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Purification and Characterization of a Recombinant *Caulobacter crescentus* Epoxide Hydrolase

Seungha Hwang[1], Hyejin Hyun[2], Byoungju Lee[2], Youngseub Park[1], Eun Yeol Lee[3], and Chayong Choi[2]*

[1] Department of Chemical Engineering, Pohang University of Science and Technology, Pohang 790-784, Korea
[2] Interdisciplinary Program for Biochemical Engineering and Biotechnology, College of Engineering, Seoul National University, Seoul 151-742, Korea
[3] Department of Food Science and Technology, Kyungsung University, Busan 608-736, and The Marine and Extreme Genome Research Center, Korea

Abstract A *Caulobacter crescentus* epoxide hydrolase (CCEH) from a recombinant *Escherichia coli* was purified to homogeneity using a three-step procedure. The CCEH protein was purified 7.3-fold with a 22.9% yield in overall activity. The optimal reaction temperature and pH were determined to be 37°C and pH 8.0, respectively. The addition of 10% (v/v) dimethylsulfoxide as a cosolvent improved the enantioselectivity of CCEH for a batch kinetic resolution of racemic indene oxide.

Keywords: chiral styrene oxide, chiral indene oxide, *Caulobacter crescentus*, epoxide hydrolase, enzyme purification

INTRODUCTION

Enantiomerically pure epoxides and diols are valuable intermediates in the preparation of more complex enantiopure bioactive compounds such as pharmaceuticals and agrochemicals [1,2]. A number of chemical and biological routes have been developed to prepare enantiopure epoxides and diols [3]. In biological production routes, enantioselective hydrolysis of racemic epoxides using epoxide hydrolases (EHs), lipases, monooxygenases [4,5], and dehydrogenases [6] is expected to be commercially feasible because enantiopure epoxides with high enantiomeric purities can be obtained from relatively cheap and readily available racemic epoxides.

One of the biological routes for obtaining optically pure epoxides and their corresponding diols is enantioselective hydrolysis of racemic epoxides by EHs. EHs are cofactor-independent enzymes that can convert epoxides to their corresponding diols through the addition of a water molecule to the oxirane ring. The EHs from mammalian sources have been studied extensively due to their involvement in the metabolism of toxic xenobiotics [7]. The potential of mammalian EHs as chiral biocatalysts on a preparative scale is limited due to difficulties in cell culture, cloning, and heterologous expression of eukaryotic genes in a prokaryotic host. Several numbers of recombinant EHs from fungi and microorganisms have been reported recently [8-14]. The recombinant EHs are presently available from various sources, including yeast, fungi, and bacteria, offering an adequate supply of highly active recombinant EH biocatalysts to process chemists.

Many functionally unknown open-reading frames (ORFs) having sequence similarities with EHs have been found from various genome databases. As the structures of the mammalian, fungal, and microbial EHs have been determined during the past few decades [10,12,15], it is now known that EHs contain the characteristic domain of α/β hydrolase fold enzymes [16]. The putative EHs can be readily screened based on the conserved sequence information of the α/β hydrolase fold domains. The several genes of putative EHs have been screened from genome databases [9,17]. In our previous work, we screened the *Caulobacter crescentus* epoxide hydrolase (CCEH) from the several functionally unknown ORFs by genome data mining [17]. It is interesting that the enantiopreference of the CCEH toward styrene oxide in a whole cell reaction is opposite that of most of the bacterial epoxide hydrolases. It has been reported that most epoxide hydrolases are (*S*)-preferential, but only the whole cells of *Beauveria sulfurescens* and cell-free extract of *Solanum tuberosum* L showed (*R*)-preferential enantioselectivity to styrene oxide [15,16]. To the best of our knowledge, CCEH is the first reported bacterial EH that shows (*R*)-preferential enantioselectivity to styrene oxide. Additionally, CCEH is the first reported bacterial EH that is capable of enantioselectively hydrolyzing racemic indene oxide to form (1*S*, 2*R*)-indene oxide in a manner opposite to that of other fungal EHs [14]. Therefore, the purification and characterization of CCEH at the enzyme level is of significant interest. In this study, we purified recombi-

***Corresponding author**
Tel: +82-2-880-7071 Fax: +82-2-880-7071
e-mail: choicy@snu.ac.kr

nant CCEH in order to study its kinetic characteristics. The optimal reaction temperature, pH, and ratio of organic solvent were determined for batch kinetic resolution of styrene oxide and indene oxide using the purified recombinant CCEH.

MATERIALS AND METHODS

Materials

Indene oxide was synthesized from trans-2-bromo-1-indanol according to the previously reported method [18]. All other chemicals were of analytical or of reagent grade and were obtained from Sigma-Aldrich (MO, USA).

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 styrene oxide were determined by chiral GC using a β-DEX 250 column (60 m length, 0.25 mm I.D., 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 I.D., and 0.25 μm film thickness, Supelco Inc.). Absolute configuration was determined according to the previously reported method [19].

Purification of CCEH

The epoxide hydrolase gene from *C. crescentus* was cloned and overexpressed in *E. coli* as described previously [17]. All purification steps were carried out at 4°C and were monitored by SDS-PAGE. Column chromatography was performed using the ÄKTA FPLC system (Phamarcia). A cell pellet harvested from 1 L of *E. coli* culture broth was resuspended in 25 mL of 25 mM sodium phosphate buffer (pH 8.0) containing 0.2 mM EDTA and 10% (v/v) glycerol, and was then subjected to ultrasonic disruption for 10 min. The supernatant solution was obtained after centrifugation (17,000 g, 30 min.) and concentrated using an Amicon PM-10 ultrafiltration unit.

The concentrated crude extract was loaded on a butyl-Sepharose FF column (10 × 300 mm, Pharmacia, USA) pre-equilibrated with 20 mM sodium phosphate (pH 8.0) in 0.6 M ammonium sulfate (buffer A1). The proteins were eluted with 20 mM sodium phosphate buffer (pH 8.0, buffer B1) using a 0.6∼0.0 M reverse linear gradient of ammonium sulfate. Active fractions were concentrated using an Amicon PM-10 ultrafiltration unit.

The sample was applied to a DEAE-Sepharose column (5 × 72 mm, Pharmacia) pre-equilibrated with 20 mM sodium phosphate buffer (pH 7.0, buffer A2). The proteins were eluted with 20 mM sodium phosphate buffer in 1 M NaCl (pH 7.0, buffer B2) using a 0∼1 M linear gradient of sodium chloride. Active fractions were also concentrated using an Amicon PM-10 ultrafiltration unit.

The concentrated sample was applied to a HiPrep 16/60 Sephacryl S-200 HR column (Pharmacia) pre-equilibrated with 20 mM sodium phosphate buffer (pH 8.0, buffer A3) containing 150 mM sodium chloride. The proteins were isocratically eluted using the same buffer at a flow rate of 1.5 mL/min. The active fractions were concentrated using an Amicon PM-10 ultrafiltration unit.

The isoelectric point of CCEH was deduced from a pH 3∼9 isoelectric focusing (IEF) gel with a broad pI calibration kit (Pharmacia), and this electrophoresis was carried out using a Phast System (Pharmacia).

Enzyme Assay

The purified enzyme was suspended in 20 mM sodium phosphate buffer (pH 8.0) in 50 mL screw-cap bottles and incubated for 5 min at 37°C. The enantioselective reaction was started by the addition of substrate, and was carried out at 250 rpm in a shaking incubator. Samples were periodically withdrawn from the reaction mixtures, extracted with the same volume of ethyl acetate, and the organic extract was then analyzed by GC. One unit (U) is the amount of enzyme that catalyzes the degradation of 1 μmol of substrate per minute. Conversion was determined by the ratio of the remaining substrate concentration to the initial substrate concentration.

Effect of pH, Temperature on Enantioselective Hydrolysis

The effect of pH on enzyme activity was determined within a pH range of 6.0 to 9.0. The optimum temperature of the recombinant CCEH was determined at temperatures between 25 and 40°C. All experiments were carried out according to the procedure described in the enzyme assay section.

Cosolvent Effects on Kinetic Resolution of Styrene Oxide and Indene Oxide

The effect of the addition of cosolvent on the kinetic resolution of styrene oxide and indene oxide using purified CCEH was evaluated. The purified enzyme (2.5 U) was suspended in 20 mM sodium phosphate buffer (pH 8.0) containing various organic solvents at a concentration of 10% (v/v). The reaction was started by the addition of 10 mM substrate, and was carried out at 250 rpm in a shaking incubator. Samples were taken from the reaction mixtures at 30 min, extracted with the same volume of ethyl acetate, and the organic extract was then subjected to chiral GC.

RESULTS AND DISCUSSION

Purification of Recombinant CCEH

Recombinant CCEH was purified to homogeneity using the three-step procedure described in Materials and Methods. The overall purification factor and yield were

Table 1. Purification of recombinant *C. crescentus* epoxide hydrolase

Fractions	Total activity (U)	Yield (%)	Total protein (mg)	Specific activity (U/mg)	Purification factor
Crude extract	51,006	100.0	384.00	132.8	1.0
Butyl-Sepharose	17,008	33.3	41.28	421.1	3.2
DEAE-Sepharose	15,747	30.9	27.85	569.1	4.3
Sephacryl S-200 HR	11,682	22.9	11.92	980.4	7.3

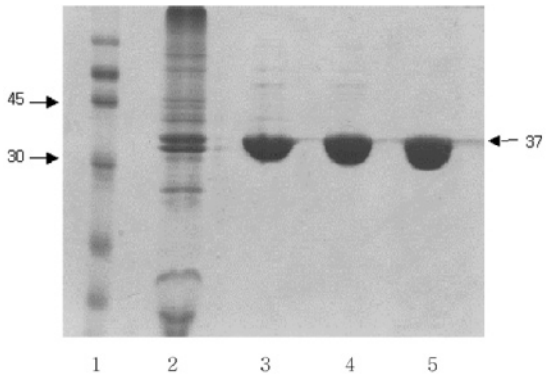

Fig. 1. SDS-polyacrylamide gel of pooled fractions during the purification of recombinant CCEH. Lane 1: marker with protein masses, lane 2: crude extract, lane 3: pooled Butyl-Sepharose, lane 4: pooled DEAE-Sepharose, lane 5: pooled Sephacryl S-200 HR.

7.3-fold and 22.9%, respectively (Table 1). The active pools were eluted with 67% buffer B1 in the butyl-Sepharose FF column procedure and 90% buffer B2 in the DEAE-Sepharose column procedure.

SDS-PAGE of the purified CCEH showed a single band after Coomassie blue staining (Fig. 1). The butyl-Sepharose column was the most efficient step, with a purification factor of 3.2. The two-step procedure without a DEAE-Sepharose column step, the least efficient step in the purification procedure, was carried out. However, the enzyme was not purified to homogeneity (data not shown). The isoelectric point of the recombinant CCEH determined by IEF was estimated to be 5.3, which is in good agreement with the calculated value of 5.12.

Determination of Optimal pH, Temperature, and Substrate/Enzyme Ratio

The theoretical maximum yield of EH-catalyzed production of enantiopure epoxides is 50%, limiting the commercial feasibility of the process and requiring the reaction conditions to be optimized. The effects of reaction conditions, reaction temperature, pH, substrate/enzyme ratio, initial substrate concentration, and cosolvent addition, on the enantioselectivity and yield were analyzed and optimized. As shown in Fig. 2A, the optimum temperature for enzyme activity was 37°C. The recombinant CCEH was prone to thermal inactivation at temperatures above 40°C. The enzyme was active in the

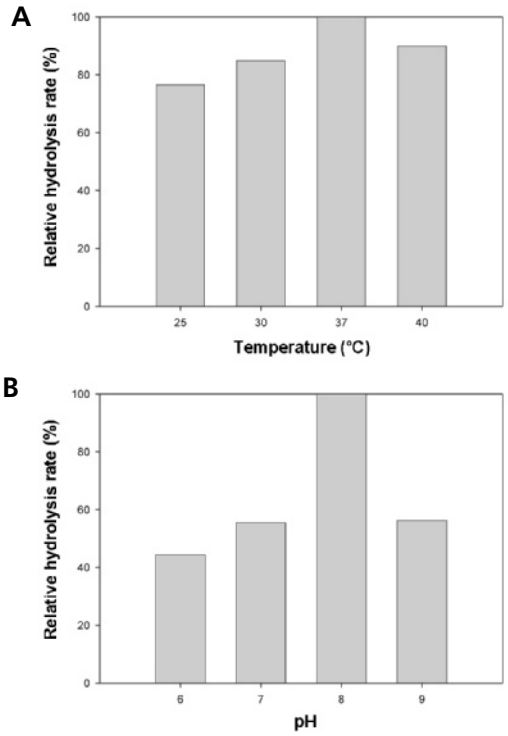

Fig. 2. Effect of reaction temperature (A) and pH (B) on the enantioselective hydrolysis of styrene oxide by purified recombinant CCEH.

pH range 6~9, with a maximum at pH 8.0 (Fig. 2B). The optimum pH and temperature of CCEH was similar to those of EH from *Aspergillus niger* strain LCP 521, with an optimum pH of 7 and an optimum temperature of 40°C [20].

The effect of the substrate/enzyme ratio on the reaction time required to reach 90% *ee* of the substrate at an initial substrate concentration of 10 mM was investigated to minimize the reaction time (Fig. 3). In the cases of kinetic resolution of 10 mM of either racemic styrene oxide or indene oxide, the reaction times required to reach 90% *ee* decreased markedly an enzyme concentration of around 20 U/mL. It was concluded that the optimal substrate/enzyme ratio was approximately 2 U/mL·mM substrate.

Effect of Initial Substrate Concentration on Enantioselective Hydrolysis

We investigated the initial substrate concentration on

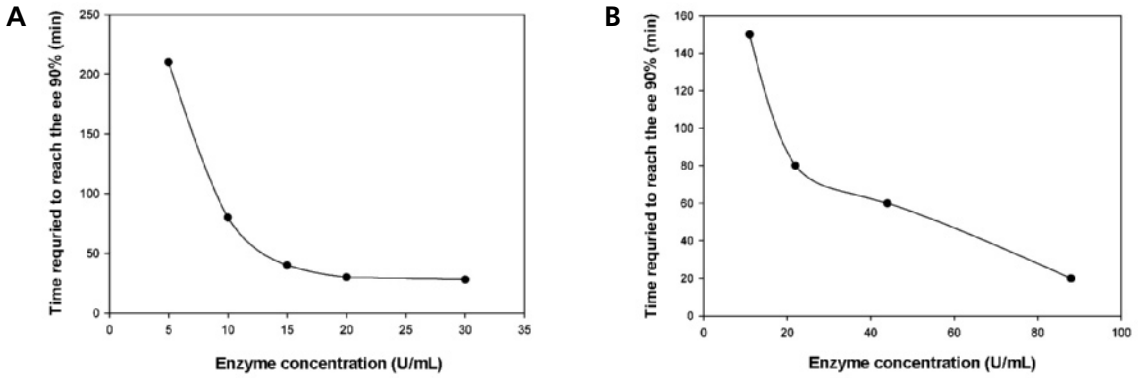

Fig. 3. Effect of the substrate/enzyme ratio on the kinetic resolution of styrene oxide (A) and indene oxide (B) by recombinant CCEH.

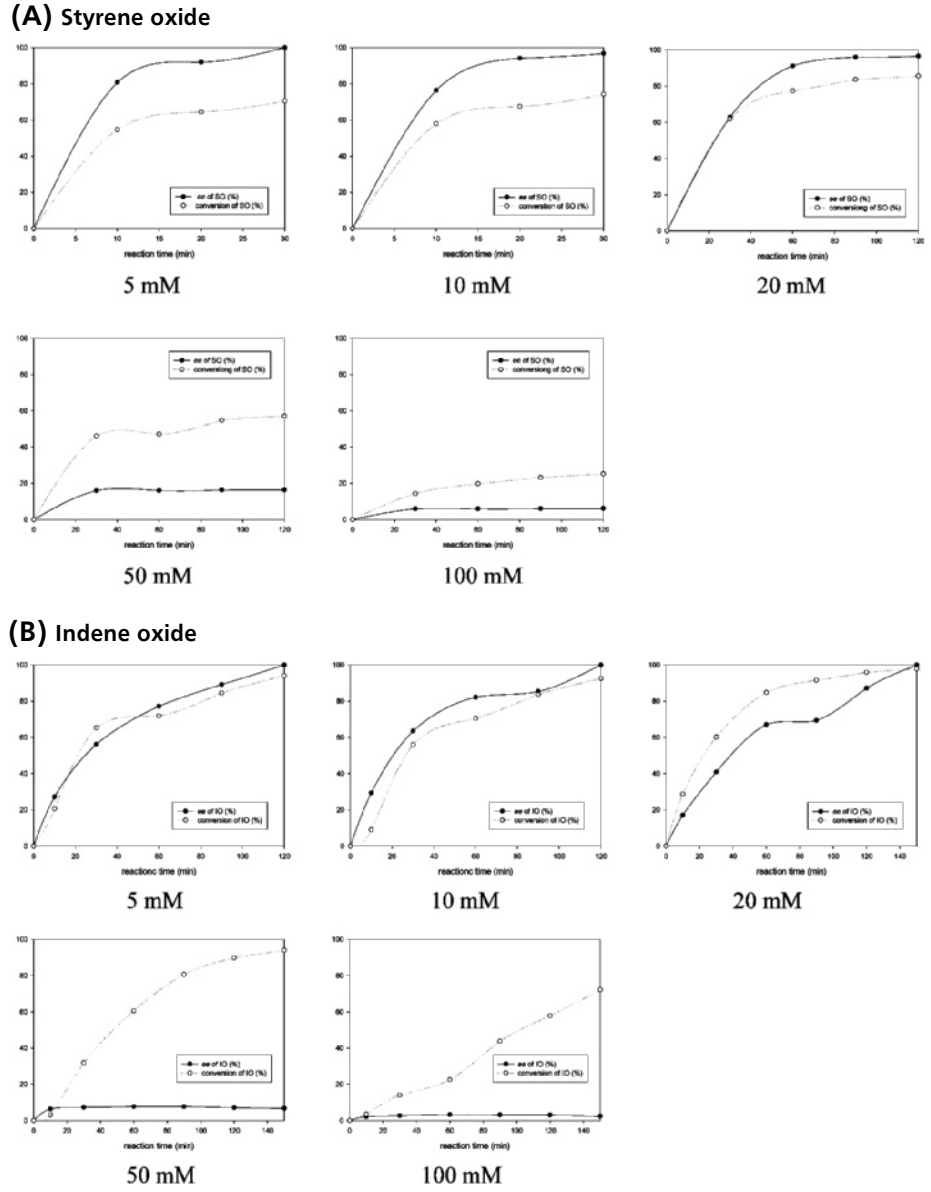

Fig. 4. Kinetic resolution of styrene oxide (A) and indene oxide (B) by recombinant CCEH (2.5 U) at various initial substrate concentrations.

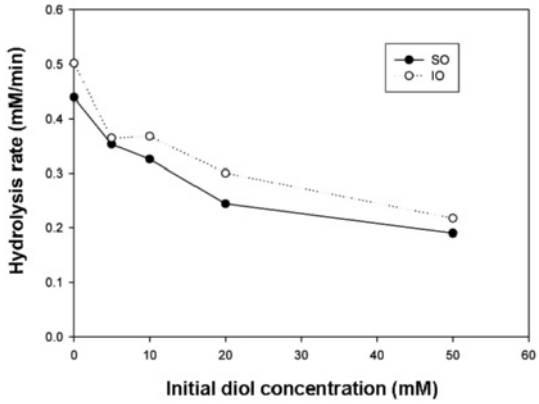

Fig. 5. Product inhibition of kinetic resolution of 10 mM styrene oxide (●) and 10 mM indene oxide (○) usingpurified recombinant CCEH (2.5 U) at 37℃ and pH 8.0.

the enantiopurity and yield of CCEH-catalyzed batch kinetic resolution of racemic styrene oxide and indene oxide. The hydrolysis reactions at various initial substrate concentrations ranging from 5 to 100 mM were preformed using the recombinant CCEH at 37°C and pH 8.0. As shown in Fig. 4, the high initial substrate concentration above 50 mM markedly impeded the progression of the CCEH-catalyzed-enantioselective hydrolysis reaction. Inhibition of the hydrolysis rate at a high substrate concentration was also observed when other EHs were used [19,21]. EHs are generally known to be sensitive to product inhibition. To confirm whether the inhibition at a high substrate concentration is caused by product inhibition, the initial hydrolysis rates were determined at various initial concentrations of the corresponding diols, ranging from 0 to 50 mM at 37°C and pH 8.0. As shown in Fig. 5, the initial hydrolysis rate of styrene oxide and indene oxide decreased radically when corresponding diol concentrations were increased above 10 mM.

Effect of Cosolvents on the Enzyme Activity

While addition of organic cosolvent can inhibit the activity of EH enzymes, it can improve the solubility and chemical stability of epoxide substrates, resulting in an increase in volumetric productivity. At a concentration of 10% (v/v), the effect of various cosolvents on the activity of recombinant CCEH was investigated, and results are shown in Fig. 6. In the case of batch resolution of styrene oxide, the recombinant CCEH retained its activity when methanol and dimethylsulfoxide (DMSO) were used as a cosolvent. However, the enantioselectivities (*E* value by Shi's equation [22]) were not improved or lowered (6.0 in aqueous buffer, 5.8 in MeOH and 5.5 in DMSO). The addition of ethanol and acetone as a cosolvent was inhibitory to CCEH. In contrast, in the case of indene oxide resolution, the enantioselectivity of CCEH toward indene oxide was slightly increased with DMSO (*E* value = 9.0) as a cosolvent. DMSO as a cosolvent slightly raised the enantioselectivity in the case of enantioselective resolution of indene oxide.

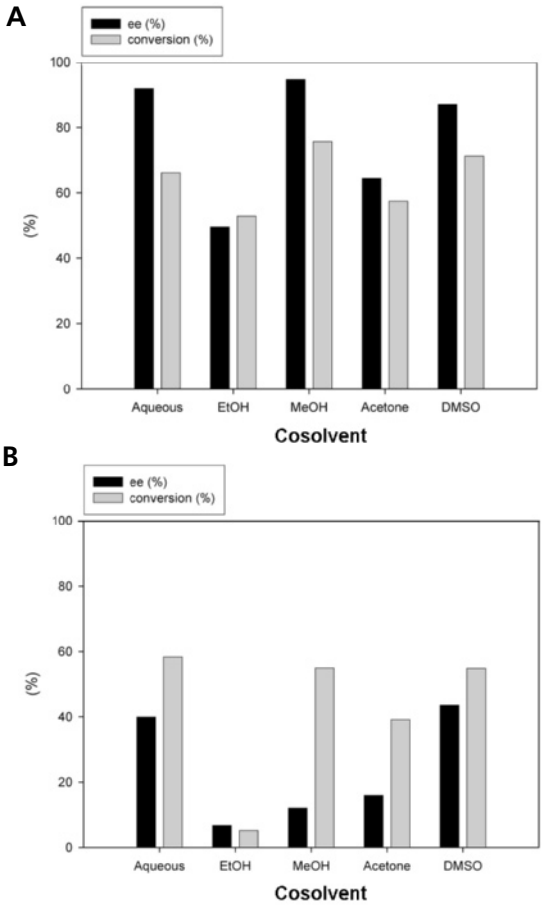

Fig. 6. Cosolvent effect on kinetic resolution of 10 mM styrene oxide (A) and 10 mM indene oxide (B) by recombinant CCEH (2.5 U) at 37°C and pH 8.0.

In the optimized conditions of enantioselective hydrolysis of racemic styrene oxide and indene oxide using purified recombinant CCEH, both the styrene oxide and indene oxide were optically purified with ~99% *ee* below the initial substrate concentration, 20 mM (Fig. 4).

CONCLUSION

Although a number of EHs have been purified for use as enzyme catalysts, purified EH loses some of its activity, especially membrane-associated EHs such as the EHs from *Rhodotorula glutinis* and *Rhodosporidium toruloides* [23,24]. The recombinant *C. crescentus* epoxide hydrolase (CCEH) was purified to homogeneity without loss of enzyme activity through simple three-step chromatography. The enzymatic characteristics of CCEH, including temperature optimum, pH optimum, isoelectric point, and product inhibition, were analyzed and optimized. This study demonstrated that the recombinant CCEH possessing the characteristic (*R*)-preferential-enantioselectivity toward racemic styrene oxide could be readily purified and used for large scale production of enantiopure styrene oxide and indene oxide.

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