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Trigger factor assisted folding of the recombinant epoxide hydrolases identified from *C. pelagibacter* and *S. nassauensis*

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ABSTRACT

Epoxide hydrolases (EHs), are enantioselective enzymes as they catalyze the kinetic resolution of racemic epoxides into the corresponding enantiopure vicinal diols, which are useful precursors in the synthesis of chiral pharmaceutical compounds. Here, we have identified and cloned two putative epoxide hydrolase genes (*cpeh* and *sneh*) from marine bacteria, *Candidatus pelagibacter ubique* and terrestrial bacteria, *Stackebrandtia nassauensis*, respectively and overexpressed them in pET28a vector in *Escherichia coli* BL21(DE3). The CPEH protein (42 kDa) was found to be overexpressed as inactive inclusion bodies while SNEH protein (40 kDa) was found to form soluble aggregates. In this study, the recombinant CPEH was successfully transformed from insoluble aggregates to the soluble and functionally active form, using pCold TF vector, though with low EH activity. To prevent the soluble aggregate formation of SNEH, it was co-expressed with GroEL/ES chaperone and was also fused with trigger factor (TF) chaperone at its N-terminus. The TF chaperone-assisted correct folding of SNEH led to a purified active EH with a specific activity of 3.85 μmol/min/mg. The pure enzyme was further used to biocatalyze the hydrolysis of 10 mM benzyl glycidyl ether (BGE) and α-methyl styrene oxide (MSO) with an enantiomeric excess of the product (ee_p) of 86% and 73% in 30 and 15 min, respectively. In conclusion, this is the first report about the heterologous expression of epoxide hydrolases using TF as a molecular chaperone in pCold TF expression vector, resulting in remarkable increase in the solubility and activity of the otherwise improperly folded recombinant epoxide hydrolases.

Introduction

Epoxide hydrolases (EHs[1]: EC 3.3.2.x) belong to α/β hydrolase fold family [1] and catalyze the hydrolytic conversion of epoxides into corresponding enantiopure vicinal diols, which are useful in the synthesis of chiral pharmaceutical drugs [2–5]. Since, chemical methods are limited in their applications for the synthesis of chiral molecules, therefore biocatalyst like EH, being an enantio- and regio-selective enzyme, has drawn much attention in the past few years [6]. EHs are also co-factor independent and ubiquitously found enzymes [7,8]. Microbial EHs are increasingly recognized as highly versatile biocatalysts due to their abundance, high enantioselectivity, efficiency and easy scale up [9]. Therefore, the discovery and utilization of epoxide hydrolases from microorganisms is of great interest.

In this study, we have identified epoxide hydrolase encoding genes from marine (*Candidatus pelagibacter ubique* HTCC 1062 {*cpeh*}) and terrestrial bacteria (*Stackebrandtia nassauensis* DSM 4478 {*sneh*}), using microbial genome database mining approach [10,11]. The open reading frames of both *cpeh* and *sneh* (locus tag SAR11_0803 and Snans_6199, respectively) were found to be annotated as α/β hydrolase fold proteins, but their biochemical function was unknown. Their phylogenetic analysis showed that *cpeh* belongs to microsomal EH class and *sneh* belongs to soluble EHs. In an earlier study, we have successfully overexpressed and characterized a soluble epoxide hydrolase from *Cupriavidus metallidurans* CH34 (*cmeh*), which was identified using the similar bioinformatic

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[1] *Abbreviations used:* EH, epoxide hydrolase; TF, trigger factor; LB, Luria–Bertani; CFE, cell free extract; NBP, 4-(4-nitrobenzyl) pyridine; Ni–NTA, nickel nitrilotriacetic acid; MW, molecular weight; PPIase, peptidyl-prolyl isomerase; BGE, benzyl glycidyl ether; BPD, benzyloxy propane diol; MSO, α-methyl styrene oxide; MPED, α-methyl phenyl ethane diol; SO, styrene oxide; PED, phenyl ethane diol; ee_p, enantiomeric excess of the product.

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Table 1
The chaperone combinations used for co-expression.

Combination	Vector name	Chaperone gene	Antibiotics	Molecular weight of chaperones (kDa)
3	pBB528 + pBB541	groESL	C + S	GroES (10.4), GroEL (58.3)
4	pBB540 + pBB542	dnaK, dnaJ, grpE, clpB, groEL/ES (high conc.)	C + S	dnaK (69.1), dna J (40), grpE (21.8), clpB (100), GroES (10.4), GroEL (58.3)
8	pBB540 + pBB550 + pBB572	ibpAB, dnaK, dnaJ, grpE, clpB, groEL/ES	C + S + A	IbpAB (16), dnaK (69.1), dnaJ (40), grpE (21.8), clpB (100), GroES (10.4), GroEL (58.3)
–	pKY206	groEL/GroES	T	GroES (10.4) , GroEL (58.3)

Antibiotics: Chloramphenicol (C), Spectinomycin (S), Ampicillin (A), Tetracycline (T) [24,38].

approach [12]. *C. pelagibacter ubique* grows in a low nutrient medium and is one of the most abundant organisms in the ocean. It is a Gram negative proteobacteria originally isolated from the Oregon coast and has an optimum growth temperature of 16 °C [13], while *S. nassauensis* is an aerobic, Gram positive, mesophilic actinomycete originally isolated from roadside soil sample in Nassau, Bahamas [14].

The putative EHs, *cpeh* and *sneh* were then cloned and overexpressed in the heterologous host, *Escherichia coli*. Both the overexpressed proteins either led to inclusion bodies (CPEH) or soluble aggregates (SNEH). Protein misfolding is the major problem encountered when overexpressing recombinant genes in *E. coli* [15], which is the most commonly used expression host [16]. The CPEH was found to be completely inactive as insoluble aggregates, while a low epoxide hydrolase activity was detected in the soluble aggregates of SNEH. In order to solubilize [17] the recombinant protein CPEH and to prevent soluble aggregate formation of SNEH in *E. coli*, we followed two approaches. First was to co-express folding modulators [18], such as, chaperone combinations 3, 4, 8 and pKY206 (Table 1) with CPEH and SNEH proteins. Using the chaperone combination 3 as the folding modulator, CPEH was still found to be incorporated within the GroEL/ES chaperone cage on Ni-affinity purification, indicating the inability of the chaperones to release the improperly folded and thus inactive protein.

In case of SNEH protein, although the co-expressed pKY206 prevented the soluble aggregation to some extent, but with little improvement in the specific activity of the purified protein.

In our second approach, both the recombinant proteins CPEH and SNEH, were expressed with trigger factor (TF) fused to their N-terminus in pCold TF vector, resulting in TF-CPEH and TF-SNEH fusion proteins. The TF fusion led to 35-fold improvement in the specific activity of purified TF-SNEH in comparison to the SNEH expressed in the soluble aggregate form without any chaperone.

Materials and methods

Microbial strains, plasmids, enzymes and reagents

Genomic DNA of *C. pelagibacter ubique* HTCC 1062 was procured from Dr. Stephen Giovannoni, Department of Microbiology, Oregon State University, Corvallis, OR 97331, USA. The mesophilic actinomycete *S. nassauensis* DSM 44728 was obtained from United States Department of Agriculture (USDA), Agricultural Research Service (ARS, NRRL) Culture Collection. The strains and plasmids used in this study are listed in Table 2. The *E. coli* strains DH10B and BL21(DE3) were grown in Luria–Bertani (LB) medium. The media and antibiotics were purchased from Himedia (India). Restriction enzymes were obtained from New England Biolabs (USA) and Fermentas Life Sciences (Thermo Fisher Scientific, USA). The chemicals used were of analytical grade purchased from Sigma–Aldrich (USA), Merck (USA) or Bio-Rad (USA). Epoxides and diols used in the study were from Sigma–Aldrich.

BLAST search of microbial genome database

Using BLAST program, the putative epoxide hydrolase (EH) genes were searched from the genome database of the two respective microbes, at the website www.ncbi.nlm.nih.gov [12]. The selected sequence hits were manually screened for the presence of conserved motifs, HGXP and GXS_mXS/T, catalytic triad residues (Asp-His-Asp) and two ring opening tyrosines. Multiple sequence alignment of the identified putative EHs with the reported epoxide hydrolases was performed using the CLUSTAL W program to find the bit score [19]. The resulting putative EHs having locus tag SAR11_0803 of *C. pelagibacter ubique* HTCC 1062 and Snans_6199 of *S. nassauensis* DSM 44728, were selected. The phylogenetic tree of both the gene sequences was constructed and analyzed using the MEGA 6 software [20].

PCR amplification of the genes

The *cpeh* gene from the genomic DNA of *C. pelagibacter ubique* was PCR amplified using the forward primer 5′ATG**GCTAGC**ATGATTAAGCCTTTTAAATTAGATATTCCCG3′ and reverse primer 5′GCG**AAGCTT**CTATCGTACAGATCTTGAAAAC3′, containing the restriction sites *Nhe*I and *Hin*dIII, respectively. Nucleotide sequences bold and underlined indicate the restriction sites. The PCR amplification of 1143 bp *cpeh* gene was done using the PCR program as follows: 3 min at 94 °C, 30 s at 94 °C, 30 s at 55 °C, 2 min at 68 °C and final extension step for 10 min at 72 °C.

S. nassauensis was grown in 5 ml of N-Z-Amine-medium containing glucose (1% w/v), soluble starch (2% w/v), yeast extract (0.5% w/v), N-Z-Amine (0.5% w/v) and $CaCO_3$ (0.1% w/v), pH 7.2 for 48 h at 28 °C. The genomic DNA of *S. nassauensis* was isolated using the standard protocol [12] and 891 bp of *sneh* gene was amplified by PCR using the forward primer 5′G**GAATTC**GTGACGGGAACCGTCGTTTCCGC3′ and reverse primer 5′GGC**AAGCTT**TCAGTGGGATTCCAGGTGAGCCTG3′ containing restriction sites *Eco*RI and *Hin*dIII, respectively which are bold and underlined. PCR amplification of the *sneh* gene was done using the PCR program: 4 min at 94 °C, 45 s at 94 °C, 40 s at 59 °C, 1 min at 72 °C and final extension step of 10 min at 72 °C.

DNA manipulation

PCR amplified fragments were eluted from the agarose gel after electrophoresis by Fermentas gel extraction kit and plasmids were isolated using Fermentas plasmid isolation kit as recommended by the manufacturer's protocol. Restriction digestion and ligation was done using standard procedures [21].

PCR amplified *cpeh* gene fragment could not be properly restriction digested, probably due to short overhangs. Therefore, *cpeh* (having "A" overhang with *Taq* polymerase activity) was first ligated with pTZ57R/T cloning vector (having "T" overhang) generating pTZ*cpeh* and transformed into *E. coli* DH10B (cloning host) by following standard chemical transformation protocol [21]. The