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### ► To cite this version:

Inès Belhaj, Sawsan Amara, Goetz Parsiegl, Priscila Sutto-Ortiz, Moulay Sahaka, et al.. Galactolipase activity of *Talaromyces thermophilus* lipase on galactolipid micelles, monomolecular films and UV-absorbing surface-coated substrate. *Biochimica et Biophysica Acta Molecular and Cell Biology of Lipids*, 2018, 1863 (9), pp.1006-1015. 10.1016/j.bbalip.2018.05.016 . hal-01819569

**HAL Id: hal-01819569**

**<https://amu.hal.science/hal-01819569>**

Submitted on 20 Jun 2018

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

6  
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9

10 *Running title:* Galactolipase activity of *Talaromyces thermophilus* lipase  
11

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25 *Keywords:* enzyme assay, galactolipid, galactolipase, *in silico* docking, lipase, monomolecular  
26 films ;

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

33 **Abstract**

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

## 54 **1. Introduction**

55 Glycolipids are present in almost all biological membranes. Among them,  
56 galactolipids are the most abundant membrane lipids in plants, especially in green tissues  
57 where they generally represent about 75% of total membrane lipids [1]. They are especially  
58 abundant in the photosynthetic membranes of the thylakoids in the chloroplast. These  
59 membranes contain mainly monogalactosyl diacylglycerols (MGDG) and digalactosyl  
60 diacylglycerols (DGDG) (Fig.1), which represent 50% and 30% of total lipids, respectively  
61 [2]. Plant galactolipids are characterized by long chain fatty acids, typically varying from C16  
62 to C20 with one to three unsaturations [3] and they are particularly rich in polyunsaturated  
63 fatty acids like C16:3 and C18:3 (ALA,  $\alpha$ -linolenic acid) [4]. Although galactolipids are not  
64 present at high levels in normal human diet compared to triglycerides, they may represent the  
65 main source of the essential ALA in the absence of specific supplementation with ALA-rich  
66 vegetable oils. Indeed, ALA can represent as much as 60 % w/w of the total fatty acid in  
67 galactolipids from plant leaves, like in spinach [4], which is higher than what is found in  
68 flaxseed oil [5]. The uptake of galactolipid fatty acids is made possible by the action of  
69 galactolipases (1,2-Di-*O*-acyl-1-*O*-( $\beta$ -D-galactosyl)-D-glycerol acylhydrolases; EC 3.1.1.26)  
70 that hydrolyze the ester bonds at *sn*-1 and *sn*-2 positions of galactolipids. In humans and other  
71 mammals, this function is mainly fulfilled by pancreatic lipase-related protein 2 (PLRP2)  
72 produced by the exocrine pancreas and acting in the GI tract [6-8].

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

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

87 In contrast to the large biochemical and structural knowledge on lipases and  
88 phospholipases, galactolipases remain poorly studied and characterized so far. This is mainly  
89 due to the fact that galactolipid substrates are not commercially available at a large scale and  
90 low cost. Natural galactolipids purified from plant green materials have been used for the  
91 characterization of some galactolipases by thin layer chromatography analysis of lipolysis  
92 products or free fatty acid titration [4, 18] but their limited availability does not allow  
93 thorough kinetic studies. Nevertheless, medium galactolipid substrates have been synthesized  
94 to develop continuous and sensitive assays using the pHstat [19] and the monomolecular film  
95 [7, 20] techniques

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

104 lipid used as the carbon source [22], we wondered whether TTL displays a galactolipase  
105 activity, as previously identified with homologous enzymes of this fungal lipase family [17,  
106 18].

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

118

119

## 120 **2. Materials and methods**

121

### 122 *2.1. Enzymes*

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

131

### 132 *2.2. Potentiometric pHstat assay of galactolipase activity*

133 Dioctanoyl galactolipid substrates, C8-MGDG (3-*O*-β-D-galactopyranosyl-1,2-di-*O*-  
134 octanoyl-*sn*-glycerol) and C8-DGDG (3-*O*-(6-*O*-α-D-galactopyranosyl-β-D-  
135 galactopyranosyl)-1,2-di-*O*-octanoyl-*sn*-glycerol), were synthesized as previously described  
136 [4, 19]. The galactolipase activity was measured potentiometrically at 37 °C and at a constant  
137 pH value of 8.0 by continuously measuring the release of free fatty acids from mechanically  
138 stirred dispersions of galactolipids, using 0.1 N NaOH as titrant and a pHstat apparatus (718  
139 STAT Titrino, Metrohm). To prepare the galactolipid dispersion, 25 mg of C8-MGDG or C8-  
140 DGDG were mixed with 5 mL of 0.33 mM Tris-HCl buffer, containing 0.1 M NaCl and  
141 various concentrations of sodium taurodeoxycholate (NaTDC) and then subjected to  
142 ultrasonic treatment for 6-8 min in a water bath (HF-Frequency 35 kHz; SONOREX SUPER  
143 compact ultrasonic bath model RK 31, BANDELIN electronic) [19]. One international  
144 galactolipase unit (U) corresponds to the release of one μmol of fatty acid released per

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

147

### 148 *2.3. Potentiometric pHstat assay of phospholipase and lipase activities*

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

155

### 156 *2.4. Monomolecular film experiments for measuring galactolipase activities*

157 The galactolipase activity was measured using monomolecular films of 1,2-di-*O*-  
158 dodecanoyl-3-*O*- $\beta$ -D-galactopyranosyl-*sn*-glycerol (C12-MGDG) and 1,2-di-*O*-dodecanoyl-  
159 3-*O*-(6-*O*- $\alpha$ -D-galactopyranosyl- $\beta$ -D-galactopyranosyl)-*sn*-glycerol (C12-DGDG) as  
160 substrates. C12-MGDG and C12-DGDG were synthesized as previously reported [7, 20]. All  
161 experiments were performed using the KSV 5000 barostat equipment (KSV, Helsinki,  
162 Finland) and a “zero order” Teflon trough [32]. The reaction compartment had a surface area  
163 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  
164 area of 156.5 cm<sup>2</sup> and a volume of 203 mL. Lipid monomolecular films were formed at the  
165 air/water interface by spreading the lipid solution (1 mg/mL C12-MGDG or C12-DGDG in  
166 chloroform). The enzyme solution was injected into the subphase at a final concentration of  
167 0.02 nM for rGPLRP2, 5nM for TLL and cutinase and 0.45 nM for TTL. The aqueous  
168 subphase was composed of 10 mM Tris-HCl, 100 mM NaCl, 21 mM CaCl<sub>2</sub>, and 1 mM  
169 EDTA, pH 8.0 and was prepared with double-distilled water. Residual surface-active

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

183

#### 184 2.5. Synthesis of 1,2-Di-O- $\alpha$ -eleostearoyl-3-O- $\beta$ -D-galactopyranosyl-*sn*-glycerol ( $\alpha$ E-MGDG)

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

192

193 3-O-[2,3,4,6-Tetra-O-levulinoyl- $\beta$ -D-galactopyranosyl]-1,2-O-isopropylidene-*sn*-glycerol (**2**):

194 3-O-[2,3,4,6-Tetra-O-acetyl- $\beta$ -D-galactopyranosyl]-1,2-O-isopropylidene-*sn*-glycerol (**1**)

195 (2.60 g, 5.53 mmol) was added to dry methanol (50 mL) containing a chip of sodium and the  
196 mixture was stirred for 2 h. Amberlite IR 120 [H<sup>+</sup>] resin was added to neutralize the solution  
197 and after 2 min, the solution was filtrated and concentrated *in vacuo*. The product was  
198 coevaporated twice from toluene (2x15 mL), dissolved in ethyl acetate (50 mL) and levulinic  
199 acid (3.92 g, 33.76 mmol), dicyclohexyldicarbodiimide (8.27 g, 40.00 mmol) and a catalytic  
200 amount of 4-dimethylaminopyridine (50 mg) were successively added. The mixture was  
201 stirred overnight, and after filtration, the solid was washed carefully with ethyl acetate. The  
202 organic phase was concentrated to dryness, the crude product was purified by column  
203 chromatography (4:1 ethyl acetate-petroleum ether to pure ethyl acetate) and the pure product  
204 **2** was recovered at 70% yield: 2.70 g, oily material, *R<sub>f</sub>* 0.30 (5:1 ethyl acetate-petroleum  
205 ether); [ $\alpha$ ]<sub>D</sub> +2.1 (*c* 1.0, CHCl<sub>3</sub>); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  5.36 (dd, 1H, *J*<sub>3,4</sub> 3.4, *J*<sub>4,5</sub> 0.8 Hz, H-4),  
206 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,  
207 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),  
208 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  
209 (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>),  
210 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,  
211 1.34 (2s, 6H, (CH<sub>3</sub>)<sub>2</sub>C); <sup>13</sup>CNMR (CDCl<sub>3</sub>):  $\delta$  206.52, 206.43, 206.16, 206.04  
212 (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),  
213 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-  
214 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  
215 (OCOCH<sub>2</sub>), 26.67, 25.20 ((CH<sub>3</sub>)<sub>2</sub>C).

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

217

218 3-*O*-[2,3,4,6-Tetra-*O*-levulinoyl- $\beta$ -D-galactopyranosyl]-*sn*-glycerol (**3**): a solution of  
219 compound **2** (2.60 g, 1.46 mmol) in 70% aqueous acetic acid (20 mL) was stirred for 5 h at

220 60°C. After concentration, the residue was coevaporated from toluene (3x20 mL). The crude  
221 product was purified by column chromatography (9:1 CHCl<sub>3</sub>-EtOH). Pure product **3** was  
222 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,  
223 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, 1 H, *J*<sub>1,2</sub> 7.8  
224 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>  
225 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>,  
226 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,  
227 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>):  
228 δ 207.76, 206.89, 206.69, 206.22 (CH<sub>3</sub>CO levulinoyl), 172.28, 171.99, 171.81, 171.75  
229 (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  
230 (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  
231 (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>).

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

233  
234 1,2-Di-*O*-α-eleostearoyl-3-*O*-[2,3,4,6-tetra-*O*-levulinoyl-β-D-galactopyranosyl]-*sn*-glycerol  
235 (**5**): α-Eleostearic acid **4** (2.42 g, 8.42 mmol), dicyclohexyldicarbodiimide (3.58 g, 17.40  
236 mmol) and a catalytic amount of 4-dimethylaminopyridine (50 mg) were successively added  
237 under argon to a solution of product **3** (1.875 g, 2.90 mmol) in dichloromethane (40 mL). The  
238 mixture was stirred overnight, and methanol (0.50 mL) was added. After 2 h, the solid was  
239 removed by filtration and washed with dichloromethane. The combined organic phases were  
240 concentrated to dryness and the crude product was purified by column chromatography (1:1 to  
241 4:1 ethyl acetate-petroleum ether). A second column chromatography (9:1 CH<sub>2</sub>Cl<sub>2</sub>-EtOH) was  
242 necessary to give the pure product **5**, recovered in 73% yield: 2.47 g, oily material, *R<sub>f</sub>* 0.20-  
243 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>);  
244 <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-1a<sub>gly</sub>), 4.20 (dd, 1H,  $J_{5,6a}$  6.6,  
249  $J_{6a,6b}$  11.1, H-6a), 4.14 (dd, 1H, H-1b<sub>gly</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-3a<sub>gly</sub>), 3.89 (bdd, 1H, H-5), 3.69 (dd, 1H, H-3b<sub>gly</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, 2COCH<sub>2</sub>), 2.11-1.95  
279 (m, 8H, 4CH<sub>2</sub>CH=), 1.57-1.47 (m, 4H, 2COCH<sub>2</sub>CH<sub>2</sub> eleo), 1.32-1.17 (m, 24H, 12CH<sub>2</sub> alkyl  
280 chains), 0.81 (t, 6H,  $J$  6.5Hz, 2CH<sub>3</sub>CH<sub>2</sub>);  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  173.98, 173.68 (COCH<sub>2</sub> 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 (COCH<sub>2</sub> 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]:

307

$$308 \quad OD_{272}(t) - OD_{272}(0) = R_{ss} \times t + \tau R_{ss}(e^{-t/\tau})$$

309

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

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

316

### 317 *2.7. Molecular modelling of Talaromyces thermophilus lipase*

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

323

### 324 *2.8. Dynamic light scattering measurements*

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

### 336 **3. Results and discussion**

337

#### 338 *3.1. Galactolipase activity of TTL on C8-MGDG and C8-DGDG micelles*

339 The galactolipase activity of TTL was first tested using synthetic medium chain (C8)  
340 MGDG and DGDG mixed with bile salts (NaTDC) at molar ratios of 1.33 and 0.25,  
341 respectively, to form mixed micelles. This presentation of substrate to the enzyme was  
342 previously reported to be the most effective for various mammalian and microbial  
343 galactolipases [4, 17, 19]. These micelles were not characterized, however, and dynamic light  
344 scattering was used here to estimate their average particle size and distribution at 37°C and  
345 pH 8. The hydrodynamic diameters ( $D_H$ , z-average) of C8-MGDG (10 mM)-NaTDC (13 mM)  
346 and C8-DGDG (10 mM)-NaTDC (2.5 mM) micelles were found to be  $9.4 \pm 0.3$  and  $24.5 \pm$   
347  $0.2$  nm, respectively, with polydispersity index (PDI) of 0.303 and 0.178. These values were in  
348 the same range as those measured with mixed micelles of phospholipids and bile salts at  
349 similar concentrations [41, 42].

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

358

359 *3.2. Galactolipase activity of TTL on C12-MGDG and C12-DGDG monomolecular films*

360 The galactolipase activity of TTL was then tested on medium chain synthetic  
361 galactolipids (C12-MGDG and C12-DGDG) that form stable monomolecular films at the air-  
362 water interface, while their lipolysis products are soluble in water [7, 17, 33, 43]. This allows  
363 measuring galactolipase activities at various surface pressures using the barostat technique  
364 [32, 34].

365 TTL activity on C12-MGDG showed a bell-shaped activity profile as a function of  
366 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  
367 (Figure 3A). TTL was found to be 10 to 14-fold more active than the homologous fungal  
368 lipase TLL, that showed its maximum activity ( $14.2 \text{ mmol cm}^{-2} \text{ min}^{-1} \text{ M}^{-1}$ ) at a lower surface  
369 pressure of  $12 \text{ mN m}^{-1}$  (Figure 3A). TTL was less active than rGPLRP2 but the optimum  
370 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  
371 surface pressure of  $10 \text{ mN m}^{-1}$  (Table 1). Remarkably, TTL was found to be able to  
372 hydrolyze C12-MGDG monomolecular films at surface pressures up to  $30 \text{ mN m}^{-1}$ . Most  
373 galactolipases characterized so far are not active at such high surface pressures, except  
374 recombinant human PLRP2 (Table 1; [33]). It confirms that lipases possessing a lid domain,  
375 like TTL, TLL and rHPLRP2, are able to hydrolyze galactolipids at higher surface pressures  
376 than those without a lid, like GPLRP2 and FsC [17].

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

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

388 The surface pressure and the size/steric hindrance of the hydrophilic polar head of  
389 galactolipids are therefore two important parameters controlling the activity of galactolipases,  
390 and this activity is favoured at higher surface pressure by the presence of a lid domain.  
391 Enzymes without a lid, like GPLRP2 and cutinase show optimum activity at low surface  
392 pressures and very long lag times (40-70 min) to reach steady state kinetics above 10 mN m<sup>-1</sup>  
393 [17]. The presence of an amphiphilic lid probably favours the interaction of the lipase with the  
394 galactolipid monolayer spread at the air-water interface. These results further underline the  
395 crucial role of the lid in the interaction with the lipid substrate and the control of enzyme  
396 activity [44, 45]..

397

### 398 *3.3. Galactolipase activity of TTL on a surface-coated MGDG substrate containing $\alpha$ -* 399 *eleostearic acid*

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

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

420 The UV absorption spectrum (230-300 nm) of an ethanolic solution of  $\alpha$ E-MGDG  
421 displayed three major peaks located at 260, 270 and 282 nm (Figure 5). This profile spectrum  
422 is similar to that of pure  $\alpha$ -eleostearic acid [24], pure tung oil triglycerides [23], synthetic  $\alpha$ -  
423 eleostearic acid-containing triglycerides [25] and phosphatidylcholine [27]. In aqueous  
424 buffer, the major absorption peak was shifted from 270 nm to 272 nm, as described earlier  
425 [47].

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

434 chain fatty acid can be further solubilized by complex formation with  $\beta$ -CD present in the  
435 buffer and its concentration can be measured continuously by monitoring UV absorbance at  
436 272 nm (Figure 6A).

437 To validate the method, various amounts of substrate coated onto the plates (5, 10, 20  
438 or 50  $\mu$ g per well) and enzymes (20, 40 and 80 ng of TTL, TLL, GPLRP2 or FsC) were  
439 tested. The highest amount of substrate tested (50  $\mu$ g  $\alpha$ E-MGDG per well) was retained  
440 because it formed a stable coating and allowed measuring steady-state enzyme kinetics for  
441 longer period of time. Typical kinetics showing the increase in OD ( $\Delta$ OD = assay OD - initial  
442 OD) at 272 nm during  $\alpha$ E-MGDG hydrolysis by various amounts of TTL are shown in Figure  
443 5B. Lag times of around 6 to 8 min were observed before recording linear OD variations with  
444 time, but these variations were then proportional to TTL amounts in the 20 to 80 ng range  
445 (Figure 6B). TTL was the most active enzyme according to  $\Delta$ OD at 272 nm and compared to  
446 TLL, GPLRP2 and FsC (Figure 6C). In all cases, steady state kinetics could be obtained after  
447 various lag times (Table 2), with a good linearity of OD variations at 272 nm as a function of  
448 enzyme amounts (20 to 80 ng; Figure 5D).

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

459 lag times with TTL reaching the most rapidly steady state kinetics although it was the enzyme  
460 with the lowest specific activity (Table 2). These findings that differentiate TTL and TLL are  
461 similar to those observed with MGDG monomolecular films at high surface pressures around  
462  $20 \text{ mN m}^{-1}$  with similar ratio of enzyme activity (10 to 12) and lag times (5 to 8) between TTL  
463 and TLL (Figure 3B). The presentation of the galactolipid substrate coated onto the microtiter  
464 plate surface to the enzyme might therefore be similar to a substrate monolayer spread at the  
465 air-water interface at  $20 \text{ mN m}^{-1}$ .

466

#### 467 *3.4. TTL substrate specificity and structure-function relationships*

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

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

485 To gain more information on the structure-function relationships of TTL, we built a  
486 3D model based on the known crystal structure of the homologous TLL with the lid in the  
487 open conformation. The most remarkable difference between the two models was located in  
488 the solvent exposed part of the hydrophobic substrate binding pocket hosting the acyl chains  
489 which encompasses four amino acid substitutions in TTL vs. TLL (namely: Leu86Ile,  
490 Leu93Ile, Phe95Leu, Phe211Leu) (Figure 7A-B). This region is also involved in the  
491 interfacial recognition site (IRS) of the enzyme when the lid is in its open conformation [45],  
492 as well as in the stabilization of the lid in its closed form. The replacement of two Phe by Leu  
493 residues in TTL might explain the slower adsorption/penetration of TTL since aromatic  
494 residues like Phe have strong contributions to protein transfer from water to water-lipid  
495 interfaces [49]. The role of these residues in TTL will be investigated by site-directed  
496 mutagenesis in future studies.

497 Since electrostatic interactions also play an important role in the interaction of  
498 lipolytic enzymes with polar lipids, we calculated dipolar vectors for the TTL model and TLL  
499 3D structure. Surprisingly the positive end of TTL dipolar vector was found to be oriented  
500 from the bottom of the active site vertically to the surface (Figure 7C), while in TLL it was  
501 oriented more horizontally along the active site (Figure 7D). This might favour a better  
502 orientation of TTL towards polar or negatively charged lipid surfaces, and might explain the  
503 5.5-fold higher activity of TTL on phospholipids compared to TLL (Table 3).

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

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

510

#### 511 **4. Conclusions**

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

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

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

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

551

552

### 553 **Acknowledgements**

554         We are grateful to Ali Gargouri (Centre de Biotechnologie de Sfax, Tunisia) for his  
555 critical reading of the manuscript and constant support to this work, Rabaa Ben Ayed for her  
556 technical assistance during the purification of TTL, Deborah Byrne for DLS measurements  
557 (Institut de Microbiologie de la Méditerranée, Marseille, France) and Vanessa Point for her

558 technical assistance during monomolecular film experiments. This work received the financial  
559 support of Agence Nationale de la Recherche in the framework of the GALACTOLIPASE  
560 project (ANR-09-CP2D-06-01). This work also received financial support from the Ministry  
561 of Higher Education and Scientific Research, Tunisia, granted to the Laboratoire de  
562 Biotechnologie Moléculaire des Eucaryotes du Centre de Biotechnologie de Sfax.

563

564

565 **Conflict of interest**

566 The authors have declared no conflict of interest

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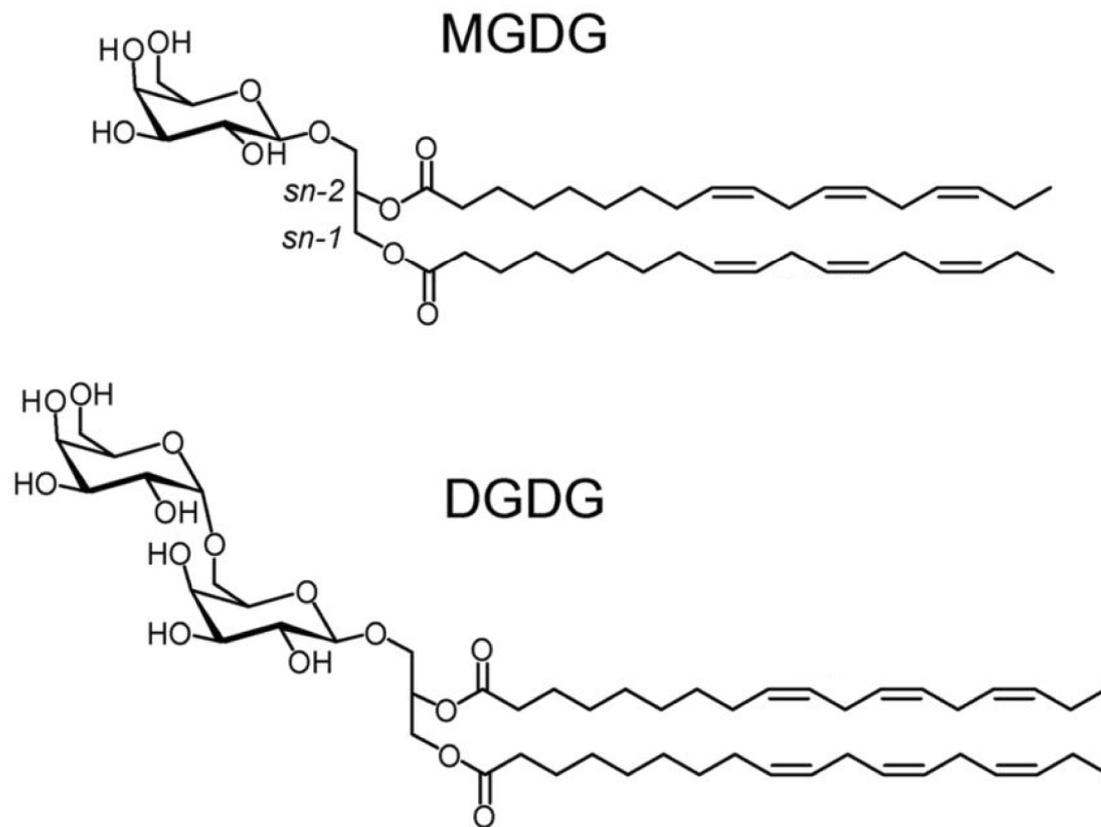
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722

723 **Figure legends**

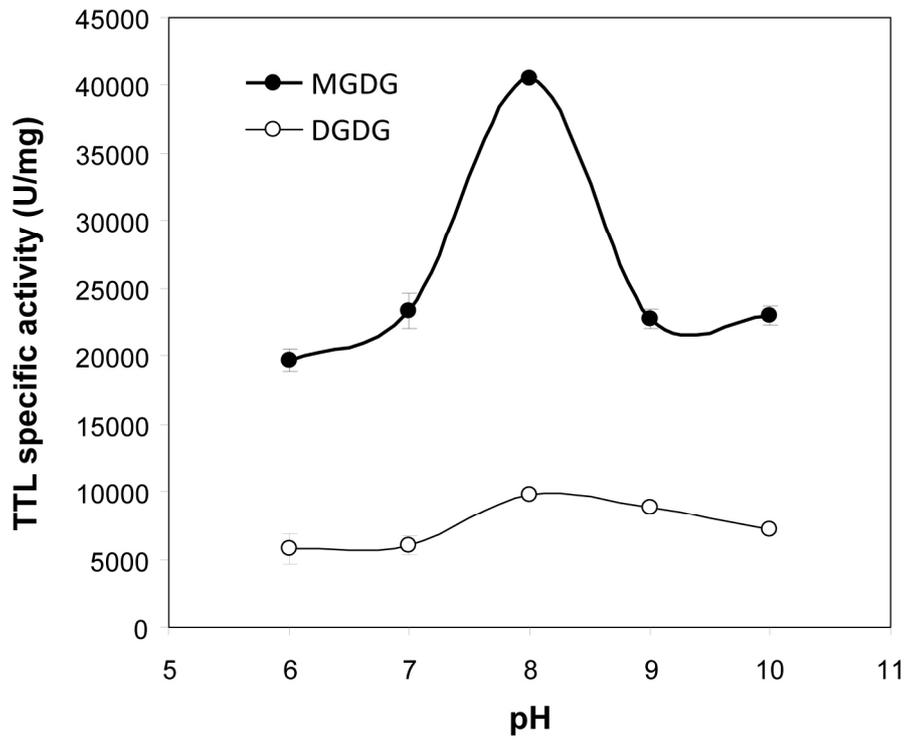
724

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



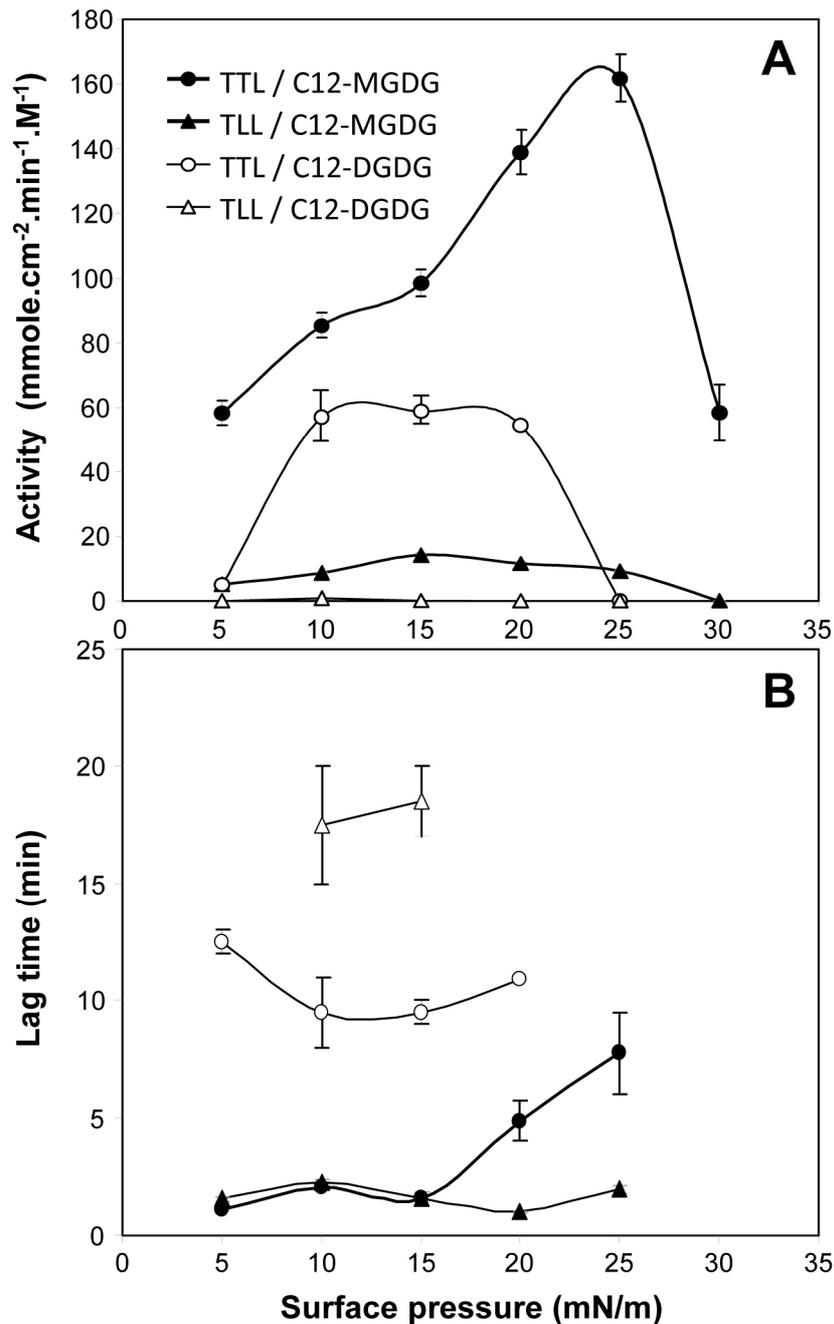
728

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.



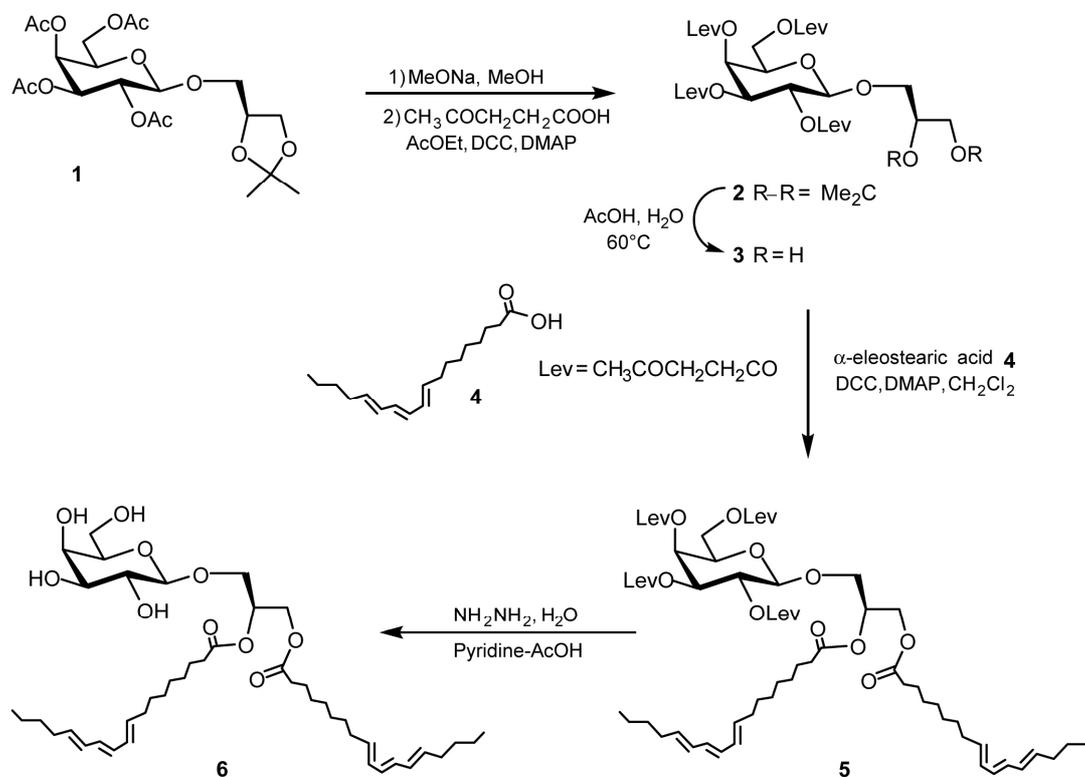
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734 **Figure 3.** Variations with surface pressure in the activity of TTL and TLL on monomolecular  
 735 films of galactolipids. (A) Steady-state activities on C12-MGDG and C12-DGDG  
 736 monomolecular films; (B) Lag times observed in the course 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).



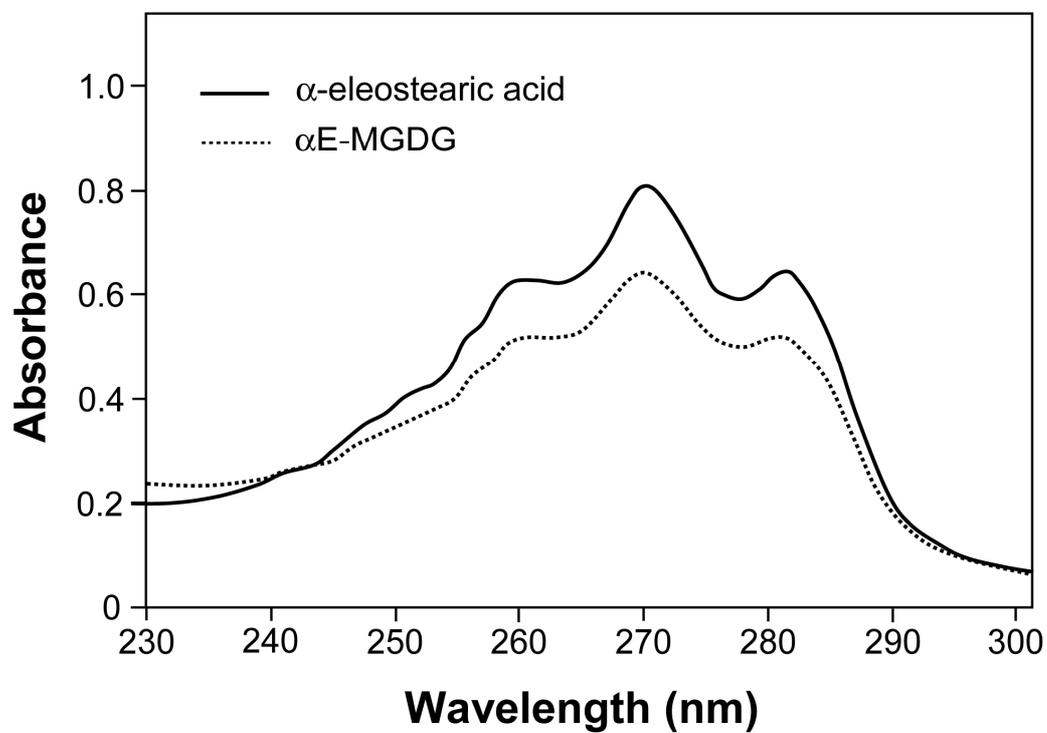
739

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



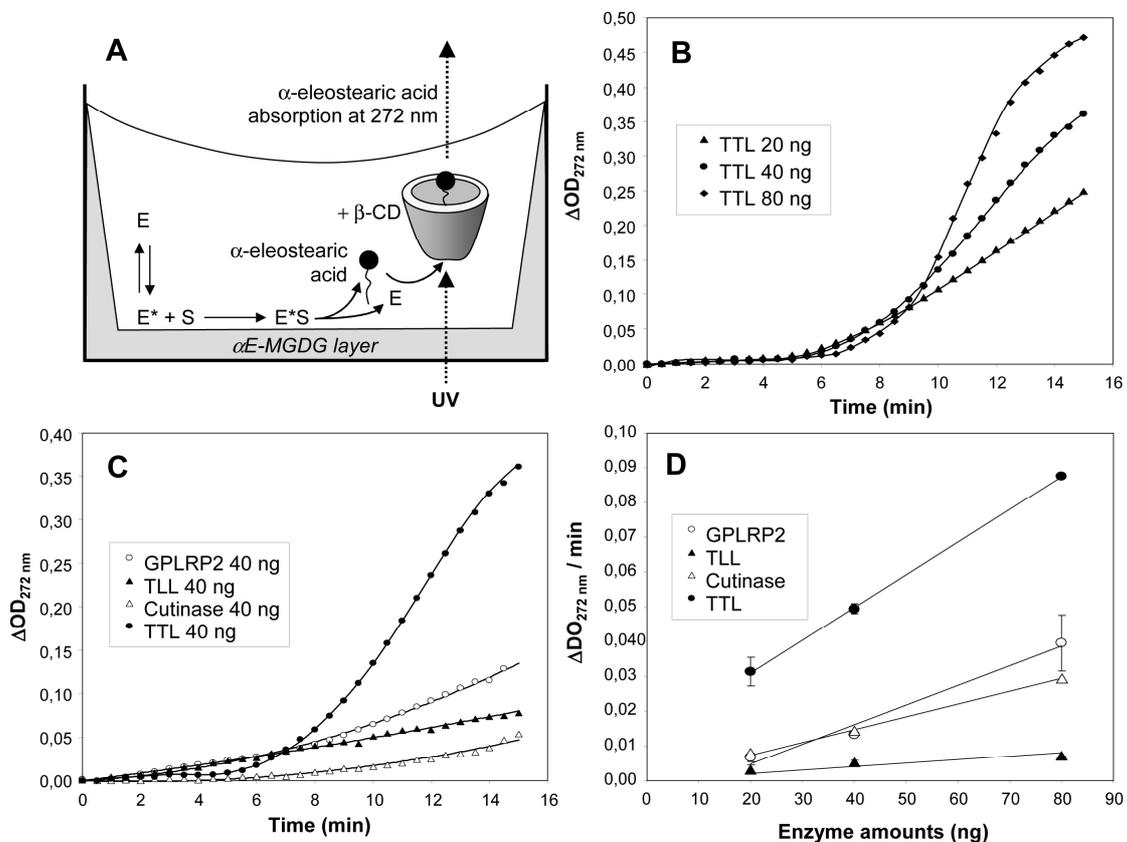
742

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%.



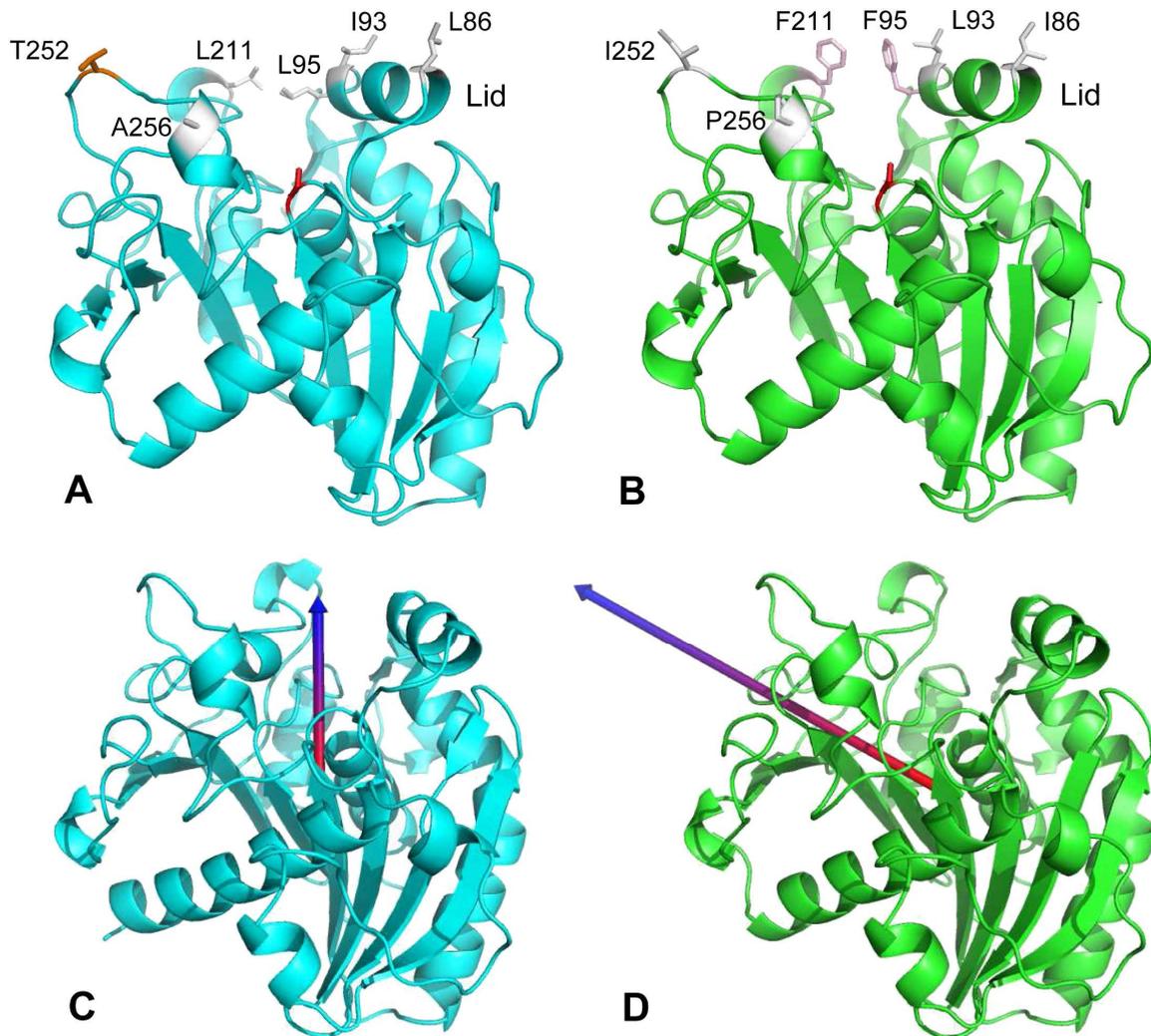
745

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



758

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



765

766

767 **Table 1.** Maximum rates of hydrolysis ( $\text{mmol cm}^{-2} \text{min}^{-1} \text{M}^{-1}$ ) of C12-MGDG and C12-  
 768 DGDG monomolecular films by TTL and other galactolipases. Values are means  $\pm$  SD (n=3).  
 769 The surface pressures ( $\pi$ ,  $\text{mN m}^{-1}$ ) at which these activities were recorded are indicated in  
 770 parenthesis.  
 771

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>	$<10^{-3}$

772

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

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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).

<b>Enzyme</b>	<b>Specific Activity</b> ( $\mu\text{moles min}^{-1} \text{mg}^{-1}$ )	<b>Lag time (min)</b>
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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779 **Table 3:** Maximum specific activities (U/mg) of TTL and other lipases on various lipid  
 780 substrates. TC4, tributyrin; TC8, trioctanoin; Egg PC, egg phosphatidylcholine; C8-MGDG,  
 781 monogalactosyldiacylglycerol; C8-DGDG, digalactosyldiacylglycerol. Values are means  $\pm$  SD  
 782 (n=3).  
 783

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>

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785 <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].

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