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Exploring the Biocatalytic Scope of a Novel Enantioselective Halohydrin Dehalogenase from an Alphaproteobacterium

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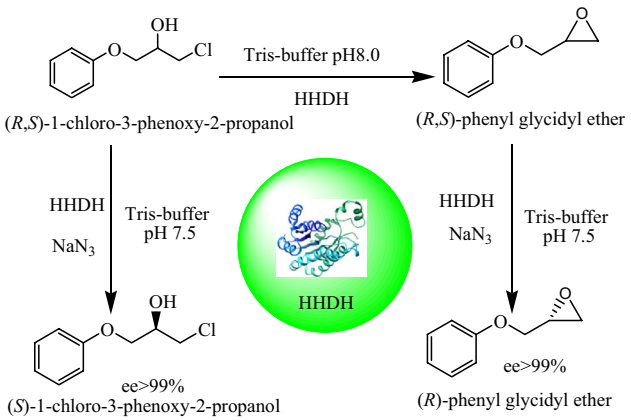
Abstract

A gene encoding halohydrin dehalogenase from an alphaproteobacterium (*Ab*HHDH) was identified, cloned and overexpressed in *Escherichia coli*. *Ab*HHDH was able to catalyze the stereoselective dehalogenation of prochiral and racemic halohydrins. It showed the highest enantioselectivity in the dehalogenation of 20 mM (*R,S*)-2-bromo-1-phenylethanol, which yielded (*S*)-2-bromo-1-phenylethanol with 99% *ee* and 34.5% yield. Moreover, *Ab*HHDH catalyzed the azidolysis of epoxides with low to moderate (*S*)-enantioselectivity. The highest enantioselectivity ($E = 18.6$) was observed when (*R,S*)-benzyl glycidyl ether was used as the substrate. A sequential kinetic resolution catalyzed by HHDH was employed for the synthesis of chiral 1-chloro-3-phenoxy-2-propanol. We prepared enantiopure (*S*)-isomer with a high enantiopurity of *ee* > 99% and a yield of 30.7% (*E*-value: 21.3) by kinetic resolution of 20 mM substrate. The (*S*)-isomer with 99% *ee* readily obtained from 40 to 150 mM (*R,S*)-1-chloro-3-phenoxy-2-propanol. Taken together, the results of this study demonstrate the applicability of this HHDH for the production of optically active compounds.



Keywords Halohydrin dehalogenase · Epoxides · Halohydrins · Enantioselectivity · Biocatalysis

1 Introduction

Halohydrin dehalogenases (HHDHs) are employed by microbes for the biodegradation of halogenated organic compounds [1]. Biochemical and structural studies have shown that HHDHs and the enzymes of the short-chain dehydrogenases/reductases family (SDR) are not only evolutionarily but also mechanistically related, catalyzing the dehalogenation of ortho-halogenated alcohols into their

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corresponding epoxides [1, 2]. In the reverse reaction, they can accept a variety of alternative anionic nucleophiles such as CN^-, NO_2^-, and N_3^-, to open the epoxide ring, which provides a novel biocatalytic strategy for the preparation of fine chemicals and pharmaceutic intermediates [2, 3].

Recent investigations focus on the biotechnological applications of HHDHs, especially regarding the stereoselective biotransformations [1]. At the early stage, HHDHs were utilized in the kinetic resolution of racemic halohydrins to obtain an optically active enantiomer [4–7]. Chiral epoxides are valuable and extremely important building blocks for the synthesis of enantiomerically pure pharmaceuticals and agrochemicals. In addition, the kinetic resolution of racemic epoxides by HHDHs is another way to obtain the optically pure isomers [3, 8–10]. The HHDH-catalyzed stereoselective ring opening of epoxides is widely used in the asymmetric synthesis of enantiopure β-substituted alcohols, which are important building blocks for a number of pharmaceuticals and biologically active compounds [11–14]. The most successful example is the synthesis of the statin side-chain precursor ethyl (*R*)-4-cyano-3-hydroxybutyrate by coupling highly engineered variant of HheC with stereoselective ketoreductase [15, 16].

Despite these diversity of reported biocatalytic applications, it is particularly surprising to remember that all the reactions studied in the past 20 years have involved only a very small number of HHDHs [2]. Before 2014, only six different HHDH sequences had been cloned, characterized and classified into three different phylogenetic subtypes A, B, and C by sequence homology and substrate specificity [1]. To explore novel promising and attractive HHDHs, many new techniques and approaches have been adopted. Among them, database mining is an effective strategy to discover new HHDHs. Using a database mining approach based on the two sequence motifs representing the nucleophile binding pocket (TX_4[FY]XG) and catalytic triad ($SX_{12}YX_3R$), the number of available HHDH sequences was greatly increased [1, 17]. Four new HHDH family subtypes, D through G, could be identified, with percentage identities below 30% for protein sequence alignments between them [1, 17]. Of these HHDHs, 21 representative proteins from six phylogenetic subtypes were characterized in detail, several of which showed broad substrate spectra [13, 18–22]. Among all the HHDHs mentioned above, HheC still seems to be the only HHDH enzyme with relatively high enantioselectivity in the conversion of various substrates, whereas most other HHDHs are either non-selective or display only low to moderate enantioselectivity toward a few halohydrins and epoxides [1, 18]. Thus, there is still a lack of enantioselective HHDHs with high enantioselectivity for various halohydrins and epoxides. Therefore, it is necessary to identify and clone more enantioselective HHDH for further characterization of their biochemical and structural properties and for further applications in organic synthesis.

Herein, a series of predicted putative HHDHs were selected as the candidates on the basis of NCBI database searches with the reported HheC and HheB as query sequences. Eight genes bearing 36–46% amino acid sequence identity with the probe HHDHs were cloned and expressed in *Escherichia coli* BL21(DE3). After one round of testing and screening, the HHDH from an alphaproteobacterium (isolate 46_93_T64), was identified as the best candidate based on its high enantioselectivity toward (*R,S*)-phenyl glycidyl ether. The substrate scope of this novel enzyme was investigated in detail. Furthermore, sequential kinetic resolution of (*R,S*)-1-chloro-3-phenoxy-2-propanol catalyzed by the halohydrin dehalogenase in conjunction with azide was performed to verify the practical applicability of HHDH.

2 Materials and Methods

2.1 Materials

The reagents 1,3-dichloro-2-propanol (**1**), 1,3-dibromo-2-propanol (**2**), (*R,S*)-2,3-dichloro-propanol (**3**), (*R,S*)-2,3-dibromo-2-propanol (**4**), (*R,S*)-2-chloro-1-phenylethanol (**5**), (*R,S*)-2-bromo-1-phenylethanol (**6**), (*R,S*)-1-chloro-3-phenoxy-2-propanol (**7**), (*R,S*)-epichlorohydrin (**8**), (*R,S*)-styrene oxide (**9**), (*R,S*)-phenyl glycidyl ether (**10**), (*R,S*)-benzyl glycidyl ether (**16**), (*R,S*)-naphthyl glycidyl ether (**17**) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All reagents were of analytical grade. The racemic epoxides **11–15** were prepared according to a previously described synthesis method [23]. Isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Yeast extract, tryptone and agar were purchased from Oxoid (Basingstoke, England). All other organic compounds were obtained from local commercial suppliers and used without further purification (Fig. 1).

2.2 Strains and Plasmid

Escherichia coli BL21 (DE3) (Novagen, Darmstadt, Germany) was used as host strains for the heterologous expression of recombinant HHDH. pET28a (+) was used as vector for the expression of HHDH gene in *E. coli* BL21 (DE3). *E. coli* transformants were grown in Luria–Bertani (LB) medium.

2.3 Database mining for HHDHs

A toolbox of HHDHs was collected by database mining. The candidates were further confirmed as putative HHDHs

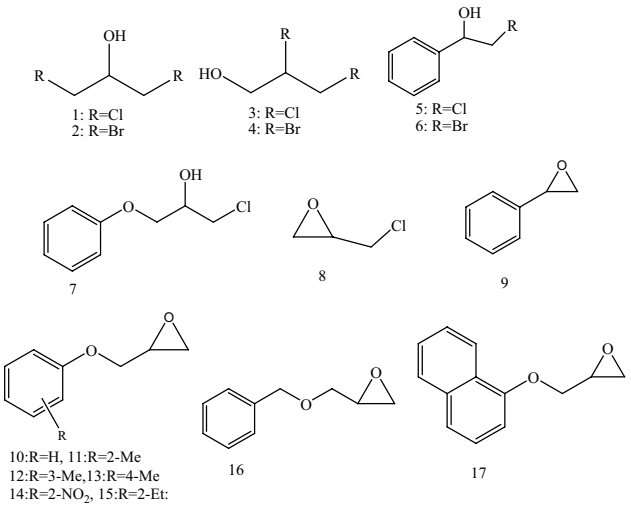

Fig. 1 Substrates used in the dehalogenation and epoxide ring opening reactions with *Ab*HHDH [**1**: 1,3-dichloro-2-propanol, **2**: 1,3-dibromo-2-propanol, **3**: (*R,S*)-2,3-dichloro-propanol, **4**: (*R,S*)-2,3-dibromo-2-propanol, **5**: (*R,S*)-2-chloro-1-phenylethanol, **6**: (*R,S*)-2-bromo-1-phentlethanol, **7**: (*R,S*)-1-chloro-3-phenoxy-2-propanol, **8**: (*R,S*)-epichlorohydrin, **9**: (*R,S*)-styrene oxide, **10**: (*R,S*)-phenyl glycidyl ether, **11**: (*R, S*)-1,2-Epoxy-3-(2-methylphenoxy)propane, 12: (*R, S*)-1,2-epoxy-3-(3-methylphenoxy)propane, **13**: (*R, S*)-1,2-epoxy-3-(4-methylphenoxy)propane, **14**: (*R, S*)-1,2-epoxy-3-(2-nitrophenoxy)propane, **15**: (*R, S*)-l-(2′-ethylphenoxy)-2,3-epoxy-propane, **16**: (*R,S*)-benzyl glycidyl ether, **17**: (*R,S*)-naphthyl glycidyl ether]

based on the presence of the conserved motifs [1]. A total of 8 putative HHDHs were selected and confirmed by multiple sequence alignment with other reported HHDHs using ClustalW2 program (Table 1). The HHDH nucleotide sequences were synthesized by Suzhou Hongxun Biotechnological Development (China) using the PCR assembly method after condons optimization for *E. coli*. The HHDH genes were cloned individually into the pET28a vector between the *Nco*I and *Xho*I restriction endonuclease sites. The ligated plasmid pET28a-HHDHs were transformed into *E. coli* BL21 (DE3). After heterogeneous overexpression in *E. coli* BL21 (DE3), the activity and enantioselectivity toward (*R,S*)-phenyl glycidyl ether were determined, as listed in Table 1.

2.4 Protein Purification

Recombinant *E. coli* cells harboring the pET28a-*Ab*HHDH were cultured in 2 L of LB with kanamycin (50 μg/mL) at 37 °C. When the optical density at 600 nm reached 0.6, the cells were induced by addition of 0.1 mM IPTG and growth was carried out at 28 °C for extra 10 h. The cells were harvested by centrifugation at 10,000×*g* for 10 min. The purification of recombinant HHDH was carried out at 0–4 °C. For preparation of the crude enzyme, 4.0 g of recombinant *E. coli* (wet cell weight) was resuspended in 40 mL Tris–SO_4 buffer (20 mM, pH 8.0). After sonification for 15 min in an ice bath, crude enzyme was prepared by centrifugation of the lysate at 15,000×*g* for

Table 1 Evaluation of HHDHs in the toolbox using (*R,S*)-phenyl glycidyl ether as a model substrate

Entry	Accession number	Source organism	Amino acid sequence identify to probe enzyme	Activity[a] (U/g cww)	ee_s(%)/configuration
1	WP_092014473.1	*Marinobacter daqiaonensis*	45% with HheB 227 aa	320 ± 7.8	18.5 ± 2.1/(*S*)
2	WP_072897053.1	*Hydrocarboniphaga daqingensis*	46% with HheB 236 aa	265 ± 2.5	17.7 ± 1.8/(*R*)
3	WP_091849011.1	*Marinobacter segnicrescens*	43% with HheB 227 aa	335 ± 3.4	46.0 ± 2.5/(*S*)
4	OJU34764.1	Alphaproteobacteria incertae sedis isolate 65–37	39% with HheC 224 aa	810 ± 6.6	-[b]
5	WP_082752032.1	*Rhodospirillaceae* incertae sedis isolate CCH5-H10	36% with HheC 244 aa	2.9 ± 0.7	-
6	WP_066115330.1	*Xylophilus ampelinus*	45% with HheB 226 aa	172 ± 2.1	64.5 ± 3.2/(*S*)
7	WP_083723433.1	*Pseudomonas pachastrellae*	41% with HheB	ND[c]	–
8	OUR79898.1	Alphaproteobacteria incertae sedis isolate 46_93_T64	41% with HheC 243 aa	550 ± 4.9	> 99/(*R*)

Highest observed ee of residual epoxide (ee_s)

[a]The specific activity was expressed in U/g cell wet weight (cww)

[b]–: no enantioselectivity

[c]*ND* no detectable activity

20 min at 4 °C. The supernatant was fractionated by the addition of ammonium sulfate between 30 and 70% saturation. The active precipitate was collected by centrifugation at 12,000×*g* for 20 min and dissolved in the buffer. The as-prepared solution was loaded onto a HiTrap Phenyl Sepharose column, which had been equilibrated with 1.0 M $(NH_4)_2SO_4$/20 mM Tris–SO_4 buffer (pH 8.0). After washing the column with the same buffer, the adsorbed proteins were eluted with Tris–SO_4 buffer (20 mM, pH 8.0) supplemented with a linear gradient of ammonium sulfate (from 1.0 to 0 M). The active fractions were combined and dialyzed against the Tris–SO_4 buffer (20 mM, pH 8.0). Finally, the fractions with HHDH activity were loaded onto a Superdex 75 column, pre-equilibrated with Tris–SO_4 buffer (20 mM, pH 8.0), and the enzyme was eluted with the same buffer at a flow rate of 0.5 mL/min. Aliquots containing pure HHDH were collected and used for subsequent studies. SDS-PAGE was applied to check the purity of the protein [24], and the protein concentration was determined using a Bradford protein assay kit with bovine serum albumin as the standard.

2.5 Substrate Specificity and Enantioselectivity of the *Ab*HHDH

The substrate specificity of recombinant *Ab*HHDH was determined by measuring the dehalogenation rates of seven different halohydrins as well as monitoring the ring-opening reaction of nine different epoxides in the presence of azide. Analytical-scale reactions were carried out in 2 mL Eppendorf tubes with a total reaction volume of 400 µL. Stock solutions comprising halohydrins 1–7 in ethanol were added to a final concentration of 20.0 mM into Tris–SO_4 buffer (100 mM, pH 8.0). The epoxide ring-opening reactions were carried out in 100 mM Tris–SO_4 buffer (pH 7.5) containing 20 mM epoxides 9–16 and 40 mM NaN_3. The reactions were started by the addition of enzyme in Tris–SO_4 buffer (pH 8.0 or 7.5) and incubated at 30 °C and 150 rpm. Samples were collected at regular time intervals, extracted twice with 1 mL ethyl acetate and dried over with anhydrous Na_2SO_4. The conversion, activity and enantiomeric excess (*ee*) values were determined using HPLC or GC analysis. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 µmol of epoxide per min under the described assay conditions. The specific activities were expressed in U/mg, and all experiments were conducted in triplicate. Activity assay of the control contained cell-free extracts obtained using the pET28a vector without the HHDH gene in *E. coli* BL21(DE3), cultured in the same way.

2.6 Analytical Methods

The *ee* and yields of halohydrins, epoxides and azido alcohol were determined by chiral GC analysis on a capillary BGB-175 column, or HPLC analysis on Chiralcel AS-H and Chiralcel OD-H columns (250×4.6 mm, particle size 5 µm, Daicel Chemical Industries, Tokyo, Japan) under the conditions described in the Supplementary information. The enantiomers of the epoxides was assigned by comparison of their elution order on the GC or HPLC columns with published data [21, 25–29]. The enantiomers of the azido alcohols were identified by analogy according to the reduced epoxides. The ee_s and ee_p were derived from the remaining epoxide and formed azido alcohols of the two enantiomers as $[ee(\%) = (S-R)/(S+R) \times 100]$. *R* and *S* are the concentrations of (*R*) and (*S*)-enantiomer, respectively. *E* values were calculated by formula $E = \ln[(1-c)(1-ee_s)]/\ln[(1-c)(1+ee_s)]$ (c, conversion) [30].

2.7 Sequential Kinetic Resolution of (*R,S*)-1-Chloro-3-Phenoxy-2-Propanol

Freshly prepared *E. coli* cells expressing *Ab*HHDH were resuspended to a density of 5 g/L cell dry weight (cdw) in 0.2 M Tris–SO_4 buffer (pH 7.0, 7.5 or 8.0) containing (*R,S*)-1-chloro-3-phenoxy-2-propanol (20–150 mM) and 1 equiv of NaN_3 to a 10 mL system in a shake-flask. Reactions were carried out at 20 °C and 150 rpm. Reaction progress was periodically monitored by taking samples (0.5 mL) from the reaction mixture. The samples were extracted with ethyl acetate (1 mL), dried over Na_2SO_4 and analyzed by HPLC.

2.8 Nucleotide Sequence Accession Number

The nucleotide sequence of *Ab*HHDH has been deposited in the GenBank database under the accession number MH782148.

3 Results and discussion

3.1 Identification and screening of HHDHs

A genome mining approach was adopted to search for robust HHDHs that might be able to catalyze the azide-mediated ring opening reaction of epoxides to corresponding azido alcohols with high enantioselectivity. In total, 8 HHDHs with 36–46% identity with HheC or HheB were selected from the NCBI database and overexpressed in *E. coli* BL21 (DE3) to form a HHDH toolbox. After testing their activities and enantioselectivities toward (*R,S*)-phenyl glycidyl ether as a representative substrate (Table 1), HHDH from an alphaproteobacterium 46_93_T64 exhibited the high specific

activity [550 U/g cell wet weight (cww)] and enantioselectivity (99% *ee*). Therefore, the *Ab*HHDH was chosen for further studies.

A protein BLAST search was conducted with *Ab*HHDH as the search sequence, which showed that it has moderate sequence similarity to known HHDH, reaching 41% identity with Hhe C from *Agrobacterium radiobacter* AD1 [31], 36% identity with HheA from *Corynebacterium* sp. N-1074 [32], 42% identity with *Tm*HHDH from *Tistrella mobilis* ZJB1405 [21], 56% identity with *Pl*HHDH from *Parvibaculum lavamentivorans* DS-1 [19]. Sequence analysis indicated that the cloned HHDH gene contained an open reading frame with 732 bp encoding a 243 amino-acid protein. Multiple alignments of *Ab*HHDH and other known HHDHs showed that it shares the N-terminal motif TX_4[FY]XG for nucleophile binding and the catalytic triad of Ser135-Tyr148-Arg152 (Fig. S1) [1]. The expression of HHDH was clearly visible in the SDS-PAGE of the soluble fraction of the *E. coli* cells taken at 10 h. When a cleavable His-tag was fused to the N- or C-terminus of *Ab*HHDH, it was almost completely inactivated. Therefore, it was purified to apparent homogeneity by salting out with ammonium sulfate, hydrophobic chromatography and gel chromatography which yielded 52 mg/L purified *Ab*HHDH. The purified enzyme had a molecular weight of 27 kDa (Fig. S2), which was in good agreement with the theoretical value (26.2 kDa) calculated using ExPASy. The specific activity of *Ab*HHDH for the azidolysis of (*R,S*)-phenyl glycidyl ether was determined to be 27.5 U/mg protein.

3.2 Substrate specificity and enantioselectivity

Substrate specificity and enantioselectivity are among the most important enzyme characteristics for biocatalysis. To map the substrate spectrum of this newly mined HHDH, various halohydrins and epoxides of diverse structure were employed. The activity assay of the control, using a cell-free extracts obtained by expression of the pET28a vector without the HHDH gene in *E. coli* BL21(DE3), did not show any activity toward any of the tested substrates. The activity and enantioselectivity of *Ab*HHDH in the dehalogenation of different halohydrins (**1–7**) are summarized in Table 2. Almost all the tested substrates were accepted by *Ab*HHDH. The highest activity was measure for the dehalogenation of 1,3-dibromo-2-propanol (17.2 U/mg), while 1,3-dichloro-2-propanol, (*R,S*)-2-bromo-1-phenylethanol and (*R,S*)-2,3-dibromo-2-propanol were converted with good specific activities, reaching 48.8%, 12.2% and 6.9% relative activity, respectively. With the substrate (*R,S*)-2-chloro-1-phenylethanol, only a low activity could be detected, and there was no detectable activity were observed with (*R,S*)-2,3-dichloro-propanol. (*R,S*)-**3** is efficiently dehalogenated by HheC but was a poor substrate for all other currently characterized HHDH. These results indicate that a halogen in the α position relative to the hydroxyl group seems to be beneficial with respect to the specific activity of the HHDHs. For instance, the activity of *Ab*HDH toward (*R,S*)-compound **4** was less than 6.9% of that toward the prochiral halohydrin **2** (Table 2). Furthermore, the activity with bromine-substituted compounds were higher than with equivalent chlorine-substituted compounds. The reason why *Ab*HHDH prefers brominated alcohols as substrates is that chlorine has a higher electronegativity than bromine, so that the strength of the carbon-bromine bond is much weaker than that of the carbon-chlorine bond [2, 18]. The specific activities toward the aromatic substrates (*R,S*)-2-chloro-1-phenylethanol and (*R,S*)-1-chloro-3-phenoxy-2-propanol were generally low. One of the reasons could be steric hindrance, which has been observed to affect the reactivity substantially.

Our interest was focused on the enantioselectivity of *Ab*HHDH in the conversion of various halohydrins. *Ab*HHDH catalyzed the transformation of prochiral 1,3-dichloro-2-propanol and 1,3-dibromo-2-propanol into (*S*)-epichlorohydrin (*ee* = 78.3%) and (*S*)-epibromohydrin (*ee* = 62.9%). Asymmetric synthesis using prochiral halohydrins is particularly attractive because the theoretical yield of one enantiomer in

Table 2 Activity and enantioselectivity of *Ab*HHDH toward various halohydrins substrates

Substrate	Activity (U/mg)	Time (min)	Conversion(%)	Halohydrins ee_s/configuration	Epoxide product ee_p(%)/configuration
1	8.4 ± 1.6	2	21.2 ± 0.5	–	78.3 ± 1.5/(*S*)
2	17.2 ± 1.9	1	30.4 ± 0.7	–	62.9 ± 1.2/(*S*)
3	ND	–	–	–	–
4	1.2 ± 0.1	5	5.7 ± 0.2	–	67.6 ± 0.9/(*S*)
5	$(2.0 \pm 0.1) \times 10^{-2}$	660	–	51.1 ± 1.7/(*S*)	59.4 ± 0.7/(*R*)
6	2.1 ± 0.2	20	65.5 ± 1.2	> 99/(*S*)	52.8 ± 1.1/(*R*)
7	$(3.7 \pm 0.2) \times 10^{-1}$	120	67.0 ± 1.3	5.7 ± 0.3/(*S*)	2.8 ± 0.3/(*S*)

The reactions were performed with halohydrins 1–7 (20 mM) and purified *Ab*HHDH (0.2 mg/mL) in Tris–SO_4 buffer (200 mM, pH 8.0) at 30 °C. The specific activity was expressed in U/mg. The *ee* value was determined by chiral HPLC and GC analysis, and the identity of the enantiomers was determined by comparison to the literature. ND: Not detectable due to very low activity

this case is 100% [33]. Compared with other HHDHs, the enantioselectivity of *Ab*HHDH toward prochiral halohydrins was similar to that of HHDH from *T. mobilis* ZJB1405 and different from that of the HheB and HheB8 from *Corynebacterium* sp. N-1074 and *Bradyrhizobium erythrophlei* [21, 22]. The epoxides produced by HheC and HheA were almost racemic, suggesting that enantioselective dehalogenation of prochiral halohydrins is not a general property of all HHDHs [34–36]. Furthermore, the enantioselectivity of *Ab*HHDH in the conversion of racemic haloalcohols **3–7** was studied by chiral GC and HPLC. *Ab*HHDH was able to convert (*R,S*)-2,3-dibromo-2-propanol into (*S*)-epibromohydrin. This indicated that the (*R*)-2,3-dibromo-2-propanol was converted preferentially, followed by a slower conversion of the (*S*)-enantiomer. Similar to the (*R*)-selective HheC, *Ab*HHDH also preferentially converted the (*R*)-enantiomer of (*R,S*)-2-chloro-1-phenylethanol [37]. So far, HheC still seems to be the only enzyme exhibiting a comparably high *R*-enantioselectivity in the conversion of (*R,S*)-2-chloro-1-phenylethanol ($E = 65$), while other reported HHDHs are either non-selective or display only low enantioselectivity toward haloalcohol **5** ($E \leq 4.7$) [18, 37]. In the dehalogenation reaction of (*R,S*)-2-chloro-1-phenylethanol, *Ab*HHDH showed a moderate enantioselectivity with an *E* value of 6.43. We also investigated the enantioselectivity of *Ab*HHDH in the dehalogenation of (*R,S*)-2-bromo-1-phenylethanol. The (*R*)-enantiomer was converted preferentially, followed by a much slower conversion of the (*S*)-enantiomer. (*S*)-2-bromo-1-phenylethanol was obtained with > 99% *ee* and 34.5% yield ($E = 27.7$). Thus, *Ab*HHDH exhibited a relatively high enantioselectivity among the reported HHDHs, with the 2nd highest *R*-enantioselectivity of all known HHDHs in literature [1, 2]. From this point of view, *Ab*HHDH may be of considerable interest in biocatalysis.

In the reverse reaction, which is epoxide-ring opening, different terminal epoxides can be accepted. However, there were significant differences in the activity and enantioselectivity among the accepted substrates. The substituents at the phenyl ring had an effect on the activity, but no general trend was observed. The position of the substituent at the phenyl ring also affected the enzyme activity, which increased from 3′<4′<2′ for the CH_3 group. *Ab*HHDH showed high activity in the kinetic resolution of (*R,S*)-phenyl glycidyl ether (27.2 U/mg) but only low activity toward (*R,S*)-1-chloro-3-phenoxy-2-propanol (0.37 U/mg). This could imply that *Ab*HHDH preferentially catalyzes epoxide ring opening of (*R,S*)-**10** using azide as a nucleophile, compared to the elimination of a chloride ion from the corresponding (*R,S*)-**7**. Although, the methyl- substituted phenyl glycidyl ether derivatives were well accepted by the *Ab*HHDH, their nitro- and methoxy- derivatives were transformed at a slower rate. Moreover, the enzyme did not perform well with (*R,S*)-naphthyl glycidyl ether (2.6 U/mg) and benzyl glycidyl ether (1.7 U/mg), which might be due to steric hindrance. Moreover, *Ab*HHDH displayed only poor activity on styrene oxide.

Table 3 Results for the enzymatic resolution of epoxides 8–17 on an analytical scale

Substrate	Activity (U/mg)	ee_s/configuration	Analytical yield (%)
8	23.7 ± 0.6	5.7 ± 0.3/(*R*)	–
9	(2.7 ± 0.1) × 10^{-1}	17.6 ± 0.6/(*S*)	–
10	27.2 ± 0.3	> 99/(*R*)	16.6 ± 0.4
11	27.6 ± 0.6	56.1 ± 1.2/(*R*)	–
12	25.1 ± 0.7	81.1 ± 1.7/(*R*)	–
13	26.0 ± 0.7	35.7 ± 0.5/(*R*)	–
14	20.1 ± 0.5	> 99/(*R*)	23.4 ± 0.3
15	5.5 ± 0.2	32.4 ± 0.8/(*R*)	–
16	2.6 ± 0.1	> 99/(*R*)	28.5 ± 0.7
17	1.7 ± 0.1	None	–

The reactions were performed with epoxides 8–17 (20 mM), 50 mM NaN_3 and purified *Ab*HHDH (0.2 mg/mL) in Tris–SO_4 buffer (200 mM, pH 7.5) at 30 °C. The specific activity was expressed in U/mg. The *ee* value was determined by chiral HPLC and GC analysis, and the identity of the enantiomers was determined by comparison to literature

To further confirm the enantiopreference of the enzyme, its selectivity in the ring-opening reaction of epoxides was also investigated, as shown in Table 3. *Ab*HHDH showed moderate enantioselectivity for substrates **10, 15**, and **16**, while poor enantioselectivity was observed for substrates **8, 9** and **17**. In contrast to (*R*)-selective HheA, *Ab*HHDH preferentially converted the (*S*)-enantiomer of epoxide **10** [10]. The substituent and its position at the phenyl ring also affected the enantioselectivity of HHDH in the biocatalytic azidolysis of epoxides. Compared to the epoxides with a substitution at the para- or ortho position (**11, 13**), the epoxide with a substitution at the meta position (**12**) was more selectively converted (higher *ee*). It is worth noting that a nitro group at the 2′-position greatly increased the enantioselectivity to 99% *ee*, while the *ee* values were 32.4% with 2′-CH_2CH_3 substituents and 56.1% *ee* with 2′-CH_3 substituents. It was also found that a larger side-chain (**17**) could decrease the enantioselectivity to some extent, which is in agreement with earlier reports [25].

Thus *Ab*HHDH was able to catalyze the enantioselective conversion of various prochiral and racemic halohydrins, as well as epoxides, yielding enantiomerically enriched (*S*)-epoxides, (*S*)-halohydrins and (*R*)-epoxides. This preference is significantly different from other well-known HHDHs such as HheC and HheA, demonstrating the unique substrate specificity and special synthetic applications of *Ab*HHDH.

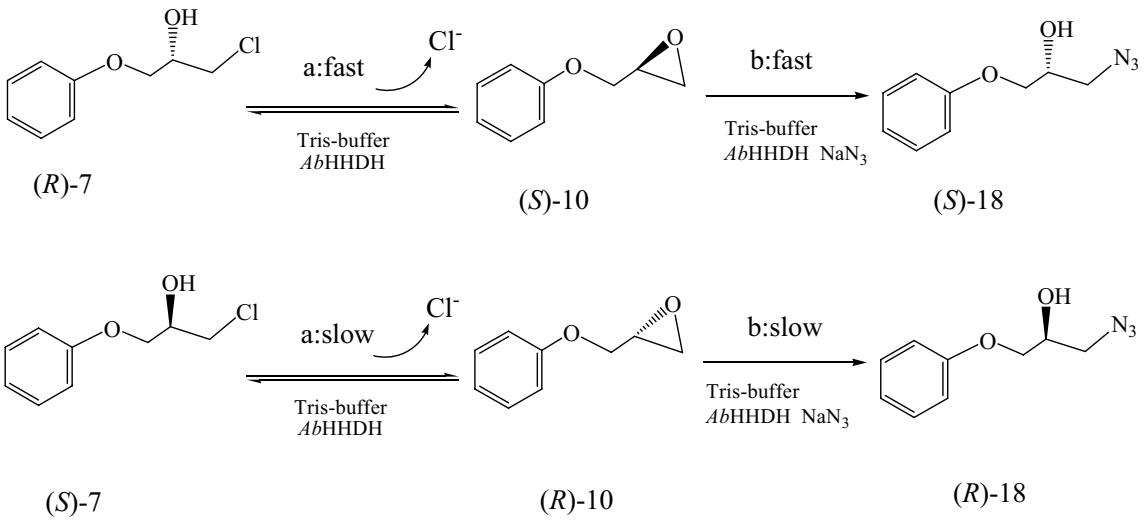

Fig. 2 Sequential kinetic resolution of (*R,S*)-1-chloro-3-phenoxy-2-propanol

3.3 Sequential kinetic resolution catalyzed by halohydrin dehalogenase

Compound **7**, (*R,S*)-1-chloro-3-phenoxy-2-propanol was chosen as the substrate to investigate the sequential kinetic resolution process. The reactions that can occur starting from a halohydrin and N_3^- are depicted in Fig. 2. The ring closure of (*R,S*)-**7** yields (*R,S*)-phenyl glycidyl ether (10), which can again act as a substrate for the enzyme. Ring opening of (*R,S*)-10 by N_3^- results in the formation of the corresponding azido alcohol.

Firstly, kinetic resolution of (*R,S*)-**10** catalyzed by *Ab*HHDH was tested in the presence of NaN_3. When the kinetic resolution was conducted at 20 mM (*R,S*)-**10**, a moderate enantioselectivity ($E = 9.98$) was obtained, leading to the formation of 16.7% (*R*)-**10** with an *ee* > 99%, and the enantiopurity of the product was very low (data not shown). The azidolysis of (*R,S*)-**10** leads to 1-azido-3-phenoxy-2-propanol, which was established through a comparison with the ^{1}H NMR spectrum reported in the literature [38]. The enantiomers of 1-azido-3-phenoxy-2-propanol were assigned by analogy according to the reduced epoxides configuration and comparing the retention times with reference materials [28]. To date, limited numbers of HHDHs were reported to catalyze azidolysis of (*R,S*)-**10**. HHDH from *Arthrobacter* sp. AD2 was reported to catalyze the ring-opening of (*R,S*)-**10** with low (*S*)-enantioselectivity ($E = 2$) [10]. In contrast to the (*S*)-selective *Ab*HHDH, the recombinant HHDH from *Bradyrhizobium erythrophlei* catalyzed the azidolysis of (*R,S*)-10, yielding (*S*)-**10** with 99% *ee* [22].

Bioconversions of (*R,S*)-**7** in the absence of azide were subsequently carried out. At the initial stage of the reaction, *Ab*HHDH converted (*R*)-**7** to the corresponding epoxide in preference to the other enantiomer. However, as the enzyme reaction is reversible, the released chlorine opens the epoxides, resulting in an equilibrium within an hour, producing racemic epoxides. This issue can be solved by removing the produced epoxide or the chloride. We tested the ring-closure reaction of *Ab*HHDH in the presence of NaN_3, allowing the *Ab*HHDH itself to eliminate the (*R,S*)-compound **10** by catalyzing a successive step. The advantage of the sequential reaction is that the enantioselectivity of the first step can be improved. The accumulation of (*R,S*)-epoxide **10** can be avoided, and (*S*)-**7** is obtained in the presence of azide in a one-pot reaction starting from (*R,S*)-**7**. When this reaction was carried out, the conversion rate of (*R,S*)-**7** increased markedly, resulting in the formation of 1-azido-3-phenoxy-2-propanol. Only a small amount of (*R,S*)-10 transiently accumulated. Since the intermediate epoxide (*S*)-**10** was efficiently and irreversibly converted by the *Ab*HHDH to (*R*)-1-azido-3-phenoxy-2-propanol, the conversion of (*R*)-**7** was drawn to completion. The reaction rate of the (*S*)-enantiomer was slower, and this resulted in a high optical purity of the remaining halohydrin (> 99% *ee*). As shown in Fig. 3, the sequential kinetic resolution of 20 mM (*R,S*)-**7** by using resting *E. coli* cells reached an *ee* of > 99% within 13 min, while the final yield of enantiopure (*S*)-**7** and the enantiomeric ratio (*E*) were 30.7% (theoretical yield = 50%) and 21.3, respectively.

The reaction steps shown in Fig. 2 can be influenced by several factors such as pH and the concentration of the organic substrates. The pH of the reaction medium influences the rate of enzyme-catalyzed ring-closure and ring-opening reactions and the position of the equilibrium between these reactions. To identify suitable reaction conditions, the influence of pH on all aspects of the sequential kinetic resolution process was investigated. Table 4 shows that the conversion rate of the (*R,S*)-**7** increased as the pH

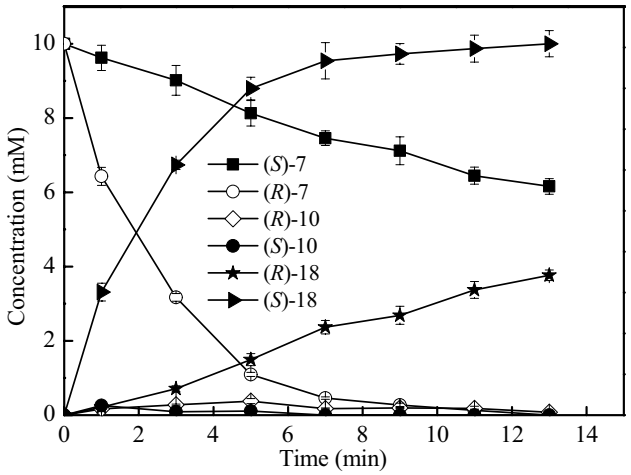

Fig. 3 Biotransformation of (*R,S*)-3-chloro-1-phenoxy-2-propanol with recombinant *E. coli* expressing *Ab*HHDH in the presence of NaN_3. The reactions were performed with (*R,S*)-3-chloro-1-phenoxy-2-propanol (20 mM), 50 mM NaN_3 and resting cells (5 g cdw/L) in Tris–SO_4 buffer (200 mM, pH 7.5) at 30 °C

Table 4 Effects of pH on the enantioselectivity and conversion rates of *Ab*HHDH

pH	Conversion rate (U/g cdw)	Time (min)	ee_s	Analytical yield	*E*
7.0	169 ± 2.9	27	> 99	30.9 ± 0.6	21.6
7.5	412 ± 3.4	13	> 99	30.8 ± 0.5	21.4
8.0	645 ± 4.6	9	> 99	26.4 ± 0.7	16.5

The specific activity was expressed in U/g cell dry weight (cdw)

E values were calculated as $E = \ln[(1-c)(1-ee_s)]/\ln[(1-c)(1+ee_s)]$

of the reaction mixture was increased from 7.0 to 8.0. The enantiomeric excess was not affected by the pH over the range 7.0–8.0, but the yield of (*S*)-**7** decreased.

To further test the feasibility of HHDH catalyzing enantioselective resolution of (*R,S*)-**7**, resting cells of *E. coli* expressing *Ab*HHDH were incubated at pH 7.0 with (*R,S*)-7 at various substrate concentrations ranging from 40 to 150 mM. Enantiopure (*S*)-**7** with a high optical purity (*ee* > 99%) was readily obtained from 40 to 150 mM (*R,S*)-**7** within 120 min. As shown in Table 5, the final yield of (*S*)-**7** decreased as more (*R,S*)-**7** was used initially, while the reaction time required for > 99% *ee* was prolonged. A yield of 27.8% enantiopure (*S*)-**7** could be obtained from 40 mM (*R,S*)-**7** after 22 min incubation, while the optical yield from the conversion of 60 mM (*R,S*)-**7** was 26.4% after 40 min incubation. When the initial concentration was further increased up to 150 mM, (*S*)-**7** with 99% *ee* was prepared with 22.1% yield. These results suggest that HHDH-catalyzed sequential kinetic resolution can be used to synthesize useful chiral building blocks.

4 Conclusions

In summary, a toolbox comprising several novel HHDHs was developed by data mining approaches. A novel HHDH, which exhibited a high activity and good enantioselectivity toward (*R,S*)-phenyl glycidyl ether, was successfully discovered from an alphaproteobacterium. *Ab*HHDH exhibits varied activities (0.02–27.6 U/mg) toward the tested halohydrins and epoxides. *Ab*HHDH catalyzed the conversion of prochiral halohydrins **1** and **2** to (*S*)-epichlorohydrin and (*S*)-epibromohydrin with 78.3% *ee* and 62.9% *ee*, demonstrating its potential for the direct synthesis of chiral epoxides. Among all the test racemic halohydrins, HHDH exhibited the highest enantioselectivity toward (*R,S*)-2-bromo-1-phenylethanol, producing (*S*)-2-bromo-1-phenylethanol with 99% *ee* and 34.5% yield. To the best of our knowledge, *Ab*HHDH has the second-highest *R*-enantioselectivity toward halohydrins of all the HHDHs reported to date, and thus may be of considerable interest in biocatalysis. In addition, *Ab*HHDH catalyzed the ring opening of epoxides with low to moderate enantioselectivity. The best result was obtained in the preparation of (*S*)-benzyl glycidyl ether, reaching 99% *ee* with 28.5% yield. Furthermore, this is also the first study to our knowledge to describe a sequential kinetic resolution process using *Ab*HHDH, which provides a green and

Table 5 Enantioselective conversion of (*R,S*)-3-chloro-1-phenoxy-2-propanol using resting cell of *E. coli* expressing *Ab*HHDH

Substrate (mM)	Time (min)	Conv. (%)	ee_s(%)/configuration	ee_p(%)/configuration	Yield of (*S*)-7 (%)
40	22	72.0 ± 0.5	> 99/*S*	38.8 ± 0.7/*S*	27.8 ± 0.4
60	40	72.2 ± 0.7	> 99/*S*	35.7 ± 0.6/*S*	26.4 ± 0.3
80	62	76.0 ± 1.0	> 99/*S*	31.8 ± 0.9/*S*	23.5 ± 0.6
100	95	77.2 ± 1.1	> 99/*S*	36.4 ± 1.0/*S*	22.7 ± 1.0
150	120	77.8 ± 0.9	> 99/*S*	37.6 ± 0.8/*S*	22.1 ± 1.2

Conditions: reactions (1 mL) were performed aerobically in Tris–SO_4 buffer (200 mM, pH 7.5) containing (*R,S*)-3-chloro-1-phenoxy-2-propanol (40–150 mM) and 1 equiv of NaN_3. The reactions were agitated at 150 rpm and 30 °C. Conversion and yield were determined by HPLC analysis. The identities of the enantiomers were determined by comparing to the literature

efficient synthesis route for (*S*)-1-chloro-3-phenoxy-2-propanol, an intermediate for the preparation of β-adrenergic blockers, in highly enantioenriched form (> 99% *ee*). These features make this a very attractive enzyme for potential applications in the biocatalytic preparation of enantiopure halohydrins, epoxides or azido alcohols.

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Compliance with Ethical Standards

Conflict of interest The authors declared that they have no conflicts of interest to this work.

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