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Epoxide hydrolase of *Trichoderma reesei*: Biochemical properties and conformational characterization

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ABSTRACT

Epoxide hydrolases (EHs) are enzymes that are present in all living organisms and catalyze the hydrolysis of epoxides to the corresponding vicinal diols. EHs have biotechnological potential in chiral chemistry. We report the cloning, purification, enzymatic activity, and conformational analysis of the TrEH gene from *Trichoderma reesei* strain QM9414 using circular dichroism spectroscopy. The EH gene has an open reading frame encoding a protein of 343 amino acid residues, resulting in a molecular mass of 38.2 kDa. The enzyme presents an optimum pH of 7.2, and it is highly active at temperatures ranging from 23 to 50 °C and thermally inactivated at 70 °C ($t_{1/2}$ = 7.4 min). The Michaelis constants (K_m) were 4.6 mM for racemic substrate, 21.7 mM for (*R*)-(+)-styrene oxide and 3.0 mM for (*S*)-(−)-styrene oxide. The k_{cat}/K_m analysis indicated that TrEH is enantioselective and preferentially hydrolyzes (*S*)-(−)-styrene oxide. The conformational stability studies suggested that, despite the extreme conditions (high temperatures and extremely acid and basic pHs), TrEH is able to maintain a considerable part of its regular structures, including the preservation of the native cores in some cases. The recombinant protein showed enantioselectivity that was distinct from other fungus EHs, making this protein a potential biotechnological tool.

1. Introduction

Chirality is a key factor in the safety and efficacy of many drugs. Regulatory requirements and the prospects of lower toxicity and higher efficacy have increased the demand for chiral compounds; thus, the production of single enantiomers of drug intermediates has become increasingly important for the chemical and pharmaceutical industries [1,2]. Among the optically active chiral compounds, enantiopure epoxides are high value-added synthons that are essential for the synthesis of pharmaceuticals, agrochemicals, and many fine chemicals and have a broad scope of market demand for their applications [2]. In this sense, epoxide hydrolases (EH) are enzymes that catalyze the addition of water to oxirane compounds (epoxides) for the preparation of enantiopure epoxides and vicinal diols (molecules with two hydroxyl groups linked to neighboring carbons) during the enantioselective hydrolysis of racemic epoxides (cyclic ethers), and this enantioselective hydrolysis is the most important feature of a bioprocess catalyzed by EHs [3].

The large majority of epoxide hydrolase isoenzymes can be grouped into two main structural families: the limonene-1,2-epoxide hydrolase (EC: 3.3.2.8; homodimeric proteins in which each subunit is composed of a six-stranded β-sheet flanked by three α-helices) [4], and the enzymes with an α/β-hydrolase fold. The epoxide hydrolases with an α/β-hydrolase fold are classified as microsomal epoxide hydrolases (EC 3.3.2.9) or soluble epoxide hydrolases (EC 3.3.2.10) according to their cellular localization in the endoplasmic reticulum or cytoplasm, respectively [5]. EHs are found in all organisms, including mammals, invertebrates, plants, fungi and bacteria. Their physiological roles include the detoxification of noxious epoxides that are taken up as xenobiotics or endogenously produced, and they are involved in blood pressure regulation and the inflammatory response in mammals [4].

The mechanism of action of EHs with an α/β-hydrolase fold involves the polarization of the substrate epoxide by two tyrosine residues (links between hydrogen atoms with the oxygen atom of the epoxide) for the nucleophilic attack of aspartate in one of the two carbons of the epoxide, which opens the epoxide ring and forms an intermediate ester (enzyme-substrate). The catalytic cycle

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is subsequently completed by hydrolysis of the intermediate ester, which is facilitated by histidine and aspartate, and the formation and release of the diol product [3,4,6,7].

Various EHs with α/β-hydrolase folds have been studied in fungi, such as *Phanerochaete chrysosporium* [8,9], *Aspergillus brasiliensis* CCT 1435 [10], *Aspergillus usamii* E001 [11], *Galactomyces geotrichum* ZJUTZQ200 [12] and *Aspergillus tubingensis* TF1 [13].

The biomass-degrading fungus *Trichoderma reesei* is a filamentous, aerobic, mesophilic fungus that has adapted to a nutrient-poor environment and has a sequenced genome and enormous biotechnological potential [14,15]. However, this organism does not have any characterized EHs. We have analyzed the genomic database of *T. reesei* [15] (http://genome.jgi.doe.gov/Trire2/Trire2.home.html) and identified a putative gene, TrEH, encoding a protein epoxide hydrolase.

In this study, we identified, for the first time, an epoxide hydrolase enzyme from *Trichoderma reesei* fungus (TrEH) that was successfully cloned, expressed, and purified. The recombinant protein was kinetically and structurally characterized, evidencing an enantioselectivity that was distinct from other fungus EHs.

2. Materials and methods

2.1. Cloning, expression and purification of TrEH

The *T. reesei* strain used in this work, QM 9414, was obtained from the American Type Culture Collection (ATCC 26921). The inoculum preparation, culture media, and growth conditions for *T. reesei* have been described previously [16]. The TrEH ORF was amplified from the *Trichoderma reesei* genomic DNA [15] by PCR with specific oligonucleotides (TrEH-F: 5′-CCGGAATTCATGGACACCAGCAAG CTCAAG-3′ and TrEH-R: 5′-ACGCGTCGACCTA CAACGCAGCCTTGGT CGCACC-3′). The PCR product was inserted into the pPROEX-HTa vector (Invitrogen, USA), and the recombinant protein was overexpressed in *E. coli* BL21. The soluble recombinant protein was loaded onto a His-Trap Chelating column connected to an ÄKTA FPLC System (GE Healthcare, USA) using a binding buffer composed of 50 mM sodium phosphate buffer, 500 mM NaCl, pH 7.5, and 0.05 M imidazole. A linear gradient of 50–300 mM imidazole in elution buffer was used to elute the bound proteins. The concentration of the purified protein was estimated using the method described by Whitaker and Einargranum [17]. The molecular mass and purity of the protein were determined by SDS–PAGE under denaturing conditions [19].

2.2. Enzymatic activity assays

The enzymatic activity was determined using the red assay, as previously described [20], using the following highly pure reagents: acetonitrile, sodium periodate, styrene oxide, (*R*)-(+)-styrene oxide, (*S*)-(−)-styrene oxide and (−)-epinephrine (L-adrenaline), which were supplied by Sigma-Aldrich (USA). The absorbance was determined on an Infinite M200 pro Tecan microplate reader (Tecan, USA). The assays were performed in triplicate.

The purified TrEH (0.04 mg/mL) was dialyzed against sodium phosphate buffer (50 mM Na_2HPO_4/NaH_2PO_4, pH 7.0). Next, the sample was incubated at 23 °C, 37 °C, 50 °C, 60 °C, and 70 °C for 20 min in thermostatted circulating water bath. Aliquots were removed at 5 min intervals (0, 5, 10, 15, and 20 min) to assay the remaining activity using racemic styrene oxide (400 mM) as the substrate.

Purified TrEH (0.04 mg/mL) was used to analyze the effect of pH on TrEH activity. These assays were performed using racemic styrene oxide (400 mM) as the substrate. Substrate solutions were prepared in 20 mM sodium phosphate, sodium borate, sodium acetate (PBA) buffer at different pH values (from 5.0 to 9.0). The pH was adjusted at 37 °C, the same temperature as the enzymatic assay.

The effect of substrate concentration on EH activity was evaluated using the purified TrEH (0.04 mg/mL) and at least 12 distinct substrate concentrations (1–60 mM). Racemic styrene oxide, (*R*)-(+)-styrene oxide and (*S*)-(−)-styrene oxide were used as substrates. The enantioselectivity was determined by comparing the kinetic parameters of the enzyme activity on the enantiopure substrates (*R*)- and (*S*)-styrene oxide. Kinetic data were analyzed using Enzfitter software (Biosoft, http://www.biosoft.com). The K_m and V_{max} values (means ± standard deviation, SD) were determined from a weighted linear regression using EnzFitter software.

2.3. Far-UV circular dichroism (CD) and secondary structure content

The TrEH stock solutions were prepared in PBA buffer at pH 4.0, 5.5, 7.2, 8.0, 9.0 and 11. The final protein concentrations were 0.212 mg/mL, and the concentration of the purified protein was determined using the method described by Whitaker and Einargranum [17]. The denaturation curves were obtained with the addition of guanidine hydrochloride (GndHCl) from a stock solution of 4.5 M, resulting in a final concentration of 3.4 M GndHCl in the sample, and upon autoclaving.

Far-UV (190–250 nm) CD spectra were recorded in a Jasco J810 (Jasco, Japan) using rectangular quartz cells (1 mm path length). The temperature was controlled using the Single Position Peltier Cell Holder PTC-423 (Jasco, Japan). All spectra were recorded after the accumulation of eight runs, with the exception of the temperature measurements, which were recorded after one run. The scan rate was 50 nm min^{-1}, with bandwidths of 0.5 nm in all measurements, and the CD spectra of the buffer solutions was subtracted to eliminate background effects. A smoothing filter, FFT (Fast Fourier Transform), was also used. Quantitative prediction of the secondary structure was performed by deconvoluting the CD spectra using the CDProWin7 package [18]. The best results were achieved with the SelconWin7, CDSStrWin7 and ContinLLWin7 programs [18,21,22,23], with a root mean square (RMS) lower than 10% for all deconvolutions.

2.4. Theoretical structural calculations

The secondary structure was predicted using the Phyre2 server [24] (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) and the tertiary structure model was calculated using the RaptorX web server [25]. Fig. 3B was generated with the program RasMol [26].

3. Results

3.1. Cloning, sequence analysis and purification of TrEH

Bioinformatic analysis of the genome of *Trichoderma reesei*, which was performed by the "Joint Genome Institute" (JGI, USA) (http://genome.jgi-psf.org/Trire2/Trire2.home.html) [15], allowed us to identify the gene with the code transcript ID: 53220, located in scaffold_1: 854,187 to 855,218, and consisting of a single 1032 bp exon. An analysis using the Interpro software (http://www.ebi.ac.uk) identifies it as a putative epoxide hydrolase enzyme (Protein ID: 53220), which will be, in this work, called soluble epoxide hydrolase of *Trichoderma reesei* (TrEH), consisting of 343 amino acids and a molecular mass of 38.2 kDa. The sequence of the protein is deposited in the GenBank database (accession n°. EGR52240.1; NCBI, http://www.ncbi.nlm.nih.gov).