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**Galactolipase activity of *Talaromyces thermophilus* lipase on galactolipid micelles, monomolecular films and UV-absorbing surface-coated substrate**

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*Running title:* Galactolipase activity of *Talaromyces thermophilus* lipase

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**Abbreviations:** BHT, butylhydroxytoluene;  $\beta$ -CD,  $\beta$ -cyclodextrin; DGDG, digalactosyldiacylglycerol ;  $\alpha$ E-MGDG, 1,2-di- $\alpha$ -eleostearoyl-3-galactopyranosyl glycerol; GPLRP2, guinea pig pancreatic lipase-related protein 2; MGDG, monogalactosyldiacylglycerol; NaTDC, sodium taurodeoxycholate; PLRP2, pancreatic lipase-related protein 2; TC4, tributyrin ; TC8; trioctanoin, TLL, *Thermomyces lanuginosus* lipase ; TTL, *Talaromyces thermophilus* lipase;

## Abstract

*Talaromyces thermophilus* lipase (TTL) was found to hydrolyze monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) substrates presented in various forms to the enzyme. Different assay techniques were used for each substrate: pHstat with dioctanoyl galactolipid-bile salt mixed micelles, barostat with dilauroyl galactolipid monomolecular films spread at the air-water interface, and UV absorption using a novel MGDG substrate containing  $\alpha$ -eleostearic acid as chromophore and coated on microtiter plates. The kinetic properties of TTL were compared to those of the homologous lipase from *Thermomyces lanuginosus* (TLL), guinea pig pancreatic lipase-related protein 2 and *Fusarium solani* cutinase. TTL was found to be the most active galactolipase, with a higher activity on micelles than on monomolecular films or surface-coated MGDG. Nevertheless, the UV absorption assay with coated MGDG was highly sensitive and allowed measuring significant activities with about ten ng of enzymes, against hundred ng to ten  $\mu$ g with the pHstat. TTL showed longer lag times than TLL for reaching steady state kinetics of hydrolysis with monomolecular films or surface-coated MGDG. These findings and 3D-modelling of TTL based on the known structure of TLL pointed out to two phenylalanine to leucine substitutions in TTL, that could be responsible for its slower adsorption at lipid-water interface. TTL was found to be more active on MGDG than on DGDG using both galactolipid-bile salt mixed micelles and galactolipid monomolecular films. These later experiments suggest that the second galactose on galactolipid polar head impairs the enzyme adsorption on its aggregated substrate.

## 1. Introduction

Glycolipids are present in almost all biological membranes. Among them, galactolipids are the most abundant membrane lipids in plants, especially in green tissues where they generally represent about 75% of total membrane lipids [1]. They are especially abundant in the photosynthetic membranes of the thylakoids in the chloroplast. These membranes contain mainly monogalactosyl diacylglycerols (MGDG) and digalactosyl diacylglycerols (DGDG) (Fig.1), which represent 50% and 30% of total lipids, respectively [2]. Plant galactolipids are characterized by long chain fatty acids, typically varying from C16 to C20 with one to three unsaturations [3] and they are particularly rich in polyunsaturated fatty acids like C16:3 and C18:3 (ALA,  $\alpha$ -linolenic acid) [4]. Although galactolipids are not present at high levels in normal human diet compared to triglycerides, they may represent the main source of the essential ALA in the absence of specific supplementation with ALA-rich vegetable oils. Indeed, ALA can represent as much as 60 % w/w of the total fatty acid in galactolipids from plant leaves, like in spinach [4], which is higher than what is found in flaxseed oil [5]. The uptake of galactolipid fatty acids is made possible by the action of galactolipases (1,2-Di-*O*-acyl-1-*O*-( $\beta$ -D-galactosyl)-D-glycerol acylhydrolases; EC 3.1.1.26) that hydrolyze the ester bonds at *sn*-1 and *sn*-2 positions of galactolipids. In humans and other mammals, this function is mainly fulfilled by pancreatic lipase-related protein 2 (PLRP2) produced by the exocrine pancreas and acting in the GI tract [6-8].

Galactolipase activity is often displayed by lipases (triacylglycerol acylhydrolases EC.3.1.1.3) with broad substrate specificity that can accept various acylglycerols as substrates like phospholipids and galactolipids. Numerous enzymes with galactolipase activity have been discovered in the plant kingdom [9-11] and more recently in microalgae [12]. Various microbial lipases are also known to degrade galactolipids. For example, Dawson et al. found that microbial lipases from rumen were able to decompose grass galactolipids in the sheep GI

tract [13]. Microbial lipases from *Rhizopus arrhizus* [14], *Mucor javanicus* [15] and *Rhizomucor miehei* (*Lipozyme TM*) [16] have been shown to hydrolyze the ester bond of galactolipids. More recently, synthetic medium chain MGDG and DGDG were used to characterize the galactolipase activity of several well-known microbial lipases, such as the lipases from *Thermomyces lanuginosus* (*TLL*), *Rhizomucor miehei* (*RML*), *Rhizopus oryzae* (*ROL*), *Candida antarctica A* (*CalA*), and the cutinase from *Fusarium solani* (*FsC*) [17]. Using these substrates and natural long chain galactolipids, a new galactolipase from *Fusarium solani* was also identified [18].

In contrast to the large biochemical and structural knowledge on lipases and phospholipases, galactolipases remain poorly studied and characterized so far. This is mainly due to the fact that galactolipid substrates are not commercially available at a large scale and low cost. Natural galactolipids purified from plant green materials have been used for the characterization of some galactolipases by thin layer chromatography analysis of lipolysis products or free fatty acid titration [4, 18] but their limited availability does not allow thorough kinetic studies. Nevertheless, medium galactolipid substrates have been synthesized to develop continuous and sensitive assays using the pHstat [19] and the monomolecular film [7, 20] techniques

Here we used these assays to search for a possible activity of *Talaromyces thermophilus* lipase (*TTL*) on galactolipids. This enzyme was previously purified from a newly isolated *Talaromyces thermophilus* fungal strain and was shown to have a high activity towards short, medium and long chain triacylglycerols [21]. The use of wheat bran as carbon source for cultivating *T. thermophilus* was found to be more effectively than olive oil to induce the production of *TTL* [21], suggesting that other types of lipids present in wheat bran, like galactolipids, could boost the level of *TTL* expression in this fungus. Since the expression of microbial lipases is in general induced by the fatty acids released upon digestion of the

lipid used as the carbon source [22], we wondered whether TTL displays a galactolipase activity, as previously identified with homologous enzymes of this fungal lipase family [17, 18].

The galactolipase activity of TTL was investigated using medium chain galactolipids presented in the form of mixed micelles with bile salts (pHstat assay; [19]) and monomolecular films (Barostat assay; [7]). We also used the characterization of TTL for developing a novel UV spectrophotometric galactolipase assay in microtiter plates using a synthetic MGDG substrate containing  $\alpha$ -eleostearic acid as chromophore. Various lipase assays have been developed for high throughput screening using  $\alpha$ -eleostearic acid esterified into natural [23, 24] and synthetic triglycerides [25, 26] or phospholipids [27] but not galactolipids so far. Using this combination of galactolipase assays, we showed that TLL is active on both MGDG and DGDG and we compared its kinetic properties with those of other galactolipases, the homologous fungal lipase from *Thermomyces lanuginosus* (TLL), guinea pig pancreatic lipase-related protein 2 (GPLRP2) and cutinase from *Fusarium solani* (FsC).

## 2. Materials and methods

### 2.1. Enzymes

*TTL* was produced by a newly isolated thermo-tolerant fungal strain identified as *Talaromyces thermophilus* Stolk and purified according to [21]. Recombinant GPLRP2 was expressed in *Aspergillus oryzae* and purified according to [28]. Cutinase from *Fusarium solani* (FsC) was expressed in *E.coli* and purified as previously described [29]. *Thermomyces lanuginosus* lipase (TLL; Lipolase™) was purchased from Sigma (St-Quentin-Fallavier, France). The homogeneity of the various enzymes was routinely assessed by performing SDS-PAGE on 12% gels using Laemmli's procedure [30]. The protein concentration was determined with a good accuracy using the BCA kit (Pierce) and BSA as standard.

### 2.2. Potentiometric pHstat assay of galactolipase activity

Diocanoyl galactolipid substrates, C8-MGDG (3-*O*-β-D-galactopyranosyl-1,2-di-*O*-octanoyl-*sn*-glycerol) and C8-DGDG (3-*O*-(6-*O*-α-D-galactopyranosyl-β-D-galactopyranosyl)-1,2-di-*O*-octanoyl-*sn*-glycerol), were synthesized as previously described [4, 19]. The galactolipase activity was measured potentiometrically at 37 °C and at a constant pH value of 8.0 by continuously measuring the release of free fatty acids from mechanically stirred dispersions of galactolipids, using 0.1 N NaOH as titrant and a pHstat apparatus (718 STAT Titrino, Metrohm). To prepare the galactolipid dispersion, 25 mg of C8-MGDG or C8-DGDG were mixed with 5 mL of 0.33 mM Tris-HCl buffer, containing 0.1 M NaCl and various concentrations of sodium taurodeoxycholate (NaTDC) and then subjected to ultrasonic treatment for 6-8 min in a water bath (HF-Frequency 35 kHz; SONOREX SUPER compact ultrasonic bath model RK 31, BANDELIN electronic) [19]. One international galactolipase unit (U) corresponds to the release of one μmol of fatty acid released per

minute. The specific activities were expressed in international units per milligram of enzyme (U/mg).

### *2.3. Potentiometric pHstat assay of phospholipase and lipase activities*

Phospholipase activities were measured potentiometrically at 37 °C and pH 8.0 by automatically titrating the free fatty acids released from purified egg L- $\alpha$ -phosphatidylcholine (Sigma) as substrate, as previously described [31]. Lipase activities were measured with mechanically stirred triglyceride emulsions according to [21]. Specific activities were expressed in international units (U) per milligram of enzyme. One U corresponds to 1  $\mu$ mol of fatty acid released per minute.

### *2.4. Monomolecular film experiments for measuring galactolipase activities*

The galactolipase activity was measured using monomolecular films of 1,2-di-*O*-dodecanoyl-3-*O*- $\beta$ -D-galactopyranosyl-*sn*-glycerol (C12-MGDG) and 1,2-di-*O*-dodecanoyl-3-*O*-(6-*O*- $\alpha$ -D-galactopyranosyl- $\beta$ -D-galactopyranosyl)-*sn*-glycerol (C12-DGDG) as substrates. C12-MGDG and C12-DGDG were synthesized as previously reported [7, 20]. All experiments were performed using the KSV 5000 barostat equipment (KSV, Helsinki, Finland) and a “zero order” Teflon trough [32]. The reaction compartment had a surface area of 38.5 cm<sup>2</sup> and a volume of 43 ml. The reservoir (24-cm long  $\times$  7.5-cm wide) had a surface area of 156.5 cm<sup>2</sup> and a volume of 203 mL. Lipid monomolecular films were formed at the air/water interface by spreading the lipid solution (1 mg/mL C12-MGDG or C12-DGDG in chloroform). The enzyme solution was injected into the subphase at a final concentration of 0.02 nM for rGPLRP2, 5nM for TLL and cutinase and 0.45 nM for TTL. The aqueous subphase was composed of 10 mM Tris-HCl, 100 mM NaCl, 21 mM CaCl<sub>2</sub>, and 1 mM EDTA, pH 8.0 and was prepared with double-distilled water. Residual surface-active



impurities were removed by simultaneous sweeping and suction of the surface before spreading the lipid solution [32]. The reaction compartment was stirred with a 1-cm magnetic bar rotating at 250 rpm. The reactions were performed at 25°C. Surface pressure was measured using a Wilhelmy plate (perimeter, 3.94 cm) attached to an electromicro balance. The trough was equipped with a mobile Teflon barrier to keep the surface pressure constant during enzymatic hydrolysis of the substrate film and desorption of the soluble lipolysis products (monododecanoyl-galactopyranosyl-glycerol, dodecanoic acid). Enzyme activity was estimated from the surface of the trough covered by the mobile barrier and the known molecular area of the substrate molecule. The molecular areas of the C12-MGDG and C12-DGDG substrates were previously determined by performing compression isotherms [33]. The galactolipase activity was expressed in moles of substrate hydrolyzed per surface unit (cm<sup>2</sup>) per minute and referred to the overall molarity of the enzyme initially injected into the aqueous subphase (mol.cm<sup>-2</sup>.min<sup>-1</sup>.M<sup>-1</sup>).

#### 2.5. Synthesis of 1,2-Di-O- $\alpha$ -eleostearoyl-3-O- $\beta$ -D-galactopyranosyl-sn-glycerol ( $\alpha$ E-MGDG)

Dry dichloromethane was prepared by successive washing with water, drying with calcium chloride, and distillation from calcium hydride. Thin layer chromatography (TLC) was carried out on aluminium sheets coated with silica gel 60 F<sub>254</sub> (Merck). TLC plates were inspected by UV light ( $\lambda$  = 254 nm). Column chromatography was performed on Silica-gel (Silicycle). <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded at 293 K using Bruker ALS300 or DRX300 spectrometers. High resolution (HR-ESI-QToF) mass spectra were recorded using a Bruker MicroToF-Q II XL spectrometer.

3-O-[2,3,4,6-Tetra-O-levulinoyl- $\beta$ -D-galactopyranosyl]-1,2-O-isopropylidene-sn-glycerol (**2**):

3-O-[2,3,4,6-Tetra-O-acetyl- $\beta$ -D-galactopyranosyl]-1,2-O-isopropylidene-sn-glycerol (**1**)

(2.60 g, 5.53 mmol) was added to dry methanol (50 mL) containing a chip of sodium and the mixture was stirred for 2 h. Amberlite IR 120 [H<sup>+</sup>] resin was added to neutralize the solution and after 2 min, the solution was filtrated and concentrated *in vacuo*. The product was coevaporated twice from toluene (2x15 mL), dissolved in ethyl acetate (50 mL) and levulinic acid (3.92 g, 33.76 mmol), dicyclohexyldicarbodiimide (8.27 g, 40.00 mmol) and a catalytic amount of 4-dimethylaminopyridine (50 mg) were successively added. The mixture was stirred overnight, and after filtration, the solid was washed carefully with ethyl acetate. The organic phase was concentrated to dryness, the crude product was purified by column chromatography (4:1 ethyl acetate-petroleum ether to pure ethyl acetate) and the pure product **2** was recovered at 70% yield: 2.70 g, oily material, *R*<sub>f</sub> 0.30 (5:1 ethyl acetate-petroleum ether); [α]<sub>D</sub> +2.1 (*c* 1.0, CHCl<sub>3</sub>); <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 5.36 (dd, 1H, *J*<sub>3,4</sub> 3.4, *J*<sub>4,5</sub> 0.8 Hz, H-4), 5.17 (dd, 1H, *J*<sub>1,2</sub> 7.9, *J*<sub>2,3</sub> 10.4 Hz, H-2), 5.01 (dd, 1H, H-3), 4.57 (d, 1H, H-1), 4.26 (dddd, 1H, H-2<sub>gly</sub>), 4.20 (dd, 1H, *J*<sub>5,6a</sub> 6.7, *J*<sub>6a,6b</sub> 11.2 Hz, H-6a), 4.10 (dd, 1H, *J*<sub>5,6b</sub> 6.4 Hz, H-6b), 4.04 (dd, 1H, *J*<sub>1agly,2gly</sub> 6.4, *J*<sub>1agly,1bgly</sub> 6.4 Hz, H-1a<sub>gly</sub>), 3.92-3.86 (m, 2H, H-5, H-3a<sub>gly</sub>), 3.83 (dd, 1H, *J*<sub>1bgly,2gly</sub> 6.2 Hz, H-1b<sub>gly</sub>), 3.62 (dd, 1H, *J*<sub>2gly,3bgly</sub> 6.2, *J*<sub>3agly,3bgly</sub> 10.5 Hz, H-3b<sub>gly</sub>), 2.80-2.40 (m, 16H, 4COCH<sub>2</sub>CH<sub>2</sub>COCH<sub>3</sub>), 2.19, 2.18, 2.17, 2.16 (4s, 12H, 4CH<sub>3</sub>CO), 1.41, 1.34 (2s, 6H, (CH<sub>3</sub>)<sub>2</sub>C); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 206.52, 206.43, 206.16, 206.04 (4CH<sub>3</sub>CO), 172.15, 171.90, 171.85, 171.41 (4OCOCH<sub>2</sub>), 109.26 (C(CH<sub>3</sub>)<sub>2</sub>), 101.23 (C-1), 74.25 (C-2<sub>gly</sub>), 70.81, 70.73 (C-3, C-5), 69.38 (C-3<sub>gly</sub>), 68.85 (C-2), 67.24 (C-4), 66.36 (C-1<sub>gly</sub>), 61.38 (C-6), 37.84, 37.77, 37.76, 37.67 (CH<sub>2</sub>COCH<sub>3</sub>), 29.76, 29.67 (CH<sub>3</sub>COCH<sub>2</sub>), 27.79 (OCOCH<sub>2</sub>), 26.67, 25.20 ((CH<sub>3</sub>)<sub>2</sub>C).

HRMS calculated for C<sub>32</sub>H<sub>46</sub>NaO<sub>16</sub> [M+Na]<sup>+</sup> 709.2678; found 709.2651.

3-*O*-[2,3,4,6-Tetra-*O*-levulinoyl-β-D-galactopyranosyl]-*sn*-glycerol (**3**): a solution of compound **2** (2.60 g, 1.46 mmol) in 70% aqueous acetic acid (20 mL) was stirred for 5 h at

60°C. After concentration, the residue was coevaporated from toluene (3x20 mL). The crude product was purified by column chromatography (9:1 CHCl<sub>3</sub>-EtOH). Pure product **3** was obtained in 90% yield: 2.20 g, oily material, *R*<sub>f</sub> 0.46 (9:1 CHCl<sub>3</sub>-EtOH); [α]<sub>D</sub>-0.5 (*c* 2.0, CHCl<sub>3</sub>); <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 5.31 (bd, 1H, *J*<sub>3,4</sub> 3.4, *J*<sub>4,5</sub> 0.2 Hz, H-4), 5.12 (dd, 1H, *J*<sub>1,2</sub> 7.8 Hz, *J*<sub>2,3</sub> 10.5 Hz, H-2), 5.00 (dd, 1H, H-3), 4.49 (d, 1H, H-1), 4.16 (dd, 1H, *J*<sub>5,6a</sub> 7.1, *J*<sub>6a,6b</sub> 11.3, H-6a), 4.06 (dd, 1H, *J*<sub>5,6b</sub> 6.1 Hz, H-6b), 3.89 (bd, 1H, H-5), 3.88-3.55 (m, 5H, H-1a<sub>gly</sub>, H-1b<sub>gly</sub>, H-2<sub>gly</sub>, H-3a<sub>gly</sub>, H-3b<sub>gly</sub>), 3.17-3.10 (m, 2H, 2OH), 2.85-2.35 (m, 16H, 4COCH<sub>2</sub>CH<sub>2</sub>COCH<sub>3</sub>), 2.19, 2.18, 2.17, 2.16 (4s, 12H, 4CH<sub>3</sub>CO); <sup>13</sup>C NMR (CDCl<sub>3</sub>): δ 207.76, 206.89, 206.69, 206.22 (CH<sub>3</sub>CO levulinoyl), 172.28, 171.99, 171.81, 171.75 (OCOCH<sub>2</sub> levulinoyl), 101.57 (C-1), 72.03 (C-3<sub>gly</sub>), 70.89 (C-5), 70.66 (C-3), 70.48, 69.02 (C-2, C-2<sub>gly</sub>), 67.35 (C-4), 63.35 (C-1<sub>gly</sub>), 61.61 (C-6), 37.87, 37.87, 37.80, 37.69 (CH<sub>3</sub>COCH<sub>2</sub>), 29.87, 29.85, 29.81, 29.72 (CH<sub>3</sub>CO), 27.82, 27.80 (OCOCH<sub>2</sub>).

HRMS calculated for C<sub>29</sub>H<sub>42</sub>NaO<sub>16</sub> [M+Na]<sup>+</sup> 669.2365; found 669.2344.

1,2-Di-*O*-α-eleostearoyl-3-*O*-[2,3,4,6-tetra-*O*-levulinoyl-β-D-galactopyranosyl]-*sn*-glycerol (**5**): α-Eleostearic acid **4** (2.42 g, 8.42 mmol), dicyclohexyldicarbodiimide (3.58 g, 17.40 mmol) and a catalytic amount of 4-dimethylaminopyridine (50 mg) were successively added under argon to a solution of product **3** (1.875 g, 2.90 mmol) in dichloromethane (40 mL). The mixture was stirred overnight, and methanol (0.50 mL) was added. After 2 h, the solid was removed by filtration and washed with dichloromethane. The combined organic phases were concentrated to dryness and the crude product was purified by column chromatography (1:1 to 4:1 ethyl acetate-petroleum ether). A second column chromatography (9:1 CH<sub>2</sub>Cl<sub>2</sub>-EtOH) was necessary to give the pure product **5**, recovered in 73% yield: 2.47 g, oily material, *R*<sub>f</sub> 0.20-0.25 (1:1 ethyl acetate-petroleum ether), 0.70 (9:1 CH<sub>2</sub>Cl<sub>2</sub>-EtOH); [α]<sub>D</sub> +3.7 (*c* 1.0, CHCl<sub>3</sub>); <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 6.42-6.33 (m, 2H, 2H-11<sub>eleo</sub>), 6.20-6.13 (m, 2H, 2H-12<sub>eleo</sub>), 6.13-6.06 (m,

245 2H, 2H-13<sub>eleo</sub>), 6.02-5.95 (m, 2H, 2H-10<sub>eleo</sub>), 5.75-5.65 (m, 2H, 2H-14<sub>eleo</sub>), 5.43-5.34 (m, 1H,  
 246 H-9<sub>eleo</sub>), 5.37 (bd, 1H,  $J_{3,4}$  3.4,  $J_{4,5}$  0.7 Hz, H-4), 5.20 (dddd, 1H,  $J_{1\text{agly},2\text{gly}}$  3.0,  $J_{1\text{bgly},2\text{gly}}$  6.2,  
 247  $J_{2\text{gly},3\text{agly}}$  4.9,  $J_{2\text{gly},3\text{bgly}}$  5.9 Hz, H-2<sub>gly</sub>), 5.16 (dd, 1H,  $J_{1,2}$  7.9 Hz,  $J_{2,3}$  10.5 Hz, H-2), 5.02 (dd,  
 248 1H, H-3), 4.49 (d, 1H, H-1), 4.41 (dd, 1H,  $J_{1\text{agly},1\text{bgly}}$  12.0 Hz, H-1<sub>agly</sub>), 4.20 (dd, 1H,  $J_{5,6a}$  6.6,  
 249  $J_{6a,6b}$  11.1, H-6a), 4.14 (dd, 1H, H-1<sub>bgly</sub>), 4.10 (dd, 1H,  $J_{5,6b}$  6.6 Hz, H-6b), 3.95 (dd, 1H,  
 250  $J_{3\text{agly},3\text{bgly}}$  10.9 Hz, H-3<sub>agly</sub>), 3.89 (bdd, 1H, H-5), 3.69 (dd, 1H, H-3<sub>bgly</sub>), 2.85-2.45 (m, 16H,  
 251 8CH<sub>2</sub> levulinoyl), 2.35-2.26 (m, 4H, 2COCH<sub>2</sub> eleo), 2.19, 2.18, 2.17, 2.16 (4s, 12H, 4CH<sub>3</sub>CO  
 252 levulinoyl), 2.14-2.07 (m, 4H, 4H, 2CH<sub>2</sub>CH=), 1.65-1.55 (m, 4H, 2COCH<sub>2</sub>CH<sub>2</sub> eleo), 1.40-  
 253 1.25 (m, 24H, 12CH<sub>2</sub> alkyl chains), 0.91 (t, 9H,  $J$  6.5Hz, 2CH<sub>3</sub>CH<sub>2</sub>); <sup>13</sup>C NMR (CDCl<sub>3</sub>):  $\delta$   
 254 206.58, 206.49, 206.25, 206.09 (CH<sub>3</sub>CO levulinoyl), 173.36, 172.88 (COCH<sub>2</sub> eleo), 172.26,  
 255 171.94, 171.94, 171.47 (OCOCH<sub>2</sub> levulinoyl), 135.27 (C-14<sub>eleo</sub>), 132.94 (C-12<sub>eleo</sub>), 131.84 (C-  
 256 9<sub>eleo</sub>), 130.63 (C-13<sub>eleo</sub>), 128.82 (C-10<sub>eleo</sub>), 126.01 (C-11<sub>eleo</sub>), 101.54 (C-1), 70.89 (C-5), 70.76  
 257 (C-3), 69.76 (C-2<sub>gly</sub>), 68.78 (C-2), 67.75 (C-3<sub>gly</sub>), 67.24 (C-4), 62.39 (C-1<sub>gly</sub>), 61.41 (C-6),  
 258 37.93, 37.87, 37.87, 37.76 (CH<sub>3</sub>COCH<sub>2</sub>), 34.27, 34.13 (COCH<sub>2</sub> eleo), 32.57 (C-15 eleo),  
 259 31.53 (C-16 eleo), 29.82 (CH<sub>3</sub>CO), 29.73, 29.25, 29.18, 29.14, 29.11 (C-4, C-5, C-6, C-7),  
 260 27.88, 27.86 (OCOCH<sub>2</sub> lev, C-8), 24.93 (C-3 eleo), 22.30 (C-17 eleo), 14.03 (C-18 eleo).  
 261 HRMS calculated for C<sub>65</sub>H<sub>98</sub>NaO<sub>10</sub> [M+Na]<sup>+</sup> 1189.6645; found 1189.6661.

262  
 263 1,2-Di-*O*- $\alpha$ -eleostearoyl-3-*O*- $\beta$ -D-galactopyranosyl-*sn*-glycerol (**6**): a solution of hydrazine  
 264 hydrate (1.00 mL, 20.60 mmol) in 3:2 pyridine-acetic acid (20 mL) was added drop wise to a  
 265 solution of galactolipid **5** (1.167 g, 1.00 mmol) in pyridine. The mixture was stirred for 15  
 266 min, and poured in chloroform (150 mL). The organic phase was washed with water (75 mL)  
 267 and with saturated NaHCO<sub>3</sub> solution (2x50 mL). The aqueous phases were extracted with  
 268 chloroform (6x40 mL) and the combined organic phases were dried (Na<sub>2</sub>SO<sub>4</sub>) and  
 269 concentrated. Pure product **6** was recovered in 60% yield after purification by column

270 chromatography (10:1 ethyl acetate-methanol): 0.465 g, oily material,  $R_f$  0.70 (10:1 ethyl  
271 acetate-methanol), 0.70 (9:1  $\text{CH}_2\text{Cl}_2$ -EtOH);  $[\alpha]_D +3.8$  ( $c$  1.0, 4:1  $\text{CHCl}_3$ -MeOH);  $^1\text{H}$  NMR  
272 ( $\text{CDCl}_3$ ):  $\delta$  6.33-6.25 (m, 2H, 2H-11<sub>eleo</sub>), 6.12-6.05 (m, 2H, 2H-12<sub>eleo</sub>), 6.04-5.97 (m, 2H, 2H-  
273 13<sub>eleo</sub>), 5.93-5.86 (m, 2H, 2H-10<sub>eleo</sub>), 5.66-5.57 (m, 2H, 2H-14<sub>eleo</sub>), 5.34-5.26 (m, 2H, 2H-  
274 9<sub>eleo</sub>), 5.19 (dddd, 1H,  $J_{1\text{agly},2\text{gly}}$  3.2,  $J_{1\text{bgly},2\text{gly}}$  6.5,  $J_{2\text{gly},3\text{agly}}$  5.4,  $J_{2\text{gly},3\text{bgly}}$  4.1 Hz, H-2<sub>gly</sub>), 4.28  
275 (dd, 1 H,  $J_{1\text{agly},1\text{bgly}}$  12.1 Hz, H-1<sub>agly</sub>), 4.14 (d, 1H,  $J_{1,2}$  7.1 Hz, H-1), 4.14 (dd, 1H, H-1<sub>bgly</sub>),  
276 3.95 (dd, 1H,  $J_{3\text{agly},3\text{bgly}}$  10.9 Hz, H-3<sub>agly</sub>), 3.81(bd, 1H,  $J_{3,4}$  3.4,  $J_{4,5}$  0.7 Hz, H-4), 3.76 (dd,  
277  $J_{5,6a}$  6.2,  $J_{6a,6b}$  11.9, H-6a), 3.67 (dd, 1H,  $J_{5,6b}$  5.2 Hz, H-6b), 3.63 (dd, 1H, H-3<sub>bgly</sub>), 3.47 (dd,  
278 1H,  $J_{2,3}$  9.6 Hz, H-2), 3.44-3.40 (m, 2H, H-3, H-5), 2.26-2.20 (m, 4H, 2 $\text{COCH}_2$ ), 2.11-1.95  
279 (m, 8H, 4 $\text{CH}_2\text{CH=}$ ), 1.57-1.47 (m, 4H, 2 $\text{COCH}_2\text{CH}_2$  eleo), 1.32-1.17 (m, 24H, 12 $\text{CH}_2$  alkyl  
280 chains), 0.81 (t, 6H,  $J$  6.5Hz, 2 $\text{CH}_3\text{CH}_2$ );  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  173.98, 173.68 ( $\text{COCH}_2$  eleo),  
281 135.18 (C-14<sub>eleo</sub>), 132.85 (C-12<sub>eleo</sub>), 131.68 (C-9<sub>eleo</sub>), 130.53 (C-13<sub>eleo</sub>), 128.72 (C-10<sub>eleo</sub>),  
282 125.88 (C-11<sub>eleo</sub>), 103.98 (C-1), 74.93 (C-3), 73.32 (C-5), 71.15 (C-2), 70.31 (C-2<sub>gly</sub>), 68.73  
283 (C-4), 67.83 (C-3<sub>gly</sub>), 62.78 (C-1<sub>gly</sub>), 61.42 (C-6), 34.19, 34.05 ( $\text{COCH}_2$  eleo), 32.44 (C-15  
284 eleo), 31.41 (C-16 eleo), 29.60, 29.12, 29.05, 29.01(C-4<sub>eleo</sub>, C-5<sub>eleo</sub>, C-6<sub>eleo</sub>, C-7<sub>eleo</sub>), 27.74 (C-  
285 8<sub>eleo</sub>), 24.79 (C-3 eleo), 22.17 (C-17<sub>eleo</sub>), 13.81 (C-18<sub>eleo</sub>).  
286 HRMS calculated for  $\text{C}_{45}\text{H}_{75}\text{O}_{10}$   $[\text{M}+\text{H}]^+$  775.5353; found 775.5327.  
287

288 2.6. *Spectrophotometric assay of galactolipase activities using 1,2-Di-O- $\alpha$ -eleostearoyl-3-O-*  
 289  *$\beta$ -D-galactopyranosyl-sn-glycerol in microtiter plates*

290 Microtiter plates were coated with the UV-absorbing galactolipid substrate using an  
 291  $\alpha$ E-MGDG solution (0.5 mg mL<sup>-1</sup>) prepared in ethanol and containing 0.01% BHT as an  
 292 antioxidant. The wells of UV-transparent microtiter plates (Corning, Inc., Corning, NY,  
 293 catalog No. 3635) were filled with the substrate solution (100  $\mu$ L/well) and left to stand under  
 294 a fume hood until the solvent had completely evaporated (for around two hours). The wells  
 295 containing the coated galactolipids were washed three times with 0.2 mL of the assay buffer  
 296 (10 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 6 mM CaCl<sub>2</sub>, 1 mM EDTA, and  
 297 3 mg mL<sup>-1</sup>  $\beta$ -cyclodextrin ( $\beta$ -CD)) and left to equilibrate at 37°C for at least 5 min with 200  
 298  $\mu$ L of the assay buffer. The  $\beta$ -CD was used in the reaction buffer in order to solubilize the  
 299 long-chain of fatty acids released upon substrate hydrolysis. Assays were performed by  
 300 adding the lipase solutions (2–10  $\mu$ L) into the wells, and the optical density (OD) at 272 nm  
 301 was recorded continuously at regular time intervals of 30 s for 15 min using a Powerwave<sup>TM</sup>  
 302 200 microtiter plate-scanning spectrophotometer (Bio-Tek Instruments Winooski, VT)  
 303 running using the KC4 software. OD measurements included pathlength correction and OD  
 304 values are given for an optical pathlength of 1cm. The steady-state rate of OD increase ( $R_{ss}$ )  
 305 as well as the lag time ( $\tau$ ) required to reach the steady state were calculated by fitting the OD  
 306 variation with time to the following equation adapted from Verger et al. [34]:

$$OD_{272}(t) - OD_{272}(0) = R_{ss} \times t + \tau R_{ss}(e^{-t/\tau})$$

310 where  $OD_{272}(t)$  and  $OD_{272}(0)$  are the optical densities recorded at 272 nm at reaction time  $t$   
 311 (min) and zero (enzyme injection), respectively,  $R_{ss}$  is the steady-state reaction rate ( $\Delta OD$   
 312 min<sup>-1</sup>), and  $\tau$  is the lag time (minutes). The specific activity of galactolipases was estimated

from the steady-state reaction rate using an apparent molar extinction of  $5320 \text{ M}^{-1} \text{ cm}^{-1}$  for  $\alpha$ -eleostearic acid [27] and was expressed as  $\mu\text{moles}$  of fatty acid released per minute per mg of enzyme, under the assay conditions.

## 2.7. Molecular modelling of *Talaromyces thermophilus* lipase

A 3D model of TTL was built based on its sequence homology with TLL (88% sequence identity), and a known 3D structure of TLL with the lid in the open conformation (PBD code 1GT6 [35]) using the swiss model server [36]. A dipolar vector for the constructed model of TTL and the structure of TLL was calculated using partial Gasteiger charges obtained with the Chimera program [39].

## 2.8. Dynamic light scattering measurements

Dynamic light scattering (DLS) experiments on C8-MGDG and C8-DGDG dispersions in 0.33mM Tris buffer, pH 8, 100mM NaCl and 5mM  $\text{CaCl}_2$  were carried out using a Zetasizer Nano S (Malvern Instruments) at  $37^\circ\text{C}$ . Each measurement with mixtures of galactolipids and bile salts was performed in triplicate and consisted in 10-15 runs of 10 seconds at a scattering angle of  $173^\circ$ . The determination of the hydrodynamic diameter ( $D_H$ ) was based on the Einstein-Stokes relation to obtain the intensity averaged size distribution. A viscosity of 0.6684 cP and a refractive index of 1.332 (at  $37^\circ\text{C}$ ) were used for the dispersion medium, while a value of 1.49 was used as an approximation of the refractive index for micelles [40]. Changes in the viscosity and in the refractive index induced by the temperature were taken into account by the software. Collected data were analyzed by applying a customized method using 70 classes with a size-range analysis of 0.6 to 10000 nm.

### 3. Results and discussion

#### 3.1. Galactolipase activity of TTL on C8-MGDG and C8-DGDG micelles

The galactolipase activity of TTL was first tested using synthetic medium chain (C8) MGDG and DGDG mixed with bile salts (NaTDC) at molar ratios of 1.33 and 0.25, respectively, to form mixed micelles. This presentation of substrate to the enzyme was previously reported to be the most effective for various mammalian and microbial galactolipases [4, 17, 19]. These micelles were not characterized, however, and dynamic light scattering was used here to estimate their average particle size and distribution at 37°C and pH 8. The hydrodynamic diameters ( $D_H$ , z-average) of C8-MGDG (10 mM)-NaTDC (13 mM) and C8-DGDG (10 mM)-NaTDC (2.5 mM) micelles were found to be  $9.4 \pm 0.3$  and  $24.5 \pm 0.2$  nm, respectively, with polydispersity index (PDI) of 0.303 and 0.178. These values were in the same range as those measured with mixed micelles of phospholipids and bile salts at similar concentrations [41, 42].

The optimum activity of TTL was found to occur at pH 8 on both C8-MGDG and C8-DGDG (Figure 2). TTL specific activity on C8-MGDG was found to be  $40,500 \pm 125$  U/mg, compared to  $4,658 \pm 146$  U/mg for *Fusarium solani* lipase [18],  $5,420 \pm 85$  U/mg for GPLRP2,  $984 \pm 62$  U/mg for FsC and  $450 \pm 41$  U/mg for TLL [17]. Thus, to our knowledge, TTL activity on C8-MGDG is the highest galactolipase activity measured. This activity is 7-fold higher than that of GPLRP2, the most active mammalian galactolipase characterized so far. The maximum specific activity of TTL on C8-DGDG was also found to be the highest galactolipase activity measured with DGDG ( $9,800 \pm 125$  U/mg; Figure 2).



### 3.2. Galactolipase activity of TTL on C12-MGDG and C12-DGDG monomolecular films

The galactolipase activity of TTL was then tested on medium chain synthetic galactolipids (C12-MGDG and C12-DGDG) that form stable monomolecular films at the air-water interface, while their lipolysis products are soluble in water [7, 17, 33, 43]. This allows measuring galactolipase activities at various surface pressures using the barostat technique [32, 34].

TTL activity on C12-MGDG showed a bell-shaped activity profile as a function of surface pressure, with a maximum activity of  $161.7 \pm 7.3 \text{ mmol cm}^{-2} \text{ min}^{-1} \text{ M}^{-1}$  at 25 mN/m (Figure 3A). TTL was found to be 10 to 14-fold more active than the homologous fungal lipase TLL, that showed its maximum activity ( $14.2 \text{ mmol cm}^{-2} \text{ min}^{-1} \text{ M}^{-1}$ ) at a lower surface pressure of 12 mN m<sup>-1</sup> (Figure 3A). TTL was less active than rGPLRP2 but the optimum activity of the latter ( $2047 \pm 237 \text{ mmol cm}^{-2} \text{ min}^{-1} \text{ M}^{-1}$ ; Table 1) was found at a much lower surface pressure of 10 mN m<sup>-1</sup> (Table 1). Remarkably, TTL was found to be able to hydrolyze C12-MGDG monomolecular films at surface pressures up to 30 mN m<sup>-1</sup>. Most galactolipases characterized so far are not active at such high surface pressures, except recombinant human PLRP2 (Table 1; [33]). It confirms that lipases possessing a lid domain, like TTL, TLL and rHPLRP2, are able to hydrolyze galactolipids at higher surface pressures than those without a lid, like GPLRP2 and FsC [17].

Similar features were observed with C12-DGDG but with optimum activities at lower surface pressures (Figure 3A and Table 1). Remarkably, TLL only showed a very weak activity of  $0.8 \pm 0.03 \text{ mmol cm}^{-2} \text{ min}^{-1} \text{ M}^{-1}$  on C12-DGDG at 10 mN m<sup>-1</sup> (Figure 3A). The presence of a second galactose unit on the polar head of galactolipids has therefore a significant effect on the penetration and activity of galactolipases as a function of surface pressure, and this effect is particularly marked with TLL. This is also shown by the lag times for measuring steady state kinetics of galactolipid hydrolysis using the barostat technique.

With C12-MGDG, the lag time values were low (1-2 min) below 15 mN m<sup>-1</sup> and only increased above and till 25 mN m<sup>-1</sup> for TTL (Figure 3B). Lag times for both enzymes were much higher (10-20 min) with C12-DGDG and this feature was already observed at low surface pressure (Figure 3B).

The surface pressure and the size/steric hindrance of the hydrophilic polar head of galactolipids are therefore two important parameters controlling the activity of galactolipases, and this activity is favoured at higher surface pressure by the presence of a lid domain. Enzymes without a lid, like GPLRP2 and cutinase show optimum activity at low surface pressures and very long lag times (40-70 min) to reach steady state kinetics above 10 mN m<sup>-1</sup> [17]. The presence of an amphiphilic lid probably favours the interaction of the lipase with the galactolipid monolayer spread at the air-water interface. These results further underline the crucial role of the lid in the interaction with the lipid substrate and the control of enzyme activity [44, 45].

### *3.3. Galactolipase activity of TTL on a surface-coated MGDG substrate containing $\alpha$ -eleostearic acid*

The conjugated triene present in  $\alpha$ -eleostearic acid confers strong UV absorption properties on both pure fatty acid and TAGs containing this fatty acid, as in tung oil in which it represents around 70% of total fatty acids [23]. These UV absorption properties have been used for developing lipase and phospholipase spectrophotometric assays in microtiter plates using tung oil [24], synthetic TAG [25, 26] and phospholipid [27] containing  $\alpha$ -eleostearic acid as chromophore. Following a similar approach, we synthesized a monogalactosyldiglyceride containing  $\alpha$ -eleostearic acid ( $\alpha$ E-MGDG) to establish a new UV spectrophotometric assay of galactolipases in microtiter plates, in which the substrate is coated on the well surface.

For the synthesis of  $\alpha$ E-MGDG (compound **6** in Figure 4), we used 3-*O*-[2,3,4,6-Tetra-*O*-acetyl- $\beta$ -D-galactopyranosyl]-1,2-*O*-isopropylidene-*sn*-glycerol (compound **1**) that was obtained in a previous study [7] and  $\alpha$ -eleostearic acid (compound **4**) prepared according to Mendoza et al. [25] or O'Connor et al. [46]. Compound **1** was *O*-deacetylated under Zemplén conditions (catalytic sodium methylate in methanol), the tetraol was esterified by treatment with levulinic acid in ethyl acetate in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP), affording compound **2** in 70% yield. After cleavage of the isopropylidene group under acidic medium (70% acetic acid, 60°C), the diol **3** was reacted with  $\alpha$ -eleostearic acid **4** (DCC, DMAP, CH<sub>2</sub>Cl<sub>2</sub>) affording the product **5** in 73% yield. Finally, the levulinoyl protecting groups were cleaved by hydrazine hydrate in a 3:2 pyridine-acetic anhydride mixture yielding product **6** in 60% yields (Figure 4).

The UV absorption spectrum (230-300 nm) of an ethanolic solution of  $\alpha$ E-MGDG displayed three major peaks located at 260, 270 and 282 nm (Figure 5). This profile spectrum is similar to that of pure  $\alpha$ -eleostearic acid [24], pure tung oil triglycerides [23], synthetic  $\alpha$ -eleostearic acid-containing triglycerides [25] and phosphatidylcholine [27]. In aqueous buffer, the major absorption peak was shifted from 270 nm to 272 nm, as described earlier [47].

Assays of galactolipase activities were performed after coating UV-transparent microtiter plates with  $\alpha$ E-MGDG that was first added as a solution in ethanol before the alcohol was evaporated. After coating the wells of microtiter plates, the absorbance at 272 nm was recorded for 20 min in the presence of buffer without enzyme to determine background absorbance. A constant baseline with optical density (OD) not exceeding 0.3 was recorded, indicating that the substrate coating was not altered by the addition of buffer. TTL and other galactolipases were then tested, assuming that these enzymes will bind to the surface-coated  $\alpha$ E-MGDG substrate, will hydrolyze it and release  $\alpha$ -eleostearic acid (Figure 6A). This long

chain fatty acid can be further solubilized by complex formation with  $\beta$ -CD present in the buffer and its concentration can be measured continuously by monitoring UV absorbance at 272 nm (Figure 6A).

To validate the method, various amounts of substrate coated onto the plates (5, 10, 20 or 50  $\mu$ g per well) and enzymes (20, 40 and 80 ng of TTL, TLL, GPLRP2 or FsC) were tested. The highest amount of substrate tested (50  $\mu$ g  $\alpha$ E-MGDG per well) was retained because it formed a stable coating and allowed measuring steady-state enzyme kinetics for longer period of time. Typical kinetics showing the increase in OD ( $\Delta$ OD = assay OD - initial OD) at 272 nm during  $\alpha$ E-MGDG hydrolysis by various amounts of TTL are shown in Figure 5B. Lag times of around 6 to 8 min were observed before recording linear OD variations with time, but these variations were then proportional to TTL amounts in the 20 to 80 ng range (Figure 6B). TTL was the most active enzyme according to  $\Delta$ OD at 272 nm and compared to TLL, GPLRP2 and FsC (Figure 6C). In all cases, steady state kinetics could be obtained after various lag times (Table 2), with a good linearity of OD variations at 272 nm as a function of enzyme amounts (20 to 80 ng; Figure 5D).

Based on a calibration with  $\alpha$ -eleostearic acid, variations in OD at 272 nm could be correlated with  $\alpha$ -eleostearic acid concentration and further used for the estimation of enzyme specific activities. The apparent molar extinction coefficient ( $\epsilon_{app}$ ) of  $\alpha$ -eleostearic acid has been previously determined in microtiter plates by recording the absorbance at 272 nm of various amounts of  $\alpha$ -eleostearic acid dispersed in the buffer with  $\beta$ -CD at 37°C and it was found to be 5320 M<sup>-1</sup> cm<sup>-1</sup> [27]. Under these conditions, the increase with time of OD at 272 nm was converted into  $\mu$ moles of  $\alpha$ -eleostearic acid released per min and per mg of enzyme (Table 2). TTL was found to be the most active galactolipase on  $\alpha$ E-MGDG under these conditions with a specific activity of  $50.3 \pm 8.9 \mu\text{moles min}^{-1} \text{mg}^{-1}$ . TLL was 10-fold less active while GPLRP2 and FsC were 3-fold less active. These enzymes also showed distinct

lag times with TTL reaching the most rapidly steady state kinetics although it was the enzyme with the lowest specific activity (Table 2). These findings that differentiate TTL and TLL are similar to those observed with MGDG monomolecular films at high surface pressures around 20 mN m<sup>-1</sup> with similar ratio of enzyme activity (10 to 12) and lag times (5 to 8) between TTL and TLL (Figure 3B). The presentation of the galactolipid substrate coated onto the microtiter plate surface to the enzyme might therefore be similar to a substrate monolayer spread at the air-water interface at 20 mN m<sup>-1</sup>.

#### *3.4. TTL substrate specificity and structure-function relationships*

TTL possesses broad substrate specificity and was found to be active on triglycerides with various acyl chain lengths, phospholipids and galactolipids (Table 3). It was however more active on galactolipids than on triglycerides and phospholipids, with a galactolipase activity on C8-MGDG micelles that is 1.68-fold, 2.4-fold and 9.5-fold higher than TTL activities on trioctanoin, olive oil, and egg phosphatidylcholine, respectively. TTL substrate preference was closer to that of GPLRP2 than to the closely related TLL (Table 3). Indeed, TLL was 6 to 20-fold more active on triglycerides than on C8-MGDG, while its activity on phospholipids was in the same order of magnitude as its activity on galactolipids (Table 3). TTL was also found to be 4-fold more active on C8-MGDG than on C8-DGDG, while TLL is slightly more active on C8-DGDG. TTL and TLL therefore display distinct substrate preference while they share 89 % amino acid identities [48]. Although TTL was globally more active on C8-MGDG than TLL at steady state, it showed longer lag times to reach the steady state, particularly at high surface pressures, which suggests a slower adsorption/penetration of TTL at the lipid-water interface compared to TLL. The presence of an additional galactose on the galactolipid polar head led to a lower activity of TTL, but it is

unclear whether this results from a steric hindrance during the interfacial adsorption step or within the active site.

To gain more information on the structure-function relationships of TTL, we built a 3D model based on the known crystal structure of the homologous TLL with the lid in the open conformation. The most remarkable difference between the two models was located in the solvent exposed part of the hydrophobic substrate binding pocket hosting the acyl chains which encompasses four amino acid substitutions in TTL vs. TLL (namely: Leu86Ile, Leu93Ile, Phe95Leu, Phe211Leu) (Figure 7A-B). This region is also involved in the interfacial recognition site (IRS) of the enzyme when the lid is in its open conformation [45], as well as in the stabilization of the lid in its closed form. The replacement of two Phe by Leu residues in TTL might explain the slower adsorption/penetration of TTL since aromatic residues like Phe have strong contributions to protein transfer from water to water-lipid interfaces [49]. The role of these residues in TTL will be investigated by site-directed mutagenesis in future studies.

Since electrostatic interactions also play an important role in the interaction of lipolytic enzymes with polar lipids, we calculated dipolar vectors for the TTL model and TLL 3D structure. Surprisingly the positive end of TTL dipolar vector was found to be oriented from the bottom of the active site vertically to the surface (Figure 7C), while in TLL it was oriented more horizontally along the active site (Figure 7D). This might favour a better orientation of TTL towards polar or negatively charged lipid surfaces, and might explain the 5.5-fold higher activity of TTL on phospholipids compared to TLL (Table 3).

The comparison of TTL model with the crystal structure of TLL does not reveal specific features that can explain the higher activity of TTL on galactolipids, nor the preference for MGDG versus DGDG. The presence of a second galactose unit on DGDG polar head may lead to steric hindrance within the enzyme active site, but monomolecular

film experiments rather suggest that the additional galactose unit impairs the enzyme adsorption on its aggregated substrate (Figure 3).

#### 4. Conclusions

Besides its activity on triglycerides [21] and phospholipids (this work), the TTL lipase purified from the fungus *Talaromyces thermophilus* was found to hydrolyze a large variety of synthetic galactolipid substrates presented in various forms to the enzyme (micelles, monolayers, coating on solid surface). In all cases, it displays some of the highest lipolytic activities recorded so far whatever the substrate. It appears to be more active on galactolipid mixed micelles than on monomolecular films or surface-coated MGDG. Nevertheless, the presence of a lid in TTL favours the hydrolysis of monomolecular films of galactolipids at high surface pressure as observed with the homologous fungal lipase from *Thermomyces lanuginosus* (TLL) belonging to the same gene family [48]. Differences in lag times for reaching steady state kinetics of hydrolysis of galactolipid monomolecular films or surface-coated MGDG, and 3D modelling based on the known 3D structure of TLL, pointed out to amino acid substitutions within the IRS of TTL that could be responsible for a slower adsorption/penetration at lipid-water interface compared to TLL.

Finally, we have developed a fast, sensitive and continuous assay of galactolipases in microtiter plates using a novel synthetic galactolipid substrate containing  $\alpha$ -eleostearic acid that allows a direct detection by UV absorption, of the fatty acids released upon lipolysis of MGDG. Although the specific activities (U/mg) deduced from this assay (Table 2) are 2 to 3 order of magnitude lower than those estimated by the pHstat technique from C8-MGDG micelle hydrolysis (Table 3), the UV detection of free fatty acids released from coated MGDG is highly sensitive and allows measuring significant activities with about ten ng of enzymes, against hundred ng to ten  $\mu$ g with the pHstat. The lower galactolipase activities measured

with the UV-spectrophotometric assay and substrate coated on microtiter plates are therefore not an obstacle to the applicability of this novel assay. These lower activities are probably linked to the mode of action of lipolytic enzymes that depends on the accessible surface available for enzyme adsorption at the lipid-water interface, the first step in the overall process of interfacial catalysis. With the pHstat method and the use of mixed micelles or fine triglyceride emulsions as substrate, a very large accessible surface is created which ensures maximum enzyme adsorption and thus maximum enzyme activity. With substrate coated onto the wells of microtiter plates, as well as with monomolecular films, the accessible surface available for enzyme adsorption is much reduced and one can expect a lower enzyme turnover.

The novel UV-spectrophotometric assay using surface-coated MGDG with UV-absorbing  $\alpha$ -eleostearic acid allows the estimation of enzyme specific activities from steady state kinetics. Moreover, the lag times for reaching these conditions give some idea about the enzyme affinity for the lipid-water interface. Indeed, the differences observed between TTL and TLL are in good agreement with independent experiments performed with galactolipid monomolecular films. In addition to TTL characterization showing its potent galactolipase activity, this novel assay will be an interesting tool for screening enzymes and mutant thereof for their galactolipase activities.

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565 **Conflict of interest**

566 The authors have declared no conflict of interest

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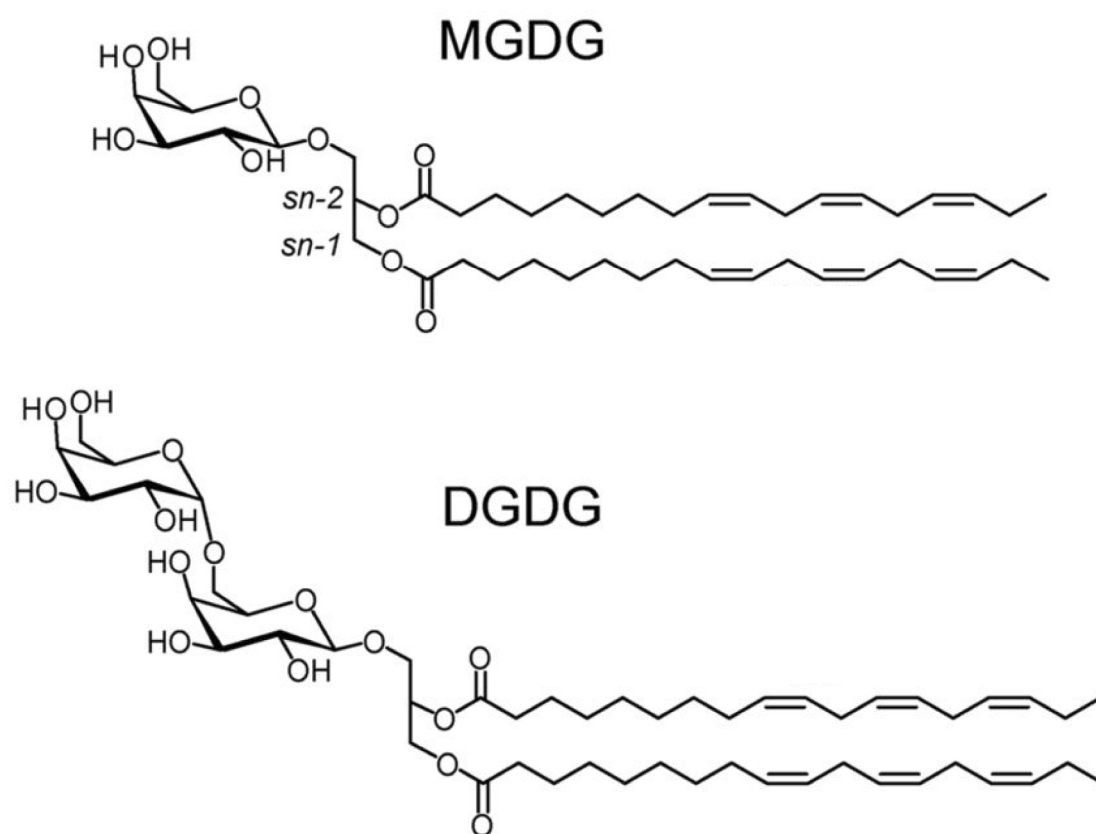
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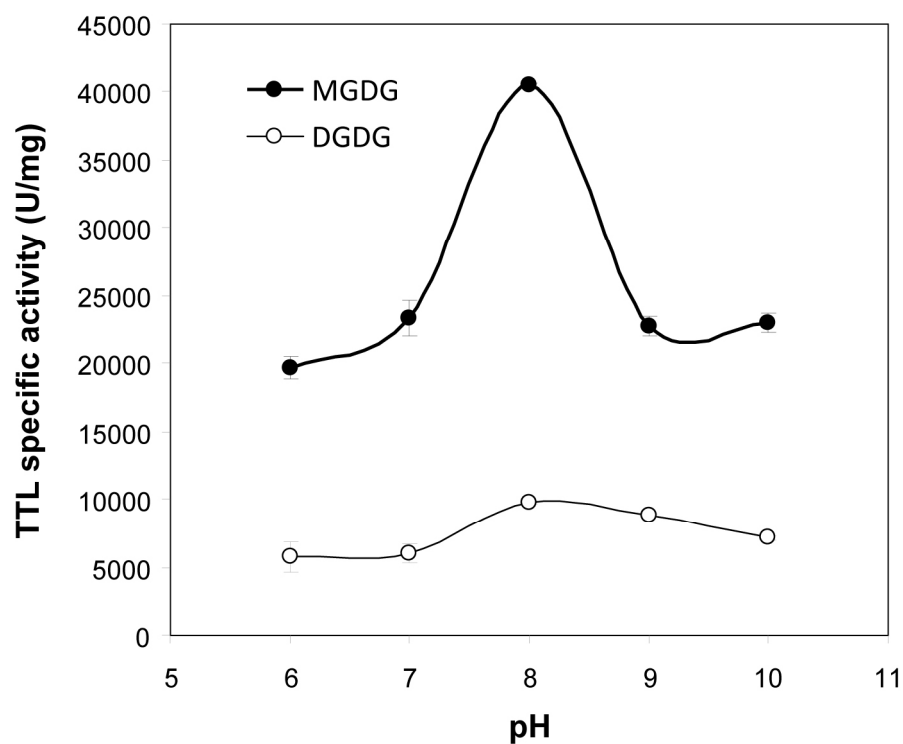
**Figure legends**

**Figure 1.** Chemical structures of monogalactosyl diacylglycerol (MGDG; 1,2-diacyl-3-*O*- $\beta$ -D-galactosyl-*sn*-glycerol) and digalactosyl diacylglycerol (DGDG; 1,2-diacyl-3-*O*-(6-*O*- $\alpha$ -D-galactosyl- $\beta$ -D-galactosyl)-*sn*-glycerol).



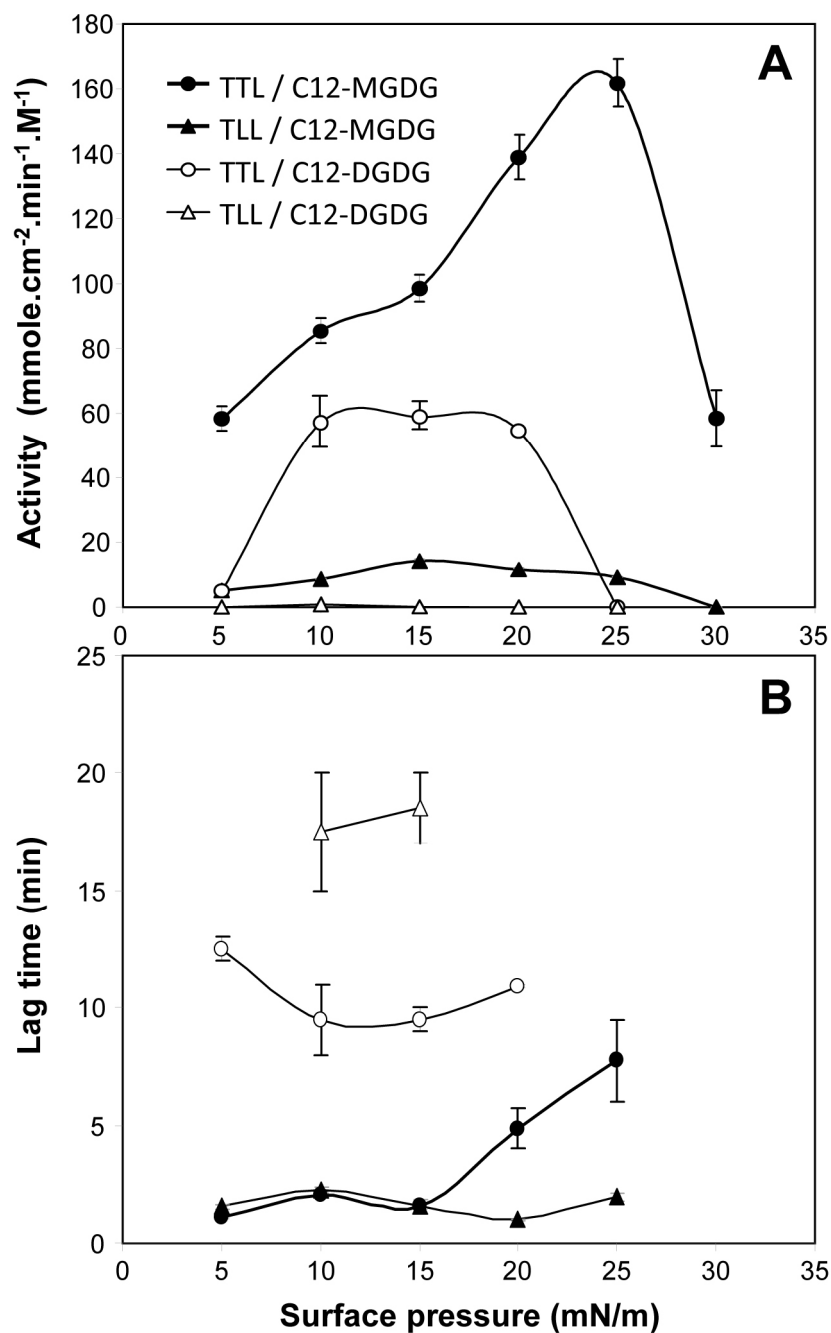


729 **Figure 2.** pH-dependent galactolipase activity of TTL on synthetic medium chain MGDG and  
730 DGDG as substrates. Activities were measured using the pHstat technique and substrate  
731 micelles with a bile salt (NaTDC) to galactolipid molar ratio of 1.33. Values (U/mg) are  
732 means  $\pm$  SD (n= 3). 1 U = 1  $\mu$ mole of free fatty acid released per min.



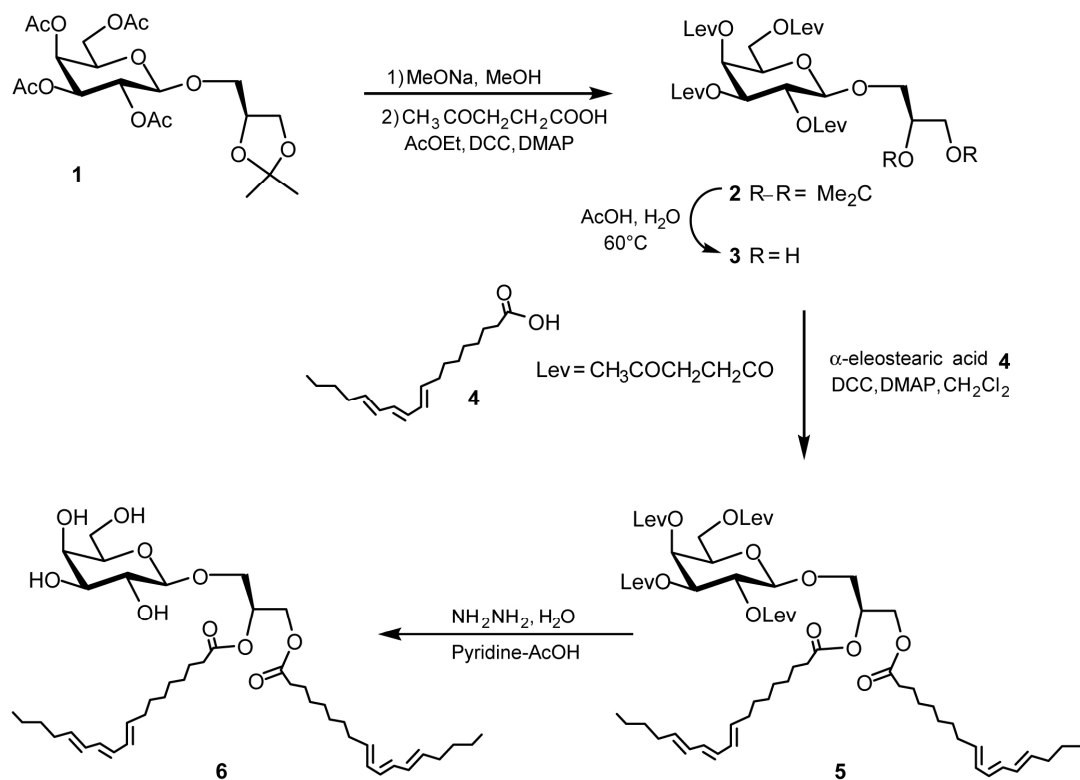
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734 **Figure 3.** Variations with surface pressure in the activity of TTL and TLL on monomolecular  
735 films of galactolipids. (A) Steady-state activities on C12-MGDG and C12-DGDG  
736 monomolecular films; (B) Lag times observed in the course of C12-MGDG and C12-DGDG  
737 monomolecular film hydrolysis by TTL and TLL. Global enzyme concentration was 0,45 nM  
738 for TTL and 5 nM for TLL. Values are means  $\pm$  SD (n=3).



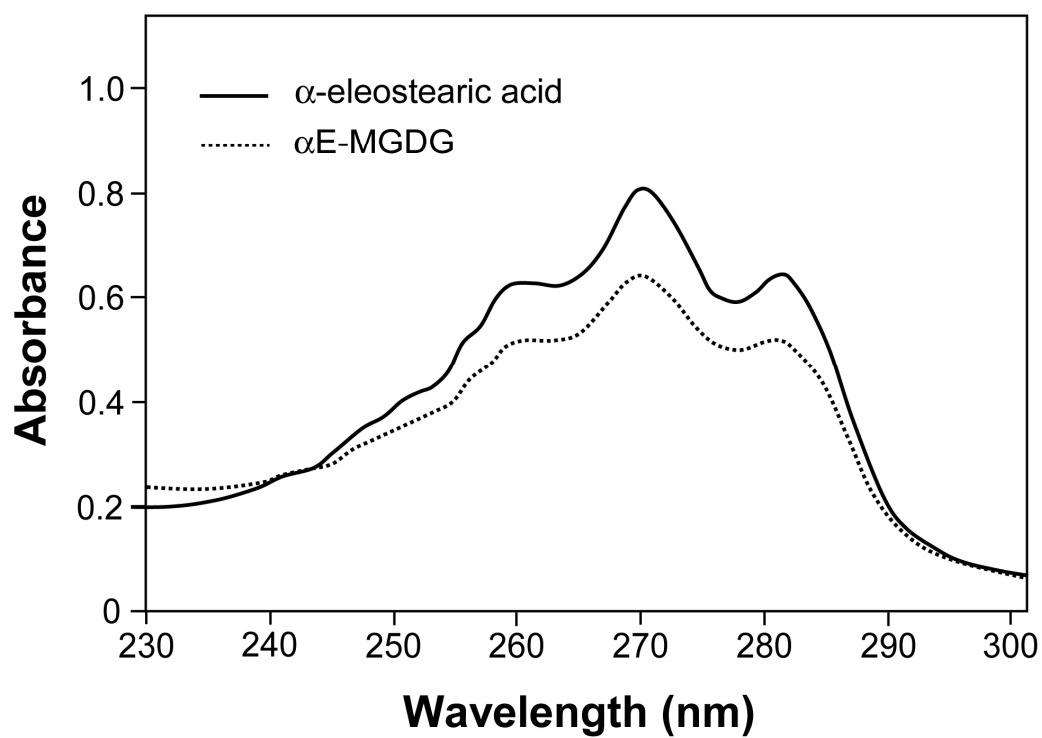
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740 **Figure 4.** Synthesis scheme of 1,2-Di-*O*- $\alpha$ -eleostearoyl-3-*O*- $\beta$ -D-galactopyranosyl-*sn*-  
 741 glycerol ( $\alpha$ E-MGDG).



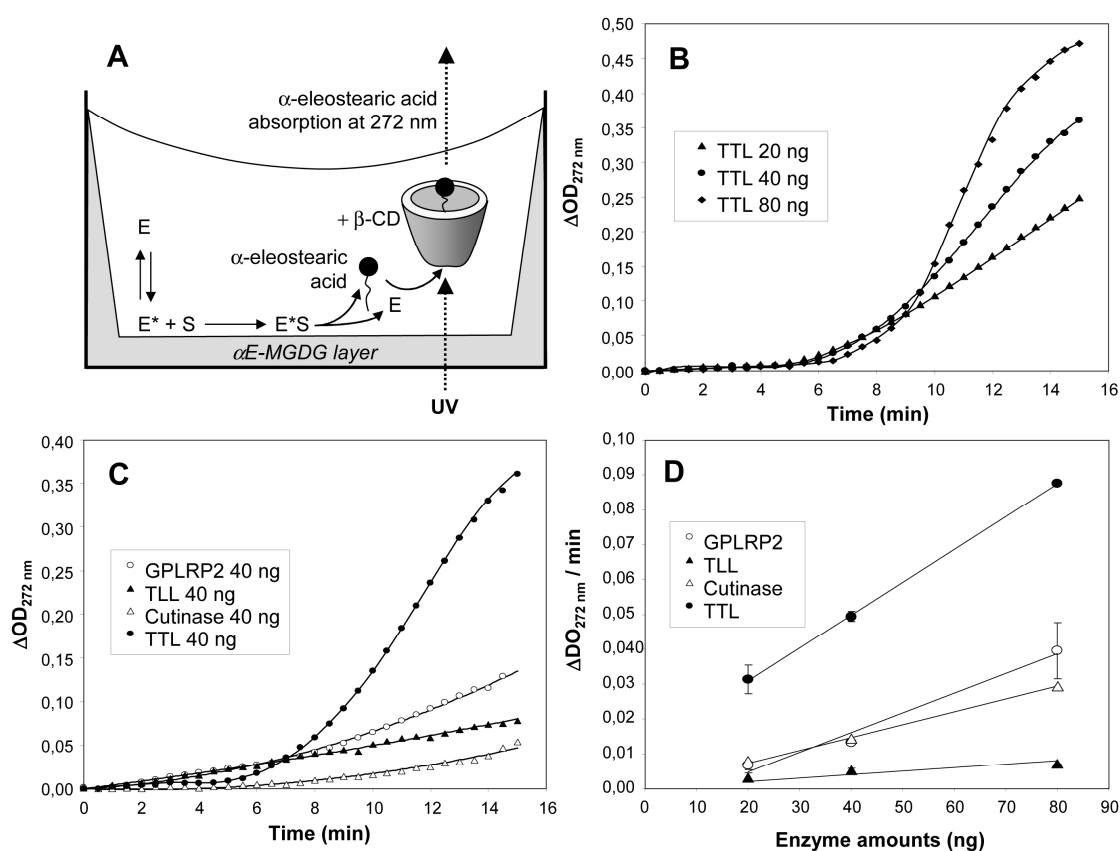
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743 **Figure 5.** UV absorption spectra of  $\alpha$ -eleostearic acid (60  $\mu\text{g/mL}$ ) and  $\alpha\text{E-MGDG}$  (60  $\mu\text{g/mL}$ )  
744 dissolved in ethanol containing BHT 0.001%.

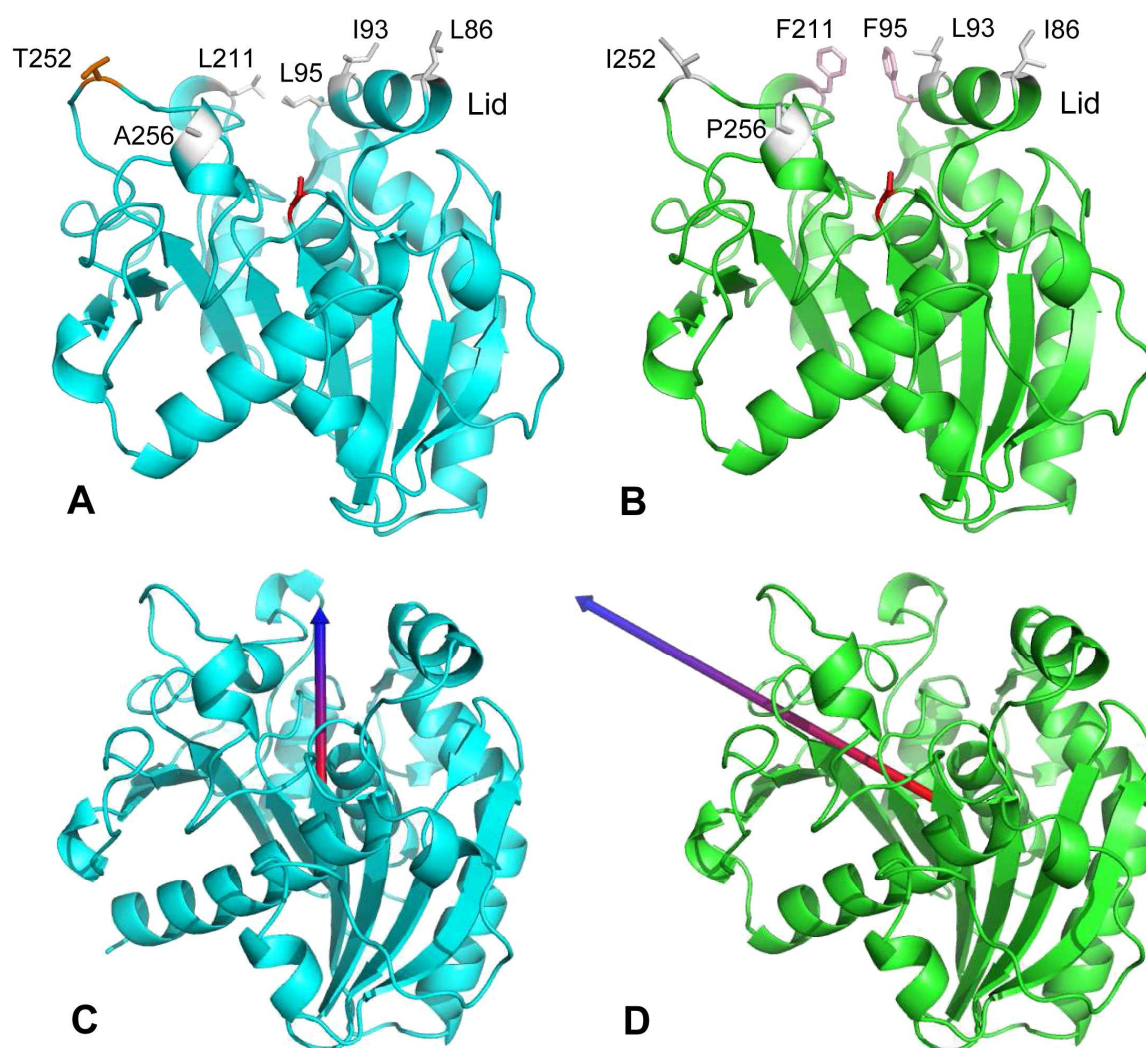


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**Figure 6.** UV-spectrophotometric assays of galactolipase activities using 1,2-Di-*O*- $\alpha$ -eleostearoyl-3-*O*- $\beta$ -D-galactopyranosyl-*sn*-glycerol ( $\alpha$ E-MGDG). (A) Schematic representation of the assay showing the enzymatic hydrolysis of the  $\alpha$ E-MGDG film coated onto the wells of a microtiter plate, followed by the solubilization of free  $\alpha$ -eleostearic acid (FFA) in the bulk phase by formation of a complex with  $\beta$ -cyclodextrin ( $\beta$ -CD); E, lipase in solution; E\*, activated lipase at the interface; S, substrate. (B) Variations with time of optical density at 272 nm (versus OD<sub>272</sub> at time zero) using various amounts of TTL. (C) Variations with time of optical density at 272 nm (versus OD<sub>272</sub> at time zero) of various enzymes (40 ng each). (D) Variations of optical density at 272 nm per min at steady state as a function of enzyme amounts. Substrate coated onto the microtiter (50  $\mu$ g/well) was incubated with 20,40 and 80 ng of GPLRP2, TLL, cutinase or TTL, respectively. Enzymes were injected into the well containing 200  $\mu$ l of buffer. The increase in OD at 272 nm was recorded for 15 min.



**Figure 7.** Comparison of TTL structural model (in cyan) and TLL X-ray structure (in green; PDB : 1GT6A). (A, B) side views of TTL and TLL ribbon models, respectively, showing the amino acid substitutions present in the interfacial recognition site (IRS) of the two enzymes. Molecules are oriented with their IRS on the top, perpendicular to the image plane and including the  $\alpha$ -helix of the lid domain. The active site serine is shown as red sticks. (C, D) calculated dipole moment vectors for TTL and TLL, respectively.



**Table 1.** Maximum rates of hydrolysis ( $\text{mmol cm}^{-2} \text{ min}^{-1} \text{ M}^{-1}$ ) of C12-MGDG and C12-DGDG monomolecular films by TTL and other galactolipases. Values are means  $\pm$  SD (n=3). The surface pressures ( $\pi$ ,  $\text{mN m}^{-1}$ ) at which these activities were recorded are indicated in parenthesis.

Enzyme	Substrate	
	C12-MGDG	C12-DGDG
TTL	$161.7 \pm 7.3$ ( $\pi=25$ )	$58.8 \pm 3.4$ ( $\pi=15$ )
TLL	$14.2 \pm 0.5$ ( $\pi=15$ ) <sup>a</sup>	$0.8 \pm 0.03$ ( $\pi=10$ )
rGPLRP2	$2047 \pm 237$ ( $\pi=10$ ) <sup>a</sup>	$2126 \pm 192$ ( $\pi=7$ ) <sup>a</sup>
rHPLRP2	$1400 \pm 500$ ( $\pi=15-25$ ) <sup>b</sup>	$3200 \pm 800$ ( $\pi=12$ ) <sup>b</sup>
Cutinase	$7.2 \pm 0.1$ ( $\pi=12$ ) <sup>a</sup>	$<10^{-3}$

<sup>a</sup> Data from [17]. <sup>b</sup> Data from [33].

775 **Table 2.** Specific activities at steady state ( $\mu\text{mole min}^{-1} \text{mg}^{-1}$ ) on  $\alpha\text{E-MGDG}$  and lag times of  
776 TTL and other galactolipases. Values are means  $\pm$  SD (n=3).

Enzyme	Specific Activity ( $\mu\text{moles min}^{-1} \text{mg}^{-1}$ )	Lag time (min)
TTL	$50.3 \pm 8.9$	$6.9 \pm 0.9$
TLL	$5.0 \pm 1.7$	$0.9 \pm 1.5$
GPLRP2	$15.0 \pm 4.0$	$5.1 \pm 1.4$
Cutinase	$14.3 \pm 1.1$	$10.9 \pm 0.5$

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778



**Table 3:** Maximum specific activities (U/mg) of TTL and other lipases on various lipid substrates. TC4, tributyrin; TC8, trioctanoin; Egg PC, egg phosphatidylcholine; C8-MGDG, monogalactosyldiacylglycerol; C8-DGDG, digalactosyldiacylglycerol. Values are means  $\pm$  SD (n=3).

Enzymes	Substrates					
	TC4	TC8	Olive oil	Egg PC	C8-MGDG	C8-DGDG
TTL	7300 $\pm$ 122 <sup>a</sup>	24110 $\pm$ 390 <sup>a</sup>	9808 $\pm$ 139 <sup>a</sup>	4250 $\pm$ 250	40500 $\pm$ 125	9800 $\pm$ 125
TLL	7834 $\pm$ 850 <sup>b</sup>	9250 $\pm$ 101	2900 $\pm$ 91	767 $\pm$ 188 <sup>b</sup>	450 $\pm$ 41 <sup>b</sup>	672 $\pm$ 61 <sup>b</sup>
rGPLRP2	2700 $\pm$ 300 <sup>b</sup>	675 $\pm$ 43 <sup>c</sup>	754 $\pm$ 151 <sup>c</sup>	500	5420 $\pm$ 85 <sup>b</sup>	4375 $\pm$ 125 <sup>b</sup>
<i>Fusarium solani</i> cutinase	2596 $\pm$ 96 <sup>b</sup>	2965 $\pm$ 50 <sup>d</sup>	688 $\pm$ 10 <sup>e</sup>	0	984 $\pm$ 62 <sup>b</sup>	300 $\pm$ 29 <sup>b</sup>

<sup>a</sup>Data from [21] ; <sup>b</sup>Data from [17]; <sup>c</sup>data from [51]; <sup>d</sup>data from [52]; <sup>e</sup>data from [53].