

Enantioconvergent Production of (R)-1-Phenyl-1,2-Ethanediol From Styrene Oxide by Combining the *Solanum tuberosum* and an Evolved *Agrobacterium radiobacter* AD1 Epoxide Hydrolases

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Abstract: Soluble epoxide hydrolase (EH) from the potato *Solanum tuberosum* and an evolved EH of the bacterium *Agrobacterium radiobacter* AD1, EchA-I219F, were purified for the enantioconvergent hydrolysis of racemic styrene oxide into the single product (R)-1-phenyl-1,2-ethanediol, which is an important intermediate for pharmaceuticals. EchA-I219F has enhanced enantioselectivity (enantiomeric ratio of 91 based on products) for converting (R)-styrene oxide to (R)-1-phenyl-1,2-ethanediol ($2.0 \pm 0.2 \mu\text{mol}/\text{min}/\text{mg}$), and the potato EH converts (S)-styrene oxide primarily to the same enantiomer, (R)-1-phenyl-1,2-ethanediol ($22 \pm 1 \mu\text{mol}/\text{min}/\text{mg}$), with an enantiomeric ratio of 40 ± 17 (based on substrates). By mixing these two purified enzymes, inexpensive racemic styrene oxide (5 mM) was converted at 100% yield to 98% enantiomeric excess (R)-1-phenyl-1,2-ethanediol at $4.7 \pm 0.7 \mu\text{mol}/\text{min}/\text{mg}$. Hence, at least 99% of substrate is converted into a single stereospecific product at a rapid rate. © 2006 Wiley Periodicals, Inc.

Keywords: epoxide hydrolase; styrene oxide; phenyl-1,2-ethanediol; enantioconvergent

INTRODUCTION

Interest in using epoxide hydrolases (EHs, EC 3.3.2.3), enzymes that hydrolyze epoxides to diols for synthesis of enantio-pure compounds (Manoj et al., 2001), is increasing for the production of pharmaceuticals (Monterde et al., 2004; Schoemaker et al., 2003). For example, the pharmaceutical intermediate, enantio-pure (R)- or (S)-mandelic acid, is used in the synthesis of β-lactam antibiotics and analytical reagents (Groger, 2001), and (R)-mandelic acid may be made from (R)-1-phenyl-1,2-ethanediol (Drummond et al., 1990; Lindstad et al., 1998).

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EchA is an EH (294 aa) (Rui et al., 2004) from the gram-negative strain *Agrobacterium radiobacter* AD1 that allows the cell to utilize epichlorohydrin as a sole carbon source (Jacobs et al., 1991). A variant of EchA was generated by saturation mutagenesis with one amino acid change I219F (EchA-I219F) (Rui et al., 2004) that hydrolyzes racemic styrene oxide twofold faster than wild-type EchA while having enhanced enantioselectivity for producing (R)-1-phenyl-1,2-ethanediol (enantiomeric ratio of 91 vs. 17 based on product) (Rui et al., 2005).

Plant soluble EHs play a central role in producing cutin, aromatics, and anti-fungal compounds (Morisseau et al., 2000); they have been isolated from at least eight plants to date (Elfstrom and Widersten, 2005; Morisseau et al., 2000). cDNA encoding a soluble EH (322 aa) from potato (*S. tuberosum*, *StEH*) was cloned and characterized (Morisseau et al., 2000; Stapleton et al., 1994). The hydrolysis of racemic styrene oxide with *StEH* has been investigated and demonstrated to be an enantioconvergent process with initial attack on the α (benzylic) carbon atom of (S)-styrene oxide to form (R)-1-phenyl-1,2-ethanediol followed by hydrolysis of the β (terminal carbon) atom of (R)-styrene oxide to form again (R)-1-phenyl-1,2-ethanediol (Fig. 1B) (Monterde et al., 2004). Therefore, this single enzyme was used to form (R)-1-phenyl-1,2-ethanediol from racemic styrene oxide at 86% enantiomeric excess at 100% conversion (Monterde et al., 2004) although the rate is 300-fold slower for the (R)-styrene oxide reaction. A mixture of EHs from the fungi *Aspergillus niger* and *Beauveria sulfurescens* have also been used for the enantioconvergent synthesis of (R)-1-phenyl-1,2-ethanediol from racemic styrene oxide (Archelas and Furstoss, 1998).

Here, we report the conversion of racemic styrene oxide to pure (R)-1-phenyl-1,2-ethanediol using a mixture of *StEH*, which produces (R)-1-phenyl-1,2-ethanediol from both (R)- and (S)-styrene oxide, and EchA-I219F, which converts (R)-styrene oxide to (R)-1-phenyl-1,2-ethanediol rapidly with

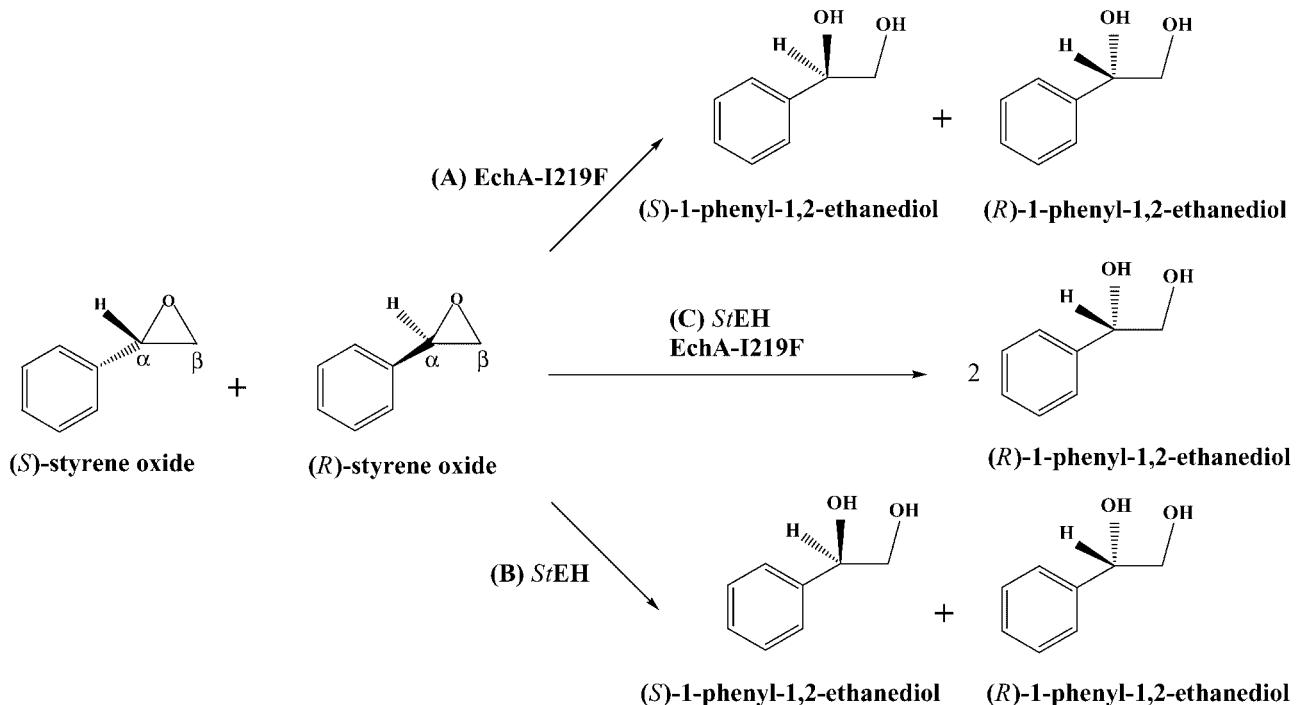


Figure 1. Schematic of enantioselective hydrolysis of racemic styrene oxide by: (A) EchA-I219F to form (R)-1-phenyl-1,2-ethanediol and (S)-1-phenyl-1,2-ethanediol, (B) StEH to form (R)-1-phenyl-1,2-ethanediol and a small amount of (S)-1-phenyl-1,2-ethanediol, and (C) a mixture of EchA-I219F and StEH to form (R)-1-phenyl-1,2-ethanediol only.

slow conversion of (S)-styrene oxide to (S)-diol (Rui et al., 2005) (Fig. 1). This is the first report of combining an EH from plant and bacterial sources for enhancing the enantioselectivity toward a racemic compound with the result: a more rapid enzyme system with both higher enantiomeric excess and yield of (R)-phenyl-1,2-ethanediol was created.

MATERIALS AND METHODS

Chemicals, Organisms, and Cultivation

Racemic styrene oxide, (R)-styrene oxide, and (S)-styrene oxide were purchased from Sigma Chemical Co. (St. Louis,

MO); all the other chemicals were obtained from Fisher Scientific Company (Pittsburgh, PA). *Escherichia coli* strain TG1 (Sambrook et al., 1989) was used as the host for cloning and functional expression of genes from the pBS(Kan) plasmids. *E. coli* strain BL21(DE3)pLysS and BL21-CodonPlus(DE3)-RIPL strain (Stratagene, La Jolla, CA) were utilized as the hosts for expression and purification of the His-tag fusion proteins. All the strains were routinely cultivated at 37°C in Luria–Bertani (LB) broth (Sambrook et al., 1989) supplemented with appropriate antibiotics (Table I). All experiments performed (unless otherwise stated) by whole cells or sonicated cells were started from single colonies, induced with 1 mM

Table I. Strains and plasmids used in this article.

Strain or plasmid	Genotype and/or phenotype	Source
Strains		
<i>E. coli</i> TG1	<i>supE hsdA5 thi Δ(lac-proAB) F' [traD36 proAB⁺ lacI^q lacZΔM15]</i>	Sambrook et al. (1989)
<i>E. coli</i> BL21 (DE3)pLysS	<i>F⁻ ompT hsdS_B (r_B m_B) gal dcm λ(DE3) pLysS Cm^r</i>	Cinquin et al. (2001)
<i>E. coli</i> BL21-CodonPlus(DE3)-RIPL	<i>B F⁻ ompT hsdS(r_B m_B) dcm⁺ Tet^r gal λ (DE3) endA Hte [argU proL Cam^r [argU ileY leuW Strep/Spec^r]</i>	Stratagene
Plasmids		
pBS(Kan)	Kan ^r , Kan ^r inserted into Amp ^r of pBluescript II KS	Canada et al. (2002)
pBS(Kan)-EchA-I219F	Kan ^r , gene for EchA-I219F inserted into pBS(Kan)	Rui et al. (2004)
pBS(Kan)-StEH	Kan ^r , gene for StEH inserted into pBS(Kan)	This study
pET-28b(+)	Kan ^r	Novagen
pET-EchA-I219F	Kan ^r , gene for EchA-I219F inserted into pET-28b(+)	This study
pET-StEH	Kan ^r , gene for StEH inserted into pET-28b(+)	This study

Kan, kanamycin (50 µg/mL); Cam, chloramphenicol (34 µg/mL); Strep, streptomycin (50 µg/mL); Tet, tetracycline; Spec, spectinomycin.

isopropyl β -D-thiogalactopyranoside (IPTG) at an optical density at 600 nm (OD_{600}) of 0.2–0.3, and harvested after 2 h of IPTG induction at an OD_{600} of 1.5–2.0.

General Molecular Techniques

Enzymes were purchased from New England Biolabs (Ipswich, MA). Polymerase chain reaction (PCR) products were purified by Wizard PCR Preps, plasmid DNA was isolated using a Midi or Mini Kit (Qiagen, Inc., Chatsworth, CA), and DNA fragments were isolated from agarose gels by using a QIAquick Gel Extraction Kit (Qiagen, Inc.). *E. coli* was electroporated using a Gene Pulser/Pulse Controller (Bio-Rad Laboratories, Hercules, CA) at 15 kV/cm, 25 μ F, and 200 Ω . A dye terminator cycle sequencing protocol based on the dideoxy method of sequencing DNA (Sanger et al., 1977) was used.

Cloning EH in *E. coli*

To stably and constitutively express the potato enzyme *StEH* in *E. coli*, an expression cassette with a typical *E. coli* ribosome-binding site (AGGAGG) and an intervening region that was used to express nitrilase in *E. coli* were used here (Kobayashi et al., 1992); this region was introduced by amplifying the gene encoding *StEH* from pEH10.1 (Stapleton et al., 1994) using primers *StEH*10.1 forward 2 with *StEH*10.1 reverse (Table II) (note pEH10.1 lacks a prokaryotic promoter). Following an initial denaturation at 96°C for 2 min, a thermal amplification profile of 30 cycles with denaturation at 94°C for 45 s, annealing at 52°C for 45 s, and extension at 72°C for 1.5 min, and then a final extension at 72°C for 7 min was used. The PCR product was double digested with *Sal*I and *Bam*H I and ligated into plasmid pBS(Kan) to form pBS(Kan)-*StEH* (Fig. 2A).

To purify both EchA-I219F and *StEH* using a histidine tag, genes encoding both EHs were amplified from the pBS(Kan) vectors by PCR using primers STEH PET forward and STEH PET reverse for *StEH*, and EHAD1 PET Front and EHAD1 PET Rear for EchA-I219F (Table II) under the same thermal amplification profile as above. The PCR products were double digested by *Nde*I and *Hind*III, and inserted into pET28b(+) (Novagen, Madison, WI) to generate the pET-*StEH* and pET-EchA-I219F (Fig. 2B and C) which yielded a

six amino-acid His-tag as well as a thrombin cleavage sites at the N-terminus of the EHs. Both of genes were sequenced completely using primers SEQ PET forward, SEQ PET reverse, EH AD1 108 Rear, and SEQ PET Front upstream (Table II) to confirm there was no amino acid change in the recombinant plasmids and to confirm the presence of the I219F mutation in EchA-I219F.

To overexpress the EchA-I219F, pET-EchA-I219F was transformed into BL21(DE3)plyS by electroporation. For pET-*StEH*, BL21-CodonPlus(DE3)-RIPL carrying genes encoding extra copies of *argU*, *ileY*, *leuW* and *proL* rare codon tRNA was used since 5 out of 10 arginines in the *StEH* gene are encoded by the rare codons AGA or AGG (Stratagene).

Protein Purification

Both EchA-I219F and *StEH* with a His-tag were purified by Ni-NTA column (Invitrogen, Carlsbad, CA). An overnight culture of BL21(DE3)plyS harboring pET-EchA-I219F was exponentially grown in 25-mL LB medium with kanamycin (50 μ g/mL) and chloramphenicol (34 μ g/mL), then diluted 1:100 in 1 L of fresh LB medium with the same antibiotics, and incubated at 37°C. IPTG (1 mM) was added at cell $OD_{600} \sim 0.3$, and the culture was incubated at 37°C overnight. One-liter cells were harvested by centrifugation at 10,000g, 4°C for 10 min and resuspended in 10 mL of the native purification buffer (50 mM NaH₂PO₄, pH 8.0, 0.5 M NaCl). Bacteria lysis was performed by passing the cell resuspension (10 mL) through a French Pressure Cell Press (Spectronic Instruments, Rochester, NY) and centrifuged at 20,000g, 4°C for 1 h to pellet the cell debris. The supernatants containing the soluble EHs were loaded into a 10-mL Ni-NTA column for purification. His-tagged enzymes were eluted with native purification buffer with imidazole (250 mM) and dialyzed against TE buffer (100 mM Tris-Cl, 0.2 mM EDTA) overnight and then dialyzed against storage buffer (10 mM Tris-SO₄, 1 mM EDTA, 1 mM β -mercaptoethanol, 0.02% sodium azide, and 20 % glycerol, pH 7.5). Purification of *StEH* was performed as above except BL21-CodonPlus (DE3)-RIPL harboring pET-*StEH* was cultured in LB with kanamycin (50 μ g/mL), chloramphenicol (34 μ g/mL), and streptomycin (50 μ g/mL) for maintaining of the pET-*StEH*, the pACYC-based plasmid with extra copies of the *argU* and *proL* tRNA genes, and the

Table II. Primers used in this work with restriction enzymes underlined and the modified ribosome-binding site in lowercase.

Primer	Sequence
<i>StEH</i> 10.1 forward 2	5'-CATGGGAGAG <u>TGTCGACA</u> AaggaggAATAGGAATGGAGAACATAGAGCACAAAGATGG-3'
<i>StEH</i> 10.1 reverse	5'-GTCATAAT <u>ATGGATC</u> CTAACGAACACTGCTTACAC-3'
STEH PET forward	5'-GGAGGAATAG <u>CATATG</u> GAGAAGATAGAGCACAAG-3'
STEH PET reverse	5'-CGCTAGAGA <u>AAAGCTT</u> AAA <u>ACTTTG</u> GAATGAAG-3'
EHAD1 PET Front	5'-AAGGAGAT <u>ATCATATG</u> GCATTGACGTCCAG-3'
EHAD1 PET Rear	5'-TCCTAG <u>CCAAGCTT</u> AGCGGAACGCGGTTTG-3'
SEQ PET forward	5'-TCATCATCACAGCAGCGG-3'
SEQ PET reverse	5'-CAGCAG <u>CCAACTCAGCTTC</u> -3'
EH AD1 108 Rear	5'-TTATGGAGGACGATGGCCGCGNNNT <u>CATGGCCAAC</u> -3'
SEQ PET Front upstream	5'-CATACCCACGCCGAAACAAG-3'

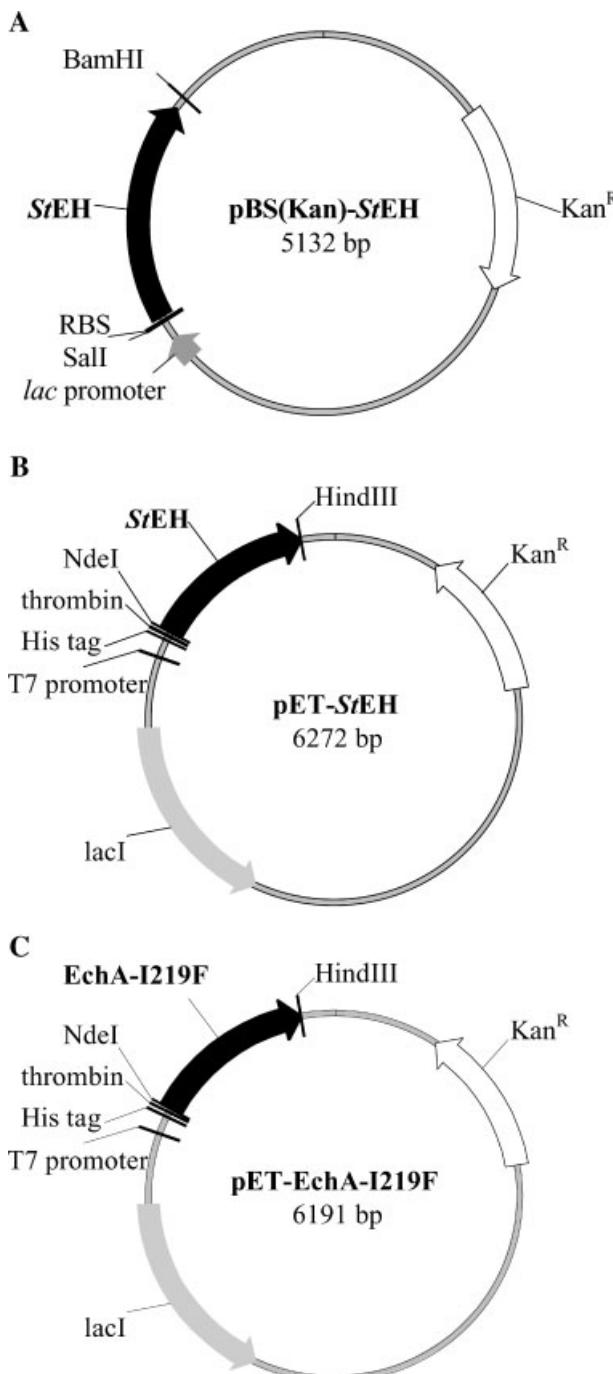


Figure 2. Schematics of plasmids (A) pBS(Kan)-*StEH*, (B) pET-*StEH*, and (C) pET-*EchA-I219F*.

pSC101-based plasmid carrying extra copies of the *argU*, *ileY*, and *leuW* tRNA genes, respectively (Stratagene).

The concentrations of the purified enzymes were evaluated by comparing standard protein samples (Precision plus protein standards, Bio-Rad Laboratories) of known concentration on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE). To corroborate these values, a method based on spectrophotometric measurement of the purified enzymes in 6 M guanidine at 280 nm was used (Edelhoch, 1967; Gill and Hoppel, 1989). Both of the

methods gave similar results for the concentration of His-*EchA-I219F* (1 µg/µL) and His-*StEH* (1 µg/µL), which was used for the calculation for the specific activity of enzymes.

Enzyme Assays

To test initial *StEH* expression in *E. coli* harboring pBS(Kan)-*StEH*, a spectrophotometric method was used (Rui et al., 2005) with whole cells. Briefly, 2 mL of exponentially-growing whole cells were incubated with 5 mM racemic styrene oxide in 15 mL glass vial at 37°C for 1 h with shaking at 250 rpm, and then the vial was incubated at 50°C for 50 min after adding 1 mL of 100 mM 4-(4-nitrobenzyl)-pyridine. To detect the remaining racemic styrene oxide, 1 mL of 50% triethylamine was added to the vial (in acetone), and the absorbance of mixture was measured at OD₆₀₀.

To make the crude extract for the enzyme assays, exponentially growing cells harboring EHs were harvested and adjusted to OD₆₀₀ to ~1 in TEM buffer (10 mM Tris-HCl, 1 mM EDTA, and 1 mM mercaptoethanol, pH 7.4), and the cells were sonicated 10 times for 10 s at 10 W (with a 1-min interval on ice between two sonifications) with a F60 sonic dismembrator (Fisher Scientific, Pittsburgh, PA). The concentration of EH in the sonicated cells was estimated from SDS-PAGE (for specific rates).

For the reactions with single purified enzymes, the hydrolysis of racemic styrene oxide, (*S*)-styrene oxide, or (*R*)-styrene oxide was performed with 60–300 µg His-*StEH* and 160–320 µg His-*EchA-I219F*. The enzymes were mixed in 4–10 mL TEM buffer and contacted with 5 mM of these substrates at 37°C with shaking (250 rpm) for 40–60 min. For the hydrolysis of racemic styrene oxide by the mixture of EHs, His-*StEH* (40 µg), and His-*EchA-I219F* (200 µg) were mixed in 5 mL TEM buffer and incubated with 5 mM racemic styrene oxide. To investigate the inhibition of (*R*)-styrene oxide hydrolysis by (*R*)-1-phenyl-1,2-ethanediol with *StEH*, (*R*)-1-phenyl-1,2-ethanediol (2.5 mM) and 2.5 mM (*R*)-styrene oxide were incubated together with purified His-*StEH* (80 µg) in 5 mL reaction at 37°C with shaking (250 rpm) for 1 h. For the control, 2.5 mM (*R*)-styrene oxide was added to the same amount of purified His-*StEH* (no (*R*)-1-phenyl-1,2-ethanediol). The initial rates of the enzymes toward the different substrates were calculated.

Identification of Products From Hydrolysis of Styrene Oxide

The reaction products were analyzed by chiral high-performance liquid chromatography (HPLC) for the production of enantiomer of diols (Rui et al., 2005). Briefly, samples (500 µL) were withdrawn by syringe from the reaction and quenched by extracting with 800 µL ethyl ether. HPLC (Waters Corp., Milford, MA) including 515 HPLC pumps, a 996 photodiode array detector, and Millenium32 Chromatography Manager Software was used to determine the absolute configuration of the enantiomers of 1-phenyl-1,2-ethanediol

formed from hydrolyzing styrene oxide. The (*R*)- and (*S*)-1-phenyl-1,2-ethanediol were separated by a Chiralcel OB (4.6 × 250 mm) column (Chiral Technologies, Inc., Exton, PA) using hexane/2-propanol (76/24, v/v) as the eluent with a flow rate of 0.5 mL/min with retention times of 10.2 min and 11.2 min, respectively. To quantify (*R*)- and (*S*)-1-phenyl-1,2-ethanediol formation and styrene oxide remaining in the reaction, UV-visible spectra were acquired using a diode array detector (scanning from 200 to 600 nm). The HPLC detection limit under these conditions for racemic styrene oxide was measured using serial dilutions of racemic styrene oxide from 5 to 0.01 mM in TEM buffer and extraction with ethyl ether.

The enantiomeric ratio was calculated based on substrate disappearance as determined by gas chromatography (GC). Samples were prepared by withdrawing 500 μL liquid from the reaction and extracting the styrene oxide with the same volume methylethene chloride with 5 mM mesitylene. A gas chromatograph (Agilent 6890 series) equipped with a 30 m × 0.25 mm Chiraldex G-TA capillary column (Astec, Whippany, NJ) and a flame-ionization detector (FID) was used. Hydrogen (40 mL/min) and air (400 mL/min) were supplied to the FID, and the carrier gas was nitrogen (0.7 mL/min). Samples injected into the GC were analyzed isothermally at 225°C, and the retention times of (*S*)-styrene oxide and (*R*)-styrene oxide were 8.16 min and 9.75 min, respectively. Concentrations were calculated by comparing the peak area with the peak area formed by the internal standard mesitylene (5 mM). The enantioselectivity (E) (Chen et al., 1982) = $\ln [(1 - C)(1 - EES)] / \ln [(1 - C)(1 + EES)]$, where conversion (C) = $1 - [\text{styrene oxide}] / [\text{initial styrene oxide}]$ and EES is the enantiomeric excess based on substrates = $([\text{S-styrene oxide}] - [\text{R-styrene oxide}]) / ([\text{S-styrene oxide}] + [\text{R-styrene oxide}])$ for EchA-I219F and $([\text{R-styrene oxide}] - [\text{S-styrene oxide}]) / ([\text{R-styrene oxide}] + [\text{S-styrene oxide}])$ for *StEH*. The enantiomeric ratio of each enzyme was calculated when roughly 50% of the racemic styrene oxide was converted (C = 0.5). EEP is the enantiomeric excess based on the products = $([\text{R-diol}] - [\text{S-diol}]) / ([\text{R-diol}] + [\text{S-diol}])$.

RESULTS

Expression of *StEH* in *E. coli*

To express *StEH* in a prokaryotic system, pBS(Kan)-*StEH* was constructed by cloning the gene for *StEH* into pBS(Kan) along with a suitable ribosome-binding site (Kobayashi et al., 1992) and transformed into *E. coli* strain TG1 under the control of a constitutive *lac* promoter (Fig. 2A). *StEH* with activity toward styrene oxide was obtained (162 nmol/min/mg protein for racemic styrene oxide using whole cells). To purify both of *StEH* and EchA-I219F, the EH genes were cloned into pET28b(+) by fusing the EH genes between the *NdeI* and *HindIII* site, so that the genes were under the control of T7 promoter and a six amino acid His-tag and thrombin site were added at the N-terminus (Fig. 2B and C).

Protein Purification

Both EHs with the His-tag at the N-terminus were purified using a Ni-NTA matrix; the purity was checked using SDS-PAGE (Fig. 3) and the enzymes were both found to be greater than 90% pure. Bands with approximately 37 kDa molecular weight were observed corresponding to both His-EchA-I219F and His-*StEH* (predicted sizes of 36.3 kDa and 38.5 kDa, respectively).

Conversion of Racemic Styrene Oxide by *StEH* and EchA-I219F

The detection limit was determined by HPLC to be 99% for racemic styrene oxide. Purified His-*StEH* demonstrated a rapid rate as racemic styrene oxide was converted to (*R*)-1-phenyl-1,2-ethanediol at an initial rate of $22 \pm 1 \mu\text{mol}/\text{min}/\text{mg}$ with the corresponding disappearance of (*S*)-styrene oxide; the rate of (*S*)-1-phenyl-1,2-ethanediol formation was 300-fold lower (Table III). Due to the 10-fold slower rate of *StEH* on (*R*)-styrene oxide, the reaction can be made to go to completion, but it took 15 h to convert $96 \pm 4\%$ racemic styrene oxide to diols with product enantiomeric excess of $87 \pm 4\%$ in favor of (*R*)-1-phenyl-1,2-ethanediol which agrees with the earlier report (100% conversion with 86% enantiomeric excess) (Fig. 4A) (Monterde et al., 2004). Similar results were obtained here by contacting crude extracts of His-*StEH* with racemic styrene oxide: at least 99%

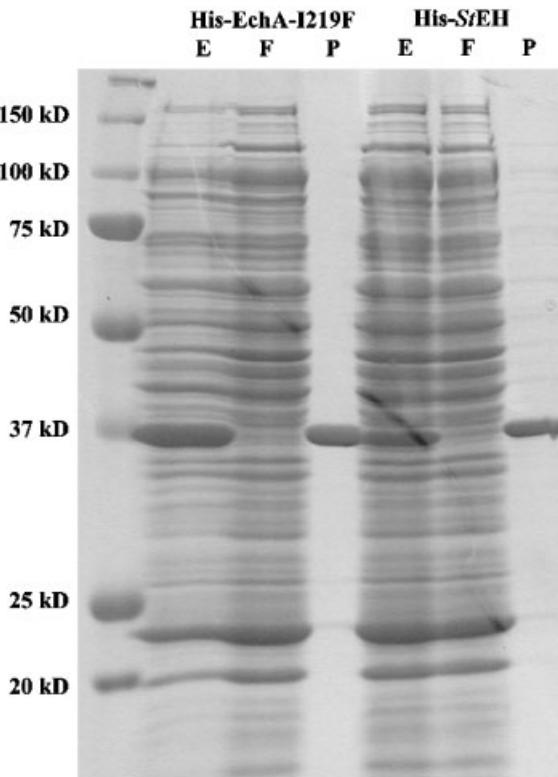


Figure 3. SDS-PAGE to check the purification of His-I219F and His-*StEH*. E, crude extract; F, flow through; and P, purified enzyme.

Table III. Specific activity of His-StEH and His-EchA-I219F toward 5 mM racemic styrene oxide.

Enzymes	(R)-1-phenyl-1,2-ethanediol ^a	(S)-1-phenyl-1,2-ethanediol ^a	Racemic styrene oxide ^a	(R)-styrene oxide	(S)-styrene oxide			
	Rate ^b (μmol/min/mg)	Rate ^b (μmol/min/mg)	Rate ^b (μmol/min/mg)	Rate ^c (μmol/min/mg)	Rate ^c (μmol/min/mg)	E	EEP (%)	C (%)
Purified His-StEH	17.2 ± 0.4	0.057 ± 0.001	22 ± 1	2.3 ± 1.3	27 ± 2	40 ± 17	87 ± 4	96 ± 4
Purified His-EchA-I219F	1.9 ± 0.1	0.17 ± 0.03	2.0 ± 0.2	1.65 ± 0.03	0.5 ± 0.1	10.0 ± 0.9	92 ± 2	51 ± 1
Mixture of purified enzymes	4.7 ± 0.7	0.04 ± 0.00	4.7 ± 0.3	1.53 ± 0.05	2.7 ± 0.6	N/A ^e	97.9 ± 0.2	99.9 ± 0.1
Crude extract of His-StEH	12.6 ± 1.2	0.2 ± 0.1	12.0 ± 5.8	N/D ^d	N/D	N/D	85.4 ± 0.4	100 ± 0
Crude extract of His-EchA-I219F	0.83 ± 0.04	0.21 ± 0.01	0.64 ± 0.08	N/D	N/D	N/D	76 ± 3	50.8 ± 0.6
Mixture of crude extract of enzymes	3.5 ± 0.9	0.13 ± 0.04	3.7 ± 0.8	N/D	N/D	N/D	92.80 ± 0.01	99.50 ± 0.01

E is enantioselectivity, EEP is product-based enantiomeric excess, and C is conversion.

^aThe formation of (R)- and (S)-1-phenyl-1,2-ethanediol and the conversion of racemic styrene oxide were determined via chiral HPLC.

^bInitial rates were calculated based on the linear range of HPLC data.

^cInitial rates were calculated based on the linear range of GC data.

^dN/D, not determined.

^eN/A, not applicable for a mixture of enzymes.

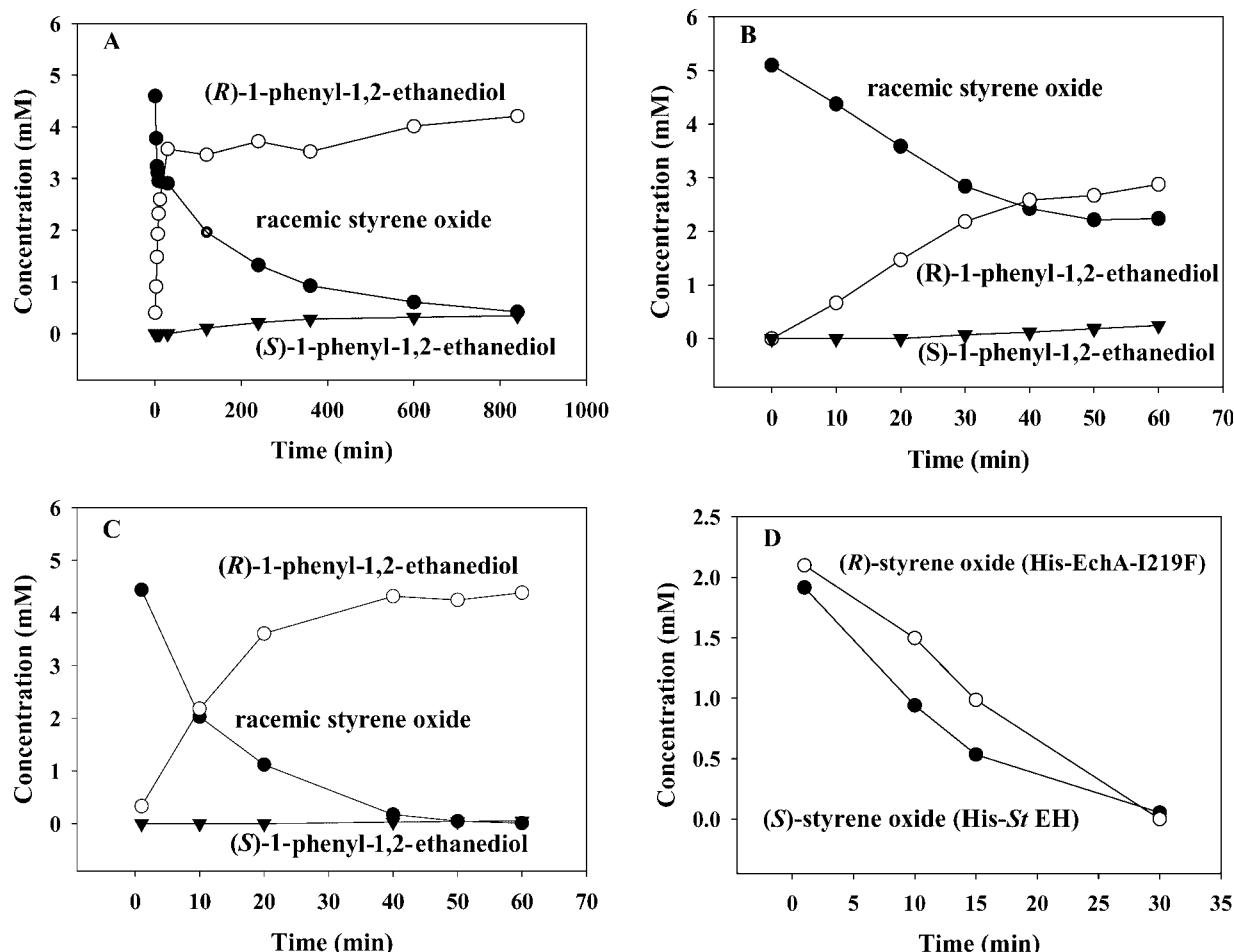


Figure 4. Enantioselective hydrolysis of 5 mM racemic styrene oxide by purified enzymes. Production of (R)-1-phenyl-1,2-ethanediol from racemic styrene epoxide by: (A) purified His-StEH (160 μg in a 10 mL reaction), (B) purified His-EchA-I219F (200 μg in a 5 mL reaction), (C) a mixture of purified His-StEH and His-EchA-I219F (40 μg and 200 μg, respectively in a 5 mL reaction), and (D) disappearance of racemic styrene oxide by the mixture of enzymes (40 μg and 200 μg, respectively in a 5 mL reaction). One of two duplicate experiments shown.

conversion of substrates with $85.4 \pm 0.4\%$ enantiomeric excess.

Purified EchA-I219F was found to convert racemic styrene oxide to (*R*)-1-phenyl-1,2-ethanediol 11 times faster than to (*S*)-1-phenyl-1,2-ethanediol with high rates (Table III). Although EchA-I219F that lacks a His-tag is more active than the wild-type enzyme (Rui et al., 2005), purified recombinant His-EchA-I219F showed lower activity toward racemic styrene oxide ($2.0 \pm 0.2 \mu\text{mol}/\text{min}/\text{mg}$) (Table III) compared with the wild-type EchA that lacks a His-tag ($5.2 \mu\text{mol}/\text{min}/\text{mg}$) (Rui et al., 2005). The same discrepancy was seen with the crude extracts, which indicates the His-tag at the N-terminus decreases the rate of reaction but did not affect enantioselectivity (Fig. 4B).

The conversion of racemic styrene oxide by purified His-*StEH* and His-EchA-I219F were also monitored by GC analysis based on the disappearance of each enantiomer of racemic styrene oxide. The enantiomeric ratio of racemic styrene oxide hydrolysis by purified His-*StEH* was 40 ± 17 (Table III), and the rate of production of (*R*)-1-phenyl-1,2-ethanediol from (*S*)-styrene oxide hydrolysis was $17.2 \pm 0.4 \mu\text{mol}/\text{min}/\text{mg}$. This shows the preference for (*S*)-enantiomer disappearance for *StEH*, while the His-EchA-I219F, which catalyzed primarily the hydrolysis of the (*R*)-enantiomer, had an enantiomeric ratio of 10.0 ± 0.9 , with the rate of (*R*)-1-phenyl-1,2-ethanediol formation as $1.9 \pm 0.1 \mu\text{mol}/\text{min}/\text{mg}$, but only $0.17 \pm 0.03 \mu\text{mol}/\text{min}/\text{mg}$ for (*S*)-1-phenyl-1,2-ethanediol formation (Table III).

Enantioconvergent Production of (*R*)-1-Phenyl-1,2-ethanediol

Since the His-*StEH* and His-EchA-I219F enzymes can hydrolyze (*S*)-styrene oxide and (*R*)-styrene oxide, respectively, to (*R*)-phenyl-1,2-ethanediol at high rates as well as high enantioselectivity, a mixture of purified His-*StEH* and His-EchA-I219F was used for hydrolyzing racemic styrene oxide. The ratio of the purified His-*StEH* and His-EchA-I219F to be used in the reaction system was based on the rate of pure styrene oxide hydrolysis by each enzyme, and the enantiomeric ratio based on substrate disappearance. To determine the rates with purified substrates, purified His-EchA-I219F or His-*StEH* was contacted with 5 mM pure (*S*)-styrene oxide and (*R*)-styrene oxide (note rates shown in Table III are with racemic mixtures). The rate of (*R*)-phenyl-1,2-ethanediol formation from (*S*)-styrene oxide by purified His-*StEH* was $18 \pm 1 \mu\text{mol}/\text{min}/\text{mg}$, while the rate of (*R*)-phenyl-1,2-ethanediol formation from (*R*)-styrene oxide by His-*StEH* was only $1.27 \pm 0.05 \mu\text{mol}/\text{min}/\text{mg}$. For purified His-EchA-I219F, the (*R*)-phenyl-1,2-ethanediol formation rate was $2.3 \pm 0.1 \mu\text{mol}/\text{min}/\text{mg}$ which is 10-fold faster than the (*S*)-phenyl-1,2-ethanediol formation from pure (*S*)-styrene oxide hydrolysis by this enzyme.

Using the rates of (*R*)-phenyl-1,2-ethanediol formation by the two enzymes as well as the ratio of enantiomeric ratio (40 ± 17 vs. 10.0 ± 0.9) as a guide, the amounts of His-*StEH* and His-EchA-I219F enzyme for the enantioconvergent

production of (*R*)-1-phenyl-1,2-ethanediol from racemic styrene oxide were 40 µg His-*StEH* and 200 µg His-EchA-I219F. Racemic styrene oxide (5 mM) was hydrolyzed by purified His-*StEH* and His-EchA-I219F to pure (*R*)-1-phenyl-1,2-ethanediol in 1 h with $99.9 \pm 0.1\%$ conversion and an enantiomeric excess of the product (*R*)-1-phenyl-1,2-ethanediol of $97.9 \pm 0.2\%$ (Fig. 4C). The initial rate of racemic styrene oxide hydrolysis was $4.7 \pm 0.3 \mu\text{mol}/\text{min}/\text{mg}$, which was corroborated by the production of (*R*)-1-phenyl-1,2-ethanediol at $4.7 \pm 0.7 \mu\text{mol}/\text{min}/\text{mg}$ (Table III). The enantiomeric ratio of the EH mixture toward racemic styrene oxide (calculated by monitoring substrate disappearance) was only 2.2 ± 0.3 , as expected, since each purified enzyme, His-EchA-I219F and His-*StEH*, reacted primarily with (*R*)- or (*S*)-styrene oxide, at the same time (Fig. 4D) (we recognize this ratio has little meaning for a dual enzyme system).

Stability of Styrene Oxide

TEM buffer and sonicated *E. coli* TG1 or BL21-Codon-Plus(DE3)-RIPL (no enzyme) were contacted with racemic styrene oxide (5 mM) to determine if styrene oxide decomposition impacts the rate of racemic styrene oxide hydrolysis by the EHs. Only 4.4% of racemic styrene oxide decomposed in TEM system after 45 min, and 10% decomposition occurred for both TG1 and BL21-Codon-Plus(DE3)-RIPL after 2 h of incubation. Because all the experiments performed by purified enzymes were reacted with substrates in the TEM system for less than 1 h and the experiments performed by sonicated cells were completed within 2 h, the decomposition of substrate had little impact on the reaction rates.

Inhibition of (*R*)-Styrene Oxide Hydrolysis by (*R*)-1-Phenyl-1,2-ethanediol With *StEH*

To investigate if (*R*)-styrene oxide hydrolysis was inhibited by the (*R*)-1-phenyl-1,2-ethanediol formed from racemic styrene oxide degradation with His-*StEH*, 2.5 mM (*R*)-1-phenyl-1,2-ethanediol and 2.5 mM (*R*)-styrene oxide were incubated together with purified His-*StEH*. The initial rate of (*R*)-1-phenyl-1,2-ethanediol production from (*R*)-styrene oxide was similar with the control at the beginning; however, the rate decreased by 36% after 15 min. Hence, (*R*)-styrene oxide hydrolysis by *StEH* is inhibited by the product (*R*)-1-phenyl-1,2-ethanediol; this causes the single potato enzyme to require large reaction times to completely convert racemic styrene oxide to (*R*)-1-phenyl-1,2-ethanediol (Fig. 4A) which is the motivation for using two enzymes.

DISCUSSION

We show clearly in this work that the mixture of EHs from a bacterial source (EchA-I219F) and a plant source (*StEH*)

produce (*R*)-1-phenyl-1,2-ethanediol rapidly (in 1 h) with both high yield ($99.9 \pm 0.1\%$) and high enantiomeric excess of (*R*)-1-phenyl-1,2-ethanediol ($97.9 \pm 0.2\%$). This process is an improvement over previous systems which utilize *StEH* alone but produce only 86% enantiomeric excess (*R*)-1-phenyl-1,2-ethanediol while requiring 15-h conversion time due to the slow rate of this enzyme for (*R*)-styrene oxide and the inhibition of (*R*)-1-phenyl-1,2-ethanediol (Fig. 4A) (Monterde et al., 2004). The EchA-I219F/*StEH* mixture is also better than the two fungal EH system, which produces only 89% enantiomeric excess and requires 2-h conversion time (Archelas and Furstoss, 1998).

The EchA-I219F was obtained from our previous work via active site engineering of the wild-type EchA by introducing the phenylalanine residue into position I219, which has van der Waals contact with both F108 and Y215. The substitution of phenylalanine for isoleucine shifts the position of (*R*)-styrene oxide to favor the enantioselectivity toward formation of (*R*)-1-phenyl-1,2-ethanediol (Rui et al., 2005). This enzyme is not suitable by itself for enantiomeric conversion since it converts (*S*)-isomer of the racemic substrate into (*S*)-1-phenyl-1,2-ethanediol of the racemic styrene oxide after consumption of (*R*)-isomer. EchA-I219F also does not completely convert racemic styrene oxide (50% yield, Fig. 4B) because the conversion of the (*S*)-isomer of the racemic substrate is inhibited by the (*S*)-diol that is formed (Rui et al., 2005). Hence, by using two enzymes, we overcame the problems of each and converted racemic styrene oxide rapidly to nearly pure (*R*)-1-phenyl-1,2-ethanediol (98% enantiomeric excess and 99% conversion). This prevents the loss of 50% of the substrate and allows an inexpensive substrate to be used. There is no chemical equivalent process known to date.

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