IMPROVING THE CATALYTIC PROPERTIES OF THE LIPASE LIPC12 THROUGH BIOIMPRINTING

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ABSTRACT

The lipase LipC12 was obtained from a metagenomic library constructed from soil contaminated with animal fat. To improve its performance, we explored bioimprinting and immobilization strategies. With free LipC12, the best increase of activity was obtained through bioimprinting with a mixture of CTAB and *t*-butanol, giving an activity 1.5-fold higher than that of a non-bioimprinted control. In the immobilization studies, LipC12 was bioimprinted and subsequently immobilized on the hydrophobic support Accurel MP-1000. However, this strategy gave a lower activity than that when non-bioimprinted LipC12 was immobilized on the same support.

Keywords: Bioimprinting. Lipases. Immobilization. Activation. Enhancement of enzymatic properties.

1 INTRODUCTION

Lipases, enzymes of industrial importance, often exhibit low activity and stability in water-restricted environments.[1,2] Their industrial application typically requires immobilization, which enables separation of the enzyme from the reaction medium and subsequent reuse and may also enhance activity, thermal stability and stability in organic solvents.[3,4] Bioimprinting with amphiphilic compounds, such as oleic acid, alcohols, or surfactants, is a further strategy that can enhance the catalytic efficiency of lipases. These so-called bioimprinting agents can induce the lipase to assume a more active conformation, often through the opening of the lid. This active conformation is preserved when the bioimprinting agent is removed by washing with an anhydrous solvent, allowing the lipase to retain a high activity in water-restricted environments.[5,6,7]

LipC12 is a lipase that was obtained from a metagenomic library constructed from soil contaminated with animal fat.[8] It has a high hydrolytic activity against long-chain triacylglycerols, comparable to that of commercial lipases, demonstrating its biotechnological potential. However, there is still room to improve its activity and stability in organic solvents. The aim of the current work is to explore the possibility of combining bioimprinting with immobilization, to generate immobilized derivatives of LipC12 with higher activity, stability, and selectivity for applications in biocatalysis.

2 MATERIAL & METHODS

Escherichia coli BL21(λDE3) cells transformed with the plasmid pET-28a(+) containing the LipC12 gene were cultured, and expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside, with further incubation at 120 rpm and 20 °C for 16 h. The proteins were purified by affinity chromatography using a nickel column.[8] Proteins were quantified by the BCA method.

The lipase preparations used were: (1) Free LipC12 (Fr-LipC12) and (2) LipC12 immobilized on Accurel MP-1000 (Ac-LipC12). Each bioimprinting solution (1 mL) used is indicated by an acronym (composition within parentheses): OA1 (29.4 nmol oleic acid and 941.18 µL of *t*-butanol); OA5 (147 nmol oleic acid and 705.9 µL of *t*-butanol); CTAB (1 mL aqueous CTAB at 50 mmol L^{-1}); CTAB-*t*-butanol (1 mL aqueous CTAB at 50 mmol L^{-1} and 1 mL of *t*-butanol); CTAB-OA1 (1 mL aqueous CTAB at 50 mmol L^{-1} solution, 29.4 nmol oleic acid and 941.18 µL of *t*-butanol); CTAB-OA5 (1 mL aqueous CTAB at 50 mmol L^{-1}, 147 nmol oleic acid and 705.9 µL of *t*-butanol); methanol (1 mL); ethanol (1 mL); *t*-butanol (1 mL); *n*-heptane (1 mL); toluene (1 mL).

Fr-LipC12 studies were done in sealed 25 mL Erlenmeyer flasks, with the addition of 4 mL of a solution containing 1 mg of purified LipC12 and 1 mL of the bioimprinting solution. The mixture was incubated on an orbital shaker at 150 rpm and 4 °C for 15 min. The olive-oil-hydrolyzing activity in aqueous medium was used to determine the activity by titration using an automatic titrator pHStat (Metrohm 718 Stat Titrino). The substrate emulsion consisted of 67 mmol L^{-1} olive oil, 3% (w v^{-1}) gum arabic, 2 mmol L^{-1} $CaCl_2$, 2.5 mmol L^{-1} Tris-HCl buffer, pH 7.5, and 150 mmol L^{-1} NaCl, dispersed in distilled water. The enzyme was added to 20 mL of the emulsion under magnetic stirring (300 rpm) at 37 °C and the reaction was followed for 5 min.[9] One unit of olive-oil-hydrolyzing activity in aqueous medium (U) corresponds to the release of 1 µmol of fatty acid per min, under the assay conditions. The results are presented as percentage hydrolytic activity relative to a control done with non-bioimprinted LipC12 (denoted "R_H").

For the Ac-LipC12 studies, the enzyme was previously bioimprinted in the manner described above for Fr-LipC12. Following the incubation, without removing the bioimprinting agent, 0.1 g of Accurel MP-1000 was added. The flasks were then incubated for 6 h at 4 ºC, following the immobilization procedure of Madalozzo.[10] The immobilized derivatives were washed with 5 mL of 50 mmol L^{-1} Tris-HCl buffer, pH 7.5, filtered through Whatman filter paper no. 15 under vacuum, and dried in a vacuum desiccator (-500

mmHg) at 4 °C for 24 h. They were then washed with 10 mL of *t*-butanol, dried again in a vacuum desiccator for 24 h, and stored at -20 °C until use. The triolein-hydrolyzing activities in organic medium of the immobilized preparations were evaluated. For this, a 125 mL Erlenmeyer flask was prepared with 70 mmol triolein, 4.6 mL *n*-hexane, 0.1 mL distilled water and 20 mg immobilized lipase and incubated on an orbital shaker at 200 rpm and 40 °C. The oleic acid concentration was determined by the method of Lowry and Tinsley.[11] The initial velocity was determined. In this assay, one unit of activity (U) corresponds to the production of 1 µmol of oleic acid per minute, under the assay conditions. The results are presented as percentage hydrolytic activity relative to a control done with immobilized, non-bioimprinted LipC12 (again, denoted "R_H").

For the immobilization, activity retention (*AR*, %) was calculated as:

$$AR = \frac{A_O}{A_T} \times 100 \qquad (1)$$

where A_O is the triolein-hydrolyzing activities activity of the immobilized preparation measured in organic medium and A_T is the theoretical triolein-hydrolyzing activity of the immobilized enzyme. The theoretical olive-oil-hydrolyzing activity was calculated as:

$$A_T = \left(M_{ip} - M_{fp}\right) \times SA_o \qquad (2)$$

where M_{ip} is the mass of protein in the supernatant before immobilization, M_{fp} is the mass of protein remaining in the supernatant after immobilization and SA_O is the specific triolein-hydrolyzing activity of the free enzyme (U mg^{-1} of protein), measured in organic medium. Values of *AR* above 100% are possible if the lipase is activated upon immobilization.

During immobilization, the disappearance of olive-oil-hydrolyzing activity from the supernatant of the aqueous lipase solution was monitored. The immobilization efficiency (*IE*, %) was calculated as:

$$IE = \frac{(A_i - A_f)}{A_i} \times 100 \qquad (3)$$

where A_i is the olive-oil-hydrolyzing activity (U), in aqueous media, of the supernatant before immobilization and A_f is the olive-oil-hydrolyzing activity (U) remaining in the supernatant after immobilization.

3 RESULTS & DISCUSSION

For Fr-LipC12 assays, the highest relative activity occurred with the CTAB-*t*-butanol combination (R_H = 148%), followed by the CTAB-AO1 and CTAB-AO5 mixtures (both with R_H = 131%)(Figure 1A). Treatment with *t*-butanol alone gave only a slight improvement in activity, with R_H = 110%. This enhancement of the activity of Fr-LipC12 might be due to the opening of the lid of LipC12, facilitating access of the substrate to the active site.[12,13] In contrast, the activity was decreased relative to the control for the treatments CTAB (R_H = 32%) and AO1 (R_H = 85%).

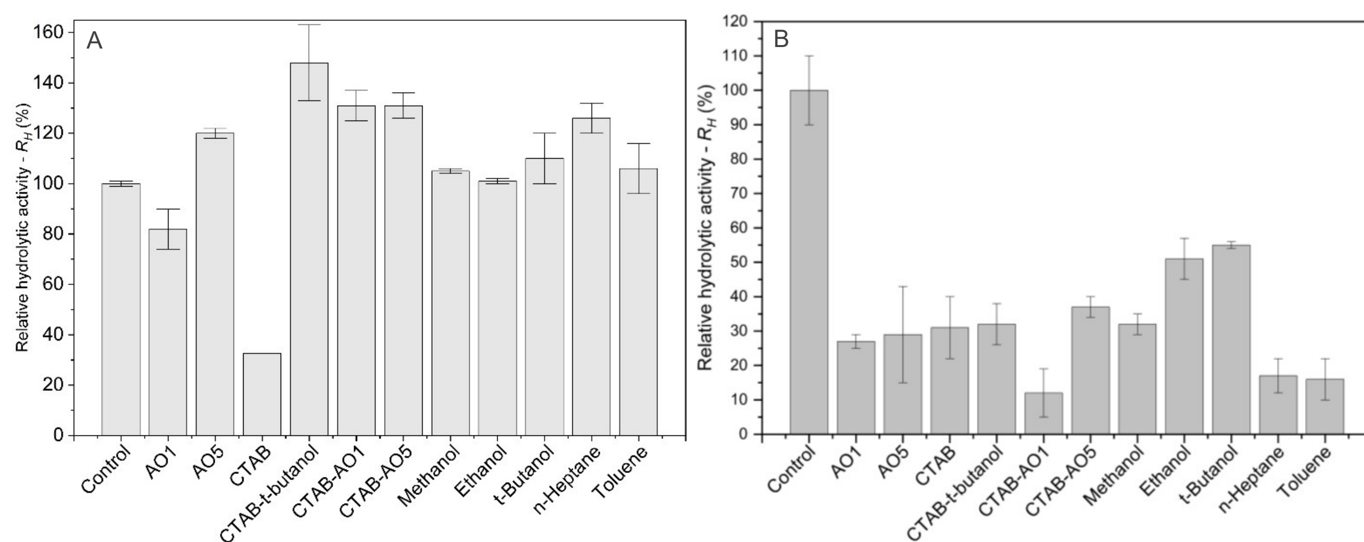

Figure 1 Effect of bioimprinting on the relative hydrolytic activity (R_H) of (A) Free-LipC12 and (B) LipC12 immobilized on Accurel MP-1000. The assays were done in duplicate and the values variations are the standard error of the mean.

For Ac-LipC12, the immobilization efficiency (*IE*) was relatively high for most treatments (Table 1), but low values of *IE* were obtained with *n*-heptane (*log P* = 4.66) and toluene (*log P* = 2.5). These hydrophobic substances may interfere with the hydrophobic interactions between LipC12 and the Accurel support.

Table 1 Activity retention (*AR*) and Immobilization efficiency (*IE*) for LipC12 immobilized on Accurel MP-1000.

Condition	Immobilization efficiency (*IE*, %)	Activity retention (*AR*, %)	Condition	Immobilization efficiency (*IE*, %)	Activity retention (*AR*, %)
Control	55	137	CTAB-AO5	62	39
AO1	62	33	Methanol	63	62
AO5	64	35	Ethanol	64	65
CTAB	59	41	*t*-Butanol	29	77
CTAB-*t*-butanol	51	46	*n*-Heptane	5	242
CTAB-AO1	68	41	Toluene	21	56

All treatments resulted in a decrease in activity and, consequently, *AR* values lower than 100%, with the exception of the treatment with *n*-heptane, which gave the highest activity retention (242%), but the *IE* was only 5%, indicating that LipC12 was activated by incubation in this solvent.

The results show that bioimprinting agents that activated Fr-LipC12, such as oleic acid and CTAB dissolved in *t*-butanol, did not lead to an increase in Ac-LipC12 activity, suggesting interference of these agents in the immobilization process. This interference is particularly pronounced for solvents with high *log P* values, such as *n*-heptane (*log P* = 4.66) and toluene (*log P* = 2.5).

4 CONCLUSION

Bioimprinting with CTAB-*t*-butanol, CTAB-AO1 and CTAB-AO5 activated free LipC12 significantly. In contrast, pretreating LipC12 with the same bioimprinting agents and then immobilizing the bioimprinted LipC12 on Accurel MP-1000 did not produce a more active immobilized preparation. These results suggest that bioimprinting agents interfere in the immobilization process, decreasing relative activities and the efficiency of enzyme immobilization. Future studies should explore different immobilization techniques in tandem with bioimprinting, such as covalent immobilization, to optimize LipC12 performance.

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