Use of naturally fluorescent triacylglycerols from *Parinari glaberrimum* to detect low lipase activities from *Arabidopsis thaliana* seedlings[1]

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Abstract **The aim of this study was to design a convenient, specific, sensitive, and continuous lipase activity assay using natural long-chain triacylglycerols (TAGs). Oil was extracted from *Parinari glaberrimum* seed kernels and the purified TAGs were used as a substrate for detecting low levels of lipase activities. The purified TAGs are naturally fluorescent because more than half of the fatty acids from *Parinari* oil are known to contain 9,11,13,15-octadecatetraenoic acid (parinaric acid) in its esterified form. The presence of detergents (sodium taurodeoxycholate, CHAPS, Sulfobetaine SB12, Tween® 20, Brij® 35, Dobanol®, n-dodecylglucoside) above their critical micellar concentration dramatically increases the fluorescence of the parinaric acid released by various lipases. This increase in the fluorescence intensity is linear with time and proportional to the amount of lipase added. This new method, performed under non-oxidative conditions, was applied successfully to detecting low lipase levels in crude protein extracts from plant seeds and could be scaled down to microtiterplate measurements. Quantities as low as 0.1 ng of pure pancreatic lipase could be detected under standard conditions (pH 8). Lipase activity can also be assayed in acidic media (pH 5) using human gastric lipase. This simple and continuous assay is compatible with a high sample throughput and might be applied to detecting true lipase activities in various biological samples.**—Beisson, F., N. Ferté, J. Nari, G. Noat, V. Arondel, and R. Verger. **Use of naturally fluorescent triacylglycerols from *Parinari glaberrimum* to detect low lipase activities from *Arabidopsis thaliana* seedlings.** ***J. Lipid Res.*** **1999.** 40: **2313–2321.**

Supplementary key words lipase assay • fluorescent lipids • pancreatic lipase • plant lipase • parinaric acid • oil seed

Lipases (EC 3.1.1.3) can be simply defined as enzymes that catalyze the hydrolysis of triacylglycerols (TAGs), releasing long-chain fatty acids (1). They occur widely among many bacteria, fungi, plants, or animals. In addition to playing an important role in fat catabolism, they have been found to have numerous applications in the food, cosmetics, detergent, and pharmaceutical industries (2). Lipases with new specificities are needed and the engineering of cloned enzymes as well as the isolation of new lipases from natural sources is therefore of increasing potential value. However, purification procedures require reliable, convenient, and sensitive assays to be able to detect a true lipase activity in cellular homogenates and culture media.

A review of some of the lipase detection and assay methods is available on our laboratory's web site (http://lle-sg.cnrs-mrs.fr). Some lipases, such as digestive lipases, are fairly abundant in animal organs or their exocrine secretions, and their catalytic activity can easily be detected using titrimetric methods. Many other lipases are present in very small amounts in biological samples (for example in mammalian cells and plant seed homogenates). To detect them, for both purification, medical diagnosis, and food quality analysis purposes, more sensitive methods are required. The methods involving the use of radiolabeled substrates (3, 4) are very sensitive but cannot be monitored continuously and need time-consuming chromatographic or organic solvent separation steps to isolate the released fatty acids. Easy and continuous assays, compatible with a high sample throughput, have been developed using chromogenic or fluorogenic substrates. As far as chromogenic substrates are concerned, resorufin ester (1,2-O-dilau-

Abbreviations: BHT, butylhydroxytoluene; Brij® 35, dodecylpoly(oxyethyleneglycoether)$_{23}$; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; coumarin, 1,2-benzopyrone; dansyl, 5-dimethylamino-naphthalene-1-sulfonyl; CMC, critical micellar concentration; DMSO, dimethyl sulfoxide; Dobanol®, tetradecylpoly(oxyethyleneglycoether)$_7$; n-dodecylglucoside, 1-O-n-dodecyl-β-d-glucopyranoside; HGL, human gastric lipase; HLL, *Humicola lanuginosa* lipase; HPL, human pancreatic lipase; TLC, thin-layer chromatography; HP-TLC, high performance TLC; IU, international unit; NaTDC, sodium taurodeoxycholate; NBD, 7-nitrobenzo-2-oxa-1,3-diazol-4-yl; SEM, standard error to the mean; Sulfobetaine SB 12, N-dodecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate; TAG, triacylglycerol; THL, tetrahydrolipstatin; Tween® 20, poly(oxyethylene)$_n$-sorbitane-monolaurate.

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ryl-rac-glycero-3-glutaric acid-resorufin ester) is a sensitive "glycerol-derived" substrate available at Hoffmann-La Roche (previously Boehringer Mannheim), that can be used conveniently under some particular experimental conditions (5). Paranitrophenol acyl esters are also widely used as substrates because of their high detection sensitivity (6–9). However, the carbonyl function of these secondary esters is electronically activated. Its carbon atom bears a partial positive charge, due to the delocalization of electrons from the aromatic ring, which is facilitated by the electronic attraction of the NO_2 substituent. These esters are therefore liable to undergo non-enzymatic alkaline hydrolysis as well as hydrolysis by the non-specific esterases often present in biological samples. Furthermore, the catalytic turn-over number of true lipases on paranitrophenol acyl esters is usually several orders of magnitude lower than that obtained with TAGs (10).

Some fluorescent lipolytic enzyme assays were reviewed by Hendrickson (11). In the case of continuous lipase assays, the fluorophore group can be either coumarin, NBD, or pyrene. A first group of fluorogenic substrates consists of coumarin fatty acyl esters (12, 13) and lauroyl pyrenemethanol (14). These molecules are chemically unrelated to TAGs and can be hydrolyzed by non-specific esterases. A second group of fluorogenic substrates consists of dansyl-phosphatidylethanolamine (15) and NBD-phospholipids (16) which were reported as substrate for the phospholipase activity of lipoprotein lipase. Therefore, although it has been established that these two groups of esters are sensitive substrates, they cannot be used systematically to detect an as yet uncharacterized lipase. The use of a TAG containing fatty acyl chains in which a pyrene residue is linked to the ω position was first described by Negre et al. (17). However, this sensitive assay requires the isolation of the released pyrene-fatty acid. A quencher residue (trinitrophenylamine residue) was introduced by Duque et al. (18) as a means of lowering the basal fluorescence of this TAG containing acyl-pyrene (1-O-hexadecyl-2-pyrenedecanoyl-3-trinitrophenylaminododecanoyl-*sn*-glycerol and its enantiomer). This intramolecularly quenched TAG containing acyl-pyrene can therefore be used in a continuous fluorescent assay (19). Unfortunately, as can be seen from the data reported in Duque et al. (18), this kind of chemically modified TAG is poorly hydrolyzed by lipases, probably for steric reasons. The pyrene group was also used as a fluorophore in continuous assays of lecithin:cholesterol acyltransferase (20), cholesteryl ester hydrolase (21), phospholipase C (22), and phospholipases A (23, 24).

Parinaric acid is a naturally fluorescent fatty acid (9,11,13,15-octadecatetraenoic acid). Rogel, Stone, and Adebonojo (25) reported a spectrophotometric assay for lipase activity based on the displacement by the released oleic acid from triolein of parinaric acid, previously bound reversibly to bovine serum albumin. This sensitive method is, however, limited by the fact that detergents and calcium ions interfere with the assay. Because Wolf, Sagaert, and Bereziat (26) used a phospholipid containing parinaric acid to monitor a phospholipase A_2 activity, we decided to investigate whether TAGs containing parinaric acid might constitute a convenient and specific lipase substrate.

Here we describe the use of fluorescent TAGs extracted from *Parinari glaberrimum* seeds to detect low lipase levels in a simple and continuous assay performed under non-oxidative conditions.

MATERIAL AND METHODS

Preparation of the fluorescent TAG stock solution

Fluorescent TAGs were extracted from the seed kernels of *Parinari glaberrimum* (previously called *Parinarium laurinum*) a tree belonging to the Rosaceae family, which grows prolifically in the Fiji Islands (27). A stabilized crude lipidic extract from one seed of *Parinari glaberrimum* was generously provided to us by Dr. Klaubert and Dr. De Giulio (Molecular Probes, Inc.) and stored in the dark at −20°C under an argon atmosphere. This crude extract is commercially available from Molecular Probes Inc. on request.

Fifty mg of the crude lipidic extract was dissolved in 1 ml of diethylether containing 0.01% (w/v) butylhydroxytoluene (BHT) as antioxidant. The TAGs were isolated by preparative TLC under an argon atmosphere and the purity was checked by TLC (see below). Purified TAGs were stored in an ethanol solution in the presence of 0.01% (w/v) BHT (stock solution), under conditions identical to those for the crude lipidic extract. The stock solution concentration was determined by measuring the dry weight after evaporating ethanol under a stream of nitrogen and found to be 1.8 ± 0.1 mg/ml.

Preparation of the emulsions

After evaporating ethanol under a nitrogen stream, 3 mg of purified TAGs was placed in a 0.5-ml polypropylene microtube, and 100 μl of the following buffer was added: 50 mm Tris-HCl (pH 8) containing 3% gum arabic, 4 mm NaTDC, 100 mm NaCl, 6 mm $CaCl_2$, 0.001% (w/v) BHT. The microtube was kept closed under argon and the mixture was sonicated for 30 s in a sonicating bath (35 kHz and 30 W). In the case of the crude oil emulsions used in pH-stat experiments, 0.15 g of almond oil or 0.15 g of the crude lipidic diethylether extract from *Parinari glaberrimum* was placed in a 2-ml polypropylene microtube with 1.50 ml of a 10% gum arabic solution and sonicated for 2 min in a sonicating bath (35 kHz and 30 W).

Thin-layer chromatography (TLC)

One-dimensional analytic or preparative TLC was carried out under an argon atmosphere, using pre-cleaned high performance thin-layer plates (HP–TLC aluminium sheets or preparative TLC plates pre-coated with silica gel 60, Merck and Co., Inc.) with the following solvent system: heptane–diethylether–acetic acid 80:20:2 (v/v/v) containing 0.01% (w/v) BHT. Lipids were detected on the analytical TLC plate by charring after spraying the plate with a mixture (50:50 v/v) of saturating copper acetate and 85.5% phosphoric acid. Alternatively, fluorescence spots were detected on the preparative TLC plate under a UV lamp, and the gel corresponding to the spots was scraped. The TAGs were extracted by adding ethanol containing 0.01% (w/v) BHT to the silica gel.

Lipase sources

Native human pancreatic lipase (HPL) was purified from pancreatic juice as described by De Caro et al. (28). Colipase was purified from pig pancreas as described by Chapus, Desnuelle, and Fogglizzo (29). HPL and colipase were premixed (at a colipase/

HPL molar ratio of 10) and diluted with the standard buffer (pH 8) to a final HPL concentration of 200 pm. This diluted colipase/HPL solution was used for both fluorescent and chromogenic assays.

Recombinant human gastric lipase (rHGL) was produced and purified from insect cells (30) and was a generous gift from M. Rivière (LLE-Marseille).

A crude protein extract was obtained by grinding 100 mg of 3 day-germinated *Arabidopsis thaliana* seedlings in 10 ml of 100 mm Tris-HCl (pH 7.5), 100 mm NaCl. The homogenate was centrifuged at 18000 *g* for 30 min and the supernatant was used for the lipase assays. Control experiments were carried out by using lipase solutions or *Arabidopsis thaliana* extracts heated at 95°C for 5 min. Whenever possible, lipase activities were expressed in international units (IU). One IU corresponds to the release of 1 μmole of free fatty acid per minute.

Parinaric acid-based lipase assays

The buffer (990 μl) and the TAG stock solution (10 μl) were added consecutively to a quartz cuvette of 1.5 ml (optic pathlength 1 cm) containing a magnetic stirrer 8 mm in length. The final TAGs concentration was 18 μg per ml. The above mixture is slightly turbid and requires continuous stirring to ensure homogeneity. The cuvette was kept under nitrogen using a Teflon cap. After gently shaking the cuvette, it was left to equilibrate at 25°C. Lipase solution (1 to 50 μl) was injected and the fluorescence was read at regular intervals under continuous stirring in a spectrofluorimeter (SFM 25 from Kontron). Excitation was at 324 nm and emission at 420 nm. With HPL and *Arabidopsis thaliana* extracts, the following standard buffer (pH 8) was used: 4 mm NaTDC, 100 mm NaCl, 6 mm $CaCl_2$, 0.001% (w/v) BHT. For the rHGL activity measurements, the following acidic buffer was used: 20 mm sodium acetate (pH 5), 150 mm NaCl, 4 mm NaTDC, 0.001% (w/v) BHT, 0.01% (w/v) BSA.

Using the 96-well microplate assays, the TAG stock solution was diluted 100 times with the standard buffer (pH 8) and vortexed for 5 sec. We added 140 μl of the above diluted mixture and 10 μl of HPL solution to each microtiterplate well. Microtiterplates were vigourously shaken for 10 sec on a rotatory agitator and fluorescence readings were taken every minute for half an hour at 420 nm (excitation at 324 nm) with a fluorescence microtiterplate reader (Fluoroskan II, Labsystems).

Resorufin ester assay

Resorufin ester (1,2-O-dilauryl-*rac*-glycero-3-glutaric acid-resorufin ester) was purchased from Boehringer Mannheim (Cat. No. 1179 934). A resorufin ester solution was prepared by dissolving 10 mg of resorufin ester made in 1 ml of dioxane and stored in the dark at −20°C until use. Lipase assay was performed in 96-microtiterplate wells. The resorufin ester solution was diluted 10 times in the standard buffer (pH 8) and vortexed for 10 sec. We used 100 μl of the above diluted solution of resorufin ester and 50 μl of HPL solution in each microtiterplate well. The microtiterplates were automatically shaken for 5 sec after adding lipase and absorbance was read every minute during half an hour at 550 nm (reference filter: 630 nm) in a microtiterplate reader (MR5000, Dynatech).

Pyrene-based lipase assay

The substrate (1,2-dioleoyl-3-(pyren-1-yl-)-decanoyl-*rac*-glycerol) was purchased from Sigma-Aldrich Co. and dissolved in DMSO at a concentration of 1.85 mm. One μl of this solution and one μl of pure DMSO were added to 100 μl of standard buffer (pH 8) in 2-ml polypropylene microtubes. Ten μl of a HPL/colipase solution in standard buffer containing 100 to 1000 ng of HPL and a 10 molar excess of colipase was added. The lipolysis was stopped after 10 min by adding 1 μl of an ethanolic solution of THL (100 μm). NaCl 150 mm (92 μl) and NaOH 1 m (15 μl) were added, along with 1.8 ml of a mixture of organic solvents: methanol–chloroform–heptane 21:19:15 (v/v/v). The microtubes were centrifuged for 5 min at 16,000 *g* and the upper aqueous phases were collected. The fluorescence intensity (excitation: 340 nm, emission: 384 nm) of the aqueous phases was read in the same spectrofluorimetric cuvettes as the parinaric acid-based assay.

A calibration curve was set up by replacing the lipase solution in the above reaction media by standard buffer and the pure DMSO (1 μl) by 1 μl of DMSO containing various amounts (10 to 100 pmole) of pure pyrene decanoic acid (Sigma-Aldrich Co.). The solvent extraction and the fluorescence intensity measurements were carried out using the same procedures as those described above.

pH-stat measurements

The continuous release of fatty acids was assayed potentiometrically with a pH-stat apparatus under mechanical stirring in a reaction vessel at 37°C, upon adding 0.1 m NaOH. The 5-ml reaction medium contained 1.65 ml of crude oil emulsion and 3.35 ml of buffer so that the final salt and bile salt concentrations were identical to those of the standard buffer. The pH was adjusted to 8, and 5 μg of HPL was added together with 5 μg of colipase at zero time after recording the background level for 2 min.

RESULTS AND DISCUSSION

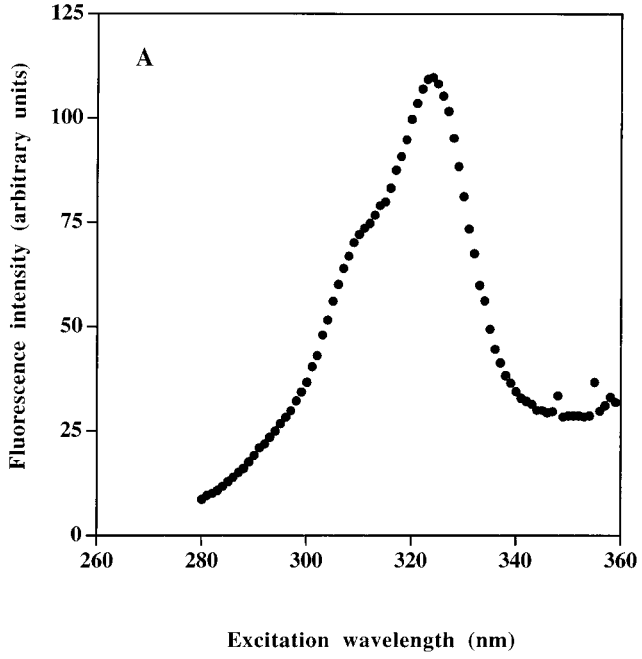
Lipolysis of TAG from *Parinari glaberrimum* can be monitored, in the presence of detergent, by recording the increase in the fluorescence intensity at 420 nm

More than half of the fatty acids extracted from *Parinari glaberrimum* oil are known to be 9,11,13,15-octadecatetraenoic acid (31), which is the *cis*, *trans*, *trans*, *cis* isomer: α-parinaric acid or *cis*-parinaric acid (32).

We determined a UV absorption spectrum of the TAG stock solution diluted 100-fold in ethanol (data not shown), which gave two peaks at 304 and 319 nm, as previously reported to occur in the case of pure α-parinaric acid in ethanol (33). Comparisons between the unique spectroscopic characteristics of pure parinaric acid and the absorption spectrum of our purified TAG confirmed that this fluorescent fatty acid was indeed present in the TAG stock solution.

Figure 1A shows the excitation spectrum of *Parinari glaberrimum* TAG dispersed in the standard buffer (pH 8). At the maximum excitation wavelength (324 nm) the emission spectrum displayed a broad bell-shaped curve centered around 420 nm (Fig. 1B). An increase in the fluorescence intensity of the emission spectrum was observed after adding HPL in the presence of 4 mm NaTDC, which was not accompanied by any wavelength shift (data not shown). To monitor the lipolysis we therefore selected 324 nm as the excitation wavelength and 420 nm as the emission wavelength.

In the absence of lipolysis (control experiment without HPL) bile salts have a negligible effect on the fluorescence measured at 420 nm (**Fig. 2A**). This observation confirms the absence of mixed micelles containing bile salts and pure TAG (34). However, when free fatty acids were released by HPL from the *Parinari glaberrimum* TAG,



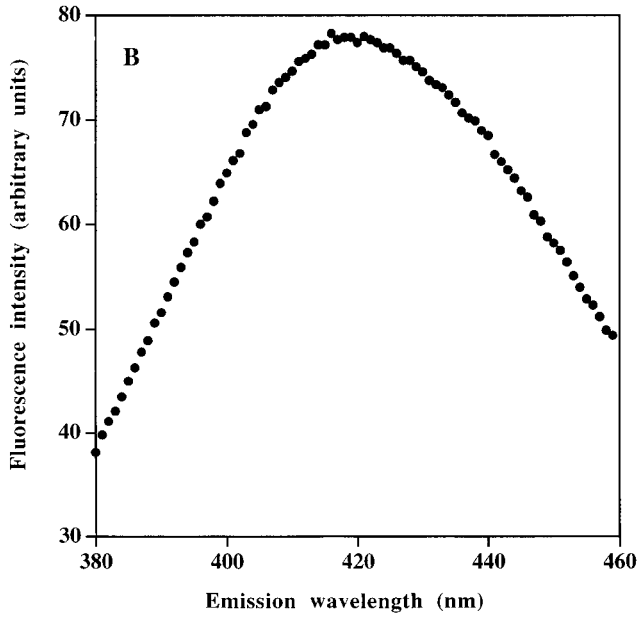


Fig. 1. Fluorescence excitation (A) and emission (B) spectra of purified *Parinari glaberrimum* TAGs dispersed by injection of the ethanolic solution in a standard buffer: 50 mm Tris-HCl (pH 8), 4 mm NaTDC, 100 mm NaCl, 6 mm $CaCl_2$, 0.001% (w/v) BHT. The final TAGs concentration was 18 μg/ml and the temperature was 25°C. Excitation wavelength was adjusted at 324 nm in the case of the emission spectrum, and emission wavelength was adjusted at 420 nm in the case of the excitation spectrum.

the emitted fluorescence intensity at 420 nm was greatly enhanced and a sigmoid-shaped curve was obtained with the half maximum intensity at around 2.5 mm NaTDC (Fig. 2A). The above results clearly show that a concentration of 4 mm NaTDC is required to be able to properly detect the fluorescent fatty acids released by HPL. In addition, one can conclude that negligible amounts of free parinaric acid were present in the purified TAG. It is worth noting that NaTDC, a ionic detergent, is not unique and can be replaced by other neutral or zwitterionic detergents (Fig. 2B). Increasing the detergent concentration did not increase the fluorescence signal (data not shown). However, subsequent addition of NaTDC (4 mm) to the media containing other detergents increased the fluorescence signal which reached about 80% (100% with CHAPS) of that obtained upon adding only 4 mm of NaTDC. This shows that NaTDC gives the most satisfactory fluorescence signal but that other detergents can be used as well.

The reasons for the increase in the fluorescence intensity in the phospholipase assay have been discussed in detail (26). The hydrolysis of a phospholipid containing parinaric acid, which occurred in the presence of albumin, was paralleled by a fluorescence hyperpolarization, which was thought to result from an increase in the quantum yield of the parinaric acid bound to albumin. The increase in the fluorescence intensity observed upon lipolysis in our assay was probably due to the change from a nonpolar medium (TAGs) to a more polar environment (mixed bile salt micelles). The requirement for detergent micelles, which can solubilize free fatty acids and not TAGs, is thus consistent with the hypothesis that the change in the fluorescence intensity may be due to a change in the polarity of the parinaric acid environment.

We further investigated the changes in the fluorescence intensity during the course of lipolysis. Upon subsequently adding a 1 μl solution containing 1 ng of HPL and 2 ng of colipase, the fluorescence intensity measured at 420 nm increased sharply (**Fig. 3A**). This increase can be stopped readily by the addition of THL, a potent HPL inhibitor (35–37). Furthermore, no changes in fluorescence intensity were detected when either heat-treated HPL and colipase, or HPL in the absence of colipase, were used. The latter results were to be expected as HPL is known to be a heat-labile enzyme and, in the presence of 4 mm NaTDC, colipase is an absolute co-factor for HPL (38). Increasing or decreasing 2-fold the final concentration of the fluorescent TAGs or using an emulsion containing gum arabic (see Material and Methods) did not affect the HPL activity (data not shown), which indicates that under these conditions the enzyme was completely adsorbed at the interface.

After rHGL injection, a continuous increase in the fluorescence intensity was observed in a buffer medium at pH 5 (Fig. 3B). The fact that the fluorescence intensity was not increased by shifting the pH from 5 to 7.5 indicates that the fluorescent free fatty acids were already completely detected at pH 5. Lipolysis was stopped, however, by shifting the pH from 5 to 7.5, as it is well known that rHGL is not active at this pH value (39). The present fluorescent assay, using TAGs from *Parinari glaberrimum*, is therefore applicable to the detection of lipase activities in acidic media.

This fluorescence assay is relatively easy to perform from the technical point of view and does not require an emulsification of the TAGs with gum arabic. Moreover, as this assay

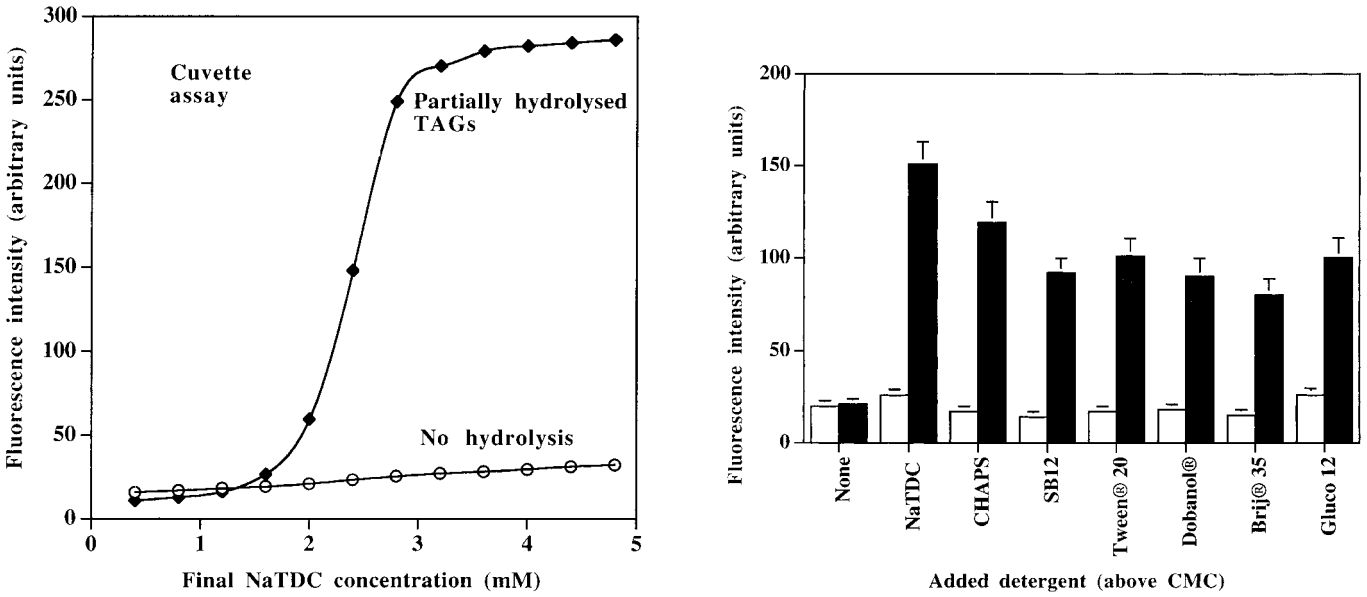


Fig. 2. A: Effect of NaTDC concentration on the fluorescence intensity of hydrolyzed TAGs. The fluorescent TAGs (18 μg) dispersed in 1 ml standard buffer (pH 8) containing 0.4 mM NaTDC were incubated for 5 min with (◆) 0.2 nm HPL and 2 nm colipase (corresponding to 17% of acyl chain hydrolysed) or without lipase (○). THL 200 nm was then added to stop the lipolysis process (enzyme/inhibitor molar ratio: 1/1000). The effect of THL on the lipolysis under our assay conditions is demonstrated in Fig. 3A. The fluorescence intensity was read (excitation 324 nm and emission 420 nm) after each NaTDC addition (1 μl), corresponding to an increase in the concentration in 0.4 mm steps. B: Effect of various detergents (see abbreviations) above their CMC on the fluorescence intensity of the hydrolyzed TAGs. Filled columns: partial hydrolysis was performed under the conditions described in Fig. 2A. Empty columns: controls with no lipase added. Ten μl of the detergent solution was added to the 1 ml cuvette before the fluorescence intensity was read (excitation 324 nm, emission 420 nm). The final concentrations of the added detergents were: NaTDC: 4 mm; CHAPS: 8 mm; Sulfobetaine SB 12: 4 mm; Tween 20: 0.2 mm; Brij 35: 0.4 mm; Dobanol: 0.4 mm; Gluco 12 (n-dodecylglucoside): 0.2 mm. Each bar gives the mean ± SEM, based on triplicate experiments.

can be performed in microtiter plates, it can be used conveniently to perform extensive screening of lipase activity in numerous samples. The tendency of parinaric acid to oxidize during the cuvette assay is likely to decrease the emitted fluorescence intensity and this drawback can be overcome by adding BHT to the buffers and by keeping the cuvettes closed under a nitrogen atmosphere. No oxidation-related problems were noted during a 30-min period of incubation in the microtiterplate assay, using BHT-containing buffer.

The above fluorescence lipase assay was compared to a commercially available (Hoffmann-La Roche, previously Boehringer Manheim) assay using resorufin ester as a synthetic chromogenic substrate. We performed the above test in microtiterplate wells using the same standard buffer (pH 8) as in the fluorescent assay. Under these conditions, with HPL concentrations ranging from 13 pm to 130 pm, the increase in the optical absorbance at 550 nm was found to be linear with time during at least ten minutes (**Fig. 4**). The sensitivity of the chromogenic resorufin assay is similar to that of the parinaric acid-based fluorescent assay. Around 0.1 ng (i.e., 13 pm) of pure HPL was detected (Fig. 4). However, in the case of the resorufin assay, it is worth mentioning that the initial rates measured did not vary linearly with the HPL concentration (see insert in Fig. 4).

Kinetics of fluorescence intensity changes are proportional to the amount of lipase used

Using TAG from *Parinari glaberrimum* as a substrate, the fluorescence intensity was recorded versus time every 10 sec for 5 min after adding variable amounts of HPL (final HPL concentrations ranging from 10 to 200 pm). The increase in the fluorescence intensity was linear with time during at least the first 2 min (data not shown). The initial rates of each kinetic curve were found to be linearly proportional to the amount of HPL used (**Fig. 5A**). When higher amounts of HPL were used, the increase in the fluorescence intensity was too fast and could not be measured accurately. The rate of variation with time in the fluorescence intensity recorded with HPL, at a final concentration of 10 pm, was ten times higher than the background level measured without any HPL. This is the lowest amount of HPL that could be detected with the cuvette assay.

Using pure parinaric acid added to standard reaction medium (i.e., in the presence of TAGs), a calibration curve was set up (not shown) to correlate the increase in the intensity of fluorescence with the added amounts of free parinaric acid. The limit of detection was about 0.1 nmole of fluorescent free fatty acid. This value is in the same range as that published by Wolf and coworkers (26) in the phospholipase A2 assay using parinaric acid-containing phospholipids as a substrate. The calibration plot was used to estimate the specific activity of HPL on the parinaric acid-containing TAGs: 310 IU · mg^{-1}.

This method was further scaled down by using microtiter plates (Fig. 5B). The lowest sensitivity limit was also found to be around 10 pm of pure HPL. In the range of 10 to 260 pm of pure HPL the fluorescence intensity varied linearly with time, at least during the first 2 min (data not

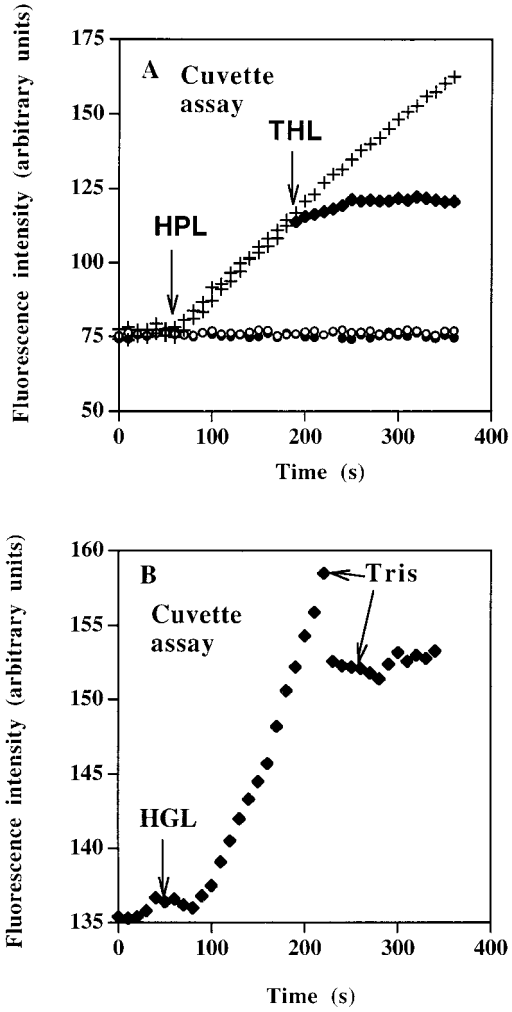


Fig. 3. Kinetics of TAGs hydrolysis followed by fluorescence intensity changes in a spectrofluorimetric cuvette (1 ml). Excitation at 324 nm and emission at 420 nm. A: To 1 ml of the standard incubation medium, 1 μl of a mixture of HPL (1 ng) and colipase (2 ng) was added at time 60 s (first arrow). The final HPL and colipase concentrations were 20 pm and 200 pm, respectively. Symbols: (+): HPL without THL; (○): No HPL; (●): heat-treated HPL. Lipolysis was stopped by adding 1 μl of an ethanolic solution (2 μm) of THL (◆) at time 190 s (second arrow), so that HPL/THL molar ratio was 1/100 (final concentration of THL: 2 nm). Control assay: 1 μl of ethanol. B: rHGL (800 ng) was injected at time 60 s (first arrow) into an acidic buffered reaction medium (pH 5.0, see Material and Methods). Lipolysis by rHGL was stopped by two consecutive additions of 10 μl of Tris buffer 1 m (pH 9). The first addition of Tris buffer shifted the pH to 7.5 and the second addition to pH 8.

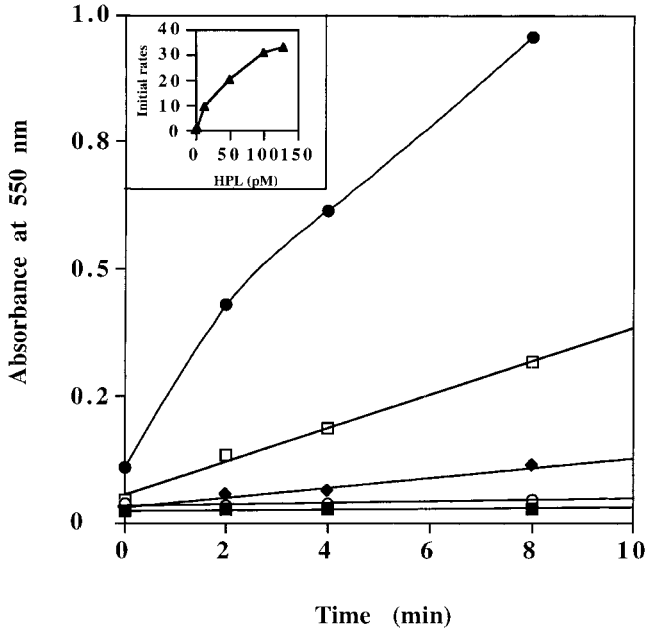


Fig. 4. Kinetic recordings of resorufin ester hydrolysis by HPL at pH 8. A molar excess of colipase to HPL of ten was used. Incubations were performed in a 150 μl reaction volume using microtiterplate wells. The release of resorufin was followed by its absorbance at 550 nm. Symbols: (■): no HPL; (○): 1.3 pm HPL; (◆): 13 pm HPL; (□): 130 pm HPL; (●): 1300 pm HPL. Insert: Initial rates (variation of absorbance × 1000 min^{-1}) versus HPL concentration (pm).

shown). The initial rates of all the kinetic curves were proportional to the HPL concentration (Fig. 5B). At higher amounts of HPL, the initial rates could not be measured. Using microtiterplates it is thus possible to detect quantities of pure HPL as low as 0.1 ng.

Application to detecting the activity of a plant lipase from seedling homogenates

Here we used a crude extract of germinated *Arabidopsis thaliana* seeds expressing a very low lipase activity level: 5 mIU per ml of homogenate, corresponding to 15 mIU per mg of protein. This lipase activity was determined and calculated using radiolabeled triolein as the substrate, under the same experimental conditions as those described by Lehner and Verger (40). This plant lipase activity can be abolished by heating the extract for 5 min at 95°C (**Fig. 6**).

With the resorufin assay, a change in optical absorbance was also detected at 550 nm using the same crude extract (Fig. 6). This change was not abolished, however, when the lipase sample was heated. This indicates that the apparent activity measured on resorufin ester was not due to the lipase activity detected in *Arabidopsis thaliana* seedling extract in the radioactive assay.

Using the fluorescent *Parinari glaberrimum* TAG in a cuvette assay, a significant level of lipase activity was detected with 50 μl of the *Arabidopsis thaliana* extract after a few hours of incubation (**Fig. 7**). The increase with time in the fluorescence intensity (arbitrary slope: 10.3) was significantly higher than the background level measured in the heat-treated extract (arbitrary slope: 1.8). The signal-to-noise ratio was found to be similar in both the assay using radiolabeled triolein and the parinaric acid-based assay (Fig. 6).

GENERAL DISCUSSION

The detection limit of the fluorescent assay developed in the present report, expressed as the minimum amount of pure lipase that can be detected, was found to be 0.1 ng of HPL using microtiterplates. Upon performing the re-

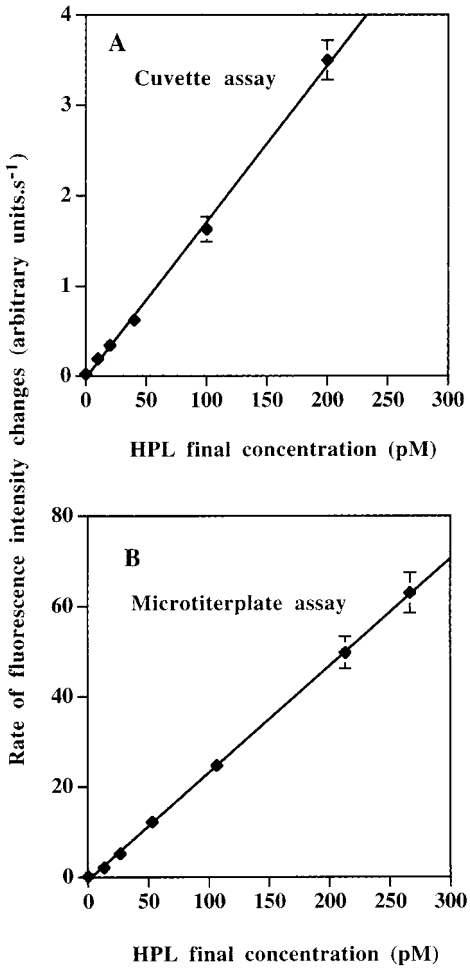


Fig. 5. Rate of fluorescence intensity changes (measured during 5 min) versus HPL concentrations in a spectrofluorimetric cuvette (A) and in microtiterplate wells (B). Each point gives the mean ± SEM of four replicates (the standards errors for most of the data points were smaller than the symbol size). A: Variable amounts of an HPL/colipase solution (10 μl) were added to 1 ml of the standard reaction medium in the cuvette, and the fluorescence was recorded for 5 min. The increase in the fluorescence intensity was linear with time for at least 2 min. Excitation at 324 nm and emission at 420 nm. B: Variable amounts of an HPL/colipase solution (10 μl) were added to 140 μl of the standard reaction medium in the microwells. The fluorescence intensity was read every minute during 30 min. Increase in fluorescence intensity was linear with time for at least 10 min. The excitation filter was at 325 nm and emission filter at 400 nm.

sorufin ester assay with microtiterplates and the radioactive assay, this minimal amount was also found to be 0.1 ng in the case of both assays. The high sensitivity of the fluorescent assay in fact results from the product of the sensitivity of the fatty acid detection (amounts of free parinaric acid as low as 0.1 nmole can be detected) and the specific activity of HPL/colipase acting on the fluorescent TAGs (310 IU·mg^{-1}). Using the pH-stat technique, we also independantly determined the specific activity of HPL/colipase on crude oil from *Parinari glaberrimum* emulsified with gum arabic (350 IU·mg^{-1}). For the sake of comparisons, the specific activity of pure HPL/colipase on long-chain TAGs

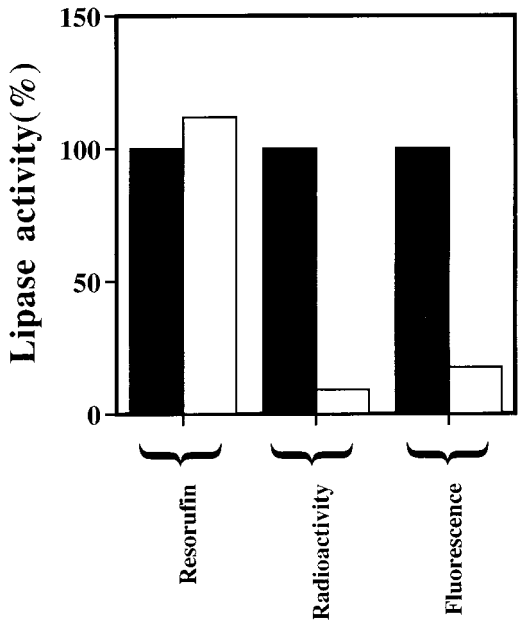


Fig. 6. Comparison between the relative lipase activities of a heat-treated (empty columns), or no heat-treated (filled columns) *Arabidopsis thaliana* extract as measured with three methods: resorufin ester (left) radiolabeled triolein (middle), and TAGs containing parinaric acid (right): see Materials and Methods. The absolute rates of lipolysis measured here were: 15 mIU·mg^{-1} (radioactivity), 1 mIU·mg^{-1} (fluorescence), 36 mIU·mg^{-1} (resorufin).

(crude almond oil emulsified with gum arabic) was found to be about 2800 IU·mg^{-1} in the same experimental conditions (data not shown). As nothing is known a priori

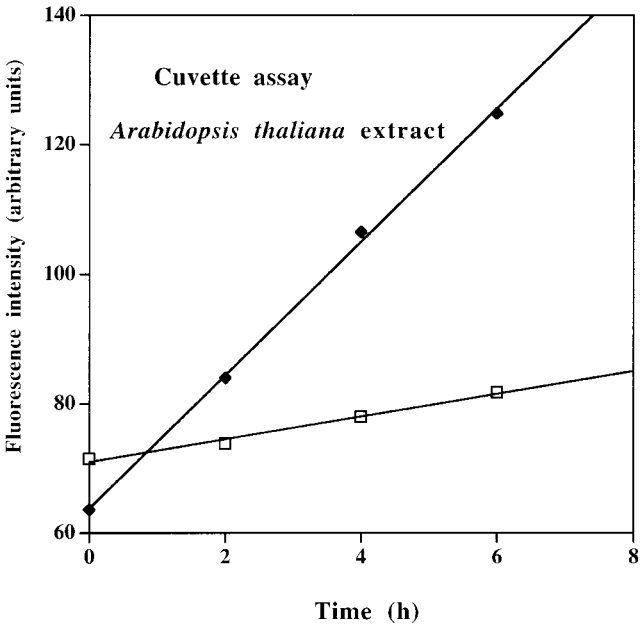


Fig. 7. Kinetic recordings of hydrolysis of naturally fluorescent TAGs from *Parinari glaberrimum* upon incubation with 50 μl of *Arabidopsis thaliana* extract (◆) added to 950 μl of the standard reaction medium. Incubation was performed at 25°C in the dark, in a cuvette kept closed under nitrogen. Fluorescence was read every 2 h. Control (■): heat-treated extract. See Materials and Methods for details.

about the exact distribution of parinaric acid between the *sn*-1, *sn*-2, and *sn*-3 positions in the above fluorescent TAGs as well as the typoselectivity of a given lipase vis-à-vis the parinaryl esters, the absolute value of HPL/colipase specific activity is obviously an approximation. For a more accurate estimation, synthetic TAGs containing parinaric acyl chains in well-defined positions will be necessary (work in progress). Furthermore, the stirring and emulsification conditions differ drastically depending on whether a cuvette, a microtiterplate or a mechanically stirred pH-stat vessel is used.

The assay involving the use of a TAG containing one acyl chain with a fluorescent pyrene residue linked to the ω position and another adjacent acyl chain containing a trinitrophenylamine residue as an intramolecular fluorescence quencher (18, 19) is a convenient assay for chiral discrimination using purified lipases. The limit of detection of the above fluorophore is highly satisfactory; it can be used to detect amounts as low as one pmole of free fatty acid, which is about a hundred times more sensitive than the parinaric acid detection. The sensitivity of this interesting quenched pyrene-based assay for lipase detection is, however, offset by the very poor rate of hydrolysis of the corresponding TAG, as compared to non-chemically modified TAGs (i.e., parinaric acid-containing TAGs). Using the data published by Duque et al. (18), we calculated that the specific activity of HPL on the quenched pyrene-modified TAG was about 3 $mIU \cdot mg^{-1}$, versus 150 $IU \cdot mg^{-1}$ measured by the same authors on tributyrin. For comparison, we also used a pyrene-modified TAG (with a decanoyl-pyrene in *sn*-1 position) to assay HPL/colipase in the standard buffer (see Material and Methods). We obtained a specific activity on this substrate of about 5 $mIU \cdot mg^{-1}$ for the pure HPL/colipase complex. Our results confirm the data by Duque et al. (18) and show that the presence of a single pyrene residue present in the ω position of one acyl chain from a TAG can dramatically decrease the HPL specific activity in comparison with a non-chemically modified TAG. Owing to the great sensitivity of the fluorescent TAGs containing quenched pyrene, along with the very low specific activity of HPL on this substrate, the effective detection limit is 2 μg of HPL (18). This amount of HPL is about 1300-fold higher than that obtained here under comparable assay conditions (3 ml reaction volume, cuvette assay) with the parinaric acid-based system. The very low specific activity of HPL/colipase on the TAG containing acyl-ω pyrene and trinitrophenylamine residues might be due to the presence of a bulky quencher group in addition to a bulky pyrene residue.

In the method described here, the fluorophore is a naturally conjugated polyene fatty acid which does not contain any chemical substituents likely to create steric hindrance. The natural TAGs extracted from *Parinari glaberrimum* seeds are therefore specific, convenient, and sensitive lipase substrates.

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