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Biocatalytic resolution of glycidyl phenyl ether using a novel epoxide hydrolase from a marine bacterium, *Rhodobacterales bacterium* HTCC2654

Jung-Hee Woo,[1,3] Ji-Hyun Kang,[1] Young-Ok Hwang,[1] Jang-Cheon Cho,[2] Sang-Jin Kim,[1] and Sung Gyun Kang[1,*]

From the Marine Biotechnology Research Centre, Korea Ocean Research and Development Institute, Ansan, P. O. Box 29, 425-600, Korea[1] Division of Biology and Ocean Sciences, Inha University, Incheon 402-751, Korea[2] and Gyeongbuk Institute for Marine Bio-Industry, Uljin 767-813, Gyeongbuk, Korea[3]

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As a continuous effort of developing highly enantioselective epoxide hydrolase from marine microorganisms, it was found that *Rhodobacterales bacterium* HTCC2654 was highly enantioselective toward racemic glycidyl phenyl ether (GPE). An open reading frame (ORF) encoding a putative epoxide hydrolase (EHase) was cloned from the genome of *R. bacterium* HTCC2654, followed by expression and purification in *Escherichia coli*. The purified EHase (REH) hydrolyzed (*S*)-GPE preferentially over (*R*)-GPE. Enantiopure (*R*)-GPE from kinetic resolution of 29.2 mM racemic GPE using the purified REH could be obtained with enantiopurity of more than 99.9% enantiomeric excess (ee) and 38.4% yield (theoretical, 50%) within 20 min (enantiomeric ratio (*E*-value): 38.4). The enantioselective activity of REH toward GPE was also confirmed by the analysis of the vicinal diol, 3-phenoxy-1,2-propanediol. To our knowledge, this study demonstrates the highest enantioselective resolution of racemic GPE using a purified biocatalyst among the known native EHases.

[**Key words:** Glycidyl phenyl ether; Enantioselective; Epoxide hydrolase; Marine microorganism; Kinetic resolution]

Enantiopure epoxides and vicinal diols are versatile synthetic intermediates for the preparation of enantiopure bioactive compounds (1, 2). Aryl epoxide and glycidyl phenyl ether (GPE) is a potentially useful intermediate for the synthesis of chiral amino alcohols (3) and bioactive compounds such as β-blockers (4). One of the most promising ways for preparing such chiral synthons under environmentally gentle conditions is the enantioselective hydrolysis of racemic epoxides using cofactor-independent epoxide hydrolase (EHase; EC 3.3.2.3) (5, 6). EHases are ubiquitous enzymes that have been isolated from a wide variety of sources such bacteria, yeast, fungi, insect, plant and mammalian (6, 7). Due to the potential application in the production of enantiopure epoxides by kinetic resolution of enantioselective EHase, several EHases have been developed (8-10). However, the limited number of enantioselective EHase demands studies to explore new enantioselective EHase for the production of enantiopure epoxides in pharmaceutical industries.

Oceans cover more than three-quarters of the earth's surface and so offer abundant resources for biotechnological research and development. Marine organisms represent a dramatically different environment for biosynthesis than do 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 (11, 12). Furthermore, the genomic sequencing of marine microorganisms mostly made by Moore foundation can facilitate a rapid cloning and overexpression for the characterization of a putative or possible EHase originated from marine environment as recent report on the screening of various genomic databases for EHases of the α/β hydrolase fold family (13).

To develop enantioselective biocatalysts from various marine environments, we have been searching for EHase activities from various marine environments by the combination of activity screening, conventional molecular engineering and genomic approach (14-16). In the present study, we characterized of a highly enantioselective EHase from *Rhodobacterales bacterium* HTCC2654. A putative EHase gene (*reh*) from *R. bacterium* HTCC2654 was cloned and expressed in *Escherichia coli*. The recombinant protein (REH) was purified by metal affinity chromatography, and the kinetic parameters of the purified REH toward GPE (Fig. 1) were further characterized.

MATERIALS AND METHODS

Materials Racemic GPE was purchased from Aldrich. Racemic 3-phenoxy-1,2-propanediol (PPD) was purchased from Acros Organics (Morris Plains, NJ, USA). All materials were of analytical or of reagent grade. The chiraldex gamma-cyclodextrin trifluoroacetyl (G-TA, 0.25 mm ID, 30 m length) gas chromatography (GC) columns

* Corresponding author. Tel.: +82 31 400 6241; fax: +82 31 406 2495.
E-mail address: sgkang@kordi.re.kr (S.G. Kang).

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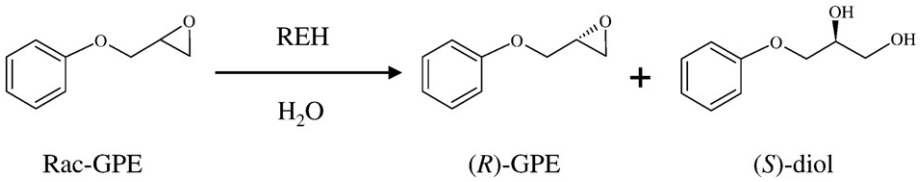

FIG. 1. The enantioselective hydrolysis of the purified REH from *Rhodobacterales bacterium* HTCC2654 toward racemic GPE.

were purchased from Supelco (Bellefonte, PA, USA). The Chiralcel OD-H (4.6 mm ID, 150 mm length) high-performance liquid chromatography (HPLC) column was purchased from Diacel Chemical industries (Tokyo, Japan). Other medium components were purchased from Merck and Difco.

Strains and growth conditions *R. bacterium* HTCC2654 was cultured at 25 °C in marine broth (Difco) for 2 days. For the storage, the bacterial cells were suspended in marine broth with 10% (v/v) glycerol and stored at −80 °C until used.

E. coli DH5α and *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene, LaJolla, CA, USA) 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 (17). Restriction enzymes and other modifying enzymes were purchased from Promega (Madison, WI, USA). 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, USA).

BLAST search and multiple sequence alignments To clone an EHase from *R. bacterium* HTCC2654, sequence searches (Sm-X-Nu-X-Sm-Sm motif and H-G-X-P) against ORFs of *R. bacterium* HTCC2654 whose genome sequence was determined by Moore foundation were performed using the ProteinFinder program of Ensoltek and the basic local alignment search tool (BLAST) program. The pairwise comparison of candidate EHase and reported EHases were performed with the CLUSTAL W program (18). The resulting candidates were manually confirmed for the presence of the putative EHase active-site residues. Sequences that contained ring-opening tyrosine, HGXP motif and Sm-X-Nu-X-Sm-Sm (Sm = small residue, X = any residue and Nu = nucleophile) motif were selected and aligned together with the known EHase sequences.

For phylogenetic analysis, EHase sequences were retrieved from SwissProt or EMBL protein database and analyzed. 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, USA) (19).

Cloning of *reh* gene from *R. bacterium* HTCC2654 Genomic DNA of *R. bacterium* HTCC2654 was isolated using the Genomic DNA extraction kit (Promega) following the manufacturer's instructions. The full-length of *reh* gene flanked by *Nde*I and *Xho*I sites was amplified by a PCR with the forward primer (rehF: 5′-CGACCCGG*CATATG*AACGACAAGACCTTTATCGAGACGAAC GGC-3′) and reverse primer (rehRH: 5′-CTCCACAT*CTCGAG*TTACAAGGCT GAAAAGAACACTCGCAAATC-3′). The underlined sequences indicate *Nde*I site in the forward primer and *Xho*I site in the reverse primer. For the expression of *reh* without His-tag, the reverse primer (rehRNH: 5′-CTCCACAT*CTCGAG*TCAAAG CGTGGCGAGCCAGTCGATGA-3′) was also designed. The amplified DNA fragment was digested with *Nde*I and *Xho*I, the fragment was ligated to *Nde*I/*Xho*I-digested plasmid pET-24a (+) and then the recombinant plasmid was used to transform *E. coli* DH5α. The recombinant plasmid was introduced into BL21-CodonPlus (DE3)-RP (Novagen) for expression after sequence confirmation.

Purification of REH from *R. bacterium* HTCC2654 A transformant was cultivated at 37 °C and overexpression was induced at 37 °C by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the optical density at 600 nm reached 0.4–0.6. After induction for 3 h, the cells were harvested by centrifugation at 5000×*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×*g* for 30 min, with a His·Bind Purification Kit (Novagen). The soluble fraction was applied to a Ni-nitrilotriacetic (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 (20). The protein concentration was measured by the method of Bradford (21) using the Bio-Rad protein assay kit with bovine serum albumin as a standard.

EHase assay Enantioselective EHase activity was measured according to Woo et al. (15) with slight modification. Purified EHase (100 μl) was mixed with racemic GPE (29.2 mM) in a 10-ml vial containing 1 ml of 100 mM Tris–HCl (pH 8.0) and then incubated at 25 °C. During incubation, the samples were withdrawn periodically, and the harvested mixtures were extracted with hexane (2 ml). The resulting extracts were analyzed on a chiraldex G-TA column using a GC system equipped with FID detector (Varian, Inc., Netherlands). The peak area corresponding to (*R*)-GPE or (*S*)-GPE was normalized by the peak area of mesitylene used as an internal standard and then converted to a residual amount of GPE in comparison with standard curve. The temperatures of oven, injector and detector in GC analysis for racemic GPE ((*S*)-GPE, t_r = 39 min; (*R*)-GPE, t_r = 40 min) were 90, 180 and 180 °C, respectively. Helium gas was used as carrier gas.

The absolute configuration of the vicinal diol of GPE, 3-phenoxy-1,2-propanediol, was also analyzed by chiral HPLC analysis as followed. Purified EHase (100 μl) was mixed with racemic GPE at a concentration of 29.2 mM in a 10-ml vial containing 1 ml of 100 mM Tris–HCl (pH 8.0) and then incubated at 25 °C. The samples were withdrawn periodically during incubation and then the reaction mixtures (500 μl) were extracted with diethyl ether (500 μl). Then the resulting extracts were analyzed on a chiralcel OD-H column with hexane:2-propanol = 9:1 as the mobile phase at a flow rate of 1 ml/min using a HPLC system (Hewlett-Packard, Avondale, PA, USA). The peak area corresponding to (*R*)- or (*S*)-3-phenoxy-1,2-propanediol (PPD, (*R*)-PPD, t_r = 10.5 min; (*S*)-PPD, t_r = 21.4 min) was converted to concentration in comparison with standard curve (22).

Determination of kinetic parameters Kinetic parameters of REH were determined by GC analysis using racemic GPE as a substrate. Purified EHase (100 μl) was mixed with various concentrations of racemic GPE in a 10-ml vial containing 1 ml of the 100 mM Tris–HCl (pH 8.0) and incubated at 25 °C with shaking at 200 rpm. The reaction mixtures were withdrawn periodically. The residual epoxides were analyzed with GC after extraction with hexane or the resulting diols were also analyzed with HPLC after extraction with diethyl ether. The enantiomeric excess (ee) was derived from remaining epoxide of the two enantiomers [ee (%) = $(S-R)/(S+R)\times 100$] and the extent of conversion (c) when terminating EHase assays before the complete consumption of epoxide [$c = \{1-(E^R\mathrm{s}+E^S\mathrm{s})/(E^R\mathrm{so}+E^S\mathrm{so})\}$], where the initial epoxide of (*R*) and (*S*) was denoted as *E*so, and the remaining epoxide of (*R*) and (*S*) was as *E*s (23). According to the method described by Chen et al. (24), the enantiomeric ratio (E) was derived from the extent of conversion (c) and the enantiomeric excess of the remaining enantiomer of the substrate (ee_s) [$E = \ln\{(1-c)(1-ee_s)\}/\ln\{(1-c)(1+ee_s)\}$]. Kinetic parameters were estimated by non-linear regression using a Sigma Plot program.

RESULTS AND DISCUSSION

Identification of the EHase gene from *R. bacterium* HTCC2654 Previously, we reported that EHases from marine microorganisms retained enantioselective activities (14-16), demonstrating an example that marine microorganisms can be possibly abundant reservoirs for developing valuable biocatalysts. Further, the recent achievement in genome sequencing of over 100 marine microorganisms is facilitating rapid cloning and characterization of putative EHases from marine microorganisms. As a continuous effort of developing enantioselective epoxide hydrolase from marine microorganisms whose genome sequences are available in public, we found an ORF in *R. bacterium* HTCC2654, showing very low similarity to known EHases, i.e. 26% identity to EEH3 (15) from *Erythrobacter litoralis* HTCC2594. The gene consisted of 963 bp (*reh*; GenBank accession number ZP_01014743), encoding 320 amino acids with molecular weight of 35,757 Da. The sequence analysis revealed that GHD^{99}WGA motif, catalytic triad (Asp^{99}, Asp^{267} and His^{299}) and oxyanion hole HGXP (HGF^{31}P) shared in most of EHases could be found in the ORF (Fig. 2) (25-29). The whole-cell extract of *R. bacterium* HTCC2654 displayed EHase activity toward GPE, indicative of the presence of an EHase gene in the genome (Fig. 3). The activity was closely associated with the cell growth (Fig. 3) and highly enantioselective to (*S*)-

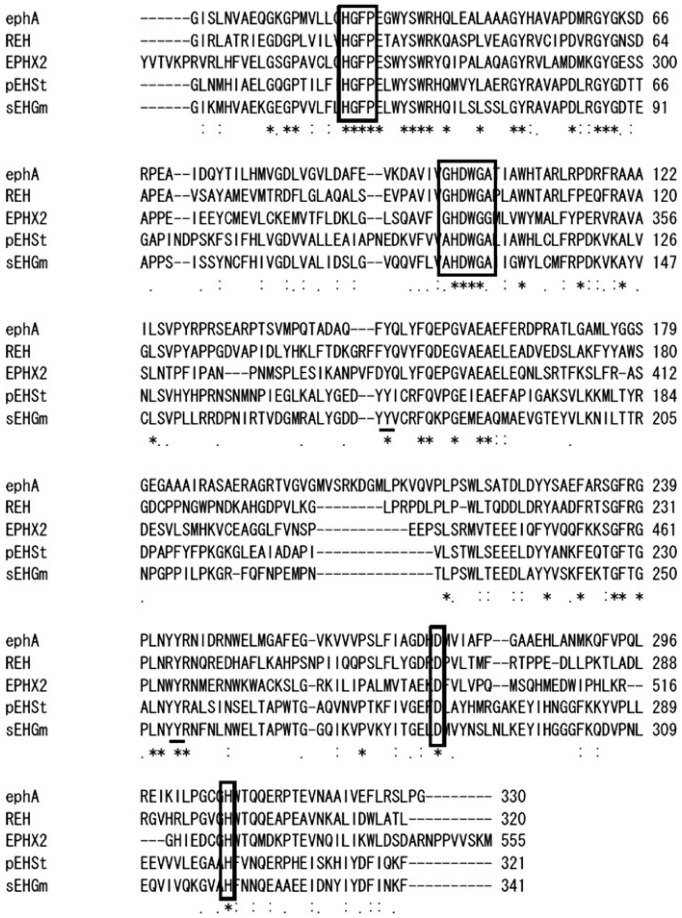

FIG. 2. Sequence alignment of EHases with REH. The protein accession numbers are *Homo sapiens* (EPHX2, Human sEH), AAH11628; *Solanum tuberosum* (pEHSt, potato; sEH), AAA81890; *Glycine max* (sEHGm, soybean sEH), CAA55293; *Bradyrhizobium japonicum* (ephA), BAC46379) and *Rhodobacterales bacterium* HTCC2654 (REH, this paper). Identical residues are marked with asterisks and residues with conserved substitutions and semiconserved substitutions are marked with colons and dots, respectively. Catalytic triad and oxyanion hole are boxed. The amino acid sequence corresponding to the equivalent positions to the two tyrosines of active-site motif is underlined.

GPE (data not shown). Since only a putative EHase was found in *R. bacterium* HTCC2654 genome through the bioinformatic analysis, the EHase seemed likely to be responsible for the enantioselective activity toward GPE. The phylogenetic analysis revealed that REH was similar mostly to eukaryotic soluble EHases (sEHase) such as human sEHase (EPHX2, 19%) and plant sEHase (pEHSt, 29%; sEHGm, 27%), grouped together with sEHases of eukaryotic origin (Fig. 4).

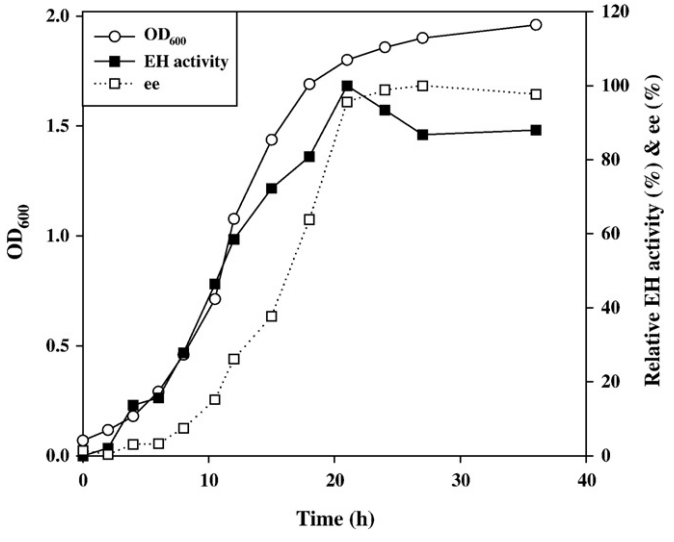

FIG. 3. Time course of the cell growth, relative EHase activity and ee of *Rhodobacterales bacterium* HTCC2654. Relative EHase activity was calculated by dividing the value of the EHase activity at the given time by that of the best result.

Kinetic resolution of racemic GPE by the purified REH To check the enantioselective hydrolysis of REH toward GPE, the *reh* gene was amplified by PCR, and the recombinant enzyme (rREH) was purified as described above. To facilitate the rapid purification, the His-tagged rREH could be purified to an apparent homogeneity by His-tag-affinity chromatography. SDS–PAGE analysis of the purified rREH showed a single band with an apparent mass of 35 kDa (Fig. 5).

Enantioselective hydrolysis of 29.2 mM racemic GPE using the purified REH (20 μg) was carried out at pH 8.0 and 25 °C. As a result, enantiopure (*R*)-GPE with high optical purity (ee, >99.9%) could be readily obtained (Fig. 6A). The hydrolysis rate of (*S*)-GPE was much faster than that of (*R*)-GPE, and the enantiopurity of the remaining (*R*)-GPE increased from 0% to >99.9% ee within 20 min. Final yield of enantiopure (*R*)-GPE was 38.4% (theoretical yield = 50%), and the enantiomeric ratio (*E*-value) was 38.4 for racemic GPE. The enantioselectivity of REH toward (*R*)-GPE was maintained through the reactions with up to 100 mM GPE even if the time to reach ee >99.9% at above 50 mM concentration of GPE was delayed (Table 1), possibly caused by substrate or product inhibition at higher concentration of GPE.

The kinetic resolution of racemic GPE was also confirmed by analyzing the vicinal diol, (*R*)- and (*S*)-PPD as described in materials

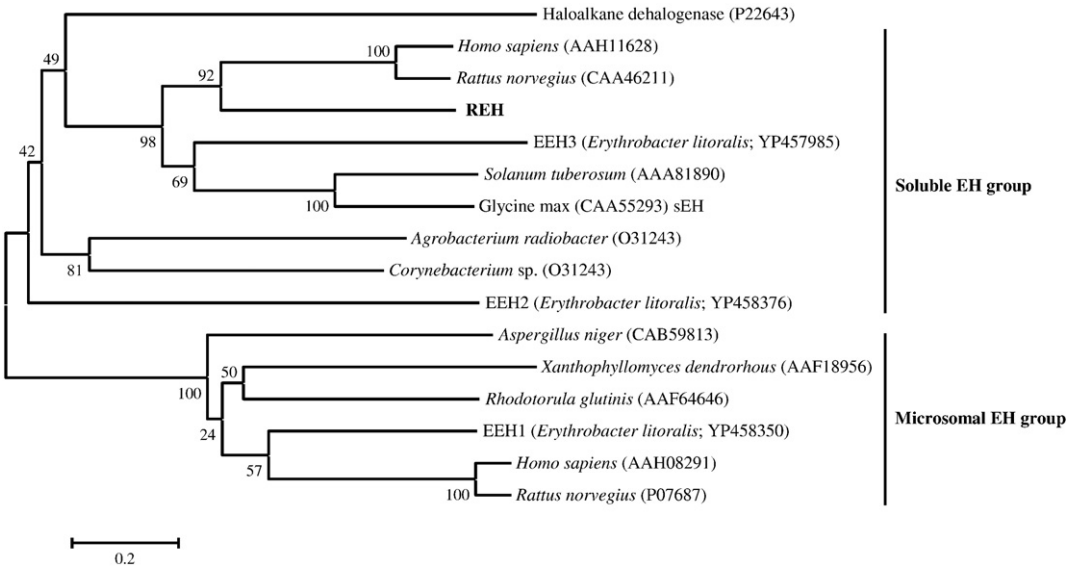

FIG. 4. Phylogenetic analysis of EHases : *Rhodotorula glutinis* (EPH1; AAF64646; (30)), *Rattus norvegicus* (Ephx1, Rat mEH; P07687; (31)), *Homo sapiens* (EPHX1, Human mEH; AAH08291; (29)), *Xanthophyllomyces dendrorhous* (Eph1; AAF18956; (32)), *Aspergillus niger* (hyl1; CAB59813; (33)), *H. sapiens* (EPHX2, Human sEH; AAH11628; (29)), *Rattus norvegicus* (Ephx2, Rat sEH; CAA46211; (27)), *Solanum tuberosum* (pEHSt, potato; sEH; AAA81890; (28)), *Glycine max* (sEHGm, soybean sEH; CAA55293; (25)), *Agrobacterium radiobacter* sEH (O31243; (34)), *Corynebacterium* sp. sEH (O52866; (35)), Haloalkane dehalogenase (P22643; (36)), EEH1 (*Erythrobacter litoralis* HTCC2594; YP458350; (15)), EEH2 (*E. litoralis* HTCC2594; YP458376; (15)), EEH3 (*E. litoralis* HTCC2594; YP457985; (15)) and REH (this paper).

and methods (Fig. 6B). As the reaction time elapsed, the amount of (*R*)- and (*S*)-PPD increased, and it was note to worthy that the increase rate of (*S*)-PPD was faster than that of (*R*)-PPD. Based on the vicinal diol configuration derived from (*S*)-GPE, it was assumed that REH might attack C-2 carbon for the ring opening with the retention of stereoisomeric configuration. As reported by Weijers and de Bont (7), the preferable carbon in epoxide ring seems to vary among EHases. The further study on the mechanism for the preference in REH would provide information on determining factors for the enantioselectivity of EHases.

Taken together, REH from *R. bacterium* HTCC2654 was highly enantioselective and very effective in GPE kinetic resolution. To our knowledge, it is the highest enantioselectivity among the known native EHases for the enantioselective hydrolysis of racemic GPE (Table 1).

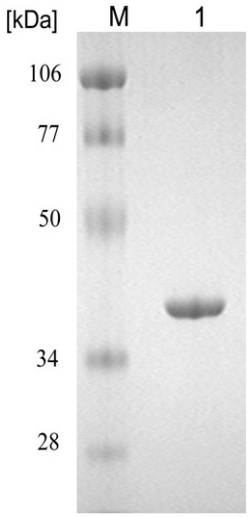

FIG. 5. Purification of REH from *Rhodobacterales bacterium* HTCC2654. M, the protein size standard; 1, the purified REH.

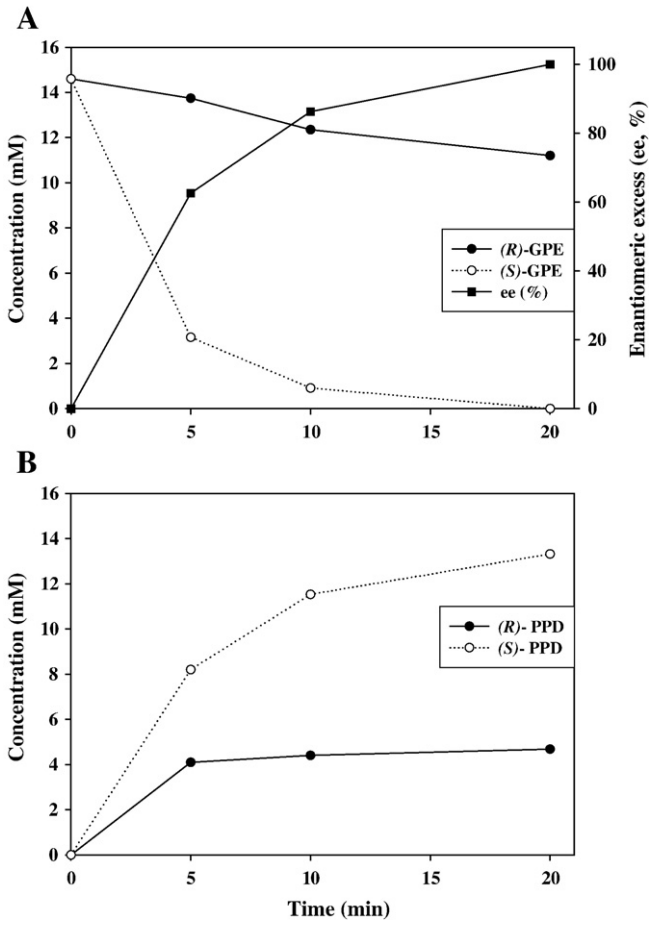

FIG. 6. Kinetic resolution of racemic GPE by the purified REH. (A) Enantioselective resolution of 29.2 mM racemic GPE by the purified REH on a chiral GC column. (B) Representative for the formation of (*R*)- and (*S*)-PPD from 29.2 mM racemic GPE catalyzed by the purified REH on a chiral HPLC column.

TABLE 1. The comparison of kinetic resolution of EHases and the purified REH toward GPE.

Epoxide hydrolase	Catalytic form	Catalytic concentration (g/L)	GPE concentration (mM)	Temperature (°C)	pH	Time (min)	ee (%)/absolute configuration	Yield (%)	*E*	Reference
Aspergillus niger	Cells	75[a]	20	27	7.5	240	100/(*R*)	26	n.i.[b]	(37)
Agrobacterium radiobacter	Enzyme	0.025–0.125	1	30	9.0	n.i.	>99/(*R*)	28	12	(22)
Bacillus megaterium ECU1001	Cells	9.1[c]	60	35	7.5	1800	100/(*S*)	25.6	47.8	(38)
B. megaterium ECU1001	Cells	7[c]	75	30	8.0	1440	100/(*S*)	n.i.	39.5	(8)
Trichosporon loubierii	Cells	50[a]	67	30	8.0	270	>99/(*R*)	35	20	(39)
Rhodobacterales bacterium HTCC2654	Enzyme (REH)	0.02	29.2	25	8.0	20	100/(*R*)	38.4	38.4[d]	This study
	Enzyme (REH)	0.02	58.4	25	8.0	100	99/(*R*)	38.2	37.8[d]	This study
	Enzyme (REH)	0.02	80.0	25	8.0	200	99/(*R*)	33.7	26.1[d]	This study

[a] Wet cell weight.
[b] No information.
[c] Dry cell weight.
[d] Calculated with an ee of 99%.

Kinetic parameters of REH To understand the reason why REH is highly enantioselective toward GPE, kinetic parameters (V_{max}, K_m and k_{cat}) of REH toward (*S*)- or (*R*)-enantiopure GPE were determined (Table 2). V^S_{max} and K^S_m of the purified REH toward (*S*)-GPE were 135.1 μmol/min/mg and 10.9 ± 0.1 mM, respectively, while V^R_{max} and K^R_m of REH toward (*R*)-GPE were 89.3 μmol/min/mg and 61.6 ± 0.1 mM, respectively (Table 2). The calculated ratio of specificity constants (k_{cat}/K_m) of both enantiomers was 8.5, consistent with the observation of high enantioselectivity of the enzyme toward the (*R*)-GPE. It seemed that the preferred substrate binding of (*S*)-GPE over (*R*)-GPE to REH was the major contributor to high enantioselectivity although the faster hydrolyzing rate toward (*S*)-GPE was also contributing.

In this paper, we characterized a novel EHase (REH) from a marine microorganism, *R. bacterium* HTCC2654. *R. bacterium* HTCC2654 displayed highly enantioselective-hydrolyzing activity toward (*S*)-GPE. In an attempt to find a candidate gene responsible for the activity, we found a putative EHase in *R. bacterium* HTCC2654 (40). Despite the low similarity to EHases, the comparative analysis of REH with an EHase from *Agrobacterium radiobacter* (34) allowed us to list REH as a candidate (22, 41, 42). For example, catalytic triad (Asp99, Asp267 and His299) and two tyrosines assisting in the opening of the epoxide ring (Tyr150 and Tyr236) could be positioned (43-45). Based on the biochemical analysis of the purified REH, the enantioselectivity and yield of REH was mainly contributed by higher affinity and catalytic constant toward the (*S*)-GPE. The structural analysis of REH will allow us to understand the mechanism of highly enantioselective hydrolysis of REH, providing information to design even better biocatalyst suitable for kinetic resolution.

This study presents the first example which discovered a highly enantioselective EHase from a marine bacterium, *R. bacterium* HTCC2654. Applying REH to kinetic resolution of 29.2 mM racemic GPE, enantiopure (*R*)-GPE could be obtained with more than 99.9% ee and yield of 38.4% (theoretical, 50%) within 20 min (enantiomeric ratio (*E*-value) = 38.4). To our knowledge, this study demonstrates the highest enantioselective resolution of racemic GPE using a purified biocatalyst among the known native EHases. These features make it a very attractive enzyme for potential application as a good biocatalyst for the preparation of enantiopure epoxides or diols.

TABLE 2. Kinetic parameters of the purified REH toward racemic GPE.

REH toward	K_m	V_{max}	k_{cat}	k_{cat}/K_m
	mM	μmol/min/mg	s^{-1}	$mM^{-1}\ s^{-1}$
(S)-GPE	10.9 ± 0.1	135.1	78.81	7.23
(R)-GPE	61.6 ± 0.1	89.3	52.09	0.85

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