Biocatalytic production of chiral epoxide: Epoxide hydrolase-catalyzed enantioselective resolution

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Abstract

A newly isolated *Aspergillus niger* possessing the novel epoxide hydrolase (EHase) activity was investigated for the enantioselective hydrolysis of racemic aromatic epoxides. The gene encoding EHase was cloned by RT-PCR, and molecular characteristics of the EHase gene were compared with other microbial EHases. The cloned gene encodes 398 amino acids with a deduced molecular mass of 44.5 kDa and pI of 4.83, and sequence homology with other microbial EHase was low. Functional recombinant EHase could be obtained by heterologous expressions in *E. coli*. Enantioselectivity of recombinant EHase was tested for valuable aromatic epoxide intermediates. Reaction conditions of EHase-catalyzed asymmetric resolution were optimized for the production of chiral styrene oxide.

Introduction

Enantiopure epoxides are important chiral synthons in organic synthesis and can be usefully used as key building blocks for the production of optically active bioactive compounds (1). Various chemical and biological methods have been developed for the production of chiral epoxide intermediates (2,3). In biocatalytic production routes, kinetic resolution of racemic epoxides based on enantioselective hydrolysis reaction by epoxide hydrolase (EHase) might be a very promising method because it is possible to obtain chiral epoxides with high optical purities from relatively cheap and readily available racemic epoxides (4). EHase is commercially potential biocatalyst because it is cofactor

independent and constitutive hydrolytic enzyme (5,6). Kinetic analysis and molecular characterization of microsomal EHase of mammalian cell have been investigated with attention to the role of microsomal EHase in the detoxification process of epoxides. Whereas microsomal EHase has some disadvantages in industrial application due to the difficulties in preparation of enzyme, microbial EHase can be used as industrial biocatalysts for the production of commercially important chiral epoxides. In this study, we isolated and characterized an *Aspergillus niger* strain possessing novel enantioselective hydrolysis activity toward racemic aromatic epoxide substrates. Enantioselectivity of EHase from *A. niger* was tested for valuable aromatic epoxide intermediates, and the reaction conditions of kinetic resolution have been analyzed and optimized. The gene encoding EHase of *A. niger* was cloned by RT-PCR and characterized at the molecular level. Recombinant EHase by functional expression in *E. coli* was developed and analyzed for their potentials for the application as biocatalysts in the biotransformation process for the production of chiral epoxides.

Materials and methods

Screening for EH activity in microorganisms was performed with racemic styrene oxide as a sole carbon source. The microorganism candidates were cultured on mineral salts medium supplemented with 20 mM styrene oxide or styrene as a sole carbon source (7). Wet cell pellets of styrene oxide or styrene-utilizing strains grown on the medium containing 20 g/l malt extract, 20 g/l glucose and 1 g/l peptone for 48 hr were suspended in 10 ml of 100 mM KH2PO4 buffer (pH 8.0) with 10 mM racemic styrene oxide. The rates of enantioselective hydrolysis of various strains were analyzed and a strain, *A. niger* LK, possessing the highest hydrolysis rates was selected for further investigation. Enantioselective resolution reaction was carried out at 30C, 250 rpm in shaking incubator.

The reaction mixture was extracted with equal volume of diethyl ether, and the organic layer was analyzed by chiral GC with fused silica capillary beta-DEX 120 column (30 m length, 0.25 mm ID, and 0.25 m film thickness, Sulpelco Inc.) and FID detector. The temperatures of column, injector, detector were 100, 220, 220C, respectively.

Total RNA was extracted from 1 ml of denaturing solution/g of mycelium (8). Reverse transcriptase (RT)-PCR for the generation of cDNA library was performed using random nonamer at 42C for 1 min. Two primers (5AN(start): 5-atgtccgctccgttcgccaagtttccctcg-3′ , 3AN(stop): 5′ -ctacttctgccacacctgctcgacaaatgc-3′) were used to amplify the gene encoding EH with Taq polymerase over 30 cycles (30 sec at 94C, 30 sec at 55C, and 2 min at 72C). The cloned gene was inserted into pET for sequence analysis and pRSET A vector for expression in *E. coli*.

For functional expression of the cloned EH gene in *E. coli*, PCR fragment of *A. niger* EH was ligated into the *XhoI/KpnI* site of pRSET. The recombinant pRSET/EH plasmid was transformed into *E. coli* BL21. The recombinant *E. coli* was grown on LB medium with ampicillin, and recombinant EH expression was induced by addition of beta-D-thiogalactose at late exponential growth phase. After 3 h, the cells were harvested, resuspended in buffer (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.4), disrupted and then the resulting solution was subjected to SDS-PAGE analysis. The proteins were stained with 0.1 %(w/v) Coomassie Blue.

Results and discussion

Various microorganisms were tested for their ability to utilize aliphatic alkene such as hexane, heptene, or octene as a sole carbon source. Alkenes are converted to a corresponding epoxide by monooxygenase and then degraded by epoxide hydrolase. The microorganisms possessing the degradation pathway of alkenes, therefore, might have an epoxide hydrolase activity. Epoxide hydrolase activity was analyzed by enantioselective hydrolysis of racemic styrene oxide. A novel *A. niger* LK strain was isolated based on its enantioselective hydrolysis of racemic styrene oxide with high enantioselectivity. *A. niger* LK was further tested for various aromatic epoxide substrates. As shown in Figure 1, the epoxide hydrolase from *A. niger* LK has a broad substrate specificity. Epoxide hydrolase of *A. niger* showed (R)-specific hydrolysis activity for styrene oxide and p-nitrostyrene oxide. (S)-specific hydrolysis, however, occurs when epoxides containing oxygen atom at C3 position were used as substrates, which showed that enantioselectivity of epoxide hydrolase from *A. niger* LK

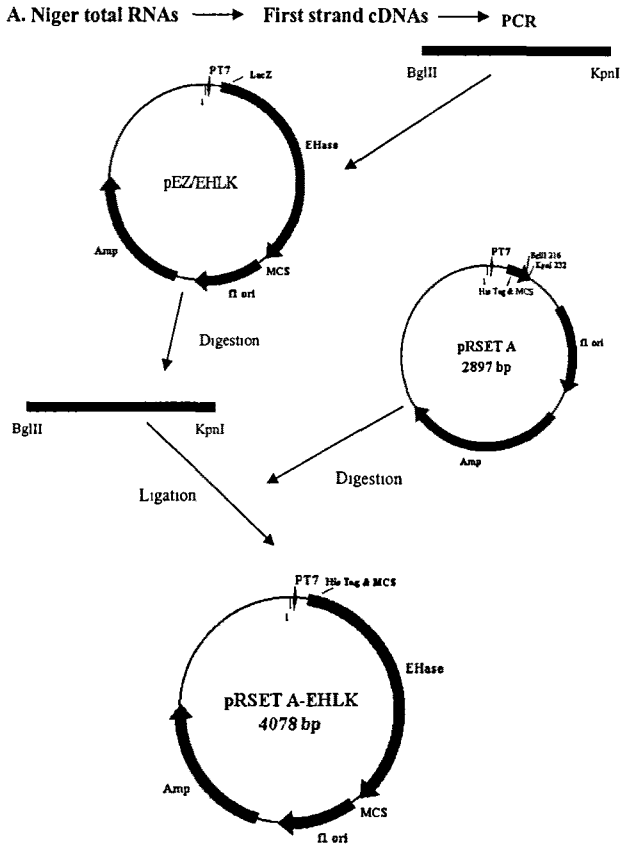


Fig. 1. Cloning of *A. niger's* EHase from total RNAs by RT-PCR in pRSET A expression vector.

depends highly on the structures of epoxide substrates. From these results, commercially important epoxides such as styrene oxide could be easily obtained using epoxide hydrolase from *A. niger* LK with excellent optical purity and reasonable yield.

cDNA library was prepared from the total mRNA using reverse transcriptase (RT)-PCR. The gene encoding epoxide hydrolase was amplified by *Taq* DNA polymerase with primer designed according to the 5- and 3-sequence from literature. This fragment was cloned into *XhoI/KpnI* site of pRSET vector. The cDNA gene encoding epoxide hydrolase contained 1197-bp open reading frame with a calculated molecular mass of 44.5 kDa and pI of 4.83. The sequence of 398 amino acids was compared with database sequence, and showed highest

homology up to 86% with epoxide hydrolase from *A. niger* LCP521 (8). Sequence analysis showed 45%, 35% and 28% identity with epoxide hydrolase from rabit, *Rhodotorula glutinis* and *Agrobacterium radiobacter*, respectively.

Epoxide hydrolase-encoding cDNA was expressed in *E. coli* BL21(DE3). The expression of epoxide hydrolase was achieved by induction with isopropyl beta-D-thiogalactoside (IPTG). The epoxide hydrolase activity was measured by sodium dodecyl sulphate/polyacrylamide gel electrophoresis with crude bacterial lysates. SDS-PAE showed a expected band of 44.5 kDa protein, but not much intense protein band. Previously, epoxide hydrolase gene from *A. niger* LCP521 and *R. glutinis* could not be successfully expressed in *E. coli*,

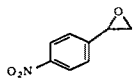
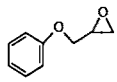
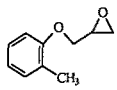
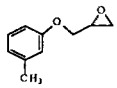
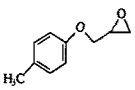
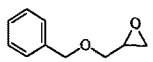
Epoxide	Initial conc. (mM)	ee (%)	Absolute configuration	Yield (%)
	10	100	(S)	30
	4	100	(S)	35
	10	100	(R)	27
	10	100	(R)	25
	10	100	(R)	18
	10	100	(R)	20
	10	100	(R)	22
	10	100	(R)	3

Fig. 2. Enantioselective hydrolysis of various aromatic epoxides by *A. niger* LK. Hydrolysis of racemic epoxide was performed in 10 ml 100 mM phosphate buffer (pH 8.0) with 300 mg dried powder of *A. niger* LK.

while epoxide hydrolase of *A. radiobacter* AD1 have been expressed up to 40% of the total protein in *E. coli* BL21, which clearly represented that functional overexpression system for epoxide hydrolase from eukaryotic cells should be developed (9,10). Resting cells of recombinant *E. coli* was tested for its ability of enantioselective hydrolysis of racemic styrene oxide substrate. Whereas no epoxide hydrolase activity could be detected using wild-type *E. coli* strain without recombinant plasmid encoding *A. nigers* epoxide hydrolase, enantioselective hydrolysis was observed with kinetic resolution using recombinant *E. coli* strain. Enantioselectivity of recombinant EHase was tested for valuable aromatic epoxide intermediates. The recombinant *E. coli* showed the same enantioselectivity for aromatic epoxide substrates as the epoxide hydrolase from *A. niger* LK.

Enantioselective hydrolysis reaction conditions including reaction temperature, pH and cosolvent concentration were optimized using surface response optimization. The optimal conditions were determined to be 28.3°C, 7.8, and 2.4% (v/v), respectively. Racemic styrene oxide of 50 mM could be enantiomerically resolved with almost 100 % ee and 35% yield (theoretical yield = 50%).

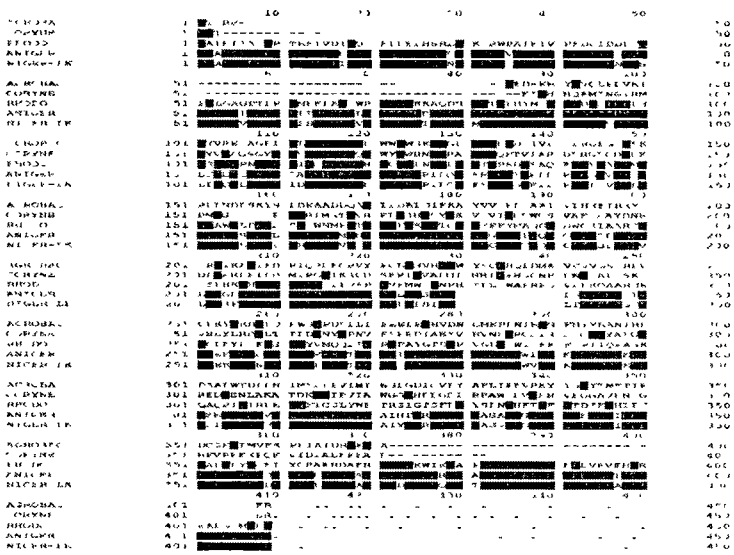


Fig. 3. Sequence alignment of the cloned EHase with fungi, yeast and bacterial EHase (Amino acid sequences of *A. niger* LK, *A. niger* LCP521, *R. glutinis* and *A. radiobacter* are aligned from top to bottom line).

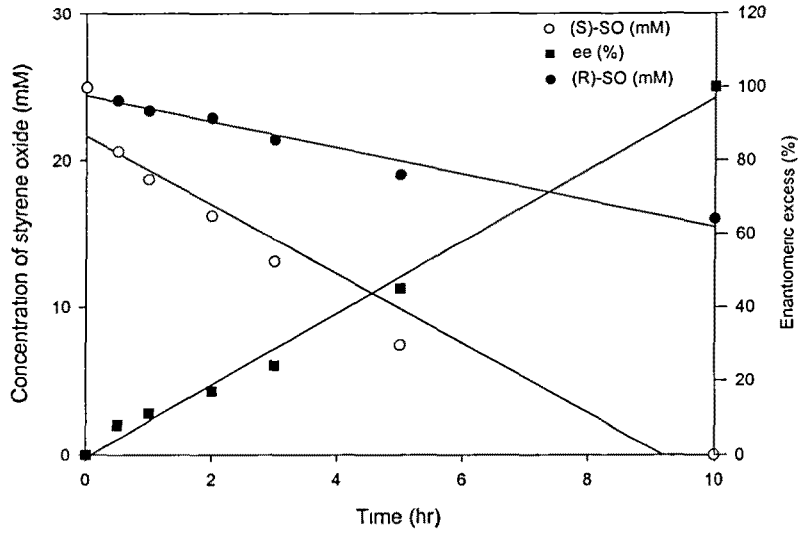


Fig. 4. Microbial enantioselective hydrolysis for the production of (S)-styrene oxide using the epoxide hydrolase activity of *A. niger* LK power. (Hydrolysis of racemic styrene oxide in 10 ml 100 mM phosphate buffer with 300 mg dried powder of *A. niger* LK)

From the sequence comparison to epoxisde hydrolase from *A. niger* LCP521 and microsomal epoxide hydrolase, epoxide hydrolase of *A. niger* LK was predicted to have a putative catalytic triad with Asp(192), His(374) and Asp(348) (8). Different enantioselectivity of epoxide hydrolase from *A. niger* LK depends on difference in amino acid sequence. Protein engineering of recombinant epoxide hydrolase based on the comparison of amino acid sequence, catalytic activity and substrate specificity can offer tailor-made catalytic activity, and overexpression of such recombinant epoxide hydrolase provides a commercially potential biocatalyst for the production of novel chiral epoxides.

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