A broad pH range indicator-based spectrophotometric assay for true lipases using tributyrin and tricaprylin[S]

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Abstract A continuous assay is proposed for the screening of acidic, neutral, or alkaline lipases using microtiter plates, emulsified short- and medium-chain TGs, and a pH indicator. The lipase activity measurement is based on the decrease of the pH indicator optical density due to protonation which is caused by the release of FFAs during the hydrolysis of TGs and thus acidification. Purified lipases with distinct pH optima and an esterase were used to validate the method. The rate of lipolysis was found to be linear with time and proportional to the amount of enzyme added in each case. Specific activities measured with this microplate assay method were lower than those obtained by the pH-stat technique. Nevertheless, the pH-dependent profiles of enzymatic activity were similar with both assays. In addition, the substrate preference of each enzyme tested was not modified and this allowed discriminating lipase and esterase activities using tributyrin (low water solubility) and tricaprylin (not water soluble) as substrates. This continuous lipase assay is compatible with a high sample throughput and can be applied for the screening of lipases and lipase inhibitors from biological samples.—Camacho-Ruiz, M. A., J. C. Mateos-Díaz, F. Carrière, and J. A. Rodriguez. **A broad pH range indicator-based spectrophotometric assay for true lipases using tributyrin and tricaprylin. *J. Lipid Res.* 2015.** 56: **1057–1067.**

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Lipases (TG ester hydrolases, EC 3.1.1.3) are lipolytic carboxylester hydrolases which catalyze the hydrolysis of the ester bonds of TGs to form FFAs and glycerol in some cases. They are widely distributed in microorganisms, plants, and animals (1–3) where they play an important role in lipid metabolism (4, 5).

One important aspect of lipolytic enzymes is the unique physicochemical character of the reactions they catalyze at lipid-water interfaces, involving interfacial adsorption and subsequent catalysis sensu stricto (6, 7). Most of the lipases are water-soluble enzymes acting on water insoluble substrates (super-substrates). The two-dimensional nature of enzyme catalysis by lipases does not obey Michaelis-Menten kinetics and critically depends on the quality of the interface (3, 8, 9). Obtaining accurate, i.e., substrate-specific, measurements of lipase activity as well as developing reliable lipase assay systems requires taking these unique features into account.

Many biotechnological applications for lipases have been described in the food, cosmetic, detergent, and pharmaceutical industries (3, 10, 11), and an interest exists for new sources of this kind of enzyme in an industrial setting.

Novel lipases can be obtained either by isolating them from various natural sources or by using classical protein engineering methods and/or directed evolution procedures (12–14). All these studies require convenient, sensitive, and specific assays for measuring lipase activity (15). In addition, screening procedures require continuous assays and substrate stability that are compatible with high sample throughput.

Considerable progress has been made in the past few years in the development of high-throughput screening (HTS) methods for carboxyl-ester hydrolases. These HTS assays have been developed using chromogenic or fluorogenic substrates such as butyrate, octanoate, and palmitate of nitrophenol or 4-methylumbelliferone. However, all of these synthetic esters are liable to undergo nonenzymatic alkaline hydrolysis as well as hydrolysis by the nonspecific carboxyl-ester hydrolases often present in biological

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Abbreviations: BCG, bromocresol green; BtA, butyric acid; β-CD, β-cyclodextrin; HTS, high-throughput screening; NaTDC, sodium taurodeoxycholate; OcA, octanoic acid; OD, optical density; PHIBLA, pH indicator-based lipase assay; *p*NPE, *p*-nitrophenyl ester; PPL, porcine pancreatic lipase; rAnFaeA, recombinant feruloyl esterase A from *Aspergillus niger*; rDGL, recombinant dog gastric lipase; RGL, rabbit gastric lipase; rHPL, recombinant human pancreatic lipase; TG(4:0), tributyrin; TG(8:0), tricaprylin; THL, tetrahydrolipstatin; TLL, *Thermomyces lanuginosus* lipase; USP, United States Pharmacopeia; YLLIP2, LIP2 lipase from *Yarrowia lipolytica*.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of text and four figures.

samples. Furthermore, the catalytic turnover number of lipases on *p*-nitrophenyl esters (*p*NPEs) is usually several orders of magnitude lower than that obtained with TGs (16). Some true lipases, like pancreatic lipase, do not display enzyme activity on these substrates, but nonspecific titration of some surface amino acid residues by *p*NPEs has often been confused with a true enzyme activity (17, 18). This pseudo-esterase activity is in the same order of magnitude as the hydrolysis of *p*NPEs by serum albumin (19), and in that case, it was shown that the pseudo-esterase activity was the result of irreversible acylation of 82 residues and was not the result of turnover (20).

To replace nonspecific chromogenic substrates, continuous methods for measuring lipase activity with real lipase substrates were developed using fluorescent long-chain TGs from *Parinari glaberrimum* (21) and UV-absorbing TGs from *Aleurites frodii* seeds or tung oil (22). TGs from *Parinari glaberrimum* are, however, very sensitive to oxidation. The TGs extracted from tung oil and used in the HTS method reported by Serveau et al. (22) are less sensitive to oxidation when they are coated on the surface of UV microtiter plate wells. Tung oil contains α-eleostearic acid (23, 24), which is a conjugated triene giving absorption in the UV. However, this method required special UV microtiter plates. TGs with fluorescent pyrene acyl chains have been employed to measure lipase activity using a continuous and sensitive (moles of product per minute) assay (25), but these substrates are not real lipase substrates and are very expensive.

The short-chain tributyrin [TG(4:0)] substrate offers several advantages as a substrate for lipases compared with natural long-chain TGs. It is readily dispersed without the need for emulsifiers like gum Arabic used with olive oil, the products formed on hydrolysis are water-soluble, and can be titrated directly in a large range of pHs. This is a major advantage for setting up continuous assays at various pH values, while the direct and continuous titration of long-chain fatty acids can only be made at alkaline pH. Synthetic TG(4:0) substrate has thus been used in many studies of lipases (16, 26–31), although it has no physiological relevance, because all known lipases are active on this substrate. However, due to its partial water solubility, it can be hydrolyzed by some esterases that are not active on insoluble TGs. The use of tricaprylin [TG(8:0)] as a totally insoluble medium-chain TG substrate is thus more appropriate to detect and assay a true lipase activity, as demonstrated with various microbial and mammalian lipases (28). Moreover, the production of the soluble caprylic acid confers advantages for direct titration compared with long-chain fatty acids.

In a previous work (32), a spectrophotometric HTS protocol for the rapid and reliable determination of lipase/esterase activity was validated using short-chain [TG(4:0)] and medium-chain [TG(8:0)] emulsified TGs and a pH indicator. The principle of the method is the indirect quantification of fatty acid released by lipase through protonation of a pH indicator, *p*-nitrophenol, and loss of its yellow coloration followed by reading absorbance at 410 nm. This method is restricted, however, to assays performed at pH 7.2 due to the chemical properties of *p*-nitrophenol, and some lipases, like acid lipases, could not be tested under these conditions. A robust HTS method for screening lipase activities should cover a wide range of pHs to become universal.

In this work, six different pH indicators were used to continuously monitor the lipase activity on emulsified TG(4:0) and TG(8:0), at pH values ranging from 5.0 to 9.2. This pH indicator-based lipase assay (PHIBLA) was validated using acidic, neutral, and alkaline lipases and the lipase inhibitor tetrahydrolipstatin (THL).

MATERIALS AND METHODS

Chemicals

TG(4:0), TG(8:0), butyric acid (BtA), octanoic acid (OcA), BSA, NaCl, $CaCl_2$, sodium taurodeoxycholate (NaTDC), β-cyclodextrin (β-CD), bromocresol green (BCG), 3,4-dinitrophenol, chlorophenol red, *p*-nitrophenol, cresol red, thymol blue, malic acid, succinic acid, MES, MOPS, Tris, CHES, 2-methyl-2-propanol (*tert*-butanol), DMSO, and THL were purchased from Sigma-Aldrich.

Lipases

Recombinant human pancreatic lipase (rHPL) was expressed in *Pichia pastoris* and purified from culture media as described by Belle et al. (33). Porcine pancreatic extract, also named pancreatin (P7545; 8× USP), was purchased from Sigma-Aldrich. Porcine pancreatic lipase (PPL) was purified according to Verger et al. (34). Porcine colipase was partly purified from lipid-free pancreatic powder using the procedure described in Fernandez et al. (35). Rabbit gastric extract and purified rabbit gastric lipase (RGL) were produced according to Moreau et al. (36). Pure recombinant dog gastric lipase (rDGL) was a generous gift of Meristem Therapeutics (Clermont-Ferrand, France). The purified *Thermomyces lanuginosus* lipase (TLL) was a generous gift from Dr. S. Patkar (Novozymes, Denmark). LIP2 lipase from *Yarrowia lipolytica* (YLLIP2) was produced and purified according to Aloulou et al. (37). Recombinant feruloyl esterase A (rAnFaeA) from *Aspergillus niger* was produced and purified from culture media as described by Record et al. (38).

Lipase activity measurements using the pH-stat technique

Activities of rHPL, PPL, RGL, rDGL, TLL, YLLIP2, and rAnFaeA were assayed potentiometrically by automatically titrating the FFAs released from mechanically stirred TG emulsions [either TG(4:0) or TG(8:0)], using 0.1 N NaOH and a pH-stat device (799 GPT Titrino, Metrohm). Each assay was performed in a thermostated (37°C) vessel containing 0.5 ml TG (3.3% v/v) and 14.5 ml of a solution containing (rHPL, PPL, RGL, rDGL, TLL, YLLIP2, rAnFaeA) 150 mM NaCl, (rHPL, PPL, TLL, YLLIP2, rAnFaeA) 6 mM $CaCl_2$, (rHPL, TLL, rAnFaeA) 0.5 mM NaTDC, (PPL, RGL, rDGL) 2 mM NaTDC, (YLLIP2) 4 mM NaTDC, (RGL, rDGL) 1.5 μM BSA. Final concentrations were 114 mM and 68 mM for TG(4:0) and TG(8:0), respectively. The TGs were added directly to the pH-stat vessel containing the assay solution and were emulsified by mechanical stirring. Pancreatic lipase kinetics were recorded in the presence of a 5-fold molar excess of colipase to lipase. Corrections were made to take into account the partial ionization of BtA and OcA occurring at pH levels below 6.0. A blank assay without any lipase was performed at each initial pH to quantify spontaneous hydrolysis of each TG tested. One unit corresponds to 1 μmol of fatty acid released per minute in the assay conditions.

Lipase activity measurements using PHIBLA

Activities of rHPL, PPL, RGL, rDGL, TLL, YLLIP2, and rAnFaeA were determined according to a HTS method implemented in our laboratory, adapted from previous work (32), by measuring the apparition of FFAs upon hydrolysis of TG(4:0) or TG(8:0). Each

substrate was prepared as follows: one volume of the substrate (50 mM dissolved in *tert*-butanol), also containing the pH indicator, was mixed vigorously on a vortex with nine volumes of buffer solution to reach a final substrate concentration of 5 mM that was found to be optimum for lipase activity measurements (32). Stock solutions (10×) of pH indicators and buffer solutions were prepared at the concentrations indicated in **Table 1**. Buffer solution included (HPL, PPL, RGL, rDGL, TLL, YLLIP2, rAnFaeA) 150 mM NaCl, (HPL, PPL, RGL, rDGL, TLL, YLLIP2, rAnFaeA) 3 mg/ml β-CD, (HPL, PPL, TLL, YLLIP2, rAnFaeA) 6 mM $CaCl_2$, (HPL, TLL, rAnFaeA) 0.5 mM NaTDC, (PPL, RGL, rDGL) 2 mM NaTDC, (YLLIP2) 4 mM NaTDC, (RGL, rDGL) 1.5 μM BSA, (HPL, PPL) colipase at an excess of approximately 5 eq. Then, 20 μl of enzyme solution at an appropriate dilution in buffer was added in each microplate well and 100 μl of substrate emulsion was quickly added using an eight-channel pipette. Subsequently, the plate was placed in a microtiter plate scanning spectrophotometer (x-Mark™, Bio-Rad) and shaken for 5 s before each reading. The decrease in absorbance at a wavelength corresponding to the λ_{max} of the pH indicator (Table 1) was recorded every 30 s at 37°C. Blanks without enzyme were performed and data were collected at least in triplicate for 15 min. Different standard curves of BtA or OcA were established for each condition. One unit corresponds to 1 μmol of fatty acid released per minute in the assay conditions.

Study of lipase inhibition using PHIBLA

The enzyme-inhibitor preincubation method was used (39) to test whether any direct interactions might occur between the lipase and the inhibitor in aqueous medium and in the absence of substrate. Recombinant HPL (0.2 mg/ml in 25 mM MOPS buffer, pH 7.2) or RGL (0.2 mg/ml in 25 mM succinate buffer, pH 5.0) were preincubated at 25°C with THL (10 mM in DMSO) at an enzyme:inhibitor molar ratio of 1:100 (4% DMSO final concentration) in the presence or absence of 4 mM NaTDC. The residual enzyme activity was then measured at various incubation times using the microtiter plate assay with TG(4:0) as substrate.

Alternatively, the inhibition-during-lipolysis method (39) was used to test whether any inhibition reaction occurred in the presence of substrate during the lipolysis reaction. Lipolysis of TG(4:0) was performed in a microtiter plate using rHPL (1.3 nM, final concentration) or RGL (6.7 nM, final concentration). THL (at an enzyme/inhibitor molar ratio of 1:100) was injected 5 min after the lipolysis reaction started. Lipase activity was continuously recorded.

RESULTS AND DISCUSSION

Principle of the PHIBLA using emulsified TGs in microtiter plates

We developed a continuous spectrophotometric lipase assay in microtiter plates using emulsified TG(4:0) and TG(8:0), that are some of the most common substrates used to measure lipase activity, apart from natural long-chain TGs. The principle of the microtiter plate lipase assay is shown in **Fig. 1**. It consists of monitoring the progress of lipolysis, which releases a proton, through the use of a pH indicator which changes color as it is protonated.

The most important variables in using a pH indicator are the buffer and the ionization properties of the pH indicator (40). Both the buffer and the indicator must have the same affinity for protons (pKa values within 0.1 units of each other) so that the relative amounts of protonated buffer and protonated indicator stay constant as the pH shifts during the reaction. Also, the pKa of the indicator has to be close to the pH of the reaction mixture to ensure that changes in pH give a large and linear color change (41). The choice of the pH indicator therefore determines what type of lipase activity can be tested (acid, neutral, or alkaline).

In order to develop a spectrophotometric method that allows measuring lipolytic activities over a broad pH range, six different indicator-buffer pairs were chosen here for measuring activity at pH 5.0, 5.5, 6.0, 7.2, 8.2, and 9.2 (Table 1). The pKa of each indicator is similar to the pKa of the respective buffer used for the assay (difference <0.2 units of pKa). It is important to note that the rate of lipolysis in an acidic environment is difficult to measure using pH indicators, because the pKa values of the FFA released by the lipase (BtA and OcA) are close to 5 (pKa 4.82 and 4.89, respectively) and protons would be hardly detectable. Nevertheless, the use of short- and medium-chain TG substrates allows the assay to be performed at lower pH values (down to 5) than long-chain TG substrates containing fatty acids with much higher pKa values ranging from 6.5 to 9 (42). Moreover, long-chain FFA tends to remain adsorbed at the interface interacting with neighboring molecules, which have an important effect on pKa (43). β-CD can be useful to desorb the fatty acids (44) and consequently to improve linearity of the assay. Using TG(4:0) and TG(8:0) provides the advantage of the formation of FFA soluble into water at the assay temperature. TG substrates with a longer chain, like tridodecanoin, were also tested, but the release C12:0 fatty acid was found to be crystallized in solution and thus could not be used for spectrophotometric assays (supplementary Fig. 1).

Another important variable in the pH indicator assay is the concentration ratio of indicator and buffer. Maximum sensitivity is obtained at maximum concentration of

TABLE 1. Final concentrations of buffer-pH indicator pairs and maximum absorption wavelength (λ_{max}) for each deprotonated indicator solution employed for the microtiter plate lipase assay, at different pH values

pH	Buffer	Buffer Concentration (mM)	Indicator	Indicator Concentration (mM)	λ_{max}	Color[a]
5.0	Malate	1.5	BCG	0.25	610	Blue
5.5	Succinate	1.5	3,4-Dinitrophenol	0.5	410	Yellow
6.0	MES	2.5	Chlorophenol red	0.125	580	Red
7.2	MOPS	2.5	4-Nitrophenol	0.5	410	Yellow
8.2	Tris	2.5	Cresol red	0.2	580	Red
9.2	CHES	2.5	Thymol blue	0.4	600	Blue

[a]Color followed during the reaction of absorbance loss.

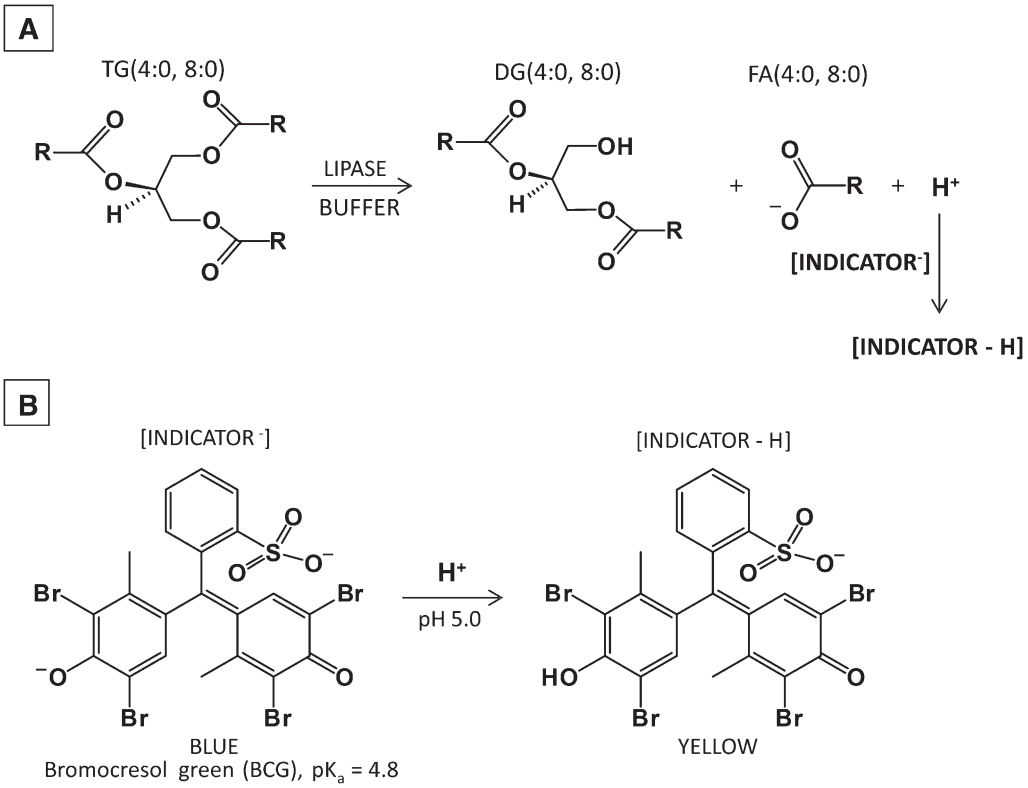

Fig. 1. Principle of PHIBLA. A: The model reaction of microtiter plate lipase assay. B: An example of the protonation of the pH indicator. Hydrolysis of a TG releases a proton, which protonates the pH indicator ion, causing a color change. DG, diacylglycerol.

indicator and minimum concentration of buffer (41). The concentration of indicator is limited by its solubility, and the concentration of buffer should be as low as possible, consistent with the condition that the pH does not change enough to cause a change in kinetic constants.

Indicator and buffer concentrations are shown in Table 1. Based on the pKa values of BtA and OcA, we chose a buffer concentration of 1.5 mM for assays at pH 5.0 and 5.5, and a buffer concentration of 2.5 mM for assays at pH 6 to 9.2. Indicator concentrations were chosen as a function of their solubility and their responsiveness to pH changes (sensitivity).

To correlate FFA levels with absorbance, the wavelength that allows detection of the highest change in the color of each indicator was first determined. Spectra of buffer solution containing indicator with and without OcA (deprotonated or protonated indicator, respectively) were done. **Figure 2A** shows an example of maximum absorption wavelength (λ_{max}) determination of BCG solution in malate

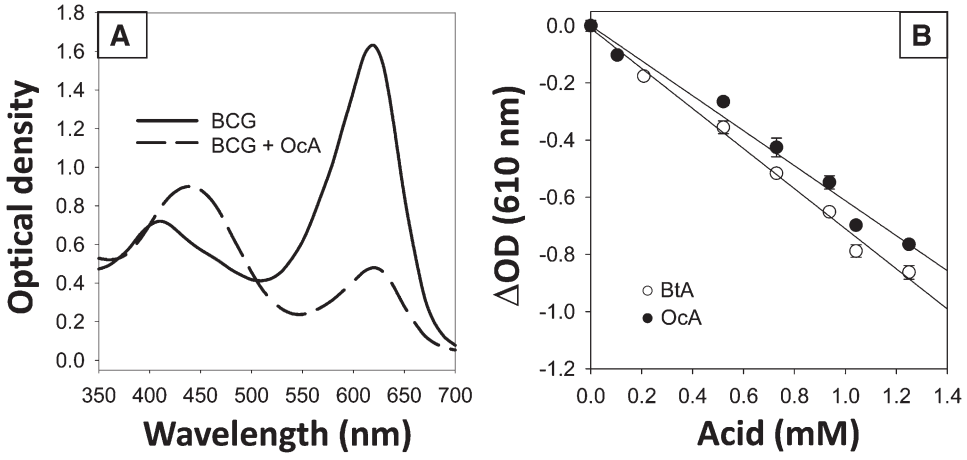

Fig. 2. Indirect detection of FFAs. A: UV absorption spectra of 2.5 mM BCG in a 1.5 mM malate buffer (pH 5.0) in the presence and absence of 5 mM OcA. B: The standard curves which correlate the decrease in absorbance at 610 nm with the concentration of BtA and OcA, emulsified in 1.5 mM malate buffer (pH 5.0) containing 2.5 mM BCG, 3 mg/ml β-CD, 150 mM NaCl, 2 mM NaTDC, and 1.5 μM BSA.

buffer (pH 5.0). Absorption spectra (350–700 nm) of deprotonated and protonated indicator were observed showing two peaks at 440 and 610 nm, corresponding to yellow and blue colors, respectively. The larger difference in the extinction coefficients of the protonated and deprotonated forms was observed at 610 nm, which gives good sensitivity in optical density (OD) variations. It was therefore decided to measure FFA release indirectly through the disappearance of the blue color. Similar absorption spectra were recorded for the other indicators and λ_{max} for observing the largest changes in absorbance upon protonation was determined for each indicator. The λ_{max} and color followed during the reaction for each indicator is shown in Table 1.

In order to develop a quantitative method, calibration curves of absorbance variations with BtA or OcA amounts were established for each experimental condition.

Figure 2B shows an example of a standard curve obtained with BtA and OcA at pH 5.0 with BCG. Linear variations in absorbance were observed while increasing fatty acid concentration, and the experimental slopes were 0.711 mM/OD and 0.6121 mM/OD for BtA and OcA, respectively. A steeper slope means more sensitivity of the method. Using linear regression, very good correlation factors (R^2) of 0.99 and 0.98 were obtained for BtA and OcA, respectively. The linearity in the standard curve implies the linearity of the enzyme assay. **Table 2** presents a variety of different slopes and R^2 values obtained from standard curves at different pH values. All standard curves were linear in experimental conditions assayed up from 0.1 to 1.5 mM FFA (final concentration), demonstrating that the quantification of enzymatic activity can be done. Each assay had to be tested against a blank sample (medium or buffer only) and lipase activity (units per milliliter) using the microtiter plate assay was calculated according to the following equation: Activity (units per milliliter) = $[(OD/min_{Reaction} - OD/min_{Blank})/S] \times$ (reaction volume/sample volume) × DF; where $OD/min_{Reaction}$ and OD/min_{Blank} are the rates for enzyme catalyzed reaction and blank sample, respectively; S is the standard curve slope in millimolar per OD; a ratio of six between the total reaction volume and the sample volume in the microtiter plate well that was chosen here; and DF is the dilution factor of the sample. One unit equals 1 μmole of FFA per minute.

Substrate preparation and emulsion stability

Emulsion quality is the main factor affecting substrate availability and reproducibility of the method. Moreover a great and stable interfacial area is needed to obtain measurable kinetic reads. This can be achieved by adding a detergent (32). NaTDC, a bile salt that is widely used in standard tests of lipase activity (45–47), can be used for this purpose at a minimum concentration of 0.5 mM. This small amount of detergent allows for the formation of a TG(8:0) emulsion that is stable for at least 60 min with a mean particle size of 2–3 μm [supplementary Fig. 2(3), (4)]. TG(4:0) emulsion (mean particle size about 25 μm) was found to be less stable and had to be used during a time interval of 15 min after being prepared [supplementary Fig. 2(1), (2)].

On the other hand, insoluble TGs, like TG(8:0), produce turbid emulsions which may interfere with the spectroscopic measurements. An overall substrate concentration of 5 mM was selected for the assays. At this concentration, turbidity may cause background noise across the visible spectrum, which increases with the concentration of detergent, but this does not affect the determination of reaction rate.

To achieve reliable and reproducible results, substrate emulsion should always be prepared just before the assay and in the same manner, considering speed and time of stirring, test tube dimensions, and the size of pipette tip used to dispense substrate drop wise into the microtiter plate wells.

Kinetics of lipases using PHIBLA

To validate PHIBLA, lipases with different pHs optima in the hydrolysis of TGs were used. RGL and rDGL are known to display an optimal activity at an acidic pH (36, 46). rHPL and PPL prefer to catalyze the hydrolysis of TG at neutral or slightly alkaline pH (48, 49). TLL is known to work optimally at alkaline pH (45). A particular case is YLLIP2, whose optimum pH of TG hydrolysis depends on the amount of detergent (NaTDC) present in the assay (45). An esterase acting at acidic pH, rAnFaeA, was also

TABLE 2. Absorbance variations with FFA concentration at different conditions and pH values

	BtA		OcA	
pH	Slope	R^2	Slope	R^2
5[a,b]	−0.711	0.99	−0.6121	0.98
5.5[a,b]	−0.3468	0.99	−0.359	0.99
6[c]	−0.9096	0.99	−0.9368	0.99
7.2[d]	−0.6552	0.99	−0.6813	0.99
8.2[a]	−0.8671	0.99	−0.9495	0.99
9.2[d]	−1.1457	0.99	−1.2368	0.99

The slope values of standard curves and correlation coefficients (R^2) were obtained by linear regression.
[a]NaTDC 2 mM.
[b]BSA 1.5 μM.
[c]NaTDC 4 mM.
[d]NaTDC 0.5 mM.

used to verify the specificity of the method. This esterase can hydrolyze the soluble fraction of TG(4:0), while it has no activity on TG(8:0).

Typical kinetics of the decrease in OD during the hydrolysis of TG(4:0) and TG(8:0) at different pHs are shown in **Fig. 3** for RGL (Fig. 3A; 39.6 ng of RGL/microwell; pH 5.0), rHPL (Fig. 3C; 7.68 ng of rHPL/microwell; pH 7.2), and TLL (Fig. 3E; 3.7 ng of TLL/microwell; pH 9.2). A greater sensitivity of the assay was observed at alkaline pH. Lag times were not observed in any case before reaching the steady-state reaction rate and linear kinetics were recorded at least for the first 5 min of the reaction. It is worth noticing that the background noise measured in blanks without enzyme increased with the pH, probably because of some spontaneous hydrolysis of substrate occurring at alkaline pH. Blank assays were therefore performed for all pH values and subtracted from the assays with enzyme.

The effects of lipase amounts on the steady-state reaction rate were tested using the same lipases (Fig. 3). Whatever the substrate [TG(4:0) or TG(8:0)], enzyme activity

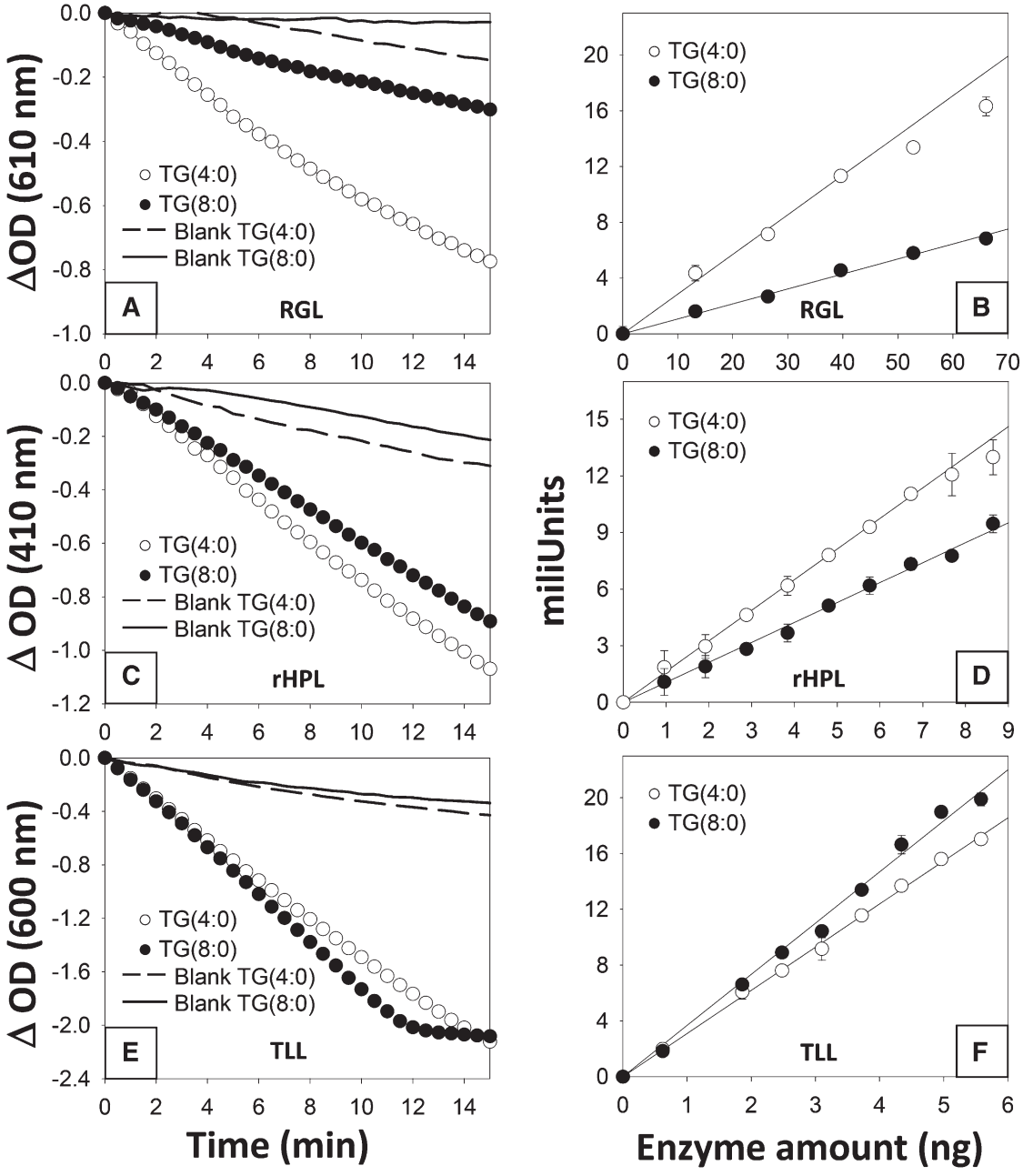

Fig. 3. Lipase activities of RGL, rHPL, and TLL using PHIBLA, TG(4:0), and TG(8:0) as substrates. A, C, E: Typical kinetics of OD decrease during the hydrolysis of TG(4:0) and TG(8:0) by RGL (39.6 ng), rHPL (7.7 ng), and TLL (8.4 ng) at pH 5.0, 7.2, and 9.2, respectively, are shown, as well as the blanks performed without enzyme for each reaction. B, D, F: The effect of the amount of enzyme on the steady-state reaction rate using TG(4:0) and TG(8:0) as substrate is shown. Reactions were carried out with variable amounts of RGL, rHPL, or TLL at pH 5.0, 7.2, and 9.2, respectively, and a substrate concentration of 5 mM. The decrease in the OD was recorded for 15 min and the maximum velocity (ΔOD per minute) was taken into account for activity determination.

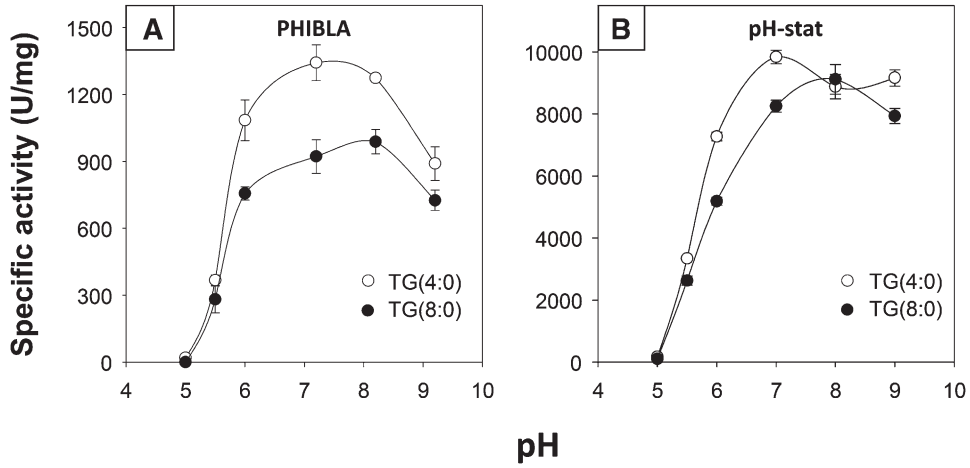

Fig. 4. pH profile of rHPL activity during hydrolysis of TG(4:0) and TG(8:0) using PHIBLA (A) and pH-stat technique (B). Reactions were carried out at 37°C. Buffer solutions contained 2.5 mM MOPS, 150 mM NaCl, 6 mM $CaCl_2$, 0.5 mM NaTDC, and 5-fold molar excess of colipase. β-CD at a concentration of 3 mg/ml was used for PHIBLA. TG(4:0) and TG(8:0) concentrations were 5 mM for both substrates using PHIBLA and 114 mM and 68 mM, respectively, using the pH-stat. Stirring was orbital for PHIBLA and with an impeller for pH-stat. Maximum velocity was taken into account for activity determination.

was found to increase linearly with enzyme amounts up to 40 ng/well for RGL (Fig. 3B; R^2 = 0.99 and 0.97, respectively; pH 5.0) and rDGL (R^2 = 0.99 and 0.99, respectively; pH 5.5), 8 ng/well for rHPL (Fig. 3D; R^2 = 0.99 and 0.99, respectively; pH 7.2), 12 ng/well for PPL (R^2 = 0.99 and 0.99, respectively; pH 8.2), 6 ng/well for TLL (Fig. 3F; R^2 = 0.99 and 0.99; pH 9.2), and YLLIP2 (R^2 = 0.99 and 0.97, respectively; pH 6.0) and 200 ng/well for rAnFaeA (R^2 = 0.99; pH 5.0). It is noteworthy that the rAnFaeA esterase was active only on TG(4:0), and the amount of enzyme required was much higher than for a lipase.

Comparison of the lipase-specific activities determined by PHIBLA in microtiter plates and by the pH-stat technique

The pH-stat method is generally used as a reference lipase assay (50, 51). This is a convenient technique for characterizing lipase activity and specificity, and it has been used with partly water-soluble substrates for studying the interfacial activation of lipases (9, 29, 52, 53). The lipase activity is determined using a mechanically stirred emulsion of a natural or synthetic TG, by continuously titrating the FFAs released upon substrate hydrolysis by NaOH, thus keeping the pH at a constant end point value.

The pH-dependent activity profiles of rHPL on TG(4:0) and TG(8:0) were recorded with PHIBLA and were compared with those obtained with the pH-stat technique at the same pH values (**Fig. 4**). The maximum specific activities on TG(4:0) were obtained at pH 7.2 using both PHIBLA (1,343 ± 80 U/mg) and the pH-stat technique (10,474 ± 654 U/mg). Similarly, the maximum specific activities on TG(8:0) were obtained at the same pH value of 8.2 using PHIBLA (922 ± 76 U/mg) and the pH-stat technique (8,949 ± 284 U/mg). Despite differences in the two methods (e.g., substrate concentration, agitation, presence of alcohols), similar pH-dependent activity profiles were obtained. Hydrolysis rates, however, were found to be around 10 times higher with the pH-stat technique than with the PHIBLA microtiter plate assay.

Table 3 shows a comparison of specific activities of pure enzymes on TG(4:0) and TG(8:0) obtained by means of PHIBLA and the pH-stat technique at distinct pH values. Although the specific activities obtained with PHIBLA were 2- to 20-fold lower than those recorded with the

TABLE 3. Comparison of the specific activities (units per milligram) of pure enzymes determined with the pH-stat technique and PHIBLA

		pH-stat			PHIBLA		
Enzyme	pH	TG(4:0)	TG(8:0)	TG(4:0)/ TG(8:0) Ratio	TG(4:0)	TG(8:0)	TG(4:0)/ TG(8:0) Ratio
RGL	5	1,200 ± 142	401 ± 25	3.0	330 ± 15	103 ± 8	3.2
rDGL	5.5	469 ± 11	853 ± 95	0.5	237 ± 15	256 ± 36	0.9
YLLIP2	6	9,239 ± 1378	16,300 ± 1,099	0.6	736 ± 42	894 ± 8	0.8
rHPL	7.2	10,474 ± 654	8,949 ± 284	1.2	1,343 ± 80	922 ± 76	1.5
PPL	8.2	6,929 ± 86	6,217 ± 645	1.1	2,476 ± 18	1,412 ± 73	1.8
TLL	9.2	6,252 ± 685	10,885 ± 183	0.6	2,356 ± 105	2,901 ± 206	0.8
rAnFaeA	5	3.7 ± 0.1	ND	ND	58 ± 3	ND	ND

ND, not determined.

pH-stat technique, the ratios of activities on TG(4:0) and TG(8:0) were found to be similar for both methods and each lipase.

The fact that purified lipases were found to be more active with the pH-stat assay than with the PHIBLA microplate method is probably related to a better emulsification of substrate with the mechanical stirring of the pH-stat device, thus generating a larger accessible surface for the lipase (54). The opposite phenomenon was observed for rAnFaeA esterase, which was 15 times more active in catalyzing the hydrolysis of TG(4:0) using PHIBLA than using the pH-stat technique. This might be attributed to the addition of co-solvent, because addition of *tert*-butanol to the emulsion was also found to increase (35-fold) the rate of TG(4:0) hydrolysis by rAnFaeA measured with the pH-stat technique (data not shown).

Study of the effect of pH on enzyme activity using PHIBLA

The effect of pH on the lipase-catalyzed hydrolysis of TG(4:0) and TG(8:0) was determined using PHIBLA (**Fig. 5**). Overall, the pH-dependent activity profiles were similar to those obtained by the pH-stat technique. PHIBLA is however limited to activity measurements in the pH 5.0–9.2 range with intervals of 0.5–1 unit, and therefore may not be adapted for determining the pH optimum of some enzymes

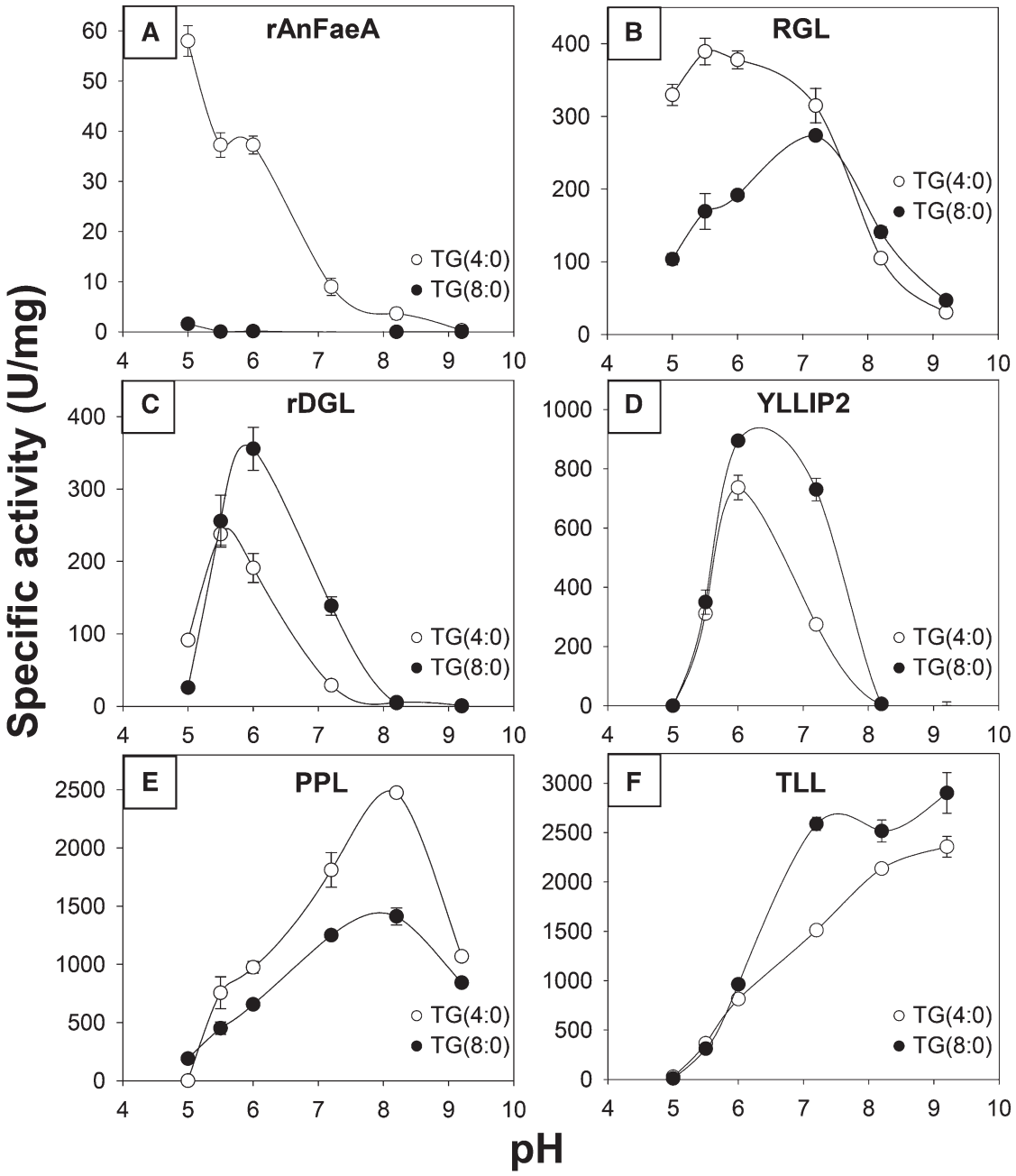

Fig. 5. Effect of pH on the activity of rAnFaeA (A), RGL (B), rDGL (C), YLLIP2 (D), PPL (E), and TLL (F) on TG(4:0) and TG(8:0) measured using PHIBLA. The decrease in the OD was recorded for 15 min and the maximum velocity (ΔOD per minute) was taken into account for activity determination.

like rAnFaeA esterase (Fig. 5A) and TLL (Fig. 5F) that have optimum pH below pH 5 and above pH 9, respectively.

The maximum activity was observed at pH 5.5 for RGL using TG(4:0) (Fig. 5B), similarly to what was reported by Moreau et al. (36), at pH 5.5 and 6 for rDGL using TG(4:0) and TG(8:0), respectively (Fig. 5C), similarly to what was reported by Carrière et al. (46), at pH 6.0 for YLLIP2 using both TGs with 4 mM NaTDC (Fig. 5D), similarly to what was reported by Aloulou and colleagues (37, 45), at pH 8.2 for PPL using both TGs (Fig. 5E), similarly to what was reported by Borgström and Erlanson (49), at pH 9.2 for TLL (Fig. 5F; in both TGs), similarly to what was reported by Aloulou et al. (45). It is worth noting that all lipases were active on both substrates [TG(4:0) and TG(8:0)], and, as expected, rAnFaeA esterase activity was observed only in the emulsion of short-chain TG [TG(4:0)], with a maximum activity at pH 5.0 (Fig. 5A). The effect of pH on the activity of rAnFaeA on TGs has not been reported so far. Only the optimum pH in the hydrolysis of methyl ferulate was found to be pH 5.0 (55).

PHIBLA was also tested with crude lipase preparations like rabbit gastric extracts containing RGL, porcine pancreatic extracts containing PPL, and the culture medium of a recombinant strain of *Pichia pastoris* producing TLL. This enabled us to confirm that highly purified RGL (Fig. 5B), PPL (Fig.5D), TLL (Fig. 5F), and their corresponding crude enzyme preparations showed similar pH-dependent activity profiles (supplementary Fig. 3). No major interference of other compounds found in crude enzyme preparations could therefore be identified.

Study of lipase inhibition using PHIBLA

In order to validate a possible application of the PHIBLA assay in the screening of lipase inhibitors, THL, a well-known covalent inhibitor of digestive lipases (56, 57), was used. Because the inhibition by rHPL and RGL by THL has been extensively studied with other methods (pH-stat technique, monomolecular films), these lipases were chosen for testing the PHIBLA assay.

Pre-incubation of either rHPL or RGL with THL (at a molar ratio of 1:100) was performed in the absence or in the presence of bile salts at 25°C and pH 7.2 or 5.0, respectively. The residual lipase activity was measured with PHIBLA

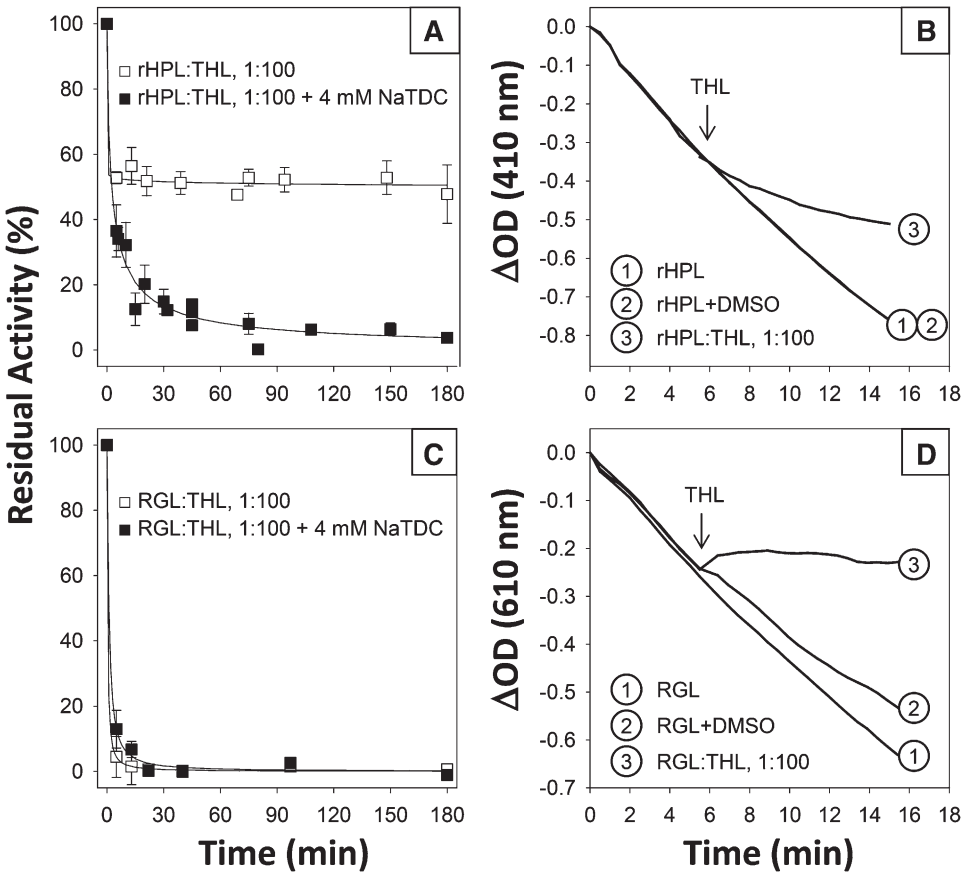

Fig. 6. Study of lipase inhibition using PHIBLA. A, C: The residual activity of rHPL and RGL, respectively, acting on a TG(4:0) emulsion, as a function of the preincubation time with THL. The incubations were carried out in the absence (empty boxes) or in the presence (filled boxes) of 4 mM NaTDC in the incubation medium. B, D: The effect of THL on the rate of hydrolysis of TG(4:0) by rHPL and RGL, respectively. Kinetic 1, enzymatic reaction without inhibitor; kinetic 2, enzymatic reaction with the injection of DMSO after 5 min; kinetic 3, enzymatic reaction with the injection of THL after 5 min. Inhibition assays were carried out at an enzyme:inhibitor molar excess of 1:100 at pH 5.0 (for RGL) or pH 7.2 (for rHPL). Curves are representative of at least three independent experiments.

and TG(4:0) emulsions at 37°C, using samples taken from the incubation medium.

In the presence of 4 mM NaTDC and colipase in the incubation medium, a rapid and nearly complete reduction of rHPL activity (initial velocity) was observed, whereas, in the absence of NaTDC in the incubation medium, THL partially inhibited HPL and a plateau value at around 50% of the initial activity was rapidly reached when using TG(4:0) as substrate (**Fig. 6A**). Similar kinetics were observed by Tiss et al. (26) in experiments carried out using the pH-stat technique at pH 8.0.

On the other hand, after a few minutes of incubation with THL, the RGL initial activity measured on TG(4:0) (Fig. 6C) was drastically reduced either in the presence or absence of NaTDC. Furthermore, RGL was completely inactivated after 30 min. Gargouri et al. (58) observed that the presence of NaTDC at a final concentration of 4 mM in the incubation medium did not affect RGL inhibition in assays carried out at pH 6.0 with the pH-stat technique. However, they observed a remaining activity of 30% after 2 h of incubation.

PHIBLA was also used to study lipase inhibition during lipolysis. The hydrolysis of TG(4:0) was recorded at pH 7.2 with rHPL and at pH 5.0 with RGL. Five minutes after the start of the reaction, THL was added at a molar ratio of 1:100. As can be seen from the kinetics shown in Fig. 6, TG(4:0) hydrolysis decreased rapidly by around 70% in reactions catalyzed by rHPL (Fig. 6B) and was completely stopped in reactions catalyzed by RGL (Fig. 6D), when adding THL. Control experiments were conducted with DMSO only in order to see the effect of the solvent in which THL solution was prepared. The rate of TG(4:0) hydrolysis decreased by around 25% in reactions catalyzed by RGL (Fig. 6D), whereas there was no effect on the reactions catalyzed by rHPL.

PHIBLA may be a convenient assay for the first line screening of lipase inhibitors before performing a more detailed study on the specificity and mechanism of action of these inhibitors.

CONCLUSION

The PHIBLA in microtiter plates is a convenient and rapid assay to measure lipase activity on short- and medium-chain TGs, by performing continuous kinetic recordings at different pH values ranging from 5.0 to 9.2. Despite the fact that the lipase-specific activities measured with this method were two to twenty times lower than those obtained with the pH-stat technique, the limit of detection was highly satisfactory and the method could be used to detect amounts as low as 1 μg of FFA, and 1 ng of enzyme. Substrate emulsion was easy to prepare and was stable for at least 15 min, the period of time needed for recording the reaction kinetics. In addition, PHIBLA was a convenient assay for studying the substrate preference of lipases between short [TG(4:0)] and medium [TG(8:0)] acyl chain TGs. Well-known lipases were able to hydrolyze both substrates while a model esterase like rAnFaeA could only hydrolyze TG(4:0), which is partly soluble in water. PHIBLA could therefore be used to discriminate lipase and esterase activities. PHIBLA also provides a useful tool for HTS of lipase and lipase inhibitor in various biological samples. It can also be used as a quick determination of enzyme activity during purification of lipases/esterases (data not shown). In addition, the principle of PHIBLA for detecting the production of FFA at various pHs should allow the use of other substrates with a carboxylic ester function (i.e., methyl esters, phospholipids, etc.) and this paves the way to future developments of PHIBLA.

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REFERENCES

1. Jaeger, K. E., S. Ransac, B. W. Dijkstra, C. Colson, M. van Heuvel, and O. Misset. 1994. Bacterial lipases. *FEMS Microbiol. Rev.* **15:** 29–63.
2. Mukherjee, K. D. 1994. Plant lipases and their application in lipid biotransformations. *Prog. Lipid Res.* **33:** 165–174.
3. Schmid, R. D., and R. Verger. 1998. Lipases: interfacial enzymes with attractive applications. *Angew. Chem. Int. Ed.* **37:** 1608–1633.
4. Carrière, F., E. Rogalska, C. Cudrey, F. Ferrato, R. Laugier, and R. Verger. 1997. In vivo and in vitro studies on the stereoselective hydrolysis of tri- and diglycerides by gastric and pancreatic lipases. *Bioorg. Med. Chem.* **5:** 429–435.
5. Lowe, M. E. 2002. The triglyceride lipases of the pancreas. *J. Lipid Res.* **43:** 2007–2016.
6. Verger, R., and G. H. de Haas. 1973. Enzyme reactions in a membrane model. 1: A new technique to study enzyme reactions in monolayers. *Chem. Phys. Lipids.* **10:** 127–136.
7. Verger, R., and G. H. de Haas. 1976. Interfacial enzyme kinetics of lipolysis. *Annu. Rev. Biophys. Bioeng.* **5:** 77–117.
8. Panaitov, I., and R. Verger. 2000. Enzymatic reactions at interfaces: interfacial and temporal organization of enzymatic lipolysis. *In* Physical Chemistry of Biological Interfaces. A. Baszkin and W. Norde, editors. Marcel Dekker, Inc., New York, Basel. 359–400.
9. Verger, R. 1997. "Interfacial activation" of lipases: facts and artifacts. *Trends Biotechnol.* **15:** 32–38.
10. Bornscheuer, U. T., J. Altenbuchner, and H. H. Meyer. 1999. Directed evolution of an esterase: screening of enzyme libraries based on pH-indicators and a growth assay. *Bioorg. Med. Chem.* **7:** 2169–2173.
11. Jaeger, K. E., B. W. Dijkstra, and M. T. Reetz. 1999. Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annu. Rev. Microbiol.* **53:** 315–351.
12. Arnold, F. H., L. Giver, A. Gershenson, H. Zhao, and K. Miyazaki. 1999. Directed evolution of mesophilic enzymes into their thermophilic counterparts. *Ann. N. Y. Acad. Sci.* **870:** 400–403.
13. Bloom, J. D., M. M. Meyer, P. Meinhold, C. R. Otey, D. MacMillan, and F. H. Arnold. 2005. Evolving strategies for enzyme engineering. *Curr. Opin. Struct. Biol.* **15:** 447–452.
14. Liebeton, K., A. Zonta, K. Schimossek, M. Nardini, D. Lang, B. W. Dijkstra, M. T. Reetz, and K. E. Jaeger. 2000. Directed evolution of an enantioselective lipase. *Chem. Biol.* **7:** 709–718.
15. Beisson, F., A. Tiss, C. Rivière, and R. Verger. 2000. Methods for lipase detection and assay: a critical review. *Eur. J. Lipid Sci. Technol.* **102:** 133–153.
16. Moreau, H., A. Moulin, Y. Gargouri, J. P. Noel, and R. Verger. 1991. Inactivation of gastric and pancreatic lipases by diethyl p-nitrophenyl phosphate. *Biochemistry.* **30:** 1037–1041.
17. De Caro, J. D., P. Rouimi, and M. Rovery. 1986. Hydrolysis of p-nitrophenyl acetate by the peptide chain fragment (336-449) of porcine pancreatic lipase. *Eur. J. Biochem.* **158:** 601–607.

18. De Caro, J., M. Chautan, P. Rouimi, and M. Rovery. 1988. Acetylation of Lys-373 in porcine pancreatic lipase after reaction of the enzyme or its C-terminal fragment with p-nitrophenyl acetate. *Biochimie.* **70:** 1785–1790.
19. Sakurai, Y., S-F. Ma, H. Watanabe, N. Yamaotsu, S. Hirono, Y. Kurono, U. Kragh-Hansen, and M. Otagiri. 2004. Esterase-like activity of serum albumin: characterization of its structural chemistry using p-nitrophenyl esters as substrates. *Pharm. Res.* **21:** 285–292.
20. Lockridge, O., W. Xue, A. Gaydess, H. Grigoryan, S. J. Ding, L. M. Schopfer, S. H. Hinrichs, and P. Masson. 2008. Pseudo-esterase activity of human albumin: slow turnover on tyrosine 411 and stable acetylation of 82 residues including 59 lysines. *J. Biol. Chem.* **283:** 22582–22590.
21. Beisson, F., N. Ferte, J. Nari, G. Noat, V. Arondel, and R. Verger. 1999. Use of naturally fluorescent triacylglycerols from Parinari glaberrimum to detect low lipase activities from Arabidopsis thaliana seedlings. *J. Lipid Res.* **40:** 2313–2321.
22. Serveau-Avesque, C., R. Verger, J. A. Rodriguez, and A. Abousalham. 2013. Development of a high-throughput assay for measuring lipase activity using natural triacylglycerols coated on microtiter plates. *Analyst.* **138:** 5230–5238.
23. Radunz, A., P. He, and G. H. Schmid. 1998. Analysis of the seed lipids of Aleurites montana. *Z. Naturforsch.* **53:** 305–310.
24. Scrimgeour, C. M., and J. L. Harwood. 2007. Fatty acid and lipid structure. *In* The Lipid Handbook. F. D. Gunstone, J. Harwood, and A. J. Dijkstra, editors. Taylor & Francis Group, LLC, Boca Raton, FL. 1–16.
25. Duque, M., M. Graupner, H. Stütz, I. Wicher, R. Zechner, F. Paltauf, and A. Hermetter. 1996. New fluorogenic triacylglycerol analogs as substrates for the determination and chiral discrimination of lipase activities. *J. Lipid Res.* **37:** 868–876.
26. Tiss, A., H. Lengsfeld, F. Carriere, and R. Verger. 2009. Inhibition of human pancreatic lipase by tetrahydrolipstatin: further kinetic studies showing its reversibility. *J. Mol. Catal., B Enzym.* **58:** 41–47.
27. Carrière, F., C. Renou, S. Ransac, V. Lopez, J. De Caro, F. Ferrato, A. De Caro, A. Fleury, P. Sanwald-Ducray, H. Lengsfeld, et al. 2001. Inhibition of gastrointestinal lipolysis by Orlistat during digestion of test meals in healthy volunteers. *Am. J. Physiol. Gastrointest. Liver Physiol.* **281:** G16–G28.
28. Chahinian, H., and L. Sarda. 2009. Distinction between esterases and lipases: comparative biochemical properties of sequence-related carboxylesterases. *Protein Pept. Lett.* **16:** 1149–1161.
29. Ferrato, F., F. Carrière, L. Sarda, and R. Verger. 1997. A critical reevaluation of the phenomenon of interfacial activation. *Methods Enzymol.* **286:** 327–347.
30. Bezzine, S., F. Ferrato, V. Lopez, A. de Caro, R. Verger, and F. Carrière. 1999. One-step purification and biochemical characterization of recombinant pancreatic lipases expressed in insect cells. *In* Lipase and Phospholipase Protocols. M. Doolittle and K. Reue, editors. Humana Press, New York. 187–202.
31. Carrière, F., K. Thirstrup, S. Hjorth, F. Ferrato, P. F. Nielsen, C. Withers-Martinez, C. Cambillau, E. Boel, L. Thim, and R. Verger. 1997. Pancreatic lipase structure-function relationships by domain exchange. *Biochemistry.* **36:** 239–248.
32. Mateos-Díaz, E., J. A. Rodríguez, M. A. Camacho-Ruiz, and J. C. Mateos-Díaz. 2012. High-throughput screening method for lipases/esterases. *In* Lipases and Phospholipases: Methods and Protocols. G. Sandoval, editor. Humana Press, New York. 89–100.
33. Belle, V., A. Fournel, M. Woudstra, S. Ranaldi, F. Prieri, V. Thome, J. Currault, R. Verger, B. Guigliarelli, and F. Carrière. 2007. Probing the opening of the pancreatic lipase lid using site-directed spin labeling and EPR spectroscopy. *Biochemistry.* **46:** 2205–2214.
34. Verger, R., G. H. de Haas, L. Sarda, and P. Desnuelle. 1969. Purification from porcine pancreas of two molecular species with lipase activity. *Biochim. Biophys. Acta.* **188:** 272–282.
35. Fernandez, S., V. Jannin, J-D. Rodier, N. Ritter, B. Mahler, and F. Carrière. 2007. Comparative study on digestive lipase activities on the self emulsifying excipient Labrasol, medium chain glycerides and PEG esters. *Biochim. Biophys. Acta.* **1771:** 633–640.
36. Moreau, H., Y. Gargouri, D. Lecat, J-L. Junien, and R. Verger. 1988. Purification, characterization and kinetic properties of the rabbit gastric lipase. *Biochim. Biophys. Acta.* **960:** 286–293.
37. Aloulou, A., J. A. Rodriguez, D. Puccinelli, N. Mouz, J. Leclaire, Y. Leblond, and F. Carriere. 2007. Purification and biochemical characterization of the LIP2 lipase from *Yarrowia lipolytica*. *Biochim. Biophys. Acta.* **1771:** 228–237.
38. Record, E., M. Asther, C. Sigoillot, S. Pages, P. J. Punt, M. Delattre, M. Haon, C. van den Hondel, J. C. Sigoillot, L. Lesage-Meessen, et al. 2003. Overproduction of the *Aspergillus niger* feruloyl esterase for pulp bleaching application. *Appl. Microbiol. Biotechnol.* **62:** 349–355.
39. Ransac, S., Y. Gargouri, F. Marguet, G. Buono, C. Beglinger, P. Hildebrand, H. Lengsfeld, P. Hadvary, and R. Verger. 1997. Covalent inactivation of lipases. *Methods Enzymol.* **286:** 190–231.
40. Janes, L. E., A. C. Lowendahl, and R. J. Kazlauskas. 1998. Quantitative screening of hydrolase libraries using pH indicators: identifying active and enantioselective hydrolases. *Chem. Eur. J..* **4:** 2324–2331.
41. Rosenberg, R. M., R. M. Herreid, G. J. Piazza, and M. H. O'Leary. 1989. Indicator assay for amino acid decarboxylases. *Anal. Biochem.* **181:** 59–65.
42. Benzonana, G. 1968. On the role of calcium ions during the hydrolysis of insoluble triglycerides by pancreatic lipase in the presence of bile salts. *Biochim. Biophys. Acta.* **151:** 137–146.
43. Egret-Charlier, M., A. Sanson, and M. Ptak. 1978. Ionization of fatty acids at the lipid–water interface. *FEBS Lett.* **89:** 313–316.
44. Alahverdjieva, V., M. Ivanova, R. Verger, and I. Panaiotov. 2005. A kinetic study of the formation of B-cyclodextrin complexes with monomolecular films of fatty acids and glycerides spread at the air/water interface. *Colloids Surf. B Biointerfaces.* **42:** 9–20.
45. Aloulou, A., D. Puccinelli, A. De Caro, Y. Leblond, and F. Carrière. 2007. A comparative study on two fungal lipases from *Thermomyces lanuginosus* and *Yarrowia lipolytica* shows the combined effects of detergents and pH on lipase adsorption and activity. *Biochim. Biophys. Acta.* **1771:** 1446–1456.
46. Carrière, F., H. Moreau, V. Raphel, R. Laugier, C. Bénicourt, J-L. Junien, and R. Verger. 1991. Purification and biochemical characterization of dog gastric lipase. *Eur. J. Biochem.* **202:** 75–83.
47. Rogalska, E., C. Cudrey, F. Ferrato, and R. Verger. 1993. Stereoselective hydrolysis of triglycerides by animal and microbial lipases. *Chirality.* **5:** 24–30.
48. Thirstrup, K., F. r. Carrière, S. Hjorth, P. B. Rasmussen, H. Wöldike, P. F. Nielsen, and L. Thim. 1993. One-step purification and characterization of human pancreatic lipase expressed in insect cells. *FEBS Lett.* **327:** 79–84.
49. Borgström, B., and C. Erlanson. 1973. Pancreatic lipase and colipase. *Eur. J. Biochem.* **37:** 60–68.
50. Desnuelle, P., M. J. Constantin, and J. Baldy. 1955. Potentiometric technic for the measurement of pancreatic lipase activity [article in French]. *Bull. Soc. Chim. Biol. (Paris).* **37:** 285–290.
51. Brockman, H. L. 1981. Triglyceride lipase from porcine pancreas. *Methods Enzymol.* **71:** 619–627.
52. Sarda, L., and P. Desnuelle. 1958. Actions of pancreatic lipase on esters in emulsions [article in French]. *Biochim. Biophys. Acta.* **30:** 513–521.
53. Entressangles, B., and P. Desnuelle. 1968. Action of pancreatic lipase on aggregated glyceride molecules in an isotropic system. *Biochim. Biophys. Acta.* **159:** 285–295.
54. Benzonana, G., and P. Desnuelle. 1965. Kinetic study of the action of pancreatic lipase on emulsified triglycerides. Enzymology assay in heterogeneous medium. *Biochim. Biophys. Acta.* **105:** 121–136.
55. Faulds, C. B., and G. Williamson. 1994. Purification and characterization of a ferulic acid esterase FAE-III from *Aspergillus niger*: specificity for the phenolic moiety and binding to microcrystalline cellulose. *Microbiology.* **140:** 779–787.
56. Hadváry, P., H. Lengsfeld, and H. Wolfer. 1988. Inhibition of pancreatic lipase in vitro by the covalent inhibitor tetrahydrolipstatin. *Biochem. J.* **256:** 357–361.
57. Ransac, S., Y. Gargouri, H. Moreau, and R. Verger. 1991. Inactivation of pancreatic and gastric lipases by tetrahydrolipstatin and alkyl-dithio-5-(2-nitrobenzoic acid). A kinetic study with 1,2-didecanoyl-sn-glycerol monolayers. *Eur. J. Biochem.* **202:** 395–400.
58. Gargouri, Y., H. Chahinian, H. Moreau, S. Ransac, and R. Verger. 1991. Inactivation of pancreatic and gastric lipases by THL and C12:0-TNB: a kinetic study with emulsified tributyrin. *Biochim. Biophys. Acta.* **1085:** 322–328.