Comparative study of kinetic and interfacial properties of a novel *Rhizopus oryzae* lipase and ROL29

Riadh BEN SALAH[1]
Habib MOSBAH[1]
Youssef GARGOURI[1]
Hafedh MEJDOUB[1,2]

[1] *Laboratoire de biochimie, ENIS, Route de Soukra, "BPW", 3038 Sfax -Tunisia <riadh_fss@yahoo.fr>*
[2] *Unité de service commun pour la recherche scientifique, FSS, Route de Soukra, Km 3.5; BP 802, 3038 Sfax-Tunisia*

Abstract: *We compared several kinetic and interfacial properties of a lipase from a novel strain of Rhizopus oryzae (ROL_w) with ROL29 lipase. In contrast to ROL29, ROL_w was able to hydrolyze triolein emulsion in the absence of any additive, like bovine serum albumin (BSA). Furthermore, unlike Rhizopus oryzae lipase (ROL29), kinetic study of ROL_w lipase shows linear dependency when using tributyrin emulsion as substrate. ROL_w can tolerate, more efficiently than ROL29, the accumulation of long-chain free fatty acids at the interface when olive oil emulsion was used as substrate. The critical surface pressure π_c of penetration into phosphatidyl choline from egg yolk films was found to be 23 mN/m with ROL_w, in contrast to a value of 10 mN/m obtained with ROL29. The effect of calcium ion and synthetic detergent on the two lipases was studied. In contrast to ROL29, ROL_w was activated in the presence of 100 μmoles TX-100. No significant difference on the two lipase activity was observed in presence or absence of calcium ion.*

Key words: *Rhizopus oryzae lipase (ROL_w), ROL29, Interfacial denaturation, Critical pressure, Detergent, Calcium ion*

Introduction

The isolation of lipases (glycerol ester hydrolases EC 3.1.1.3) from various microorganisms revealed that all these enzymes degrade the ester bonds present in substrate selectivity. Lipases are found in all living species of the animal kingdom, as well as plants and microorganisms such as yeast, bacteria, and fungi [1]. The physicochemical properties of the interface play an important role in lipolysis [2]. Lipases are ubiquitous enzymes of considerable physiological significance and industrial potential and catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an oil-water interface [3-5] and do not hydrolyse dissolved substrates in the bulk fluid. A true lipase will split emulsified esters of glycerin long-chain fatty acids such as triolein and tripalmitin. Lipases are serine hydrolases and display little activity in aqueous solutions containing soluble substrates. In contrast, esterases show Michaelis-Menten kinetics in solution.

In previous works, the lipase of *Rhizopus oryzae* (ROL29) was produced, purified to homogeneity from the culture medium and some kinetic properties were determined using classical emulsified system [6].

Rhizopus oryzae lipase (ROL_w) was recently purified, and several properties were established using the classical emulsified system [7]. The biochemical properties of lipase isolated from *Rhizopus oryzae* (ROL_w) are different to those of ROL29. The main difference observed was the kinetic of this enzyme toward the substrates and interface.

With emulsified substrates, it is not possible to control the interfacial quality and to explain the kinetic behaviour at interfaces. Then, we use the monomolecular film using Egg-PC, where some interfacial parameters like critical pressure can be controlled and measured.

In this work we compared some kinetics and interfacial properties of ROL_w and ROL29. Two techniques were used: pH-stat with emulsified substrates and monomolecular film using Egg-PC.

Materials and methods

Chemicals

Tributyrin 99% was from Fluka (Buchs, Switzerland); trioctanoin (99%, GC) was from Jansen (Pantin, France); bovine serum albumin (BSA) and arabic gum were from Mayaud Baker LTD (Dagenham, United Kingdom); phosphatidylcholine was from Sigma-Aldrich (Stenheim, Germany); pH-stat was from Metrohm (Buchs, Switzerland).

Culture conditions medium

Rhizopus oryzae were grown during 72 h at 30 °C in 250 mL shaking flasks at 80 rpm with medium A (40 g/L propanoic peptone, 10 g/L glucose, 0.1 g/L $CaCl_2$, 1.5 g/L sodium citrate, 2 g/L K_2HPO_4 (Difco; Stenheim, Germany), pH 6) [7].

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Lipase

Rhizopus oryzae lipase (ROL_w) and *Rhizopus oryzae* lipase (ROL29) were respectively purified to homogeneity in our laboratory as previously described by Ben Salah *et al.*, (2006) [7] and Sayari *et al.* (2005) [6].

Protein concentration

Protein concentrations were determined as described by Bradford [8] using BSA as standard.

Lipase activity determination

The lipase activity was measured titrimetrically at pH 8 and 37 °C with a pH-stat under standard conditions using tributyrin or trioctanoin (0.25 mL) in 30 mL of 2.5 mm Tris-HCl pH 8, or olive oil emulsion (10 mL in 20 mL of 2.5 mm Tris-HCl pH 8) as substrate [9]. When measuring lipase activity in the absence of $CaCl_2$, we added 10 mm EDTA to the lipolytic system. Lipolytic activity was expressed as units. One unit corresponds to 1 μmole of fatty acid released per minute.

Effect of temperature and pH on lipase activity and stability

The optimal temperature for the hydrolysis of olive oil emulsion by lipase was determined by measuring the rate of the reaction at temperatures ranging from 20 °C to 50 °C under standard assay conditions, as described above.

For studying thermal stability, the enzyme was incubated at temperature ranging from 20 °C to 50 °C for 30 min and residual activity was determined.

The optimal pH enzyme activity was determined by measuring olive oil emulsion hydrolysis at pH from 5 to 10 in various buffers.

The effect of pH on lipase stability was studied by measuring residual activity at pH 8 after 1 h incubation at pH ranging from 5 to 9.

Analytical methods

Polyacrylamide gel electrophoresis of proteins in the presence of 0.3 m sodium dodecyl sulfate and 0.25 M β-mercaptoethanol or 0.5 M dithiothreithol (SDS/PAGE) was performed as described by [10].

Measurement of lipase penetration into the Egg-PC monolayer

The surface pressure increase due to the penetration of lipase into the egg-PC water interface was measured in a cylindrical trough drilled in a Teflon block (surface area 7 cm^2, total volume 5 mL of 10 mm Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA).

The aqueous subphase was continuously stirred at 250 rpm with a magnetic rod. Measurements of penetration were estimated as described previously [11].

Results and discussion

General enzymatic properties

ROL_w showed similar substrate specificity as ROL29. However, ROL_w and ROL29 most preferably hydrolyzed the olive oil emulsion substrate. ROL_w was found to be 3.9 times more active on long chain triacylglycerols as compared to short-chain triacylglycerols (TC_4). The specific activity of ROL_w reaches 3500 U/mg using olive oil emulsion as substrate at pH 8 and 37 °C and 900 U/mg were obtained when tributyrin was used as substrate under the same experimental conditions [7]. However, ROL29 is able to hydrolyze long chain triacylglycerols 10-folds than short-chain triacylglycerols (TC_4) [6].

Figure 1A shows that the activity of the ROL_w against olive oil emulsion increased from 5.5 to 8 and rapidly decreased at pH above 9. Similar behavior was obtained with ROL29 [6]. Both ROL_w and ROL29 were stable at neutral pH range for 24 h at room temperature *(figure 1*A).

Figure 1B indicates that the activity of ROL_w is strongly dependent upon temperature above 40 °C. It seems from thermostability studies that the enzyme is highly inactivated at 45 °C and that almost all activity is lost at 50°C after 30 min of incubation.

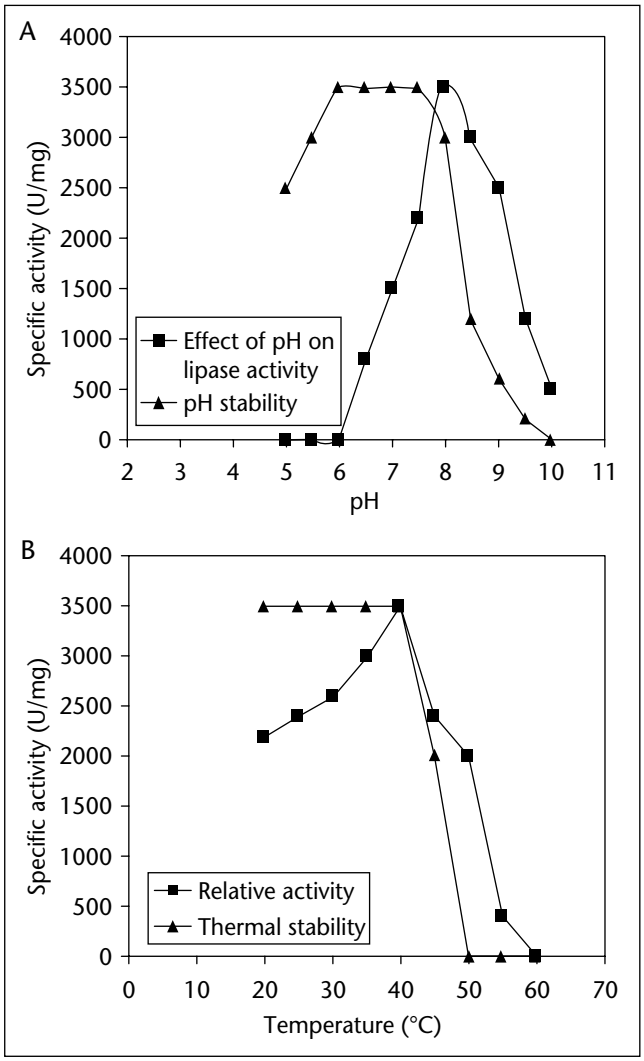

Figure 1. *A) Effect of pH on the activity of ROL_w and enzyme stability. Activity versus pH was determined against olive oil emulsion at 37 °C under standard conditions. The effect of pH on enzyme stability was studied by measuring specific activity after 24 h incubation at pH ranging from 4 to 10. B) Effect of temperature on the activity of ROL_w and enzyme stability. Activity versus temperature was determined at pH 8 using olive oil emulsion as substrate. Enzyme stability was studied by measuring activity after 30 min incubation at temperature ranging from 20 to 50 °C.*

Abbreviations: *ROL_w, Rhizopus oryzae* lipase (went and Prinsen Geerlig); *ROL29*, *Rhizopus oryzae* lipase (29 kDa); *ROL*, *Rhizopus oryzae* lipase; *RDL*, *Rhizopus delemar* lipase; *TC_4*, Tributyrin; *TC_8*, Trioctanoin; *EDTA*, Ethylene Diamine Tetraacetic Acid; *EGTA*, Ethylene Glycol-bis (β-aminoethyl ether) N,N,N′,N′-Tetraacetic Acid; *TX-100*, Triton-X100; *BSA*, Bovine Serum Albumin; *NaDC*, Deoxycholate of sodium; *Egg-PC*, Phosphatidyl Choline. π_c, Critical pressure;

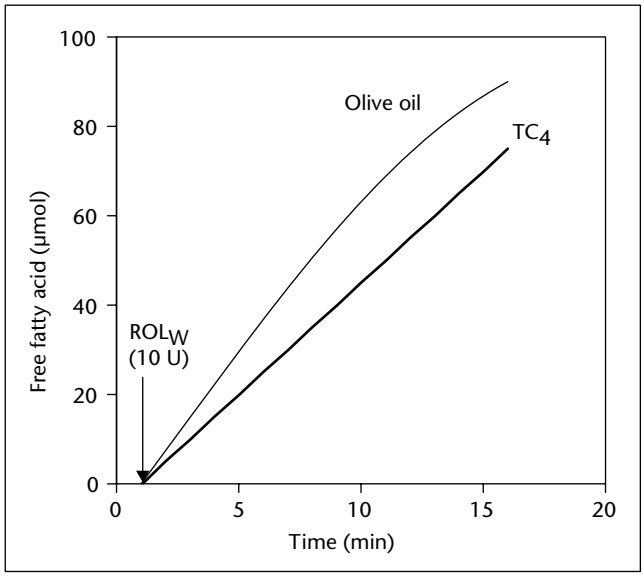

Figure 2. *Kinetic of hydrolysis of tributyrin and olive oil emulsion by ROL_w (10 U). Lipolytic activity was measured at pH 8 and 37°C.*

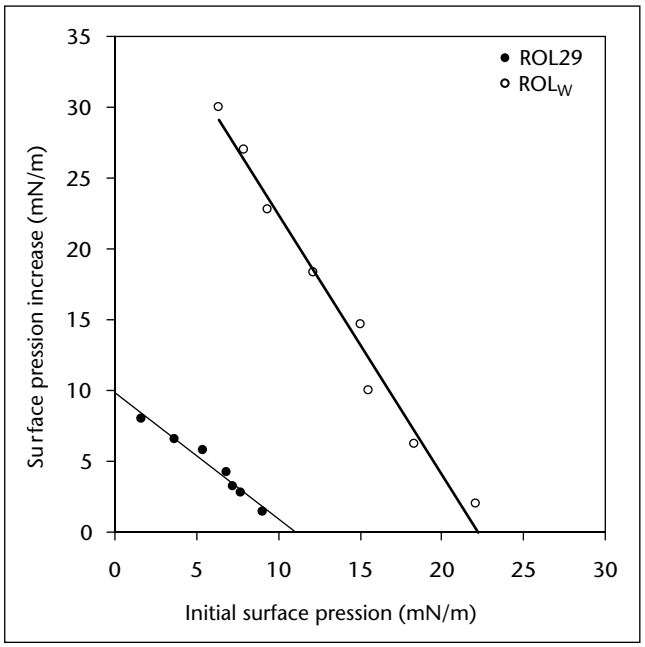

Figure 3. *Interaction of ROL_w with Egg-PC monolayers. Maximal increase in surface pressure obtained at equilibrium 40 min after injection of ROL_w under egg-PC monomolecular films spread at various initial surface pressures. Assays were carried out in a cylindrical Teflon 2 trough (volume, 5 ml; surface area, 7 cm^2); final enzyme concentration, 5 nM; and buffer: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 21 mM $CaCl_2$, 1 mM EDTA. Interaction of ROL29 with Egg-PC monolayer, reproduced from Sayari et al., (2005), is represented in dotted lines.*

Kinetic studies on tributyrin and olive oil emulsion by ROL_w

It has been established that some mammal pancreatic lipases may lack enzyme activity when TC_4 is used as substrate in the absence of bile salt and colipase. The high energy existing at the tributyrin/water interface is responsible for their irreversible denaturation [12, 13]. *Figure 2* shows that ROL_w is able to hydrolyze the TC_4 emulsion efficiently without interfacial denaturation. Kinetic remains linear more than 15 min *(figure 2)*. In contrast, under the same conditions, ROL29 fails to catalyze the hydrolysis of pure TC_4 (data not shown).

Figure 2 also shows that ROL_w, in contrast to ROL29 (data not shown), is able to hydrolyze the olive oil emulsion, in the absence of BSA. The kinetic remain linear more than 10 min *(figure 2)*. To trigger the ROL29 activity on olive oil emulsion, BSA should be added prior to the ROL29 injection (data not shown).

These results suggest that ROL_w, hydrolyze triacylglycerols efficiently at high interfacial energy (TC_4) without any denaturation and tolerate the presence of long-chain free fatty acids at the olive oil-water interface.

Interactions of ROL_w with Egg-PC monolayers

In order to check the above hypothesis, we measured the critical surface pressure π_c for ROL_w. We injected a lipase sample under a monomolecular film of egg-PC at an initial surface pressure π_i that ranged from 1 to 30 mN m^{-1}. The value of the maximal surface pressure increase $\Delta\pi_{max}$ reached equilibrium around 40 min after the injection of the lipase into the stirred aqueous subphase. It was determined and plotted as function of π_i. With ROL_w lipase, $\Delta\pi_{max}$, decreased linearly with increasing π_i *(figure 3)*. The critical surface pressure π_c for ROL_w lipase was estimated by linear extrapolation to zero surface pressure increase of the experimental points.

As can be seen from *figure 3*, π_c values of 23 mNm^{-1} was obtained with ROL_w. For further comparison, we reported the results obtained for ROL29 under the same experimental conditions [6]. The critical surface pressure of penetration π_c of ROL29 estimated by a linear extrapolation to zero surface pressure increase of the experimental points was found to be 10 mN/m. Our results, show that the π_c values is different to the one obtained with ROL29 (10 mN/m) [6] and 9.5 mN/m obtained with RDL [14]. If we use the π_c value to appreciate the capacity of a protein to penetrate into a monomolecular film, we can say that the penetration power of ROL_w is higher than ROL29 and RDL [15]. This result can be related to the kinetics of these three fungals lipases. ROL_w interacts more efficiently with substrates even at high interfacial energy and in spite of the presence of long-chain free fatty acids.

Chain length selectivity and kinetic parameters of ROL_w lipase

The enzymatic activity of lipases is very sensitive to the physical state of the substrate. In fact, the chain length selectivity constitutes an important difference between *Rhizopus oryzae* lipases. Both ROL_w and ROL29 have a strong preference for long chain substrates [6, 7], whereas ROL [16] have a strong preference for short chain substrates.

To determine the kinetic parameters of lipases, the rates of hydrolysis of different concentrations of TC_4 or TC_8, above the critical micellar concentration, were measured using ROL_w lipase. The Lineweaver-Burk curves were plotted (data not shown). From these fits, the substrate affinity constants (K_{Mapp}) and the turnover value of the enzymatic reaction (k_{cat}) were obtained. The catalytic efficiency (k_{cat}/K_{Mapp}) was deduced. These values were summarized in *table 1*.

Table 1. *Comparison of kinetic parameters of ROL_w and ROL29 on TC_4 and TC_8.*

		V_{max} (µmol/min/mg)	K_{Mapp} (mM)	k_{cat} (s^{-1})	k_{cat}/K_{Mapp} (s^{-1} mM^{-1})
TC_4	ROL_w	1250	16.6	2200	132
TC_8	ROL_w	1600	30	2823	94.1
TC_4	ROL29	900	12	435	36
TC_8	ROL29	7000	25	3338	133

With respect to lipase activity, ROL_w displays a 3.7 fold increase in k_{cat}/K_{Mapp} values compared to ROL29, when using TC_4 and 1.4 fold

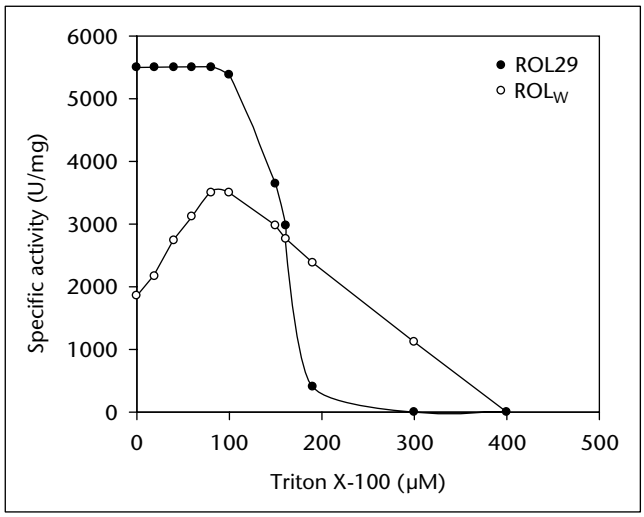

Figure 4. *Effect of synthetic detergent TX-100 on ROL_w activity. Lipolytic activities were measured at an increasing concentration of TX-100 at pH-stat using olive oil emulsion as substrate at pH 8 and 37 °C.*

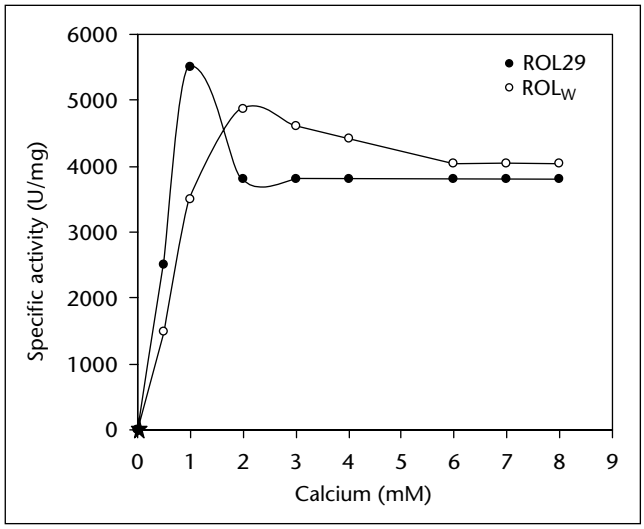

Figure 5. *Effect of increasing concentration of calcium on the rate of hydrolysis of olive oil emulsion by ROL_w. Lipolytic activity was measured under standard conditions at pH 8 and 37°C using a pH-stat technique. The star indicates the lipolytic activity measured in the absence of $CaCl_2$ and the presence of 10 mM EDTA or EGTA.*

decrease in k_{cat}/K_{Mapp} values when using TC_8 as substrate, respectively. This decrease in the catalytic efficiency (k_{cat}/K_{Mapp}) of the ROL29 enzyme is mainly due in an increase in k_{cat}, to 5.05 and 0.8 fold for TC_4 or TC_8, respectively.

Effect of synthetic detergents and amphiphilic proteins on ROL_w activity

It is well known that all detergents act as potent inhibitors of pancreatic and some microbial lipases [6, 17-19].

The effect of varying concentrations of synthetic detergent like TX-100 on ROL_w and ROL29 activity was achieved when using olive oil emulsion as substrate. *Figure 4* shows that, in contrast to ROL29 described by Sayari *et al.*, (2005) [6], the ROL_w activity increases with increasing TX-100 concentration until 100 µM. Furthermore, the addition of increasing concentration of NaDC or BSA in the reaction system containing ROL_w or ROL29 do not restores lipase activity (data not shown). In contrast, with thermophilic *Rhizopus oryzae* lipase, Hiol *et al.* (2000) [20], show that addition of NaDC in lipolysis system, slightly increased enzyme activity at concentration from 1 to 7 mM.

Effect of calcium on ROL_w activity

Hiol *et al.* [20] studied the effect of various metals (Fe^{2+}, Fe^{3+}, Hg^{2+} and Cu^{2+}) on *Rhizopus oryzae* lipase activity. These metal ions strongly inhibited the enzyme. Only the effect of Ca^{2+} ion is not studied. By the way, we studied the effect of various Ca^{2+} concentrations on the rate of hydrolysis of olive oil emulsion by ROL_w and ROL29. Our results show that in the absence of chelators such as EDTA or EGTA, the activity of ROL_w and ROL29 increases respectively at 2 mM and 1 mM of $CaCl_2$ *(figure 5)*. No lipase activity was detected with the two enzymes, when using olive oil emulsion as substrate in the absence of Ca^{2+} and in the presence of 10 mm of chelators *(figure 5)*. The maximal specific activities reached, by ROL_w and ROL29 is respectively 4500 U/mg and 5500 U/mg when using olive oil emulsion in the absence of chelators. We concluded that the enzymatic activity of *Rhizopus oryzae* lipases is stimulated by Ca^{2+} and this metal ion seems to have a structural rather than a catalytic function.

In previous work [7], we have shown the presence of two differences in the primary sequence between ROL_w and ROL29. The differences between kinetic and interfacial properties of ROL_w and ROL29 is may be due to the change of this two residues: Asn 134 (ROL_w) [7]/ His 134 [ROL29) [6], Ala 200 (ROL_w) [7]/Val 200 [ROL29) [6]. In fact theses substitutions could be responsible for a higher tolerance to the presence of long-chain free fatty acids at the lipid/water interface and then explain the differences in kinetic and interfacial behavior between the two lipases.

In the future, we attempted to overexpress the ROL_w to establish the importance of these residues in interface interaction.

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