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A novel strategy to improve the thermostability of *Penicillium camembertii* mono- and di-acylglycerol lipase

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ABSTRACT

Penicillium camembertii (PCL), a mono- and di-acylglycerol lipase (DGL), has the vital potential in the oil chemistry for food industry. However, known DGLs are mesophilic enzymes which restricts its application in the industry. To improve thermostability of PCL, we used amino acid substitution by comparison of amino acids compositions of PCL and protein sequences from typical thermophilic bacteria. Then, some conservative residues around active center were avoided to mutate according to homologous alignment analyses. Furthermore, the list was narrowed down to 28 candidate mutational sites of PCL by analyzing the hydrophobic interaction of amino acids in the structure. And among them only the mutant PCL-D25R had formed an additional salt bridge between R25-D32 and increased more hydrogen bonds interaction. Therefore, mutant PCL-D25R were constructed and expressed. Thermal inactivation assay showed that the half-life of mutant PCL-D25R at 45 °C increased 4-fold compared to that of PCL-WT. Melting temperature of mutant PCL-D25R increased to 49.5 °C from 46.5 °C by fluorescence-based thermal stability assay. This study provides a valuable strategy for engineering DGL thermostability.

1. Introduction

Microorganism mono- and di-acylglycerol lipases (DGLs) hydrolyze mono- (MAG) and di-acylglycerol (DAG) to produce free fatty acids, MAG or glycerol but without activity towards triacylgycerols [1]. *Penicillium camembertii* lipase (PCL) is a DCL and has great potential for industrial application because of its special substrate selectivity [2]. PCL is also widely used in oils and fats processing for fine chemical synthesis [3], and has the potential advantage in the peroxidation process without undesired side reactions [4]. *Malassezia globose* LIP1 (SMG1) lipase is a DGL and has a high biocatalyst potential in oil/fat modifications [5,6]. However, the optimal temperature of all reported microorganism DGLs range from 15 to 40 °C [7], which hinders their usage in industry; therefore, it is urgent to improve DGL thermostability.

Many strategies towards improving protein thermostability have been applied, including error prone PCR and consensus protein design [8,9]. However, constructing a large-scale mutant library and implementing a sensitive and effective method for high-throughput screening still pose difficult obstacles before being applied in protein engineering. Structure-based interactions of disulfide bonds, salt bridges, hydrogen bonds and hydrophobic interactions, and interaction of aromatic rings [10–12] had been widely used to improve protein thermostability. However, its efficiency is still very limited.

Computation-based comparisons of amino acid compositions in thermophilic and mesophilic proteins have been conducted in several reports [13,14]. Zhao et al. found that the hydrophobic residues L, P, M, F, W as well as the polar residue Y had higher occurrence in thermophilic lipases than thermostable ones by comparing 77 thermophilic and 65 mesophilic lipases [15]. Ludovica et al. showed that amino acids of E, K, A, I, L, M, P, V, W, Y, R and H had high ratio in hyperthermophilic proteins; while Q, C, F, G, N, Q, S, T and D were lower by sequence comparison of 378 proteins from thermophilic bacteria and 1015 from mesophilic bacteria [16]. Furthermore, the exponential growth of microbial genomic sequences in recent years provide vast protein sequences from microorganisms adapted to different environments, including

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thousands of protein sequences from thermophilic bacteria. However, computed-based comparison of thermophilic and mesophilic proteins for enzyme engineering to improve thermostability is still limited.

In this study, amino acid substitution was implemented to design mutants by comparison of known amino acid compositions in proteins of thermophilic and mesophilic microorganisms. We further performed structural analyses of these candidate mutants to screen the suitable site to mutate for enhancing its thermostability.

2. Materials and methods

2.1. Materials

Escherichia coli DH5α strain (Stratagene, LaJolla, CA, USA) and pGAPZαA plasmid (Invitrogen, Waltham, MA, USA) were used for cloning. The strain pGAPZαA-PCL-DH5α and strain pGAPZαA-PCL-X33 were stored in our lab [4]. *Pichia pastoris* X-33 strain (Invitrogen, Waltham, MA, USA) was used for expression. DAG-rich oils (50.1% of TAG, 49.2% of DAG, 0.6% MAG and 0.1% of Fatty acids) were prepared by hydrolysis of rape oil using Palatase 20000 L and purified by molecular distillation in our lab. All other chemicals were of analytical grade.

2.2. Mutants construction, expression, and purification

Site-directed mutagenesis at position D25 of PCL was carried out using the pGAPZαA-PCL plasmid as template by the site-directed mutation. Primer sequences were listed in Table S1. After confirmed by DNA sequencing, the plasmids were linearized by PCR method and transformed into *P. pastoris* X-33 strain by electroporation. The transformant of PCL-WT and mutant PCL-D25R were grown on YPD (1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose) plates supplemented with Zeocin™ (100 mg/mL) at 30 °C until colonies form.

P. pastoris X-33 cells containing the recombinant plasmids were grown and expressed in YPD liquid medium at 30 °C with shaking of 200 rpm for 60 h. Cell suspension was centrifuged at 10,000 × g for 20 min at 4 °C and proteins were concentrated by ammonium sulfate and buffer-exchanged to sodium phosphate buffer (20 mM, pH 7.0) through a desalting column (GE Healthcare, China) at 4 °C. PCL-WT and mutant PCL-D25R were purified using anion exchange chromatography (Q-Sepharose FF, GE Healthcare, China).

2.3. Sequence alignment, construction of mutant models and structure analysis

Multiple-sequence alignment was performed with MultAlin (http://multalin.toulouse.inra.fr/multalin/) [17]. Homologous modeling structures of 28 PCL mutants were conducted based on crystal structure of PCL (PDB ID: 5CH8) using the MODELLER [18].

2.4. Determination of apparent melting temperatures

A fluorescence-based thermal stability assay was used to determine Tm (melting temperature) using a CFX connect Real-Time PCR instrument (Bio-Rad, Hercules, CA, USA) [19]. A sample of 20 μl of purified protein in buffer (20 mM phosphate buffer, pH 6) was mixed with 5 μl of 100-fold diluted Sypro Orange dye (Molecular Probes, Life Technologies, USA) in a thin wall 96-well PCR plate. Samples were sealed with Optical-Quality Sealing Tape and heated in a CFX 96 Real Time PCR System from 25 to 90 °C with increasing at every 0.5 °C and holding for 5 s. The wavelengths for excitation and emission were 490 and 575 nm, respectively. Tm is the temperature in the peak point of the fluorescence curve.

2.5. Biochemical characterizations of recombinant lipases

Lipase activity assay was performed by titration. PCL-WT and mutant PCL-D25R were added to the substrate solution containing 4 g of emulsified DAG-rich oil (1 g DAG-rich oil and 3 g 4.0% (w/v) polyvinyl alcohol were mixed with a blender) and 5 mL buffer. After incubation for 10 min at certain temperature, the reaction was terminated by the addition of 15 mL of ethanol. The level of fatty acid release was determined by titration with 50 mM NaOH solution. One unit of lipase activity was defined as the amount of releasing 1 μmol of fatty acid per minute.

To determine the optimal temperature, lipases activity was detected under temperatures ranging from 30 to 55 °C. The effect of pH on activity of PCL was investigated over a pH range 4.0–9.0 at 40 °C. For the determination of temperature and pH stability, the residual activity was measured at optimal condition. Purified enzymes were incubated in 20 mM phosphate buffer at temperatures ranging from 30 to 50 °C for 30 min, and in 20 mM buffer at pH from 4.0 to 9.0 at 4 °C for 40 h, respectively. PCL-WT and mutant PCL-D25R were pre-incubated in 45 °C for 1 h, and the residual activity of these proteins were measured at optimal condition to determine their half-life. The deactivation constant k_d was calculated from the slope of Ln plot (residual activity) versus time. Formula of $T_{1/2} = \ln 2/k_d$ was used for calculating the half-life of PCL-WT and mutant PCL-D25R.

Kinetic constants were determined using p-nitrophenyl caprylate (C8) as substrate. V_{max} and K_m values were determined under different concentration of pNP-C8 esters ranging from 0.05 to 1 mM of p-NP esters by Lineweaver–Burk plots method. k_{cat} was subsequently calculated using different enzyme concentration.

2.6. MD simulation for PCL-WT and D25R

MD simulation was performed to analyze the thermal fluctuation of PCL-WT and mutant PCL-D25R at the molecular mechanics level at 320 K for 50 ns using GROMACS 5.1.4 [20] with OPLS-AA force field [21]. The structures were initially cleaned to optimize potential for liquid simulation by adding hydrogen or incomplete side-chain atoms. In the MD simulation, the structures were put in a cubic box with a volume of 8 × 8 × 8 Å, and the TIP4P model of water [22] was used to solvate the protein. The system was neutralized by adding 0.02 mol/L Na^+ and Cl^-, respectively. Trajectory analysis of data was performed with GROMACS. And the RMSD and RMSF values for backbone atoms and distance were calculated.

3. Results

3.1. Rational design of PCL mutants

A combination of amino acids substitution and structural analyses was used to design and screen the mutational sites of PCL for improving thermostability. First, we compared amino acid compositions of PCL and common compositions of proteins from hyperthermophilic microorganisms [23] (Fig. S1). The contents of amino acid residues A, D, S and T in PCL were higher than in protein sequences from hyperthermophilic microorganisms, and the contents of residues E, I, K, L and R in PCL were lower than in protein sequences from hyperthermophilic microorganisms. The residues of A, D, S, and T in PCL were replaced, obtaining 93 candidate mutational sites. Sequence alignment of PCL with homologous thermostable lipases, *Rhizomucor miehei* (PDB ID: 4TGL) and *Thermomyces lanuginosa* (PDB ID: 1DT3) was performed to avoid the