CHARACTERIZATION OF LipC12V261F, A VARIANT OBTAINED THROUGH SITE-DIRECTED MUTAGENESIS OF LIPC12

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ABSTRACT

Lipases play a fundamental role in various biotechnological sectors, with applications extending across the food, detergent, oleochemical, and pharmaceutical industries. Driven by the desire to improve the performance of lipases in these industries, there is an ongoing search for new lipase variants with enhanced properties. Our group has been using site-directed mutagenesis to modify the lipase LipC12, which has a resolved crystal structure, making it an ideal candidate for protein engineering. In the present study, we characterize the variant LipC12V261F, which has a mutation in the catalytic cleft, at position 261. The variant had increased hydrolytic activity against *p*-nitrophenyl esters, compared to wild-type LipC12 (LipC12WT), with a 6-fold higher activity against *p*-nitrophenyl myristate (*p*-NPM). The optimum pH of LipC12V261F was unaffected by the mutation at position 261, but LipC12V261F had a higher optimum temperature for activity (45 ºC) than did LipC12WT (35 ºC). Molecular docking indicated that LipC12V261F has more interactions with *p*-NPM than does LipC12WT, which may explain the increased hydrolytic activity of LipC12V261F against this ester.

Keywords: Lipases. Protein engineering. Site-directed mutagenesis. Molecular docking.

1 INTRODUCTION

Lipases (EC 3.1.1.3) catalyze the hydrolysis of triglycerides in aqueous environments and esterification, transesterification, and interesterification reactions in organic media. Their many applications in the food, detergent, oleochemical, and pharmaceutical, industries[1] have stimulated an ongoing search for new lipases with enhanced properties. While bioprospecting is a viable approach, protein engineering methods, such as rational design, are preferred, due to their effectiveness in generating optimized variants, accelerating the process of enhancing enzymatic activity[2].

In this context, the metagenomic lipase LipC12 is an ideal candidate for improvement of catalytic properties through site-directed mutagenesis, given its well-known crystallographic structure[3]. In addition to its high activity against triglycerides, LipC12 has good activity and stability over a wide pH range and in organic solvents[4]. Recently, our research group identified promising mutation sites of LipC12 through molecular docking[5] and then generated several variants with site-specific mutations in the catalytic cleft. The resulting variants were characterized regarding the kinetic resolution of (*RS*)-1-phenylethanol and the hydrolysis of *p*-nitrophenyl esters[6]. Among these variants, LipC12V261F, in which the valine at position 261 was replaced by phenylalanine, had a significantly increased hydrolytic activity *p*-nitrophenyl octanoate. The objective of this work is to determine the activity of LipC12V261F against *p*-nitrophenyl esters of different carbon chain lengths, and to determine the effect of pH and temperature on the activity of LipC12V261F.

2 MATERIAL & METHODS

LipC12WT and LipC12V261F were expressed in *Escherichia coli* BL21 (λDE3) transformed with pET-28a(+) at 120 rpm, 20 °C for 16 h, induced by isopropyl-β-D-thiogalactopyranoside (IPTG)[4]. The cells were harvested by centrifugation, lysed by sonication, and the target proteins were purified using Ni^{2+}-affinity chromatography. Protein was quantified using the Bradford method[7].

Hydrolytic activity was determined using *p*-nitrophenyl (*p*-NP) esters as substrates[8]. For the assay, a 1 mL cuvette was prepared with 860 μL of distilled water, 50 μL of solution A (1 M Tris-HCl pH 7.5, 20 mM $CaCl_2 \cdot 2H_2O$, 6% Triton X-100), 50 μL of solution B (20 mM *p*-NP ester in isopropanol:acetonitrile (4:1, v/v), and 40 μL of enzyme solution. Activities were measured by monitoring the release of *p*-NP at 410 nm at 25 °C using a spectrophotometer, over 2 min. The effect of pH on activity was determined by the hydrolysis of *p*-nitrophenol myristate (*p*-NPM) from pH 6.0 to 9.0, using the following buffers: citrate-phosphate (pH 6.0–7.0), phosphate (pH 7.0–7.5), and Tris-HCl (pH 7.5–9.0), all at 1 mM. The effect of temperature on activity was determined by the hydrolysis of *p*-NPM, using phosphate buffer at pH 7.0, from 25 to 50 °C. One unit of enzymatic activity (U) was defined as the production of 1 μmol of *p*-NP per min under the assay conditions.

Molecular docking was used to investigate the interact of LipC12WT and LipC12V261F with the substrates. The *p*-NPM ligand was designed using Chimera[9], and the lowest energy conformation was identified. The region defined for molecular docking included all the amino acid residues of the catalytic cleft of LipC12WT and LipC12V261F. Molecular docking was done using Autodock Vina[10]. Identification of regions with the highest substrate affinity was based on ΔG binding values. Enzyme-substrate complexes were analyzed with LigPlot+ to identify hydrophobic interactions and hydrogen bonds, assessing substrate affinity and the residues involved[11].

3 RESULTS & DISCUSSION

The hydrolytic activities of LipC12WT and LipC12V261F were evaluated using *p*-nitrophenyl esters with different carbon chain lengths (Fig. 1). Both enzymes preferred longer-chain esters. Notably, LipC12V261F exhibited higher activity than LipC12WT against all tested substrates, with a 6-fold higher activity against *p*-NPM (C14).

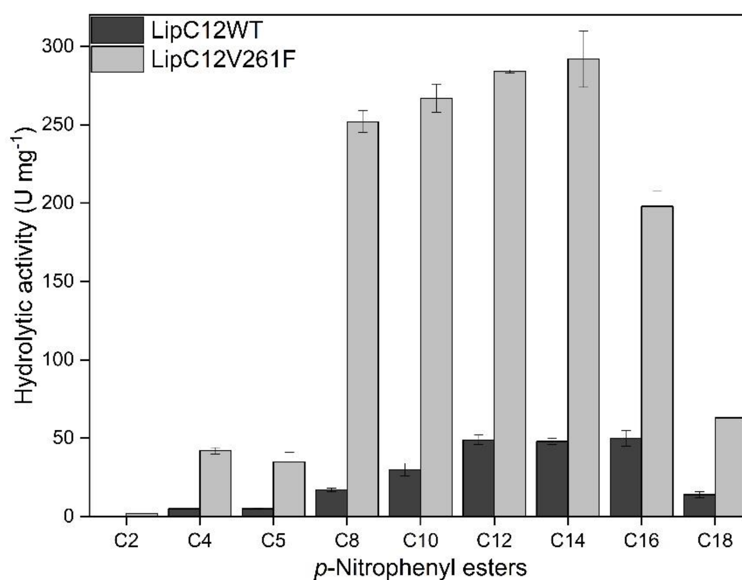

Figure 1 Hydrolytic activity of lipase Lipc12WT and LipC12V261F against different *p*-nitrophenyl esters.

The effect of pH on the activity of LipC12WT and LipC12V261F was evaluated in the pH range of 6.0 to 9.0 (Fig. 2A). The optimal pH for the activity of both LipC12WT and LipC12V261F is 7.0. The effect of temperature on the activity of LipC12WT and LipC12V261F was evaluated from 25 to 50 °C (Fig. 2B). LipC12WT exhibits higher activity in the range of 30 to 45 °C, with maximum activity at 35 °C, while LipC12V261F shows maximum activity at 45 °C. Across all tested pH and temperature ranges, the activity of LipC12V261F against *p*-NPM was higher than that of LipC12WT.

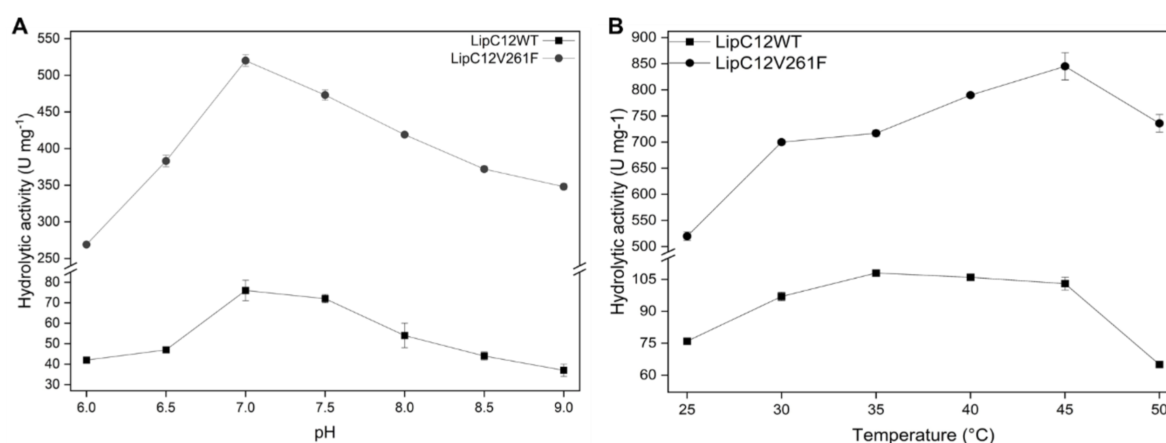

Figure 2 Effect of pH and temperature on the hydrolytic activity of lipase LipC12WT and LipC12V261F.

The enzyme-substrate interactions between *p*-NPM and the structural models of LipC12WT and LipC12V261F were investigated by molecular docking (Fig. 3A). LipC12V261F has a greater number of interactions with *p*-NPM than does the wild-type enzyme. LipC12V261F presents a greater number of interactions between *p*-NPM and the mutated amino acid residue at position 261 (Fig. 3B), which can be explained by the π-π stacking effect between the aromatic rings present in the phenylalanine side chain and *p*-NPM.

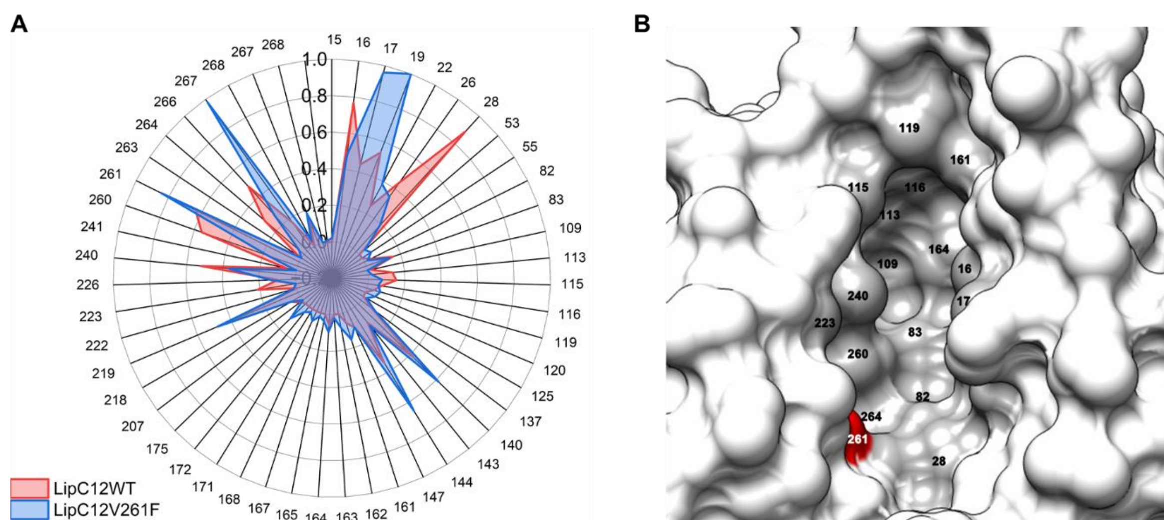

Figure 3 A) Degree of relative interaction between *p*-nitrophenyl myristate and the lipases LipC12WT and LipC12V261F. B) Numbered amino acid residues that compose the catalytic cleft of LipC12.

4 CONCLUSION

LipC12V261F, a variant obtained by site-directed mutagenesis at residue 261 in the catalytic cleft of LipC12WT, had significantly higher activities than LipC12WT in the hydrolysis of *p*-nitrophenyl esters, with 6-fold higher activity against *p*-NPM. With respect to reaction conditions, LipC12V261F had maximum activity at 45 °C, while LipC12WT had maximum activity at 35 °C; both had maximum activity at pH 7.0. Docking analyses revealed a greater number of interactions between *p*-NPM and LipC12V261F, including interactions with the mutated amino acid residue. This research highlights how mutations in the catalytic cleft impact the activity and substrate preference of lipases.

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