CHARACTERIZATION OF THE LIPC12G185C-Y244C VARIANT WITH DISULFIDE BOND INSERTION

Rafaela Kriczinski[1], David A. Mitchell[1] & Nadia Krieger[2]*

[1] *Department of Biochemistry and Molecular Biology, Federal University of Paraná, Curitiba, Brazil.*
[2] *Department of Chemistry, Federal University of Paraná, Curitiba, Brazil.*
** Corresponding author's email address: nkrieger@ufpr.br*

ABSTRACT

Lipases play a crucial role in biocatalysis due to their efficiency in catalyzing a broad range of reactions. However, their activity and stability in both organic solvents and relatively high temperatures pose challenges for their industrial application. This study aimed to enhance the catalytic properties of the wild-type lipase LipC12 (LipC12WT) by using site-directed mutagenesis to insert cysteine residues, aiming to form a disulfide bridge in the protein structure. The hydrolytic activity of the resulting variant, LipC12G185C-Y244C, was determined against *p*-nitrophenyl esters and triglycerides of varying chain lengths. The activity increased significantly ($p < 0.05$) for all tested substrates compared to LipC12WT, with the best results obtained for tributyrin (C4:0) and *p*-nitrophenyl stearate (C18:0), showing increases of 24% and 55%, respectively. However, the stability of LipC12G185C-Y244C at high temperatures was no different from that of LipC12WT. The high hydrolytic activity of LipC12G185C-Y244C shows the potential of using site-directed mutagenesis to improve the performance of lipases.

Keywords: Lipases. Protein engineering. Disulfide bonds.

1 INTRODUCTION

Lipases are widely used in the food, cosmetics, detergents, pharmaceuticals and biofuel industries[1,2]. However, many lipases have low activity and stability under conditions commonly used in industrial processes, such as high temperatures and organic solvents, which hinders the industrial implementation of lipase catalyzed processes[3]. To overcome this problem, protein engineering, along with techniques such as immobilization and reactor engineering, seek to improve the catalytic properties and stability of lipases.

The metagenomic lipase LipC12, studied in this work, is an ideal model for the application of site-directed mutagenesis, since its crystallographic structure is already defined[4]. However, the activity and stability of LipC12 in organic solvents and at relatively high temperatures need to be improved. Molecular dynamics studies suggested that the thermostability of LipC12 could be enhanced if two cysteine residues were introduced into its structure, facilitating the formation of a disulfide bridge at position Y244-G185[4]. Thus, the variant LipC12G185C-Y244C was produced by site-directed mutagenesis[5]. The present study aims to characterize the hydrolytic activity and thermostability of this variant compared to wild type LipC12.

2 MATERIAL & METHODS

Escherichia coli BL21(λDE3) cells transformed with the plasmid pET-28a(+) containing the gene for either LipC12WT or LipC12G185C-Y244C were cultured, and expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). Expression occurred at 120 rpm and 20 °C for 16 h[6]. The proteins were purified by affinity chromatography using a nickel column. Protein was quantified using the Bradford method[7].

The hydrolytic activities of LipC12G185C-Y244C and LipC12WT were determined using *p*-nitrophenyl esters (*p*NP) as substrates. For the assay, 810 µL of distilled water, 50 µL of solution A (1 mol L^{-1} Tris-HCl pH 7.5, 20 mmol L^{-1} $CaCl_2.2H_2O$, 6% (v/v) Triton X-100), and 50 µL of solution B (20 mmol L^{-1} *p*NP in isopropanol:acetonitrile (4:1, v/v)) were added to a 1 mL cuvette. After removing the turbidity at 60 °C, the solutions were equilibrated to 25 °C for activity measurement. The reaction was initiated by adding 40 µL of the enzyme solution (7 µg of protein), and release of *p*-nitrophenol was monitored at 410 nm using a UV-1800 spectrophotometer for 2 min[8]. One unit of hydrolytic activity (U) was defined as the release of 1 µmol of *p*-nitrophenol per min. All experiments were performed in triplicate, with the error bars representing the standard deviation of the means.

The hydrolytic activity against triglycerides was determined by the titrimetric method[9]. The substrates used were tributyrin, tricaprylin, and olive oil (67 mmol L^{-1}). A substrate emulsion was prepared with Tris-HCl (2.5 mmol L^{-1}, pH 7.0), $CaCl_2$ (2 mmol L^{-1}), NaCl (150 mmol L^{-1}), and gum arabic (3%, w/v)[10]. For each assay, 20 mL of the homogenized emulsion was added to a thermostated vessel at 37 °C. The reaction was initiated by adding 20 µL of the enzyme solution (5 µg of protein), under agitation in an automatic pH-Stat titrator, maintaining the pH at 7.0 at 37 °C, using NaOH (0.05 mol L^{-1}), and was monitored for 5 min. One unit (U) of triglyceride-hydrolyzing activity was defined as the release of 1 µmol of fatty acid per min. All experiments were performed in triplicate, with the error bars representing the standard deviation of the means.

To determine thermostability, 12.5 µL (5 µg of protein) of LipC12WT and LipC12G185C-Y244C were incubated in 112.5 µL of 50 mmol L^{-1} Tris-HCl buffer pH 7.5, 150 mmol L^{-1} NaCl, and 2 mmol L^{-1} $CaCl_2$. The incubation was done in a thermostated bath at

65 °C for 24 h, using 1.5 mL Eppendorf tubes with 125 µL of the enzyme solution. After incubation, the tubes were cooled on ice, and the residual activity was measured by the hydrolysis of tributyrin in an aqueous medium. The residual activities were calculated relative to controls that were treated identically but without incubation. All experiments were performed in triplicate, with the error bars representing the standard deviation of the means.

3 RESULTS & DISCUSSION

LipC12G185C-Y244C had a significantly higher ($p < 0.05$) activity than LipC12WT for all *p*-nitrophenyl esters (Fig. 1A). For both lipases, using *p*-nitrophenyl esters, the activity increased with the acyl chain length from C4:0 to C16:0, but decreased at C18:0, with maximum activity (115±3 U mg^{-1} for LipC12WT and 128±1 U mg^{-1} for LipC12G185C-Y244C) against *p*-nitrophenyl palmitate (C16:0).

Regarding triglyceride-hydrolyzing activities, both LipC12WT and LipC12G185C-Y244C gave their highest activities against tributyrin (4372±145 U mg^{-1} and 5428±174 U mg^{-1}, respectively) and lower activities against tricaprylin and olive oil (Fig. 1B). LipC12G185C-Y244C had significantly higher activities ($p < 0.05$) than LipC12WT for all three substrates (24% higher for tributyrin, 14% higher for tricaprylin, and 11% higher for olive oil).

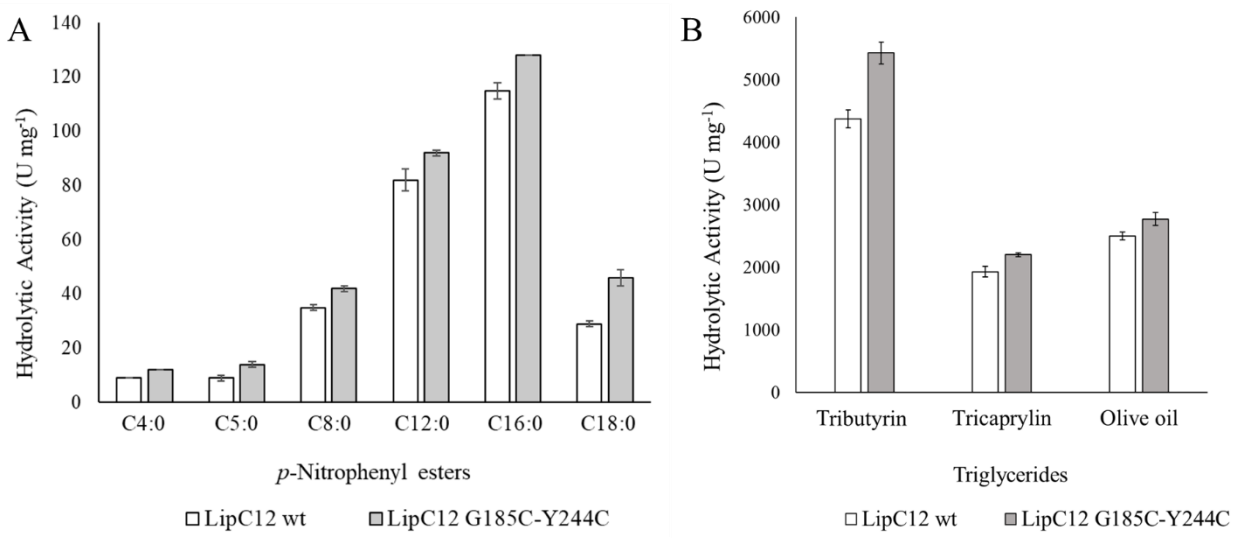

Figure 1 Hydrolytic activity of LipC12WT and LipC12G185C-Y244C against (A) *p*-nitrophenyl esters and (B) triglycerides.

Regarding thermostability, after incubation at 65 °C for 24 h, the residual activities were 54% for LipC12G185C-Y244C and 50% for LipC12WT (Fig. 2), demonstrating that the variant is not more stable than LipC12WT.

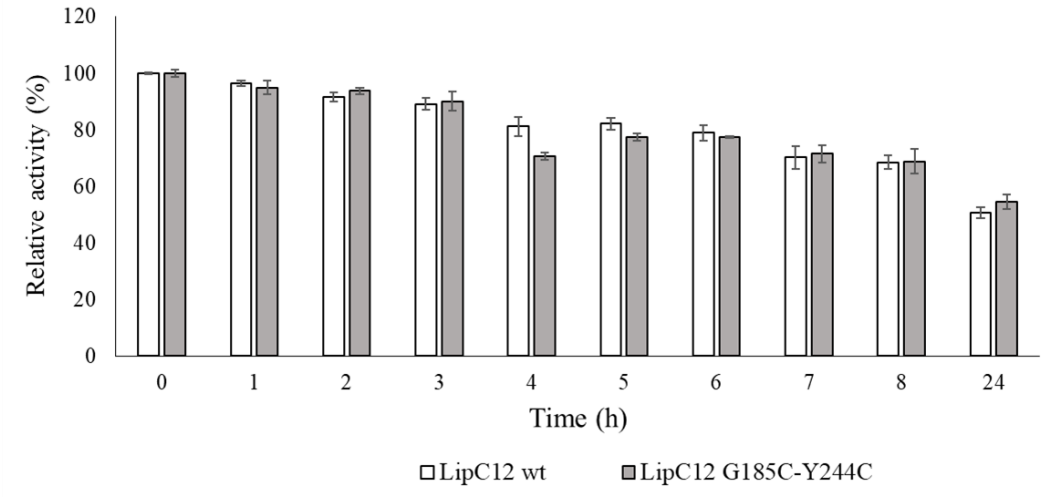

Figure 2 Stability of LipC12WT and LipC12G185C-Y244C at 65 ºC.

4 CONCLUSION

LipC12G185C-Y244C exhibited significantly higher hydrolytic activity than LipC12WT for most of the tested *p*NP esters. Hydrolytic activity increased with the acyl chain length of the substrates, reaching the highest activity against p-nitrophenyl palmitate (C16:0). LipC12G185C-Y244C also had a higher hydrolytic activity against tributyrin, triolein, and olive oil. However, LipC12G185C-Y244C was not more thermostable than LipC12WT. The higher hydrolytic activity makes LipC12G185C-Y244C a promising variant for biotechnological applications.

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