Enhancement of Soluble Expression and Biochemical Characterization of Two Epoxide Hydrolases from Bacillus

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Abstract

**Background:** Enantiopure epoxides are important intermediates in the synthesis of high-value chiral chemicals. Epoxide hydrolases have been exploited in biocatalysis for kinetic resolution of racemic epoxides to produce enantiopure epoxides and vicinal diols. It is necessary to obtain sufficient stable epoxide hydrolases with high enantioselectivity to meet the requirements of industry.

**Objectives:** Enhancement of soluble expression and biochemical characterization of epoxide hydrolases from Bacillus pumilus and B. subtilis.

**Material and Methods:** Homologous genes encoding epoxide hydrolases from B. pumilus and B. subtilis were cloned and expressed in Escherichia coli. The recombinant epoxide hydrolases were characterized biochemically.

**Results:** Low temperature induction of expression and a C-terminal-fused His-tag enhanced soluble expression of the epoxide hydrolases from the two Bacillus species in E. coli. These epoxide hydrolases could hydrolyze various epoxide substrates, with stereoselectivity toward some epoxides such as styrene oxide and glycidyl tosylate.

**Conclusions:** The position of the His-tag and the induction temperature were found to play a vital role in soluble expression of these two epoxide hydrolases in E. coli. In view of their catalytic properties, the epoxide hydrolases from Bacillus have potential for application in kinetic resolution of some epoxides to prepare enantiopure epoxides and vicinal diols.

**Keywords:** Bacillus; Epoxide Hydrolase; Hydrolysis

1. Background

Enantiopure epoxides are attractive intermediates for use in synthesis of high-value-added chiral chemicals, like pharmaceuticals. Therefore, chemical and biocatalytic procedures have been developed to prepare enantiopure epoxides from racemic mixtures by kinetic resolution (1-3). With the development of green chemistry, biocatalysis is recognized as one of the most promising strategies for hydrolytic kinetic resolution of racemic epoxides (4, 5).

Epoxide hydrolases (EHs; EC 3.3.2.3), present in bacteria, fungi, insects, plants and human (6, 7), can catalyze the hydrolysis of epoxides into corresponding vicinal diols via a trans opening of the oxirane with a varied range of stereoselectivities (8, 9). Therefore, racemic epoxides can be kinetically resolved by EHs in biocatalytic reactions to produce enantiopure epoxides and vicinal diols. Several microbial EHs have been exploited on the laboratory scale for this purpose (10-14), but commercial availability of EHs is limited, although EHs from Aspergillus niger and Rhodococcus rhodochrous are available from Sigma-Aldrich as lyophilized powder (5). Great efforts are still being made to exploit novel EHs from various sources (14-16) and to improve the properties of EHs by protein engineering (17-19). Many recombinant EHs have been expressed in Escherichia coli (20-22); unfortunately,
these usually form inclusion bodies (23). Optimization of expression conditions, especially to enhance the enzyme solubility, is a prerequisite to obtain enough active and stable EH for biocatalytic industrial applications.

2. Objective

The primary objectives of this study were to recombinantly express and characterize homologous EHs from Bacillus pumilus and B. subtilis. Soluble expression of these two EHs in E. coli was improved by using a lower induction temperature and a C-terminal-fused His-tag. Biochemical characterization indicated that these Bacillus EHs have potential for application in chiral resolution of certain epoxides.

3. Materials and Methods

3.1. Materials

Racemic styrene oxide (SO) and its (R)/(S)-enantiomers, (R)/(S)-epichlorohydrin (ECH), and (R)/(S)-1,2-epoxybutane (EPB) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Racemic ECH and EPB, as well as (R)/(S)-glycidyl tosylate (GT), were obtained from Kemiou Chemical Reagent Co. (Tianjin, China). Pfu DNA polymerase was purchased from Tiangen Biotech Co. (Beijing, China). Restriction enzymes and T4 DNA ligase were obtained from Thermo Electron Co. (Waltham, MA, USA). An E.Z.N.A. Pure Kit was from Omega Bio Tek Co. (Norcross, GA, USA). HisTrap FF Crude 1 mL columns were purchased from GE Healthcare (Boston, MA, USA).

3.2. Cloning and Construction of Expression Vectors

In the genomes of B. subtilis S1-4 (GenBank accession no. NZ_ANIPO00000000) (24) and B. pumilus BA06 (GenBank accession no. NZ_AMDH00000000) (25), homologous genes were found to encode EHs. These two EHs were assigned as BsuEH (GenBank accession no. WP_014479220) and BpuEH (GenBank accession no. WP_008342154).

Based on the coding sequences of BpuEH and BsuEH, primers were designed to amplify these genes (Table 1). The PCR reaction was set up in 50 μL mixtures containing 10 ng genomic DNA, 200 nM dNTP, 40 nM each primer (B06-2817F/R; S-3919F/R), and 2.5 U Pfu DNA polymerase. The reaction conditions were: 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products were purified using the E.Z.N.A. Cycle Pure Kit and digested with restriction enzymes Hind III and Bam HI. The DNA fragments were ligated with vector pET28 digested with the same restriction enzymes, resulting in the expression vectors pET28BpuEH.N.1(Hin) and pET28BsuEH.N.1(Hin) with a His-tag at the N-terminus of the recombinant protein. The recombinant vectors were confirmed by DNA sequencing using BigDye Terminator v3.1 (Life Technologies Co., Carlsbad, CA, USA).

Table 1. Primers used in cloning epoxy hydrolyase genes from B. pumilus and B. subtilis

| Primers   | Sequence (5’-3’)                                      | Remarks          |
|-----------|-----------------------------------------------------|------------------|
| B06-2817F | GGATTCGATGAGGATCAGTGAATTCGAGTTCAAT                  | Hind III         |
| B06-2817R | ATAGGATCCATGAGGATCAGTGAATTCGAGTTCAAT                | Bam HI           |
| S-3919F   | GGATTCGATGAGGATCAGTGAATTCGAGTTCAAT                  | Hind III         |
| S-3919R   | ATAGGATCCATGAGGATCAGTGAATTCGAGTTCAAT                | Bam HI           |
| S3919MF   | CTTGCTCGACGGATTCTCGAGTTCTAGTGTTGTCCTG             | Deletion of Nco I|
| S3919MR   | AAATCCCCGAGGAGGAGGACAAATGACG                     | Deletion of Nco I|
| B28171NcF | GCGTTCTCCGAGCTTTGCATGAGGATCAGTGAATTCGAGTTCAAT     | Nco I            |
| B28171XhR | CGGTTCTCCGAGCTTTGCATGAGGATCAGTGAATTCGAGTTCAAT     | Xho I            |
| S3919NcF  | CGTTCTCCGAGCTTTGCATGAGGATCAGTGAATTCGAGTTCAAT     | Nco I            |
| S3919XhR  | CGTTCTCCGAGCTTTGCATGAGGATCAGTGAATTCGAGTTCAAT     | Xho I            |

For construction of expression vectors pET28BpuEH.C(Hin) and pET28BsuEH.C(Hin) with the His-tag at the C-terminus, similar experimental procedures were carried out as above, but primers B28171Nc.F/B28171Xh.R and S3919Nc.F/S3919Xh.R were used respectively. Before constructing pET28BsuEH.C(Hin), PCR-based site-directed mutagenesis was performed using primers S3919M.F/R to make a substitution of T with C, which led to deletion of the Nco I site present in the coding sequence of BsuEH. This substitution did not change the encoded amino acid residue.

3.3. Recombinant Protein Expression and Purification

Each expression vector was transformed into competent cells of E. coli BL21 (DE3), which were purchased from Tiangen Biotech Co. An overnight culture (5 mL) was inoculated into 1 L Luria-Bertani broth containing 50 μg mL⁻¹ kanamycin, which was incubated at 37 °C with shaking at 200 rpm in a ZWT-211B Incubator Shaker (Shanghai Zhicheng Co. Shanghai, China). After the cells reached an optical density at 600 nm (OD₆₀₀) of 0.6–0.8, 0.5 mL of 1 M isopropyl-β-D-thiogalactoside was added to the culture (final concentration 0.5 mM). The culture was then incubated at 18 °C for an additional 24 h. The cells were harvested by centrifugation using a Centrifuge 5804R (Eppendorf Co., Hamburg, Germany) at 4,600 × g for 10 min at 4 °C. The cell pellet was resuspended in 25 mL disruption buffer (20 mM K₂HPO₄/KH₂PO₄, pH 7.5, 250 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol), and sonicated using an Ultrasonic Homogenizer Sonicator (Cole-Parmer Co., Vernon Hills, USA) in an ice bath.
The lysate was clarified at 15,500 × g for 10 min at 4 °C. The supernatant was loaded onto a HisTrap FF Crude column with immobilized nickel ions using an ÄKTA Prime System (Amersham, San Francisco, USA). The recombinant proteins were eluted with 0–1 M imidazole.

The fractions with an absorption peak at 280 nm were pooled and dialyzed against 100 volumes of dialysis buffer [10 mM K3HPO4/KH2PO4, pH 7.5, 250 mM NaCl, 10% glycerol, 0.1 mM ethylenediaminetetraacetic acid (EDTA)] at 4 °C overnight. The protein sample was concentrated to 1 mL by addition of polyethylene glycol (molecular weight, 20,000). Protein purity was determined by 12% SDSPAGE and protein concentration was quantified using AlphaImager software (Alpha Innotech Co., San Leandro, USA) with bovine serum albumin (BSA) as a standard.

3.4. Epoxide Hydrolase Activity Assay
The EH assay was assayed with 4-nitrobenzylpyridine reagent as described previously (26). Briefly, the standard reaction was set up in 100 μL mixture (0.1 M phosphate buffer, pH 7.5) containing 30 mM substrate and 5 μg recombinant EH protein. The catalytic reaction was performed at 35 °C for 10 min or the indicated time. Reagent A (100 μL, 50 mM 4-nitrobenzylpyridine in 80% ethylene glycol, 20% acetonitrile [v/v]) was then added. The mixture was heated at 80 °C for 10 min. After cooling to room temperature, 180 μL of the mixture was diluted into 2,520 μL of 0.1 M phosphate buffer (pH 7.5), and then 900 μL of reagent B (50% triethylamine and 50% acetonitrile [v/v]) were added. Finally, the residual substrate was determined using OD280 on a UV-2450 spectrophotometer (Shimadzu, Tokyo, Japan).

The enzymatic activity was calculated according to a standard curve made using the same procedures as above with a range of concentrations of each substrate. For determination of catalytic progress over time, a master mixture (600 μL) was set up which contained 30 μg enzyme and 30 mM substrate. The reaction conditions were as above. At indicated time points, 100 μL reaction sample was withdrawn and the residual substrate was determined as described above.

3.5. Optimal pH and Temperature for Hydrolytic Reaction
Racemic GT was selected as the substrate to determine the optimal pH and temperature for enzyme activity. The activity assay was performed as described above but using 2.5 μg purified enzyme and 30 mM substrate. The pH values were 6.0 to 9.0 in 0.1 M phosphate buffer (pH 6.0–7.8) or 0.1 M Tris-HCl buffer (pH 7.8–9.0). To examine the effect of temperature on the catalytic activity, the reaction was incubated for 30 min at 20, 25, 30, 35, 40, 45, and 50 °C.

3.6. Effects of Various Chemicals on Hydrolytic Reaction
The effects of various chemicals on enzyme activity were determined using racemic SO as substrate. The reaction was set up in 100 μL 0.1 M phosphate buffer (pH 7.5) containing 2.5 μg purified enzyme and 30 mM substrate. The tested chemicals were: metal ions (Mg2+, Ca2+, Mn2+, Cu2+, Zn2+ and Ba2+) at 2.0 mM; organic cosolvents (acetonitrile and ethanol, 10% v/v); 5 mM EDTA-Na2; and 2% SDS. The enzyme activity was calculated relative to that in the absence of added chemicals, which was defined as 100%.

3.7. Determination of Enantioselectivity
Enantioselective hydrolysis of several enantiopure epoxides by the recombinant EHs was investigated. Briefly, (R)- and (S)-enantiomers of SO, ECH, EPB and GT were chosen as the substrates to examine the enantioselective hydrolysis using 5 μg enzyme and 30 mM substrate in 100 μL 0.1 M phosphate buffer (pH 7.5). The hydrolytic reaction was performed at 35 °C for 30 min.

All the above activity assays were performed with three repeats. The data are presented as the average value with the experimental deviation.

3.8. Sequence Analysis of Epoxide Hydrolases
Sequence similarity searches were performed using BLASTp (https://www.ncbi.nlm.nih.org). A multiple sequence alignment of EHs from various organisms was constructed using the software Clustal X v1.83 (www.clustal.org) and BioEdit v7.2.5 (https://bioedit.updatestar.com/).

4 Results

4.1. Sequence Analysis of the Epoxide Hydrolases from two Bacillus Species
The EHs from various *Bacillus* strains were previously exploited in kinetic resolution of racemic epoxides (27-29).

When examining the genomes of *B. subtilis* S1-4 (24) and *B. pumilus* BA06 (25), homologous genes were found to encode EHs and their coding sequences were different from the EH-encoding genes of other *Bacillus* species such as *B. megaterium* and *B. cereus* (Fig. 1). However, BpuEH and BsuEH share conserved sequence motifs of EHs, including a catalytic triad (Asp-Asp-His), an oxygen ion hole (HGFP), and two Tyr residues that are involved in substrate binding and assist in the ring-opening of epoxide by acting as proton donor to the epoxide oxygen (6, 7, 10).

4.2. Recombinant Expression and Purification of BsuEH and BpuEH
Our initial results showed that recombinant BsuEH and BpuEH with a His-tag at the N-terminus were almost entirely expressed in insoluble form at 37 °C (Fig. 2A, left panel).
Figure 1. Multiple sequence alignment of epoxide hydrolases (EHs) from *Bacillus subtilis* (BsuEH, GenBank accession no. WP_014479220), *B. pumilus* (BpuEH, GenBank accession no. WP_008342154), *B. cereus* (BceEH, GenBank Accession no. KXY30655), and *B. megaterium* (BmeEH, GenBank Accession no. ADV36302). Solid triangles indicate putative active site residues (Asp-Asp-His); empty triangles indicate two Tyr residues involved in catalysis; stars indicate the conserved oxyanion hole (HGFP motif).

**Figure 2.** Overexpression of epoxide hydrolases (BpuEH and BsuEH) from *B. pumilus* and *B. subtilis* and their hydrolytic reactions toward various epoxide substrates. A: SDS-PAGE analysis of recombinant BpuEH and BsuEH with a His-tag fused to the N- or C-terminus. CK, not induced with isopropyl-β-D-thiogalactoside; P, precipitate fraction of the whole cell lysate; S, supernatant fraction of the whole cell lysate; WC, whole cell lysate. The arrows indicate the recombinant proteins. B: Hydrolysis of racemic epoxides by BpuEH and BsuEH. C and D: Hydrolytic progress of BpuEH and BsuEH toward racemic epoxides. The reaction was performed in 0.1 M phosphate buffer (pH 7.5) using 5 μg enzyme and 30 mM substrate at 35°C. EPB, 1,2-epoxybutane; ECH, epichlorohydrin; SO, styrene oxide; GT, glycidyl tosylate.
When the induction temperature was changed to 18 °C, soluble EHs could be detected by SDS-PAGE (Fig. 2A, right panel). However, the amount of soluble EHs was still limited.

Since His-tag position significantly influences the soluble expression of some heterologous genes in E. coli (30), expression vectors were constructed with the His-tag moved to the C-terminus [pET28BpuEH.C(His) and pET28BsuEH.C(His)] and were used to express the EHs with induction at 18 °C. Figure 2A (right panel) shows that the amount of soluble protein was greatly enhanced for both EHs in comparison with that with His-tag at N-terminus, indicating that the position of the His-tag indeed played an important role in soluble expression of these EHs. The recombinant proteins BsuEH and BpuEH were purified by nickel affinity chromatography and obtained with a C-terminal-fused His-tag. The purified EHs were quantified based the density of protein bands on SDS-PAGE with BSA as a standard.

4.3. Catalytic Hydrolysis of Various Epoxide Substrates

Figure 2B shows that both EHs (BpuEH and BsuEH) could hydrolyze the four selected substrates. However, these two EHs exhibited some differences in substrate specificity, which might be due to sequence differences (Fig. 1). The kinetics of the hydrolysis reactions were determined (Fig. 2C, 2D). BpuEH could hydrolyze GT and ECH faster than SO and EPB. BsuEH exhibited higher reaction rates in hydrolysis of EPB and ECH. BsuEH hydrolyzed EPB faster than BpuEH. In hydrolysis of GT, BsuEH was slower than BpuEH.

4.4. Optimal pH and Temperature of Catalytic Reaction

The effects of pH and temperature on hydrolytic activity were determined with GT as the substrate. As shown in Figure 3A and 3B, BsuEH and BpuEH exhibited similar optimal pH and temperature for the hydrolytic reaction, with the highest catalytic activity at pH 7.2 and 30 °C, respectively. However, at pH >7.8 or temperature >35 °C, the hydrolytic activity of BsuEH and BpuEH markedly decreased.

4.5. Effects of Various Chemicals on Hydrolytic Reaction

The effect of various chemicals (including metal ions and cosolvents) on enzyme activity was determined using racemic SO as the substrate. As shown in Figure 3C, the hydrolytic activity of both EHs was strongly inhibited by addition of Ca²⁺ and Zn²⁺ to the reaction mixture. Mg²⁺ and Cu²⁺ caused less inhibition of the EHs. Mn²⁺ and Ba²⁺ more strongly inhibited the catalytic activity of BpuEH than that of BsuEH. The hydrolytic activity of both BpuEH and BsuEH was tolerant to 10% acetonitrile and ethanol, but sensitive to EDTA-Na₂ and SDS.

4.6. Enantioselectivity of Hydrolytic Reaction

To probe the catalytic stereoselectivity of the EHs toward various substrates, hydrolysis of (R) and (S)-enantiomers of four epoxides was performed. Figure 4 indicates that the two EHs showed similar stereoselectivity. BpuEH and BsuEH exhibited higher enantioselectivity toward substrates with a larger side chain, like SO and GT, but almost no enantioselectivity toward ECH. These results suggest that both EHs have the potential to be used in kinetic resolution of some racemic epoxides for preparation of enantiopure diols and epoxides.
The reaction was performed in 0.1 M activity toward different epoxides by BpuEH (A) and BsuEH (B). EPB, 1,2-epoxybutane; ECH, epichlorohydrin; SO, styrene oxide; GT, glycidyl tosylate. The reaction was performed in 0.1 M phosphate buffer (pH 7.5) using 5 μg enzyme and 30 mM substrate at 35 °C for 30 min.

5. Discussion

In this work, genes from B. subtilis S1-4 and B. pumilus BA06 encoding EHs were successfully cloned and expressed in E. coli. Our results indicated that the induction temperature and the position of the His-tag had a significant impact on soluble expression of these two EHs. A His-tag fused to the C-terminus enhanced the soluble expression of these enzymes (Fig. 2). Similar results were reported in inductive expression of other heterologous genes in E. coli (30). An EH from marine bacterium Sphingophyxis alikeness was largely expressed in soluble form when a His-tag was fused to the C-terminus (31, 32). In contrast, soluble recombinant proteins with an N-terminal fused His-tag could not be obtained in seven out of eight cases when expressing EH-encoding cDNAs from the white-rot fungus Phanerochaete chrysosporium (33). Accordingly, various fusion tags have been adopted as a general strategy to produce recombinant proteins, which may not only enhance solubility of expressed proteins but also simplify the purification procedures (34). The His-tag is more popular fusion used in recombinant protein expression in E. coli. The position and even the sequence of His-tag could affect the solubility and expression efficiency of a given protein (30, 35). The reason may be ascribed to modulation of the protein 3-D structure by the His-tag. For example, by 3-D structural comparison of a bacterial laccase Lac15 with or without a His-tag fusion, the His-tag was recognized as the main reason for fold disturbance and formation of inclusion body (36). On the contrary, a 3-D structural homology modeling showed that a His-tag added at the N-terminus of (+)-γ-lactamase had no effect on the formation of inclusion bodies (37). Therefore, effect of the position of a His-tag on solubility of the expressed proteins is different, perhaps depending on their 3-D structure. In the cases of EHs, a His-tag added at the N-terminus of microbial EHs may disturb the 3-D structure, favoring aggregation into inclusion bodies when recombinantly expressed in E. coli. Further, overexpression of EHs from microbes as maltose binding protein fusions is not always successful (23). However, coexpression of molecular chaperones was reported to enhance soluble expression of EH from Rhodotorula glutinis in E. coli (38). Therefore, enhancement of soluble expression of EHs is worth investigating in the future, so as to obtain sufficient EH to meet the requirements of biocatalysis. BpuEH and BsuEH exhibited wide substrate specificity since they could hydrolyze four epoxides. The EH from B. subtilis 168 was reported to have narrower substrate specificity (23), but this may have been due to poor expression in E. coli. Further, BpuEH and BsuEH showed different stereoselectivity toward the enantiomers of some epoxides, i.e., selective hydrolysis of the epoxides with larger side chains such as SO and GT (Fig. 4). This phenomenon is often observed for EHs (10). For example, the EH from Novosphingobium aromaticivorans was reported to better hydrolyze the (R)-enantiomer of SO and ECH (22). In contrast, some EHs show low enantioselectivity toward different enantiomers (31).

The catalytic properties of BpuEH and BsuEH were also analyzed. They catalyzed the hydrolytic reaction in a narrow range of pH and temperature. Metal ions had various effects on the EH activity (13, 26). Hydrolytic activity of the both EHs was strongly inhibited by some divalent cations such as Ca²⁺ and Zn²⁺. On the other hand, Mg²⁺, Mn²⁺ and Cu²⁺ had less effect on the catalytic reaction (Fig. 3C). Similar results were reported for the other EHs. For example, the recombinant potato soluble EH was inhibited by Zn²⁺, Ca²⁺ and the other metal ions (39). Nevertheless, seldom explanations have been given for the loss in catalytic activity caused by the metal cations for the EHs. Zn²⁺ is known to interact with free thiol groups, which may cause inhibition as in case of 6-phosphogluconate dehydrogenase and yeast glutathione reductase (40, 41). In fact, there is only one cysteine residue in the amino acid sequence of BpuEH and BsuEH (Fig. 1), which may be chosen for site-directed mutagenesis to test this hypothesis. In addition, in terms of the catalytic kinetics, inhibition of human soluble EH by Zn²⁺ was non-competitive, but noncompetitive for mouse soluble
EH, suggesting that the metal binding sites in the EHs may be different from the enzyme active center (39). It is known histidine residues in proteins can interacted with divalent metal ions. Several histidine residues including one of the active sites exist in all the EHs. Therefore, it is reasonable to conclude that these divalent cations like Zn$^{2+}$ may also disturb the 3-D structure of the EHs through coordinating with these amino acid residues. Most recently, human histidine triad nucleotide binding protein 1 (hHint1) was reported to be inhibited by several divalent cations, and the crystal structure of hHint1 revealed that Cu$^{2+}$ could disrupt the second structure of the C-terminus, leading to irreversible inactivation (42). However, Ca$^{2+}$ does not inhibit many other EHs (13, 26, 39). Therefore, the mechanism of inhibition by divalent cations appears to be species-specific and the metal binding site is different from the active site. In view of their sensitivity to metal ions, BpuEH and BsuEH are different from the EHs from other sources. However, both EHs reported here were tolerant to organic solvents, which is a prerequisite to perform catalytic reactions in biphasic systems (43).

6. Conclusions

In conclusion, homologous EHs from B. pumilus and B. subtilis were cloned and expressed in E. coli. Lower temperature and the position of a His-tag were vital in successful soluble expression. The recombinant EHs could catalyze hydrolysis of various epoxides and showed enantioselectivity in the ring-opening reaction of some epoxides, especially SO and GT with larger side chains. The two EHs were tolerant to organic solvents. However, protein engineering technologies like in vitro directed-evolution should be applied to the EHs to increase the stereoselective hydrolysis of racemic epoxides and improve soluble expression of the enzymes. Taken together, the EHs from two Bacillus species show potential for application in kinetic resolution of racemic epoxides to prepare enantiopure epoxides and vicinal diols.

Conflict of Interest

There is no conflict of interest with this study.

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