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Conversion of a *Monascus ruber* esterase into a lipase by disrupting a salt bridge

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ABSTRACT

Cold-active lipases have emerged as an important class of biocatalysts for chemical and food industries due to their high efficiency at low temperature and long-chain substrate preference. In an effort to explore the feasibility of converting a cold-active esterase from *Monascus ruber* (Lip10) into a cold-active lipase, an Y264F variant in which the salt bridge between K243 and Y264 was disrupted has been constructed and characterized. The interfacial kinetic parameter, K_m^{app} for pNP-laurate (C12) and pNP-palmitate (C16), of Lip10 esterase was 4.2 and 5.7 times higher than those of the Y264F variant, respectively. Substrate specificity of the Y264F variant changed from shot-chain length substrate to medium- and long-chain length substrates, indicating that the Y264F variant turned into a lipase. Meanwhile, the Y264F variant displayed 48.6% maximum activity at 4 °C and 3.2 kcal/mol activation energy in the range of 5–30 °C, suggesting that it was still cold-active. Based on analysis of the structure-function relationships, it suggests that the shape of substrate channel controlled by the conserved salt bridge was very important for the substrate specificity. This study provides a way to alter the substrate preference of the Lip10 esterase as well as new insight into the structural basis of esterase substrate specificity.

1. Introduction

Esterases and lipases catalyze the hydrolysis of carboxylic esters, and thus have wide applications in food, pharmacy, and chemical industries [1]. There is a significant difference in activity between the two enzymes, with the esterase catalyzing water-soluble short-chain fatty acid esters, and the lipase catalyzing the water-insoluble long-chain fatty acid esters [2,3]. Among these enzymes, cold-active esterases and lipases are of interest to industry in recent years because they can exhibit high catalytic activities at low temperatures allowing for energy savings, the preparation of thermo-sensitive compounds, and reducing the side-reaction products generated under high-temperature conditions [4–6]. However, compared with the cold-active lipases, the cold-active esterases can only hydrolyze the short-chain fatty acid esters. This obvious disadvantage imposed great restrictions on the use of cold-active esterases in industry.

There are differences between esterases and lipases on the amino acid sequence, spatial structure, and active site, but each has one substrate channel through which the substrates enter into the active site [7,8]. The properties of substrate channel amino acid residues played key role in enzyme catalytic performance [3]. The entry of a substrate to the active center was mainly affected by the properties of amino acid side chains in the substrate channel, with bulky amino acid side chains hindering large substrate molecules from entering into the active center [9,10]. In addition, the hydrophilic nature of the substrate channel in esterase could alter the movement of hydrophobic substrates through the substrate channel [11]. Thus, it is possible that the catalytic performance can be changed through replacing the amino acids in substrate channel.

In previous work, a novel cold-active esterase (Lip10) was cloned from *Monascus ruber* M7 and expressed in *Escherichia coli* [12]. The characteristics of Lip10 suggested a potential application in food and chemical industries. However, its substrate specificity with respect to the chain length in the substrate is not optimal for the manufacture of a particular product. Therefore, the molecular manipulation of Lip10 esterase to alter its chain-length specificity is of industrial and theoretical interest.

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In this study, we designed and characterized a *M. ruber* esterase variant by disrupting a salt bridge, to explore the feasibility of converting an esterase into a lipase. The *M. ruber* esterase Y264F variant was successfully converted into a lipase. Furthermore, the thermostability of variant was also studied.

2. Materials and methods

2.1. Materials

p-Nitrophenyl derivatives (pNP-derivatives) and triacylglycerols were purchased from Sigma Co. Ltd (St. Louis, USA). DNA polymerase and T4 DNA ligase were purchased from Takara Co. Ltd (Dalian, China). Plasmid pET-30a(+) (Invitrogen, USA) was used as the expression vector. *Escherichia coli* TOP10 and BL21(DE3) (Novagen, USA) were used as the hosts for plasmid amplification and heterologous expression, respectively. The *Lip10* esterase gene (GenBank accession no. KT862834) was described in our previous work [12].

2.2. Site-directed mutagenesis and expression of Lip10

Recombinant vector pET30a(+)-Lip10, prepared previously [12], was utilized as the mutagenesis template. Site-directed mutagenesis experiments were carried out as described previously [13]. All sequences were confirmed by DNA sequencing. The resulting vectors were used for expression of the mutations. Transformants were grown in LB medium (50 μg/mL Kan^R) at 37 °C and 200 rpm. When the optical density at 600 nm reached 0.6, IPTG was added to the medium to a final concentration of 0.5 mM, and the culture was incubated at 18 °C and 200 rpm for 12 h. The purification protocol was used to purify His-tagged protein, as described in previous work [12]. The protein obtained by purification was tested on a 12% SDS-PAGE gel. Protein concentration was estimated by the Bradford method using bovine serum albumin as the standard.

2.3. Hydrolytic activity assays

An esterase activity assay for WT Lip10 esterase and the Y264F mutant was performed by a spectrophotometric method at 410 nm on a UV-160 spectrophotometer (Shimadzu, Japan) [14]. 20 μL of pNP ester solution (40 mM) and 960 μL of 50 mM Tris–HCl buffer (pH 8.0) was mixed. The reaction was initiated by adding 20 μL of the WT Lip10 esterase at 40 °C or mutation Y264F solution at 30 °C. One unit of esterase activity by WT Lip10 and mutatant Y264F was defined as the amount of enzyme releasing 1.0 μmol of pNP per minute in 50 mM Tris–HCl (pH 8.0) at 40 and 30 °C, respectively.

The hydrolysis activity for triacylglycerol was determined as previously described with few modifications [2]. The hydrolytic activity was measured by monitoring the production of free fatty acids in 2.5 mM Tris–HCl buffer (pH 8.0) in an automatic pH-stat titrator (Metrohm, Herisau, Switzerland) at the titration end point of pH = 8.0. One unit of esterase activity of WT Lip10 esterase and the Y264F mutant was defined as the amount of esterase that released 1.0 μmol of fatty acid per minute at 40 and 30 °C, respectively.

2.4. Characterization of Lip10 and its mutations

2.4.1. Effect of temperature on esterases

The optimum temperature for wild-type Lip10 esterase and the Y264F mutant was measured by determining the hydrolytic activity at various temperatures (4–60 °C) in 50 mM Tris-HCl buffer (pH 8.0) using pNP butyrate and pNP caproate as substrate, respectively. To study thermostability, purified enzymes were incubated at various temperatures (4–60 °C) for up to 30 min. Aliquots of WT Lip10 and the Y264F mutant were withdrawn and adjusted to 40 and 30 °C, respectively. The residual hydrolytic activity of WT Lip10 and the Y264F mutant was measured by the colorimetric method at 40 and 30 °C using pNP butyrate and pNP caproate as substrates, respectively.

2.4.2. Circular dichroism (CD) spectra

To perform CD analysis, purified protein samples were diluted to 3.0 μmol/L in 50 mM Tris-HCl buffer (pH 8.0). CD spectroscopy was performed on a Jasco 1500 spectropolarimeter equipped with a peltier-type temperature controller with a 1.0-mm path length cell (Jasco, Inc., Easton, MD). Variable wavelength measurements of protein solutions were scanned from 180 to 260 nm at 20 °C and data points were collected every 0.1 nm. Thermal denaturation experiments were conducted as follows: 3.0 μmol/L protein samples were heated from 5 to 55 °C at a rate of 1.0 °C/min in a 1.0-mm quartz cuvette. The results were analyzed with Standard Analysis software (JACSO) and expressed as mean residue molar ellipticity [θ]. Thermal denaturation profiles were displayed in molar ellipticity at 222 nm.

2.4.3. Substrate specificity

To determine the effect of the acyl chain length of the ester substrate on esterase activity, the pNP-derivatives and triacylglycerols were selected as substrates. Activity of the WT Lip10 esterase and the Y264F mutant for pNP-derivatives was assayed by the colorimetric method (50 mM Tris-HCl, pH 8.0) at 40 and 30 °C, respectively. The hydrolytic activity of WT Lip10 esterase and mutant Y264F toward triacylglycerols was measured in 2.5 mM Tris–HCl buffer at pH 8.0 by the titrimetric method at 40 and 30 °C, respectively.

2.4.4. Interfacial kinetics

The interfacial kinetic parameters (K_m and k_{cat}) were measured in a heterogeneous medium (pH 8.0) at 40 °C for WT Lip10 esterase and 30 °C for mutation Y264F using various concentrations of pNP-acetate, pNP-caprylate, pNP-laurate or pNP-palmitate as substrate according to the method described by Burdette and Quinn [15].

2.5. Homology modeling

A 3D model of Lip10 esterase was built by SWISS-MODEL, an automated protein program [16]. First, different template structures from the PDB database were superimposed using an iterative least squares algorithm. A pair-wise alignment of the Lip10 sequence to the template, an esterase crystal structure (EST2) from *Alicyclobacillus acidocaldarius* (PDB ID: 1evq) [17] was calculated. Then, the backbone atom positions of the template structure were averaged to generate the core of the Lip10 model. To generate insertions or deletions in the model, an ensemble of fragments compatible with the neighboring backbone was constructed and loops was added in the model. Thirdly, the construction of side chains was performed on the basis of the weighted residues positions in the template structures. Lastly, geometric deviations in the Lip10 model were standardized by steepest descent energy minimization using the GROMOS96 force field for 10 ps at 298 K. For validation of the model, the stereochemical properties of the model were analyzed by the PROCHECK, GMQE, QMEAN, Solvation and Torsion program [18]. PyMOL was used to visualize the protein conformation.