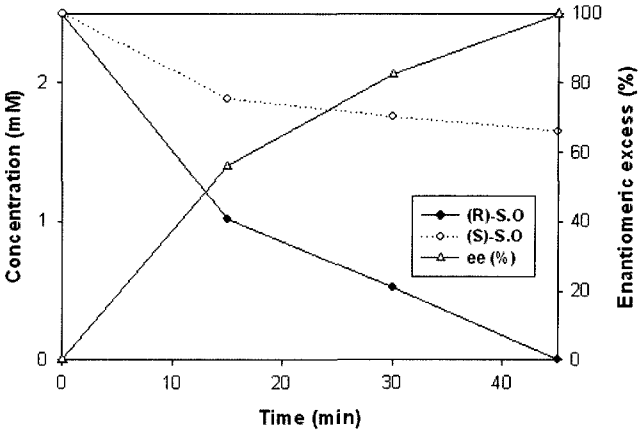

Fig. 5. Batch kinetic resolution of racemic styrene oxides by the recombinant *E. coli* containing the mEH gene of *D. rerio*. Hydrolytic reaction was carried out at 100 mM potassium phosphate (pH 7.0, temperature 30°C). Symbols: (●) (*R*)-styrene oxide, (○) (*S*)-styrene oxide and (△) enantiomeric excess.

CONCLUTION

We identified and characterized a fish mEH based on the genomic data mining. The mEH protein of *D. rerio* had the conserved sequence and domains with mammalian mEHs, annotated by cloning and expression of the putative gene from the cDNA library of *D. rerio*. Molecular modeling and structural alignment of the putative mEH based on known crystallographic templates showed that the putative mEH has a similar active site region and a lid domain. Enantiopure (*S*)-styrene oxide with an enantiomeric excess (*e.e.*) higher than 99% was obtained using the recombinant *E. coli* possessing the fish mEH gene of *D. rerio*. We expect that the fish mEH can be used as a biocatalyst for preparation of enantiopure epoxides.

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국문초록

생물정보학을 이용한 Zebrafish Microsomal Epoxide Hydrolase 클로닝 및 특성연구

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Zebrafish (*Danio rerio*)의 microsomal epoxide hydrolase (mEH)로 추정되는 유전자를 클로닝하고 그 특성을 연구하였다. *D. rerio*의 mEH 추정단백질은 포유동물의 mEH 및 세균의 EH들과 아미노산서열 상동성을 보였으므로 결정분자구조(1qo7 및 1ehy)를 template로 하여 homology modelling을 행하였다. 클론된 단백질은 Asp^{233}, Glu^{413} 및 His^{440}으로 구성된 catalytic triad와 2개의 tyrosine 잔기 및 oxyanion hole이 보존되어 있었다 생물정보학적인 분석 및 EH 활성시험은 추정단백질이 *D. rerio*의 mEH라는 것을 보여주었다. Racemic styrene oxide를 기질로 하여 활성시험을 행한 결과, 재조합 *D. rerio* mEH는 (*R*)-styrene oxide을 입체선택적으로 가수분해하였으며 45분 반응시간에 99%ee의 광학순도를 가진 (*S*)-styrene oxide를 33.5% 얻을 수 있었다.

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