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Protein termini relocation plus random mutation: A new strategy for finding key sites in esterase evolution

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ABSTRACT

A pharmaceutically relevant esterase, *Rh*Est1, could catalyze the hydrolysis of (*R,S*)-ethyl-2,2-dimethyl cyclopropane carboxylate [(*R,S*)-DmCpCe] with excellent enantioselectivity, producing (*S*)-(+)-2,2-dimethyl cyclopropane carboxylic acid [(*S*)-DmCpCa], which is a key chiral building block for the synthesis of Cilastatin. In our previous work, a mutant *Rh*Est1-M2 was identified with 6.4-fold higher activity than the wild-type. Additionally, the termini of *Rh*Est1 protein were altered by circular permutation (CP), resulting in a mutant CP-176 which still maintains the catalytic activity of esterase. In this work, to improve the catalytic properties of *Rh*Est1, the mutant CP-176 was taken as the parent of directed evolution. Consequently, a new mutant designated as CP-M1 (=CP-176_{G282S}) was identified, indicating 3.2-fold catalytic efficiency enhancement and nearly 7 ℃ improvement in melting temperature (T_m) as compared with CP-176. Furthermore, the beneficial mutation "G282*S*" of CP-M1 was reversely introduced into *Rh*Est1-M2, generating the best mutant M3 (=*Rh*Est1-$M2_{G167S}$), with 1.8-fold catalytic efficiency improvement and nearly 10 ℃ improvement of T_m, as compared with *Rh*Est1-M2. This is the first report that the circular permutation and random mutagenesis were combined to reshape a protein, affording distinctly improved activity and thermostability.

1. Introduction

Cilastatin, combining with carbapenem antibiotic Imipenem is essential for the clinical treatment of serious infected patients [1–3]. (*S*)-(+)-2,2-Dimethyl cyclopropane carboxylic acid (abbreviated hereafter as (*S*)-DmCpCa) is a key chiral building block for the synthesis of Cilastatin. Highly efficient and enantioselective synthesis of optically pure organic acids, especially (*S*)-DmCpCa, represents a greater challenge than the synthesis of chiral alcohols by either chemical or enzymatic methods [4]. Various strategies have been developed recently. Enzymatic preparation of chiral synthons provides a direct, efficient, green, and highly chemoselective/enantioselective alternative, due to the excellent catalytic properties of diverse enzymes.

An α/β-fold hydrolase *Rh*Est1 from *Rhodococcus* sp. strain ECU1013, could catalyze the asymmetric hydrolysis of racemic ethyl (*R,S*)-2,2-dimethyl cyclopropane carboxylate [(*R,S*)-DmCpCe] to produce (*S*)-DmCpCa [4,5] (Scheme 1), and is the first reported esterase with such catalytic function. However, enzymatic characterization of *Rh*Est1 indicated that this enzyme showed relatively low catalytic activity towards the unnatural substrate DmCpCe, which is a principal bottleneck against industrial application. Therefore, various engineering strategies were proposed and adopted for improving the activity of *Rh*Est1.

Directed evolution as an effective engineering-strategy was frequently applied to improve the performance of an enzyme, such as activity, thermostability and substrate specificity [6–10]. In our previous work, a quadruple mutant, *Rh*Est1-M2 (*Rh*$Est1_{A143T/A147I/V148F/G254A}$), with 6.4-fold higher activity than the wild-type was obtained by substrate channel evolution plus random mutagenesis [11] and cap domain engineering [12]. Nevertheless, there was no significant improvement even if additional random mutagenesis was further performed. The variant *Rh*Est1-M2 seems to have little improvement potential since it might become stuck in a local optimum of the evolution landscapes [13]. Circular permutation (CP) is a typical protein engineering method also inspired from nature [14,15], which rearranges the order of polypeptide sequence by altering the locations of new termini. We also tried to tailor *Rh*Est1 with the inspiring strategy [16], resulting in a functionally active CP variant, CP-176, where the number "176" indicates the cleavage site of circular *Rh*Est1 sequence which is located in the loop region between α5 and α6 helices of *Rh*Est1 protein. Unfortunately, the catalytic activity of CP-176 towards DmCpCe declines to 0.10 U/mg protein from 0.17 U/mg protein of native *Rh*Est1,

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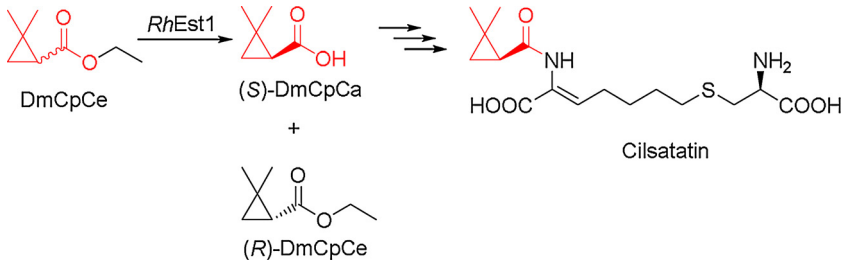

Scheme 1. *Rh*Est1-catalyzed enantioselective hydrolysis of (*R*,*S*)-2,2-DmCpCe.

and its thermostability also decreases significantly [16]. However, it was believed that the decline of CP-176 activity might move into troughs before navigating a new higher peak due to the nature of rugged fitness evolution landscapes [13].

Considering both random mutagenesis and circular permutation are effective protein engineering strategies inspired from nature, we speculate that the proper combination of the two useful strategies may be beneficial. Hence the new strategy by combining directed evolution with circular permutation was designed. The variant CP-176 with altered termini was employed as a parental protein of directed evolution to reshape the protein structure for improving its catalytic function. Although the resultant mutants did not reach any higher activity peak than *Rh*Est1-M2, a sensitive point (CP-176$_{G282S}$) hidden previously was uncovered by this cycle of random mutagenesis, with 3.4-fold activity and nearly 7 °C of T_m improvements compared with CP-176. Subsequently, the integrative effect of this positive mutation on *Rh*Est1-M2 was further examined. Interestingly, when the beneficial mutation G282S of CP-M1, which is equivalent to G167S in *Rh*Est1, was introduced into *Rh*Est1-M2, the resultant variant *Rh*Est1-M3 gave the best performance, with 80% catalytic efficiency increase and nearly 10 °C improvement of T_m compared with *Rh*Est1-M2, indicating the effectiveness of the newly proposed strategy of circular permutation plus directed evolution.

2. Materials and methods

2.1. Materials

Tryptone and yeast extract were obtained from Oxoid (Shanghai, China). r*Taq* polymerase, T4 DNA ligase, and PrimeSTARHS were all purchased from TaKaRa (China). Restriction endonucleases (*Dpn*I, *Eco*RI, *Hin*dIII) were purchased from New England Biolabs (Beijing, China). All oligonucleotide primers were synthesized by Generay (Shanghai, China). DNA Purification Kits were purchased from Tiangen (Beijing, China) for DNA isolation and purification. Plasmid Extraction Mini Kits were purchased from Favorgen (Taiwan, China) for plasmid extraction. Racemic ester (*R*,*S*)-DmCpCe was prepared from the acid (*R*,*S*)-DmCpCa and ethanol by chemical esterification in our laboratory [12]. The acid (*R*,*S*)-DmCpCa was obtained commercially with an analytical grade (Zhejiang Hisoar Pharmaceutical Co., Ltd.).

2.2. Construction of CP-176 mutant libraries by error-prone PCR

To obtain mutated CP-176 gene, error prone PCR was carried out on the CP-176 gene (873 bp) and the amino acid mutagenesis rate was controlled at 0.3%–0.7%. For amplifying the CP-176 gene, the forward primer (containing an *Eco*RI restriction site) and the reverse primer (containing a *Hin*dIII restriction site) are as follows:

5′-ATCCGGAATTCGAAGGGTGCAGCGTCGCAG-3′

3′-GAGTGGCCGGGTGGGCTCCCGTTCGAACCTT-5′

The PCR system contained 60 ng template plasmids (pRSFDute-CP-176), dNTP mix (0.2 mM each), 0.2 μM of each primer, 150 μM Mn^{2+}, PCR buffer and 2.5 U r*Taq* polymerase in a total volume of 50 μL. The PCR was implemented under the following conditions: initial heating at 95 °C for 3 min then 35 cycles of heating at 96 °C for 30 s, 57 °C for 40 s and 72 °C for 1 min, followed by an extension step at 72 °C for 10 min. The PCR products were separated by agarose gel electrophoresis and purified by using a DNA recovery kit (Qiagen, shanghai), digested with *Eco*RI and *Hin*dIII, and ligated into pRSF-Dute plasmid which was also digested with the same two enzymes.The recombined vector was transformed into *E. coli* BL21 (DE3) competent cells, and plated onto LB agar medium with 50 μg mL^{-1} of kanamycin.

2.3. Site-directed saturation mutagenesis

For site-directed saturation mutagenesis, the codon of corresponding target amino acid was replaced by NNK degenerate codon. The PCR amplification products were treated with *Dpn*I for 2 h to digest the template plasmids before being transformed into the competent cells. Colonies harboring the CP-176 mutant genes were transferred into 96-deep well plates and cultivated at 37 °C and 220 rpm for 12 h, with each well containing 300 μL LB fresh medium and 50 mg L^{-1} kanamycin. These plates were preserved as master plates with glycerol at a final concentration of 10% (v/v).

2.4. Libraries screening

The libraries screening was conducted for finding the variants with improved activity compared with CP-176 by measuring the increase in absorbance of *p*-nitrophenol at 405 nm with a Microplate Reader [4]. The reaction mixture was consisted of 1 mM *p*NPP (*p*-nitrophenol propionate) and an appropriate amount of cell free extract in KPB (potassium phosphate buffer, pH 8.0,100 mM).

2.5. Activity assay of the variants towards (R,S)-DmCpCe

To determine the enzymatic activity toward (*R*,*S*)-DmCpCe, a 500-μL scale reaction containing a certain amount of the purified enzyme (0.1 mM or 0.2 mM) and 10 mM (*R*,*S*)-DmCpCe (with 10% v/v of DMSO) was performed at 30 °C and 1000 rpm on a mini-shaker (Eppendorf, Germany) [4]. After a certain period of time, 20 μL of 20% (w/v) H_2SO_4 was added to terminate the reaction. Then the acidulated product was extracted with an equal volume of ethyl acetate including 0.5 mM dodecane as an internal standard and detected by gas chromatography (GC).

The quantitative analysis of the product enantiomers (*R*,*S*-DmCpCa) were determined by using a gas chromatography instrument (GC-2014; Shimadzu) equipped with a flame ionization detector and a CP-Chirasil-Dex CB capillary column (25 m by 0.25 mm; Varian Co., Palo Alto, CA, USA). The analysis method by GC is the same as described previously [4]. The retention times of (*S*)-DmCpCa and (*R*)-DmCpCa were 9.28 and 9.55 min, respectively.

2.6. Kinetic parameter determination

The kinetic parameters of the purified variants toward (*R*,*S*)-DmCpCe were determined by measuring the activity under the varied substrate concentrations (0.05–10 mM). More details about the kinetic