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Lipase activity in biphasic media: Why interfacial area is a significant parameter?

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ABSTRACT

This work focused on lipase-catalyzed triglyceride hydrolysis in biphasic media. The effect of specific interfacial area of oil-in-water emulsions on the hydrolysis activity of lipase was particularly investigated following a rigorous methodology and using two different oils, tributyrin and olive oil. The specific interfacial area was varied over several orders of magnitude by changing either the amount of emulsified oil or the average diameter of oil droplets. This work particularly focused on the effect of changing droplet size (at given amount of oil) on lipase activity. When the specific interfacial area was varied over several orders of magnitude, the specific activity of the enzyme exhibited a non-monotonic variation with a pronounced maximum. At low specific interfacial area, initial velocity increased with specific interfacial area. Inhibition of enzyme activity at a high interfacial concentration of triglyceride was observed. Experimental results were interpreted on the basis of a theoretical mechanism assuming Michaelis–Menten mechanism for enzyme catalysis, Langmuir-type adsorption isotherm for enzyme and limitation of enzyme–substrate formation by enzyme adsorption process.

1. Introduction

The unique interfacial properties of lipases (triacylglycerol hydrolase, EC 3.1.1.3) make them a perfect catalyst for the industrial fat cleavage process in which the natural fat/oil is dispersed into a benign aqueous phase which initially contains the lipase enzymes [1,2]. It is expected that such reactions should occur with remarkable and phenomenal rate accelerations since such enzymatic processes implicate the hydrolysis reaction of the natural substrate in an aqueous system that is the most favorable for lipase selectivity and activity [3]. Moreover, performing an enzymatic reaction in an emulsion system represents a particular advantage in the substrate load, a key criterion for technical applications, which is no longer limited by the substrate solubility as encountered in homogeneous reactions. The oil droplets contained within the process play a key role as surface micro or nanoreactors and the substrate concentration is strongly related to the surface area which can be increased by decreasing the droplet size in the emulsion [4].

One of the major requisite for industrial process is fast reaction rate to comply with technological and competitiveness requirements [5,6]. This is not an easy task for the biotechnologist dealing with lipase application due to the lack of knowledge concerning interfacial kinetics together with a systematic method to determine enzyme activity and to optimize the reaction conditions in a biphasic system. Since lipases catalyze reactions in biphasic media, it is evident that their activities as well as specificities depend not only on the molecular properties of the enzyme, but also on the type of substrates, and more particularly on the physico-chemical properties of the interface [7]. Conflicting data have been reported in the literature resulting from the difference in emulsion preparation, the difficulties to characterize the interface of the emulsion and the part of which the lipolytic reaction was investigated [8]. Even though many theoretical models have been established in the past ten years to relate the rate of hydrolysis to enzyme and reactant concentrations as well as interfacial properties, they focus, however, only on the specific situation where the interfacial area is controlled by the amount of oil in the feed and not by varying the droplet diameter at a constant amount of the oil [9–14]. Moreover, no direct relation was established between the reaction rate and the interfacial area and compared to the experimental data. In addition, the possible competition between the lipase and interfacial products of the reaction for adsorption at the oil/water interface was not analyzed, while it is known that such a competition occurs when long chain fatty acids are produced [15]. Tributyrin was used in several examples, for which such competition does not take place

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Nomenclature

A	fatty acid molecule located in bulk aqueous phase
A*	fatty acid molecules located at oil–water interface
D	diglyceride molecule located in bulk oil phase
D*	diglyceride located at oil–water interface
d_H	Sauter diameter (d_{32}, μm) or *Z*-average diameter (d_z, nm)
d_m	molecular diameter (μm)
E	enzyme molecule located in bulk aqueous phase
E*	enzyme molecule located at oil–water interface
$[E^*]$	superficial concentrations of adsorbed enzyme (mol/m^2)
$[E^*]_{max}$	maximum superficial concentration of adsorbed enzyme (mol/m^2)
ES*	enzyme–substrate complex located at oil–water interface
$[E]_{tot}$	total enzyme concentration in the feed (mol/l)
$[IA]$	specific interfacial area (m^2/l)
k_1	rate constant of enzyme–substrate formation (l mol^{-1} min^{-1})
k_{-1}	rate constant of enzyme–substrate dissociation (min^{-1})
k_2	rate constant of product formation (min^{-1})
k_a	rate constants of enzyme adsorption (l mol^{-1} min^{-1})
k_d	rate constants of enzyme desorption (min^{-1})
K_d^*	interfacial affinity constant (mol/l)
K_m	Michaelis–Menten constant (mol/l)
m_{oil}	mass of the oil (g)
$N_{particle}$	number of particles
$[S]$	substrate concentration (mol/l)
S*	substrate (triglyceride) located at oil–water interface
V	volume of the oil (ml)
v_i	initial rate of hydrolysis reaction (mol/ml min)
v_m	molecular volume (μm^3/mol)
V_{max}	maximum rate of reaction in a homogeneous medium (mol/l min)
$V_{max\,IA}$	maximum initial rate at a given enzyme concentration (mol/l min)
$V_{max\,E}$	maximum initial rate at a given interfacial area (mol/l min)

Greek letters

α	free site concentration (mmol/l)
ρ_{oil}	density of the oil (g/ml)

since the product of the reaction (i.e. butyric acid) is fully soluble in the bulk aqueous phase.

It is the aim of the present work to test a methodology for studying the interfacial kinetic, specifically investigating lipase-catalyzed hydrolysis of tributyrin and olive oil in oil-in-water reaction media. For the sake of precluding the complication resulting from the specificity of enzymes, a lipase with broad fatty acid chain length specificity, lipase AY (lipase from *Candida rugosa*, Amano Enzymes Co.) was used as a model enzyme [16,17]. From this model study, insight was gained into the influence of the interfacial area on lipase AY activity, the effect of the fatty acid product and the effect of specific interfacial area.

2. Experimental

2.1. Materials

The lipase (EC 3.1.1.3) from *Candida rugosa* (Lipase AY) was purchased from Amano Enzyme Co. (Nagoya, Japan). According to the specification sheets, the activity and the molecular weight were 32,800 U/g and 60,000 g/mol, respectively (Lot No. LAY E0151016). Olive oil highly purified and with a low acidity grade was purchased from Sigma–Aldrich (Buchs, Switzerland). All other chemicals were of highest commercial purity and used without further purification.

2.2. Methods

2.2.1. Protein determination and fatty acid profile analysis

The protein content of lipase AY was 2.95 ± 0.33% as determined by the Lowry method [18]. The olive oil molecular weight was 875.77 g/mol as calculated based on the fatty acid profile analyzed by gas chromatography [19].

2.2.2. Determination of the initial rate of lipase-catalyzed hydrolysis reactions

Tributyrin and olive oil are commonly used triglycerides. A modified assay using a pH-stat method was carried out [20,21]. Triglyceride emulsion in 2% gum arabic was prepared at 37 °C as described by Kaewprapan et al. [21]. A 0.28 mM Tris–HCl solution was used as a reaction buffer containing 150 mM of NaCl and 1.4 mM of $CaCl_2$. A cup containing 20 ml of emulsion was connected to a pH-stat titrator (Mettler Toledo DL50, Schwerzenbach, Switzerland). The pH and temperature of the emulsion was adjusted to 7.5 and 37 °C and the reaction was started by the addition of one ml of lipase solution containing a predetermined amount of protein. Liberated fatty acid was titrated continuously with 0.01 M NaOH to a constant pH of 7.5. The initial velocity was determined from the slope at the origin of the fatty acid concentration against the time between 0 and 10 min. The same experiment was repeated twice and the average initial velocity (μmol/min) was determined.

Because of the low water-solubility of palmitic acid and oleic acid liberated from the olive oil hydrolysis reaction, care was taken to ensure that they were well dispersed in this emulsion system and not underestimated. In order to check that assumption, 23.15 μmol and 46.30 μmol of oleic acid (generally liberated from the hydrolysis reaction in our study) were spiked into the standard olive oil emulsion and then titrated back by pH-stat. The experimental data obtained from triplicate experiments were 22.37 ± 0.63 μmol and 45.34 ± 0.89 μmol, corresponding to error percentages of back titration only of 3.37% and 2.07%, respectively.

2.2.3. Emulsion preparation

A fixed volume of 20 ml of triglyceride emulsion was prepared at room temperature. As an example, Table 1 shows the preparation of olive oil emulsions with specific interfacial area experimentally varying from 4.15×10^{-1} to 600×10^{-1} m^2/l. These were achieved by changing either the amount of the emulsified oil or the energy supplied over a controlled period of time. Olive oil emulsions of average droplet sizes ranging from tens of micrometers down to hundreds of nanometers were prepared using two different procedures: mechanical mixing by Ultra-Turrax T-25 (IKA laboratechnik) and application of TT13 ultrasonic homogenizers SONOPLUS HD 2200 series (Ultrasound horn 20 KHz, 200 W, BANDELIN electronic GmbH&Co. KG), respectively. In regard to the emulsification by TT13 ultrasonic homogenizers, the triglyceride/water mixture needs to be chilled in ice bath during the emulsification.

Table 1
Conditions used to emulsify olive oil with the specific interfacial area varying from 4.15×10^{-4} to 600×10^{-4} m^2/ml.

Olive oil concentration (mM)	Droplet size (μm)	Specific interfacial area ($\times 10^{-1}$ m^2/l)	Condition of emulsification
1.00	13.9	4.15	Ultra-Turrax, 1 min at 11,000 rpm
1.25	14.8	4.86	
1.50	15.7	5.55	
1.75	15.2	6.65	
2.50	15.8	9.00	
5.00	12.2	23.75	
10.00	7.7	74.50	
21.44	9.0	138.00	
40.00	8.5	272.00	
1.25	0.173	417.50	TT13 ultrasonic homogenizers, 5 min at 50% amplitude
1.25	0.155	466.00	TT13 ultrasonic homogenizers, 5 min at 75% amplitude
1.75	0.187	541.00	TT13 ultrasonic homogenizers, 5 min at 50% amplitude
2.50	0.241	600.00	

The same principle was applied to prepare tributyrin emulsions with specific interfacial area varying from 0.50×10^{-1} to 226.74×10^{-1} m^2/l.

The stability of triglyceride emulsions was evaluated by following the variation of average droplet sizes (d_{32} or d_z) of emulsion over a period of 1 h at 37 °C. The average sizes of triglyceride droplets used for stability study are 8.0 μm and 186 nm for tributyrin emulsion and 12.2 μm and 226 nm for olive oil emulsion.

2.2.4. Emulsion characterization

The droplet size distributions of the prepared emulsions were determined by means of a Mastersizer 2000 for emulsions prepared with Ultra-Turrax and Zetasizer Nano ZS for emulsions prepared by ultrasounds. Both apparatuses were from Malvern (Malvern Instrument Co., Ltd.). The emulsions were diluted with Tris–HCl buffer prior to analysis. Results are reported as the average of three measurements on freshly prepared emulsions. With the Mastersizer 2000, the reported diameters d_{10}, d_{50} and d_{90} were diameters at 10, 50 and 90% volume of the cumulated distribution. The average droplet size, expressed as the Sauter diameter ($d_{32} = \sum n_i d_i^3 / \sum n_i d_i^2$, representing a surface average value) was extracted from these data. With Zetasizer Nano ZS, an intensity average diameter (d_z, usually called "*Z*-average diameter") was calculated from the raw data following cumulated analysis.

The number of particles ($N_{particle}$) can be calculated from Eq. (1):

$$V = \frac{m_{oil}}{\rho_{oil}} = \frac{4}{3}\pi\left[\frac{d_H}{2}\right]^3 N_{particle} \quad (1)$$

where V, m_{oil}, ρ_{oil} and d_H stand for volume of the oil (ml), mass of the oil (g), density of the oil (g/ml) and Sauter diameter (d_{32}, μm) or *Z*-average diameter (d_z, nm), respectively.

The number of particles obtained from Eq. (1) was used to calculate the specific interfacial area ([*IA*], m^2/l) (Eq. (2)).

$$[IA] = \frac{4\pi\,[d_H/2]^2 N_{particle}}{20} \quad (2)$$

Here 20 is the volume in ml of the emulsion used for the lipase assay.

The specific interfacial area can be converted into a volume concentration of superficial substrate molecules (mole/l) by assuming that the enzymatic reaction occurs only on the outer spherical crown of spherical droplets of triglycerides. According to this assumption, the concentration of superficial ester bonds should be directly proportional to that of surface molecules of triglyceride of which the procedure of estimation was described in detail by Jurado et al. [11]. The values of the molecular volume (v_m) and the molecular diameter (d_m) of the triglycerides used for the estimation of the interfacial triglyceride concentration are listed in Table 2.

3. Results and discussion

In what follows, we will consider biphasic reaction media comprising a triglyceride oil phase emulsified into an aqueous phase in which the enzyme is initially dissolved. *Initial enzyme concentration* will be defined as the amount of enzyme added in the feed divided by the overall volume of biphasic medium (in mol/ml). The *specific interfacial area* ([*IA*]) will be defined as the ratio of the total interfacial area to the overall volume of biphasic medium (in m^2/l). Consistently, the *free site concentration* will be calculated as the amount of superficial sites available for enzyme adsorption and catalysis (on droplet surface) divided by the total volume of biphasic medium (in mmol/l).

3.1. Theoretical model for the kinetics of interfacial lipolysis catalyzed by lipases

In what follows, we will focus on the initial rate of reaction. Thus we will limit to hydrolysis of triglyceride molecules. Any reaction involving di- or monoglyceride molecules will be ignored which implies that this kinetic model is only valid for discussing initial reaction rate. The mechanism of hydrolysis at the interface of triglyceride-water is represented by three successive steps, (1) adsorption of enzyme (E) at the oil–water interface (I) following Langmuir equilibrium, (2) formation of enzyme–substrate superficial complex following the Michaelis–Menten mechanism and (3) the formation of the products of hydrolysis (diglyceride and fatty acid) followed by a fourth step which is their eventual desorption from the oil–water interface and release of the activated enzyme (Fig. 1) [11,22]. The assumption of Langmuir isotherm for adsorption of enzyme at interface was shown to be reasonable in several experimental studies [23,24].

Because we consider the initial stages of hydrolysis, the question of accumulation of fatty acid molecules at oil–water interface will not be considered in details and its consequences on the reaction rate will not be included in the kinetic equations derived below.

In his kinetic treatment of lipase catalysis at oil–water interfaces, Marangoni proposed to consider that the rate-limiting step was the formation of products (step (3)) and that the formation of superficial enzyme–substrate complex (step (2)) was very fast as compared to enzyme adsorption (step (1)) [22,25]. Consequently, enzyme adsorption determines the superficial concentration of enzyme–substrate complex and the initial rate of hydrolysis reac-

Table 2
Values of the molecular weight (*Mw*), density, molecular volume (v_m) and the molecular diameter (d_m) of the triglycerides used for the estimation of the volume concentration of superficial molecules of triglyceride.

Triglycerides	*Mw* (g/mol)	Density (g/ml)	v_m[b] (μm³/mol)	d_m[b] (μm/mol)
Tributyrin	302.37	1.04	4.85×10^{-10}	9.75×10^{-4}
Olive oil	875.77[a]	0.91	1.59×10^{-9}	1.49×10^{-3}

[a] The olive oil molecular weight used for v_m calculation was 875.77 g/mol. This value was calculated on the basis of the fatty acid profile analyzed by gas chromatography.
[b] The v_m and d_m of triglycerides were estimated using the method of Jurado et al. [11].

tion (v_i in mol/ml min) should be expressed as

$$v_i = k_2[E^*][IA] \tag{3}$$

In Eq. (3), k_2 is the rate constant (in min^{-1}), $[E^*]$ and $[IA]$ are the superficial concentrations of adsorbed enzyme (in mol/m^2) and the specific interfacial area (in m^2/l).

Considering that the rates of enzyme adsorption and desorption processes are exactly equal and that a maximum surface coverage exists for enzyme adsorption (Langmuir equilibrium isotherm), it comes

$$[E] = \frac{K_d^*[E^*]}{[E^*]_{max} - [E^*]} \tag{4}$$

In Eq. (4), $[E^*]_{max}$ is the maximum superficial concentration of adsorbed enzyme (in mol/m^2) and $K_d^* = k_d/k_a$ (in mol/l).

The mass balance of enzyme in the biphasic medium with preceding assumptions gives

$$[E]_{tot} = [E] + [E^*][IA] \tag{5}$$

In Eq. (5), $[E]_{tot}$ is the total enzyme concentration in the feed (in mol/l). In the mass balance of Eq. (5), enzyme–substrate complex does not appear since its concentration is exactly that of adsorbed enzyme molecules because of its instantaneous formation as compared to the adsorption process.

In what follows (up to Eq. (9)), it will be assumed that $[E]_{tot}$ is lower than $[E^*]_{max}[IA]$. Making use of Eqs. (4) and (5), the initial rate of reaction can be re-expressed as

$$v_i = \frac{k_2[E]_{tot}[E^*][IA]}{[E] + [E^*][IA]} = \frac{k_2[E]_{tot}[E*][IA]}{(K_d^*[E^*]/([E^*]_{max} - [E*])) + [E*][IA]}$$
$$= \frac{k_2[E]_{tot}([E^*]_{max} - [E^*])[IA]}{K_d^* + ([E^*]_{max} - [E^*])[IA]} \tag{6}$$

Finally, the initial rate of enzyme catalyzed lipolysis can be expressed as follows

$$v_i = \frac{V_{max\ IA}\alpha}{K_d^* + \alpha} \tag{7}$$

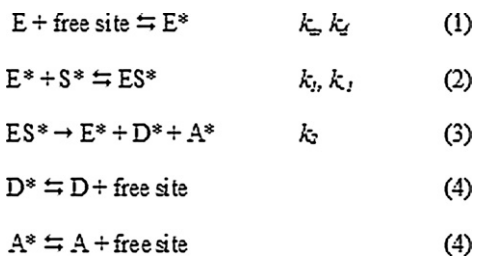

Fig. 1. Kinetic scheme of interfacial lipolysis catalyzed by lipase at very low triglyceride conversion. E, D and A are enzyme, diglyceride and fatty acid molecules (respectively) located in bulk aqueous phase (E and A) or bulk oil phase (D). E*, ES*, S*, D*, A* are enzyme, enzyme–substrate complex, substrate (triglyceride), diglyceride and fatty acid molecules located at oil–water interface (respectively). "Free site" stands for an available space at oil–water interface. k_a, k_d, k_1, k_{-1}, k_2 are the rate constants of enzyme adsorption and desorption, enzyme–substrate formation and dissociation, product formation (respectively).

In Eq. (7), $V_{max\,IA} = k_2[E]_{tot}$ is the maximum initial rate (in mol/l min) corresponding to a total adsorption of enzyme at oil–water interface for high enough values of $[IA]$ (with the assumption that the interface is not saturated by the adsorbed enzyme) and $\alpha = ([E^*]_{max} - [E^*])[IA]$ represents the free site concentration (in mmol/l).

Slightly rearranging Eq. (7) leads to

$$\frac{1}{v_i} = \frac{1}{V_{max\ IA}} + \frac{K_d^*}{V_{max\,IA}}\frac{1}{\alpha} \tag{8}$$

Eq. (8) gives the variation of initial rate of lipolysis as a function of free site concentration. A linear variation of $1/v_i$ versus $1/\alpha$ should be observed provided that the total amount of enzyme ($[E]_{tot}$) is kept unchanged. In case the surface coverage by enzyme is far from saturation, $\alpha \approx [E^*]_{max}[IA]$ and Eq. (8) reduces to

$$\frac{1}{v_i} \approx \frac{1}{V_{max\ IA}} + \frac{K_d^*}{[E^*]_{max}V_{max\,IA}}\frac{1}{[IA]} \tag{9}$$

Eqs. (8) and (9) can be considered as equivalents of the Lineweaver–Burk equation which corresponds to the Michaelis–Menten mechanism in a homogeneous reaction medium.

Finally, Eq. (9) allows an evaluation of the enzyme affinity towards the interface (K_d^*) when the velocity measurement is performed during the initial stage of the lipolytic reaction (in which adsorbed species and interfacial area are well-defined).

At a fixed interfacial area and varying the amount of enzyme in the feed ($[E]_{tot}$), the initial velocity of lipolysis varies as follows:

$$v_i = \frac{V_{max\,E}[E]}{K_d^* + [E]} \tag{10}$$

In Eq. (10), $V_{max\,E} = k_2[E^*]_{max}[IA]$ is the maximum initial rate corresponding to a saturated oil–water interface. When $[E] \gg K_d^*$, Eq. (10) gives $v_i = V_{max\,E}$. Conversely, when $[E] \ll K_d^*$, Eq. (10) gives $v_i \approx V_{max\,E}[E]/K_d^* \approx V_{max\,E}[E]_{tot}/(K_d^* + [E^*]_{max}[IA])$ which shows that v_i is expected to increase linearly with $[E]_{tot}$. Eq. (10) demonstrates that a graph representing v_i as a function of $[E]_{tot}$ should reach an asymptotic maximum value which is directly proportional to $[E^*]_{max}$ [22]. Obviously, $[E]_{tot}$ is linked to $[E]$ via the mass balance of enzyme and adsorption isotherm (Eq. (5)).

3.2. Characteristic of triglyceride emulsions

The reliability of the interfacial kinetic analysis done in emulsion system depends on the emulsion composition and the exactness of two variables which are the specific interfacial area and the initial reaction velocity. Consequently, the emulsion composition should be designed in order to (a) favor the binding of the targeted lipase on the interface, (b) form a stable emulsion at least over the time needed for determining the initial velocity and (c) provide an interface free of emulsifier or any other surface active compound [8].

3.2.1. Design of the emulsion composition

Lipase AY was chosen particularly because the influence of the buffer system and ions on the reaction rate had been well investigated previously [26,27]. The binding of lipase AY on the interface of

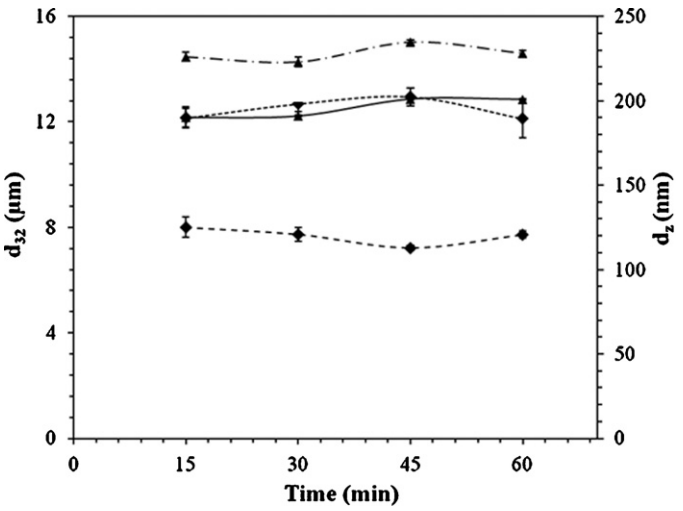

Fig. 2. Variation of d_{32} (μm) and d_z (nm) of tributyrin (bold diamonds) or olive oil (bold triangles) droplets as a function of time. The sizes of triglyceride droplets used for stability study are 8.00 μm (– – –) and 185 nm (- - -) for tributyrin emulsion and 12.17 μm (—) and 226 nm (—·—) for olive oil emulsion.

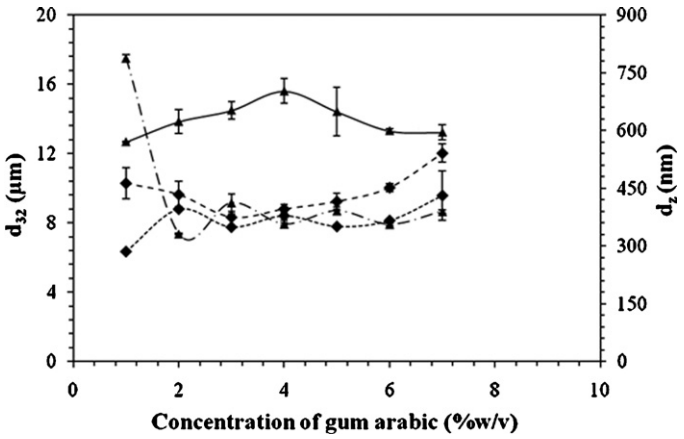

Fig. 3. Dependence of d_{32} (μm) (– – – and —) and d_z (nm) (- - - and —·—) of tributyrin (bold diamonds) and olive oil (bold triangles) droplets as a function of gum arabic concentration. Triglycerides were emulsified in 20 ml of 0.28 mM Tris–HCl buffer containing 150 mM NaCl and 1.4 mM $CaCl_2$ and gum arabic percentages varying from 1 to 7%. Each value represents the mean diameter ± SD of triplicate measurements.

tributyrin-buffer and olive oil-buffer was optimized by modifying the ionic strength and considering the zeta potential of oil–water interface (a detailed investigation will be published elsewhere). It turned out that the type of buffer had no effect on the interfacial adsorption behavior of lipase AY once the ionic strength was adjusted to 0.154 M and the zeta potential to −10.76 mV, respectively. The optimized buffer system was used to prepare the emulsion in which 21.44 mM of triglyceride was emulsified in the presence of 2 wt% of gum arabic.

The accuracy of the specific surface area calculation and the knowledge of the surface area available for enzyme adsorption were essential for the reliability of the interfacial kinetic study. Such precision was based on two parameters, the colloidal stability of droplets during the reaction and the absence of emulsifiers or other surface active compounds on the interfaces. The stabilities of the emulsions with the average droplet size diameter in the range of μm and nm were assessed by monitoring the variation of the average droplet diameter as a function of time during the first hours following emulsification (Fig. 2).

For each emulsion studied, it can be seen that the average droplet diameter did not vary during the first hours. Consequently, their specific interfacial areas remained unchanged during this period. These chosen emulsions were, thus, suitable for determination of the initial rate of lipase AY catalyzed lipolysis of tributyrin and olive oil in the interval of the first hours following emulsification.

The second consideration was that the oil–water interfaces must be free from gum arabic used as a stabilizer. The eventual adsorption of gum arabic on the interface was verified by following the average diameter (d_{32} or d_z) of the oil droplet prepared by a varying percentage of the gum arabic concentration used as a stabilizer (Fig. 3). In fact, a decrease of droplet size upon increasing gum arabic concentration, especially in the first part of the curve, was expected if the latter was surface active polymer.

As seen in Fig. 3, at least 2% of gum arabic was necessary to prepare a triglyceride emulsion of a desirable droplet size. Then, the d_{32} and d_z of the stable emulsion remained constant over the whole range of the tested concentrations indicating that gum arabic was not adsorbed on the surface layer of the oil droplets. This result was consistent with the common idea that gum arabic is a hydrocolloid that stabilizes oil-in-water emulsions by increasing the viscosity of the continuous phase but not by adsorbing at the interface [28,29]. A minimal concentration of gum arabic was preferred and 2 wt% was considered as suitable for preparing triglyceride emulsions throughout this study.

3.2.2. Determination of the initial velocity using a model of the reaction system

The measurement of the velocity in the initial region avoided many difficulties in the treatment of interfacial catalysis such as the loss of enzyme activity at the interface over time, any randomization phenomena caused by the stepwise hydrolysis of diglycerides and monoglycerides and the inhibition by fatty acid products. To test the validity of the initial velocity measurement, 21.44 mM of triglyceride was emulsified in the optimal condition in 2 wt% of gum arabic, as mentioned previously. The rates of lipase AY-catalyzed olive oil hydrolysis and tributyrin hydrolysis were determined by monitoring the amount of liberated fatty acid with time (Fig. 4).

The chosen emulsion was suitable for determination of the initial rate of lipase AY-catalyzed lipolysis of tributyrin and olive oil and the linear kinetics were obtained in the interval of 15 min for both triglycerides (Fig. 4, right). The initial lipolysis rate towards tributyrin ($0.28 \pm 0.02 \times 10^{-6}$ mol/ml min) was close to that towards olive oil ($0.23 \pm 0.04 \times 10^{-6}$ mol/ml min), a result which was attributed to the broad fatty chain length specificity of lipase AY, as mentioned above. In regard to the hydrolysis of olive oil, the progress curve deviated from linearity after 15 min and the reaction slowed down. This might be explained by the involvement of the surface active long chain fatty acids that were produced by olive oil hydrolysis and accumulated on the oil–water interface. The hydrolysis of olive oil mainly produced fatty acids with long hydrocarbon chains (palmitic acid and oleic acid) which exhibited a significant surface activity comparable to that of the used lipase itself. Thus, these hydrolysis products strongly adsorbed at the oil–water interface and competed with the lipase [15]. In the case of tributyrin, the deviation from linearity was much less pronounced and almost not observed. This can be rationalized by considering that the butyric acid formed during hydrolysis did not remain at the interface, but almost instantaneously dissolved in the bulk aqueous medium, thus not competing at the interface with the lipase molecules.

It was necessary to ensure that no autolysis of triglycerides occurred in the emulsions containing high specific interfacial areas which were prepared using the high energy emulsification technique (ultrasounds). Those emulsions were selected and their fatty acid functions were quantified immediately after emulsification

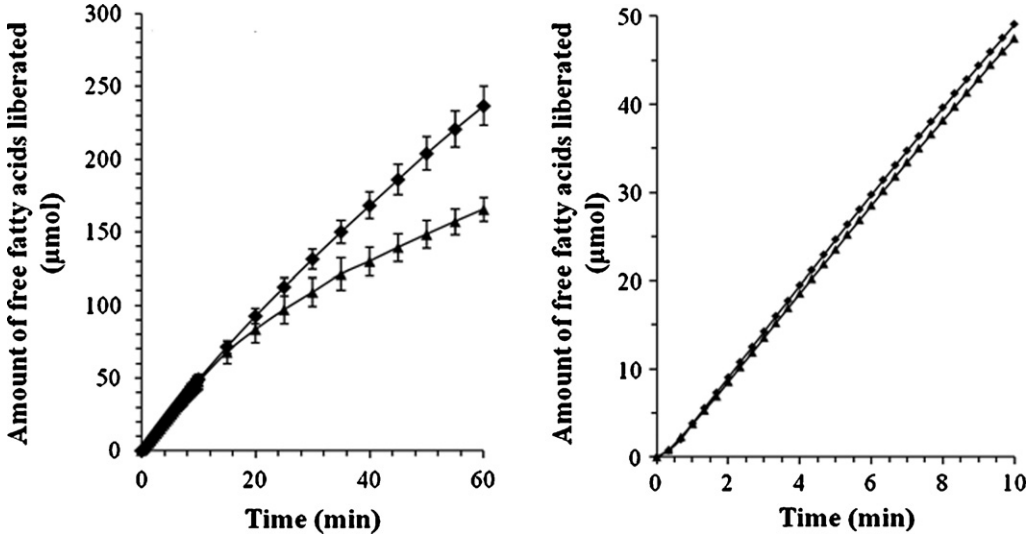

Fig. 4. Progress curve of lipase AY-catalyzed hydrolysis of tributyrin (bold diamonds) and olive oil (bold triangles) during 1 h (left) and the zoom of the progress curve at the beginning 15 min of the reaction (right). 20 ml of emulsion was prepared in 0.28 mM Tris–HCl buffer pH 7.5 containing 150 mM of NaCl, 1.4 mM of $CaCl_2$, 21.44 mM of triglyceride and 2% gum arabic using Ultra-Turrax at 11,000 rpm for 1 min. The concentration of lipase AY used was 1.48 μg protein/ml.

Table 3
Properties of selected triglyceride emulsions and their corresponding amount of fatty acid determined immediately after emulsification.

Triglycerides emulsion	Concentration (mM)	Emulsification method	[*IA*] ($\times 10^{-1}$ m^2/l)	Amount of fatty acid* (μmol) ± SD
Tributyrin	40 (Control)	Stirred	N/D	3.56 ± 0.45[a]
	2.5	TT13 ultrasonic homogenizer, 5 min at 50% amplitude	119.9	1.91 ± 0.22 [b]
	1.75	TT13 ultrasonic homogenizer, 5 min at 50% amplitude	173.5	2.04 ± 0.11 [b]
Olive oil	40 (Control)	Stirred	N/D	1.19 ± 0.16 [c]
	40	Ultra-Turrax homogenizer, 1 min at 11,000 rpm	272.0	2.13 ± 0.15 [c]
	1.25	TT13 ultrasonic homogenizer, 5 min at 50% amplitude	417.5	1.75 ± 0.20 [c]

N/D stands for not determined.
* For tributyrin emulsion, values within a column with different letters (a and b) differ significantly ($p < 0.05$). Results compared using Duncan, one-way ANOVA; for olive oil emulsion, values within a column with letter (c) do not differ significantly ($p > 0.05$). Results compared using Duncan, one-way ANOVA.

and compared with those of 40 mM triglyceride solution used as control (Table 3). For all emulsions studied, autolysis did not occur since the amounts of fatty acid after emulsification were lower or not statistically different. In case of tributyrin emulsion, lower fatty acid concentration after emulsification comparing to that of the control was a result of lower tributyrin concentration used (Table 3). For the control experiment using stirred method, higher concentration of substrate was required so that the fatty acid obtained could be detected by pH-stat.

3.3. Effect of the interfacial triglyceride concentration on lipase AY activity

The double reciprocal of initial velocity (v_i) versus specific interfacial area of triglyceride ([*IA*]) plot for lipase AY-catalyzed hydrolysis of tributyrin and olive oil emulsions are shown in Fig. 5.

As evident from Fig. 5, the trend given by Eq. (9) is consistent with the way how specific interfacial area of triglycerides acts on the reaction rate. The linear variation observed is similar to Lineweaver–Burk plots. The interfacial kinetic parameters including $K_d^*/[E^*]_{max}$ and the apparent maximum velocity ($V_{max\,IA}$) can be, thus, determined from such a double-reciprocal plot (Table 4).

For the sake of enzyme affinity comparison, the $K_d^*/[E^*]_{max}$ (in m^2/l) need to be converted into K_d^* (in mol/l). To that, the $[E^*]_{max}$ (in mol/m^2) need to be determined precisely. This is very difficult

Table 4
Kinetic parameters; $K_d^*/[E^*]_{max}$, $V_{max\,IA}$, deduced from the double reciprocal plot of data rate of lipase AY-catalyzed hydrolysis of emulsified tributyrin and olive oil.

Substrate	$K_d^*/[E^*]_{max}$ (m^2/l)	$V_{max\,IA}$ ($\times 10^{-6}$ mol/ml min)
Tributyrin	7.57×10^{-2}	0.38
Olive oil	3.74×10^{-1}	0.23

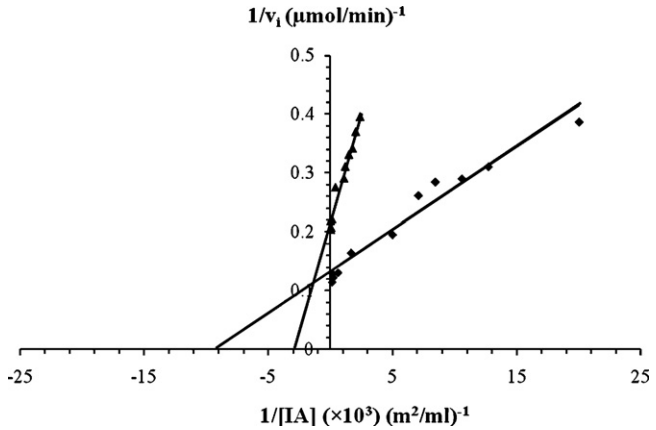

Fig. 5. Double-reciprocal plot of rate data obtained from lipase AY-catalyzed hydrolysis of emulsified tributyrin (bold diamonds) and emulsified olive oil (bold triangles) when the specific interfacial area of triglycerides was varied from 0.5×10^{-1} m^2/l to 74×10^{-1} m^2/l and 4×10^{-1} m^2/l to 272×10^{-1} m^2/l, respectively.

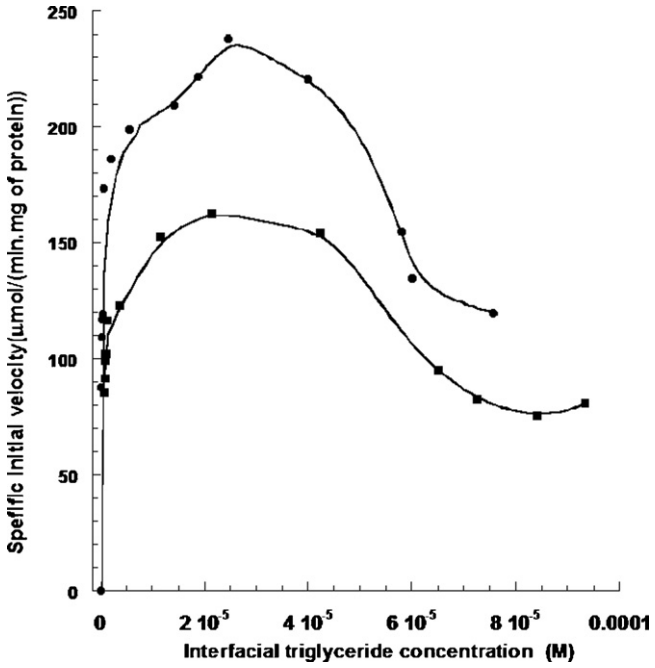

Fig. 6. Changes in initial velocity of lipase AY-catalyzed hydrolysis of emulsified tributyrin (bold circles) and olive oil (bold squares) as a function of the volume concentration of superficial substrate molecules of olive oil and tributyrin.

for our study due to the use of a crude enzyme that makes the determination $[E^*]_{max}$ difficult and inaccurate. As the described process should be applicable for industrial use, a purification of enzyme should be unrewarding. Therefore, only an estimated value of $[E^*]_{max}$ will be determined after the variation of initial velocity was evaluated over several orders of magnitude of specific interfacial area. Thus, the preference of lipase AY towards tributyrin-water interface and olive oil–water interface cannot be concluded at this stage. In contrast, a larger value of $V_{max\,IA}$ for tributyrin hydrolysis than for olive oil hydrolysis implies that the conversion of butyric acid followed by its desorption from the tributyrin-water interface is approximately two times faster than the one of oleic acid/palmitic acid from the interface of olive oil–water. This is in agreement with the much stronger hydrophobic character of fatty acids produced from olive oil as compared to butyric acid.

3.4. Inhibition at high specific interfacial areas

In order to shed further light on the effect of the interfacial concentration of triglyceride on lipase AY activity, the variation of initial velocity was evaluated over several orders of magnitude of specific interfacial area. The additional calculation described by Jurado et al. [11] is performed in order to convert the specific interfacial area $[IA]$ in (m^2/l) into the volume concentration of superficial substrate molecules (in mol/l) (Fig. 6).

Rather than asymptotically approaching a maximum as expected for Michaelis–Menten kinetics, Fig. 6 demonstrates that the specific activity of lipase AY exhibited a non-monotonic variation with a specific maximum. The shape of the velocity versus the interfacial triglyceride concentration curve obtained experimentally illustrates the theoretical calculations carried out by Vasilevskaya et al. describing the concept of surface nanoreactors [30]. These authors studied the variation of the rate of a simple model reaction taking place at the interface between an adsorbed substrate and an adsorbed catalyst. They theoretically demonstrated that a maximum reaction rate was attained as a function of interfacial area. In the case of that model reaction, the maximum was explained by the decrease of both substrate and catalyst surface concentrations (in mol/m^2) at large interfacial areas. This was slightly different from our experiments in which the surface concentration of substrate (expressed in mol/m^2) was independent of the interfacial area and was only fixed by molecular size of oil. Nevertheless, the concept of an optimal interfacial area was evidenced by their calculations.

Another explanation for the decrease of the velocity at a high interfacial triglyceride concentration is the inhibition of the enzyme reaction by an excess of superficial substrate molecules in comparison to adsorbed enzyme molecules. It was shown previously in Section 3.2 that the specific interfacial area represents the reactant concentration. The increase of specific interfacial area at a fixed amount of lipase AY in the kinetic study resulted in a strong excess of superficial substrate molecules as compared to the available enzyme. This situation led to the binding of more than one substrate molecule to the same active site to form a dead-end (non-productive) complex, resulting in the decrease of the enzyme activity. Only a few publications reported this inhibition effect for lipases [31–33]. Nevertheless, such inhibition, in contrast to our study, was attributed to substrate inhibition but not related to interfacial characteristics. Because of the order of magnitude of enzyme molecule size (several nanometers) and that of droplet radius around the maximum of enzyme activity (8 μm), any effect of surface curvature should be discarded. Another possible reason for the decrease of reaction rate could be a phase inversion for high enough oil volume fractions. Nevertheless, in all our experiments, oil-in-water emulsions were formed. In addition, phase inversion has been reported to occur for oil volume fractions exceeding 60% while in our experiments, oil volume fractions were lower than 4%.

To confirm that the inhibition was caused by the excess of interfacial area, the assessment of the Langmuir adsorption isotherm was explored.

3.5. Determination of the maximum superficial concentration of lipase AY

The amounts of lipase AY required to saturate the highest specific interfacial area of tributyrin ($225 \times 10^{-1}\ m^2/l$) and olive oil ($600 \times 10^{-1}\ m^2/l$) (data of Fig. 6) were determined by measuring the initial reaction velocity versus enzyme concentration (Fig. 7).

The trend indicated by Eq. (10) is consistent with the experimental variation of initial velocity v_i with $[E]_{tot}$ for both oils. Velocity profiles of tributyrin and olive oil hydrolysis followed the same hyperbolic trend as predicted by the Langmuir model. The velocity varied as a function of the ratio of the free available interfacial area to the amount of enzyme. At the beginning this ratio was very high, thus, increasing the amount of lipase AY allowed all enzyme molecules to adsorb and catalyze hydrolysis of triglycerides at the interface leading to a rapid increase in the velocity of the reaction related directly to the enzyme concentration. Increasing the concentration of the enzyme to a certain extent, the interface/enzyme ratio decreased and the interface became saturated with enzyme

Table 5
Kinetic parameters $K_d^*/[E^*]_{max}$ (reported previously from Table 4 $[E^*]_{max}$ and $K_d{}^*$ of lipase AY-catalyzed hydrolysis of emulsified tributyrin and olive oil.

Substrates	$K_d^*/[E^*]_{max}$ (m^2/l)	$[E^*]_{max}$ ($\times 10^{-6}$ mol/m^2)	K_d^* ($\times 10^{-6}$ mol/l)
Tributyrin	7.57×10^{-2}	3.26×10^{-3}	2.46×10^{-4}
Olive oil	3.74×10^{-1}	1.16×10^{-3}	4.34×10^{-4}

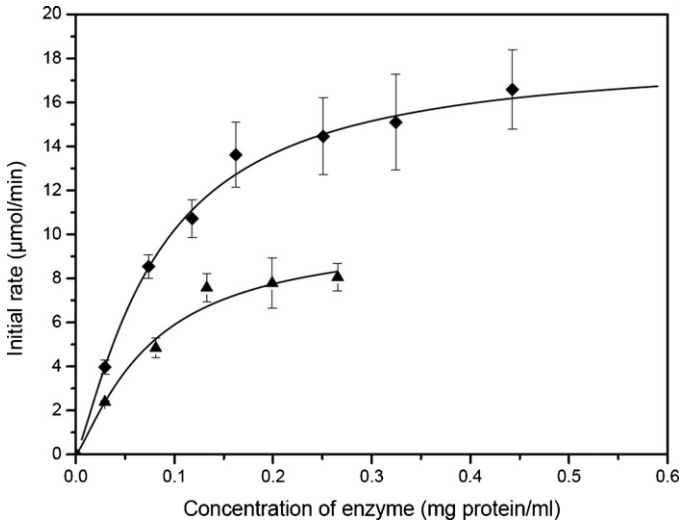

Fig. 7. Initial velocity versus total amount of enzyme plot used in the determination of maximum superficial concentration of adsorbed enzyme (lipase AY) ($[E^*]_{max}$, in mol/m^2) on the tributyrin (bold diamonds) and olive oil (bold triangles) droplets. The specific interfacial area of tributyrin and olive oil was fixed at 225×10^{-1} m^2/l and 600×10^{-1} m^2/l, respectively. The assay was carried out at 37 °C.

leading to the slowdown of the relative initial velocity since the amount of new enzyme able to partition to the interface will progressively decrease relative to the total amount of enzyme present in the system. A maximum value of velocity should be expected when almost all the initial enzymes are adsorbed at the interface (i.e. the quantity corresponding to the equilibrium value at the plateau of the adsorption isotherm).

For an efficient use the lipase in emulsion, the saturation concentration of the enzyme needs to be determined. Nevertheless, a large excess of surface area as compared to the available amount of enzyme in the feed is not positive since inhibition takes place by surface excess. These results demonstrate that the decrease of initial reaction rate at high specific interfacial areas is due to the low surface coverage by enzyme and can be compensated by increasing enzyme concentration at a given specific interfacial area.

3.6. Determination of lipase AY specificity towards the interfacial triglycerides

At a fixed specific interfacial area, the $[E]_{tot}$ was linked to $[E]$ via the mass balance of enzyme and adsorption isotherm (Eq. (5)). Considering that the concentration of $[E]_{tot}$ was sufficiently high comparing to the adsorbed enzyme, the value of $[E^*]_{max}$ could be estimated from a double-reciprocal plot of data rate shown previously in Fig. 7. The K_d^* that shows the preference of lipase AY towards interfacial triglycerides can be, thus estimated from the values of $[E^*]_{max}$ (in mol/m^2) (Table 5).

The K_d^* of lipase AY towards tributyrin-water interface and olive oil–water interface are similar. This infers its broad fatty acid chain length specificity, results in accordance with those reported in the literature [16,17].

4. Conclusions

The results that emerge from this study demonstrate clearly that the interfacial area is a significant parameter that influences the lipase activity in biphasic media. With this well defined system, insight was gained into the influence of specific interfacial area on lipase AY, the effect of fatty acid products and the interfacial substrate inhibition. The specific interfacial area acts on the reaction rate in a similar manner as the reactant concentrations and can be described conveniently by the Michaelis–Menten model. Extending currently available literature data, this work particularly focused on the influence of droplet size on lipase activity. The reaction rate at the interface in the emulsion could be increased simply by decreasing the droplet size at fixed concentration of substrate and fixed volume of continuous phase. A direct relationship between substrate concentration at the interface and enzyme activity could be described. Nevertheless, beyond a specific substrate interface/enzyme concentration ratio, substrate inhibition occurred similarly to what has been observed in homogenous reactions. Our study appeared to be the first systematic study that has examined this concept. In this respect, it was demonstrated that the specific interfacial area of triglyceride should be considered as a key parameter in the design of biotechnological reactors involving enzyme-catalyzed reaction in emulsion. For biotechnological reactors, it is worth decreasing the droplet size in order to obtain maximum interfacial areas for enzyme catalysis. Nevertheless, non-monotonic variation should be taken into account. For the titration of the enzyme activity, it seems essential to normalize the mode of agitation as well as the amount of oil in order to control the specific interfacial area. Furthermore, it seems essential to standardize the determination of lipase activity at a given specific interfacial area, rather than relating it only to the volumetric substrate concentration.

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