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Enhanced activity of *Thermomyces lanuginosus* lipase by site-saturation mutagenesis for efficient biosynthesis of chiral intermediate of pregabalin

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ABSTRACT

Thermomyces lanuginosus lipase (TLL) variants with enhanced activity for kinetic resolution of 2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester (CNDE) were constructed by site-saturation mutagenesis. Single mutant S83T and double mutant S58L/S83T exhibited 2.69 and 5.46-fold improvement in their specific activity for CNDE over the wild type TLL. The catalytic efficiency of S83T and S58L/S83T mutants were significantly increased, with k_{cat}/K_m values of 11.3 and 27.3 mM^{-1} min^{-1}, which was 2.97 and 7.18 times higher than that of the wild type. The whole cell catalysis of 3 M CNDE by *Escherichia coli* harboring mutant S58L/S83T (5% w/v) resulted in 44.8% yield and >96% ee_P within 24 h. These encouraging results demonstrated the great potential of the modified TLL for efficient production of (*S*)-2-carboxyethyl-3-cyano-5-methylhexaoic acid used as chiral intermediate for pregabalin.

1. Introduction

Thermomyces lanuginosus lipase (TLL) is a kind of noticeable thermostable lipase and commercialized as lipolase® by Novo Nordisk [1]. With its high catalytic efficiency, strict enantioselectivity and broad specificity, the enzyme has been found to catalyze a diversity of reactions toward a broad range of natural and unnatural substrates [2,3]. It has been widely applied in many different industrial areas such as the production of biodiesel, detergent, cosmetic, and other organic chemicals [3–5]. Recently, TLL was reported for the kinetic resolution of 2-carboxyethyl-3-cyano-5-methylhexanoic acid ethyl ester (CNDE) to produce (*S*)-2-carboxyethyl-3-cyano-5-methylhexanoic acid, a chiral intermediate of pregabalin [6,7].

Developed as a new blockbuster drug for the treatment of central nervous system disorders, pregabalin has attracted widespread attention and occupied a large market [8,9]. Previously, the synthesis of pregabalin with pure single enantiomer was mainly achieved by chemical processes such as asymmetric synthesis, which required drastic conditions and produced unwanted by-products and considerable amount of inorganic wastes [10,11]. The development of biocatalysts like nitrilase and lipase paved the way for preparation of chiral intermediates of pregabalin under mild and environment-friendly conditions [12–14]. Currently, the lipase-catalyzed resolution of CNDE was demonstrated as the most effective strategy for scalable production of desired (*S*)-enantiomer, and TLL was reported as the main responsible lipase [6,7].

Although commercially available, the practical use of TLL requires large quantities and as a result, there is an urgent need to achieve enhanced activity and enantioselectivity of TLL for a cost-effective and economically viable process. Genetic modification including directed evolution and rational design of proteins has progressed and become a ubiquitous technique to engineer existing enzymes to be compatible with the target processes [15–17]. The structures of TLL family enzymes have been discovered and based on their well-known structural and catalytic information [18], site-

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saturation mutagenesis becomes an ideal choice to modify TLL with desired properties.

In this study, the TLL enzyme was first cloned and expressed in *E. coli*. The site-saturation mutagenesis was designed on the amino acid residues nearby the active site, in the lid hinge regions, and affecting the formation of oxyganion hole and disulfide bond, to screen mutants with enhanced activity and enantioselectivity toward CNDE. The mutants with highest activity were further purified and used for the analysis of kinetics, thermostability and other catalytic characteristics. The kinetic resolution of CNDE by whole cell of *E. coli* harboring TLL mutant aims to find out a new promising biocatalyst for industrial production of chiral intermediate of pregabalin.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The TLL gene was amplified using RT-PCR from the total RNA of *T. lanuginosus* DSM 10635 with the method as described previously [7] and digested with *Nco*I and *Xho*I restriction endonuclease. It was ligated to the pre-digested pET-28b(+) with the same restriction enzymes, to construct the expression vectors pET-28b-TLL and transformed into *E. coli* BL21 (DE3) competent cells. *E. coli* harboring the recombinant plasmid was grown at 37 °C, 150 rpm in Luria-Bertani (LB) medium (NaCl 10 g/L, Yeast extract 5 g/L, Tryptone10 g/L) supplemented with 50 μg/mL kanamycin. When the OD_{600} of the fermentation broth reached 0.6–0.8, the expression of TLL was induced by 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) for 9 h at 28 °C.

2.2. Site-saturation mutagenesis

Plasmid pET-28b-TLL was extracted and used as template DNA for site-saturation mutagenesis. The target mutagenesis sites were designed at amino acid residues at different locations of the structure. The forward and backward primers are listed in Table 1. The PCR was carried out with high-fidelity thermostable DNA polymerase (PrimeSTAR) as follows: 98 °C for 2 min; followed by 25 cycles at 98 °C for 10 s, 50–60 °C for 10 s and 72 °C for 6 min; the reaction was further kept at 72 °C for 10 min and the temperature was finally decreased to 4 °C. The PCR products were extracted and purified from 0.9% agarose DNA gel and digested by *Dpn*I endonuclease at 37 °C for 3 h, to remove the parental plasmid. The treated products were then transformed into *E. coli* BL21 (DE3) competent cells and spread on LB agar plates containing 50 μg/mL kanamycin.

2.3. Screening of TLL variants for enhanced activity

The preliminary screening of TLL mutants for enhanced activity was carried out using a high-throughput screening method with a pH-based indicator described in a previously report [7]. The positive variants with improved activity were further verified by gas chromatography (GC) analysis. The most active mutant was sequenced and used as parental strain for the next round of site-saturation mutagenesis and the same screening method was applied for the mutagenesis library of each round (Fig. 1).

2.4. Expression and purification of TLL variants

Recombinant *E. coli* harboring the wild type and mutant TLL were cultured at 37 °C in 100 mL LB medium supplemented with 50 μg/mL kanamycin until the optical density at 600 nm reached 0.5–0.6. The expression of the recombinant TLL was initiated by adding 0.1 mM IPTG. After induction at 28 °C for 10 h, the cells were harvested by centrifugation at 8000*g* for 10 min. The collected cells were re-suspended in Tris-HCl buffer (50 mM, pH 8.0) and disrupted using an ultrasonic oscillator. Cell debris was removed by centrifugation at 4 °C, 12,000*g* for 10 min. Solid ammonium sulfate was added into the cell-free extract to a final concentration of 35% and the precipitate was removed again by centrifugation at 4 °C, 12,000*g* for 10 min. The supernatant was then fractionated with ammonium sulfate to a concentration of 60%. The resulted precipitate was collected and subsequently dissolved in Tris-HCl buffer (50 mM, pH 8.0) containing 1.3 M ammonium sulfate. The dissolved solution was loaded onto a Phenyl Sepharose 6 Fast Flow column (1.6 × 20 cm, GE) pre-equilibrated with 1.3 M ammonium sulfate in Tris-HCl buffer (50 mM, pH 8.0). The enzyme was eluted with a linear gradient of ammonium sulfate from 1.3 to 0 M in Tris-HCl buffer (50 mM, pH 8.0) with a flow rate of 1 mL/min. The fractions containing TLL activity were dialyzed against in Tris-HCl buffer (50 mM, pH 8.0) at 0 °C overnight. The dialyzed fraction was then loaded onto a DEAE-Sepharose FF column (1.6 × 20 cm, GE) and successively eluted first with washing buffer (150 mM NaCl, pH 8.0) and then with eluting buffer (350 mM NaCl, pH 8.0) in Tris-HCl buffer (50 mM, pH 8.0). Fractions containing TLL activity were pooled and dialyzed against Tris-HCl buffer (50 mM, pH 8.0) at 0 °Covernight.

To detect the purity of the enzyme, 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The protein concentration was measured according to the Bradford method.

2.5. Activity assay with purified enzyme

Purified enzyme was applied to measure the lipase activity toward CNDE. The reaction was carried out at 40 °C in 10 mL Tris-HCl buffer (100 mM pH 8.0) containing 10 mM calcium acetate, 100 mM CNDE and certain amount of the purified enzyme. One unit of lipase activity (U) was defined as the required amount of enzyme releasing 1 μmol of 2-carboxyethyl-3-cyano-5-methylhexanoic acid per min under the given assay conditions.

2.6. Kinetic analysis

The purified wild type and mutant TLL were dissolved in Tris-HCl buffer (100 mM, pH 8.0) at 40 °C, 150 rpm and different concentrations of CNDE ranging from 5 to 200 mM were added as substrate. The kinetic model of double reciprocal plot was applied as described previously [19]. The kinetic parameters K_m and V_{max} were calculated by building Linewaver-Burk type plots. The value of catalytic rate constant (k_{cat}) was measured according to the equation $k_{cat} = V_{max}/[E]$, where [E] was defined as the molar concentration of enzymes.

2.7. Effect of pH and temperature on TLL activity

The effect of pH on TLL activity was investigated in 50 mM Na_2HPO_4-NaH_2PO_4 buffer with pH values varying from 6.0 to 8.0, 50 mM Tris-HCl buffer with pH values varying from pH 8.0–9.0 and 50 mM Glycine-NaOH buffer with pH values varying from 9.0 to 11.0. The effect of temperature on TLL activity was investigated in 50 mM Tris-HCl buffer (pH 8.0) at temperatures ranging from 30 °C to 60 °C.

2.8. Whole cells-catalyzed kinetic resolution of CNDE

The recombinant *E. coli* harboring mutant TLL gene was cultured, induced and harvested by centrifugation at 8000*g* for 10 min. The resting cells (5% w/v) were suspended in 10 mL Tris-HCl buffer (100 mM, pH 9.0) containing 150 mM calcium acetate. Different concentrations of CNDE were added as substrate. The reactions were carried out at 40 °C with shaking at 180 rpm. Samples were