Catalysis Communications 16 (2011) 133–139

Contents lists available at SciVerse ScienceDirect

Catalysis Communications

journal homepage: www.elsevier.com/locate/catcom

Short Communication

Characterization of a newly synthesized epoxide hydrolase and its application in racemic resolution of (*R*,*S*)-epichlorohydrin

Zhi-Qiang Liu, Li-Ping Zhang, Feng Cheng, Li-Tao Ruan, Zhong-Ce Hu, Yu-Guo Zheng *, Yin-Chu Shen

Institute of Bioengineering, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China
Engineering Research Center of Bioconversion and Biopurification, Ministry of Education, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China

ARTICLE INFO

Article history:
Received 17 July 2011
Received in revised form 1 September 2011
Accepted 12 September 2011
Available online 20 September 2011

Keywords:
Epoxide hydrolase
Characterization
Racemic resolution
Epichlorohydrin

ABSTRACT

In the current study, an epoxide hydrolase (EH) gene from *Rhodosporidium toruloides* was synthesized and expressed in *Escherichia coli*. After purification, we found that the optimal pH and temperature of this enzyme were 7.5 and 35 °C, respectively. The recombinant EH obtained in this study was temperature-sensitive and the activity decreased significantly above 45 °C. The values of apparent K_m and V_{max} were 0.5953 mol/l and 0.0105 mol/(L·min). In addition, enantiomeric excesses value of (*R*)-epichlorohydrin could reach 100% after 40-min reaction. Moreover, this EH showed a broad substrates specificity toward epoxides. To the best of our knowledge, this is the first time to report the application of *R. toruloides* EH in racemic resolution of (*R*,*S*)-epichlorohydrin to produce (*R*)-epichlorohydrin.

1. Introduction

Epoxide hydrolases (EHs, EC 3.3.2.x) catalyze the hydrolysis of epoxides to corresponding vicinal diols with the addition of a H_2O molecule, without any cofactors and metal ions [1,2]. EHs have been found in various sources [3–6] and most of the EHs belong to the α/β hydrolase fold family [7,8]. Previous studies demonstrated that microbial EHs had a broad substrate specificity, high enantioselectivities, and reaction rates, which has received more attention due to its promising potential for the enantioselective resolution of racemic epoxides [3,9–11]. Recently, the discovery and production of EHs in microorganisms allowed applying EHs to a large scale.

Enantiopure epoxides are key intermediates of many bioactive compounds [12,13]. As a promising enantiopure epoxide, (*R*)-epichlorohydrin was widely used for synthesis of β-blockers, *L*-cartine, and ferroelectric liquid crystals [14,15]. Choi et al. have reported the preparation of chiral epichlorohydrin in organic solvents using *Aspergillus niger* cells harboring EH, the enantiomeric excesses (*e.e.*) value of (*R*)-epichlorohydrin could reach 100% and yield was around 18% [16]. *Rhodotorula glutinis* EH has been cloned by Kim's group and applied into resolution of (*R*,*S*)-epichlorohydrin to provide (*R*)-epichlorohydrin with 25% yield [17]. However, the yield and *e.e.* with existed EHs are low and not desirable for industrial production in large scale by far [12]. Therefore, finding new EHs or employing engineering enzymes is getting more and more attraction in the production of (*R*)-epichlorohydrin [18]. With the recent development in bioinformatics and gene engineering, it is possible to obtain novel EHs by analysis of putative EHs sequence and activity information [18,19].

To explore the production of (*R*)-epichlorohydrin by biological method, we synthesized an EH gene from *Rhodosporidium toruloides* CBS14 and then successfully expressed it in *Escherichia coli.* After purification, the catalytic properties of the recombinant EH and its application in racemic resolution of (*R*,*S*)-epichlorohydrin were investigated in this study.

2. Materials and methods

2.1. Strains and plasmid

E. coli BL21(DE3) was used as a host to express recombinant EH. pET28b(+) was used as vector for the expression of EH gene in *E. coli* BL21 (DE3). *E. coli* transformants were grown in Luria–Bertani (LB) medium. All the chemicals used were of analytical grade and commercially available.

2.2. Expression and purification of EH

A nucleotide sequence of EH from *R. toruloides* CBS14 (Genbank accession No. AAN32662) [20] with a length of 1227 bp was synthesized using PCR assembly method [21] after optimization of the codons by a gene designer software against *E. coli* as host [22]. The synthesized gene with 6× His-tag was inserted into expression vector pET28b(+) between *Nco*I and *Xho*I restriction endonuclease sites. The ligated plasmid pET28b-EH was transformed into *E. coli* BL21 (DE3).

* Corresponding author. Tel./fax: +86 571 88320630.
E-mail address: zhengyg@zjut.edu.cn (Y.-G. Zheng).

1566-7367/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.catcom.2011.09.010

Permanent frozen stock of *E. coli* cells BL21(DE3) harboring the plasmid pET28b-EH was used to inoculate into 50 ml LB broth medium containing 50 μg ml^{-1} Kanamycin (Kan), and cultures were grown at 250 rpm and 37 °C. When the cultures reached optical densities of 0.6–0.8 at 600 nm, the temperature was lower to 28 °C, and isopropyl-1-thio-β-D-galactoside (IPTG) was added to a final concentration of 0.1 mM. After 7 h induction, the cells were harvested by centrifugation at 9,000 rpm for 20 min.

All purification steps were carried out at 4 °C. The wet cell paste was disrupted by sonication in buffer (20 mM NaH_2PO_4, pH 8.0; 300 mM NaCl). And then the cell debris was removed by centrifugation at 12,000 rpm for 20 min. Subsequently, the soluble fraction was loaded onto a Nickel-NTA superflow column (10 ml) equilibrated with a binding buffer (20 mM NaH_2PO_4, pH 8.0; 300 mM NaCl). The column was eluted with 2 volumes of washing buffer (20 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 50 mM imidazole) and unbounded proteins were washed out from the column. Later, the expected protein was eluted from the column with an elution buffer (20 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 500 mM imidazole). These proteins were analyzed using a 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.3. SDS-PAGE

Enzyme fractions and the molecular mass under denaturing conditions were determined by SDS-PAGE performed using a Mini-gel system (Bio-Rad, Hercules, USA). SDS-PAGE was performed according to Laemmli's discontinuous Tris–glycine buffer system [23]. Proteins in the gel were stained with Coomassie brilliant blue R-250 for 1 h, and then destained with 0.5 M NaCl aqueous solution.

2.4. Influence of factors on EH and its substrate specificity

The effects of pH, temperature and chemicals on the EHs were systematically investigated. The optimal pH for the reaction was determined with 100 μl epichlorohydrin in a 10 mL reaction system at 30 °C, in a pH range of 3.0–9.0 using citric acid–sodium citrate buffer (pH 3.0–6.0), K_2HPO_4–KH_2PO_4 buffer (pH 6.0–8.0) and Tris–HCl buffer (pH 7.0–9.0). The optimal temperature of the enzyme was determined by assaying enzyme activities at temperatures ranging from 25 to 55 °C. Thermal stability was investigated by incubating enzymes at temperatures ranging from 35 to 65 °C in 10 mL of 100 mM phosphate buffer at pH 7.5 in water bath. The enzyme activities were assayed at 30 °C after incubation at regular time intervals (30–600 min). The residual activity was calculated by comparing final activity to initial activity of EHs before incubation. The half-life of EH at each temperature was calculated by plotting the natural logarithm of residual activity (ln RA) at each temperature against time. The effects of different chemicals were assessed by suspending 0.01 g of purified EH in 9 mL of 100 mM phosphate buffer at 30 °C with series of metal ions (final concentration of 5 mM) and EDTA (final concentration of 10 mM) in 10 ml of pH 7.5 buffers containing 100 μl epichlorohydrin. The substrate specificity of the EH was evaluated using different epoxides, each presented at a concentration of 20 mM.

2.5. Enzyme assay

0.01 g recombinant EH was added into 10 mL phosphate buffer (pH 7.5) containing 100 μl epichlorohydrin. The reaction was carried out at 30 °C for 10 min in a thermomixer (Eppendorf, Hamburg, Germany). After reaction, 1.0 ml biotransformation sample was taken and centrifuged at 12,000 rpm for 5 min and then 200 μl of supernatant was extracted with 800 μl of ethyl acetate and centrifuged at 10,000 rpm for 5 min. The organic layer was taken and dried by anhydrous sodium sulfate. 1 μl of the reaction mixture was analyzed by GC and chiral GC for enzyme activity and *e.e.*, respectively. The GC system was equipped with a capillary HP-5 column (0.35 mm ID × 30 m, 0.25 μl film thickness) and a FID detector with N_2 as carrier gas. And then the 1 μl organic layer was subjected to chiral GC analysis to calculate the *e.e.* value. The chiral GC system was equipped with a chiral capillary BGB-175 column (0.25 mm ID × 30 m, 0.25 μl film thickness) fitted with a FID detector and He was used as carrier gas. The temperatures of the oven, injector, and detector for analysis of racemic epichlorohydrin were 90, 220, and 220 °C, respectively. The retention times of racemic epichlorohydrin, (*R*)-epichlorohydrin and (*S*)-epichlorohydrin were 2.70, 5.69, 5.50 min, respectively.

One unit of EH activity was defined as the amount of enzyme required to convert 1 μmol (*S*)-epichlorohydrin at 30 °C. Specific enzyme activities were defined as units per mg enzyme.

2.6. Determinations of kinetics parameters

The values of K_m and V_{max} for the EH were determined by assaying purified enzyme at increasing substrate concentrations ranging from 0.2% to 2.0% (v/v). The temperature, pH and quantity of the enzyme were kept the same as the standard enzyme activity assay described above. Apparent K_m and V_{max} were calculated according to Lineweaver–Burk plots.

2.7. Homology modeling and docking

The three-dimensional homology model of EH was generated using Build Homology Models (MODELER) in Discovery Studio (DS) 2.1 (Accelrys Software, San Diego, USA) using crystal structures of EH from *A. niger* at 1.8 Å resolution (PDB accession code 1QO7) and crystal structure of EH from *Agrobacterium radiobacter* AD1 at 2.1 Å resolution (PDB accession code 1EHY) as templates. The generated structures were improved by subsequent refinement of the loop conformations by assessing the compatibility of an amino acid sequence to known PDB structures using the Protein Health module in DS 2.1. The geometry of loop regions was corrected using Refine Loop/MODELER. Finally, the best quality model was chosen for further calculations, molecular modeling, and docking studies by Autodock 4.0 [24].

3. Results

3.1. Expression of EH gene

Based on the reported nucleotide sequence, the *R. toruloides* EH gene was synthesized after optimization of the codons. Subsequently, the EH gene and expression plasmid pET28b were treated with restriction

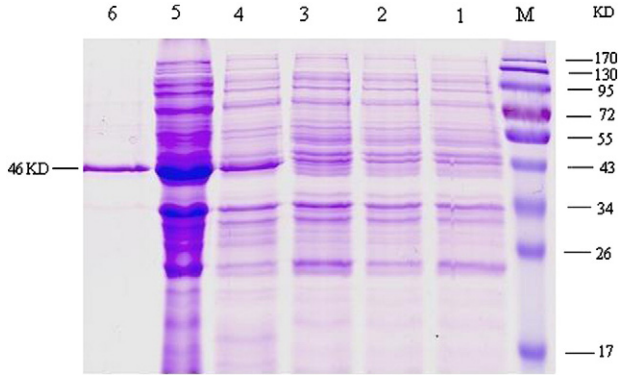


Fig. 1. SDS-PAGE analysis of recombinant EH. Lane M, the protein size standard; Lane 1, *E. coli* BL21(DE3); Lane 2, *E. coli* BL21(DE3)/pET28b; Lane 3, uninduced *E. coli* BL21 (DE3)/pET28b-EH; Lane 4, *E. coli* BL21(DE3)/pET28b-EH induced by IPTG; Lane 5, crude extract; Lane 6, the purified EH.

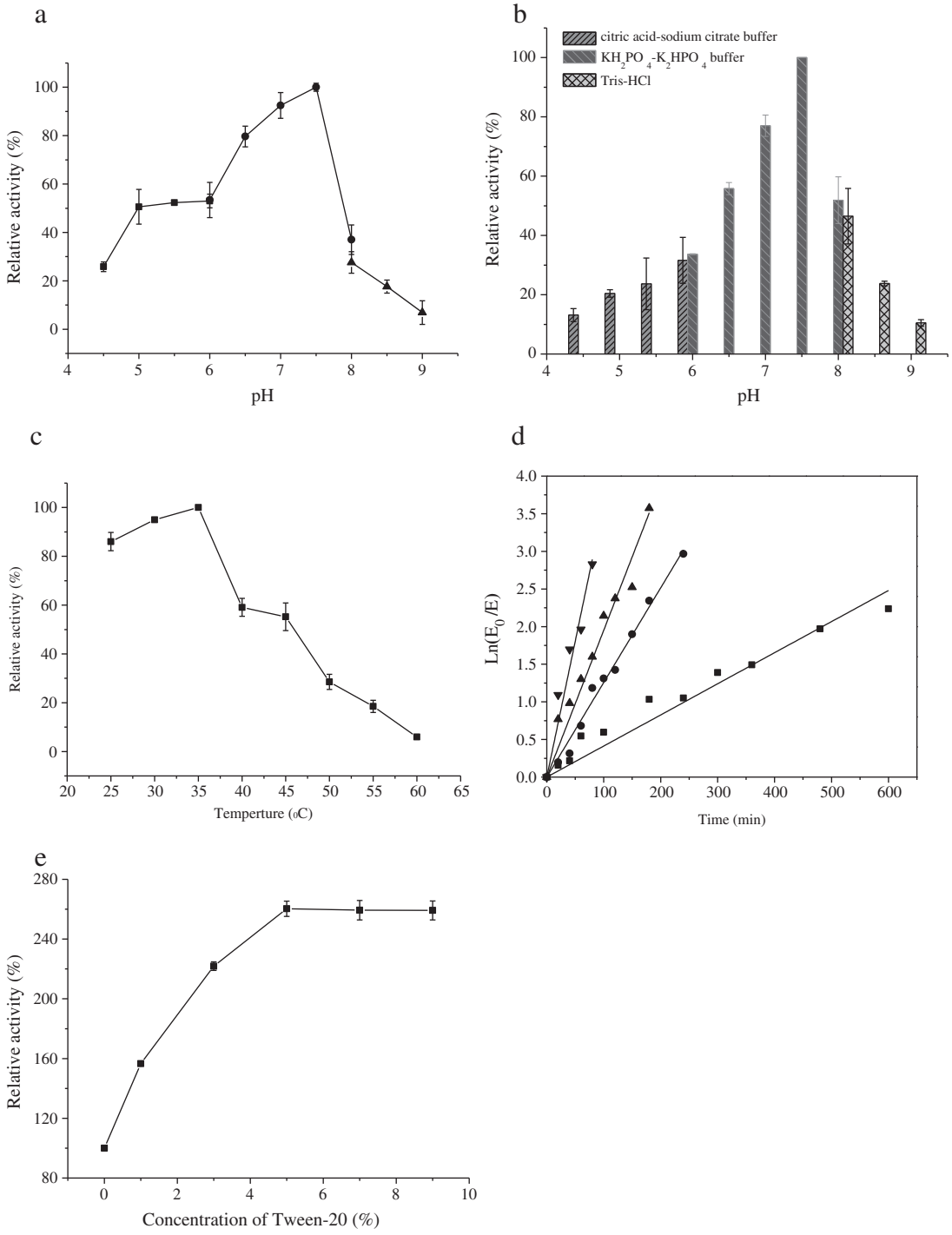


Fig. 2. a) Effect of pH on epoxide hydrolase activity. b) pH stability of epoxide hydrolase. The purified EH was pretreated in different buffers with pH ranging from 4.5 to 9.0 at 30 °C for 1 h, and then the EH activities were determined according to the standard enzyme assay method. The activities of enzymes that assayed under the standard reaction were taken as 100%. c) Effect of temperature on epoxide hydrolase activity. d) The thermo-stability of epoxide hydrolase activity. The purified enzymes were incubated at different temperatures for 30–600 min, and then the EH activities were determined according to the standard enzyme assay method. The activities of enzymes that assayed under the standard reaction were taken as 100%. ■ the liner of 25 °C; ● the liner of 35 °C; ◆ the liner of 45 °C; ▼ the liner of 55 °C. e) Effect of Tween-20 on the activity of epoxide hydrolase. Tween-20 with concentration from 0 to 9% was added to the reaction mixture, and then the EH activities were determined according to the standard enzyme assay method. The activities of enzymes that assayed under the standard reaction without addition of Tween-20 were taken as 100%.

endonucleases of *Nco*I and *Xho*I, and ligated by T4 DNA ligase to construct recombinant plasmid. And then the recombinant plasmid was transformed into *E. coli* BL21 (DE3) and several clones were selected. The recombinant plasmids were extracted and further sequenced to verify the existence of the EH gene. The positive transformant harboring recombinant pET28b-EH was cultivated and induced by 0.1 mM of IPTG. The cells were harvested and disrupted by sonication, after which the recombinant EH was purified by His-tag-affinity chromatography. The SDS-PAGE analysis (Fig. 1) clearly shows that a band with molecular mass of around 46 kDa on Lane 6 is obtained, which is in agreement with the predicted value based on the amino acid sequence of EH. The specific activity and amount of protein of the recombinant EH were calculated to be 113.1 U/mg and 0.79 g/l, respectively.

3.2. Influence of factors on EH and its substrate specificity

3.2.1. Effect of pH on EH activity

Fig. 2a shows that the maximum activity of the EH is around pH 7.5, but the lowest enzyme activity was found at pH 9.0. Moreover, a rapid decrease in the enzyme activity was observed under acidic and basic conditions, while neutral pH condition seemed to favor the enzyme activity. To study the pH stability, EH was pretreated in different buffers with pH ranging from 4.5 to 9.0 at 30 °C for 1 h and the results showed that the EH was stable at pH 7.5. In addition, pH higher or lower than 7.5 resulted in the significant decrease in the relative activity of EH, and only 10% of the residual enzyme activity was left at pH 9.0 (Fig. 2b), which showed that the pH stability of this enzyme is not satisfied and needs further improvements.

3.2.2. Effect of temperature on EH activity

Fig. 2c shows that the activity gradually increased with the increasing of temperature from 20 to 35 °C, and reached its maximum at 35 °C, which indicates that the optimal temperature of the EH is around 35 °C. After this apex point, the activity sharply decreased. Only negligible EH activity was observed at 60 °C. In order to test the thermo-stability of EH, the purified enzyme was incubated at various temperatures (25–55 °C) for various times and then residual activity was determined according to the standard enzyme assay method. The residual activity was obtained in accordance with the activity at the 0th h and its natural logarithm value (Ln E_0/E) was plotted against time. Fig. 2d shows the results of the thermo-stability of EH. The *K*d of the enzyme for temperatures at 25, 35, 45, and 55 °C were 0.0041, 0.0126, 0.0195, and 0.0362 min^{-1}, respectively. Accordingly, the half-life ($t_{1/2}$) of the purified enzyme at 25, 35, 45, and 55 °C were calculated to be 2.820, 0.917, 0.592, and 0.319 h, respectively, which demonstrates that this enzyme was not thermo-stable.

3.2.3. Effect of metal ions and other reagents on EH activity

To investigate the effects of the metal ions and chemicals on EH activity, 0.01 g of the purified EH was preincubated according to the method described in Materials and methods section 2.4. The results showed that EH activity was slightly inhibited by Zn^{2+} and strongly inhibited by Hg^{2+}, Fe^{2+}, Ag^{+}, Ba^{2+}, Mg^{2+}, Al^{3+}, Cu^{2+} and Mn^{2+}. Moreover, EDTA, Co^{2+}, Ca^{2+} and Ni^{2+} did not show any significant effects on the EH activity (Table 1). However, it appears that there are no metal ions tested in this study that could activate EH activity, which illustrated that the EH obtained in the current study was not metal ion-dependent in terms of performing its catalysis.

3.2.4. The effect of lyoprotectants and detergents on EH activity

It is reported that lyoprotectants and detergents have a very important effect on the EH activity [25]. In this study, the effects of lyoprotectants and detergents including SDS, Tween-20, Tween-80 and *D*-sorbitol on the EH activity were also investigated, the results showed that the addition of Tween-20 has a positive effect on EH activity for the production of (*R*)-epichlorohydrin because of a correlation between the effects of the Tween-20 and the substrate used. Further investigation illustrated that enzyme with 5% of Tween-20 exhibited the highest hydrolysis activity. However, further increase in the concentration of Tween-20 did not improve the enzyme activity.

Table 1
Effect of metal ions and other reagents on epoxide hydrolase activity.

Chemicals	Concentration (mM)	Relative activity (%)
Control		100
EDTA	10	106.0 ± 2.1
$HgCl_2$	5	0
$AlCl_3$	5	55.0 ± 3.3
$AgNO_3$	5	51.5 ± 1.7
$ZnCl_2$	5	83.1 ± 2.6
$BaCl_2$	5	50.7 ± 1.3
$MgCl_2$	5	42.9 ± 2.9
$CaCl_2$	5	101.7 ± 3.1
$NiCl_2$	5	100.2 ± 2.0
$CoCl_2$	5	104.5 ± 2.7
$CuCl_2$	5	31.3 ± 2.1
$FeCl_2$	5	45.9 ± 1.3
$MnCl_2$	5	63.8 ± 1.8

3.2.5. Substrate specificity

Table 2 shows the hydrolytic activity of the recombinant EH towards a set of substrates. The purified enzyme had no activity towards 1,3-chlorophenyl glycidol ether, 2-[(4-chlorophenoxy)methyl] oxirane and oxirane-2,3-dicarboxylic acid, and very low activity with 2-[(4-methylphenoxy)methyl]oxirane, but showed high activity with 2-(chloromethyl)oxirane, 2-[(benzyloxy)methyl]oxirane, and 2-[(2-methylphenoxy)methyl]oxirane. The substrate specificity study demonstrated that this recombinant enzyme could be explored for the productions of multiple diols with high values, besides enantiopure (*R*)-epichlorohydrin.

3.3. Determination of kinetic parameters

The catalytic efficiency on (*S*)-epichlorohydrin hydrolysis and associated kinetic parameters of the recombinant EH were measured using Lineweaver–Burk plots. The (*R*)-epichlorohydrin productivity was determined at concentrations of (*R*,*S*)-epichlorohydrin from 0.2% to 2% and the Michaelis constant K_m and V_{max} values were estimated as well. As illustrated in Fig. 3, the values of apparent K_m and V_{max} are 0.5953 mol/L and 0.0105 mol/(L min) for the EH, respectively. In addition, the *e.e.* value of (*R*)-epichlorohydrin reached 100%, which suggests that the EH in this study had a relatively satisfied substrate specificity on (*S*)-epichlorohydrin.

3.4. The time course of racemic resolution of (R,S)-epichlorohydrin

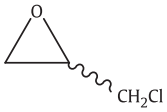
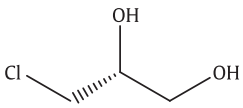
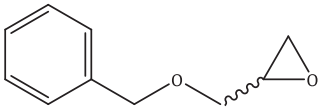
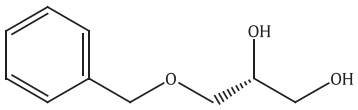
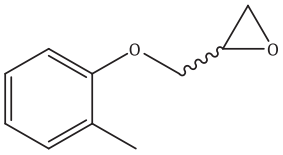
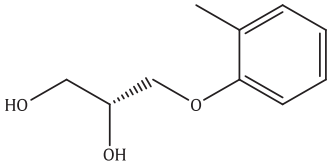
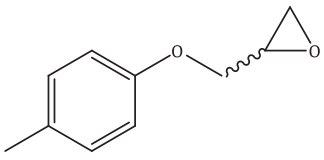
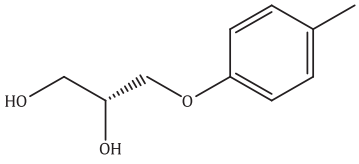
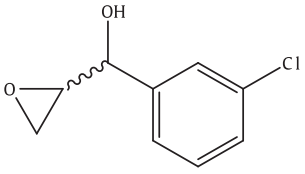
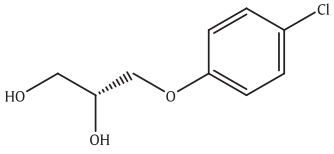
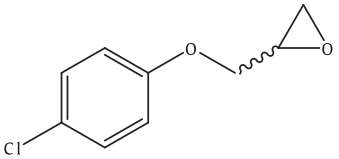
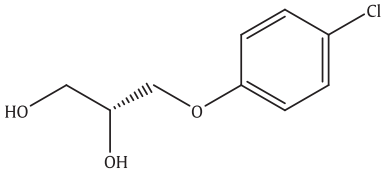
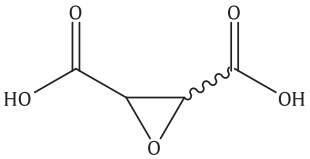
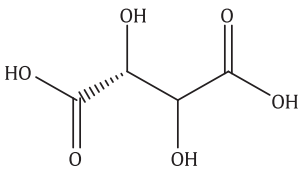
The time course of the racemic resolution of epichlorohydrin was investigated using 1% (v/v) of (*R*,*S*)-epichlorohydrin as substrate under the optimal condition. After 40 min resolution, the *e.e.* value reached 100%. The enantiopure (*R*)-epichlorohydrin was remained and the yield could be 18% (Fig. 4).

3.5. Homology modeling and molecular docking

The secondary structure and tertiary structure analysis showed that EH obtained in this study consisted of 19 α-helix and 8 β-sheet. In addition, one lid-like structure was formed by 7 α-helix. Crystal structures of *A. niger* EH (PDB accession code 1QO7, resolution 1.80 Å) [26] and *A. radiobacter* EH (PDB accession code 1EHY, resolution 2.10 Å) [27] were used as templates for homology modeling of *R. toruloides* EH. The best quality model as shown in Fig. 5a was chosen for further calculations, molecular modeling, and docking studies. The modeling results showed that the EH belongs to α/β enzyme family containing a core motif and lid-like motif. The NC-loop combined core motif and hat-like motif are different with the one in the lid-like motif. The conserved catalytic residues Asp 190, Glu 359 and His 385 from EH protein have similar orientations and locations in the EH model.

The molecular docking studies of the EH could show substrate interactions in the active site (Fig. 5b and c), which showed that both of (*R*)- and (*S*)-epichlorohydrin could dock into the catalytic pocket of EH, however the distances of hydrogen bonds with Tyr 261 were different. The mechanism of EH to catalyze (*S*)-epichlorohydrin can be proposed as follows: two tyrosines, 261 and 262, bind and concomitantly activate the substrate by forming hydrogen bonds (both 2.8 Å) at the epoxide O-atom, and the Asp 190 then initiates the nucleophilic attack at the sterically less hindered C-atom of substrate,

Table 2
Substrate specificity of the recombinant EH.

Substrates	Products	Relative activity (%)	Conversion rate (%)
2-(chloromethyl)oxirane 	3-chloropropane-1,2-diol 	100	46.7
2-[(benzyloxy)methyl]oxirane 	3-(benzyloxy)propane-1,2-diol 	164	76.6
2-[(2-methylphenoxy)methyl]oxirane 	3-(2-methylphenoxy)propane-1,2-diol 	129	60.3
2-[(4-methylphenoxy)methyl]oxirane 	3-(4-methylphenoxy)propane-1,2-diol 	59.3	27.7
1,3-Chlorophenyl glycidol ether 	3-(4-chlorophenoxy)propane-1,2-diol 	0	0
2-[(4-chlorophenoxy)methyl]oxirane 	3-(4-chlorophenoxy)propane-1,2-diol 	0	0
oxirane-2,3-dicarboxylic acid 	2,3-dihydroxybutanedioic acid 	0	0

resulting in ring-opening and the formation of a covalently bound ester intermediate. Subsequently, the Glu 359 located in low position would help His 385 to activate the H_2O molecules which makes the intermediate reacts with water and formed ester hydrolyzed, and then 3-chloro-1,2-diol released.

4. Discussion

As a promising biocatalyst, EH has been widely put into industrial applications for the preparation of enantiopure pharmaceuticals and other fine chemicals because of its high enantioselectivity [3]. Many

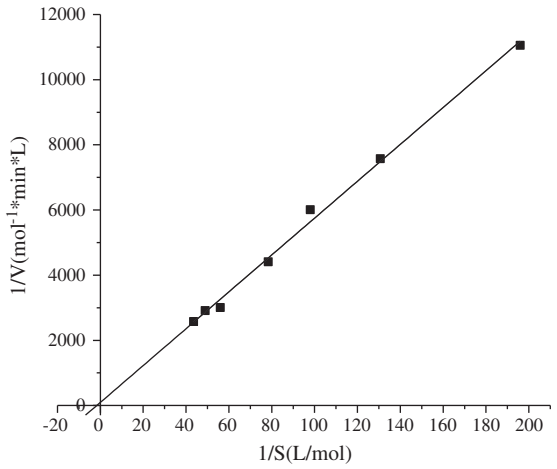


Fig. 3. Lineweaver–Burk double reciprocal plots of epoxide hydrolase.

EHs from microbial origins had been researched and some of them were promising for preparative scale biotransformation [2]. This work focused on cloning the EH gene and investigating its characteristics for exploring industrial applications.

Though the *R. toruloides* CBS14 EH has been well studied [28,29], its application in production of (*R*)-epichlorohydrin is still not investigated and reported yet. In the current work, an EH gene from *R. toruloides* CBS14 was synthesized and expressed in *E. coli*. The recombinant EH showed higher activity in neutral or basic condition than in acidic condition and it was sensitive to temperature, which is similar to the previous reports [1]. The racemic resolution of epichlorohydrin showed that this EH could catalyze the hydrolysis of (*S*)-epichlorohydrin to produce enantiopure (*R*)-epichlorohydrin, however its productivity is lower as compared to EH from *R. glutinis* [17]. In addition, the substrate specificity study suggested that this recombinant EH has potential use for the production of diols with high values.

It is reported that not only *e.e.* value could increase, but also the spontaneous hydrolysis could be inhibited, and the substrate solubility would be improved as well in the organic media [30]. In this study, series of organic solvents were tested to investigate the influence of organic solvents on EH and its catalytic process using DEAE-cellulose immobilized enzyme as catalyst [16]. It is found the EH functioned very well and the *e.e.* of product could reach 100%. In addition, the spontaneous hydrolysis was avoided when hexane was used as organic medium, which is similar to previous reports [31]. Further studies are undergoing in our lab to investigate the detailed function of EH in the organic media.

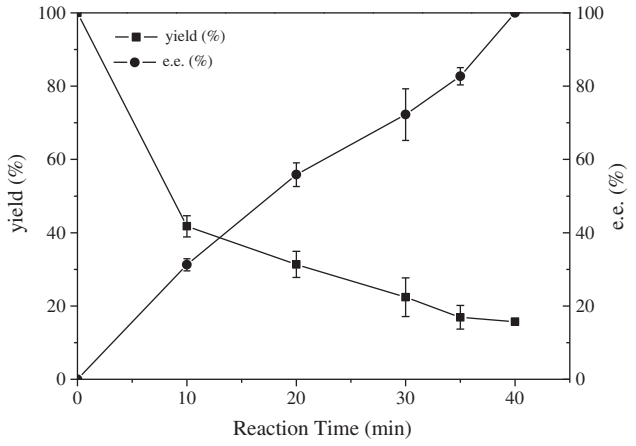


Fig. 4. Time course of racemic resolution of epichlorohydrin.

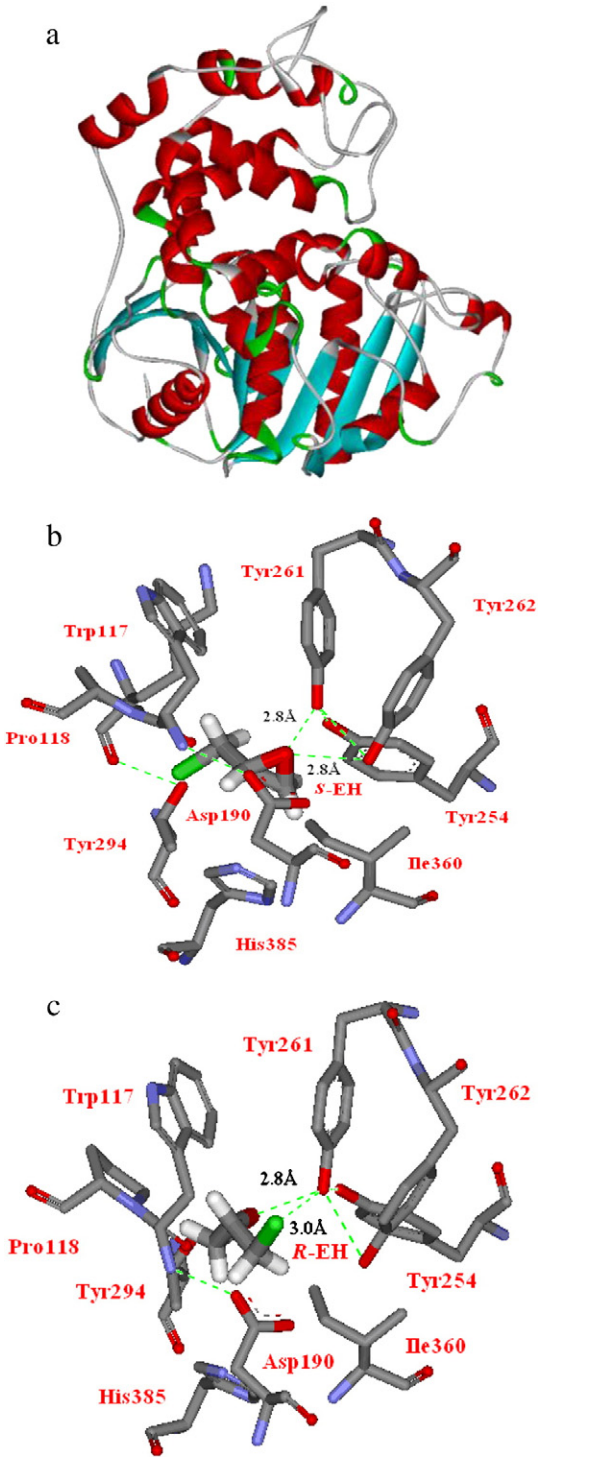


Fig. 5. Homology modeling and molecular docking. a. Homology protein model of epoxide hydrolase. b. (*S*)-epichlorohydrin docking into the active site of epoxide hydrolase. c. (*R*)-epichlorohydrin docking into the active site of epoxide hydrolase.

Analysis of catalytic characteristics based on structures modeling and molecular docking provides a new insight to this synthesized EH and paves the fundamental for its further application [32]. The energies of binding were expected to depend on several factors including hydrogen bonds with the two tyrosines (Tyr 261 and Tyr 262, activate the substrate), the docked energy and the distance (d) between the attacking O-atom of Asp 190 and the epoxide C-atom. It is reported that d value is a key factor influencing the activity and enantioselectivity of EH [33]. In this study, we found that though the docked energies for

(*R*)-(−2.944 kcal/mol) and (*S*)-epichlorohydrin (−2.928 kcal/mol) were close, there was still a big difference in d values between (*R*)-(5.7 Å) and (*S*)-epichlorohydrin (4.3 Å), which leads to the EH obtained in this study was more active on (*S*)-epichlorohydrin. Regulating the d value and making the distance closer between (*S*)-epichlorohydrin and Asp 190 would help to improve the activity and enantioselectivity of this enzyme on (*S*)-epichlorohydrin. In addition, to improve the thermo-stability, enantioselectivity, productivity and activity of EH, other strategies such as rational design or by directed molecular evolution of proteins are also powerful tools [34] to combine advantages of EH from different origins [35] based on the investigation of the structure modeling and docking.

In conclusion, *R. toruloides* CBS14 EH gene was successfully synthesized, cloned and expressed in *E. coli*. The optimal pH and temperature of purified EH were found to be 7.5 and 35 °C, respectively. The recombinant EH was sensitive to temperature, and the activity decreased significantly at 45 °C. The values of apparent K_m and V_{max} were calculated to be 0.5953 mol/l and 0.0105 mol/(L min) with the epichlorohydrin as substrate. The application of recombinant EH in racemic resolution of (*R*,*S*)-epichlorohydrin showed that the *e.e.* value and productivity of (*R*)-epichlorohydrin reached around 100% and 18%. After further modification by rational or irrational protein design methods, this recombinant EH could be a potential candidate for upscale production of (*R*)-epichlorohydrin.

Acknowledgements

The support of this work by the National Basic Research Program of China (973 Program) (No. 2011CB710806), National Natural Science Foundation of China (No. 21176224) and the Research Project of Natural Science Foundation of Zhejiang Province (Nos. Z4080032 and R3110155) is gratefully acknowledged.

References

[1] Z.Q. Liu, Y. Li, Y.Y. Xu, L.F. Ping, Y.G. Zheng, Applied Microbiology and Biotechnology 74 (2007) 99–106.
[2] A. Steinreiber, K. Faber, Current Opinion in Biotechnology 12 (2001) 552–558.
[3] E. Blee, F. Schuber, European Journal of Biochemistry 230 (1995) 229–234.
[4] R.J. Linderman, E.A. Walker, C. Haney, R.M. Roe, Tetrahedron 51 (1995) 10845–10856.
[5] I. Osprian, W. Kroutil, M. Mischitz, K. Faber, Tetrahedron: Asymmetry 8 (1997) 65–71.
[6] C.A.G.M. Weijers, J.A.M. de Bont, Journal of Molecular Catalysis B: Enzymatic 6 (1999) 199–214.
[7] M. Nardini, I.S. Ridder, H.J. Rozeboom, K.H. Kalk, R. Rink, D.B. Janssen, B.W. Dijkstra, The Journal of Biological Chemistry 274 (1999) 14579–14586.
[8] R. Rink, J.H.L. Spelberg, R.J. Pieters, J. Kingma, M. Nardini, R.M. Kellogg, B.W. Dijkstra, D.B. Janssen, Journal of the American Chemical Society 121 (1999) 7417–7418.
[9] K. Faber, M. Mischitz, W. Kroutil, Acta Chemica Scandinavica 50 (1996) 249–258.
[10] S. Hwang, C.Y. Choi, E.Y. Lee, Biotechnology and Bioprocess Engineering 13 (2008) 453–457.
[11] K.E. Jaeger, T. Eggert, A. Eipper, M.T. Reetz, Applied Microbiology and Biotechnology 55 (2001) 519–530.
[12] W.J. Choi, Applied Microbiology and Biotechnology 84 (2009) 239–247.
[13] M. Kotik, V. Stepanek, M. Grulich, P. Kyslik, A. Archelas, Journal of Molecular Catalysis B: Enzymatic 65 (2010) 41–48.
[14] Y.C. Jiang, J.Y. Wu, C. Liu, M.C. Hu, S.N. Li, Q.G. Zhai, Catalysis Communications 11 (2010) 727–731.
[15] I.K. Song, S.H. Lee, S.H. Song, D.R. Park, J.C. Jung, J.H. Song, S.Y. Woo, W.S. Song, M.S. Kwon, Catalysis Communications 10 (2008) 160–164.
[16] W.J. Choi, E.Y. Lee, S.J. Yoon, S.T. Yang, C.Y. Choi, Journal of Bioscience and Bioengineering 88 (1999) 339–341.
[17] H.S. Kim, J.H. Lee, S. Park, E.Y. Lee, Biotechnology and Bioprocess Engineering 9 (2004) 62–64.
[18] H.S. Kim, S.J. Lee, E.J. Lee, J.W. Hwang, S. Park, S.J. Kim, E.Y. Lee, Journal of Molecular Catalysis B: Enzymatic 37 (2005) 30–35.
[19] H.B. Zheng, M.T. Reetz, Journal of the American Chemical Society 132 (2010) 15744–15751.
[20] H. Visser, C.A.G.M. Weijers, A.J.J. van Ooyen, J.C. Verdoes, Biotechnology Letters 24 (2002) 1687–1694.
[21] R. Rydzanicz, X.S. Zhao, P.E. Johnson, Nucleic Acids Research 33 (2005) W521–W525.
[22] A. Villalobos, J.E. Ness, C. Gustafsson, J. Minshull, S. Govindarajan, BMC Bioinformatics 7 (2006) 285–292.
[23] U.K. Laemmli, Nature 227 (1970) 680–685.
[24] G.M. Morris, D.S. Goodsell, R.S. Halliday, R. Huey, W.E. Hart, R.K. Belew, A.J. Olson, Journal of Computational Chemistry 19 (1998) 1639–1662.
[25] N.A.E. Kronenburg, J.A.M. de Bont, Enzyme and Microbial Technology 28 (2001) 210–217.
[26] N. Sakai, Y. Tajika, M. Yao, N. Watanabe, I. Tanaka, Proteins 57 (2004) 869–873.
[27] C.L. Hung, J.H. Liu, W.C. Chiu, S.W. Huang, J.K. Hwang, W.C. Wang, The Journal of Biological Chemistry 282 (2007) 12220–12229.
[28] J. Maritz, H.M. Krieg, C.A. Yeates, A.L. Botes, J.C. Breytenbach, Biotechnology Letters 25 (2003) 1775–1781.
[29] M.S. Smit, Trends in Biotechnology 22 (2004) 123–129.
[30] S. Karboune, A. Archelas, R. Furstoss, J. Baratti, Journal of Molecular Catalysis B: Enzymatic 32 (2005) 175–183.
[31] S. Karboune, A. Archelas, J. Baratti, Enzyme and Microbial Technology 39 (2006) 318–324.
[32] Z.Q. Liu, Y. Gosser, P.J. Baker, Y. Ravee, Z.Y. Lu, G. Alemu, H.G. Li, G.L. Butterfoss, X.P. Kong, R. Gross, J.K. Montclare, Journal of the American Chemical Society 131 (2009) 15711–15716.
[33] M.T. Reetz, M. Bocola, L.W. Wang, J. Sanchis, A. Cronin, M. Arand, J.Y. Zou, A. Archelas, A.L. Bottalla, A. Naworyta, S.L. Mowbray, Journal of the American Chemical Society 131 (2009) 7334–7343.
[34] L.Y. Rui, L. Cao, W. Chen, K.F. Reardon, T.K. Wood, The Journal of Biological Chemistry 279 (2004) 46810–46817.
[35] J.H. Woo, Y.O. Hwang, J.H. Kang, H.S. Lee, S.J. Kim, S.G. Kang, Journal of Bioscience and Bioengineering 110 (2010) 295–297.