

A Cold-Adapted Epoxide Hydrolase from a Strict Marine Bacterium, *Sphingophyxis alaskensis*

Kang, Ji-Hyun, Jung-Hee Woo, Sung Gyun Kang, Young-Ok Hwang, and Sang-Jin Kim*

Marine Biotechnology Research Centre, Korea Ocean Research and Development Institute, P.O. Box 29, Ansan 425-600, Korea

Received: January 8, 2008 / Accepted: February 19, 2008

An open reading frame (ORF) encoding a putative epoxide hydrolase (EHase) was identified by analyzing the genome sequence of *Sphingophyxis alaskensis*. The EHase gene (*seh*) was cloned and expressed in *E. coli*. To facilitate purification, the gene was fused in-frame to 6× histidine at the C-terminus. The recombinant EHase (rSEH) was highly soluble and could be purified to apparent homogeneity by one step of metal affinity chromatography. The purified SEH displayed hydrolyzing activities toward various epoxides such as styrene oxide, glycidyl phenyl ether, epoxyhexane, epoxybutane, epichlorohydrin, and epifluorohydrin. The optimum activity toward styrene oxide was observed at pH 6.5 and 35°C. The purified SEH showed a cold-adapted property, displaying more than 40% of activity at low temperature of 10°C compared with the optimum activity. Despite the catalytic efficiency, the purified SEH did not hydrolyze various epoxides enantioselectively. K_m and k_{cat} of SEH toward (*R*)-styrene oxide were calculated as 4 ± 0.3 mM and 7.42 s⁻¹, respectively, whereas K_m and k_{cat} of SEH toward (*S*)-styrene oxide were 5.25 ± 0.3 mM and 10.08 s⁻¹, respectively.

Keywords: Epoxide hydrolase, genome sequence, cold-adapted, marine microorganism

Epoxide hydrolases (EHases; E.C. 3.3.2.3) are ubiquitous enzymes that have been isolated from a wide variety of sources, such as bacteria, yeast, fungi, insect, plant, and mammalian [5, 38], and hydrolyze an epoxide to its corresponding vicinal diol with the addition of a water molecule to the oxirane ring [38]. Enantiopure epoxides and vicinal diols are versatile synthetic intermediates for the preparation of enantiopure bioactive compounds [4]. Because of potential application in the production of enantiopure epoxides by kinetic resolution of enantioselective EHase, several

EHases from microbial sources have been developed [32]. However, the limited number of enantioselective EHases demands new enantioselective EHases for the production of enantiopure epoxides in pharmaceutical industries.

Most EHases are members of the α/β hydrolase fold family [20, 21, 25], which includes lipases, esterases, and haloalkane dehalogenases [20, 33]. α/β domains consist of a central, parallel, or mixed β sheet surrounded by α helices with a variable cap domain sitting on top [21]. These enzymes characteristically employ a two-step mechanism in which a catalytic nucleophile of the enzyme attacks a polarized electrophile substrate of the covalent intermediate, followed by hydrolysis (Fig. 1A) [40, 41]. The conserved catalytic triad of α/β hydrolase fold enzymes consists of a nucleophilic residue (Asp or Ser or Cys), an acidic residue (Asp or Glu), and a conserved histidine residue [3]. The nucleophile fits the conserved amino-acid-sequence motif Sm-X-Nu-X-Sm-Sm (Sm=small residue, X=any residue, and Nu=nucleophile). Another conserved amino acid sequence is the HGXP motif containing the oxyanion hole of the enzyme [21]. The active site of EHase contains further two tyrosine residues located in the cap domain, which are involved in substrate binding and assist in the ring opening of the epoxide by acting as proton donor to the epoxide oxygen [25, 26].

Oceans cover more than three-quarters of the earth's surface, thus offering abundant resources for biotechnological research and development. Marine organisms represent an environment for biosynthesis dramatically different from that for terrestrial organisms and, therefore, represent a vast untapped resource with potential benefits in many different areas such as medicine, aquaculture and fisheries, industry, research tools, and environmental applications. Marine organisms, in particular, represent great phylogenetic diversity, making them reservoirs of unique genetic information and important natural resources for possible development [35]. Furthermore, the genomic sequencing of marine microorganisms, done mostly by the Gordon and Betty Moore Foundation (<http://www.moore.org>), can rapidly facilitate

*Corresponding author

Phone: 82-31-400-6240; Fax: 82-31-406-2495;
E-mail: s-jkim@kordi.re.kr

cloning and overexpression for the characterization of a putative or possible EHase originated from a marine environment, as recented report on the screening of various genomic databases for epoxide hydrolases of the α/β hydrolase fold family [10, 33].

We have been collecting samples from various marine environments and the isolating microorganisms, followed by a partial identification and systematic deposition to the Korea Ocean Research and Development Institute (KORDI) collection, and then developing enantioselective biocatalysts by combination of conventional molecular engineering and genomic approaches [11, 13, 14, 18, 23, 39]. In the present study, we characterized an EHase originated from the genome of *S. alaskensis*. A putative EHase gene from *S. alaskensis* was cloned and expressed in *E. coli*. The recombinant protein was purified by metal affinity chromatography, and the hydrolyzing activity of the purified enzyme toward various epoxide substrates was then determined.

MATERIALS AND METHODS

Materials

The epoxides used in this study are shown in Fig. 1B. Racemic styrene oxide (SO) was purchased from Fluka. Pure (*R*)-SO and (*S*)-SO and all other racemic epoxides were purchased from Aldrich. All materials were of analytical or of reagent grade. The silica cyclodextrin beta-DEX 120 (0.25 mm ID, 30 m length) and Chiraldex gamma-cyclodextrin trifluoroacetyl (G-TA, 0.25 mm ID, 30 m length) gas chromatography (GC) columns were purchased

from Supelco (Bellefonte, PA, U.S.A.) and Astec (Whippany, NJ, U.S.A.), respectively. Other medium components were purchased from Merck and Difco.

Strains and Growth Conditions

S. alaskensis was cultured at 30°C in ZoBell 2216E broth [22] consisting of 0.5% peptone, 0.1% yeast extract, and 75% seawater (pH 7.5) for 1 day. For the storage, the bacterial cells were suspended in ZoBell 2216E broth with 20% glycerol and stored at -80°C.

E. coli DH5 α and *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene, La Jolla, CA, U.S.A.) were used for plasmid propagation and gene expression, respectively. *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37°C, and appropriate antibiotic was added.

DNA Manipulation and DNA Sequencing

DNA manipulations were performed using standard procedures [27]. Restriction enzymes and other modifying enzymes were purchased from Promega (Madison, WI, U.S.A.). Small-scale preparation of plasmid DNA from *E. coli* cells was performed with a plasmid mini kit (Qiagen, Hilden, Germany). DNA sequencing was performed with an automated sequencer (ABI3100) using a BigDye terminator kit (PE Applied Biosystems, Foster City, CA, U.S.A.).

BLAST Search and Sequence Analysis

To clone a putative EHase from *S. alaskensis*, sequence searches (Sm-X-Nu-X-Sm-Sm motif and H-G-X-P) against ORFs of *S. alaskensis*, whose genome sequence was determined by the Gordon and Betty Moore Foundation (<http://www.moore.org>), were performed using the ProteinFinder program of Ensoltek (<http://www.ensoltek.com>) and the BLAST program. The pair-wise comparison of candidate EHases and reported EHases was performed using the CLUSTAL

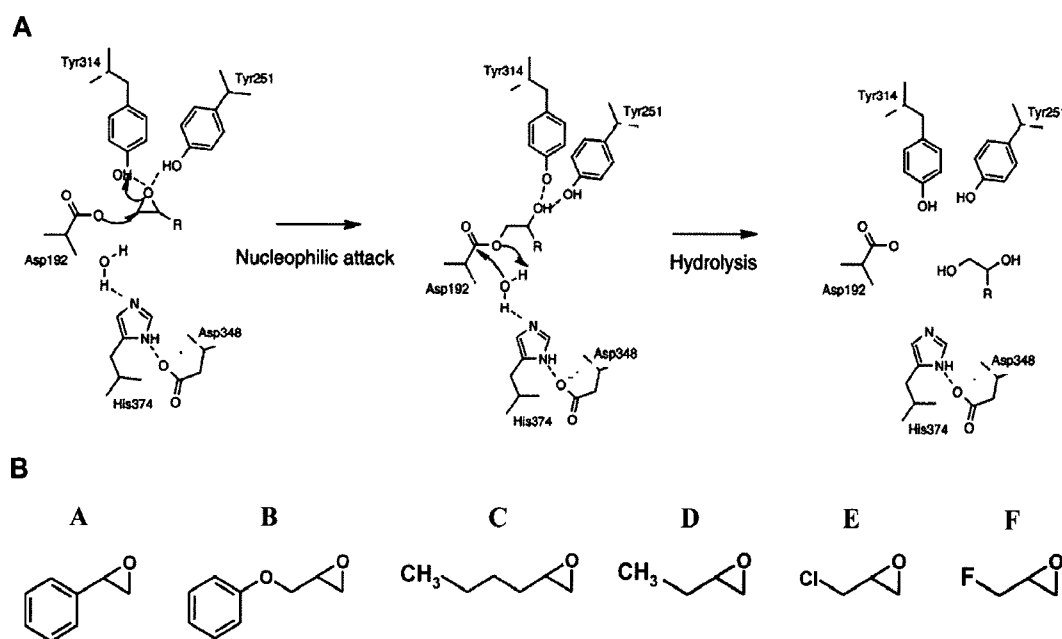


Fig. 1. A. Schematic of the reaction mechanism of epoxide hydrolysis by EHase from *Aspergillus niger* [41]. B. Epoxide substrates used in this study.

A, Styrene oxide (SO); B, glycidyl phenyl ether (GPE); C, 1,2-epoxyhexane (EX); D, 1,2-epoxybutane (EB); E, epichlorohydrin (ECH); F, epifluorohydrin (EF).

W program [31]. The resulting candidates were manually confirmed for the presence of ring-opening tyrosine, HGXP motif, and Sm-X-Nu-X-Sm-Sm (Sm=small residue, X=any residue, and Nu=nucleophile) motif.

For phylogenetic analysis, EHase sequences were retrieved from the SwissProt or EMBL protein databases, and analyzed in comparison with SEH. Phylogenetic distances were calculated by using the CLUSTAL W program, and phylogenetic trees were drawn by the Molecular Evolutionary Genetics Analysis 3.1 software (The Biodesign Institute, Tempe, AZ, U.S.A.) [16].

Cloning of *seh* Gene

Genomic DNA of *S. alaskensis* was isolated using the Genomic DNA extraction kit (Promega) following the manufacturer's instructions. The full-length *seh* gene flanked by NdeI and NotI sites was amplified by PCR with a forward primer (*seh*F: 5'-CGACCCGG-CATATGTCCTCCCGCCAAATCAATTTTCGC-3') and a reverse primer (*seh*RH: 5'-CTCCACATGCGGCCGCTTCTTCTCGCGCAAGGG-3'). The underlined sequences indicate the NdeI site in the forward primer and NotI site in the reverse primer. For the expression of *seh* without His-tag, another reverse primer (*seh*RNH: 5'-CTCCACATGCGGCCGCTCACTTCTTCTCGCGCAAGGG-3') was also designed. The amplified DNA fragment was digested with NdeI and NotI, the fragment was ligated to NdeI/NotI-digested plasmid pET-24a (+), and then the recombinant plasmid was used to transform *E. coli* DH5 α . The recombinant plasmid was introduced into BL21-CodonPlus (DE3)-RP (Novagen) for expression after sequence confirmation.

Purification of SEH

A transformant was cultivated at 37°C and overexpression was induced at 37°C by the addition of 1 mM IPTG when optical density at 600 nm reached 0.4–0.6. After induction for 3 h, the cells were harvested by centrifugation at 5,000 \times g for 20 min, resuspended in a buffer [50 mM phosphate (pH 7.0), 0.5 M KCl, and 10% glycerol], and disrupted by sonication. Cell debris was removed by centrifugation at 15,000 \times g for 30 min with a His-Bind Purification Kit (Novagen). The soluble fraction was applied to a Ni-nitrilotriacetic acid (Ni-NTA) column equilibrated with binding buffer [500 mM NaCl, 20 mM phosphate (pH 7.0), and 5 mM imidazole]. After washing with washing buffer [500 mM NaCl, 20 mM phosphate (pH 7.0), and 60 mM imidazole], the bound enzyme was eluted with elution buffer [500 mM NaCl, 20 mM phosphate (pH 7.0), and 1 M imidazole], and then dialyzed against 50 mM phosphate buffer (pH 7.0). The purity of the protein was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions, as described by Laemmli [17]. The protein concentration was measured by the method of Bradford [7] using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

EHase Assay

EHase activity was determined by a spectrophotometric assay based on the extraction of remaining epoxide from the reaction mixture, followed by spectrometric quantification of the non-extracted diol [6]. Four mM styrene oxide [100 mM stock in dimethyl formamide (DMF)] was mixed with 100 ml of purified EHase in a buffer (10 mM sodium phosphate, pH 6.8), and the mixture was incubated for 15 min at 25°C. Then, 40 ml of the NaIO₄ stock solution (200 mM stock in DMF) was added and vortexed immediately for 2 min.

After centrifugation at 16,500 \times g for 90 s, the supernatant was quantified at 290 nm.

Enantioselective EHase activity was measured according to Woo *et al.* [39] with slight modifications. One hundred μ l of purified EHase showing EHase activity in the spectrophotometric measurement was mixed with 17 mM racemic styrene oxide, (*R*)-styrene oxide, or (*S*)-styrene oxide at a concentration of 8.5 mM in a 10-ml vial containing 1 ml of 100 mM Tris-HCl (pH 7.5), and incubated at 25°C. Then, the samples were withdrawn periodically during incubation, and the reaction mixtures were extracted with hexane (2 ml). The resulting extracts were analyzed on a silica cyclodextrin beta-DEX120 column and a Chiraldex G-TA column [34] using a GC system equipped with a FID detector (Varian, Inc., The Netherlands). The peak area corresponding to (*R*)-styrene oxide or (*S*)-styrene oxide was normalized by the peak area of mesitylene used as an internal standard, and then converted to a residual amount of styrene oxide in comparison with the standard curve. The temperatures of the oven, injector, and detector in GC analysis for racemic styrene oxide were 100°C, 180°C, and 180°C, respectively. The hydrolysis toward other substrates depicted in Fig. 1B was also analyzed similarly. Glycidyl phenyl ether (GPE), epoxyhexane (EX), epoxybutane (EB), epichlorohydrin (ECH), and epifluorohydrin (EF) were analyzed on a Chiraldex G-TA column using a GC system. The oven temperature for the compounds was 90°C, 50°C, 30°C, 60°C, and 28°C, respectively. Helium gas was used as the carrier gas.

Effects of pH and Temperature on EHase Activity

The pH dependence of EHase activity was investigated with the following buffers: 50 mM sodium acetate-acetic acid buffer (pH 4.0 and 6.0), 50 mM MES buffer (pH 6.0 to 7.0), 50 mM Tris-HCl buffer (pH 7.0 to 9.0), and 50 mM glycine buffer (pH 9.0 and 10.0). For determination of the optimal reaction temperature, EHase activities were measured at pH 7.5 over a temperature range of 4 to 70°C.

Determination of Kinetic Parameters

Kinetic parameters of SEH was determined by a GC analysis using racemic styrene oxide, and (*R*)- and (*S*)-styrene oxides as substrates. One hundred μ l of purified EHase was mixed with various concentrations of racemic styrene oxide, or (*R*)- or (*S*)-styrene oxides, in a 10-ml vial containing 1 ml of 100 mM Tris-HCl (pH 7.5), and incubated at 25°C with shaking at 200 rpm. Aliquots of the reaction mixtures were withdrawn periodically. The residual epoxides were analyzed with GC after extraction with hexane. The enantiomeric excess (ee) was derived from the remaining epoxide of the two enantiomers [ee (%) = $(S-R)/(S+R) \times 100$] and the conversion and the extent of conversion when terminating EHase assays before the complete consumption of epoxide [Con. (%) = $\{1 - (E^R_s + E^S_s/E^R_{so} + E^S_{so})\} \times 100$] (theoretical yield = 50%), where the initial epoxide of (*R*) and (*S*) was denoted as *Eso*, and the remaining epoxide of (*R*) and (*S*) as *Es* [29]. Kinetic parameters were estimated by nonlinear regression using a Sigma Plot program. Various substrates, shown in Fig. 1B, were also tested for enantioselective hydrolysis by SEH.

RESULTS

Identification of an EHase Gene from *S. alaskensis*

Previously, we reported that 10 strains belonging to the *Erythrobacter* clan showed epoxide hydrolase (EHase) activity

toward various epoxide substrates [11], and 3 EHases from *E. litoralis* HTCC2594 showed EHase activities toward various epoxide substrates [39], implying that there are potential epoxide hydrolases in marine microorganisms. Currently, the genome sequencing of over 100 microorganisms is under progress by the Gordon and Betty Moore Foundation, facilitating cloning and characterization. To search and characterize epoxide hydrolases from marine microorganisms, we examined epoxide hydrolase activities in various marine microorganisms whose genome sequence has been determined. Consequently, it was found that *S. alaskensis* displayed hydrolyzing activity toward racemic styrene oxide (Fig. 2). As shown in Fig. 2, the EHase activity increased after 10 h with the maximum activity at 21 h, and then it slowly decreased. The cell growth increased significantly after 8 h, reaching the highest at 21 h, and then entered a stationary phase, indicating that the activity of EHase is well associated with the cell growth.

The above observation indicates that *S. alaskensis* retained epoxide hydrolase activity, thus allowing us to

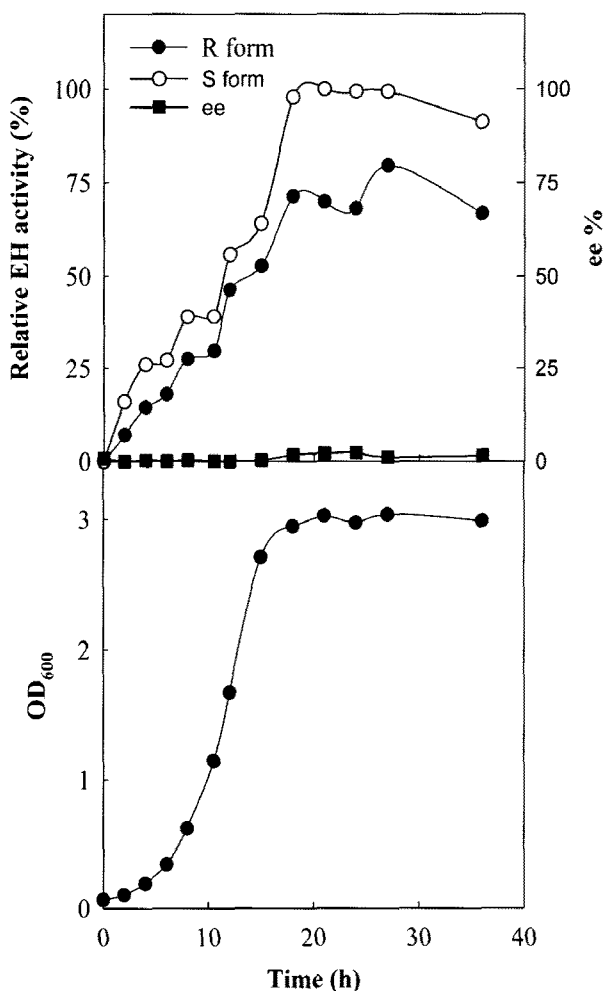


Fig. 2. Time course of cell growth, relative EHase activity, and enantiomeric excess (ee) of *Sphingophyxis alaskensis*. Relative EHase activity was calculated by dividing the value of EHase activity at a given time by that of the best result.

analyze the ORFs of *S. alaskensis* as described in Materials and Methods. Consequently, a putative EHase gene from *S. alaskensis* (42%) was selected, similar to EEH1 from *E. litoralis* HTCC2594. The gene consisted of 1,347 bp (*seh*; GenBank Accession No. YP_615140), encoding 448 amino acids with a molecular mass of 49,030 Da. The *seh* gene showed similarities to microsomal epoxide hydrolases such as human microsomal EHase (mEHase; EPHX1 (*Homo sapiens*), 33%; [30]), Rat mEHase (Ephx1 (*Rattus norvegicus*), 34%; [8]), *Xanthophyllomyces dendrorhous* EHase (Eph1, 28%; [36]), and *Aspergillus niger* EHase (hyl1, 28%; [2]). The sequence analysis of selected ORFs showed that the Sm-X-Nu-X-Sm-Sm motif (GGD²³¹WGA), catalytic triad (Asp²³¹, Glu³⁹⁶, and His⁴²³), and oxyanion hole HGXP (HGW¹⁵⁶P), which are shared in most EHases, were found (Fig. 3). Phylogenetic analysis showed that SEH could be grouped together with microsomal EHases, even

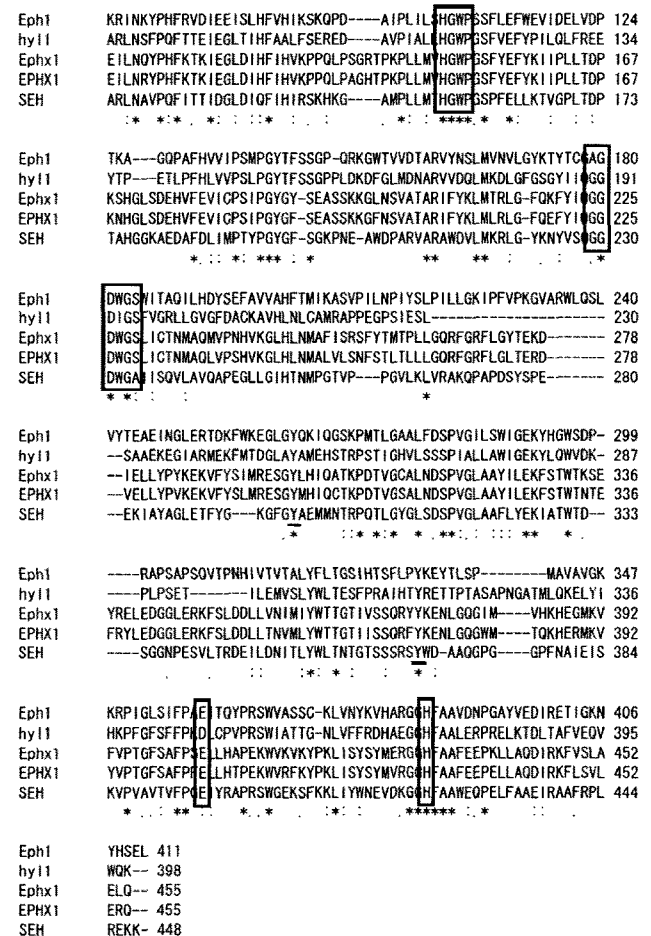


Fig. 3. Sequence alignment of EHases. The protein accession numbers are *Rattus norvegicus* (Ephx1), P07687; *Homo sapiens* (EPHX1), AAH08291; *Xanthophyllomyces dendrorhous* (Eph1), AAF18956; *Aspergillus niger* (hyl1), CAB59813; *Sphingophyxis alaskensis* (SEH, this paper). The identical and conserved residues are highlighted by nucleophilic residue, acidic residue, oxyanion hole, and histidine. Regions of a putative motif are boxed. The amino acid sequence corresponding to the equivalent positions to the two tyrosines of the active site motif is underlined.

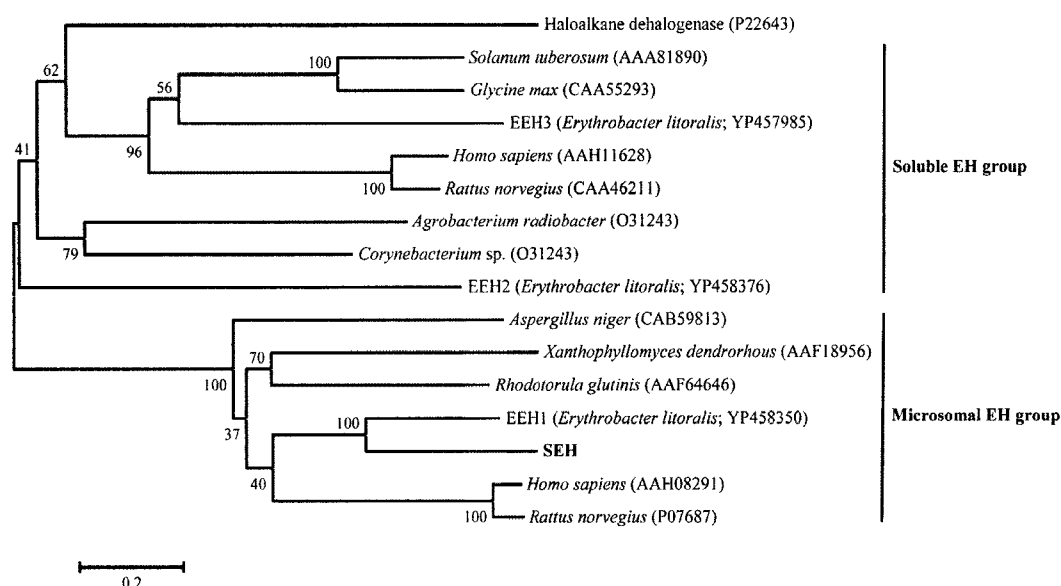


Fig. 4. Phylogenetic analysis of EHases.

Sequence alignment on the desired amino acid sequence was performed using the CLUSTAL W software package. *Rhodotorula glutinis* (EPH1; AAF64646; [37]), *Rattus norvegicus* (Ephx1, Rat mEH; P07687; [8]), *Homo sapiens* (EPHX1, Human mEH; AAH08291; [30]), *Xanthophyllomyces dendrorhous* (Eph1; AAF18956; [36]), *Aspergillus niger* (hyl1; CAB59813; [2]), *Homo sapiens* (EPHX2, Human sEH; AAH11628; [30]), *Rattus norvegicus* (Ephx2, Rat sEH; CAA46211; [15]), *Solanum tuberosum* (pEHSt, potato; sEH; AAA81890; [28]), *Glycine max* (sEHGm, soybean sEH; CAA55293; [1]), *Agrobacterium radiobacter* sEH (O31243; [24]), *Corynebacterium* sp. sEH (O52866; [19]), Haloalkane dehalogenase (P22643; [12]), EEH1 (*Erythrobacter litoralis* HTCC2594; YP458350; [39]), EEH2 (*Erythrobacter litoralis* HTCC2594; YP458376; [39]), EEH3 (*Erythrobacter litoralis* HTCC2594; YP457985; [39]), and SEH (this paper).

though SEH lacked the common membrane anchor found in most members of mEHases (Fig. 4).

Cloning and Expression of the EHase Gene from *S. alaskensis*

To confirm the functionality of the *seh* gene, the full ORFs were amplified by PCR, and the recombinant enzyme

(rSEH) was purified as described above. To facilitate purification of the protein, the *seh* gene was fused in-frame to 6 \times histidine in a T7lac promoter-driven pET system. The recombinant SEH was expressed as a soluble form in the cytosol of *E. coli* (data not shown), and the His-tagged rSEH was purified to an apparent homogeneity by His-tag-affinity chromatography. SDS-PAGE analysis of the purified rSEH showed a single band with an apparent mass of 49 kDa (Fig. 5).

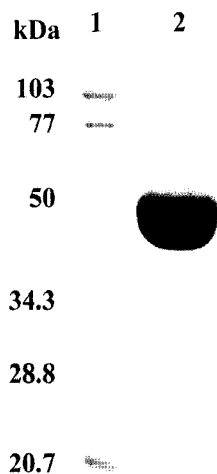


Fig. 5. Purification of the EHase from *Sphingophyxis alaskensis* (the purified SEH).

EHase was purified from recombinant *E. coli* cells by His-tag affinity column chromatography. Lane 1, the protein size standard; lane 2, the purified SEH.

Effects of pH and Temperature on the EHase Activity

The EHase activity of the purified recombinant SEH (rSEH) was determined by measuring the hydrolysis of styrene oxide as a substrate. The purified rSEH could hydrolyze styrene oxide, thus proving the functionality. The effect of pH on the EHase activity was investigated by varying the pH from 4.0 to 10.0. Optimum activity of rSEH toward styrene oxide was observed at pH 6.5 (Fig. 6A). The rSEH was stable at neutral pHs, but unstable below pH 6.0 and above pH 9.0.

The effect of temperature on the activity of the EHase was determined in the range of 4 to 70°C. The hydrolysis rate of rSEH toward styrene oxide was optimal at 35°C. EHase activity increased as the temperature was increased from 4 to 35°C, and then decreased sharply above the optimum temperature (Fig. 6B). The rSEH showed a cold-adapted property, displaying more than 40% activity at low temperature of 10°C compared with the optimum activity.

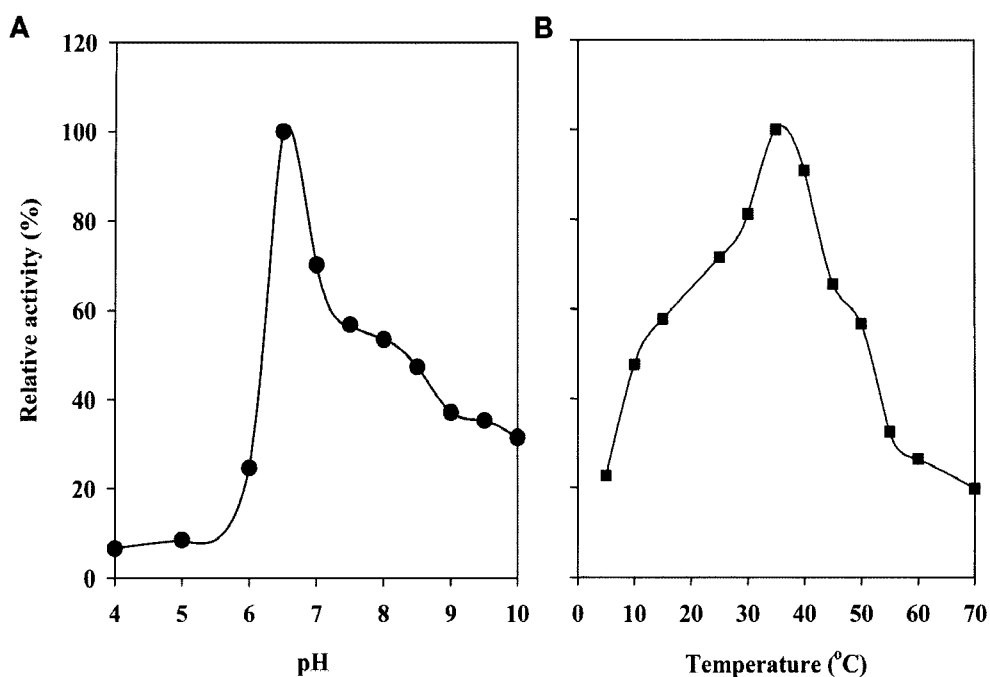


Fig. 6. Effects of pH and temperature on the purified SEH activity.

A. Enzyme activity was determined with 2 mM styrene oxide in 50 mM sodium acetate-acetic acid buffer (pH 4.0 to 6.0), 50 mM MES buffer (pH 6.0 to 7.0), 50 mM Tris-HCl buffer (pH 7.0 to 9.0), and 50 mM glycine buffer (pH 9.0 and 10.0). **B.** Residual enzyme activity was determined after pre-incubation of the enzyme at temperatures between 4 and 70°C in 50 mM Tris-HCl buffer (pH 7.5).

Catalytic Parameters and Substrate Selectivity

To determine the enantioselective hydrolyzing activity of the purified enzyme, the hydrolysis rates of SEH toward (*S*)- or (*R*)-enantiopure styrene oxide, were determined, and kinetic parameters (V_{\max} , K_m , and k_{cat}) were determined by non-linear regression using a Sigma Plot program. The V_{\max}^R and K_m^R of the purified SEH toward (*R*)-styrene oxide were 8.9 $\mu\text{mol}/\text{min}/\text{mg}$ and 4 ± 0.3 mM, respectively, whereas V_{\max}^S and K_m^S of SEH toward (*S*)-styrene oxide were 12.1 $\mu\text{mol}/\text{min}/\text{mg}$ and 5.25 ± 0.3 mM, respectively, indicating that SEH could hydrolyze both (*R*)-styrene oxide and (*S*)-styrene oxide at a similar rate (Table 1). These results were consistent with our previous observation that whole cells of *S. alaskensis* displayed the hydrolyzing activity without enantioselectivity toward racemic styrene oxide (Fig. 2).

Table 2 shows the substrate selectivity of SEH toward various epoxide substrates, depicted in Fig. 1B. The purified SEH could also hydrolyze all the epoxide substrates tested,

Table 1. Kinetic parameter of hydrolysis of (*S*)- and (*R*)-styrene oxides with the SEH.

Enzyme	K_m mM	V_{\max} $\mu\text{mol}/\text{min}/\text{mg}$	k_{cat} s^{-1}	k_{cat}/K_m $\text{mM}^{-1} \text{s}^{-1}$
(S)-enantiomer				
SEH	5.25 ± 0.3	12.1	10.08	1.92
(R)-enantiomer				
SEH	4.00 ± 0.3	8.9	7.42	1.86

even though the enantioselectivity was not observed. The hydrolysis rates of the purified SEH toward GPE, ECH, and EF were higher than those toward SO, EB, and EX.

DISCUSSION

This study reports the characterization of an epoxide hydrolase from a marine bacterium, *S. alaskensis*. The previous report on EHases from *E. litoralis* HTCC2594 strain showed that EEH1 among three epoxide hydrolases belongs to microsomal epoxide hydrolase with high enantioselective hydrolyzing activity toward styrene oxide [39], raising the possibility that the homologous proteins would be a valuable resource to develop enantioselective EHase. By analyzing the whole genome sequence of *S. alaskensis* ([9, 35]; <http://www.moore.org>), we found the *seh* gene encoding a putative epoxide hydrolase, showing similarity to EEH1. The whole cells could hydrolyze styrene oxide, and the activity was closely related to the cell growth (Fig. 2). Unlike *E. litoralis* HTCC2594, however, there were no other genes retaining the typical motif conserved in epoxide hydrolases such as the ring-opening tyrosine, HGXP motif, and Sm-X-Nu-X-Sm-Sm, implying that the presence of the three epoxide hydrolase genes in *E. litoralis* HTCC2594 is not common in marine microorganisms.

The C-terminal fusion of 6 \times histidine was effective in purifying the recombinant protein without affecting the activity. Despite the similarity to EEH1 and other microsomal

Table 2. Enantioselective EHase activity of the SEH toward various epoxide substrates.

Enzyme	Hydrolysis rate ($\times 10^{-2}$) mg/min											
	SO		GPE		EX		EB		ECH		EF	
	(S)	(R)	(S)	(R)	(S)	(R)	(S)	(R)	(S)	(R)	(S)	(R)
SEH	1.16	1.12	7.15	7.59	1.90	1.70	0.09	0.06	9.10	9.24	4.11	5.76

epoxide hydrolases, the purified SEH did not show any enantioselectivity toward various epoxide substrates. Based on a three-dimensional model and comparison with the EHase from *Aspergillus niger* [2, 41], the following mechanism of hydrolysis could be proposed. The residues of the catalytic triad can be positioned with great confidence and Glu108 involved in water activation was found. Tyr231 and Tyr300 appeared in equivalent positions to the two tyrosines of the active sites, assisting in ring opening. The residues, Gln235 (equivalent to His255) and Trp256 (equivalent to Trp276), supporting the two tyrosines could be aligned. It is worthy to note that there was a significant change in the lid region between 6 and 7 beta strands, affecting the active site and substrate binding domain. The swapping experiment of the region between SEH and EHase from *Aspergillus niger* would be informative in understanding the determinants of enantioselective hydrolysis, even though it is not easy by looking at the predicted structure to clearly identify the residues responsible for losing the enantioselectivity in SEH. Nonetheless, the purified SEH showed a cold-adapted property, displaying more than 40% of optimum activity at the low temperature of 10°C. It appears to be quite possible that rSEH could be applied to the hydrolysis of heat-labile epoxides. Screening of a novel EHase in marine microorganisms presented in this study emphasizes the fact that marine microorganisms could provide natural resources of the enzyme with new biophysical properties.

Acknowledgments

This work was supported by the Marine and Extreme Genome Research Center Program, Ministry of Land, Transportation and Marine Affairs, Republic of Korea and a KORDI in-house program (PE98230). We appreciate the Gordon and Betty Moore Foundation for the great effort on the Microbial Genome Sequencing Project (Moore 155).

REFERENCES

- Arahira, M., V. H. Nong, K. Udaka, and C. Fukazawa. 2000. Purification, molecular cloning and ethylene-inducible expression of a soluble-type epoxide hydrolase from soybean (*Glycine max* [L.] Merr.). *Eur. J. Biochem.* **267**: 2649–2657.
- Arand, M., H. Hemmer, H. Durk, J. Baratti, A. Archelas, R. Furstoss, and F. Oesch. 1999. Cloning and molecular characterization of a soluble epoxide hydrolase from *Aspergillus niger* that is related to mammalian microsomal epoxide hydrolase. *Biochem. J.* **344**: 273–280.
- Arand, M., H. Wagner, and F. Oesch. 1996. Asp333, Asp495, and His523 form the catalytic triad of rat soluble epoxide hydrolase. *J. Biol. Chem.* **271**: 4223–4229.
- Archelas, A. and R. Furstoss. 1997. Synthesis of enantiopure epoxides through biocatalytic approaches. *Annu. Rev. Microbiol.* **51**: 491–525.
- Archelas, A. and R. Furstoss. 2001. Synthetic applications of epoxide hydrolases. *Curr. Opin. Chem. Biol.* **5**: 112–119.
- Bhatnagar, T., K. M. Manoj, and J. C. Baratti. 2001. A spectrophotometric method to assay epoxide hydrolase activity. *J. Biochem. Biophys. Methods* **50**: 1–13.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248–254.
- Falany, C. N., P. McQuiddy, and C. B. Kasper. 1987. Structure and organization of the microsomal xenobiotic epoxide hydrolase gene. *J. Biol. Chem.* **262**: 5924–5930.
- Giovannoni, S. J. and U. Stingl. 2005. Molecular diversity and ecology of microbial plankton. *Nature* **437**: 343–348.
- Hwang, S. H., H. J. Hyun, B. J. Lee, Y. S. Park, C. Y. Choi, J. Han, and H. Joo. 2006. Screening from the genome database: Novel epoxide hydrolase from *Caulobacter crescentus*. *J. Microbiol. Biotechnol.* **16**: 32–36.
- Hwang, Y.-O., S. G. Kang, J.-H. Woo, K. K. Kwon, T. Sato, E. Y. Lee, M. S. Han, and S.-J. Kim. 2008. Screening enantioselective epoxide hydrolase activities from marine microorganisms: Detection of activities in *Erythrobacter* spp. *Marine Biotechnol.* (in press).
- Janssen, D. B., F. Pries, J. van der Ploeg, B. Kazemier, P. Terpstra, and B. Witholt. 1989. Cloning of 1,2-dichloroethane degradation genes of *Xanthobacter autotrophicus* GJ10 and expression and sequencing of the *dhlA* gene. *J. Bacteriol.* **171**: 6791–6799.
- Kim, J.-T., S. G. Kang, J.-H. Woo, J.-H. Lee, B. C. Jeong, and S.-J. Kim. 2007. Screening and its potential application of lipolytic activity from a marine environment: Characterization of a novel esterase from *Yarrowia lipolytica* CL180. *Appl. Microbiol. Biotechnol.* **74**: 820–828.
- Kim, Y. J., H. S. Lee, S. S. Bae, J. H. Jeon, J. K. Lim, Y. Cho, et al. 2007. Cloning, purification, and characterization of a new DNA polymerase from a hyperthermophilic archaeon, *Thermococcus* sp. NA1. *J. Microbiol. Biotechnol.* **17**: 1090–1097.
- Knehr, M., H. Thomas, M. Arand, T. Gebel, H. D. Zeller, and F. Oesch. 1993. Isolation and characterization of a cDNA encoding rat liver cytosolic epoxide hydrolase and its functional

- expression in *Escherichia coli*. *J. Biol. Chem.* **268**: 17623–17627.
16. Kumar, S., K. Tamura, and M. Nei. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* **5**: 150–163.
 17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
 18. Lim, J. K., H. S. Lee, Y. J. Kim, S. S. Bae, J. H. Jeon, S. G. Kang, and J.-H. Lee. 2007. Critical factors to high thermostability of an alpha-amylase from hyperthermophilic archaeon *Thermococcus omurineus* NA1. *J. Microbiol. Biotechnol.* **17**: 1242–1248.
 19. Misawa, E., C. K. Chion, I. V. Archer, M. P. Woodland, N. Y. Zhou, S. F. Carter, D. A. Widdowson, and D. J. Leak. 1998. Characterisation of a catabolic epoxide hydrolase from a *Corynebacterium* sp. *Eur. J. Biochem.* **253**: 173–183.
 20. Nardini, M. and B. W. Dijkstra. 1999. α/β hydrolase fold enzymes: The family keeps growing. *Curr. Opin. Struct. Biol.* **9**: 732–737.
 21. Ollis, D. L., E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S. M. Franken, *et al.* 1992. The α/β hydrolase fold. *Protein Eng.* **5**: 197–211.
 22. Oppenheimer, C. H. and C. E. ZoBell. 1952. The growth and viability of sixty-three species of marine bacteria as influenced by hydrostatic pressure. *J. Mar. Res.* **11**: 10–18.
 23. Park, S.-Y., J.-T. Kim, S. G. Kang, J.-H. Woo, J.-H. Lee, H.-T. Choi, and S.-J. Kim. 2007. A new esterase showing similarity to putative dienelactone hydrolase from a strict marine bacterium, *Vibrio* sp. GMD509. *Appl. Microbiol. Biotechnol.* **77**: 107–115.
 24. Rink, R., M. Fennema, M. Smids, U. Dehmel, and D. B. Janssen. 1997. Primary structure and catalytic mechanism of the epoxide hydrolase from *Agrobacterium radiobacter* AD1. *J. Biol. Chem.* **272**: 14650–14657.
 25. Rink, R., J. H. L. Spelberg, R. J. Pieters, J. Kingma, M. Nardini, R. M. Kellogg, B. W. Dijkstra, and D. B. Janssen. 1999. Mutation of tyrosine residues involved in the alkylation half reaction of epoxide hydrolase from *Agrobacterium radiobacter* AD1 results in improved enantioselectivity. *J. Am. Chem. Soc.* **121**: 7417–7418.
 26. Rink, R., J. Kingma, J. H. Spelberg, and D. B. Janssen. 2000. Tyrosine residues serve as proton donor in the catalytic mechanism of epoxide hydrolase from *Agrobacterium radiobacter*. *Biochemistry* **39**: 5600–5613.
 27. Sambrook, J. and D. W. Russell. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd Ed. Vol 2, pp. 8.65–8.71. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 28. Stapleton, A., J. K. Beetham, F. Pinot, J. E. Garbarino, D. R. Rockhold, M. Friedman, B. D. Hammock, and W. R. Belknap. 1994. Cloning and expression of soluble epoxide hydrolase from potato. *Plant J.* **6**: 251–258.
 29. Straathof, A. J. J. and J. A. Jongejan. 1997. The enantiomeric ratio: Origin, determination and prediction. *Enzyme Microb. Tech.* **21**: 559–571.
 30. Strausberg, R. L., E. A. Feingold, L. H. Grouse, J. G. Derge, R. D. Klausner, F. S. Collins, *et al.* 2002. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. *Proc. Natl. Acad. Sci. USA* **99**: 16899–16903.
 31. Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673–4680.
 32. Tokunaga, M., J. F. Larrow, F. Kakiuchi, and E. N. Jacobsen. 1997. Asymmetric catalysis with water: Efficient kinetic resolution of terminal epoxides by means of catalytic hydrolysis. *Science* **277**: 936–938.
 33. van Loo, B., J. Kingma, M. Arand, M. G. Wubbolts, and D. B. Janssen. 2006. Diversity and biocatalytic potential of epoxide hydrolases identified by genome analysis. *Appl. Environ. Microbiol.* **72**: 2905–2917.
 34. van Loo, B., J. H. L. Spelberg, J. Kingma, T. Sonke, M. G. Wubbolts, and D. B. Janssen. 2004. Directed evolution of epoxide hydrolase from *A. radiobacter* toward higher enantioselectivity by error-prone PCR and DNA shuffling. *Chem. Biol.* **11**: 981–990.
 35. Venter, J. C., K. Remington, J. F. Heidelberg, A. L. Halpern, D. Rusch, J. A. Eisen, *et al.* 2004. Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**: 66–74.
 36. Visser, H., J. A. M. de Bont, and J. C. Verdoes. 1999. Isolation and characterization of the epoxide hydrolase-encoding gene from *Xanthophyllomyces dendrorhous*. *Appl. Environ. Microbiol.* **65**: 5459–5463.
 37. Visser, H., S. Vreugdenhil, J. A. M. de Bont, and J. C. Verdoes. 2000. Cloning and characterization of an epoxide hydrolase-encoding gene from *Rhodotorula glutinis*. *Appl. Microbiol. Biotechnol.* **53**: 415–419.
 38. Weijers, C. A. G. M. and J. A. M. de Bont. 1999. Epoxide hydrolases from yeasts and other sources: Versatile tools in biocatalysis. *J. Mol. Catal. B Enzym.* **6**: 199–214.
 39. Woo, J.-H., O.-K. Hwang, S. G. Kang, H. S. Lee, J. Cho, and S.-J. Kim. 2007. Cloning and characterization of three novel epoxide hydrolases from a marine bacterium, *Erythrobacter litoralis* HTCC2594. *Appl. Microbiol. Biotechnol.* **76**: 365–375.
 40. Yamada, T., C. Morisseau, J. E. Maxwell, M. A. Argiriadi, D. W. Christianson, and B. D. Hammock. 2000. Biochemical evidence for the involvement of tyrosine in epoxide activation during the catalytic cycle of epoxide hydrolase. *J. Biol. Chem.* **275**: 23082–23088.
 41. Zou, J., B. M. Hallberg, T. Bergfors, F. Oesch, M. Arand, S. L. Mowbray, and T. A. Jones. 2000. Structure of *Aspergillus niger* epoxide hydrolase at 1.8 Å resolution: Implications for the structure and function of the mammalian microsomal class of epoxide hydrolases. *Structure* **15**: 111–122.