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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; β -CD, β -cyclodextrin; DGDG,
28 digalactosyldiacylglycerol ; α E-MGDG, 1,2-di- α -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 α -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 μ 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, α -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*-(β -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* (*CaIA*), 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 α -eleostearic acid as chromophore. Various lipase
112 assays have been developed for high throughput screening using α -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- α -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 μ 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*- β -D-galactopyranosyl-*sn*-glycerol (C12-MGDG) and 1,2-di-*O*-dodecanoyl-
159 3-*O*-(6-*O*- α -D-galactopyranosyl- β -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 cm² and a volume of 43 ml. The reservoir (24-cm long \times 7.5-cm wide) had a surface
164 area of 156.5 cm² 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 CaCl₂, 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 (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- α -eleostearoyl-3-O- β -D-galactopyranosyl-*sn*-glycerol (α 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 F₂₅₄ (Merck). TLC plates were
188 inspected by UV light ($\lambda = 254 \text{ nm}$). Column chromatography was performed on Silica-gel
189 (Silicycle). ¹H and ¹³C 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- β -D-galactopyranosyl]-1,2-O-isopropylidene-*sn*-glycerol (**2**):

194 3-O-[2,3,4,6-Tetra-O-acetyl- β -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⁺] 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*_f 0.30 (5:1 ethyl acetate-petroleum
205 ether); [α]_D +2.1 (*c* 1.0, CHCl₃); ¹H NMR (CDCl₃): δ 5.36 (dd, 1H, *J*_{3,4} 3.4, *J*_{4,5} 0.8 Hz, H-4),
206 5.17 (dd, 1H, *J*_{1,2} 7.9, *J*_{2,3} 10.4 Hz, H-2), 5.01 (dd, 1H, H-3), 4.57 (d, 1H, H-1), 4.26 (dddd,
207 1H, H-2_{gly}), 4.20 (dd, 1H, *J*_{5,6a} 6.7, *J*_{6a,6b} 11.2 Hz, H-6a), 4.10 (dd, 1H, *J*_{5,6b} 6.4 Hz, H-6b),
208 4.04 (dd, 1H, *J*_{1a_{gly},2_{gly}} 6.4, *J*_{1a_{gly},1b_{gly}} 6.4 Hz, H-1a_{gly}), 3.92-3.86 (m, 2H, H-5, H-3a_{gly}), 3.83
209 (dd, 1H, *J*_{1b_{gly},2_{gly}} 6.2 Hz, H-1b_{gly}), 3.62 (dd, 1H, *J*_{2_{gly},3b_{gly}} 6.2, *J*_{3a_{gly},3b_{gly}} 10.5 Hz, H-3b_{gly}),
210 2.80-2.40 (m, 16H, 4COCH₂CH₂COCH₃), 2.19, 2.18, 2.17, 2.16 (4s, 12H, 4CH₃CO), 1.41,
211 1.34 (2s, 6H, (CH₃)₂C); ¹³C NMR (CDCl₃): δ 206.52, 206.43, 206.16, 206.04
212 (4CH₃CO), 172.15, 171.90, 171.85, 171.41 (4OCOCH₂), 109.26 (C(CH₃)₂), 101.23 (C-1),
213 74.25 (C-2_{gly}), 70.81, 70.73 (C-3, C-5), 69.38 (C-3_{gly}), 68.85 (C-2), 67.24 (C-4), 66.36 (C-
214 1_{gly}), 61.38 (C-6), 37.84, 37.77, 37.76, 37.67 (CH₂COCH₃), 29.76, 29.67 (CH₃COCH₂), 27.79
215 (OCOCH₂), 26.67, 25.20 ((CH₃)₂C).

216 HRMS calculated for C₃₂H₄₆NaO₁₆ [M+Na]⁺ 709.2678; found 709.2651.

217

218 3-*O*-[2,3,4,6-Tetra-*O*-levulinoyl-β-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 CHCl₃-EtOH). Pure product **3** was
222 obtained in 90% yield: 2.20 g, oily material, *R*_f 0.46 (9:1 CHCl₃-EtOH); [α]_D-0.5 (*c* 2.0,
223 CHCl₃); ¹H NMR (CDCl₃): δ 5.31 (bd, 1H, *J*_{3,4} 3.4, *J*_{4,5} 0.2 Hz, H-4), 5.12 (dd, 1 H, *J*_{1,2} 7.8
224 Hz, *J*_{2,3} 10.5 Hz, H-2), 5.00 (dd, 1H, H-3), 4.49 (d, 1H, H-1), 4.16 (dd, 1H, *J*_{5,6a} 7.1, *J*_{6a,6b}
225 11.3, H-6a), 4.06 (dd, 1H, *J*_{5,6b} 6.1 Hz, H-6b), 3.89 (bd, 1H, H-5), 3.88-3.55 (m, 5H, H-1a_{gly},
226 H-1b_{gly}, H-2_{gly}, H-3a_{gly}, H-3b_{gly}), 3.17-3.10 (m, 2H, 2OH), 2.85-2.35 (m, 16H,
227 4COCH₂CH₂COCH₃), 2.19, 2.18, 2.17, 2.16 (4s, 12H, 4CH₃CO); ¹³C NMR (CDCl₃):
228 δ 207.76, 206.89, 206.69, 206.22 (CH₃CO levulinoyl), 172.28, 171.99, 171.81, 171.75
229 (OCOCH₂ levulinoyl), 101.57 (C-1), 72.03 (C-3_{gly}), 70.89 (C-5), 70.66 (C-3), 70.48, 69.02
230 (C-2, C-2_{gly}), 67.35 (C-4), 63.35 (C-1_{gly}), 61.61 (C-6), 37.87, 37.87, 37.80, 37.69
231 (CH₃COCH₂), 29.87, 29.85, 29.81, 29.72 (CH₃CO), 27.82, 27.80 (OCOCH₂).

232 HRMS calculated for C₂₉H₄₂NaO₁₆ [M+Na]⁺ 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 CH₂Cl₂-EtOH) was
242 necessary to give the pure product **5**, recovered in 73% yield: 2.47 g, oily material, *R*_f 0.20-
243 0.25 (1:1 ethyl acetate-petroleum ether), 0.70 (9:1 CH₂Cl₂-EtOH); [α]_D +3.7 (*c* 1.0, CHCl₃);
244 ¹H NMR (CDCl₃): δ 6.42-6.33 (m, 2H, 2H-11_{eleo}), 6.20-6.13 (m, 2H, 2H-12_{eleo}), 6.13-6.06 (m,

245 2H, 2H-13_{eleo}), 6.02-5.95 (m, 2H, 2H-10_{eleo}), 5.75-5.65 (m, 2H, 2H-14_{eleo}), 5.43-5.34 (m, 1H,
246 H-9_{eleo}), 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_{gly}), 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_{gly}), 4.20 (dd, 1H, $J_{5,6a}$ 6.6,
249 $J_{6a,6b}$ 11.1, H-6a), 4.14 (dd, 1H, H-1b_{gly}), 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_{gly}), 3.89 (bdd, 1H, H-5), 3.69 (dd, 1H, H-3b_{gly}), 2.85-2.45 (m, 16H,
251 8CH₂ levulinoyl), 2.35-2.26 (m, 4H, 2COCH₂ eleo), 2.19, 2.18, 2.17, 2.16 (4s, 12H, 4CH₃CO
252 levulinoyl), 2.14-2.07 (m, 4H, 4H, 2CH₂CH=), 1.65-1.55 (m, 4H, 2COCH₂CH₂ eleo), 1.40-
253 1.25 (m, 24H, 12CH₂ alkyl chains), 0.91 (t, 9H, J 6.5Hz, 2CH₃CH₂); ¹³C NMR (CDCl₃): δ
254 206.58, 206.49, 206.25, 206.09 (CH₃CO levulinoyl), 173.36, 172.88 (COCH₂ eleo), 172.26,
255 171.94, 171.94, 171.47 (OCOCH₂ levulinoyl), 135.27 (C-14_{eleo}), 132.94 (C-12_{eleo}), 131.84 (C-
256 9_{eleo}), 130.63 (C-13_{eleo}), 128.82 (C-10_{eleo}), 126.01 (C-11_{eleo}), 101.54 (C-1), 70.89 (C-5), 70.76
257 (C-3), 69.76 (C-2_{gly}), 68.78 (C-2), 67.75 (C-3_{gly}), 67.24 (C-4), 62.39 (C-1_{gly}), 61.41 (C-6),
258 37.93, 37.87, 37.87, 37.76 (CH₃COCH₂), 34.27, 34.13 (COCH₂ eleo), 32.57 (C-15 eleo),
259 31.53 (C-16 eleo), 29.82 (CH₃CO), 29.73, 29.25, 29.18, 29.14, 29.11 (C-4, C-5, C-6, C-7),
260 27.88, 27.86 (OCOCH₂ lev, C-8), 24.93 (C-3 eleo), 22.30 (C-17 eleo), 14.03 (C-18 eleo).
261 HRMS calculated for C₆₅H₉₈NaO₁₀ [M+Na]⁺ 1189.6645; found 1189.6661.

262
263 1,2-Di-*O*- α -eleostearoyl-3-*O*- β -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 NaHCO₃ solution (2x50 mL). The aqueous phases were extracted with
268 chloroform (6x40 mL) and the combined organic phases were dried (Na₂SO₄) 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 CH_2Cl_2 -EtOH); $[\alpha]_D +3.8$ (c 1.0, 4:1 CHCl_3 -MeOH); ^1H NMR
272 (CDCl_3): δ 6.33-6.25 (m, 2H, 2H-11_{eleo}), 6.12-6.05 (m, 2H, 2H-12_{eleo}), 6.04-5.97 (m, 2H, 2H-
273 13_{eleo}), 5.93-5.86 (m, 2H, 2H-10_{eleo}), 5.66-5.57 (m, 2H, 2H-14_{eleo}), 5.34-5.26 (m, 2H, 2H-
274 9_{eleo}), 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_{gly}), 4.28
275 (dd, 1 H, $J_{1\text{agly},1\text{bgly}}$ 12.1 Hz, H-1_{agly}), 4.14 (d, 1H, $J_{1,2}$ 7.1 Hz, H-1), 4.14 (dd, 1H, H-1_{bgly}),
276 3.95 (dd, 1H, $J_{3\text{agly},3\text{bgly}}$ 10.9 Hz, H-3_{agly}), 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_{bgly}), 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, 2COCH₂), 2.11-1.95
279 (m, 8H, 4CH₂CH=), 1.57-1.47 (m, 4H, 2COCH₂CH₂ eleo), 1.32-1.17 (m, 24H, 12CH₂ alkyl
280 chains), 0.81 (t, 6H, J 6.5Hz, 2CH₃CH₂); ^{13}C NMR (CDCl_3): δ 173.98, 173.68 (COCH₂ eleo),
281 135.18 (C-14_{eleo}), 132.85 (C-12_{eleo}), 131.68 (C-9_{eleo}), 130.53 (C-13_{eleo}), 128.72 (C-10_{eleo}),
282 125.88 (C-11_{eleo}), 103.98 (C-1), 74.93 (C-3), 73.32 (C-5), 71.15 (C-2), 70.31 (C-2_{gly}), 68.73
283 (C-4), 67.83 (C-3_{gly}), 62.78 (C-1_{gly}), 61.42 (C-6), 34.19, 34.05 (COCH₂ eleo), 32.44 (C-15
284 eleo), 31.41 (C-16 eleo), 29.60, 29.12, 29.05, 29.01(C-4_{eleo}, C-5_{eleo}, C-6_{eleo}, C-7_{eleo}), 27.74 (C-
285 8_{eleo}), 24.79 (C-3 eleo), 22.17 (C-17_{eleo}), 13.81 (C-18_{eleo}).

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- α -eleostearoyl-3-O-
289 β -D-galactopyranosyl-sn-glycerol in microtiter plates

290 Microtiter plates were coated with the UV-absorbing galactolipid substrate using an
291 α E-MGDG solution (0.5 mg mL⁻¹) 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 μ 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 CaCl₂, 1 mM EDTA, and
297 3 mg mL⁻¹ β -cyclodextrin (β -CD)) and left to equilibrate at 37°C for at least 5 min with 200
298 μ l of the assay buffer. The β -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 μ 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 PowerwaveTM
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 (τ) 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 (ΔOD
312 min⁻¹), and τ 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 α -
314 eleostearic acid [27] and was expressed as μmoles 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 CaCl_2 were carried out
327 using a Zetasizer Nano S (Malvern Instruments) at 37°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° . 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°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 ± 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 ± 62 U/mg for FsC and 450 ± 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 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 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 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 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⁻¹ and only
385 increased above and till 25 mN m⁻¹ 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⁻¹
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 α -* 399 *eleostearic acid*

400 The conjugated triene present in α -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 α -eleostearic
405 acid as chromophore. Following a similar approach, we synthesized a
406 monogalactosyldiglyceride containing α -eleostearic acid (α 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 α E-MGDG (compound **6** in Figure 4), we used 3-*O*-[2,3,4,6-
410 Tetra-*O*-acetyl- β -D-galactopyranosyl]-1,2-*O*-isopropylidene-*sn*-glycerol (compound **1**) that
411 was obtained in a previous study [7] and α -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 α -eleostearic acid **4** (DCC, DMAP, CH₂Cl₂) 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 α 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 α -eleostearic acid [24], pure tung oil triglycerides [23], synthetic α -
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 α 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 α E-MGDG substrate, will hydrolyze it and release α -eleostearic acid (Figure 6A). This long

434 chain fatty acid can be further solubilized by complex formation with β -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 μ 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 μ g α 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 (Δ OD = assay OD - initial
442 OD) at 272 nm during α 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 Δ 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 α -eleostearic acid, variations in OD at 272 nm could be
450 correlated with α -eleostearic acid concentration and further used for the estimation of enzyme
451 specific activities. The apparent molar extinction coefficient (ϵ_{app}) of α -eleostearic acid has
452 been previously determined in microtiter plates by recording the absorbance at 272 nm of
453 various amounts of α -eleostearic acid dispersed in the buffer with β -CD at 37°C and it was
454 found to be 5320 M⁻¹ cm⁻¹ [27]. Under these conditions, the increase with time of OD at 272
455 nm was converted into μ moles of α -eleostearic acid released per min and per mg of enzyme
456 (Table 2). TTL was found to be the most active galactolipase on α 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 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 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 α -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 μ 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 α -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

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563

564

565 **Conflict of interest**

566 The authors have declared no conflict of interest

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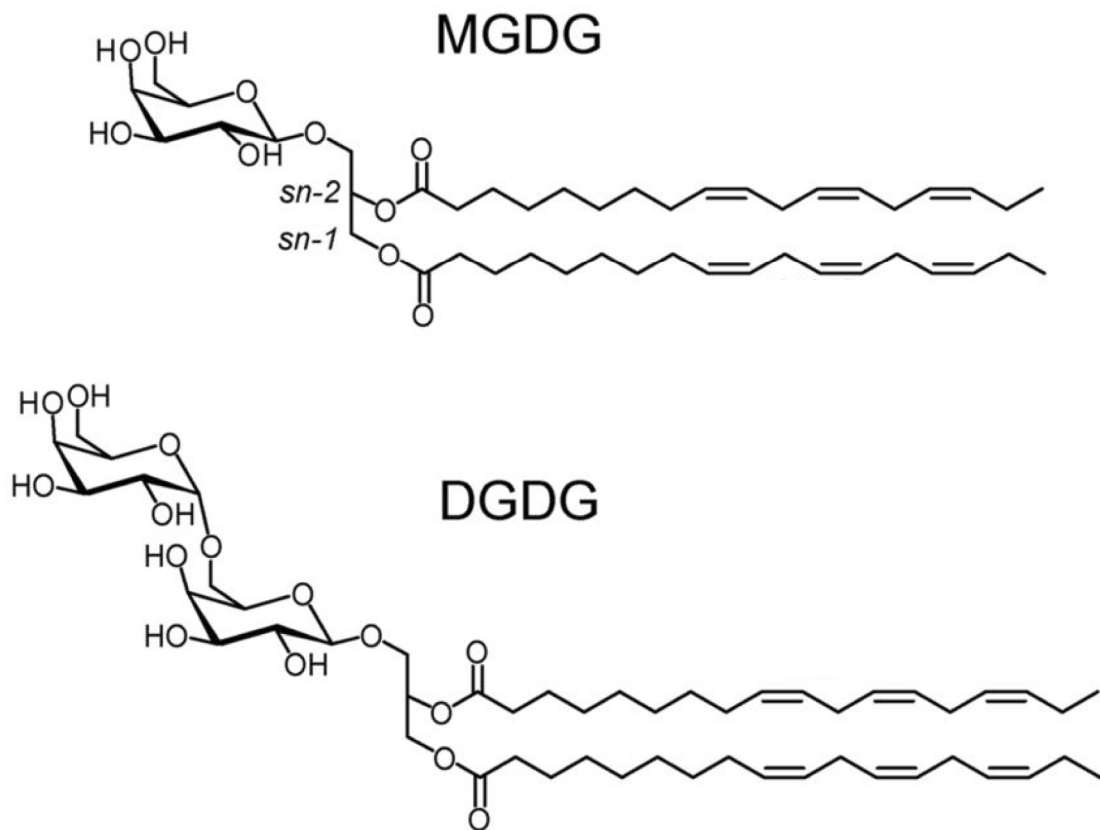
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722

723 **Figure legends**

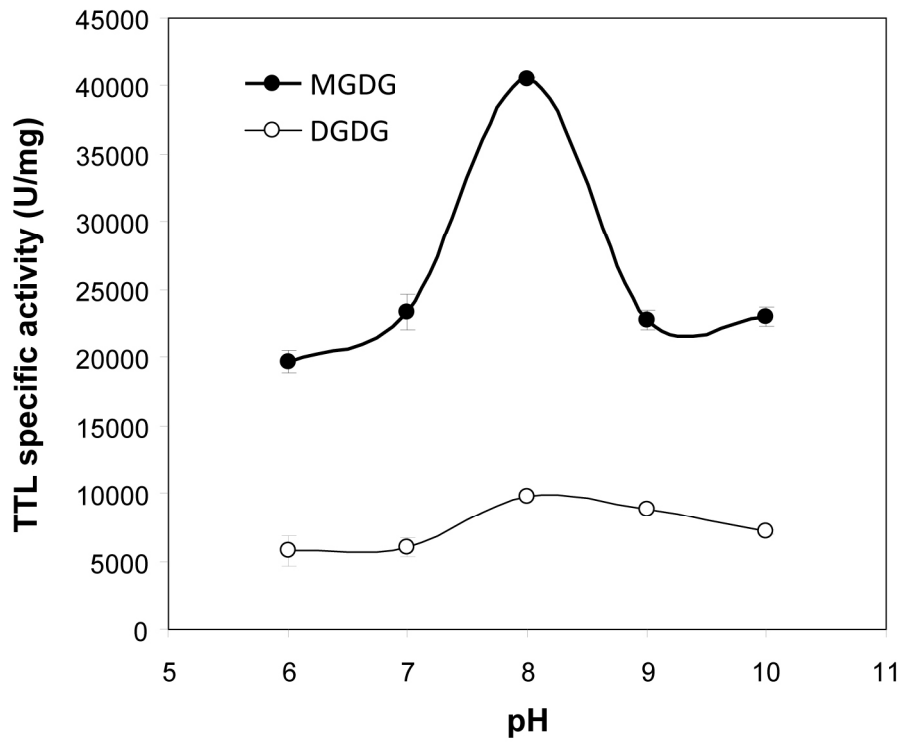
724

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



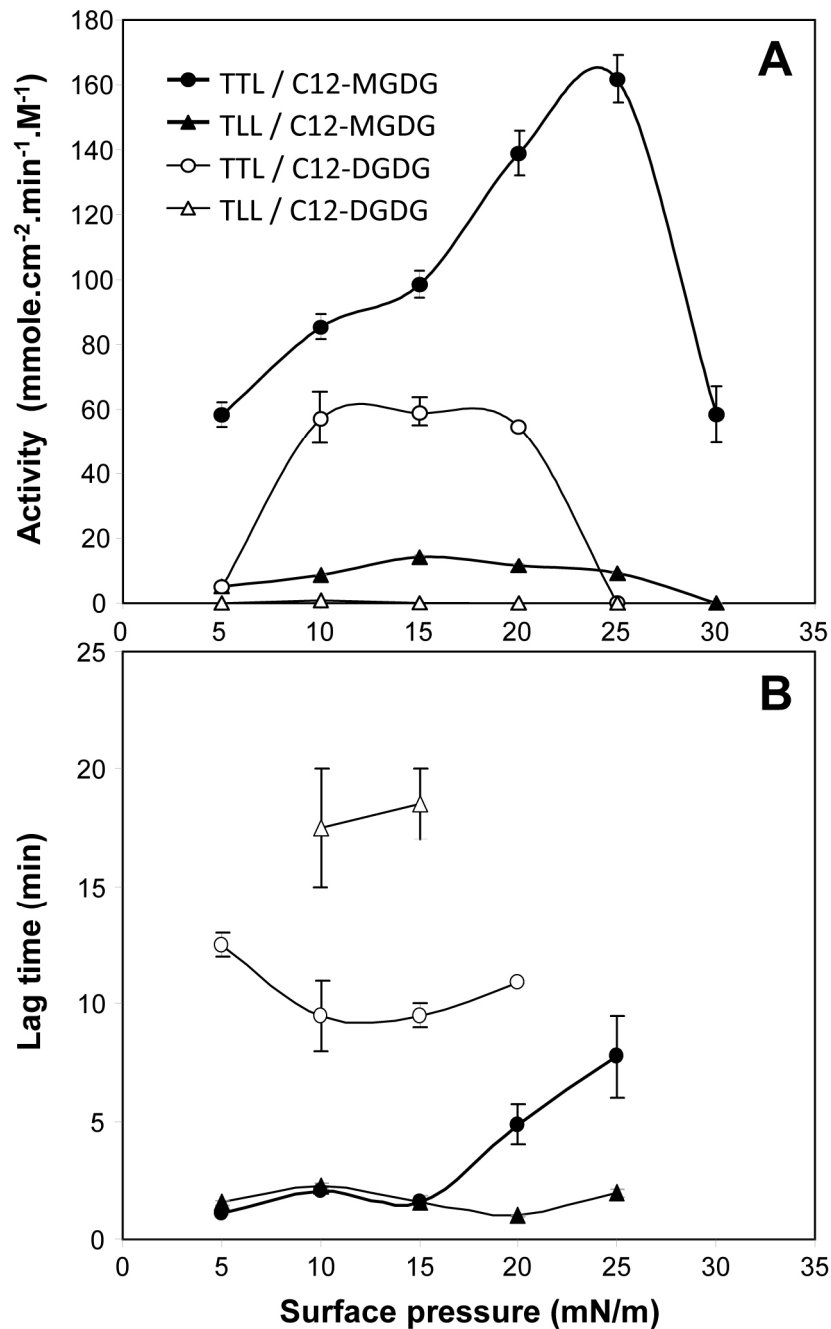
728

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 μ mole of free fatty acid released per min.



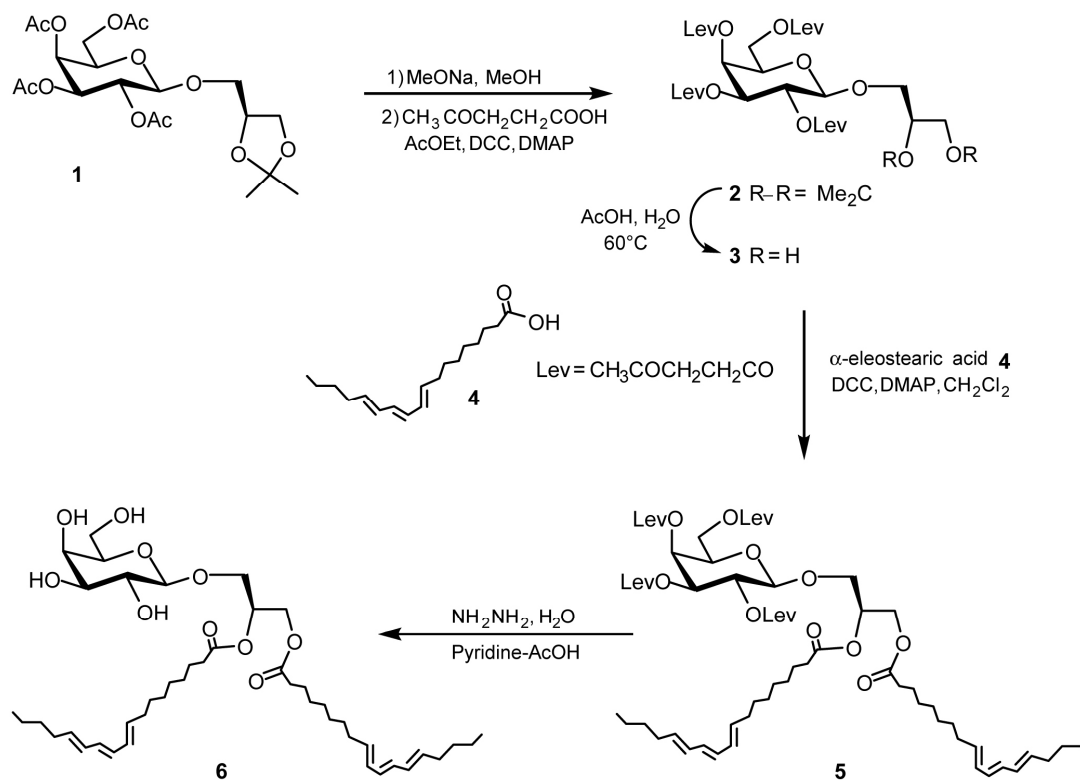
733

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).



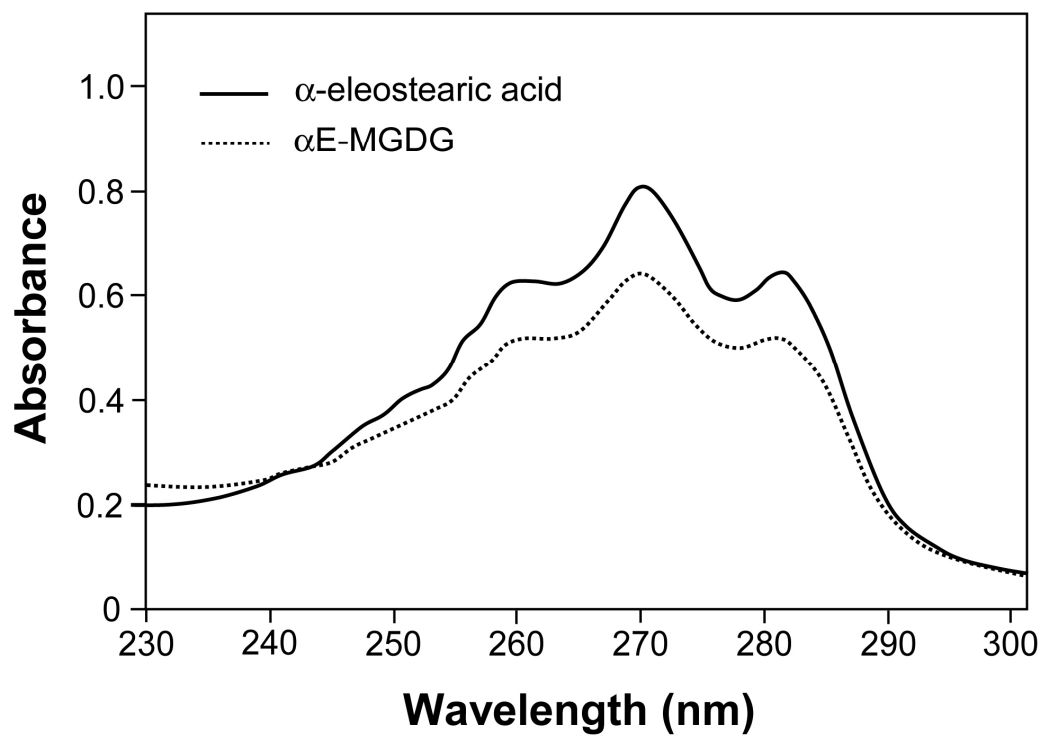
739

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



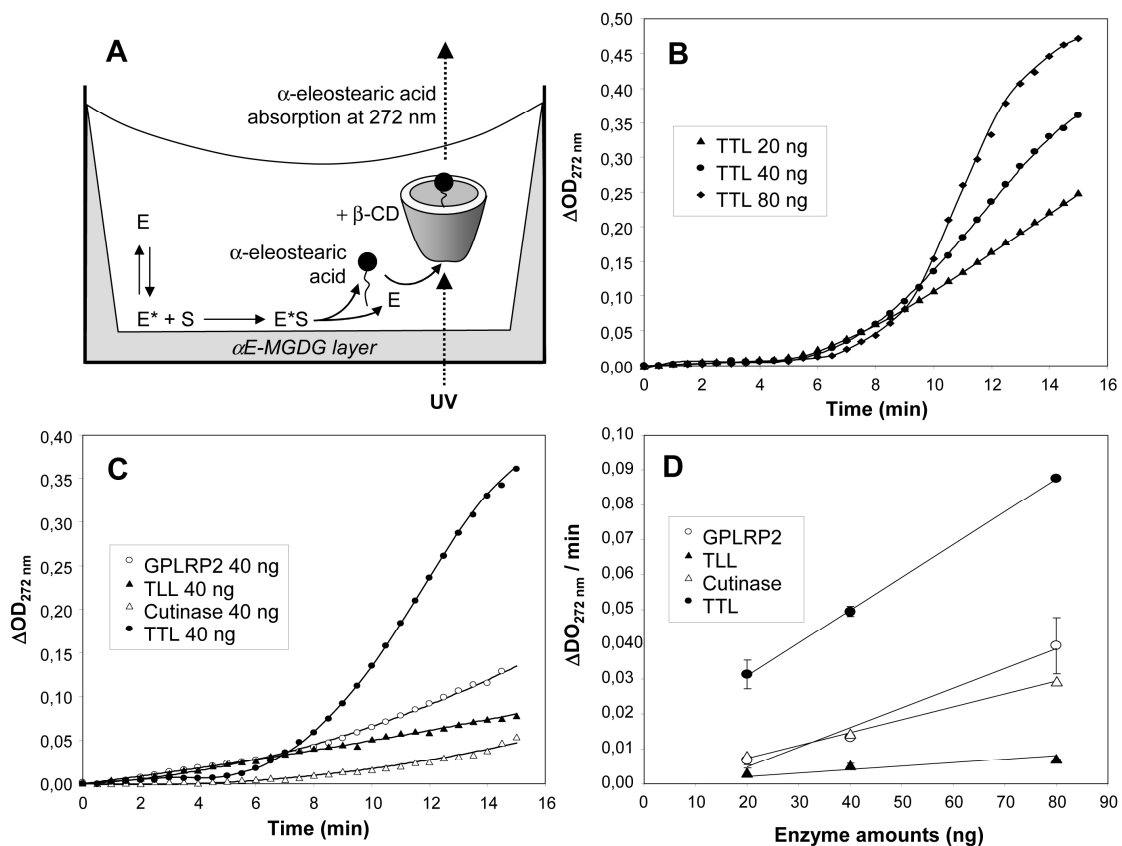
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743 **Figure 5.** UV absorption spectra of α -eleostearic acid (60 $\mu\text{g/mL}$) and $\alpha\text{E-MGDG}$ (60 $\mu\text{g/mL}$)
744 dissolved in ethanol containing BHT 0.001%.



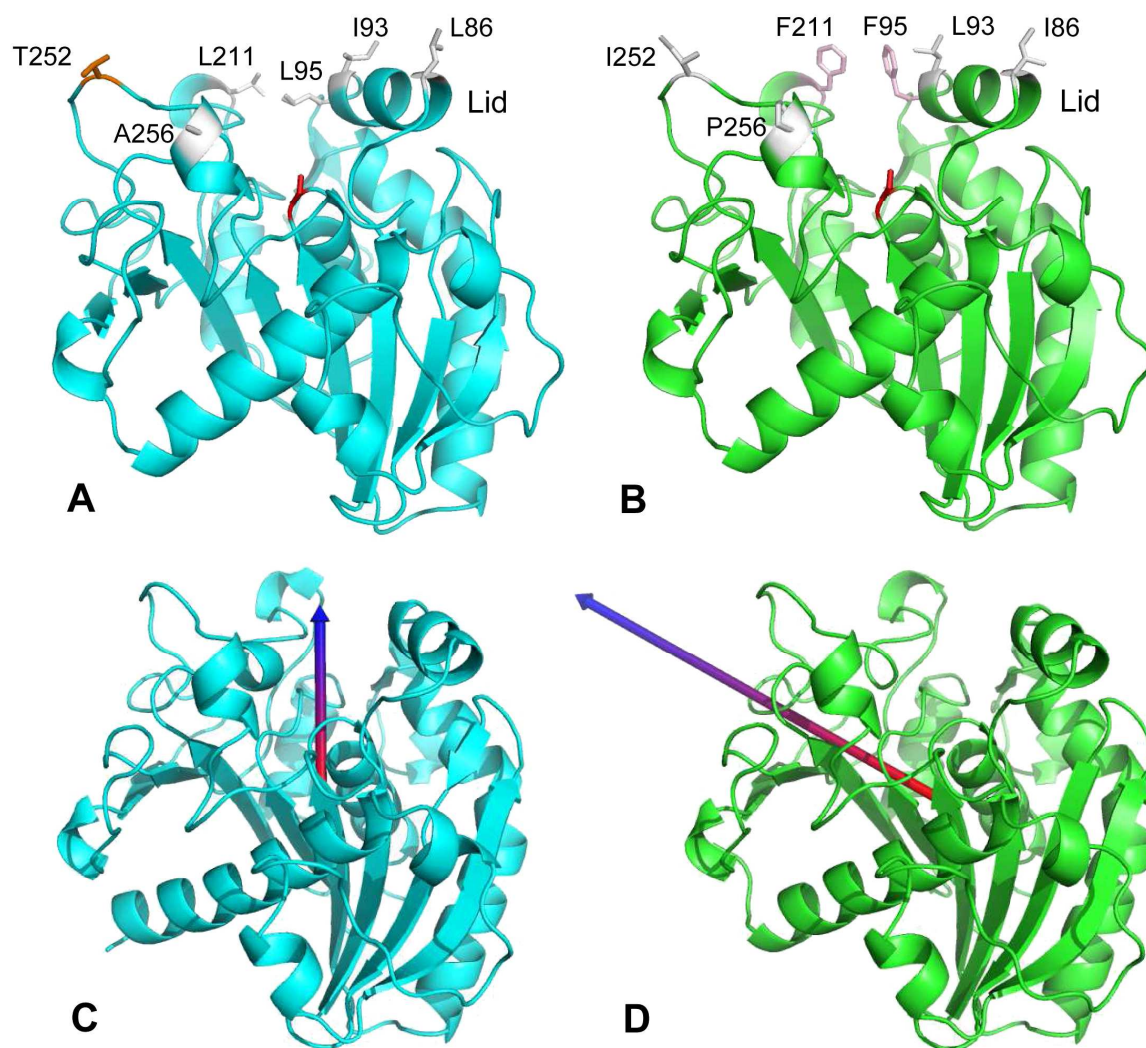
745

746 **Figure 6.** UV-spectrophotometric assays of galactolipase activities using 1,2-Di-*O*- α -
 747 eleostearoyl-3-*O*- β -D-galactopyranosyl-*sn*-glycerol (α E-MGDG). (A) Schematic
 748 representation of the assay showing the enzymatic hydrolysis of the α E-MGDG film coated
 749 onto the wells of a microtiter plate, followed by the solubilization of free α -eleostearic acid
 750 (FFA) in the bulk phase by formation of a complex with β -cyclodextrin (β -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 OD₂₇₂ at time zero) using various amounts of TTL. (C) Variations
 753 with time of optical density at 272 nm (versus OD₂₇₂ 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 μ 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 μ 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 α -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 (π , mN m^{-1}) at which these activities were recorded are indicated in
770 parenthesis.
771

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

772

773 ^a Data from [17]. ^b Data from [33].

774

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

777

778

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 ^a	24110 \pm 390 ^a	9808 \pm 139 ^a	4250 \pm 250	40500 \pm 125	9800 \pm 125
TLL	7834 \pm 850 ^b	9250 \pm 101	2900 \pm 91	767 \pm 188 ^b	450 \pm 41 ^b	672 \pm 61 ^b
rGPLRP2	2700 \pm 300 ^b	675 \pm 43 ^c	754 \pm 151 ^c	500	5420 \pm 85 ^b	4375 \pm 125 ^b
<i>Fusarium solani</i> cutinase	2596 \pm 96 ^b	2965 \pm 50 ^d	688 \pm 10 ^e	0	984 \pm 62 ^b	300 \pm 29 ^b

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785 ^aData from [21] ; ^bData from [17]; ^cdata from [51]; ^ddata from [52]; ^edata from [53].

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