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Engineering of *Yarrowia lipolytica* lipase Lip8p by circular permutation to alter substrate and temperature characteristics

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Abstract Applications of lipases are mainly based on their catalytic efficiency and substrate specificity. In this study, circular permutation (CP), an unconventional protein engineering technique, was employed to acquire active mutants of *Yarrowia lipolytica* lipase Lip8p. A total of 21 mutant lipases exhibited significant shifts in substrate specificity. Cp128, the most active enzyme mutant, showed higher catalytic activity (14.5-fold) and higher affinity (4.6-fold) (decreased K_m) to *p*-nitrophenyl-myristate (*p*NP-C_{14}) than wild type (WT). Based on the three-dimensional (3D) structure model of the Lip8p, we found that most of the functional mutation occurred in the surface-exposed loop region in close proximity to the lid domain (S112–F122), which implies the steric effect of the lid on lipase activity and substrate specificity. The temperature properties of Cp128 were also investigated. In contrast to the optimal temperature of 45 °C for the WT enzyme, Cp128 exhibited the maximal activity at 37 °C. But it is noteworthy that there is no change in thermostability.

Keywords Lipase · Circular permutation · Lid · Substrate specificity · K_m

Introduction

Lipases, one of the hydrolases, have been widely applied in food making, fine chemistry, pharmaceutical industries and waste treatment [1, 33]. Although lipases from different sources showed distinct enzyme characteristics, catalytic efficiency and substrate specificity are still the key factors for commercial applications. Therefore, how to improve an enzyme by protein engineering to achieve the desired property is attracting tremendous interest among molecular biologists and biochemical engineers [23, 30].

It has been proved that most of the lipases have a catalytic triad usually formed by Ser, His and Asp/Glu residues [2, 28]. In addition, a mobile alpha-helical surface loop, the so-called lid, covers the catalytic triad to prevent direct access of the substrate [3, 5, 22]. During activation, the lid undergoes a conformational rearrangement, which provides ample space around the catalytic triad and allows access of the substrate to the active site [4, 7, 13]. Like *Rhizomucor miehei* lipase, in activation, the backbone of the central part of the lid moves by more than 7 Å [10]. Obviously, an incompletely opened lid usually became the hindrance preventing bulky substrates from encountering the catalytic triad. The importance of the lid domain has made it a target for tailoring substrate specificity and improving catalytic performance [14, 22, 28].

Yarrowia lipolytica, a "generally recognized as safe" (GRAS) microorganism with no adverse effect on humans, has attracted much attention. A search of the *Y. lipolytica* genome database showed that it possesses 16 lipase paralogs of genes. Among these isoenzymes, Lip8p (accession no. AJ549520) is one of the most commonly used reagents in detergents and food industry [12]. It showed a maximal activity toward esters of *p*-nitrophenyl-caprate (*p*NP-C_{10}), but the efficiency to hydrolyze esters with shorter and longer chains decreased rapidly [12, 29]. We assume that if the structural constraints of the enzyme could be relaxed, the overall flexibility of Lip8p would be enhanced. Then, at the same activation energy, the lid would move farther away from the catalytic triad, and the long chain substrates and products could easily enter and exit the active site. Then,

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Lip8p could accommodate a range of substrates. To test our idea, we employed a technique called circular permutation (CP) to explore the effects of catalytic triad accessibility on enzyme activity. CP is a method to create mutant proteins by connecting the native protein termini via a covalent soft linker and introducing new ends through the cleavage of an existing peptide bond [14, 19, 32]. Along with the new termini relocation, the local tertiary and quaternary structures of the protein were usually changed, which resulted in topological changes of the active site [16, 17, 27]. Since the first report about circularly permuted protein was published in 1979, many artificial CPs, inclusive of T4 lysozyme, phosphoglycerate kinase, lipase and alkaline phosphatase, have been generated. These mutant proteins showed a distinct biological function [9, 22, 24, 32, 35]. Inspired by these works, we constructed a random CP library of lipase Lip8p with a flexible Gly-rich (-GGGSGGG-) linker. Finally, a total of 21 mutant lipases exhibited significant shifts in substrate specificity. Cp128, one of the most active mutant enzymes, showed an improved catalytic efficiency and affinity relative to the wild type (WT). This work indicated that CP is a valuable method for protein engineering.

Materials and methods

Strains and media

Escherichia coli DH5α was used in gene manipulations. *E. coli* was grown in Luria–Bertani (LB) liquid media or on LB agar plates supplemented with the appropriate kanamycin. The *Pichia pastoris* strain GS115 (Invitrogen), harboring the recombinant plasmids, was used for expressing the mutated Lip8p gene. *P. pastoris* was cultured in BMGY medium and transferred to BMMY medium for protein expression. All media were prepared according to the Yeast Expression Guidelines (Invitrogen).

Construction and expression of the circularly permuted gene

Gly-rich oligonucleotides were obtained from Sangon (Sangon, China). A 1,116-bp ORF of the Lip8p gene was amplified by PCR using the primers Lip8F 5′-ggaggaggatccggaggaggaatggtatccctctctgc-3′ and Lip8R 5′-catgtcaactcgttca-3′. The DNA fragment encoding the soft linker was designed at the 5′-terminal of the forward primer (underlined). The PCR products were treated with T4 DNA ligase for 12 h at 16 °C. After intramolecular ligation, the circular DNA was linearized in random positions using DNaseI. Reaction conditions were chosen such that, on average, only a single cut per DNA strand was introduced. Since DNaseI not only creates blunt ends but also protruding ends, the circularly permuted Lip8p genes were subsequently treated with T4 DNA polymerase/dNTPs and T4 DNA ligase at 16 °C in order to repair nicks and gaps and to create uniformly blunt-ended molecules. The circularly permuted genes were loaded on an agarose gel, and DNA fragments, 1,000 to 1,200 nucleotides in size, were recovered by gel excision and DNA extraction. The recovered DNA was then cloned into vector pPIC9K and expressed in *P. pastoris* GS115. The permuted nucleotide sequence cloned into pPIC9K was checked using an ABI PRISM 377XL DNA Sequencer (Applied Biosystems).

Expression and purification of circularly permuted lipases

Pichia pastoris transformants were initially grown in BMGY medium at 28 °C with shaking (250 rpm) until the OD_{600} reached 2-6. Then the cultures were supplemented with 0.5 % (v/v) methanol to induce the expression of lipase. To maintain expression, 100 % methanol was added every 24 h to the final concentration of 0.5 %. After 72 h (optimal induction period), the culture was centrifuged, and the supernatant was concentrated by ultrafiltration (10-kDa cutoff). Then, the concentrated enzyme solution was applied to a Q-Sepharose Fast Flow column (10 ml volume) pre-equilibrated with buffer A (20 mM phosphate buffer, pH 8.0). After the column was washed with the same buffer, the bond proteins were then eluted with a linear gradient of NaCl solution over the range of 0–0.5 M in buffer B (20 mM phosphate buffer, 0.8 M NaCl, pH 8.0). All these steps were performed at 4 °C. The peak with lipase-positive was collected and further purified by gel filtration chromatography using a Sephacryl®S-100 column (60 cm × 1.8 cm) with buffer A.

Screening by halo assay

Screening of mutants was performed using a Victoria Blue polyvinyl alcohol (PVA)-*p*-nitrophenyl-myristate (*p*NP-C_{14})-agarose (VPMA) plate. The VPMA plate was prepared as follows: 0.1 % (v/v) *p*NP-C_{14}, 7.5(v/v) PVA with 1,750 ± 50 degrees of polymerization and 0.2 % Victoria Blue were subjected to sonication for 3 min for emulsification purposes. Then, 2 % of agar was added. All media were buffered with 50 mM phosphate (pH 7.0). A 5 μl volume of culture supernatant (sterile-filtered) induced by methanol was poured into each preformed 3-mm-diameter hole, and the plate was incubated at 35 °C overnight. Transformants of expressing active lipase were determined by a blue halo on the VPMA plate.

Determination of lipase activity and kinetic parameters

Lipase activity was determined by the methods described by Liu et al. [18]. An aliquot of 0.1 ml purified enzyme

(final lipase concentration was 8 mg/ml) was added to 1.5 ml substrate solution (pH 8.0), and the mixture was incubated for 10 min in a water bath at 35 °C. Then, the mixture was put into ice. and the OD value at 410 nm was read using a spectrophotometer. One unit (U) of enzyme activity was defined as the amount of enzyme required for the liberation of 1.0 μmol *p*-nitrophenol per minute under the assay conditions. Kinetic parameters: affinity constant (K_m), maximal velocity (V_{max}) and the turnover number (k_{cat}) were determined by a line Weaver-Burke plot of the Michaelis-Menten equation using *p*NP esters as substrate.

Effects of temperature on lipase mutant activity and thermostability

Using *p*-nitrophenyl-laurate (*p*NP-C_{12}) as substrate, the optimal temperature for lipase mutants was determined in the range of 20–60 °C in 0.1 M phosphate buffer at pH 8.0. Thermal stability of the mutants was tested by pre-incubating for 120 min at different temperatures ranging from 15 to 60 °C, and the residual activity was measured immediately as described above. The activity of the pre-incubated lipase at 4 °C was taken as 100 %.

Results and discussion

Construction and expression of the circularly permuted gene and screening of the mutants

Based on the Lip8p 3D modeling, the distance between the amino and carboxyl termini of Lip8p is 23.9 Å. As a general rule, six or seven residues are sufficient to span this distance because of an estimated length of 3.5 Å per amino acid. So, we selected the flexible Gly-rich (-GGGSGGG-) fragment as a soft linker to accommodate emerging conformational strains. With the intramolecular ligation and the DNaseI digestion, the new termini over the entire length of the Lip8p gene sequence have unbiased distribution. Following transformation into *P. pastoris*, the size of the Lip8p circularly permuted library was estimated as 3.0×10^4 mutants. This greatly exceeds the number of possible "perfect" circular permutations of the Lip8p gene (1,116 bp). Functional mutants were screened using *p*NP-C_{14} as substrate because the WT enzyme has almost no hydrolytic activity for it [12, 29]. As indicated by a blue halo surrounding the hole (Fig. 1), a total of 21 mutant lipases that showed catalytic activity toward *p*NP-C_{14} were screened from generated mutant library.

The functional mutants were identified and sequenced. We found that the new terminals of five functional mutants are located in α-helices and β-strands, while those of the other mutants are all located in the surface-exposed loop region and in close proximity to the lid domain, which we called "easy permutation region." For example, the termini of four most active mutant lipases (Cp101, Cp126, Cp128 and Cp137) are all located in these regions (Fig. 2). Why was such a high concentration of functional mutation focused on these regions? From a structural point of view, we speculate that the surface-exposed loop region is flexible and mutable, it is not critical for protein folding, and backbone cleavage on this location does not perturb the protein's overall structure, so the mutants could retain native biological functions. This phenomenon has been observed by several authors from different laboratories [25, 26]. In addition, the cleavage of the peptide backbone at the site close to the lid appears to result in a relaxation of structural constraints of the lid, which reduce the steric effect within the molecule and enhance the ability of conformational changes of the lid in the catalytic process. At the same activation energy, the lid could move farther, and the bulky substrates and product could easily enter and exit the active site.

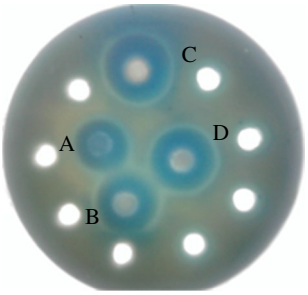

Fig. 1 VPMA plate showing different lipase mutants. Fatty acids produced through hydrolysis of *p*NP-C_{14} by functional mutants reacted with Victoria Blue and formed *blue holes*. *a*, *b*, *c* and *d* are functional lipase mutants Cp101, Cp126, Cp128 and Cp137, respectively; the rest of the mutants are inactive

Some inactive mutants were also sequenced. We found that the regions' proximity to Ser195, Asp258 and His318, which make up the catalytic triad, are "forbidden" regions. The mutants whose new termini are located in these regions have almost no catalytic activity.

Substrate specificity and kinetic parameters

Cp128, one of the most active mutants, was selected for testing substrate specificity. Because all the examined esters showed low solubility in water, we evaluated the effect of the presence of isopropanol in determining the lipase activity. No negative effect on lipase catalytic activity was detected when isopropanol was added up to 1 % (v/v). As shown in Fig. 3, Cp128 showed maximal activity toward *p*NP-C_{12} (33.2 U/mg), followed by *p*NP-C_{10} (26.8 U/mg). The activity of Cp128 for *p*NP-C_{14} (14.2 U/mg)

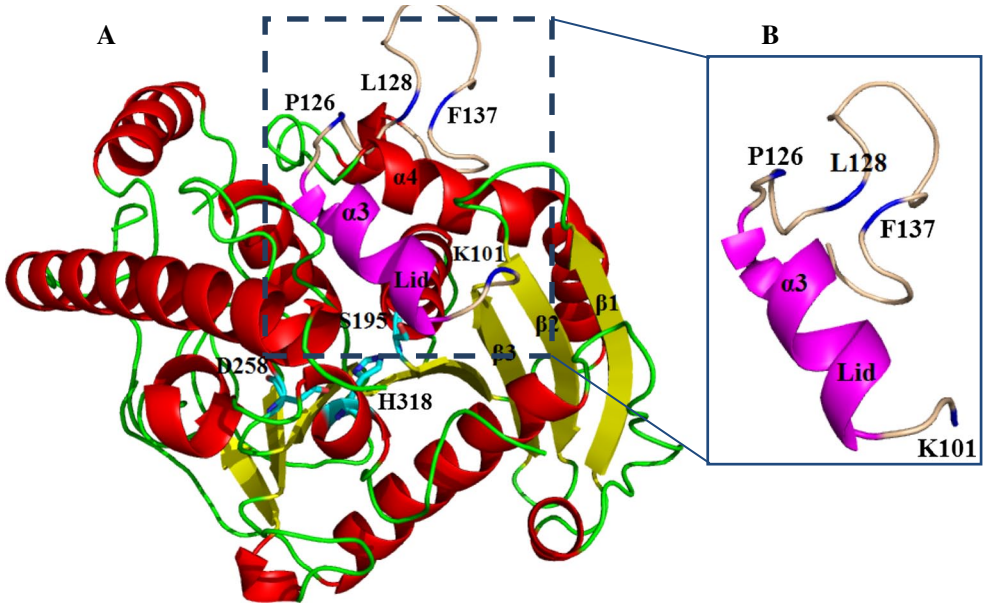

Fig. 2 Lip8p structure (**a**) and the lid region (**b**). The structure of this figure was generated by Pymol, shown as a cartoon. The lid (Ser112-F122) is in *purple*. The "easy permutation region," which is surface-exposed and in close proximity to the lid domain, is indicated in *wheat* (**b**). K101, P126, L128 and F137 (in *blue*), the new termini of the four most active lipase mutants Cp101, Cp126, Cp128 and Cp137, respectively, are all located in these regions

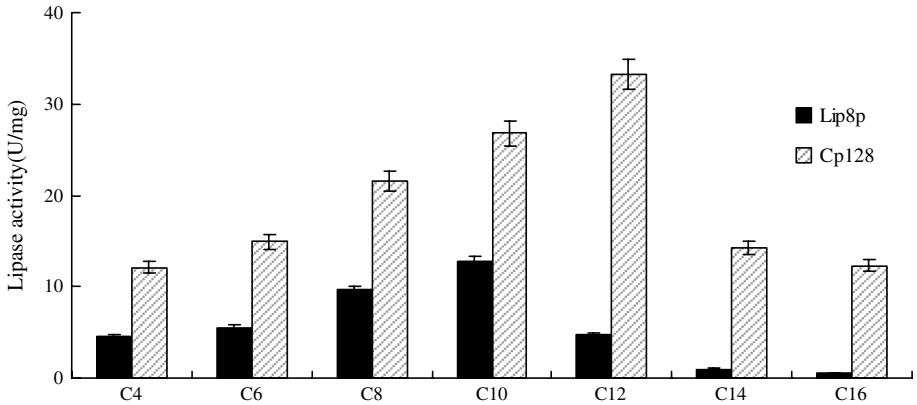

Fig. 3 Lipase activity of Cp128 and Lip8p on *p*NP esters of varying carbon chain lengths at 35 °C and pH 8.0. Data are given as the mean ± SD, $n = 3$

is 14.5-fold higher than that of Lip8p. By contrast, Lip8p showed maximal activity toward *p*NP-C_{10}; the efficiency of Lip8p in bulky substrates decreased rapidly, and it only retained 5 % relative activity when using *p*-NP-palmitate (*p*NP-C_{16}) as substrate [11, 29]. These results suggest that the ability of Cp128 to hydrolyze long chain fatty acid esters was improved. The K_m of purified Lip8p and Cp128 toward different *p*NP esters was also determined and summarized (Table 1). Cp128 showed a higher affinity (decreased K_m) for longer chain substrates than Lip8p lipase. The affinity of Cp128 for *p*NP-C_{14} and *p*NP-C_{16} was enhanced approximately 4.65 and 4.03 fold, respectively. With respect to catalytic efficiency, Cp128 displays

Table 1 K_m of Cp128 and Lip8p on *p*NPs

Substrate	Cp128/K_m*(μM)	Lip8p/K_m*(μM)
*p*NP-C_4	0.963	0.957
*p*NP-C_6	0.852	0.813
*p*NP-C_8	0.413	0.442
*p*NP-C_{10}	0.260	0.354
*p*NP-C_{12}	0.315	0.916
*p*NP-C_{14}	1.437	6.692
*p*NP-C_{16}	2.513	10.138

* K_m was calculated by monitoring initial reaction velocities at several substrate concentrations from 5 μM to 1 mM at 35 °C (pH 8.0)

Table 2 Kinetic constants of Lip8p and Cp128 for *p*NP-C_{12}

	V_{max} (mmol min^{-1} ml^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})	E_a (KJ/mol)
Lip8p	0.153	0.916	125	136	30.2
Cp128	0.182	0.315	149	473	12.6

Kinetic parameters were determined at 35 °C (pH 8.0) using *p*NP-C_{12} as substrate

a 2.47-fold increase in k_{cat}/K_m values compared to Lip8p when using *p*NP-C_{12} as substrate (Table 2). This increase in the catalytic efficiency of the Cp128 is mainly due to the affinity increase (1.9-fold) for *p*NP-C_{12} compared to Lip8p.

Effects of temperature on CpLip8 lipase activity and thermostability

By contrast to the optimal temperature of 45 °C for Lip8p [29], Cp128 exhibited the highest activity at 37 °C. The relative activity reached 64 % even at 20 °C. Cp128 presented a higher catalytic activity at low temperature (under 40 °C) than Lip8p (Fig. 4). Activation energies (E_a) were calculated from the Arrhenius plots of enzymes and are shown in Table 2. They followed Arrhenius' law between 20 and 40 °C (data not shown). The activation energy of Cp128 was about 12.6 kJ/mol using *p*NP-C_{12} as substrate, which was 2.4-fold lower than that of Lip8p lipase. It seems that the increased number and clustering of glycine residues provided a plasticity of the molecular structure, which allows them to counteract the decrease of catalytic efficiency at low temperature through low-energy cost interactions between the Cp128 active site and the substrates. In the previous reports, a high flexibility of protein is almost always associated with low stability [8, 20]. Remarkably, with the flexibility increase of Cp128, there is no change on thermostability. Both Cp128 and Lip8p retained over 70 % of its maximum activity after incubation at a wide range of temperatures from 15 to 60 °C (data not shown). This result suggested that the relationship between enzymatic rigidity and temperature stability is more complicated than initially believed. The high flexibility of the molecular structure is sometimes not at the cost of enzyme stability. To some extent, localized areas of flexibility and rigidity may be compatible with thermostability.

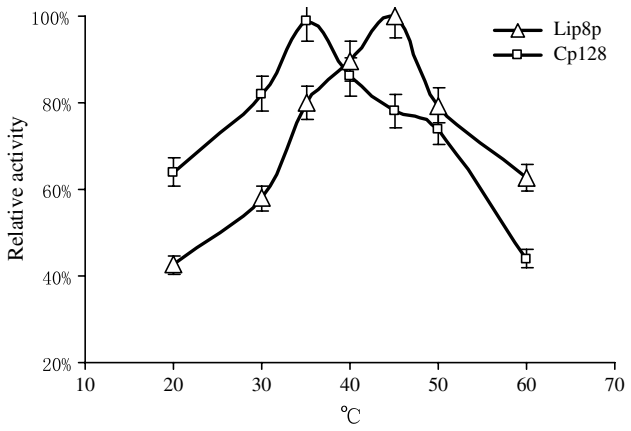

Fig. 4 Effects of different temperatures on Cp128 (*filled squares*) and Lip8p activity (*filled triangles*). Data are given as the mean ± SD, $n = 3$

Conclusion

The substrate binding site, hydrophobic tunnel and lid domain are key factors affecting the substrate specificity of lipase. During the last decade, many molecular modification techniques, including site-directed mutagenesis and DNA shuffling, have been applied to lipase for exploring the relationship between the structure and activity [2, 34]. In this study, we only discussed the effect of structural constraints of the lid on lipase. By circular permutation method, we created some functional mutants. Cp128, one of the most active mutant lipases, showed significant shifts in substrate specificity and catalytic efficiency. With further research, we found that most of the new termini of the functional mutants are located in the surface-exposed loop region and in close proximity to the lid domain. This implies the importance of these regions on the protein fold and function. The thermal properties of the mutants were also investigated. The optimal temperature of Cp128 was shifted from 45 to 37 °C, but there was no change in thermostability. According to our knowledge, most of the circularly permuted proteins show a slightly reduced conformational stability compared to natural protein [6, 15]. This observation will help in understanding the mechanism of the thermal stability of enzymes. In conclusion, the work presented here indicated that circular permutation is an effective method for protein engineering.

In spite of Cp's interesting properties and applications, there is still some uncertainty affecting the prevalence of CP. First, up to now, there have only been five studies in the literature that have observed improvements in the catalytic performance of enzyme mutants over their parents [21, 22, 28, 31, 35]. We lack comprehensive CP resources that can serve as a good base for studying it. Second, the design strategy of the linker is still a formidable challenge for a successful CP experiment. A successful linker can not only minimize potential strain from native termini topology, but also maintain overall protein stability. Therefore, the linker length and composition, protein surface regional hydrophobicity, electrostatics and curvature are all key factors we should consider.

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