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Screening from the Genome Databases: Novel Epoxide Hydrolase from *Caulobacter crescentus*

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Abstract The genome sequences from several microbes have led to the discovery of numerous open reading frames of unknown functionality. The putative bacterial epoxide hydrolase (EH) genes selected from the genome databases were examined for their activities toward various epoxides. Among the nine open reading frames (ORFs) from four microbial species, the ORF from *Caulobacter crescentus* showed an epoxide hydrolase activity. The kinetic resolution, using *C. crescentus* EH (CCEH) of the aryl epoxides such as styrene oxide, could be performed more efficiently than short aliphatic epoxides. The resolution of racemic indene oxide, which could previously be resolved only by fungal epoxide hydrolases, was effectively accomplished by CCEH.

Key words: Epoxide hydrolase, *Caulobacter crescentus*, kinetic resolution, styrene oxide, indene oxide

Enantiopure epoxides are versatile chiral synthons in organic synthesis. They are valuable chiral building blocks in the preparation of more complex enantiopure bioactive compounds such as pharmaceuticals and agrochemicals [5]. Chemical methods for producing epoxides are often specific only for a narrow range of substrates and require environmentally hazardous catalytic compounds such as heavy metals. In biological production routes, enantioselective hydrolysis of racemic epoxides by the use of epoxide hydrolases (EHs; EC 3.3.2.3) may be commercially feasible, because it is possible to obtain enantiopure epoxides with high enantiomeric purities from relatively cheap and readily available racemic epoxides.

EHs are cofactor-independent enzymes that can convert epoxides to their corresponding diols by addition of a water molecule to the oxirane ring. The EHs from mammalian sources have been extensively studied because of their involvement in the metabolism of toxic xenobiotics [6]. The potential of mammalian EHs as chiral biocatalysts on a preparative scale is limited because of the low availability of these enzymes. Several EHs from microbial sources have recently been discovered. High enantioselectivity was obtained with the hydrolysis of styrene oxide using fungal cells [4, 10] and with a broad variety of aryl and aliphatic epoxides with yeast *Rhodotorula glutinis* [14]. During the last few years, enantioselective bacterial EHs have been detected in *Rhodococcus* sp. [7], *Norcardia* sp. [9], *Corynebacterium* sp. [3], *Agrobacterium* sp. [11], and some other genera. Biotransformations of epoxides on a preparative scale are hampered by the limited supply of EHs. Bacterial enzymes are more easily expressed in *E. coli* than mammalian and fungal enzymes. This heterologous expression leads to an unlimited supply of these enzymes for biotransformation applications. Recently, recombinant EHs from yeast [13] and bacteria [11] have been reported. With the genome sequences from some microbes determined in the genome projects, many functionally unknown open reading frames that have many sequence similarities with EHs have been found. Because of the structures of mammalian, fungal, and microbial EHs unveiled during the past few decades [2, 8, 16], it is now known that the EHs contains the α/β hydrolase fold domains, which is also found in haloalkane dehydrogenases, esterases, and carboxypeptidases [1]. The EHs shared a weak but significant sequence similarity to each other, leading the conclusion that the EHs share the same overall tertiary structure.

In this paper, we searched for probable bacterial EH genes, which were annotated by sequence homology, from the genome databases and examined their activities toward various epoxides.

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MATERIALS AND METHODS

Materials

All DNA modifying enzymes were purchased from New England Biolabs (Hertfordshire, U.K.) unless otherwise indicated. Styrene oxide, epichlorohydrin, glycidol, 1,2-epoxy-3-phenoxypropane, and antibiotics were from Sigma-Aldrich (MO, U.S.A.). Indene oxide was synthesized from trans-2-bromo-1-indanol, according to published reference [10]. All other chemicals were of analytical or of reagent grade. Wild-type *C. crescentus* (KCTC 3405) was purchased from Korean collection for type cultures.

Analytical Methods

Gas chromatography (GC) was performed on a Hewlett-Packard 6890 series GC system equipped with a flame ionization detector. The enantiomeric purities of glycidol and 1,2-epoxy-3-phenoxypropane were determined by chiral GC using a fused silica cyclodextrin capillary β-DEX 120 column (30 m length, 0.25 mm ID, and 0.25 μm film thickness, Supelco Inc.), and the enantiomeric purities of styrene oxide and epichlorohydrin was determined by chiral GC using a β-DEX 250 column (60 m length, 0.25 mm ID, and 0.25 μm film thickness, Supelco Inc.). The enantiomeric purity of indene oxide was determined by chiral GC using an α-DEX 120 column (30 m length, 0.25 mm ID, and 0.25 μm film thickness, Supelco Inc.). Absolute configuration was determined, based on published reference [4].

Cloning of Probable EH Genes

To amplify 9 open reading frames from 4 microbial sources in the chromosomal DNA of the *Mesorhizobium loti*, *Bacillus subtilis*, *Agrobacterium tumefaciens*, *Caulobacter crescentus*, and *Agrobacterium radiobacter* EH (AREH) gene from pEH20 [11], PCR was performed using the primers summarized in Table 1. PCR amplifications were carried out for 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, with a final extension stage at 72°C for 7 min using *Pfu* polymerase (Genenmed, Korea). The fragments were digested with appropriate restriction enzymes and ligated into the IPTG-inducible expression vector, pET28b.

Expression and Analysis of Recombinant EHs

The plasmids were introduced into *E. coli* BL21(DE3), and the transformants were grown in Luria-Bertani (LB) broth containing 50 mg/ml of kanamycin at 37°C. *E. coli* BL21(DE3) harboring a putative EH gene was grown in 1 l broth containing 50 mg/ml of kanamycin at 37°C. When OD_{600} reached 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cell broth. After induction, the cells were cultured overnight at 20°C. The cells harvested from 1 l of culture broth of recombinants *E. coli* BL21(DE3) by centrifugation (1,000 ×*g*, 10 min, 4°C) were washed with 50 ml of 20 mM phosphate buffer (pH 8.0), and a small amount of cells was then sampled for SDS-PAGE analysis. Harvested cells were freeze-dried for the enzyme reactions. Wild-type *Caulobacter crescentus* was grown in peptone-yeast extract (PYE) media at 25°C. The culture of wild-type *C. crescentus* was harvested by centrifugation (1,000 ×*g*, 10 min, 4°C), washed with 20 mM phosphate buffer (pH 8.0), and lyophilized. BugBuster® protein extraction reagent (Novagen) was used for the extraction of soluble and particulate fraction of proteins according to manufacturer's instructions. The prepared samples of soluble and particulate fraction of proteins were applied to 12% SDS-PAGE.

Table 1. Putative bacterial epoxide hydrolase genes selected from the genome database and their PCR primers.

Source	ORF identifier	Primer sequences (5' → 3')
M. loti	mlr1789	ggaattccatatgttccgtgattttcagacg[a]
		cccgctcgagtcactaggcgcgatctgg[b]
	mlr3288	ggaattccatatgagcacagctggcg[a]
		ccccaagctttcacgaaccggcaacg[c]
	mlr6682	ggaattccatatgttggcggcaatcctgg[a]
		cccgctcgagtcaggacgcgcgcagc[b]
	mlr6683	ggaattccatatgcgtgttgcagctctg[a]
		ccccaagctttcatgacaaatggtcaac[c]
B. subtilis	BG12888	ggaattccatatggacggagttaaatgc[a]
		cccgctcgagtcattggttcttaagatattc[b]
A. tumefaciens	atu1814	ggaattccatatgcttcgtagaaccttc[a]
		cccgctcgagtcactatatgctttcc[b]
	atu3664	ggaattccatatgaaagacactatcatttcg[a]
		cccgctcgagtcattaggcgctttccac[b]
C. crescentus	ccr1229	ggaattccatatgacggacacccctttc[a]
		ccccaagctttcactaaagcgggaagcggcg[c]
	ccr3091	ggaattccatatgccaaagccgttcgag[a]
		ccccaagctttcaactaaagctctctggccc[c]

[a]NdeI, [b]XhoI, [c]HindIII.

Kinetic Study on Various Epoxides

The prepared freeze-dried whole cells (0.5 mg/ml) were preincubated in 20 mM phosphate buffer (pH 8.0) at 37°C. In the case of 1,2-epoxy-3-phenoxypropane, the buffer contained 20% dimethylsulfoxide (DMSO). The epoxide was added to a final 10 mM concentration. The reaction mixture was sampled at several time points and was extracted with ethyl acetate for quantitative analysis by GC.

RESULTS AND DISCUSSION

Cloning and Heterologous Expression of Probable EH Genes

There were 58 ORFs from about 20 bacterial sources, which had been annotated by sequence similarities with EHs, in the Kyoto encyclopedia of genes and genomes (KEGG) genome database (http://www.genome.jp/kegg/kegg2.html).

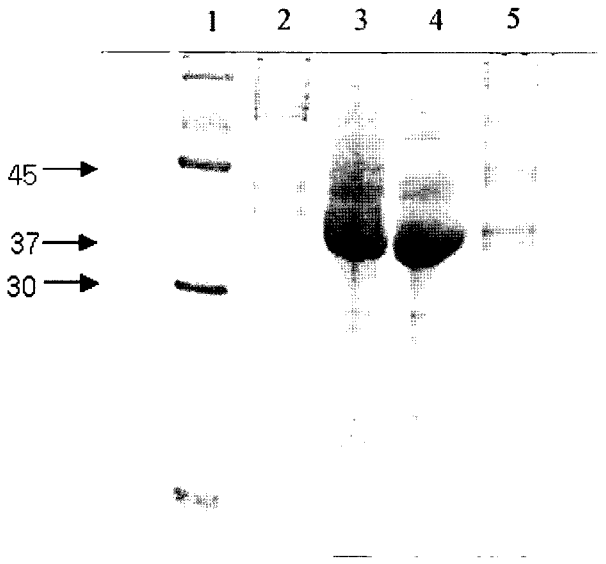

Fig. 1. Heterologous expression of CCEH in *E. coli* BL21(DE3). Lane 1, protein mass markers; lane 2, total proteins of wild-type *C. crescentus*; lane 3, total proteins of recombinant *E. coli* BL21(DE3); lane 4, soluble fraction of recombinant *E. coli* BL21(DE3); lane 5, particulate fraction of recombinant *E. coli* BL21(DE3).

Among these ORFs, 9 ORFs from 4 commercially available bacterial sources (Table 1) were selected and amplified in a PCR using primers listed in Table 1. Amplified fragments were cloned into an IPTG-inducible expression vector, pET28b (Novagen), and overexpressed in *E. coli* BL21(DE3) under control of a T7 promoter. The 9 recombinants were tested for the ability to hydrolyze styrene oxide in 20 mM phosphate buffer (pH 8.0) containing 10 mM styrene oxide. The expression of each ORF was verified by SDS-PAGE. Only 1 ORF from *C. crescentus* (identifier: CC1229) among the 9 ORFs showed activity toward styrene oxide, when reacted with whole cell. The inactive 6 ORFs, except BG12888 and ccr3091, were expressed in *E. coli*, but they had no activities toward epoxides, including styrene oxide, indene oxide, and 1,2-epoxy-3-phenoxypropane. The ORFs of BG12888 and ccr3091 were not expressed.

According to the genome database, the ORF CC1229 codes a polypeptide of 330 amino acids (calculated molecular mass of 37,037 Da), in good agreement with our data of SDS-PAGE analysis (Fig. 1). Overexpressed epoxide hydrolase from *C. crescentus* (CCEH) constituted 75% of total protein, corresponding to about 85% of the soluble protein estimated by densitometric analysis [GS-690 densitometer (Bio-Rad)] of SDS-PAGE gel.

Kinetics of Various Aromatic Epoxides by EH from *C. crescentus*

The results of the kinetic study of various epoxides, using the lyophilized whole cells of the recombinant CCEH, are listed in Table 2. The enantioselectivities and yields were highly dependent on the structures of substrate. Hydrolytic activities and enantioselectivities for short aliphatic epoxides (epichlorohydrin and glycidol), useful C3 chiral synthons, were very low (data not shown). However, whole cells of recombinant CCEH showed higher enantioselectivities and yields for aromatic epoxides than for short aliphatic epoxides (Table 2). The whole cells of wil-type *C. crescentus* had no activity to hydrolyze the styrene oxide. Therefore, the expression level of CCEH in normal culture condition appeared to be quite low.

Chiral styrene oxide could be obtained through bacterial resolution of racemic styrene oxide, using the whole cells of recombinant CCEH at higher enantioselectivity (>99% *ee*, E=8.8) with a reasonable yield (20%), than that using the whole cells of recombinant AREH (31% *ee*, E=4.3, yield=40.8%) at the same time point (60 min) (Fig. 2). It is of interest to note that the enantiopreference of the CCEH toward styrene oxide in whole-cell reaction was opposite to that of the AREH. It has been reported that most epoxide hydrolases are (*S*)-preferable; however, the CCEH showed (*R*)-preferable enantioselectivity to styrene oxide, similar to the kinetic resolution using the whole cells of *B. sulfurescens* [10].

The interest for the production of enantiopure indene oxide is due to its possible usage as (1*S*,2*R*)-indene oxide, which is a precursor of the side chain of the HIV protease inhibitor, indinavir [14]. Kinetic resolution of indene oxide has so far been reported only with fungal epoxide hydrolases [10, 15]. The bacterial resolution of the racemic indene oxide, using the lyophilized whole cells of recombinant CCEH, produced (1*R*,2*S*)-indene oxide with >99% *ee*, and 23% yield, E=6.

Table 2. Hydrolysis of aryl epoxides by the recombinant EH from *C. crescentus*.

Epoxide	ee (%)	Yield (%)	E	Abs. conf.[d]	Reaction time (min)
Styrene oxide	>99(31[b])	20(40.8[b])	8.8[c] (4.3[b])	R(S[b])	60
Indene oxide	>99	23	6[c]	(1R,2S)	30
1,2-Epoxy-3-phenoxypropane[a]	>99	7.8	3.5[c]	S(none[f])	180

[a]20% DMSO as a co-solvent.
[b]Kinetic resolution parameters using whole cells of recombinant *A. radiobacter* EH in the same reaction condition.
[c]Calculated from Shi's equation.
[d]The absolute configurations were determined by chiral GC.
[f]AREH has no enanatioselectivity to 1,2-epoxy-3-phenoxypropane.

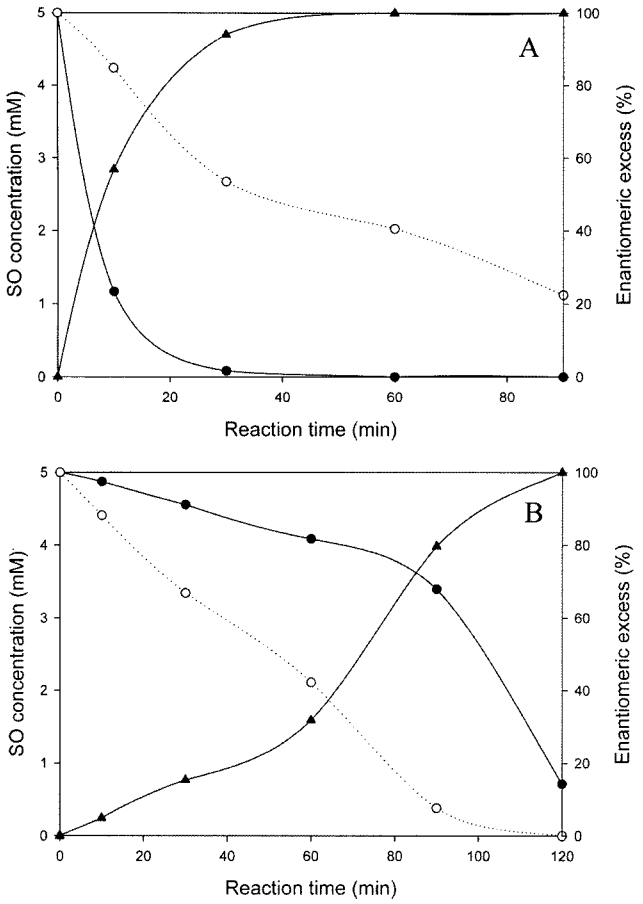

Fig. 2. Kinetic resolution of 10 mM racemic styrene oxide (SO) using lyophilized whole cells of recombinant CCEH (A) and lyophilized whole cells of recombinant AREH (B).
(○), (*R*)-enantiomer; (●), (*S*)-enantiomer; (▲), enantiomeric excess.

1,2-Epoxy-3-phenoxypropane is one of the aryloxy epoxides for the synthesis of chiral amino alcohols and β-blockers [12]. Only a few biocatalysts with sufficiently high enantioselectivity for the kinetic resolution of this compound has been found among bacteria and fungi [4, 12]. The lyophilized whole cells of recombinant CCEH could preferentially hydrolyze the (*R*)-enantiomer of 1,2-epoxy-3-phenoxypropane, yielding (*S*)-epoxide with enantioselectivity (*E*=3.5).

The bacterial resolution of the epoxides using whole cells of recombinant CCEH needs to be optimized to improve the yield and the enantioselectivity. In addition, the properties of CCEH, such as stability, enantioselectivity, and substrate specificity, could be improved through directed evolution technology. Compared with other bacterial epoxide hydrolases, CCEH shows characteristic properties, such as different enantiopreference to styrene oxide and ability to resolve racemic indene oxide. Therefore, CCEH could provide clues to unravel the function of the residues around the active site of epoxide hydrolases, if the 3D structure of the CCEH is determined.

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