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Cloning and characterization of a fish microsomal epoxide hydrolase of *Danio rerio* and application to kinetic resolution of racemic styrene oxide

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Abstract

Gene mining of the genome database of the zebra fish *Danio rerio* revealed the presence of a putative microsomal epoxide hydrolase (mEH)-like protein containing the characteristic catalytic triad composed of Asp^{223}, Glu^{402}, and His^{429} as well as the oxyanion hole common to all mEH. Based on the sequence information, a new EH gene was cloned by PCR amplification of cDNA of the zebra fish *Danio rerio* and expressed heterologously in *Escherichia coli*. The recombinant *E. coli* exhibited the enantiopreference toward (*R*)-styrene oxide with the maximum hydrolytic activity of 11.4 μmol min^{-1} (mg dcw)$^{-1}$. When the kinetic resolution was conducted with 40 mM of racemic styrene oxide, enantiopure (*S*)-styrene oxide was obtained with an enantiomeric excess (ee) higher than 99 and 23.5% yield at 30 min. These results demonstrate that the recombinant fish EH has the possible application as a biocatalyst for the production of enantiopure epoxides.

Keywords: Enantiopure styrene oxide; *Danio rerio*; Epoxide hydrolase; Gene mining; Kinetic resolution

1. Introduction

Epoxide hydrolase (EH) can be used for the hydrolytic kinetic resolution of racemic epoxides for the production of enantiopure epoxides [1–3]. The reaction has been studied extensively since enantiopure epoxides are versatile synthetic intermediates for the preparation of enantiopure bioactive compounds and they can be obtained from cheap and readily available racemic epoxides [4–10]. For example, enantiopure 2-, 3-, and 4-pyridyloxiranes, key building blocks for the synthesis of β-adrenergic receptor agonists or anti-obesity drugs, can be produced by EH from their corresponding racemic mixtures [11]. The reaction is known to proceed via two steps as follows; first, the nucleophilic attack of an aspartic residue in the active site to yield a covalent 'glycol-monoester' intermediate, and next, hydrolysis of the covalent intermediate by water, assisted by histidine residue in charge relay system [12].

EHs are ubiquitous enzymes found in bacteria, yeast, fungi, insect, plant, and mammalian cells [13,14]. EHs are involved in the catabolizing of hydrocarbon in various microorganisms and the synthesis of secondary metabolites in plant cells [15]. EHs are also responsible for the cellular detoxification of xenobiotics in mammalian cells [16].

Although various EHs with novel activities are needed to explore for the just-in-time application in the preparation of enantiopure epoxides, only a limited number of recombinant EHs have been characterized in detail [17–20]. Due to the recent advances in bioinformatics and rapidly accumulating genome sequencing data, it became possible to obtain novel EHs by genome data mining and analysis of putative EHs sequence and activity information [21]. The putative EHs could be readily screened based on the conserved sequence information of a cat-

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alytic triad consisting of a catalytic nucleophile and a charge relay system. However, in most cases, the protein function cannot be annotated by sequence information alone. Therefore, the genes of putative EHs exploited by a conserved sequence motif should be cloned and expressed to prove the function.

While various EHs from microorganisms, plant and mammalian cells have been investigated, no fish EHs have been cloned and characterized to our best knowledge. In this paper, we conducted data mining on the genome sequences of a zebra fish, *Danio rerio*, in order to obtain a fish EH. Then, the fish EH gene was cloned from a cDNA library of total mRNA generated by RT-PCR and expressed heterologously in *Escherichia coli*. The recombinant cells were evaluated for potential use as biocatalysts in the preparation of enantiopure epoxides via enantioselective hydrolytic resolution.

2. Experimental

2.1. Identification of a putative EH in D. rerio and sequence homology analysis

Bioinformatic searches of nucleotide or protein sequence for fish EHs were performed using NCBI, NGIC, and Predict Protein servers [22]. Comparison of the conserved features of microsomal EH (mEH) and mEH-like protein from *D. rerio* with other mammalian mEHs was performed by multiple sequence alignment in ClustalW.

2.2. Strains, plasmids, and DNA manipulations

Tissue extract of *D. rerio* was used as the source of mRNA to clone the putative EH gene. The pGEM-T Easy vector (Promega, USA) was used for the gene cloning and the pCold I vector (Takara, Japan) was used for the expression of EH in *E. coli* [23]. 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).

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

Fresh zebra fish was frozen in liquid nitrogen, ground in a mortar, and used for extraction of total RNA. The broken tissues were suspended in 20 ml of Zol B solution (4 M guanidium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% lauryl sarcosine sodium salt, 0.1% 2-mercaptoethanol, 2 M sodium acetate (pH 4), and 10 ml phenol saturated with DDW), and extracted with 2 ml of chloroform/isoamylalcohol twice. The aqueous phase was then added with 10 ml of isopropanol, incubated at −20 °C for 30 min, and centrifuged. The RNA pellet was washed once with 70% ethanol and dried at room temperature.

The cDNA library of *D. rerio* was synthesized from the total RNA by RT-PCR using the First Strand cDNA Kit (Invitrogen, USA). After adding reverse transcriptase (RTase) to the reaction mixture, the reaction was carried out at 42 °C for 50 min. Afterwards, the RTase was inactivated by raising the temperature to 70 °C for 15 min and RNA was hydrolyzed by treating with RNase H at 37 °C for 20 min.

2.4. Cloning and expression of a mEH-like protein of D. rerio

The putative EH gene was amplified from cDNA library using two primers; forward, 5′-gtctcgagatgtacctagaggtgatagtgg-3′ and backward, 5′-gtgaattctcatttccgcttcctgttctctac-3′. Primers with *Xho*I and *Eco*RI restriction sites (underlined in the primers) were designed from the nucleotide sequence of mEH-like protein of *D. rerio*. The PCR operation conditions were as follows: 3 min at 94 °C, 30 cycles of 45 s at 94 °C, 30 s at 58 °C, 1 min at 72 °C, and ending with 10 min at 72 °C. The PCR product was cloned into pGEM-T easy vector, subsequently inserted into pCold I expression vector after digestion with *Xho*I and *Eco*RI, and the resulting recombinant plasmid was transformed into *E. coli* BL21(DE3). 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 EH after adding of 1 mM IPTG.

To analyze the expressed gene product, proteins were extracted from the recombinant cells, 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) solution (Pierce, USA).

2.5. Enantioselective hydrolysis by the recombinant E. coli possessing the EH gene of D. rerio

The EH activity of the recombinant *E. coli* was studied by hydrolysis of enantiopure (*R*)- or (*S*)-styrene oxide (Aldrich Chemical Inc., USA). The reaction was conducted at 30 °C in 5 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 40 mM racemic styrene oxide, and stopped by adding an equal volume of diethyl ether for extraction of unreacted styrene oxide. The progression of enantioselective hydrolysis was investigated in a 10 ml glass enzyme reactor with a magnetic stirrer. The reaction was followed by analyzing the samples withdrawn periodically from the reaction mixture.

2.6. Analyses

Cell concentration was measured by a spectrophotometer at 600 nm (Lambda 20, Perkin-Elmer, USA). 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 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 i.d. × 30 m, 0.25 μm film thickness, Sulpelco Inc., USA) fitted with a FID detector. The temperatures of the column, injector, and detector were 100, 220, and 220 °C, respectively.

3. Results and discussions

3.1. Identification of fish mEH from genome of D. rerio

Zebra fish, *D. rerio*, has been used as an in vivo fish model for many pharmacokinetic and toxicological studies, and the whole genome sequences of *D. rerio* are available [22]. BLAST searches on protein sequences with the query of *D. rerio* EH hit one putative microsomal EH (mEH, Ephx1 protein, GenBank ID: AAH55594) and one mEH-like protein (similar to epoxide hydrolase 1, GenBank ID: AAH45930). These two sequences showed 61% identity to each other, and were further characterized by multiple sequence alignment with other mEHs using ClustalW (Fig. 1). Most of EHs belong to α/β-hydrolase fold enzymes, which share the common features in that they have a catalytic nucleophile and a charge relay system. These characteristic amino acids sequences were used to compare the putative EH and EH-like protein of *D. rerio*. Both protein sequences possessed a catalytic triad consisted of aspartic, glutamic, and histidine residues (Asp^{233}, Glu^{413}, His^{440} for mEH; Asp^{223}, Glu^{402}, His^{429} for mEH-like protein). Two tyrosine residues that bind to oxide oxygen and act as proton donor were highly conserved (Tyr^{308}, Tyr^{383} for mEH; Tyr^{298}, Tyr^{373} for mEH-like protein) (Fig. 1). The formation of a hydrogen bond between the oxide oxygen atom and the tyrosine residue has been known to assist in the proper positioning of the substrate in the catalytic active site of EH and to activate the epoxide moiety [12,13]. A multiple sequence alignment with known EHs revealed that the mEH and mEH-like protein of *D. rerio* have about 60% sequence identity with mammalian mEH at protein level. A phylogenetic analysis was conducted based on neighbor-joining method as shown in Fig. 2. The mEH-like protein was closely related with the mEHs from rat, human, mouse, and rabbit.

The presence of EH enzymatic activity can be confirmed by measuring the conversion of epoxide to vicinal diol [24]. When the tissue extract of *D. rerio* was incubated with racemic styrene oxide, a clear hydrolytic activity was observed although the rate was very low (data not shown). This indicates that *D. rerio* has EH enzyme which might be encoded from the genes selected by data mining.

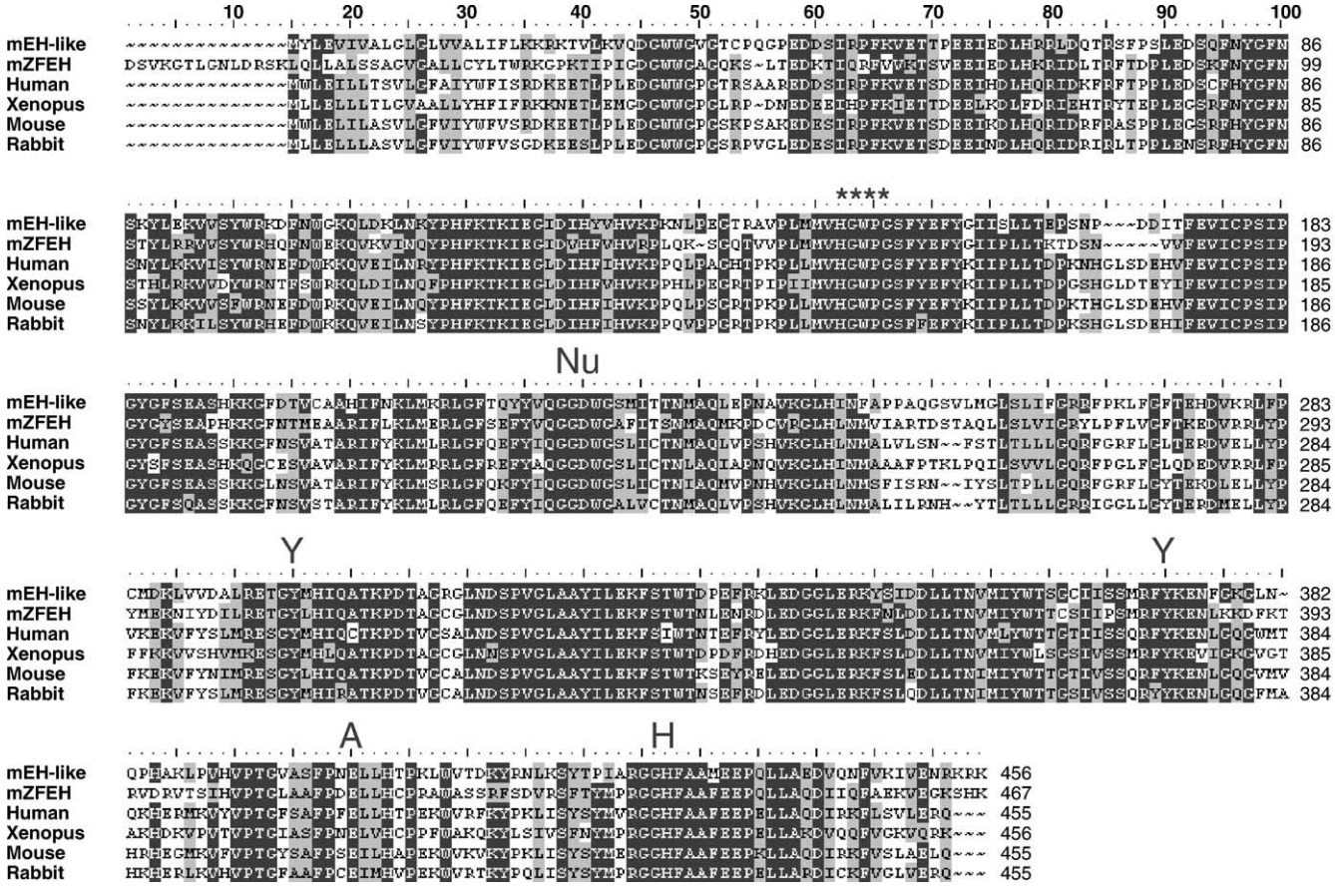

Fig. 1. Multiple alignment of amino acid sequences of fish EHs of *D. rerio* with those of several organisms extracted from SwissProt. The conserved domain regions of a catalytic triad and an oxyanion hole are represented with abbreviations. The residues that form the catalytic triad are indicated by Nu (nucleophile), A (acidic residue), and H (histidine). The amino sequences corresponding to the oxyanion hole are indicated by (****). Conserved amino acid residues are boxed in dark shaded and similar amino acid residues are boxed in pale shaded.

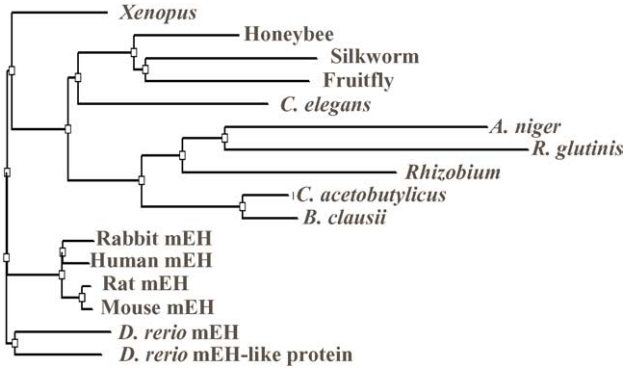

Fig. 2. Phylogenetic relationship between fish EHs of *D. rerio* and EHs from various species. Based on the multiple sequence comparison of the functional and structural domain sequence (shown as Fig. 1), phylogenetic tree was obtained by using neighbor-joining method Version 3.573c. Protein distance matrix was made from BLOSUM62.

3.2. Cloning and characterization of mEH-like protein of *D. rerio*

It is impractical to annotate protein function by sequence information alone. Therefore, it is of interest to clone and characterize the gene of mEH-like protein exploited by bioinformatics to annotate the function. The gene encoding mEH-like protein was cloned from a cDNA library generated by RT-PCR reaction of the isolated total mRNA. The gene for mEH-like protein was amplified by PCR with specific primers from a cDNA library of *D. rerio* (see Section 2). A 1.37 kb DNA fragment of PCR product was obtained and cloned into pGEM-T Easy vector. The DNA sequences of this fragment were practically the same (99.9% identity) as the mEH-like protein mined from *D. rerio* genome. The cloned gene encodes 456 amino acids with a deduced molecular mass of 51.9 kDa. SDS-PAGE and immunoblotting analyses were also performed for the recombinant colony to confirm the expression of the cloned mEH-like protein (Fig. 3). The expressed mEH-like protein showed a major band of about 52 kDa on SDS-PAGE, which agrees well with the calculated value. Whereas mEH of *Aspergillus niger* was not highly expressed in a soluble form [25], the mEH-like protein of *D. rerio* was overexpressed as a soluble form in *E. coli*. This indicates that the recombinant *E. coli* can exhibit a high whole-cell EH activity per unit cell mass.

3.3. Hydrolysis kinetics of styrene oxide

To verify the enantioselective hydrolytic activity of the recombinant mEH-like protein, kinetic resolution of racemic styrene oxide was carried out at 10 mM. When incubated for 10 min with the recombinant *E. coli* containing the mEH-like protein, the (*R*)-enantiomer was preferentially degraded, leaving most of the (*S*)-enantiomer unreacted (data not shown). This result indicates that the mEH-like protein of the zebra fish has EH activity and the activity can be successfully expressed in the prokaryotic *E. coli* cell. Also, it indicates that the kinetic preference of the fish EH is toward (*R*)-enantiomer as in most EHs reported thus far.

The hydrolysis kinetics by the recombinant *E. coli* were further studied using enantiopure (*R*)- and (*S*)-styrene oxides, and the results were shown in Fig. 4. The hydrolysis rates of the (*R*)- and (*S*)-enantiomers increased linearly with increasing in substrate concentrations at low substrate concentrations, and slowed down at higher concentrations. The Michaelis-Menten kinetic model could explain the kinetic behaviors well.

$$v = \frac{V_{\max} s}{K_{\mathrm{m}} + s} \tag{1}$$

The maximum hydrolysis rates ($V_{\max}$) and affinity constants (K_{m}) for both enantiomers were determined from Lineweaver-Burke plots. The maximum hydrolysis rate ($V^S_{\max}$) and saturation constant (K^S_{m}) for (*S*)-enantiomer were 1.1 μmol min^{-1} (mg dcw)$^{-1}$ and 3.2 mM, respectively, while the maximum rate ($V^R_{\max}$) and saturation constant (K^R_{m}) for (*R*)-enantiomer were 11.4 μmol min^{-1} (mg dcw)$^{-1}$ and 7.3 mM, respectively. The value $V^R_{\max}$ was 10-fold higher than $V^S_{\max}$, and the ratio of the first-order reaction rate, $(V^R_{\max}/K^R_{\mathrm{m}})/(V^S_{\max}/K^S_{\mathrm{m}})$,

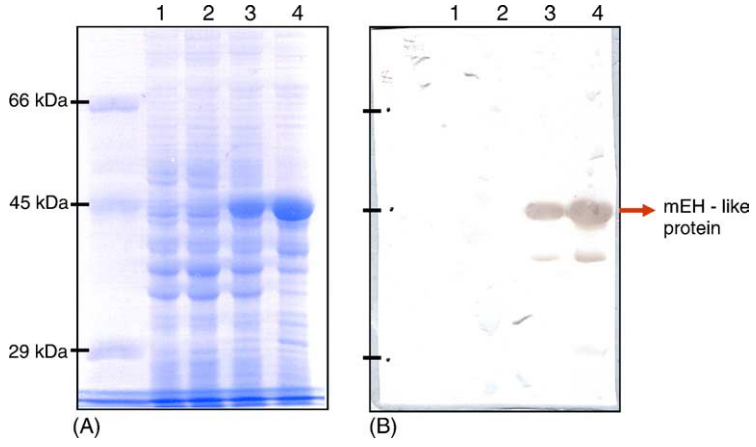

Fig. 3. SDS-PAGE (A) and immunoblot (B) analysis of the expressed mEH-like protein in *E. coli*. Proteins of cell extract were separated on 12% gel and electrotransferred on to nitrocellulose paper for immunoblotting. Primary Ab and secondary Ab were polyclonal anti-hexahistidine antibody and peroxidase conjugated anti-rabbit IgG, respectively. Lanes 1 and 2: pColdI vector-transformed *E. coli* without or with induction, lanes 3 and 4: the recombinant plasmid-transformed *E. coli* without or with induction.

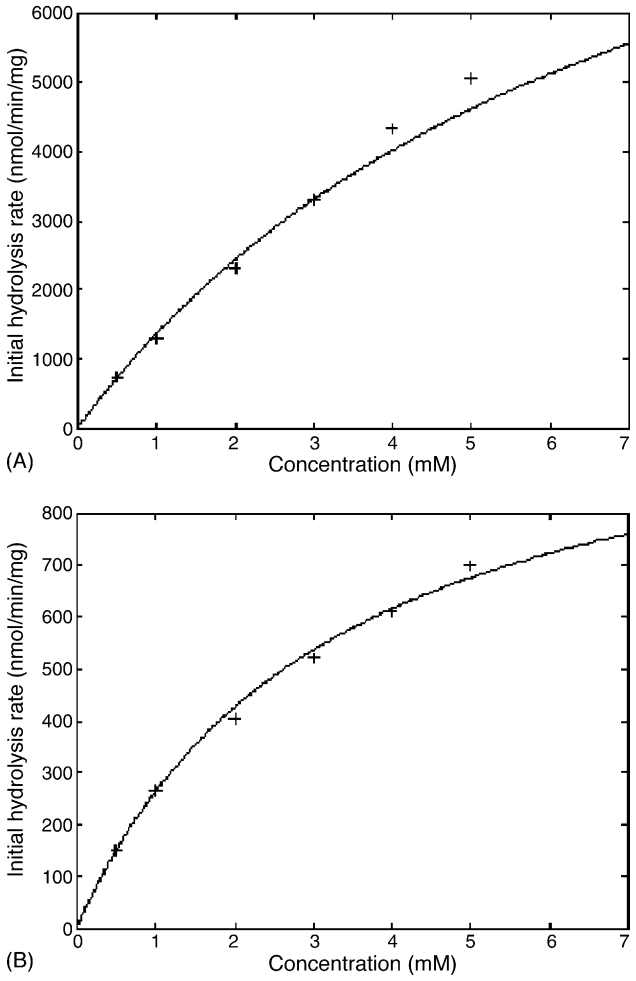

Fig. 4. Hydrolysis of racemic styrene oxides by the recombinant *E. coli* containing the putative mEH gene of *D. rerio*. Both (*R*)-styrene oxide (A) and (*S*)-styrene oxide (B) were used as substrates, and initial hydrolytic rate was determined in the range of 0.5 and 6.0 mM in 100 mM potassium phosphate (pH 7.0).

was about 4.5. This ratio is usually used to represent the degree of preferential degradation of one enantiomer over the other at low substrate concentrations. It should be noticed that the present recombinant *E. coli* has very high whole-cell specific hydrolytic activities for both (*S*)- and (*R*)-styrene oxide compared to other strains reported thus far. For example, the wild-type *Rhodotorula glutinis* possessing an active EH enzyme exhibited V^S_{max} and V^R_{max} as 10.2 and 34.5 nmol min^{-1} (mg dcw)$^{-1}$, respectively. The recombinant yeast *Pichia pastoris* haboring the EH gene cloned from *R. glutinis* exhibited V^S_{max} and V^R_{max} as 111.3 and 358.4 nmol min^{-1} (mg dcw)$^{-1}$, respectively [10]. The comparison indicates that the $V^S_{max}$and V^R_{max} values of the present recombinant *E. coli* are about 10–100 (V^S_{max}) and 30–300 fold (V^R_{max}) higher than the recombinant *P. pastoris* or wild-type *R. glutinis.* However, the saturation constants lay in the same order of magnitude (3–8 mM) for all the whole-cell EH. The high maximum degradation rates for both (*S*)- and (*R*)-enantiomers indicates that the present *E. coli* can be a better whole-cell biocatalyst than the recombinant *P. pastoris* or wild-type *R. glutinis*. The

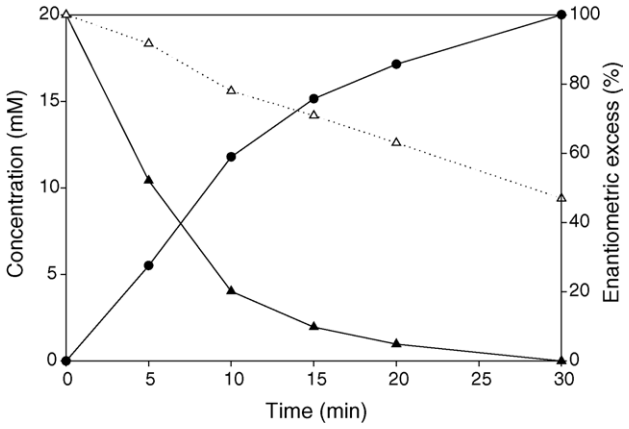

Fig. 5. Batch kinetic resolution of racemic styrene oxide at 40 mM. *Symbols*: (▲) (*R*)-styrene oxide, (△) (*S*)-styrene oxide, and (●) enantiomeric excess.

higher activity is probably attributed to the higher expression of the EH as a soluble form in this recombinant strain.

3.4. Batch kinetic resolution of racemic styrene oxide by the recombinant cells possessing EH of D. rerio

Kinetic resolution of 40 mM by the recombinant *E. coli* was carried out in a batch mode at 30 °C, pH 7.0. Initial concentration of racemic styrene oxide was 40 mM and cell concentration was 0.4 mg dcw ml^{-1}. During 30 min incubation, reaction mixtures were withdrawn periodically and the residual epoxides were analyzed with GC after extraction with diethyl ether. As shown in Fig. 5, the hydrolysis rate of (*R*)-styrene oxide became slow with time, but the enantiopurity of the remaining (*S*)-styrene oxide increased from 0 to 99% ee after 30 min. In general, epoxides are hydrolyzed spontaneously in phosphate buffer, but the hydrolysis of styrene oxide in this reaction was not noticeable during 30 min (data not shown). Final yield of enantiopure (*S*)-styrene oxide was 23.5% (theoretical yield = 50%). These results indicate that the recombinant *E. coli* possessing the *D. rerio* EH is a good whole-cell biocatalyst for production of enantiopure (*S*)-styrene oxide from its racemic mixture. The *D. rerio* EH is the first EH derived from fish, and this study has extended the sources of EH to fish. Further investigations on the enantioselective hydrolysis of various racemic epoxides using the present EH are under progress.

4. Conclusions

Most of enzymes have been obtained by screening specific microorganisms that have the desired activity. The respective genes could be cloned to express in recombinant form. Nowadays, many genomes have been sequenced, and we can easily mine the sequences information to search novel enzymes. Although it is very difficult to predict substrate specificity and enantioselectivity of EH by analyzing the amino acid sequences, genome mining can be an efficient approach to discover novel EH from fish bioresources. Therefore, the putative genes should be cloned and expressed to characterize the functions of the putative enzymes.

Although a number of microbial EHs have been purified for the use as enzyme catalysts, the purified EH loose the activity especially for membrane-associated EH like EHs from *R. glutinis* and *Rhodosporidium toruloides* [26,27]. Purification procedure is time-consuming and requires extra cost. Therefore, from a practical point of view, more efficient production of enantiopure epoxides can be achieved using recombinant cells expressing highly active EH [10]. In this study, a new EH gene was cloned by PCR amplification of the cDNA of the zebra fish *D. rerio* and expressed heterologously in *E. coli*. Enantiopure (*S*)-styrene oxide with an enantiomeric excess (ee) higher than 99% was readily obtained using the recombinant fish EH of *D. rerio*. In conclusion, discovery of novel fish EHs by genome mining is expected to extend the range of industrial epoxides and to meet just-in-time supply of highly active recombinant EH biocatalysts to organic chemists and bioengineers.

Acknowledgments

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