An ultraviolet spectrophotometric assay for the screening of sn-2-specific lipases using 1,3-O-dioleoyl-2-O-α-eleostearoyl-sn-glycerol as substrate

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Abstract

In the present study, we propose a continuous assay for the screening of sn-2 lipases by using triacylglycerols (TAGs) from Aleurites fordii seed (tung oil) and a synthetic TAG containing the α-eleostearic acid at sn-2 position and the oleic acid at sn-1 and sn-3 positions (sn-OEO). Each TAG was coated into microplate well and the lipase activity was measured by optical density increase at 272 nm due to transition of α-eleostearic acid from the adsorbed to the soluble state. sn-1,3-regioselective lipases (human pancreatic lipase (HPL) and LIP2 from Yarrowia lipolytica (YLLIP2) and a known sn-2 lipase (Candida antarctica lipase A, CALA) were used to validate this method. Thin layer chromatography analysis of lipolysis products showed that the lipases tested were able to hydrolyze the sn-OEO and the tung oil TAGs, but only CALA hydrolyzed the sn-2 position. The ratio of initial velocities on sn-OEO and tung oil TAGs was used to estimate the sn-2 preference of lipases. CALA was the enzyme with the highest ratio (0.22±0.015), while HPL and YLLIP2 showed a much lower ratio (0.072±0.026 and 0.038±0.016, respectively). This continuous sn-2 lipase assay is compatible with a high sample throughput and thus can be applied to the screening of sn-2 lipases.

Keywords: Lipases, Candida antarctica A Lipase, β-cyclodextrin, stereoselectivity, sn-2 specificity, Aleurites fordii, Tung oil, α-eleostearic acid, polyunsaturated fatty acid, high-throughput screening.
Abbreviations used:

BHT, butylhydroxytoluene; BSA, Bovine serum albumin; CALA, *Candida antarctica* lipase A; β-CD, β-cyclodextrin; CRL, *Candida rugosa* lipase; CRLIP3, LIP3 lipase from *Candida rugosa*; DAG, diacylglycerol; DCC, \(N,N\)'-Dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)-pyridine; ee, enantiomeric excess; FFA, free fatty acid; HPL, human pancreatic lipase; MAG, monoacylglycerol; NaTDC, sodium taurodeoxycholate; OA, oleic acid; \(sn\)-OEO, 1,3-\(O\)-dioleoyl-2-\(O\)-α-eleostearoyl-\(sn\)-glycerol; RHL, *Rhizopus homothallicus* lipase; TAG, triacylglycerol; TLC, Thin-layer chromatography; TLL, *Thermomyces lanuginosus* lipase. YLLIP2, LIP2 lipase from *Yarrowia lipolytica*. 
Introduction

Lipases (triacylglycerol ester hydrolase, EC 3.1.1.3) are lipolytic carboxylester hydrolases which catalyze the hydrolysis of the ester bonds of long-chain triacylglycerols (TAGs). They are widely distributed in microorganisms, plants, and animals (1-3) where they play an important role in lipid metabolism (4, 5).

Since lipases are water-soluble enzymes hydrolyzing insoluble long chain TAG substrates, the cleavage reaction has to occur at the lipid-water interface (6-8). The mechanisms involved in the enzymatic lipolysis depend strongly on the mode of organization of the lipid substrate in interfacial structures such as monolayers, micelles, liposomal dispersions and oil-in-water emulsions. Lipases interact with these lipid complexes, or “super-substrates”, via hydrophobic domains which are exposed upon contact as the result of a substrate-induced conformational change which sometimes has been called “interfacial activation” (3, 9). The two-dimensional nature of this lipase reaction does not obey Michaelis-Menten kinetics and depends critically 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 take these unique features into account.

Most lipases show a chemo-preference for TAG rather than partial acylglycerols (e.g. monoacylglycerol, MAG and diacylglycerol, DAG). They also show enantio- and regio-selectivity during hydrolysis, alcoholysis, acidolysis and transesterification reactions with a wide range of natural and synthetic substrates (10, 11). Many biotechnological applications for lipases have been described in the food, cosmetic, detergent, and pharmaceutical industries (3, 12, 13). Lipases with new properties (such as high levels of chemo-specificity, stereo-selectivity and
thermo-stability) are of particular interest in the biotransformation of oils and fats and structured lipid engineering (14).

These lipases can be obtained either by isolating them from various natural sources or by using classical protein engineering methods, and directed evolution procedures can also now be used for this purpose (15-17). All these studies require convenient, sensitive, and specific assays for measuring lipase activity (18). In addition, screening procedures require continuous assays and substrate stability that are compatible with high sample throughput.

A continuous lipase assay using naturally occurring fluorescent TAGs isolated from *Parinari glaberrimum* as substrates has been developed (18). This substrate is however too sensitive to oxidation to be used under high-throughput assay conditions. Synthetic octadeca-9,11,13,15-tetraenoic-3-hydroxy-octadecyloxypropylester, a 1-acyl-2-alkyl glycerol from parinaric acid is a DAG analogue which provides an efficient substrate for hormone-sensitive lipase (19). But its pronounced sensitivity to oxidation precludes its use under routine conditions. Petry *et al.* (20) developed a continuous, sensitive *in vitro* lipase assay using a MAG containing a fluorescent label, *p*-nitrobenzofurazan, as lipase substrate. An alternative ultraviolet (UV) spectrophotometric assay based on the use of TAG from *Aleurites fordii* seeds, which is less sensitive to oxidation, was introduced by our group (21). *A. fordii* is a tree originating from China, where its seed oil, known as tung oil, is one of the main primary products used in the lacquer industry. Crude tung oil contains up to 70% \(\alpha\)-eleostearic acid (22, 23), an octadecatrienoic fatty acid with a conjugated triene containing double bonds having \(\Delta^9\)cis, \(\Delta^{11}\)trans, \(\Delta^{13}\)trans configurations. The other main fatty acids present in tung oil are linoleic acid (around 15%) and oleic acid (around 10%). Position analysis (23) showed that \(\alpha\)-eleostearic
acid is esterified at the sn-1 and sn-3 positions in the TAGs present in tung oil. The conjugated triene present in α-eleostearic acid is an intrinsic chromophore which confers strong UV absorption properties on both the pure fatty acid (24) and the TAGs present in tung oil (21). More recently, the UV spectrophotometric lipase assay with tung oil was adapted to microtiter plates for high throughput screening procedures (25).

Intensive research is being carried out to make use of the specificities and preferences of some lipases for synthesizing lipids with well-defined fatty acid compositions and patterns of distribution, which could be used in the production of human dietary supplements and edible oils (26, 27). Most lipases can be classified in one of two large groups: sn-1,3 regiospecific lipases, which hydrolyse acylglycerol only at the external positions of glycerol, and non-regiospecific (or random) lipases, which act on all three positions of acylglycerols. Only a few lipases show a preference for the sn-2 (or internal) position of acylglycerols. Candida antarctica lipase A (CALA) (28, 29) and Geotrichum candidum lipase (30) were reported to show sn-2 preference during the hydrolysis of TAGs. A review on CALA illustrates the increasing attention being paid to this unique biocatalyst and the many applications which have been developed, especially in the synthesis of structured TAGs (31). Sn-2 specific lipases are particularly interesting for tailoring the fatty acids at the internal position of structured TAGs.

In a recent study, Horchani et al. (32) used three pairs of enantiomers from diacylglycerol analogs (didecanoyldeoxyamino-O-methyl glycerol) containing a single hydrolysable decanoyl ester group and two lipase-resistant groups in order to study the stereoselectivity of nine staphylococcal lipases. The lipases tested by Horchani et al. (32), using the monomolecular film technique, were found to significantly
hydrolyse the secondary ester group of diacylglycerol analogs, with a strong preference for the $R$ configuration (32). Furthermore, all the staphylococcal lipases tested efficiently hydrolysed triolein at the $sn$-2 position (32). It was concluded that these diacylglycerol analogs might therefore provide useful initial screening tools for its use in future searches for strictly $sn$-2 specific lipases.

In the present study, we used a synthetic TAG, 1,3-O-dioleoyl-2-O-α-eleostearoyl-$sn$-glycerol ($sn$-OEO) containing α-eleostearic acid as a chromophore only at the $sn$-2 position to establish a specific assay of lipases having specificity for the hydrolysis of the ester bond at the $sn$-2 position of TAGs. This continuous lipase assay is compatible with a high sample throughput and thus can be applied to the screening of strictly $sn$-2 specific lipases.
Materials and methods

Chemicals

Crude tung oil, trioleoyl (triolein), 1,3-sn-dioleoyl (1,3-sn-diolein), 1,2/2,3-sn-dioleoyl (1,2/2,3-sn-diolein), monooleoyl (monoolein), oleic acid (OA), bovine serum albumin (BSA), CaCl$_2$, taurodeoxycholic acid sodium (NaTDC), butylhydroxytoluene (BHT), β-cyclodextrin (β-CD), 4-(dimethylamino)-pyridine (DMAP), N,N'-Dicyclohexylcarbodiimide (DCC) and anhydrous MgSO$_4$ were purchased from Sigma-Aldrich (St Quentin-Fallavier, France). All the solvents were purchased from SDS (Peypin, France) and were of HPLC grade. Pure monoolein and diolein were purified from a commercial low-grade DL-α-monoolein from Fluka. High-performance thin-layer chromatography (TLC) glass plates pre-coated with silica gel 60 F254 were from Merck. All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers.

Lipases

Recombinant HPL and its inactive mutant in which the active site serine is mutated to glycine (HPL S152G) were expressed in the yeast Pichia pastoris and purified from culture media as described by Belle et al. (33). The purified Thermomyces lanuginosus lipase (TLL), CALA from Candida Antarctica and LIP3 from Candida rugosa (CRLIP3) were a generous gift from Dr. S. Patkar (Novozymes, Denmark). LIP2 from Yarrowia lipolytica (YLLIP2) was produced and purified according to (34). Rhizopus homothallicus lipase (RHL) was produced and purified according to (35). Candida rugosa lipase (CRL) AY30 was purchased from Amano Pharmaceuticals Ltd. (Nagoya, Japan) and was used to produce FFA from tung oil.
Thin-Layer Chromatography

Neutral lipids were separated by performing preparative and analytic TLC on glass plates coated with 0.5-mm and 0.2-mm silica gel 60, respectively. In both cases the following chromatographic solvent system was used: heptane:diethylether:acetic acid, 55:45:1 (v/v/v) containing 0.001% (w/v) BHT as an antioxidant. For preparative TLC, samples were applied in the form of 4-cm long spots. The various lipids were showed up under a UV lamp in the form of fluorescent spots which were then scraped off. The lipids were then extracted from the silica gel with diethylether containing 0.001% BHT. For analytic TLC, the various lipids were revealed by charring the plate after spraying it with a mixture (50:50, v/v) of saturated solution copper acetate in water and 85.5% phosphoric acid. Triolein, diolein (1,2(2,3) and 1,3-isomers), monololein and oleic acid were used as reference standards for the TLC analysis.

Preparation of purified TAGs from Tung Oil

A 50 mg.mL\textsuperscript{-1} solution of crude oil was prepared in diethylether containing 0.01% (w/v) BHT as an antioxidant. The purified TAGs were isolated by performing preparative TLC (sample application: 400 µl of the stock solution, \textit{i.e.}, 20 mg of crude oil) as described above. Water was removed from the TAGs solution in diethyl ether using an anhydrous MgSO\textsubscript{4} column packed into a Pasteur pipette. The amount of purified TAGs was determined by measuring a constant dry weight after evaporating diethylether under a stream of nitrogen. Around 12 mg of purified TAGs were recovered. The purified TAGs were finally dissolved in ethanol containing 0.01% (w/v) BHT. The final concentration was 1 mg.mL\textsuperscript{-1}, which corresponds to the solubility limit of the purified TAGs from tung oil in ethanol. The stock solution was stable (no variations occurred in the UV spectrum) for at least 1 month.
Preparation of purified total FFA from Tung Oil

Twenty grams of crude tung oil was mixed with 12 mL of water and 500 mg of a CRL AY30 from Amano Pharmaceuticals Ltd. (Nagoya, Japan) at 40°C and pH 7.3 for 1 h under agitation. One hundred mL of 3 N HCl was then added and the mixture was transferred into a decantation vial. The lipids were extracted with 100 mL of diethylether containing 0.01% BHT. The upper organic phase was recovered and dried by adding anhydrous MgSO₄, which was thereafter removed by filtration using a paper filter. The mixture contained mainly FFA and residual TAGs and DAGs, as checked by TLC (data not shown). The total FFAs were further purified by performing preparative TLC as described above. The amount of total FFAs recovered was determined by measuring a constant dry weight after the solvent had evaporated. The ethanolic stock solution was prepared at 1 mg.mL⁻¹ as final concentration. The stock solution was stable (no changes were observed in the UV spectrum) for at least 1 month.

Alternatively, α-eleostearic acid was purified directly from tung oil using an adapted method from O’Connor et al. (36). Tung oil (7.2 g) was dissolved in ethanol (27 mL). Potassium hydroxide (2.9 g) was added and the resulting mixture refluxed in the dark for two hours under inert atmosphere. After cooling to 0°C, 13.5 mL of 15% aqueous sulfuric acid were added to acidify the medium (pH = 2). The resulting mixture was extracted with diethyl ether (2 times 20 mL), the organic layer dried with Na₂SO₄, filtered and evaporated to dryness. The crude solid residue obtained was then purified by column chromatography on silica gel (Diethyl ether /petroleum ether: 9/1, v/v), to provide a waxy yellow solid. Pure crystalline sample could be obtained by recrystallization in cold acetone (29% yield). TLC analysis (Rᵢ= 0.38; eluent: petroleum ether/diethyl ether/acetic acid, 5.5/1.5/0.1, v/v/v), UV analysis (Ethanol, 95
% $\lambda_{\text{max}}$ = 260, 270, 280 nm) and $^1$H NMR (200 MHz; deuterated chloroform) confirm the purity of the solid in full agreement with earlier reported characterizations (37).

**Synthesis of 1,3-O-dioleoyl-2-O-$\alpha$-eleostearoyl-sn-glycerol**

Pure $\alpha$-eleostearic acid (13.6 mg, 0.049 mmol) was mixed with 1.2 equivalents (36.6 mg, 0.059 mmol) of 1,3-diolein and 0.2 equivalents of DMAP (1.19 mg, 0.0098 mmol) in 0.2 ml of CH$_2$Cl$_2$ by stirring under argon atmosphere and in the absence of light to avoid oxidation, isomerization and polymerization of $\alpha$-eleostearic acid (36). The mixture was stirred at 0°C for 25 minutes and 1.2 equivalents of DCC (12.2 mg, 0.059 mmol) in 0.5 ml of CH$_2$Cl$_2$ were then slowly added. The reaction was allowed to come back to room temperature and the conversion into sn-OEO was followed by TLC revealed by UV exposition (to reveal $\alpha$-eleostearic acid and sn-OEO) and by charring the plate after spraying it with a mixture (50:50, v/v) of saturated solution copper acetate in water and 85.5% phosphoric acid (to reveal all acyl species). After 5 days, the reaction was stopped as no change in the composition of the reactive mixture could be observed after that period. The reaction mixture was concentrated under nitrogen atmosphere and the products were purified by flash chromatography on silicagel (eluent: CH$_2$Cl$_2$) to afford 10 mg (0.0113 mmol) sn-OEO with 23% yield. (Molecular mass = 881; $R_f$ = 0.60; eluent: CH$_2$Cl$_2$). $\lambda_{\text{max}}$ (Ethanol 95 %) = 258, 268, 278 nm.

**Coating microtiter plates with tung oil TAGs or sn-OEO**

Microtiter plates were coated with either purified tung oil TAGs or sn-OEO as described by Verger et al. (25). A TAG solution (0.5 mg.mL$^{-1}$) was prepared in hexane containing 0.01% BHT serving as an anti-oxidant. The wells of UV-transparent microtiter plates (Corning Inc., Corning, NY, catalog No. 3635) were
filled with the TAG in hexane solution (100 µL/well) and left to stand under a fume hood until the solvent had completely evaporated (for around 30 min). After hexane evaporation, the coated TAGs were found to be stable in the dark for at least 1 week at 4°C.

**UV Spectrophotometric lipase assay using coated TAGs**

The wells containing the coated TAGs were washed three times with 0.2 mL of the assay buffer (10 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl, 6 mM CaCl₂, 1 mM EDTA, and 3 mg.mL⁻¹ β-CD) and left to equilibrate at 25 °C for at least 5 min with 200 µL of the assay buffer. A lipase solution (2-10 µL) was added to each well, and the optical density (OD) at 272 nm was recorded continuously at regular time intervals of 30 s against the buffer alone using a microtiter plate scanning spectrophotometer (PowerWave™, Bio-Tek Instruments).

Alternatively, after various periods of time, the hydrolytic reaction was stopped by acidification (final pH 3.5), using 20 µL of 0.1 M HCl. The total aqueous phase (220 µL) was transferred to an Eppendorf tube. The lipids remaining in each well were extracted five times using 200 µL of hexane (containing BHT 0.001%). These hexane extracts (1 ml) were added to the aqueous phase (220 µL) in order to maximize total lipid recovery. The Eppendorf tube was centrifuged for 10 min at 10,000 rpm. The hexane phase recovered was evaporated under a nitrogen flow and the lipids were dissolved in 30 µL of a mixture of chloroform/methanol (2/1, v/v) containing 0.001% BHT. TLC analysis of neutral lipids was carried out by using TLC pre-coated plates 60 F254 as described above.
Results and discussion

Synthesis of 1,3-O-dioleoyl-2-O-α-eleostearoyl-sn-glycerol (sn-OEO) and spectroscopic properties

The synthesis of sn-OEO was conducted with pure and fully characterized reactants. The purified α-eleostearic acid was characterized by $^1$H NMR (Fig. 1). The protons carried by the trienic system resonate as a complex set of multiplets between 5.3 and 6.4 ppm, the methylene and methyl groups born in the C-beta and C-omega position as characteristic triplets at 2.35 ppm and 0.9 ppm, respectively (Fig. 1). Chemical shifts, coupling constants and integrations are in full agreement with the values reported by Cao et al. (37) confirming the purity of the α-eleostearic acid obtained.

The synthesis sn-OEO was performed as indicated in Fig. 2. DCC was used a chemical coupling agent in the presence of DMAP at room temperature for 5 days in dichloromethane to introduce α-eleostearic acid into the mid-position of the 1,3-sn-diolein adduct (adapted from (38)). Pure sn-OEO was obtained with a 23% yield after purification on silicagel. This low conversion yield, which is in agreement with previous data obtained with the same type of reaction but other fatty acids, might be attributed to a lower reactivity of the sn-2 secondary alcohol group as compared to sn-1/3 primary counterparts. The length of the fatty acids at the sn-1 and sn-3 positions does not seem to have a major effect since the synthesis of 1,3-O-dilauroyl-2-O-α-eleostearoyl-sn-glycerol from 1,3-sn-dilaurin was performed with a similar yield of 26% (data not shown). Additionally, previous studies confirmed that under such coupling conditions, no acyl migration between sn-1/3 and sn-2 positions could be observed (39). Finally, performing the synthesis, purification steps and
storage in the dark ensured to avoid any isomerization of the trienic moiety from an α- to β-eleostearic acyl chain. Nevertheless, the purity of the sn-OEO was controlled by analytical chromatography and UV spectrometry while 1D-NMR spectroscopy proved to be uninformative.

The UV spectrum of sn-OEO could be compared with those of pure α-eleostearic acid and TAGs from tung oil in ethanol (Fig. 3). The profile of the spectrum appeared to be similar (240–300 nm), displaying 3 typical peaks around 260, 270 and 280 nm (21). Still, a slight solvochromic effect, which was pointed out earlier (40), could be observed in the α-eleostearic acid-containing products and provided some information about the local polarity surrounding the trienic fatty acid moiety. While the maxima in the UV spectrum of pure α-eleostearic acid are precisely located at 260, 270 and 280 nm in ethanol, a red shift of 2 nm was reported when the spectrum was recorded in an aqueous media, hence a more polar environment. Interestingly, the maxima of the spectra obtained herein for sn-OEO (respectively TAGs in tung oil) undergo a reproducible 2 nm (respectively 1 nm) blue shift. This correlates with the positive solvatochromism observed earlier (21) and with a reduction in the polarity surrounding the α-eleostearic acid moiety while shifting from ethanol to sn-1/3 position in tung oil and to sn-2 position in between two oleic side chains in sn-OEO (40). For the sake of comparison, the spectrum of OA in ethanol was also determined. As shown in Fig. 3, slight absorbance was observed in the range of 240-260 nm and doesn’t interfere with the three typical peaks of α-eleostearic acid.

Using an apparent molar extinction coefficient of 13,900 M⁻¹.cm⁻¹ at 292 nm for α-eleostearic acid in ethanol (21), the contents in α-eleostearic acid could be estimated to be 100%, 58%, 64% and 26% in pure α-eleostearic sample, purified
tung oil FFAs, tung oil TAGs and sn-OEO, respectively. The value obtained with sn-OEO was therefore similar to the expected one (33%, 1 acyl chain out of 3), the deviation to this theoretical value is presumably due to a potential hypochromic effect (40) accompanying the insertion of the α-eleostearic acid in the sn-2 position of the DAG backbone, reminiscent of what was observed, and to a lesser extent, for DNA denaturation. An apparent molar extinction coefficients (ε\textsubscript{app}) for the three compounds were also estimated and found to be 8,123 ± 372, 26,500 ± 486 and 3,656 ± 57 M\textsuperscript{-1}.cm\textsuperscript{-1} for purified tung oil FFAs, tung oil TAGs and sn-OEO, respectively. As mentioned earlier, the spectra obtained in the reaction buffer were slightly different from those obtained in ethanol (data not shown) and the ε\textsubscript{app} of pure α-eleostearic acid in the reaction conditions at 272 nm was estimated to be 6,519 ± 642 M\textsuperscript{-1}.cm.

Principle of the lipase assay using TAGs coated in microtiter plates

The TAGs were coated into the wells of microtiter plates as indicated in the Materials and Methods section. Using UV spectrum measurement, the coated TAGs were found to be stable for at least 1 week at 4 °C prior the hydrolysis reaction was launched. Fig. 4 shows the principle of the reaction for sn-OEO. Once injected in the microwell, the lipase in solution (E, enzyme in solution) can bind to the interface (E*, enzyme at the interface) where the hydrolysis of TAGs is carried out by releasing FFAs, DAGs and MAGs as products (9). The long chain FFAs (R\textsubscript{1} = oleic acid; R\textsubscript{2} = α-eleostearic acid) can then be solubilized by the β-CD present in the buffer (41, 42). The R\textsubscript{2}/β-CD complex can be measured continuously by recording the increase in the UV absorbance at 272 nm due to the conjugated triene of the α-eleostearic acid present in solution (21, 25). When using sn-OEO, both 1,3-sn-regioselective and non-regioselective lipases can release R\textsubscript{1} which is present at sn-1 and sn-3
positions, but only lipases with the capacity to hydrolyze the sn-2 position can release R₂ and theoretically generate an increase in UV absorbance. When using the natural TAGs from tung oil (about 64% of R₂), we assumed that R₂ is equally present at the 3 positions of TAGs to when estimating the lipase activity from the increase in UV absorbance. To determine whether a lipase had a sn-2 preference, we used the ratio between the initial rates of sn-OEO and tung oil TAGs hydrolysis.

Stability of the TAGs coating in microtiter plates

For the validation of the assay, the stability of the multilayer of TAGs (tung oil or sn-OEO) coated in the microtiter plate well was tested. After coating the TAGs, the absorbance at 272 nm was recorded for 40 min in the presence of the reaction buffer and without enzyme. A constant base line was recorded for both sn-OEO and tung oil TAGs (Fig. 5A), indicating that the coating of TAGs was not disrupted by an interaction with the buffer. To test the hypothesis whether a protein without catalytic activity could disrupt the TAGs coating, BSA (4.8 µg per well; 24 µg.ml⁻¹, final concentration) was injected into the wells containing sn-OEO or tung oil TAGs and buffer. As it can be seen from Fig. 5B, only a slight increase in the OD at 272 nm was observed immediately after addition of BSA, probably due to the natural absorbance of BSA, and then a constant absorption was recorded with time, indicating that no TAGs were released from the surface. We therefore demonstrated that the addition of a large quantity of protein does not induce a large background noise that could mask the OD increase due to the lipolysis products and the solubilization of α-eleostearic acid by β-CD.

Most lipases have however, the ability to interact more strongly with lipid interface than BSA and this might affect the stability of TAGs film coated on the microtiter plates. This would result in a change in the base line of substrate. To verify
this possibility, high amounts of inactive lipases (HPL heat-inactivated at 95°C for 5 min and the inactive HPL S152G mutant) were used as controls to determinate the change of the base line of coated tung oil TAGs absorption. After injection of HPL S152G (195 µg.ml⁻¹, final concentration), the UV base line increases of about 0.2 and remains practically constant during the time course of reaction (Fig. 5C), showing that a large amount of lipase increases slightly the absorbance at 272 nm due to intrinsically lipase UV absorption and don’t disrupt the tung oil TAGs coating. Furthermore, insignificant changes in the absorbance at 272 nm were observed after the injection of heat-inactivated HPL (20 ng) in coated tung oil TAGs (Fig. 5D), whereas, under the same experimental conditions, a higher hydrolysis rate (ΔOD/min) of 0.75 was observed (Fig. 5D). It is worth noticing that it was not possible to test quantities of heat-inactivated HPL as high as those of HPL S152G because of a precipitate of denatured HPL was observed at high concentrations. Together, these results clearly indicate that inactive lipases don’t change significantly the substrate surface optical properties and structure.

Method validation using various purified lipases

To validate the assay method, various lipases with different selectivities towards TAGs were used. CALA is known to display a stereopreference for the sn-2 position of TAGs (29, 43). HPL and YLLIP2 are known to be 1,3-sn-regioselective lipases (4, 29, 44). Typical kinetics of the increase in the OD at 272 nm during the hydrolysis of purified TAGs from tung oil and sn-OEO are shown in Fig. 6 for CALA (Fig. 6A; 340 ng of CALA/microwell), HPL (Fig. 6C; 20 ng of HPL/microwell) and YLLIP2 (Fig. 6E; 12 ng of YLLIP2/microwell). The respective amounts of lipases were chosen to obtain similar velocities of hydrolysis using tung oil TAGs. The final OD variation (ΔOD_{272}) was close to 0.75 in all three cases. Lag times of 3 min, 2 min
and 4 min for the steady-state to be reached after sample injection were observed for CALA, HPL and YLLIP2, respectively. These lag phases were probably due to the time required for efficient mixing of the enzyme to occur in the microwells.

Under similar conditions but using \( sn \)-OEO as substrate, a higher hydrolysis rate (\( \Delta \text{OD/min} \)) was observed with CALA (Fig. 6A) when compared to HPL (Fig. 6C) and YLLIP2 (Fig. 6E). As expected, these results indicated that CALA was the lipase with the higher ability to release the \( \alpha \)-eleostearic acid at the \( sn \)-2 position of \( sn \)-OEO. As also expected for a \( sn \)-1,3-regioselective lipase, no significant increase in the OD was observed with YLLIP2 when \( sn \)-OEO was used as substrate (Fig. 6E). A slight linear increase in the OD was however observed after 10 min of reaction with HPL when \( sn \)-OEO was used as substrate (Fig. 6C). Although HPL is well known to be a \( sn \)-1,3-regioselective lipase, these findings suggested that HPL was able to release free \( \alpha \)-eleostearic acid from the \( sn \)-2 position of \( sn \)-OEO. Alternatively, another lipolysis product released during the reaction and containing \( \alpha \)-eleostearic acid might be also sequestered by \( \beta \)-CD and solubilized, thus contributing to the increase in the OD at 272 nm. One possibility could be that MAGs were also produced and formed complexes with \( \beta \)-CD (42, 45).

To check this hypothesis, the lipolysis products were extracted from the wells of the microtiter plates and analyzed by TLC. This was performed during the hydrolysis of both tung oil TAGs and \( sn \)-OEO by CALA, HPL and YLLIP2. For the sake of comparison, all reactions with tung oil TAGs were stopped after reaching a \( \Delta \text{OD}_{272} \) of 0.75. Experiments with \( sn \)-OEO were performed with the same amounts of enzymes and for the same time period that allows the \( \Delta \text{OD}_{272} \) to reach 0.75 when using tung oil TAGs as substrate.
The effects of the lipase amounts on the steady-state reaction rate are shown in Fig. 6. Using tung oil TAGs or sn-OEO, the steady-state reaction rate ((ΔOD/min) was shown to increase linearly with the enzyme amounts. This linear relationship was observed to a maximum amount of 500 ng/well for CALA (R² = 0.999, Fig. 6B), 100 ng/well for HPL (R² = 0.995, Fig. 6D), and 50 ng/well for YLLIP2 (R² = 0.987, Fig. 6F).

The TLC analysis of tung oil hydrolysis by CALA, HPL and YLLIP2 is showed in Fig. 7A. The products were first revealed under UV light (254 nm, non-destructive conditions), a picture was taken and then the plate was further stained with cupric acetate/phosphoric acid 50:50 and charring at 180°C for 15 min as indicated in Materials and Methods. The lipid bands observed with UV light and charring revelation were found to have the same intensity for HPL and YLLIP2, showing a decrease of TAGs content and the appearance of FFAs, 1,2-rac-DAGs and MAGs. The presence of MAGs was however more important for HPL than YLLIP2. The hydrolysis profile of tung oil TAGs by CALA was different than those observed with HPL and YLLIP2, with a slight MAG accumulation and the clear appearance of a band corresponding to 1,3-sn-DAG and that was slightly more intense that of 1,2-rac-DAG. These results clearly showed that CALA can hydrolyze the sn-2 position of TAGs and generate 1,3-sn-DAG, whereas HPL et YLLIP2 did not. The acyl migration of FFA from the sn-2 to sn-1 or sn-3 position was considered as non-significant since the same treatment was used for the three lipases and only weak bands of 1,3-sn-DAGs were observed in the case of HPL and YLLIP2.

When similar TLC analysis was applied to sn-OEO hydrolysis (Fig. 7B), the apparent lipolysis product profiles determined by UV light and charring revelation were different. In the case of the UV revelation, an appearance of 1,2-rac-DAG and
2-sn-MAG containing the $\alpha$-eleostearic acid was observed with HPL and YLLIP2, whereas no band corresponding to FFAs could be seen. The band of MAGs was more important for HPL than for YLLIP2. These experiments clearly demonstrated that MAGs containing $\alpha$-eleostearic acid could be produced and could therefore be solubilized by $\beta$-CD. Conversely and still with the UV revelation, only the appearance of a band corresponding to FFAs could be observed with CALA, indicating that CALA was not only able to hydrolyze the $sn$-2 position, but also that no other lipolysis product contained $\alpha$-eleostearic acid were seen. Since CALA is not entirely specific for the $sn$-2 position, these results suggest that the other lipolysis products (DAGs and MAGs) generated by CALA and containing $\alpha$-eleostearic acid have probably a very short life time and are rapidly hydrolyzed in their turn by CALA to generate ultimate lipolysis products, *i.e.* FFAs and glycerol. When TLC plates corresponding to the same experiments with $sn$-OEO were analyzed by the charring revelation, the appearance of FFAs was clearly observed for HPL and YLLIP2, indicating that these FFAs were mostly oleic acid liberated by the action of these enzymes on the $sn$-1 and $sn$-3 position. For CALA, no significant bands of DAGs and MAGs were observed, thus confirming our hypothesis that these products had a short life time and were hydrolyzed by CALA.

All the TLC experiments confirmed the specificities of CALA, YLLIP2 and HPL (29, 43, 44). They show however, that MAGs with $\alpha$-eleostearic acid could be produced by HPL and to a lesser extent by YLLIP2. This means that these MAGs might be solubilized by $\beta$-CD like FFAs. Similar levels of 2-MAGs generation were observed for HPL and YLLIP2 (Fig. 7B), however, the increase of OD$_{272}$ with $sn$-OEO is more important for HPL than YLLIP2 (see Figs. 6C and E). These results support the hypothesis that HPL induces 2-MAGs desorption to a greater extent than
YLLIP2. Haiker et al. (46) reported a rapid exchange of HPL in triacylglycerol droplets, in which the HPL/collipase complex can be exchanged quickly between the bulk and the substrate surface and this phenomenon is done via micellar structures that serve as shuttles. This could explain the increase of 2-MAG desorption from substrate by HPL and its solubilization by βCD into the buffer. They would therefore contribute to the increase in the OD at 272 nm. If this is the case, the variation of the OD at 272 nm could still be used for a global measurement of lipolysis (production of both FFAs and MAGs) but its direct relationship with FFA release would not be valid anymore.

**Determination of the sn-2 preference of lipases**

The TLC analysis performed in this study clearly showed that the synthetic sn-OEO substrate can already be used for screening sn-2-specific lipases. A continuous assay using microtiter plates would be however much more interesting for high throughput screening of large amounts of samples. Taken into account the previous results, the variation of the OD at 272 nm using sn-OEO as substrate cannot be used as an absolute measurement of the release of the fatty acid present at the sn-2 position. One can see however that a lipase like CALA that has a high specificity for the sn-2 position can be clearly distinguished from the sn-1,3-regioselective HPL and YLLIP2 using sn-OEO as substrate. Based on this statement and to compare the preference for the sn-2 position of several lipases, we measured the initial velocities of these lipases on both sn-OEO and tung oil TAGs and calculated their ratio. For this purpose, the OD variations at 272 nm were converted into mole.min\(^{-1}\) using the \(\varepsilon_{\text{app}}\) of \(\alpha\)-eleostearic acid in the reaction conditions (6,519 ± 642 M\(^{-1}\).cm\(^{-1}\)). The theoretical ratio for non-specific lipases able to hydrolyze each position at the same rate was calculated with the ratio of \(\varepsilon_{\text{app}}\) of sn-OEO between the \(\varepsilon_{\text{app}}\) purified tung oil
TAGs in ethanol resulting to be 0.14. Lipases showing a preference for the sn-2 position should theoretically display a higher ratio. This was confirmed with CALA that shows the higher ratio (0.22 ± 0.015; Fig. 8). Ratio of 0.10 ± 0.007, 0.072 ± 0.026 and 0.038 ± 0.016 were obtained for the sn-1,3-regioselective TLL, HPL and YLLIP2, respectively (Fig. 8, panel B). Although these ratios are not zero due to the interference of MAGs in the UV absorption, they are lower than 0.14 and this is consistent with a lower attack of the sn-2 position. Other non specific lipases that have been reported to hydrolyze the sn-2 position were also tested (Fig. 8, panel C). RHL (35) gives a value of 0.094 ± 0.037 that is close to the values obtained with sn-1,3-regioselective lipases. The ratio obtained with CRL AY30 and CRLIP3 were 0.11 ± 0.047 and 0.14 ± 0.02, respectively. Both values are close to the value of 0.14 assigned theoretically to non-specific lipases.

**Conclusion**

The synthetic sn-OEO substrate appears to be a convenient tool for studying the sn-2 position specificity of lipases by using TLC analysis and UV detection. On the contrary, the method for the high throughput screening of sn-2 lipases tentatively developed in this work using TAGs coated on microtiter plates suffer from the interference of the MAGs produced in lipolysis reaction and probably solubilized by β-CD, although the assay is sensitive and the substrate coating was found to be stable. The comparison of the initial velocities for the hydrolysis of sn-OEO and tung oil TAGs allows however to rank lipases in various groups as shown in Fig. 8, with CALA being clearly distinguished. This method can therefore be applied for the screening of lipases with high sn-2 specificity like CALA and this is currently what researchers in this field are looking for. Using a lipase already selected for some sn-2 preference, the method can be used during directed evolution or mutagenesis
processes for improving the sn-2 preference. As a general rule with this methodology, the lipases with a higher preference for the sn-2 position than CALA, must show a ratio higher than 0.22. Finally, these results open the way for the synthesis of other lipid substrates with \( \alpha \)-eleostearic acid at various positions.
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References


Figure legends:

**Figure 1.** $^1$H NMR (200 MHz) spectrum of pure $\alpha$-eleostearic acid in deuterated chloroform. Chemical shifts are given in $\delta$-values in ppm downfield from tetramethylsilane ($\delta_{\text{TMS}} = 0$).

**Figure 2.** Synthesis of 1,3-O-dioleoyl-2-O-$\alpha$-eleostearoyl-sn-glycerol.

**Figure 3.** UV absorption spectra of $\alpha$-eleostearic acid, purified TAGs from tung oil, sn-OEO and OA dissolved in ethanol + BHT 0.001%. The final concentration of $\alpha$-eleostearic acid, TAGs, sn-OEO and OA were 4, 2.7, 40 and 1000 µg.mL$^{-1}$, respectively.

**Figure 4.** Schematic representation of the assay reaction showing the hydrolysis of tung oil TAGs or sn-OEO coated in a microwell. E, lipase in solution; E*, lipase at the interface; S, substrate (tung oil TAGs or sn-OEO); P, lipolysis products ($R_1$ = oleic acid; $R_2$ = $\alpha$-eleostearic acid).

**Figure 5.** Stability of sn-OEO and/or tung oil TAGs coated in the microtiter plate wells. After TAGs coating, the absorbance at 272 nm was recorded for 40 min in the presence of the reaction buffer alone (A), in the reaction buffer containing 4.8 µg of BSA/microwell (B), in the reaction buffer containing 39 µg of HPL S152G/microwell (C) and in the reaction buffer containing 20 ng of HPL or heat HPL inactivated HPL/microwell (D).

**Figure 6.** Assays of the lipase activities of CALA, HPL and YLLIP2 using TAGs from tung oil and sn-OEO coated on microtiter plates. Panels A, C and E show typical kinetics of the increase in the OD at 272 nm during the hydrolysis of tung oil TAGs and sn-OEO by CALA (340 ng), HPL (20 ng) and YLLIP2 (12 ng), respectively.
Panels B, D and F show the effects of the enzyme amounts on the steady-state reaction rate using TAGs from tung oil as substrate. For enzyme reaction, 50 µg/well of coated tung oil TAGs or sn-OEO were incubated with variable amounts CALA, HPL and YLLIP2 injected into the well containing 200 µl of standard buffer. The increase in the OD at 272nm was recorded for 40 min and the initial velocity (ΔDO/min) was taken into account for reaction rate determination.

**Figure 7.** Analysis by TLC of tung oil (A) and sn-OEO (B) hydrolysis revealed with UV light at 254 nm (left panels) and with cupric acetate/phosphoric acid 50:50 followed by charring at 180°C for 15 min (right panels). The standards used (20 µg of each compound) were monoolein (a), 1,2(2,3)-sn-diolein (b), 1,3-sn-diolein (c), oleic acid (d) and triolein (e). Lanes 1, TAGs before hydrolysis; lanes 2, TAGs hydrolyzed by HPL; lanes 3, TAG hydrolyzed by YLLIP2 and lanes 4, TAG hydrolyzed by CALA. The chromatographic solvent system was heptane:diethylether:acetic acid, 55:45:1 (v/v/v) containing 0.001% (w/v) BHT.

**Figure 8.** Ratio of initial reaction rate of sn-OEO and tung oil TAGs hydrolysis by various lipases. Panel A, BSA used as control for a non-enzymatic protein, panel B, sn-1,3-regioselective lipases, and panel C, lipases that can hydrolyze the sn-2 position. Dashed line indicates the theoretical ratio (0.14) for non-specific lipases able to hydrolyze each position at the same rate.
Fig. 1:
Fig. 2:

\[
\text{1,3-sn-diolein} + \alpha\text{-eleostearic acid} \rightarrow \text{sn-OEO}
\]

1. DMAP, CH₂Cl₂, 25 min, 0°C
2. DCC, CH₂Cl₂, 5 days, 25°C, 23% yield
Fig. 3:
Fig. 4:
Fig. 5:

A: Buffer
- Tung oil
- \textit{sn}-OEO

B: BSA
- Tung oil
- \textit{sn}-OEO

C: HPL S152G
- Sample Injection

D: HPL
- Active
- Denatured

\textbf{OD}_{272} vs \textbf{Time (min)}
Fig. 7:
Fig. 8: Ratio of initial reaction rate (sn-OEO/Tung oil)

Enzyme: BSA, TLL, HPL, YLLIP2, CALA, CRLAY30, CRLIP3, RHL.