

Biochemical Study of the Effect of Free Fatty Acids in the Interface on a Novel Lipase from *Bacillus pumilus* Strain

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Abstract: The main objective of this work was to identify novel lipases of industrial interest; under alkaline conditions and at high temperatures. The sequenced BPL having a molecular mass of about 27 kDa and its activity was maximal at pH 8.0 and 45 °C (Accepted article). In this paper, BPL kept their stability even in the presence of high concentrations of detergent that vary from 0 to 10 mM NaDC. Bile salts showed no inhibitory effect on the lipolytic activity, whereas the calcium salts showed a stimulating action of the lipase activity. The accumulation of free fatty acids with long chains at the oil / water interface does not affect the activity of the enzyme which still effectively hydrolyzes the emulsified olive oil even in the absence of bile salts. This study allowed us to prove that BPL presents the interfacial activation phenomenon. A 3D structure model of BPL was built using PDB structures as template. We have concluded that the ratio hydrophobic surface/hydrophilic surface is (55% vs 45%), could be responsible for a higher tolerance to the presence of long-chain free fatty acids at the lipid/water interface.

Key words: *Bacillus pumilus* lipase • Detergent • Interfacial activation • Free fatty acids • 3D structure

INTRODUCTION

The development of new biotechnologies, bio-industries and biorefineries is a major challenge for the future. However such developments require the focus of biocatalysts (whether microorganisms or enzymes) increasingly efficient but also suited for implementation in industrial conditions [1]. The fatty substances or lipids are very energetic and insoluble compounds consisting primarily of triglycerides (TG). The lipids are a major part of the biomass and lipolytic enzymes provide the metabolic turnover of these lipids [2]. Lipases are ubiquitous enzymes that are found both in the inferior organisms such as bacteria, fungi or yeasts that in higher organisms such as plants and animals. They are able to catalyze the hydrolysis of glyceride esters in an aqueous medium and the synthesis of esters in non-aqueous

medium [3, 4]. Lipolytic enzymes are a class of enzymes perfectly soluble in water. They are responsible for the hydrolysis of lipids. They act on insoluble lipid substrates in water but which are organized spontaneously on contact with water to form emulsions, micelles, liposomes or mono molecular film [2]. The esterase acts on dissolved esters and is unable to attack the emulsified esters [5]. The study of these enzymes has contributed to the development of interfacial enzymology, catalysis occurs in a heterogeneous medium with oil-water interface. It follows that the biochemical properties of these enzymes depend much on the quality of the interface that certain conventional parameters such as pH or ionic strength. [2]. Lipases and esterases are important biocatalysts and are particularly suitable for industrial applications, as they are very stable and active in organic solvents [6].

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MATERIALS AND METHODS

Lipase Activity Determination: The lipase activity was measured titrimetrically at pH 8.5 and 40 °C with a pH-stat under standard conditions using tributyrin (0.25 ml) in 30 ml of 2.5 mM Tris-HCl pH 8.2, 2 mM CaCl₂, 2 mM NaDC or olive oil emulsion (10 ml in 20 ml of 2.5 mM Tris-HCl pH 8.2, 2 mM CaCl₂, 4 mM NaDC) [7] as substrate. Assays were carried out in 30 ml of 2.5 mM Tris-HCl buffer pH 7.0 containing 0.1 M NaCl. Standard conditions for measuring enzyme activity at increasing esters concentrations have been described previously [8]. When measuring BPL activity in the absence of CaCl₂, we added EDTA or EGTA to the lipolytic system. Lipolytic activity was expressed as units. One unit corresponds to 1 μ mol of fatty acid released per minute.

Determination of Protein Concentration: Protein concentration was determined as described by Bradford [9] using BSA ($E_{1\text{cm}}^{1\%} = 6.7$) as reference.

Effect of Free Fatty Acids: The lipase activity was measured according to various substrate (TC₄, TC₈, TC₁₈, olive oil) assigns concentrations ranging from 0-40 mM. The Michaelis-Menten (KMapp) and the maximum velocity (Vmax) for the reaction were calculated by Lineweaver-Burk.

Effect of Detergents: The lipase activity was measured using tributyrin and olive oil as a substrate in the presence of increasing concentrations of NaDC ranging from 0 to 10 mM, under optimum conditions of pH and temperature.

Effect of Calcium: The lipase activity was measured using tributyrin and olive oil as a substrate in the presence of increasing concentrations of calcium from 0 to 6 mM and under optimum conditions of pH and temperature. In the absence of calcium, the lipase activity is measured in the presence of 10 mM EDTA or EGTA.

3D Structure Prediction: We searched the grounds signing lipolytic enzymes especially around the catalytic serine and through the tool ScanProsite available online (<http://expasy.org/tools/scanprosite/>) by bringing the raw sequence or code identification (PDB code or UniProtKB (Swiss-Prot and TrEMBL code)...). The BPL structure was modelled using the 3D coordinates of the closed form of

the Bacillus lipases (BL) (PDB code 1I6W). The method of minimization by (Gromos96) (located in the server Swiss-PDB-Viewer) was used with the version of force field GROMOS43B1. This method allows the evaluation of minimizing energy and the geometry qualities of the final models were checked using PROCHECK program.

RESULTS AND DISCUSSION

Kinetic Studies of BPL: BPL is able to hydrolyse the TC₄ or the olive oil emulsion alone (Figure 1). The kinetic of substrate hydrolysis remains linear for more than 20 min. Accordingly; BPL probably presents a tridimensionnel structure allowing it to hydrolyse its substrate efficiently and without any denaturation at interfacial. Also to tolerate the presence of long-chain free fatty acids, at the olive oil/water interface without any addition of amphipathic reagent (NaDC, Triton X-100...). Another difference between Bacillus lipase and others microbial lipases, which have a strongly preference for long-chain substrates [10-12].

Effects of Calcium on BPL Activity: Metal cations, particularly Ca²⁺, play important roles in influencing the structure and function of enzyme and the calcium-stimulated the lipases have been reported [13]. Previously, it has been demonstrated that the activity of Aspergillus lipases may depend on the presence of Ca²⁺ ions like the staphylococcal lipases [14]. The effect of various Ca²⁺ concentrations on the rate of hydrolysis of BPL was studied. Our results showed that BPL activity can be detected in the absence of Ca²⁺. A specific activity of 1600 U/mg was measured in the presence of 10 mM of chelator such as EDTA or EGTA, when using olive oil emulsion as substrate. In the absence of chelators, the specific activity of BPL reached 2100 U/mg at 3 mM CaCl₂ (Figure 2). The enzymatic activity of BPL is stimulated by Ca²⁺. It has been reported that the lipases from *P. Glumae* and *S. hyicus* [14, 15] contain a Ca²⁺ binding site which is formed by two conserved aspartic acid residues near the active-site and that binding of the Ca²⁺ ion to this site dramatically enhanced the activities of these enzymes.

Effect of Detergents: From the results as shown in the figure 3, we notice that the NaDC has no inhibitory effect on activity of lipolytic enzymes even at a high concentration (10 mM). This result confirms that, the pure BPL is able to reach its substrate even in the presence of

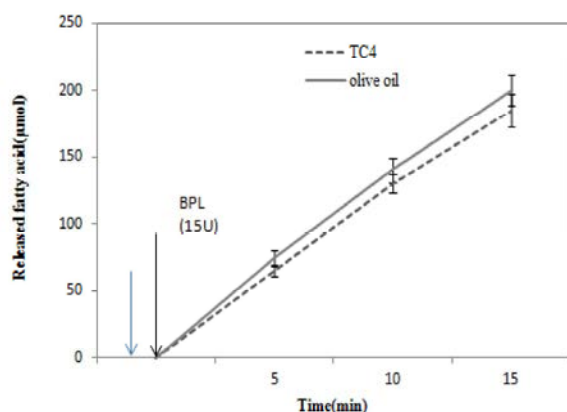


Fig. 1: Kinetic of hydrolysis of olive oil or tributyrin emulsions by BPL (15 U). Lipolytic activity was followed at pH 8.5 and 40 °C.

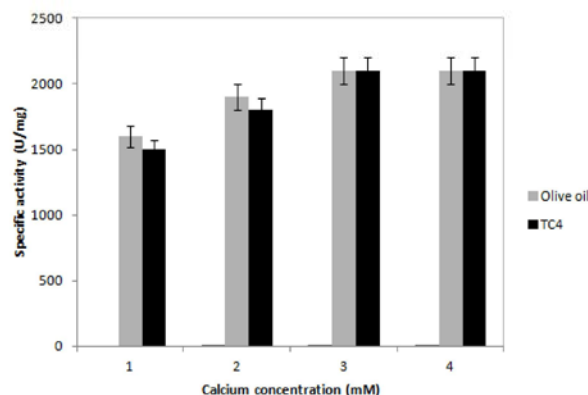


Fig. 2: Effect of increasing concentrations of calcium on the rate of hydrolysis of tributyrin and olive oil emulsion by BPL.

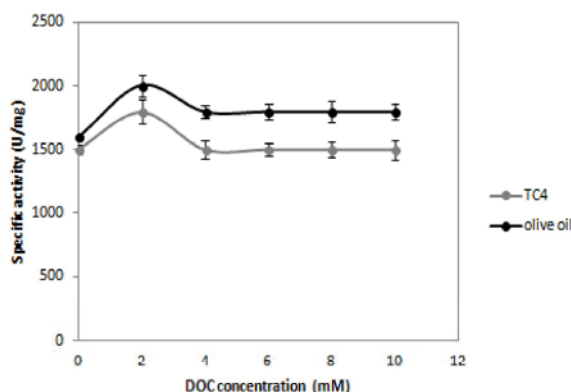


Fig. 3: Effect of increasing concentration of NaDC on the rate of hydrolysis of tributyrin and olive oil emulsion by BPL. Lipolytic activity was measured under standard conditions at pH 8.5 and 40 °C using a pH-stat.

Table 1: Alignment of the N-terminal sequence of *B. pumilus*

<i>Bacillus pumilus</i>		
Lipase	total score	% identity
<i>Bacillus subtilis</i> TU-B-10	35.8%	100%
<i>Bacillus subtilis</i> RO-NN-1	35.8%	100%
<i>Bacillus subtilis</i> Bsn5	35.8%	100%

certain active agents' surface such as bile salts. Similar results were obtained by Sayari [12]. Similarly, Simons *et al.* [16] showed that SSL was not inhibited by anionic detergents such as NaDC. Therefore, it can be inferred that probably BPL has a higher penetrating power than those of other microbial lipases that enables it to hydrolyse the olive oil or TC₄ in presence of bile salts.

3D Structure Model of BPL: The research of homologous with the BPL sequence was made in the database using the BLASTp program and the results are shown in Table 1.

Sequence analysis allowed us to reveal a 100% of homology with *Bacillus subtilis* TU-B-10, *Bacillus subtilis* RO-NN-1 and *Bacillus subtilis* BSn5. A sequence alignment of the N-terminal of BPL was completed. From Figure 4, we see that the catalytic triad of BPL is constituted of Ser132, Asp260 and His298. The three amino acids are located on the C-terminal side of β core lamina (Figure 4). Indeed, the catalytic triad (Ser-His-Asp) is a characteristic structure of a well known serine proteases [17]. These amino acids of the catalytic triad of lipases are generally determined by chemical modification studies [18] or by site directed mutagenesis [19]. Mhetras, *et al.*, (2009) showed the cause of catalysis of the purified lipase. This later composed of, residues Ser and carboxylates [20]. Supachok, *et al.*, (2001) also reported that the model of the *Bacillus stearothermophilus* lipase P1 constructed by the use of the basis of secondary structure predictions, shows an organization in fold and this enzyme α / β -hydrolase is identified by the catalytic triad of Ser-113, Asp-317 and His-358, in close proximity to each other at distances of hydrogen bonding and the serine residue which plays a key role in the catalytic mechanism [21].

In order to create a sample of the closed form of the *B. pumilus* lipase, we used the automatic modeling by the Swiss-Model server (<http://www.expasy.org/spdvb>). The model was then subjected to several cycles of energy minimization using Gromos96. The superposition of the two closed structures gives an average standard deviation (rmsd) equal to 1.66 Å. In addition, the stereochemical quality of the closed model of BPL and

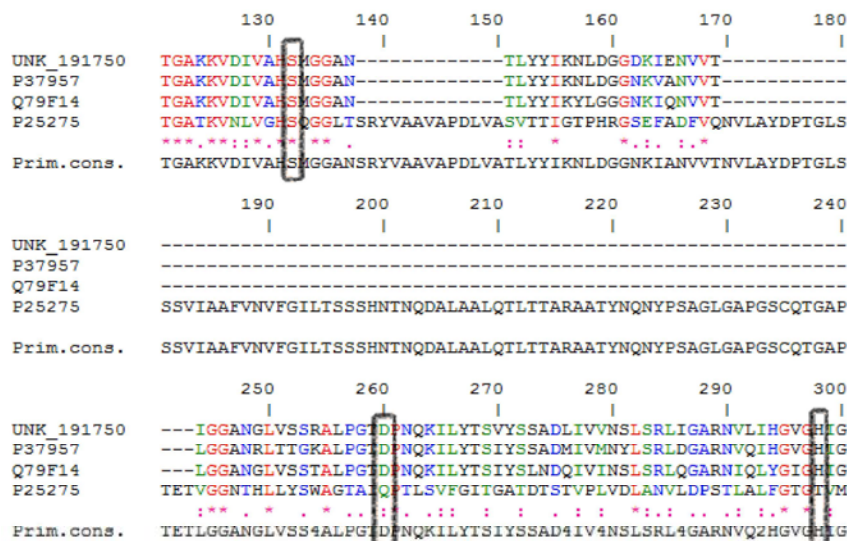


Fig. 4: The alignment of the three sequences named respectively, chain (P37957), chain (Q79F14) and chain (P25275) with the additional sequence (prim. cons) and the sequence of BPL (UNK_191750).

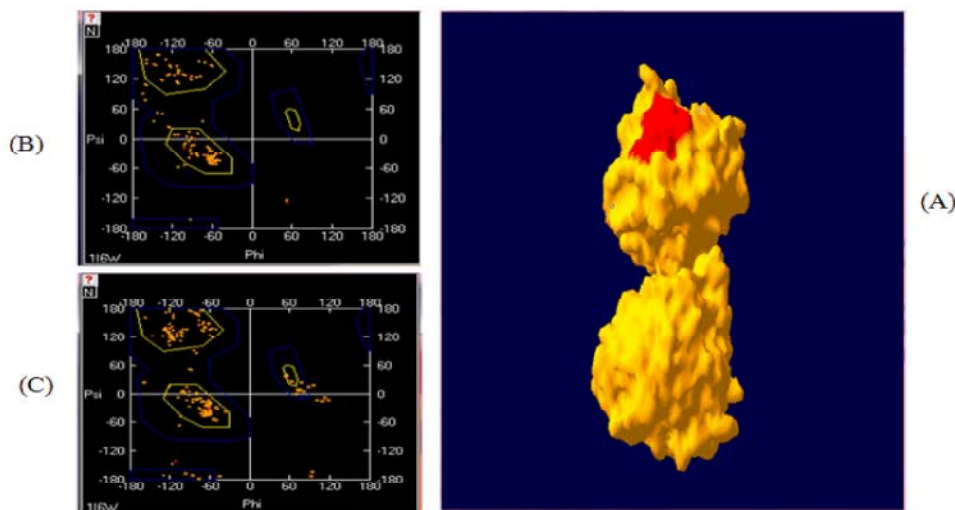


Fig. 5: Modeling the closed model of BPL and surface structure. The catalytic triad is colored red (A). Hydrophobic amino acids, belonging to the polar N-terminal domain are located in the Ramachandran plot (B). Hydrophobic residues of BLP involved in increasing the accessible surface interacting with an interface is indicated (C).

statistical analysis of the distribution of amino acids in the Ramachandran plot were also tested by the program PROCHECK [22]. According to the Ramachandran plot (Figure 5), we found that 95% and 93% of non-polar and polar amino acids respectively, are located in suitable areas (Figure 5). Indeed, Rengachari, *et al.*, (2012), have reported the crystal structures of *Bacillus sp.* H257 lipase in its free form at 1.2 Å which is found complexed with phenylmethylsulfonyl fluoride at 1.8 Å [23]. Furthermore, the study of the total surface accessible (N-terminal domains) of the closed form of BPL, showed that the ratio

hydrophobic surface/hydrophilic surface is (55% vs 45%), this could explain the fact that BPL tolerates accumulation of long chain fatty acids in the lipid/water interface and have a linear kinetics over 15 min, during the hydrolysis of an emulsion of oil olive.

Rengachari, *et al.*, (2012), confirmed that the two structures of *Bacillus sp.* H257 lipase adopts a hydrolase α / β in an open conformation. Indeed, the catalytic residues are buried at the bottom of a long chain ~ 22 Å, from the surface to the active site and therefore this maintains the active center at a distance from a polar

environment. Similar results have also been reported by Liu [24]. They have determined the structure of the open conformation for *Aspergillus Niger* lipase and the prediction of secondary structure clearly showed that they shared a common arrangement of secondary structure elements along the amino acid sequence.

CONCLUSION

The interest of microbial lipases for biotechnological applications has taken a meteoric rise in recent years. Therefore, the industry requires new enzymes, which meet the criteria for use, particularly in terms of thermostability. The *B. pumilus* lipase kept their stability even in the presence of high concentrations of detergent (NaDC). Calcium salts showed a stimulating action of the lipase activity. Molecular modeling of the 3D structure was also carried out and the results have allowed us to demonstrate that BPL tolerates the accumulation of long chain fatty acids in the lipid / water interface. These results show that BPL has biochemical properties attractive for various industrial applications.

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