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Short communication

Efficient synthesis of (*S*)-epichlorohydrin in high yield by cascade biocatalysis with halohydrin dehalogenase and epoxide hydrolase mutants

Feng Xue [a,b], Zhi-Qiang Liu [a,b], Ya-Jun Wang [a,b], Hang-Qin Zhu [a,b], Nan-Wei Wan [a,b], Yu-Guo Zheng [a,b,*]

[a] Institute of Bioengineering, Zhejiang University of Technology, Hangzhou 310014, PR China
[b] Engineering Research Center of Bioconversion and Biopurification of the Ministry of Education, Zhejiang University of Technology, Hangzhou 310014, PR China

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ABSTRACT

Enantioselective biotransformation of prochiral 1,3-DCP by halohydrin dehalogenases (HHDHs) is particularly attractive since 100% yield of chiral epichlorohydrin (ECH) may be obtained. HheC mutant (P175S/W249P) displayed greatly improved enantiomeric excess (*ee*) of (*S*)-ECH from 5% to 95.3% in the catalyzed dehalogenation of 1,3-DCP at pH 8.0. (*S*)-ECH was enantioselectively biotransformed from 40 mM 1,3-DCP with 92.3% *ee* and 93.2% yield at pH 10.0. To increase the *ee* of (*S*)-ECH, the catalysis was carried out using HheC mutant coupled with epoxide hydrolase mutant and the maximum yield and *ee* of (*S*)-ECH reached 91.2% and >99%.

1. Introduction

Enantiopure epichlorohydrin (ECH) is a valuable intermediate for producing optically active pharmaceuticals such as atorvastatin, β-blockers, L-carnitine, and ferroelectric liquid crystals [1]. Chiral ECH can be prepared based on chemical and biological approaches [2–4]. Hydrolytic kinetic resolution of racemic ECH catalyzed by cobalt-salen complexes provides an effective way to the production of chiral ECH [4]. In recent years, biological methods for enantiopure ECH preparation have been paid much attention with respect to high enantioselectivity, extensive enzyme sources, low production costs and green environmental protection [5]. Biocatalytic transformations include epoxide hydrolase (EH) mediated kinetic resolution, leading to the formation of 3-chloro-1,2-propanediol and enantiopure remaining substrate [6]. With the nucleophiles that are known to be accepted by halohydrin dehalogenases (HHDHs), chiral ECH can also be obtained by non-hydrolytic enantioselective ring opening [7]. A disadvantage of optical resolution methodology based on enantioselective resolution is that the yield of the desired enantiomer is less than 50%. An alternative strategy to the biocatalytic production of chiral ECH from prochiral 1,3-dicholoro-2-propanol (1,3-DCP) has been previously reported [8,9]. Stereoselective conversion of this prochiral 1,3-DCP by HHDH is particularly attractive, because 100% of the starting material can be converted to product, in contrast to a resolution approach where 50% of raw material is unused.

Halohydrin dehalogenases (HHDHs, EC 4.5.1.X) are microbial enzymes that catalyze the intramolecular nucleophilic displacement of the halogen atom by a hydroxyl group in halohydrin to generate the corresponding epoxide [10,11]. The HHDH from *Agrobacterium radiobacter* AD1 (HheC) has been studied extensively [12,13]. Its crystal structure and catalytic mechanism have been determined [14]. In the previous report, it is indicated that HheC and its mutants were efficient biocatalysts for the enantioselective formation of optically pure epoxides, halohydrins and chiral β-substituted alcohols from racemic halohydrins and epoxides [15,16], but the production ECH from prochiral 1,3-DCP using HheC was racemic [17,18].

In this paper, to obtain stereoselective enzymes for asymmetric conversions of 1,3-DCP, we set out to accomplish laboratory evolution of HHDH into mutants selective for the chiral ECH with use of HheC as the starting point. To overcome the relative low enantiomeric excess (*ee*) of (*S*)-ECH (<99%), it seemed reasonable to employ HheC mutant combined with a (*R*)-EH mutant from *Agromyces mediolanus* ZJB120203 in a cascade process starting from the corresponding prochiral 1,3-DCP.

* Corresponding author at: Institute of Bioengineering, Zhejiang University of Technology, Hangzhou 310014, PR China.
E-mail address: zhengyg@zjut.edu.cn (Y.-G. Zheng).

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Table 1
The stereoselective dehalogenation of 1,3-DCP with recombinant halohydrin dehalogenase.

Halohydrin	Enzyme	Specific activity[a]	*ee* epoxide (%)	Analytical yield (%)[b]	Abs. config.[c]
1,3-DCP	WT	34.7	5.2	43.8	(*S*)
	W249P	24.3	10.4	36.2	(*S*)
	P175S	43.9	89.3	90.9	(*S*)
	P175S/W249P	40.6	95.3	93.7	(*S*)

[a] In Na_2HPO_4–NaH_2PO_4, pH 8.0, substrate concentration is 20 mM, activity is expressed as μmol/min/mg.
[b] The maximum analytical yield is 100%.
[c] Absolute configuration of the epoxide.

2. Materials and methods

2.1. Strains and plasmids

Escherichia coli BL21(DE3), pET-28b(+), pCDFDuet-1 were used for expression experiments. The plasmid pET28b-HheC hosting the gene encoding HHDH gene from *A. radiobacter* AD1 was used as the template for construction of the mutation genes [18]. The *E. coli* strain expressing AmEH mutant VDF (W182F/S207V/N240D) from *A. mediolanus* ZJB120203 was developed as an efficient catalyst for the preparation of enantiopure ECH by kinetic resolution [19].

2.2. Analytical methods

The optical purity and conversion were determined by GC-14C gas chromatography (Shimadzu, Tokyo, Japan) equipped with FID detector and BGB-175 chiral column using He as carrier gas. The initial column temperature was set at 90 °C and the inlet and detector temperatures were both 220 °C. The retention times of (*S*)-ECH, (*R*)-ECH were 5.4 and 5.7 min. The *ee* was derived from the remaining epoxide of the two enantiomers [$ee\ (\%) = (S - R) / (S + R) \times 100$]. Optical rotation was measured on an Autopol IV automatic polarimeter (Rudolph Research Analytical, USA).

See Supplementary data for other experimental details.

3. Results and discussion

3.1. Construction and screening of mutant libraries of HheC

In order to choose appropriate randomization sites, the published X-ray crystal structure and catalytic mechanism of HheC from *A. radiobacter* AD1 was used as template for comparison and analysis [14]. It is well recognized that residues F12, P175, N176, Y177, L178, Y185, F186, Y187 and W249 suggested to play critical roles in catalytic activity and enantioselectivity [20], thus will commonly act as the target sites for rational design. The mutant libraries were screened for the enantioselectivity toward 1,3-DCP by using automated chiral GC to obtain the respective conversion and *ee* values. The cell free extracts of wild-type (WT) HheC and mutants were purified by one-step nickel affinity chromatography on Ni-NTA resin as described in our previous work [21]. It is found that there is no difference in the molecular mass between the WT and mutant HheC (Fig. S1).

For most of the mutants, the resulting ECH from 1,3-DCP was racemic, suggesting that no positive mutant with improved enantioselectivity was isolated at position F12, N176, Y177, L178, Y185 and F186. Among site-saturation mutagenesis libraries at position P175 and W249, two mutants P175S and W249P, were identified, displaying higher enantioselectivity toward 1,3-DCP (Table 1). W249P and P175S increased the *ee* value of (*S*)-ECH from 5.2% to 10.4% and 89.3%. Combination of mutations can have additive effects in the case of enantioselectivity. The double-mutant mutant (P175S/W249P) exhibited higher enantioselectivity (up to 95.3% *ee*) than either of the two single-mutated mutants (Table 1). The enhanced enantioselectivity is caused by the decreasing steric hindrance of one of the halogen-bearing carbon atoms of 1,3-DCP, resulting in asymmetric dehalogenation.

Table 2
Optimization of reaction conditions for production of (*S*)-ECH from 1,3-DCP using HheC mutant (P175S/W249P).

Reaction conditions	Analytical yield (%)[a]	(*S*)-ECH (*ee* %)
pH[b]		
8.0 (Na_2HPO_4–NaH_2PO_4)	50.7	94.2
9.0 (Gly-NaOH)	71.4	94.5
10.0 (Gly-NaOH)	89.3	92.3
Temperature (°C)[c]		
20	90.2	93.5
30	90.7	92.4
40	91.1	92.2
1,3-DCP concentration (mM)[d]		
20	91.4	92.7
40	90.2	92.1
80	64.9	91.3
100	58.0	90.4

[a] The maximum analytical yield is 100%.
[b] 40 mM 1,3-DCP, 35 °C, 3 min.
[c] 40 mM 1,3-DCP, 3 min pH 10.0, 200 mM Gly-NaOH.
[d] 200 mM Gly-NaOH (pH 10.0), 35 °C.

3.2. Synthesis of (S)-ECH by mutant HheC

The HheC mutant P175S/W249P was then evaluated for its asymmetric conversions of 1,3-DCP (Table 2). The *ee* of (*S*)-ECH produced was affected by the initial pH over the range of 8.0–10.0, and the yield of (*S*)-ECH decreased from 89.3% to 50.7% as the pH of the reaction mixture was changed from 10.0 to 8.0. The effect of 1,3-DCP concentration on the optical purity of the (*S*)-ECH was also investigated. The *ee* and yield of (*S*)-ECH respectively decreased from 92.7% and 91.4% to 90.4% and 58.0% as the 1,3-DCP concentration was increased from 20 to 100 mM. A slight decrease in the optical purity of (*S*)-ECH from 93.5% to 92.2% has been observed, due to spontaneous dehalogenation rate increases with the elevated temperature.

The production profile of (*S*)-ECH from 40 mM 1,3-DCP with time by HheC mutant (P175S/W249P) with initial pH at 10.0 is shown in Fig. 1. (*S*)-ECH was obtained with 93.2% yield and 92.3% *ee* determined by GC in a 5-min reaction. Prochiral 1,3-DCP was transformed into optically active ECH by HHDHs, which has been reported in the literature. (*S*)-ECH with *ee* > 60% was obtained and a conversion was 95.2% in a 2-min reaction at pH 10.0 by $HHDH_{Tm}$ from *Tistrella mobilis* ZJB1405

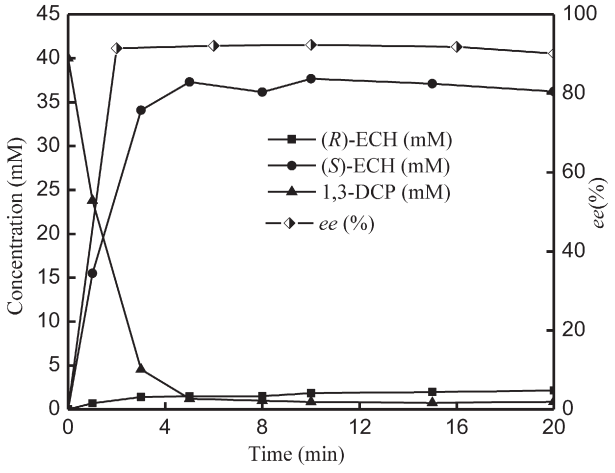

Fig. 1. Time course of the transformation of 1,3-DCP into (*S*)-ECH catalyzed by HheC mutant (P175S/W249P). The reaction was performed at 35 °C in 200 mM glycine-NaOH buffer (pH 10.0).

Table 3
Preparation of (*S*)-ECH from 1,3-DCP by two-step biocatalysis using HheC mutant (P175S/W249P) and AmEH mutant (W182F/S207V/N240D).

1,3-DCP concentration (mM)	Method 1[a]		Method 2[b]		Method 3[c]	
	Analytical yield (%)	(*S*)-ECH (*ee* %)	Analytical yield (%)	(*S*)-ECH (*ee* %)	Analytical yield (%)	(*S*)-ECH (*ee* %)
20	91.0	99.3	87.3	99.2	91.2	99.9
40	89.2	97.5	83	96.1	89.6	99.9
60	69	95.2	64.9	95.5	72.4	99.9
80	58.9	94.1	58.0	94.3	61.4	99.9
100	52.1	93.2	51.2	91.4	54.2	99.9

[a] One-pot enantioselective conversion of 1,3-DCP to (*S*)-ECH via cascade biocatalysis with the mixtures of resting cells of *E. coli* (HheC mutant) and *E. coli* (AmEH mutant).
[b] One-pot enantioselective conversion of 1,3-DCP to (*S*)-ECH via cascade biocatalysis with the resting cells of *E. coli* (HheC mutant-AmEH mutant).
[c] Enantioselective cascade conversion of 1,3-DCP to (*S*)-ECH using *E. coli* (HheC mutant) and *E. coli* (AmEH mutant) in two reaction vessels.

[22]. The HheB was shown to dehalogenate 1,3-DCP and stereoselectively form (*R*)-ECH with *ee* > 90% in the first 10-min reaction, however, the optical purity also decreased quickly with prolonged incubation [8]. The HHDH from *A. mediolanus* ZJB120203 ($HheA_{Am}$) also can transform 1,3-DCP into (*S*)-ECH, but the *ee* and the activity of $HheA_{Am}$ was very low [23]. Compared to the previous report, it showed that HheC mutant P175S/W249P is a powerful chiral tool for the production of (*S*)-ECH with an almost theoretical yield of 100%.

3.3. Establishment of a cascade system by HheC mutant and AmEH mutant

To obtain the (*S*)-ECH with *ee* > 99%, the HheC mutant (P175S/W249P) was used for the stereoselective dehalogenation of 1,3-DCP to the corresponding (*S*)-ECH with *ee* of less than 99%, and then the minority (*R*)-ECH was preferentially hydrolyzed by the AmEH mutant (W182F/S207V/N240D). Resting cells of *E. coli* (HheC mutant) and *E. coli* (AmEH mutant) were used at a ratio range of 1:1–3:1 for the one-pot cascade biocatalysis to demonstrate the process, (*S*)-ECH were obtained in good *ee* (93.2–99.3%) at a concentration of 20–100 mM 1,3-DCP and good yield (52.1–91.0%) (Table 3).

The use of single recombinant strain coexpressing all necessary enzymes could avoid the cell cultivation of multiple strain and reduce the total cell density for the cascade biocatalysis [24]. The co-expression plasmid construction is shown in Fig. S2A. Fig. S2B shows the SDS-PAGE analysis of the protein extract from *E. coli* (DE3) harboring the plasmids. The expression level of HheC mutant and AmEH mutant in the co-expression recombinants were lower than those expressed alone. Resting cells of *E. coli* (HheC mutant-AmEH mutant) were thus used for the one-pot conversion of 1,3-DCP to (*S*)-ECH. As shown in Table 3, reactions of 20–100 mM 1,3-DCP with *E. coli* (HHDH-AmEH) at 10–30 g/L (wet cell weight) afforded (*S*)-ECH in 91.4–99.2% *ee* and 51.2–87.3% yield.

A practical, two-pot, two-step catalytic method is successfully constructed for conversion of 1,3-DCP to (*S*)-ECH. 1,3-DCP at a concentration of 20–100 mM was first converted to (*S*)-ECH with *ee* < 99% by using HheC mutant as the catalyst. Subsequently, the remained (*R*)-ECH was hydrolyzed by *E. coli* cells harboring AmEH mutant. The final concentration of (*S*)-ECH with >99% of *ee* was 18.2, 35.8, 43.4, 49.1 and 54.2 mM from 20, 40, 60, 80 and 100 mM 1,3-DCP (Table 3). The yield and optical purity was found to be the highest level for conversion of 1,3-DCP to (*S*)-ECH by HheC mutant and AmEH mutant. This suggests that the stepwise procedure is more effective than "one pot" conversion, since higher optical purity of (*S*)-ECH was obtained.

4. Conclusions

In this study, we have engineered HheC to give a significantly improved enantioselectivity from 5% to 95.3% *ee* for asymmetric conversions of 1,3-DCP by using saturation mutagenesis. The best HheC mutant (P175S/W249P) catalyzed the conversion of 1,3-DCP to (*S*)-ECH with 92.3% *ee* and 93.2% yield at pH 10.0, demonstrating its potential for the direct synthesis of (*S*)-ECH. To increase the optical purity of (*S*)-ECH, an efficient two-step enzymatic process for production of (*S*)-ECH from 1,3-DCP was developed by using recombinant *E. coli* cell separately or simultaneously expressing a mutant HheC gene and a mutant AmEH gene, giving the corresponding (*S*)-ECH in 91.4–99.3% *ee* and 51.2–91.0% yield. A biocatalytic cascade reaction system involving a two-step, two-enzyme reaction was successfully implemented to give (*S*)-ECH in >99% *ee* and 54.2–91.2% yield.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.catcom.2015.09.025.

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