A novel and enantioselective epoxide hydrolase from *Aspergillus brasiliensis* CCT 1435: Purification and characterization

Lilian L. Beloti\(^a\), Bruna Z. Costa\(^b\), Marcelo A.S. Toledo\(^a\), Clelton A. Santos\(^a\), Aline Crucello\(^a\), Marianna T.P. Fávaro\(^a\), Juliano S. Mendes\(^a\), Anita J. Marsaioli\(^b\), Anete P. Souza\(^a, c, *\)

\(^a\) Centro de Biologia Molecular e Engenharia Genética (CBMEG), Universidade Estadual de Campinas, Campinas, São Paulo, Brazil
\(^b\) Instituto de Química (IQ), Universidade Estadual de Campinas, São Paulo, Brazil
\(^c\) Instituto de Biologia, Departamento de Biologia Vegetal, Universidade Estadual de Campinas, São Paulo, Brazil

**Abstract**

A novel epoxide hydrolase from *Aspergillus brasiliensis* CCT1435 (AbEH) was cloned and overexpressed in *Escherichia coli* cells with a 6xHis-tag and purified by nickel affinity chromatography. Gel filtration analysis and circular dichroism measurements indicated that this novel AbEH is a homodimer in aqueous solution and contains the typical secondary structure of an α/β hydrolase fold. The activity of AbEH was initially assessed using the fluorogenic probe O-(3,4-epoxybutyl) umbelliferone and was active in a broad range of pH (6–9) and temperature (25–45°C), showing optimum performance at pH 6.0 and 30°C. The Michaelis constant (\(K_M\)) and maximum rate (\(V_{max}\)) values were 495 \(\mu\)M and 0.24 \(\mu\)M/s, respectively. Racemic styrene oxide (SO) was used as a substrate to assess the AbEH activity and enantioselectivity, and 66% of the SO was hydrolyzed after only 5 min of reaction, with the remaining (S)-SO exceeding 99% in a typical kinetic resolution behavior. The AbEH-catalyzed hydrolysis of SO was also evaluated in a biphasic system of water:isooctane; (R)-diol in 84% ee and unreacted (S)-SO in 36% ee were produced, with 43% conversion in 24 h, indicating a discrete enantioconvergent behavior for AbEH. This novel epoxide hydrolase has biotechnological potential for the preparation of enantiopure epoxides or vicinal diols.

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**Introduction**

Epoxide hydrolases (EHs)\(^1\) catalyze the hydrolytic ring opening of racemic epoxides to yield enantiopure epoxides and vicinal diols [1,2]. EHs are enzymes that do not utilize cofactors and are ubiquitously found in all living organisms. Several EH genes have been sequenced and described (more than 100 EH genes), including EHs from *Aspergillus niger* [3], *Solanum tuberosum* [4], humans [5], and *Agrobacterium radiobacter* [6], providing useful information that assists and encourages further research.

EHs (EH; EC.3.3.2.3) belong to the α/β hydrolase fold family [7,8] and have a structurally conserved catalytic triad (Asp-His-Asp/Glu) exhibiting similar epoxide-opening biocatalytic mechanisms [2,3,6,9,10].

Epoxides and vicinal diols are valuable chiral intermediates for the preparation of biologically active molecules, such as agrochemicals, drugs, and other pharmaceutical compounds [11,12]. Therefore, the stereoselective syntheses of epoxides and vicinal diols are of commercial interest. Chiral epoxides and vicinal diols can be prepared by several chemical procedures (most of which are based on transition-metal catalysis); however, these processes have several limitations, including low substrate-to-catalyst ratios, low efficiency, and waste generation [13,14]. Indeed, such environmental issues are important to the fine chemical industry, which is facing severe regulatory constraints, and these facts have encouraged the search for mild and cleaner synthetic processes, such as the application of epoxide hydrolase, to produce chiral epoxides and vicinal diols [13,14].

Due to the potential application of EHs, it is important to search for novel enzymes in addition to the application of protein engineering to improve the performance of existing EHs. Thus, microbial EHs have attracted great interest due to their easy manipulation and numerous potential applications in the asymmetric hydrolysis of epoxides on a preparative scale [15,16].

The present work addresses a full-length epoxide hydrolase-encoding EH cDNA from *Aspergillus brasiliensis* CCT1435. The cDNA encoding the EH was cloned into the pET28a vector and heterologously overexpressed in *E. coli* BL21(DE3) Rosetta cells. The catalytic performance of this new EH, named AbEH, was further characterized using a fluorogenic probe and styrene oxide (SO) as a substrate.
Materials and methods

Materials

The *E. coli* DH10B strain was used as the host strain, whereas *E. coli* BL21(DE3) Rosetta (Novagen, USA) was used for heterologous protein expression. The oligonucleotide primers were synthesized at Sinapace Technologies (Brazil). The pET28a(+) expression vector was obtained from Novagen (USA), and the *NdeI* and *XhoI* restriction enzymes were purchased from Fermentas (USA). The protease inhibitor phenylmethanesulfonyl fluoride (PMSF), β-mercaptoethanol, Tween-20, and lysozyme were obtained from Sigma Chemical (USA). The Ni–NTA affinity resin was obtained from Qiagen (Germany). The molecular mass standard (low molecular weight (LMW) and high molecular weight (HMW)) for the gel filtration calibration kit and Superdex 200 10/300 GL chromatography column were purchased from GE Healthcare (Sweden). The total protein concentration was determined using a micro BCA™ protein assay kit (Thermo Scientific, USA). Fluorogenic probe 1 and product 2 were readily synthesized by our group [17]. Racemic SO (4) and enantiopure (R)- and (S)-SO were purchased from Sigma Aldrich.

Blast search and sequence analysis of *Aspergillus* epoxide hydrolases

The EH sequences of *Aspergillus* were used to search the microbial database at NCBI (http://www.ncbi.nlm.nih.gov). The DNA sequence of the *A. niger* LCP521 epoxide hydrolase (AnEH LCP531), which has previously been described (PDB IN: 1QO7_A), was used as the model for the analysis of other homologs. Based on these data, primers were screened and used to amplify the complete EH gene from *A. brasiliensis* (AbEH CCT1435).

Alignment of AbEH CCT1435 with AnEH LCP521

The amino acid sequence of epoxide hydrolase from *A. niger* LCP521 (GenBank accession number: AJ238460) was obtained from the NCBI databank (http://www.ncbi.nlm.nih.gov). The AbEH CCT1435 sequence presented in this work was aligned with the AnEH LCP521 sequence using ClustalW software (http://www.ebi.ac.uk/Tools/msa/clustalw2/); the editing of the alignment was performed using GENEDOC software [18].

Isolation of nucleic acids from *A. brasiliensis*

*A. brasiliensis* (strain CCT1435), deposited at the Coleção de Culturas Tropical Fundação André Tosello-Brazil, was grown in malt extract medium (ME) at a concentration of 20 g/L of culture. The culture medium (300 mL) at 28 °C was inoculated with *A. brasiliensis* spores and incubated for 72 h in a shaking flask. The mycelium was harvested by filtration through Miracloth (Millipore, Germany) and stored at −80 °C. RNA isolation was performed as described by Jones [19]. The EH-cDNA was synthesized using the SuperScript II kit (Invitrogen, USA) according to the manufacturer’s instructions.

cDNA amplification and cloning

The cDNA of *A. brasiliensis* was amplified by polymerase chain reaction (PCR). The forward and reverse AbEH primers (forward, 5′-ATACATATGATGTCGCTCCTGCGC-3′, and reverse, 5′-ATACTCGAGTCTACTGCCACGCTGTC-3′) contained *NdeI* and *XhoI* restriction sites (underlined), respectively. The PCR amplification product and pET28a(+) plasmid (Novagen, USA) were both digested with *NdeI* and *XhoI* and ligated using T4 DNA ligase (Invitrogen, USA). The constructed plasmid was transformed by electroporation into *E. coli* BL21(DE3) Rosetta cells, and the inserted sequences were confirmed by DNA sequencing.

Heterologous overexpression of AbEH in *E. coli*

AbEH was overexpressed in the *E. coli* BL21(DE3) Rosetta strain. The cells were grown at 37 °C and 300 rpm in 1 L of LB broth containing kanamycin (30 μg/mL) and chloramphenicol (34 μg/mL). When the cells reached an optical density at 600 nm (OD600) of 0.6–0.8, IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to a final concentration of 0.4 mM to initiate the overexpression of the EH protein. The cells were then incubated at 25 °C and 200 rpm for 16 h. The cells were harvested by centrifugation (3000g for 10 min at 4 °C). The cell pellets were resuspended in 50 mL of buffer A (50 mM phosphate buffer, pH 7.2) supplemented with 0.2% Tween-20, 12 mM β-mercaptoethanol, 1 mg/mL lysozyme, and 1 mM PMSF and subsequently incubated on ice for 30 min. The cells were disrupted by sonication (Ultrasonic Homogenizer 4710; the output control was set to six, and the duty cycle was set to 70%), and the soluble fraction was collected by centrifugation (27,000g for 50 min at 4 °C). The soluble fraction was loaded to an Ni–NTA chromatography column (Qiagen, Germany) equilibrated with buffer B (50 mM phosphate buffer and 250 mM NaCl, pH 7.2). The EH protein was purified with an imidazole gradient (20, 50, 75, 250, and 500 mM). The purified EH protein was eluted with five volume columns of buffer B containing 250 mM imidazole and then dialyzed against buffer C (50 mM phosphate buffer, 1 mM dithiothreitol (DTT), and 0.2% Tween-20, pH 7.2). The purity of the protein was determined by SDS–PAGE under denaturing conditions, as described by Laemmli [20]. The protein concentration was measured with a micro BCA™ protein assay kit using bovine serum albumin as the standard.

Analytical size-exclusion chromatography

The molecular mass of the purified AbEHHis-tag was determined under native conditions by size-exclusion chromatography using a Superdex 200 10/300 GL column. The column was equilibrated with buffer B. The protein samples (250 μL) were injected at a flow rate of 0.5 mL/min. HMW and LMW gel-filtration calibration kits (GE Healthcare) were used as the calibration standards. Ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa), and blue dextran 2000 (2000 kDa) were used as the standards.

Circular dichroism measurements

The circular dichroism (CD) spectra of the purified AbEHHis-tag protein were obtained using a Jasco J-810 spectropolarimeter dichrograph (Japan Spectroscopic, Tokyo, Japan). The far-UV CD spectra were generated at 25 °C using 6 μM of AbEH protein under two conditions: 10 mM sodium phosphate buffer (pH 7.2) and 10 mM sodium phosphate buffer plus 1 mM of TCEP (tris(2-carboxyethyl)phosphine) as a reducing agent. The assays were performed using a quartz cuvette with a path length of 2 mm. Ten accumulations within the 270- to 190-nm range at a rate of 50 nm/min were recorded. The deconvolution and statistical analyses of the CD spectra were performed using the DichroWeb server [21,22].

EH fluorescence-based assay

The substrate O-(3,4-epoxybutyl)umbelliferone (1) was used as a fluorogenic probe for the detection of AbEH hydrolytic activity. The assays were performed in flat-bottom polystyrene 96-well
microtiter plates and incubated at 30°C in a plate reader spectrophotometer (FlashScan 530, Analytik Jena, Germany). The fluorescence intensities were measured every 5 min for 90 min using 390 and 460 nm as the excitation and emission wavelengths, respectively. The enzymatic reaction conversions (in percentages) were obtained by comparing the fluorescence intensities of the enzymatic assays with the positive (100% conversion) and negative (0% conversion) controls according to the following equation:

\[
\text{Conversion (\%)} = \left( \frac{\text{FI}(A) - \text{FI}(NC)}{\text{FI}(PC)} \right) \times 100
\]

where FI(A), FI(NC), and FI(PC) are the fluorescence intensities of the enzymatic assay, negative control, and positive control, respectively.

The reaction conditions were as follows:

- **Enzymatic assay:** 1 (5 μL of a 2 mM solution in acetonitrile:water [1:1]), NaIO4 (5 μL of a 2 mM solution in deionized water), BSA (40 μL of a 5 mg/mL solution in buffer D [50 mM sodium phosphate, 1 mM DTT, pH 7.2, and 0.1% Tween-20]), and AbEH (5 μL of a 1 mg/mL solution in buffer D with 1 mM DTT).

- **Positive control:** 2 (5 μL of a 2 mM solution in acetonitrile:water [1:1]), NaIO4 (5 μL of a 2 mM solution in deionized water), BSA (40 μL of a 5 mg/mL solution in buffer D), and AbEH (5 μL of a 1 mg/mL solution in buffer D with 1 mM DTT).

- **Negative control:** 1 (5 μL of a 2 mM solution in acetonitrile:water [1:1]), NaIO4 (5 μL of a 2 mM solution in deionized water), and BSA (40 μL of a 5 mg/mL solution in buffer D).

**pH, temperature, and Tween-20 concentration dependence of EH activity**

The effects of pH, temperature, and Tween-20 concentration on EH activity were determined using the fluorogenic assay described above by varying the pH from 4 to 10 (pH 4–5, acetate buffer; pH 6–8, sodium phosphate buffer; pH 9–10, glycine–NaOH buffer; each at 50 mM and 0.1% Tween-20), temperature from 25 to 45°C, and Tween-20 concentration from 0.1% to 5%.

**Kinetic parameters**

The enzyme kinetic parameters were also determined using the above fluorogenic assays by varying the substrate (1) concentration from 10 to 1500 μM and reaction time from 0 to 90 min. The apparent kinetic parameters, Michaelis constant \((K_m)\) and maximum rate \((V_{\text{max}})\), were estimated from the initial reaction rates at each substrate concentration and the subsequent non-linear regression of the Michaelis– Menten equation using Origin (OriginLab, Northampton, MA). The catalytic constants, turnover number \((k_{\text{cat}} = V_{\text{max}}/E)\) and specificity constant \((v = k_{\text{cat}}/K_m)\), were calculated taking 0.22 μM as the enzyme concentration.

**AbEH hydrolysis of styrene oxide**

Hydrolysis of racemic SO (R/S-SO, 4) by AbEH was assayed by monitoring the epoxide consumption and diol formation. The enzymatic reaction conditions were as follows. AbEH (2 mL of a 1 mg/mL solution in buffer D with 1 mM DTT) was added to buffer E (7.5 mL of sodium phosphate buffer at 70 mM [pH 6.0] and 0.1% Tween-20), and the mixture was preincubated at 30°C for 5 min. SO (0.5 mL of a 160 mM solution in DMSO) was added to obtain a final concentration of 8 mM in a total reaction volume of 10 mL. During the incubation, samples were periodically collected (every 5 min for 40 min), saturated with NaCl, and extracted with ethyl acetate (1 × 1 mL), adding benzophenone as an internal standard at a final concentration of 0.05 mg/mL. All samples were analyzed by GC–MS and chiral GC–FID to evaluate the conversions and enantiomeric excesses, respectively.

The SO conversion (%) into diol was calculated by comparing the peak areas of reagents, products, and benzophenone in the chromatogram considering the calibration curves. SO spontaneous hydrolysis under the reaction conditions was evaluated in the absence of EH.

The enantiomeric ratio of (R/S)-SO hydrolysis by AbEH was calculated based on the enantiomeric excess (ee) of the remaining epoxide and the conversion of racemic SO according to Sih’s equation [23], as follows:

\[
E = \frac{\ln[(1 - C)(1 - ee)]}{\ln[(1 - C)(1 + ee)]}
\]

where \(c = (A + B)/(A_0 + B_0)\), \(A\) and \(B\) are the concentration of fast- and slow-reacting enantiomers, and \(A_0\) and \(B_0\) are the initial concentrations.

The reaction enantioselectivity was further evaluated using the same reaction conditions described above with enantiomeric pure SO (R-SO or S-SO). In this case, the enantiomeric ratio \((E)\) was estimated based on the initial reaction rate of each SO enantiomer according to the equation:

\[
E = \frac{v_0(\text{fast enantiomer})}{v_0(\text{slow enantiomer})}
\]

**Reactions in liquid–liquid biphasic systems**

Reactions in liquid–liquid biphasic systems were performed in closed vials (10 mL) containing buffer D (0.5 mL, 50 mM sodium phosphate buffer [pH 7.2] and 0.1% Tween-20), AbEH (0.5 mL of a 1 mg/mL solution in buffer D), a water-immiscible organic solvent (3 mL), and (R/S)-SO (10 μL, final concentration of 22 mM). The reaction mixtures were incubated at 30°C, with stirring (200 rpm), for 24 h.

The best organic solvent among heptane, iso-octane, toluene, ethyl acetate, and iso-amyl alcohol was selected.

The remaining epoxide in the organic phase of the reaction was analyzed by GC–MS. The diol was recovered from the aqueous phase by saturation with NaCl, followed by extraction with ethyl acetate (2 × 1 mL) by mixing in a vortex and centrifugation at 2000 rpm for 5 min. The organic layers were combined, dried over anhydrous MgSO4, and filtered for further GC–MS and GC–FID analyses.

Control experiments without AbEH were performed in tandem with the enzymatic reactions to monitor the spontaneous hydrolysis of (R/S)-SO. The conversions (%), enantiomeric excesses, and enantiomeric ratios were assessed as previously described.

**Chromatographic analyses**

The enzymatic reaction conversions were monitored by GC–MS using an Agilent 7890 gas chromatograph coupled to a Hewlett Packard 5975C-MSD (70 eV) spectrometer equipped with a fused silica capillary column (HP-5MS, 30 m × 0.25 mm i.d. × 0.25 μm film thickness). The GC–MS analyses were conducted using a helium flow of 1 mL/min, the splitless mode, and the following temperature program: initial temperature of 80°C (3 min) increasing to 200°C at 10°C/min and to 300°C at 20°C/min, holding for 5 min.

The enantiomeric discriminations were performed using an Agilent 6850 gas chromatograph coupled to a flame ionization detector (GC–FID) using a Chirasil-Dex CB β-cycloextrin chiral column (Chrompack, 25 m × 0.25 mm i.d. × 0.25 μm film thickness). The
GC–FID analyses were conducted using 1 mL/min H2 flow, the split mode (10:1), and the following temperature program: initial temperature of 50 °C increasing to 100 °C at 10 °C/min and to 180 °C at 5 °C/min, holding for 5 min.

Results and discussion

Blast search and EH identification

Progress in the area of bioinformatics and cloning techniques plays an important role in the identification of novel EHs and other enzymes from metagenomic and genomic databases [5,11,24]. Thus, nucleotide sequence of AnEH LCP521 and other EHs deposited in NCBI GenBank were used as templates for the screening of primers. Although most of the known EHs have low sequence identity with each other, the EHs from the genus Aspergillus have high identity (45–99%), which allows for the design and screening of primers for gene amplification and characterization. After the amplification of the EH cDNA by PCR, the gene was cloned and sequenced and the identity of the gene was confirmed by sequencing (data not shown).

Alignment of AbEH with A. niger LCP 521 EH

The alignment of the amino acid sequence of A. brasiliensis EH strain CCT1453 with that of A. niger EH strain LCP521 indicated a high degree of conservation, with 89% identity. The alignment of the EH sequence cloned in this study with those of other strains deposited in the NCBI GenBank database revealed the presence of the conserved motifs that are characteristic of these enzymes (data not shown) [3,8]. A catalytic triad, Asp124–Asp260–His289, found in the EH from A. niger LCP 521 [2,3] was also present at the same positions in EH from A. brasiliensis CCT1435 (Fig. 1). The two active-site tyrosines (Tyr251 and Tyr314) that function as proton donors to the epoxide ring oxygen and aid in the ring-opening reaction are also present in AbEH [6].

Expression and purification of recombinant AbEH6His-tag in E. coli

The full-length AbEH cDNA was cloned into the pET28a(+) vector and expressed in E. coli BL21(DE3) Rosetta cells. High levels of expression were reached after the cells were incubated in the presence of 0.4 mM IPTG at 25 °C and 200 rpm for 16 h. The purification of the recombinant AbEH was performed by nickel affinity chromatography according to the described methodology. The elution gradient (20–500 mM imidazole) resulted in the optimal elution of AbEH from the resin in a buffer with 250 mM imidazole. The SDS–PAGE analyses indicated a single protein band of approximately 44 kDa (Fig. 2). Recombinant AbEH6His-tag was obtained at a satisfactory purity level and concentration (0.8 mg/mL). Analysis by mass spectrometry confirmed the identity of the recombinant protein (data not shown).

Preliminary structural characterization of AbEH

The secondary structure of purified AbEH was assessed by CD measurements, and a typical signal of a protein composed of α helices and β sheets was obtained (Fig. 2). This analysis indicated that the enzyme has a secondary structure that is consistent with that predicted for proteins with an α/β hydrolase fold [3,8].

The CD analysis of the purified protein was performed under oxidizing and reducing conditions. The reducing agent TCEP was

Fig. 1. ClustalW alignment of the amino acid sequences of EHs from Aspergillus brasiliensis CCT1435 and Aspergillus niger LCP 521 (PDB ID: 1QO7_A). The regions that are conserved in the two sequences (black) are highlighted. A high similarity between the two sequences can be observed (89% identity). The catalytic triad formed by Asp192-His374-Asp348 is highlighted in red in both sequences. The Tyr251 and Tyr314 residues, which act as proton donors for the epoxide ring oxygen, are highlighted in yellow.
used at a final concentration of 1 mM. However, no differences in the secondary structure were observed (data not shown). The oxy-red protein was examined because we observed that AbEH exhibits a low catalytic performance when its cysteines are oxidized (data not shown). Indeed, certain proteins tend to aggregate under oxidizing conditions, and the EH studied exhibited a low activity under these conditions [27].

The oligomeric state of the purified AbEH was examined by analytical gel filtration, which indicated that the protein is a homodimer in solution. The predicted molecular weight of the recombinant protein is 44 kDa, and we obtained an elution peak corresponding to a protein with an apparent molecular weight of 79 kDa, which is close to the expected molecular weight of the dimer (Fig. 3). These data agree with the data obtained for the EH of A. niger reported by Zou et al. [3].

Characterization of EH activity

The activity of AbEH was assessed with the fluorogenic probe O-(3,4-epoxybutyl)umbelliferone (1, Fig. 4a), which involves a cascade of chemo-enzymatic reactions in which diol 2, formed by the EH hydrolysis of epoxide 1, releases the umbelliferonyl anion (3) in the presence of NaIO₄ and BSA, producing a fluorescent signal that is measured at 460 nm after excitation at 390 nm [28].

Under the initial conditions applied (pH 7.2, 30 °C, and 0.1% Tween-20), compound 1 was 90% hydrolyzed by AbEH in 30 min (Fig. 4b). This observation indicated that AbEH has a specific activity of 0.17 U mg⁻¹, where 1 U is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of 1 per minute, at the applied reaction conditions. The determined AbEH specific activity is approximately five times greater than that of an EH from A. niger evaluated by Reymond and co-workers using the same fluorogenic probe [28].
It is well known that the protein ionization state is important for efficient enzyme catalysis, as is the temperature of an enzymatic reaction [29]. Within this context, the AbEH activity dependence on pH and temperature was investigated from pH 4.0 to 10.0 and a temperature range from 25 to 45 °C using probe 1 (Fig. 4c). The best AbEH catalytic performance was achieved at pH 6.0 and 30 °C. AbEH hydrolyzed probe 1 over a wide pH range between 5.0 and 10.0, maintaining at least 40% of its relative activity up to pH 9.0 at the optimum temperature; however, a pronounced decrease in activity was observed below pH 6.0 and above pH 9.0. AbEH was also active at all of the evaluated temperatures, though the relative activity did decrease to 11% at 25 °C and pH 6.0. This broad pH and temperature activity is reported among EHs from Aspergillus genera [30–33] and is a beneficial property for their chemical application.

The influence of surfactants on enzymatic activity is well known [34,35]; thus, AbEH activity was measured in the presence of Tween-20 (from 0.1% to 5.0% v/v). Although increased activity was observed with the addition of 0.1% of Tween-20, further increases in the Tween-20 concentration did not significantly improve AbEH activity (data not shown).

To further evaluate AbEH, the Michaelis–Menten kinetic parameters of 1 hydrolysis were determined. The plot of the initial reaction rates versus substrate concentration indicated a clear plateau within the investigated range (Fig. 4d). The non-linear regression fitting of the data to the Michaelis–Menten model produced Michaelis constant (K_m) and maximum rate (V_max) values of 495 μM and 0.24 μM/s, respectively, with a good regression coefficient of 0.983. From these data, the turnover number (k_cat) and catalytic efficiency (κ) of AbEH were also obtained, with values of 1.1 s⁻¹ and 2.3 × 10⁻¹ M⁻¹ s⁻¹, respectively. These results indicated that AbEH exhibits a Michaelis–Menten kinetic behavior and a moderate affinity for 1 under the applied conditions.

**AbEH hydrolysis of racemic styrene oxide**

Enantiopure epoxides and vicinal diols are versatile building blocks in the synthesis of various biologically active compounds and are therefore chemical intermediates of high value for the chemical and pharmaceutical industries [12]. Accordingly, there are well-established synthetic strategies to obtain these compounds, such as Sharpless asymmetric epoxidation [36] and AD-mix α- or β-mediated dihydroxylation of olefins [37]. However, the use of an EH allows for the direct preparation of enantiomerically pure epoxides and diols from the kinetic resolution of racemic epoxides in particularly mild conditions without using toxic reagents, which are principles of Green Chemistry [38].

Within this context, styrene oxide (SO) was used to assess the AbEH performance and selectivity (Fig. 5a).

The time course of the AbEH enantioselective epoxide hydrolysis was investigated using 8 mM (R/S)-SO and DMSO (5%) as the co-solvent under the determined optimal conditions (pH 6.0, 30 °C, and 0.1% Tween-20). Fig. 5b presents the AbEH conversion profile of SO and the enantiomeric excesses of the formed diol and remaining epoxide. After only 5 min of reaction, 66% of the SO was hydrolyzed, and the remaining epoxide ee exceeded 99% when (R)-SO was entirely consumed, leaving 34% of enantiopure (S)-SO; these results indicate a typical kinetic resolution behavior. Based
on Sih's equation, the estimated $E$ for this kinetic resolution of (R)/S)-SO was 9. The fast kinetic resolution of SO outweighed this relatively low $E$ value, which allows promising future applications of AbEH. Additionally, as shown in Table 1, AbEH exhibits more rapid enzyme kinetics than the listed EHs (up to 72-fold faster than the AbEH. Additionally, as shown in Table 1, AbEH exhibits more rapid consumption initial rates for the production of an enantioenriched 1,2-diol.

The EH-catalyzed hydrolysis of racemic epoxides is typically performed in an aqueous medium, as described above. However, in these systems small amounts of epoxides can be dissolved and spontaneous hydrolysis can occur. To overcome these disadvantages, biphasic systems involving an organic and aqueous layer to perform the enzymatic reactions have been applied with the additional benefit of the facile substrate and product recovery at the end of the reaction. Consequently, liquid–liquid biphasic systems for the hydrolysis of racemic SO by AbEH were evaluated using five different water-immiscible organic solvents (heptane, iso-octane, toluene, ethyl acetate, and iso-amyl alcohol) and a phosphate buffer as the aqueous phase.

The enzymatic hydrolysis of (R/S)-SO was only observed in the presence of heptane or iso-octane as the organic solvent, indicating the preferential hydrolysis of (R)-SO to produce (R)-1-phenyl-1,2-ethanediol. When using heptane as the organic solvent, the diol ee was 66% and that of the remaining epoxide was 30%, with 25% conversion after 24 h. However, the diol ee reached 84% in iso-octane, whereas that of the remaining epoxide (S)-SO was 36%, with a reaction conversion of 43% after 24 h.

Although discrete, the results obtained for the water:iso-octane system indicated a slight enantioconvergent behavior of AbEH-catalyzed hydrolysis of SO, particularly when considering the low ee observed for the remaining epoxide, which is not compatible with the reaction conversion and observed diol ee.

Additionally, the evaluated biphasic systems efficiently prevented the spontaneous hydrolysis of SO for days. This phenomenon was credited to the reduced contact time of the epoxide with the aqueous phase.

### Table 1

<table>
<thead>
<tr>
<th>Epoxide hydrolase</th>
<th>Ref.</th>
<th>Cat. Form</th>
<th>(±) SO mM</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>Time (min)</th>
<th>(S)-SO ee (%)</th>
<th>Yield (%)</th>
<th>$E$</th>
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<td>8</td>
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<td>99</td>
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</table>

* From *A. brasiliensis* CCT1435 analyzed in this research.
* Enantiomeric ratio ($E$) was calculated according to Sih's equation.
* Calculated with an ee of 90%.
* No information.

### Table 2

Summary of the AbEH-catalyzed hydrolysis of racemic SO in monophasic and biphasic liquid reaction systems.

<table>
<thead>
<tr>
<th>Medium</th>
<th>AbEH reaction system</th>
<th>Monophasic</th>
<th>Biphasic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Buffer ($\text{pH } 6.0$)</td>
<td>Buffer ($\text{pH } 7.2$):Heptane</td>
</tr>
<tr>
<td>SO concentration (mM)</td>
<td>8.0</td>
<td>22.0</td>
<td>22.0</td>
</tr>
<tr>
<td>AbEH concentration ($\mu$M)</td>
<td>4.3</td>
<td>11$^a$</td>
<td>11$^a$</td>
</tr>
<tr>
<td>Reaction time</td>
<td>5 min</td>
<td>24 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Conversion (%)</td>
<td>66</td>
<td>25</td>
<td>43</td>
</tr>
<tr>
<td>SO ee (%)</td>
<td>&gt;99/(S)</td>
<td>30/(S)</td>
<td>36 (S)</td>
</tr>
<tr>
<td>Diol ee (%)</td>
<td>58/(R)</td>
<td>66/(R)</td>
<td>84/(R)</td>
</tr>
<tr>
<td>$E$</td>
<td>9.0</td>
<td>26.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* Phosphate buffer, 50 mM (pH 6.0) and 0.1% Tween-20.
* Phosphate buffer, 50 mM (pH 7.2) and 0.1% Tween-20.
* AbEH concentration in the aqueous phase.
* Calculated based on Sih’s equation.

### AbEH hydrolysis of racemic styrene oxide in liquid–liquid biphasic systems

The EH-catalyzed hydrolysis of racemic epoxides is typically performed in an aqueous medium, as described above. However, in these systems small amounts of epoxides can be dissolved and spontaneous hydrolysis can occur. To overcome these disadvantages, biphasic systems involving an organic and aqueous layer to perform the enzymatic reactions have been applied with the additional benefit of the facile substrate and product recovery at the end of the reaction [48,49]. Consequently, liquid–liquid biphasic systems for the hydrolysis of racemic SO by AbEH were evaluated using five different water-immiscible organic solvents (heptane, iso-octane, toluene, ethyl acetate, and iso-amyl alcohol) and a phosphate buffer as the aqueous phase.

The enzymatic hydrolysis of (R/S)-SO was only observed in the presence of heptane or iso-octane as the organic solvent, indicating the preferential hydrolysis of (R)-SO to produce (R)-1-phenyl-1,2-ethanediol. When using heptane as the organic solvent, the diol ee was 66% and that of the remaining epoxide was 30%, with 25% conversion after 24 h. However, the diol ee reached 84% in iso-octane, whereas that of the remaining epoxide (S)-SO was 36%, with a reaction conversion of 43% after 24 h.

Although discrete, the results obtained for the water:iso-octane system indicated a slight enantioconvergent behavior of AbEH-catalyzed hydrolysis of SO, particularly when considering the low ee observed for the remaining epoxide, which is not compatible with the reaction conversion and observed diol ee.

Additionally, the evaluated biphasic systems efficiently prevented the spontaneous hydrolysis of SO for days. This phenomenon was credited to the reduced contact time of the epoxide with the aqueous phase.
The use of biphasic systems in enzymatic reactions also simplifies the purification steps, an appreciated feature in industrial applications. In this case, the substrate (epoxide, less polar) has a greater affinity for the organic phase and the product (diol, more polar) for the aqueous phase [40]. This partitioning provides in situ purification of the reaction components; therefore, the substrate and product were recovered through the simple separation of the organic and aqueous phases. Table 2 summarizes the results of SO hydrolysis by AbEH in all of the evaluated systems. It is clear that the aqueous systems using only DMSO as the co-solvent to assist in epoxide solubilization is more efficient and productive, allowing for the kinetic resolution of racemic SO by AbEH in only 5 min. However, these biphasic systems illustrated a possible new enantioconvergent action for the recombinant wild-type EH from *A. brasilensis* CCT1435. Further studies are being performed to understand and confirm this behavior and to apply this novel EH to epoxides of industrial interest.

**Conclusion**

Our results demonstrate that the new AbEH was successfully expressed in *E. coli* and purified. AbEH has an optimum enzymatic performance at 30 °C and pH 6.0 and exhibits an increased activity with the addition of Tween-20 (0.1% v/v). This study demonstrated that recombinant AbEH has an (R)-preferential enantioselectivity in an aqueous system, leading to the complete kinetic resolution of 8 mM racemic SO within 5 min. AbEH also presents a potential enantioconvergent performance in a water:iso-octane biphasic system; however, further studies will be performed to confirm this behavior. Therefore, this novel AbEH has suitable catalytic properties, enhancing its potential for use in the preparation of enantiopure epoxides and vicinal diols.

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**References**


