Bioorganic Chemistry 77 (2018) 330–338

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Engineering of *Talaromyces thermophilus* lipase by altering its crevice-like binding site for highly efficient biocatalytic synthesis of chiral intermediate of Pregablin

Xu Ding, Ren-Chao Zheng, Xiao-Ling Tang, Yu-Guo Zheng *

Key Laboratory of Bioorganic Synthesis of Zhejiang Province, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China
Engineering Research Center of Bioconversion and Biopurification of Ministry of Education, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China

ARTICLE INFO

Article history:
Received 2 December 2017
Revised 10 January 2018
Accepted 11 January 2018
Available online 12 January 2018

Keywords:
Talaromyces thermophilus lipase
Protein engineering
Crevice-like binding site
Kinetic resolution
Pregabalin

ABSTRACT

The scissile fatty acid binding site of lipases is divided into different sub-groups and plays an important role in the catalytic properties of the enzymes. In this study, the *Talaromyces thermophilus* lipase was engineered by altering its crevice-like binding site for efficient synthesis of chiral intermediate of Pregablin through kinetic resolution of 2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester (CNDE). The substitution of residues located at the crevice-like binding site with phenylalanine (Phe) resulted in significantly increased hydrolysis activity. The variant L206F/P207F/L259F exhibited a 37.23-fold and 47.02-fold improvement in the specific activity and turnover number (k_{cat}) toward CNDE, respectively. Simultaneously, the optimum temperature and substrate preference were both altered in the variants. The study herein successfully engineered the TTL with improved catalytic properties for efficient biosynthesis of Pregablin intermediate. The investigation of structure-functional relationship provided important guidance for further modification of lipases with crevice-like binding site domain.

1. Introduction

Lipases (triacylglycerol acylhydrolase, E.C.3.1.1.3) are enzymes belonging to the α/β-hydrolase fold family characterized by the conserved catalytic triad of Ser-Asp/Glu-His. They catalyze the hydrolysis of ester bonds of water-insoluble triglyceride substrates [1] and have been widely applied in industries of pharmaceutics, detergents, food additives, and fine chemicals, due to their strict chemo-, regio-, and enantioselectivity under mild conditions [2–6].

Lipases demonstrate their great potential for efficient synthesis of fine chemicals, especially for the preparation of chiral pharmaceutical intermediate [7,8]. However, wild-type lipases themselves barley satisfied the requirements of industrial process due to the undesirable catalytic performance. As such, rational and semi-rational design were employed as effective approaches to engineer the enzymes for catalytic properties improvement [9–11]. The amino acid residues located at the regions of protein surface [12–14], surrounded the active center [15,16], oxyanion hole [17–19], scissile fatty acid binding site [20] and lid domain [21,22] are regarded as hot residues affecting their catalytic properties.

The scissile fatty acid binding site of lipases can be divided into three sub-groups, namely crevice-like binding site, funnel-like binding site and tunnel-like binding site [23]. Engineering of tunnel-like binding site of *Candida rugosa* lipase, *C. antarctica* lipase A and *Bacillus thermocatenulatus* lipase resulted in a strong discrimination of chain length specificity [20,24,25]. Study of funnel-like binding site of *C. antarctica* lipase B indicated that the modified hydrophobic domain was conducive to the improvement of its activity and enantioselectivity [26–28]. The crevice-like binding sites also played important roles in the enzyme properties [23,29,30], however, no systematic investigation was carried out to further elucidate the structure-functional relationship.

In our previous study, the lipase from *Talaromyces thermophilus* lipase (TTL) was explored for kinetic resolution of 2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester (CNDE) to (3*S*)-2-carboxyethyl-3-cyano-5-methylhexanoic acid (*S*-CCMA), the key chiral intermediate of Pregabalin, a new blockbuster drug for the treatment of several central nervous disorders [31,32]. However,

* Corresponding author at: Key Laboratory of Bioorganic Synthesis of Zhejiang Province, College of Biotechnology and Bioengineering, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China.
E-mail address: zhengyg@zjut.edu.cn (Y.-G. Zheng).

https://doi.org/10.1016/j.bioorg.2018.01.018
0045-2068/

the wild-type TTL exhibited low activity and relatively inferior catalytic efficiency, restricting its industrial applications.

In this study, the scissile fatty acid binding site of TTL was found to be the sub-group of crevice-like binding site and rational design of the domain was performed to improve its catalytic efficiency. Interestingly, the substitution of residues located at the crevice-like binding site with triple Phe resulted in mutant with significant improvement of activity toward CNDE. Simultaneously, both the optimum temperature and substrate preference of the mutant changed. The investigation of structure-functional relationship of the mutant revealed that the Phe substitution at the crevice-like binding site affected the intensive entrance of substrate and release of product. The results obtained in this study not only expanded the understanding of the effect of the crevice-like binding site on the enzyme's catalytic performance, but also provided valuable guidance for efficient engineering of lipases with improved properties for industrial applications.

2. Materials and methods

2.1. Reagents and chemicals

Isopropyl-β-d-thiogalactopyranoside (IPTG) and kanamycin were purchased from Sigma (Shanghai, China). *Pichia pastoris* X33, plasmid pPICZα-A and antibiotic Zeocin™ were purchased from Thermo Scientific (Shanghai, China). Phanta Max Super-Fidelity DNA Polymerase and *Taq* DNA polymerase for mutagenesis were purchased from Vazyme (Nanjing, China). Restriction endonucleases and T4 DNA ligase were purchased from Fermentas (Shenzhen, China). DNA gel extraction kit, plasmid extraction kit and PCR product purification kits were obtained from Axygen (Hangzhou, China). 2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester (CNDE) was donated by Zhejiang Apeloa Medical Technology Co., Ltd. (Jinhua, China). Other chemicals were of analytical grade from local suppliers.

2.2. Plasmid construction and strains

The total RNA was extracted from *T. thermophilus* ATCC 20,186 and taken as template for cDNA synthesis. The DNA sequence encoding *T. thermophilus* lipase (TTL) was obtained by PCR amplification with cDNA as template. The primers TTL-F and TTL-R (Table SI) were designed from available *T. thermophilus* lipase sequence (GenBank accession no. JF414585.1). After digested by *Nco*I/*Xho*I endonuclease respectively, vector pET-28b and PCR product were ligated by T4 DNA ligase overnight at 16 °C. The recombinant plasmid was transformed into *E. coli* BL21 (DE3) (Novagen, Darmstadt, Germany) and plated onto Luria-Bertani (LB) agar plate containing 1% (w/v) peptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl with Kanamycin (50 μg mL^{-1}). The primers Pic-F and Pic-R (Table SI of the Supplementary Information) were used to amplify the TTL sequence. The obtained fragment was digested by *Xho*I and *Xba*I endonucleases, and ligated with pPICZα-A plasmid, which was predigested by the same enzymes. After transformed into *E. coli* JM109 (Novagen, Darmstadt, Germany), the recombinant plasmid was extracted and linearized by *Sac*I, and transformed into *P. pastoris* X33 by electroporation. The positively transformed colonies were screened by colony PCR and confirmed by DNA sequencing.

2.3. Expression of the wild-type TTL and variants in E. coli and P. pastoris

The positive *E. coli* transformants containing the recombinant plasmids were inoculated into LB medium and cultivated at 37 °C. When the OD_{600} reached ~0.6, IPTG with a final concentration of 0.1 mM was added to induce the protein expression at 28 °C. After 10 h, the cells were harvested by centrifugation at 12,000*g* for 10 min at 4 °C.

The single colonies of the recombinant *P. pastoris* were grown in YPD medium (2% peptone, 1% yeast extract, 2% glucose, 50 μg mL^{-1} Zeocin™) at 30 °C. The seeds were further transferred into BMGY medium (w/v) containing 2% peptone, 1% yeast extract, 1% glycerol, 1.34% (w/v) yeast nitrogen base, 4×10^{-5}% biotin, 100 mM potassium phosphate buffer (pH 6.0). The cultivation was performed at 30 °C for 22 h and after then, the cells were harvested by centrifugation, re-suspended and cultured in BMMY media (w/v) containing 2% peptone, 1% yeast extract, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 100 mM potassium phosphate buffer (pH 6.0) at 30 °C. Methanol (2%, v/v) was fed to the medium at 24 h interval for 5 days.

2.4. Mutagenesis and screening

The DNA sequence encoding TTL was amplified by T7 primers using error-prone PCR [33]. The reaction mixture (100 μL) contained 1 × *Taq* buffer, 0.2 μM of T7 primers, 5 mM $MgCl_2$, 0.2 mM $MnCl_2$, 0.2 mM dGTP, 0.2 mM dATP, 1.0 mM dCTP, 1.0 mM dTTP, 50 ng template DNA, and 5 U of *Taq* polymerase. The PCR reaction programs were carried out as follows: 95 °C, 3 min; followed by 30 cycles of 15 s at 95 °C, 15 s at 58 °C and 1 min at 72 °C; and further elongation for 10 min at 72 °C. The PCR products were analyzed by agarose gel electrophoresis and then purified using a DNA gel extraction kit. Subsequently, the amplified PCR product was digested with *Nco* I/*Hind* III, and ligated into plasmid pET-28b (+). The recombinant plasmid was then transformed into *E. coli* BL21 (DE3) to construct the mutant library. To obtain variants with further increased activity for CDNE, site-directed mutagenesis was performed to the residues located at the crevice-like binding site with primers listed in Table SI.

Screening of TTL variants with increased activity was performed by a pH-based high-throughput screening strategy using CNDE as substrate [19]. The colonies were initially transferred into 96-well plates containing 1 mL LB medium with 0.1 mM IPTG. After cultured for 20 h at 28 °C, the cells were harvested by centrifugation at 4 °C, 5000*g* for 15 min and suspended in 200 μL Tris-HCl buffer (10 mM, pH 8.0). The reaction mixture consisted of 20 μL cell suspension, 180 μL Tris-HCl buffer (10 mM, pH 8.0), 0.01% bromothymol blue and 50 μL 7% CNDE in acetonitrile. The reaction was carried out at 37 °C for 30–60 min, and the color changed from blue to yellow was used as an indicator of substrate hydrolysis.

The activity and enantioselectivity of the positive variants were further confirmed by gas chromatography (GC) equipped with FID detector and chiral capillary column Astec CHIRALDEX™ G-TA (30 m × 0.25 mm, 0.25 μm film thickness). Helium was used as carrier gas at a flow rate of 1 mL min^{-1} [19,34]. The injector and detector temperatures were both set at 220 °C and the column temperature was set at 135 °C for 45 min.

2.5. Protein purification and enzyme activity assay

The fermentation broth was initially purified by ammonium sulfate precipitation at 0 °C and dialyzed against Tris-HCl buffer (50 mM, pH 8.0). For further purification, dialyzed enzyme solution was applied to DEAE-Sepharose FF column (1.6 × 20 cm, GE) equilibrated with Tris–HCl buffer (50 mM, pH 8.0). The target protein was eluted with a linear salt gradient of NaCl from 0 to 1.0 M. The purified enzyme was confirmed by 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentrations were measured by Coomassie brilliant blue method [35] with bovine serum albumin as standard. The activity of purified lipases toward CNDE was determined by GC.