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1 **Title:** Interfacial adsorption and activity of pancreatic lipase-related protein 2 onto
2 heterogeneous plant lipid model membrane

3 **Name(s) of Author(s)** Jeanne Kergomard^{1,2}, Frédéric Carrière³, Gilles Paboeuf^{1,4}, Lauriane
4 Chonchon¹, Nathalie Barouh^{5,6}, Véronique Vié^{1,4*} & Claire Bourlieu^{2**}

5 **Author Affiliation(s)** ¹IPR Institute of Physics, Rennes 1 University, France; ²INRAE/UM/Institut
6 Agro Montpellier UMR 1208 IATE, France; ³Aix-Marseille Université, CNRS, UMR7281
7 Bioénergétique et Ingénierie des Protéines, Marseille, France ; ⁴Univ Rennes, CNRS, ScanMAT -
8 UMS 2001, F-35042, Rennes, France ; ⁵CIRAD, UMR QUALISUD, F34398 Montpellier-France,
9 ⁶Qualisud, Univ Montpellier, Avignon Université, CIRAD, Institut Agro, Université de La
10 Réunion, Montpellier, France.

11 **Corresponding authors:**

12 ***Dr. Véronique Vié, Institut de Physique de Rennes, Campus de Beaulieu, UMR UR1 CNRS**
13 **6251, Université de Rennes, 35042 Rennes cedex**, phone number: 33 (0)2 23 23 56 45 and E-
14 mail address : veronique.vie@univ-rennes.fr;

15 ****Dr. C. Bourlieu-Lacanal, UMR 1208 IATE, 2 Place Pierre Viala, Bât. 31,**
16 **INRAE/UM/Institut Agro Montpellier, F34060 MONTPELLIER CEDEX 1**, France, phone
17 number: 33 (0)4 99 61 22 03 and E-mail address : claire.bourlieu-lacanal@inrae.fr

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23 **Abbreviations**

- 24 DGDG: digalactosyldiacylglycerol
- 25 DGG: digalactosylglycerol
- 26 DGMG: digalactosylmonoacylglycerol
- 27 DLS: dynamic light scattering
- 28 FFA: free fatty acids
- 29 GL: galactolipids (model system)
- 30 gPLRP2: guinea pig protein lipase related-protein 2
- 31 hPLRP2: human protein lipase related-protein 2
- 32 MGDG: monogalactosyldiacylglycerol
- 33 MGG: monogalactosylglycerol
- 34 MGMG: monogalactosylmonoacylglycerol
- 35 PL: phospholipids
- 36 PLRP2: protein lipase related-protein 2
- 37 PUFA: polyunsaturated fatty acids
- 38 pS: phytosterols
- 39

40 **ABSTRACT**

41 Pancreatic lipase related-protein 2 (PLRP2) exhibits remarkable galactolipase and phospholipase
42 A1 activities, which depend greatly on the supramolecular organization of the substrates and the
43 presence of surfactant molecules such as bile salts. The objective of the study was to understand
44 the modulation of the adsorption mechanisms and enzymatic activity of Guinea pig PLRP2
45 (gPLRP2), by the physical environment of the enzyme and the physical state of its substrate.
46 Langmuir monolayers were used to reproduce homogeneous and heterogeneous photosynthetic
47 model membranes containing galactolipids (GL), and/or phospholipids (PL), and/or phytosterols
48 (pS), presenting uncharged or charged interfaces. The same lipid mixtures were also used to form
49 micrometric liposomes, and their gPLRP2 catalyzed digestion kinetics were investigated in
50 presence or in absence of bile salts (NaTDC) during static *in vitro*, so called “bulk”, digestion.

51 The enzymatic activity of gPLRP2 onto the galactolipid-based monolayers was characterized with
52 an optimum activity at 15 mN/m, in the absence of bile salts. gPLRP2 showed enhanced adsorption
53 onto biomimetic model monolayer containing negatively charged lipids. However, the
54 compositional complexity in the heterogeneous uncharged model systems induced a lag phase
55 before the initiation of lipolysis. In bulk, no enzymatic activity could be demonstrated on GL-based
56 liposomes in the absence of bile salts, probably due to the high lateral pressure of the lipid bilayers.
57 In the presence of NaTDC (4 mM), however, gPLRP2 showed both high galactolipase and
58 moderate phospholipase A1 activities on liposomes, probably due to a decrease in packing and
59 lateral pressure upon NaTDC adsorption, and subsequent disruption of liposomes.

60 **KEYWORDS:** pancreatic lipase related-protein 2, heterogeneous monolayers, galactolipids,
61 monolayer, liposomes

62

63 1 INTRODUCTION

64 Galactolipids (GL) are the main lipids found in the photosynthetic membrane of plants and algae,
65 accounting for more than 70% wt. of the total membrane lipids (Dörmann, 2013; Douce et al.,
66 1973; Gurevich et al., 1997). Due to the natural abundance of plants and algae on Earth, GL
67 represent the most important class of lipids, and therefore, the most important reservoir of fatty
68 acids (80% versus 20% wt. for plant phospholipids (PL) and TAG), including some essential
69 polyunsaturated fatty acids (PUFA) (Gounaris & Barber, 1983). The two main GL composing the
70 photosynthetic membranes of plants are the neutral monogalactosyldiacylglycerol (MGDG, 53%
71 wt.) and digalactosyldiacylglycerol (DGDG, 27% wt.). MGDG possess a unique small 1- β -
72 galactose polar head bound at the *sn*-3 position to a diacylglycerol (Lee, 2000), whereas DGDG
73 has a larger polar head with an additional α -galactose, linked to β -galactose (Mizusawa & Wada,
74 2012). Both galactolipids possess two esterified acyl chains of fatty acids at the *sn*-1 and *sn*-2
75 position of the glycerol backbone, whose nature depends mainly on the synthesis pathway of GL
76 (Glöckner, 2013; Sahaka et al., 2020). In addition to these two glycolipids, photosynthetic plant
77 membranes contain smaller amounts of charged lipids, sulfoquinovosyldiacylglycerol (SQDG) and
78 phosphatidylglycerol (PG), the proportions of which vary between photosynthetic plant species.
79 GL are naturally rich in the essential α -linolenic acid (ALA, C18:3 ω 3), which is the precursor of
80 longer chain ω 3 fatty acids, the eicosapentaenoic acid (EPA, C20:5, ω 3), and the docosahexaenoic
81 acid (DHA, C22:6 ω 3), resulting from elongation and desaturation reactions (Kergomard et al.,
82 2021). In particular, these two long-chain PUFA play a crucial role in the homeostatic regulation
83 of the human body by being the precursors of signaling oxygenated lipids involved in inflammation
84 resolution processes in our body (Saini & Keum, 2018). GL also contain a significant amount of
85 hexadecatrienoic acid (HTA, C16:3 ω 3), an unusual fatty acid found mainly in green plants and

86 algae. The nutritional benefits of HTA have been scarcely studied, although it represents a unique
87 biomarker of the digestion, absorption, and accretion of GL FA. Indeed, it has been found in tissues
88 of zebrafish fed with chloroplast-rich fractions (Gedi et al., 2019), as well as in the meat of horses
89 (Belaunzaran et al., 2018), and has been identified as a potential precursor of ALA in rodents
90 (Cunnane et al., 1995). The interesting nutritional profile of GL makes them compounds of interest
91 for the development of food products rich in ω 3 PUFA. Nevertheless, in order to exploit the
92 nutritional properties of GL in potential food applications, it is necessary to determine their
93 digestibility by humans.

94 Regarding this digestibility, human pancreatic juice and duodenal contents have been shown to
95 exhibit galactolipase activity (Andersson et al., 1995). This activity was associated to PLRP2
96 (Andersson et al., 1996), as well as, to a lesser extent, to the bile salt-simulated lipase/carboxyl
97 ester hydrolase (BSSL/CEH) (Amara et al., 2013; Bakala N’Goma et al., 2012). PLRP2 shows
98 enzymatic activity on polar lipid substrates with larger heads in comparison with other classical
99 pancreatic lipases such as HPL. Indeed, in addition to lower lipase activity (1250 *versus* 8500 U/mg
100 for HPL on tributyrin), PLRP2 exhibits some phospholipase A1 (74 U/mg on purified L- α -PC) and
101 high galactolipase (~2800 U/mg on MGDG for instance) activities (Amara et al., 2009; De Caro et
102 al., 2004; Sahaka et al., 2020; Wattanakul et al., 2019). This enzymatic activity on a wider range
103 of substrates than HPL is partly explained by the unusual conformation of the lid controlling the
104 access to the active site of hPLRP2 (Eydoux et al., 2008). PLRP2 is also present in the digestive
105 system of other species, and in particular in monogastric herbivores such as the guinea pig
106 (gPLRP2), whose diet contains significant amounts of GL. Although the galactolipase activity of
107 PLRP2 has been the subject of numerous studies, they were mainly focused on the identification
108 and quantification of enzyme activity on synthetic (medium chain acyl GL) or natural substrates

109 most of the time presented in the form of micelles with bile salts. In these studies, little attention
110 was given to the local physical state, whether regarding the level of condensation, nor the presence
111 of charged molecules. PLRP2 was also found to be active on monolayers of pure PL and GL, with
112 an optimum activity at surface pressures below the lateral surface pressure of membranes, *i.e.* 10-
113 15 mN/m (Eydoux, De Caro, et al., 2007; Hjorth et al., 1993; Sias et al., 2004). These findings,
114 together with the absence of interaction and activity of PLRP2 on PL liposomes (Mateos-Diaz,
115 Bakala N’Goma, et al., 2018), suggest that PLRP2 may not be able to act directly on plant
116 membranes. In the present study, gPLRP2, whose biochemical properties are close to those of
117 hPLRP2, was used as a model of PLRP2. We proposed to investigate the adsorption mechanisms
118 of PLRP2 on plant model and natural monolayers presenting homogeneous or heterogeneous
119 physical states at the air/water interface, as well as on GL liposomes in static dispersed condition,
120 hereafter called “bulk”, in the absence and presence of bile salts (NaTDC, 4 mM). Indeed, bile salts
121 are biosurfactants that are secreted by the liver, and which play key contrasting roles in lipid
122 digestion: they adsorb onto interfaces where they can compete with lipases and inhibit lipolysis
123 (Bezzine et al., 1999; Borgström, 1975), but they also remove lipolysis products from the interface,
124 solubilizing them into micelles (Pabois et al., 2021). More importantly, they form mixed micelles
125 with polar lipids, *i.e.* PL and GL, that are the preferred substrates for pancreatic phospholipase A2
126 (Borgström, 1993) and PLRP2 (Amara et al., 2010; Mateos-Diaz, Bakala N’Goma, et al., 2018),
127 respectively.

128 We studied the organizational properties and enzymatic activity of gPLRP2 on different GL
129 substrates, controlling finely their physical state, *i.e.* on systems with or without phase
130 heterogeneities. The adsorption and enzymatic hydrolysis capacity of gPLRP2 were first tested on
131 homogeneous and heterogeneous monolayers of GL, PL, and pS (GL and GL/DPPC/pS

132 monolayers), as well as on more biomimetic system (MGDG/DGDG/SQDG/PG monolayer), in
133 order to gain a mechanistic understanding of the digestion mechanisms at the lipid interface
134 molecular level (nm). These three lipid mixtures were then formulated into liposomes and
135 incubated in the presence of gPLRP2 to determine if galactolipase and/or phospholipase A1
136 activities were displayed on these dispersed micronic objects (μm) either in the absence or presence
137 of bile salts (NaTDC).

138 **2 EXPERIMENTAL SECTION**

139 Chloroform, methanol, SQDG, and PG were purchased from Sigma Aldrich Ltd. (St. Louis, MO).
140 *1,2-dipalmitoylphosphatidylcholine* (DPPC), MGDG and DGDG were purchased from Avanti
141 Polar Lipids. Canola pS, composed of a mixture of β -sitosterol (50 mol%), campesterol (40 mol%)
142 and brassicasterol (10 mol%), were kindly donated by Cognis France (Estarac, France). pS were
143 collected from desodorization distillates of canola oil. If not stated otherwise, all biophysical
144 characterizations were conducted at least in triplicate.

145 **2.1 Preparation of lipid mixtures**

146 Binary mixture of natural long chains MGDG and DGDG (60:40, mol/mol) was prepared, namely
147 (1) GL. Heterogeneous mixture of GL, DPPC and pS, namely (2) GL/DPPC/pS (45:45:10,
148 mol/mol/mol), respectively was also prepared both to simplify the composition of natural plant
149 membrane, and to provide a pronounced phase coexistence. A biomimetic model system was also
150 prepared, reproducing more accurately the composition of plant photosynthetic membranes, by the
151 addition of charged polar lipids, SQDG (predominant species C18:3/C16:0) and PG, and hereafter
152 called (3) MGDG/DGDG/SQDG/PG (56:24:10:10, mol/mol/mol/mol). Relative compositions of
153 the model systems and fatty acid repartitions of MGDG and DGDG used in this study are given in
154 Table S1 and Figure S2, respectively.

155 **2.2 Enzyme purification and preparation of aliquots**

156 Recombinant guinea pig pancreatic lipase-related protein 2 (gPLRP2) and its inactive variant
157 gPLRP2 S125G were produced in *Aspergillus oryzae* and *Pichia pastoris*, respectively, and purified
158 as described in Hjorth et al. (1993) and Mateos-Diaz et al. (2018). For the interfacial measurements,
159 a gPLRP2 stock solution (0.15 mg/mL) was prepared in a Tris HCl buffer (10 mM Tris, 100 mM

160 NaCl, 5 mM CaCl₂, pH 7) and aliquots were prepared in the same buffer at a final concentration of
161 0.128 mg/L (2.7 nM). This value is closed to the physiological concentrations divided by 100 and
162 corresponds to the usual value used in interfacial studies to avoid saturating the interface with
163 digestive proteins. The inactive gPLRP2 S125G variant was used as a control of the protein effect
164 on lipids in the absence of any enzyme activity as previously shown with phospholipids (Mateos-
165 Diaz et al., 2018). For digestion experiments in static conditions, 100 μL aliquots were prepared at
166 a final concentration of 3.3 mg/L.

167 **2.3 Ellipsometry and surface pressure measurements at the air/water interface**

168 Kinetic measurements were performed over 2 hours using a computer controlled and user-
169 programmable LB Teflon Langmuir trough (KSV Nima, Helsinki, Finland) with a surface area of
170 35 cm² controlled by two mobile barriers. The Teflon trough has been carefully cleaned with UP
171 water and ethanol before each experiment, and ellipsometric and tensiometric measurements were
172 performed during half an hour on pH 7 buffer to check the cleaned surface.

173 The surface pressure (π) was measured every 4s with a precision of ± 0.2 mN/m using a filter paper
174 connected to a microelectronic feedback system (Nima Technology, UK), according to the
175 Wilhelmy-plate method. The ellipsometric angle (Δ) was recorded simultaneously every 4 s with a
176 precision of $\pm 0.5^\circ$, using a home-made automated ellipsometer in a “null ellipsometer”
177 configuration (Berge & Renault, 1993; Bourlieu et al., 2020). The laser beam probed a surface of
178 1 mm² and a depth in the order of 1 μm and provided insight on the thickness of the interfacial film
179 formed at the interface.

180 **2.4 Monitoring of the gPLRP2 adsorption onto mixed galactolipid monolayers at the** 181 **air/water interface**

182 The three monolayers studied were formed by spreading a few microliters of 1 mM solution of
183 lipids in CHCl₃/MeOH (2:1, v/v) over the surface of the buffer solution until an initial pressure of
184 20 ± 1 mN/m (Bénarouche et al., 2013).

185 After stabilization of the film over 5 minutes, 14.6 μ L of gPLRP2 solution (0.15 mg/mL) was
186 diluted with 30 μ L Tris buffer and injected in the sub-phase to achieve a final gPLRP2
187 concentration of 2.7 nM. The evolution of the surface pressure and ellipsometric angle due to the
188 enzyme adsorption and lipolytic activity onto the lipid monolayer was continuously monitored over
189 45 minutes to 2 hours depending on the system studied, until a final surface pressure of 6 mN/m
190 was reached, this value being the one of the gel-fluid phase transition of DPPC (Xu & Zuo, 2018).

191 **2.5 Analysis of the digestion products present at the interface and in the sub-phase**

192 The interface of the GL monolayer was collected after 1h digestion kinetic, using a home-made
193 vacuum extraction pump system. Lipids were extracted by Folch method before being analyzed by
194 thin layer chromatography (TLC) to determine the concentrations of lipolysis products. The
195 organic phase resulting from the extraction was separated and eluted on TLC plates using a mixture
196 of chloroform/methanol/water (95:20:2.5, v/v/v). The TLC plate revelation was made by dipping
197 the plate in a 50:50 v/v mixture of saturated copper acetate solution in water and 85.5% phosphoric
198 acid solution and subsequent oven drying (180°C, 10min). Revealed bands were then scanned by
199 densitometry (500 nm, TLC Scanner 4, CAMAG) and quantified using VisionCat software.

200 **2.6 Visualization of lipase distribution in heterogeneous film by atomic force microscopy**

201 For AFM imaging, interfacial films were transferred onto a freshly-cleaved mica plate using the
202 Langmuir-Blodgett method at the end of the kinetics, at a constant surface pressure and at a very
203 low speed (0.5 mm/min). For each monolayer, two samplings were performed at different times, in

204 order to observe the organization of the interface at different stages of lipase adsorption and
205 lipolysis. For the GL monolayer, sampling was performed at 35 minutes and 1 hour, respectively.
206 For the GL/DPPC monolayer, sampling was carried out at 45 minutes and 1 hour and 15 minutes,
207 respectively. For the GL/DPPC/pS monolayer, sampling was done at 45 minutes and 1 hour 45
208 minutes, respectively. Finally, for the MGDG/DGDG/SQDG/PG, sampling was carried out after 1
209 hour kinetic. AFM (Multimode Nanoscope 8, Bruker, France) was used for imaging in contact
210 mode QNM in air (20°C), using a standard silicon cantilevers (0.06 N/m, SNL-10, Bruker, France),
211 and at a scan rate of 1 Hz. The force was minimized during all scans and the scanner size was
212 100×100 μm². The processed images analyzed by the open-source platform Gwyddion were
213 representative of at least duplicated experiments.

214 **2.7 Static digestion of liposomes made from mixed GL, GL/DPPS/pS, and** 215 **MGDG/DGDG/SQDG/pS systems by gPLRP2**

216 1 μm extruded liposomes of i) GL, ii) GL/DPPC/pS, and iii) MGDG/DGDG/SQDG/PG model
217 solutions, respectively, were prepared at a final concentration of 0.4% wt. in Tris HCl buffer (10
218 mM Tris, 100 mM NaCl, 5 mM CaCl₂, pH 7).

219 **2.6.1 Size distribution of liposomes by dynamic light scattering**

220 The size (diameter, nm) distribution of liposomes was assessed by dynamic light scattering
221 (DLS) with a Malvern Panalytical Zetasizer PRO (Malvern, Worcestershire, United Kingdom)
222 fitted with a 633-nm He-Ne laser at 25 °C. ZS Explorer Software version 3.1.0. (Malvern) was used
223 to collect and analyze the data. Measurement were conducted on 1 mL of liposomes dispersion
224 (after 10 times dilution in mQ water) with equilibration time of 120 s, 10 runs of 120 s
225 measurements were performed with a refractive index of 1.45 for liposomes, respectively. The

226 intensity, diameter distribution, the hydrodynamic diameter as Z-average, and the polydispersity
227 index (PdI) were deduced from the autocorrelation fit of the data.

228 **2.6.2 Static bulk digestion of liposomes by gPLRP2 in absence and in presence of 4**
229 **mM NaTDC**

230 Liposomes were incubated under constant agitation in Tris HCl buffer (10 mM Tris, 100 mM NaCl,
231 5 mM CaCl₂, pH 7) containing gPLRP2 at 3.3 mg/L in absence or in presence of 4 mM NaTDC
232 (above CMC value). 100 μL aliquots were sampled at T₀ (control) and after 5 min of gPLRP2
233 digestion (T_{5min}), and lipids were extracted by Folch method before being analyzed by thin layer
234 chromatography (TLC) to determine the concentrations of residual substrates and lipolysis
235 products. The organic phase resulting from the extraction was separated and eluted on TLC plates
236 as detailed in section 2.5 above. It was thus possible to monitor the enzymatic activity of gPLRP2
237 on liposomes of both galactolipid mixtures in absence or presence of bile salt-related detergent
238 (NaTDC, 4 mM).

239

240 **3 RESULTS AND DISCUSSION**

241 **3.1. Interfacial behavior of model lipid monolayers**

242 Lipid-lipid interactions and molecular organization at the air/water interface were investigated at
243 20 mN/m and pH 7 and are presented in Figure 1. We were able to form stable GL based-
244 monolayers at the air/water interface. The GL interface was characterized by a fluid phase,
245 presenting some roughness due to the intercalation of the polar heads of MGDG and DGDG (Figure
246 1.A). The GL/DPPC/pS system showed a coexistence of condensed liquid/expanded liquid phases,
247 with the presence of condensed phase domains visible on the AFM images, enriched in DPPC-
248 MGDG and pS (Figure 1.B). Additionally, the presence of pS in condensed domains have induced
249 the appearance of defects, that could modulate the subsequent adsorption of lipolytic enzymes
250 (Bourlieu et al., 2016; Kergomard, Carrière, Paboeuf, Artzner, et al., 2022). For the
251 MGDG/DGDG/SQDG/PG biomimetic monolayer (Figure 1.C), small flower-shaped nanodomains
252 of 1.6 ± 0.1 nm height were evidenced at the air/water interface, coexisting with a fluid phase.

253 **3.2. Interfacial adsorption and enzymatic activity of gPLRP2 onto homogeneous galactolipid** 254 **monolayer (GL)**

255 The interfacial adsorption and enzymatic activity of gPLRP2 onto homogeneous GL monolayer
256 GL was monitored using tensiometry coupled with ellipsometric measurements. Figure 2.A shows
257 the evolution of surface pressure and ellipsometric angle over one hour after the injection of
258 gPLRP2 at 0.128 mg/L in the subphase. Right after the injection of the enzyme below the GL
259 monolayer, the surface pressure started to decrease drastically, reporting the modifications of the
260 interactions between molecules at the interface and the probable lipolysis of the acyl chains of
261 galactolipids by gPLRP2. When considering the maximal slope of this decreasing curve, it

262 coincided with a range of surface pressure from 15 to 10 mN/m, *i.e.* a surface pressure where the
263 enzymatic activity of gPLRP2 was the highest. This assumption was consistent with the evolution
264 of the ellipsometric angle, as a sharp drop of $\delta\Delta=0.8$ was obtained at $\pi=15$ mN/m (Figure 2.A).
265 Since the activity of gPLRP2 on medium chain MGDG and DGDG monolayer was previously
266 reported to be maximum between 10 to 15 mN/m (De Caro et al., 2004; Eydoux, De Caro, et al.,
267 2007), it is hypothesized that the changes occurring at the interface (π and Δ) results from gPLRP2
268 activity on the GL monolayer. Given the fact that the surface pressure did not show a significant
269 increase after gPLRP2 injection, contrary to what had been previously observed with other lipases
270 onto heterogeneous monolayers (Bourlieu et al., 2016), it is hypothesized that most gPLRP2
271 molecules are found right below the surface and do not penetrate into the monolayer. This
272 assumption was consistent with the ellipsometric angle data: no evolution was observed during the
273 first 0.6 hour of kinetic after the lipase injection in the subphase. These data suggest that gPLRP2
274 adsorption below the surface is quite discrete and limited in comparison to gastric lipase for
275 instance (Bourlieu et al., 2016).

276 In order to understand the partitioning of the enzyme and the disorganization of the interface
277 induced by the enzymatic activity, two Langmuir-Blodgett sampling of the interface were realized;
278 before and after the drop of the ellipsometric angle. The $5\times 5\ \mu\text{m}^2$ AFM images of the two samples,
279 after 35 min and 1 hour kinetic, respectively, are presented in Figure 2.B. After 35 min of enzymatic
280 kinetic, small flower-like condensed phase domains of 1.9 ± 0.1 nm height appeared at the air/water
281 interface, presumably attributed to the generation of digestion products by the degradation of
282 MGDG and DGDG by gPLRP2, in agreement with the subsequent decrease of the surface pressure.
283 Protuberances of 3.6 ± 0.3 nm height were also visible, very likely being attributed to some lipase
284 molecules adsorbed at the interface. Indeed, such height differences had previously been shown in

285 the literature to be associated with the presence of self-organized proteins at the interface of a lipid
286 monolayer (Kergomard, Carrière, Paboeuf, Barouh, et al., 2022; Sarkis & Vié, 2020). After 1 hour
287 kinetic, the resulting interface had evolved further. Surprisingly, gPLRP2 seems to have formed a
288 protein network of 3.4 ± 0.1 nm in height, in addition to the protuberances observed on the 35 min
289 images, despite the absence of increase in the surface pressure. Additionally, condensed phase
290 domains have grown, reinforcing the hypothesis of their attribution to the generation of lipolysis
291 products.

292 Indeed, gPLRP2 is known to hydrolyze the *sn*-1 position of GL, according to the reaction scheme
293 proposed in Figure 3, generating monogalactosylmonoacylglycerol (MGMG) and
294 digalactosylmonoacylglycerol (DGMG) in the case of MGDG and DGDG, respectively, as well as
295 free fatty acids (FFA) (Amara et al., 2010; Withers-Martinez et al., 1996). Due to their
296 polyunsaturated content, it is likely that MGMG and DGMG molecules remained in the fluid phase
297 at the air/water interface, and that the condensed phase domains were probably enriched in
298 saturated fatty acids released by PLRP2. Further hydrolysis of MGMG and DGMG by gPLRP2
299 can also lead to the production of water-soluble galactosylated products: monogalactosylglycerol
300 (MGG) and digalactosylglycerol (DGG) (Figure 3) (Sahaka et al., 2021). The generation of MGG
301 and DGG could explain the decrease in surface pressure after gPLRP2 injection, as well as the drop
302 in the ellipsometric angle corresponding to a loss of matter at the air/water interface. Additionally,
303 the reorganization of these lipolysis products at the interface and in the aqueous subphase may have
304 resulted in the formation of structural defects, thereby promoting lipolysis. To support the
305 hypothesis of a galactolipid degradation by gPLRP2, leading to the generation of digestion
306 products, the interface and subphase were collected after 1h of kinetics, and the lipid products were
307 analyzed by TLC. The results (supplementary data, Figure S3) indicated the presence of digestion

308 products (FFA) at the interface and in the subphase, confirming the galactolipase activity of
309 gPLRP2 on the GL model monolayer.

310 To check which part of the evolution of the surface pressure and the ellipsometric angle was
311 resulting either from the galactolipase activity of gPLRP2, or from the interactions of the protein
312 with the lipid monolayer, the experiment was reproduced using an inactive variant (S125G) of
313 gPLRP2. In this variant, the catalytic serine S152 was replaced by a glycine, resulting in the loss
314 of the enzymatic activity. The S125G variant of gPLRP2 has been previously characterized using
315 Fourier transform infrared spectroscopy (FTIR) in the study of Mateos-Diaz et al. (2018), showing
316 that the inactive variant retained his correct folding compared to active gPLRP2, and that its
317 interfacial behavior should not be affected.

318 Figure 4.A. presents the kinetic evolution of the surface pressure and ellipsometric angle over 1
319 hour after the injection of the inactive variant of gPLRP2 into the subphase of the GL monolayer.
320 After the S125G gPLRP2 injection in the subphase, there was no evolution in the surface pressure,
321 nor in the ellipsometric angle, confirming that the variations previously observed with gPLRP2
322 (Figure 2.A) were due to enzymatic activity. Additionally, AFM image Figure 4.B showed the
323 presence of the same protuberances observed with the active enzyme, with similar height of $3.8 \pm$
324 0.2 nm. Thus, it seems that, despite the lack of surface pressure increase, the enzyme gets adsorbed
325 at the interface.

326 **3.3. Modulation of the gPLRP2 adsorption and kinetic activity onto heterogeneous model** 327 **monolayer of galactolipids, phospholipids and phytosterols (GL/DPPC/pS)**

328 Figure 5.A shows the kinetic evolution of the surface pressure and ellipsometric angle after the
329 injection of gPLRP2 below the GL/DPPC/pS monolayer. A decrease with time in the surface

330 pressure similar to what was observed with the GL monolayer (Figure 2.A), was observed right
331 after the injection of the enzyme in the subphase. However, the decrease in surface pressure was
332 slower than with the GL monolayer. A drop in the ellipsometric angle ($\delta\Delta=0.9^\circ$) was also observed
333 when the surface pressure reached 15 mN/m, but it occurred at 1.4 h instead of 0.6 h (35 min) with
334 the GL monolayer, reflecting the slowing down of the lipolysis rate. As previously, we assumed
335 that these variations correspond to a loss of matter at the interface, upon lipolysis of the monolayer
336 by gPLRP2 and to the generation of water-soluble MGG and DGG. The lag phase of about 50 min
337 observed with the GL/DPPC/pS monolayer before the initiation of lipolysis could be explained by
338 the higher packing of the heterogeneous monolayer induced by DPPC and pS, and a greater
339 difficulty for gPLRP2 to reach the acyl chains of hydrolysable substrates (galactolipids and DPPC).
340 The previous characterization study of homogeneous and heterogeneous GL monolayers has
341 indeed shown that the addition of DPPC and pS to a GL monolayer led to the formation of
342 condensed phase domains enriched in DPPC and MGDG, reducing the lateral distance between the
343 acyl chains available for gPLRP2 to insert (Kergomard, Carrière, Paboeuf, Artzner, et al., 2022).
344 This higher packing could thus explain the lag phase observed before the gPLRP2 could reach its
345 optimum activity, a high packing density at the air/water interface having been proposed to explain
346 the long induction times observed for other lipases onto tightly packed short-chained phospholipids
347 (Verger et al., 1973) and diacylglycerols (Wieloch et al., 1982) monolayers. The initiation of
348 lipolysis of the GL/DPPC/pS led however to the formation of lipolysis products and to the
349 subsequent decrease in surface pressure and lipid packing, that accelerate the activity of gPLRP2.

350 Two Langmuir-Blodgett transfers of the monolayer were taken after 45 minutes and 1h45 of
351 kinetics, respectively, and AFM images of the interfacial organization were recorded (Figure 5.B).
352 After 45 minutes of kinetics, condensed domains of 1.5 ± 0.2 nm in height were visible at the

353 air/water interface. Given the low drop in surface pressure observed at 45 min, these domains are
354 probably not related to the generation of digestion products. Furthermore, the interfacial
355 organization and heights observed were similar to those obtained at T_0 before the injection of
356 gPLRP2 into the subphase (Figure 1.B), supporting the hypothesis that lipolysis is probably not yet
357 initiated at this stage of the kinetics. At 1h45 minutes of kinetics, the surface pressure had reached
358 $\pi=6.3$ mN/m, and AFM images of the film interface revealed a very different interfacial
359 organization, consistent with the evolution of surface pressure and the drop in the ellipsometric
360 angle. Thin and discontinuous lines of $h_1=3.1 \pm 0.2$ nm in height were visible in the fluid phase and
361 around the condensed domains, that could correspond to gPLRP2 molecules adsorbed at the
362 monolayer interface. Condensed domains of three different heights were also identified. First, small
363 flower-like shaped condensed domains were visible in the fluid phase, with a height h_2 of 2.0 ± 0.1
364 nm, probably attributed to the generation of lipolysis product MGMG and DGMG, as observed for
365 the GL monolayer. Fragmented condensed phase domains were also revealed, composed of at least
366 three different height levels (h_3 , h_4 , fluid bottom). This observed fragmentation could be due to
367 the disorganization caused by the adsorption and enzymatic activity of gPLRP2, but also to the
368 low-pressure value ($\pi=6.3$ mN/m), causing phase segregation within the condensed phase domains
369 thought to be enriched in DPPC-MGDG-pS. Indeed, the lateral pressure was probably no longer
370 sufficient to ensure the miscibility of DPPC and pS with MGDG, causing phase segregation which
371 could explain the observed height differences.

372 These observations nevertheless confirm the miscibility of these three compounds and the phase
373 heterogeneity in the condensed phase domains at 20 mN/m, as well as the condensation effect of
374 DPPC and pS on MGDG chains observed in our previous study (Kergomard, Carrière, Paboeuf,
375 Artzner, et al., 2022). Given the molar composition of the GL/DPPC/pS monolayer, the central

376 rounded domain ($h_3=1.7 \pm 0.2$) observed at 1h45 of kinetic could be attributed to the presence of
377 condensed DPPC. The smaller domains of h_3 and h_4 heights, coexisting with the fluid phase, could
378 be attributed to the presence of FFA and pS, coexisting with MGDG and MGMG in the fluid phase.
379 In the considered range of surface pressure, it is indeed unlikely that gPLRP2 shows significant
380 enzymatic activity on DPPC, since monolayer studies have shown that gPLRP2 was only active on
381 this substrate at low surface pressure ($\pi < 5$ mN/m) and was totally inactive at $\pi > 10$ mN/m (Hjorth
382 et al., 1993). However, it remains difficult to attribute each type of domain to a species of molecule,
383 given the complex interactions and differences in miscibility observed in this type of ternary
384 mixture. Surface composition studies will be needed to answer these questions, but the small
385 quantities used for interfacial characterizations do not facilitate such analyses.

386 **3.4. Influence of the presence of charged lipids on the adsorption capacity and enzymatic** 387 **activity of gPLRP2 in model biomimetic lipid monolayer (MGDG/DGDG/SQDG/PG)**

388 The impact of a charged interface on the adsorption capacities and enzymatic activity of gPLRP2
389 was studied using MGDG/DGDG/SQDG/PG biomimetic model monolayer. The evolution of
390 surface pressure and ellipsometric angle upon the adsorption of gPLRP2 at the air/water interface
391 is presented Figure 6.A. A continuous decrease of the surface pressure was observed right after the
392 injection of gPLRP2 in the sub-phase until it reached a value of $\pi=8.5$ mN/m after 1h of kinetic. In
393 contrast to the GL/DPPC/pS complex system, no lag phase was observed before the onset of
394 lipolysis, and the decrease was continuous, revealing a constant enzymatic activity of gPLRP2 over
395 the 1h kinetic. This observation could be explained by the presence of negatively charged lipids at
396 the interface (SQDG, PG), which could facilitate the adsorption of gPLRP2 underneath the
397 monolayer, and the subsequent degradation of galactolipids. The facilitated adsorption onto a
398 charged surface was previously observed for recombinant dog gastric lipase (rDGL) at the level of

399 heterogeneous monolayers of polar dairy lipids, with the establishment of electrostatic interactions
400 between the interface and the interfacial recognition site facilitating the orientation and approach
401 of the active site onto the lipid substrates (Bourlieu et al., 2016). In our case however the surface
402 potential electrostatic distribution of charge is very different between rDGL and gPLRP2, but
403 seems to result in favorable interactions with negatively charged lipid interface. The ellipsometric
404 angle did not significantly evolved during the first 30 minutes of the kinetics. After this, it
405 decreased again ($\delta\Delta=-0.7^\circ$) at $\pi=15$ mN/m, in the range of the optimal surface pressure for the
406 activity of gPLRP2, as previously observed on GL and GL/DPPC/pS monolayers. The
407 ellipsometric angle then slowly decreased until it reached a value of $\Delta=5.2^\circ$ after 1h kinetic,
408 reflecting a decrease in the thickness of the monolayer due to the degradation of GL and the
409 progressive release of polar lipolysis products into the subphase.

410 Langmuir-Blodgett transfer of the interface was performed on the MGDG/DGDG/SQDG/PG
411 monolayer after 1 kinetic. AFM image (Figure 6.B) after 1h kinetic of incubation with gPLRP2
412 showed the coexistence of LC snowflake-shape domains of 2.2 ± 0.1 in height in the fluid phase.
413 These domains shared a similar morphology with those obtained after 35 minutes of digestion of
414 the GL monolayer by gPLRP2, and can therefore be attributed to the generation of FFA digestion
415 products by galactolipid degradation. As previously observed, small protuberances of 3.3 ± 0.3 nm
416 in height were also observed, attributed to the adsorbed gPLRP2 molecules in the fluid phase.

417 **3.5. Interaction of liposomal structures (GL, GL/DPPC/pS, MGDG/DGDG/SQDG/PG) with** 418 **bile salts**

419 Since the interfacial characterization of gPLRP2 interaction with mixed galactolipid monolayer
420 revealed some lipolytic activity, we then evaluate the ability of gPLRP2 to interact with liposomes

421 made with the same lipid mixture, in the presence and absence of bile salts, to mimic the conditions
422 found in the gastrointestinal tract. We first characterized the effects of bile salts on the liposomal
423 dispersions using DLS.

424 In the absence of bile salts, GL liposomes showed a monomodal distribution centered at 198 nm
425 while GL/DPPC/pS liposomes were much larger with a monomodal distribution centered at 2990
426 nm (Figure 7), although both objects had been extruded 10 times over filters of 1 μm pore diameter.
427 Upon the addition of NaTDC (4 mM), bimodal distributions appeared with peaks at 894 and 117
428 nm for GL liposomes and at 2990 and 146 nm for GL/DPPC/pS. The presence of NaTDC had
429 therefore a strong impact on lipid organization with changes in particle size distribution, a major
430 shift towards larger objects but also the appearance of smaller populations. Ultimately, GL and PL
431 mixed with micellar concentrations of bile salts are known to form mixed micelles with diameter
432 of 10 to 40 nm (Mazer et al., 1980). In that case, DLS is not the most appropriate techniques for
433 covering such large variations in particle size distribution. Nevertheless, it allowed showing lipid
434 re-organization upon the addition of bile salts. The size increase observed with the larger objects
435 could be partially explained by a destabilization of the liposomes during the adsorption of NaTDC
436 at the interface, leading to their fusion. Additionally, the adsorption of NaTDC onto liposomes
437 could have resulted in a diminished GL packing, explaining the larger diameter observed. The
438 smallest objects observed could be related to the desorption of some lipid molecules from the
439 bilayer stabilizing the liposomes. Indeed, previous studies had already investigated the interfacial
440 behavior of NaTDC at the level of assembled lipid structures, and have highlighted its desorption
441 capacities. As an example, (Pabois et al., 2019) have studied the adsorption behavior of NaTDC at
442 the air/water interface, and its interaction with a monolayer of phospholipids (DPPC), mimicking
443 the organization of physiological compounds present at the interface of fat droplets. Firstly, the

444 results showed a very fast adsorption of NaTDC at the air/water interface at low concentration (<
445 1 mM), forming stable but irregular film, which was attributed to its unusual polar planar structure
446 and large surface area (Maldonado-Valderrama et al., 2011, 2014). However, these bile salt
447 concentrations were below the critical micellar concentration (CMC) (Roda et al., 1983). At higher
448 concentrations (> 5 mM; *i.e.* > CMC), the addition of bile salts was shown to lead to a decrease in
449 thickness, demonstrating that NaTDC partially desorbs from the interface. The interaction of
450 NaTDC with the DPPC was then studied. Results showed the strong desorption of DPPC molecules
451 (to approximately 40%) from the interface upon the NaTDC adsorption, resulting in the formation
452 of domains with distinct organization. Additionally, increasing the amount of NaTDC have been
453 shown to decrease the DPPC monolayer packing. These results illustrate the well-known micellar
454 solubilization effect of bile salts, leading to the formation of mixed micelles in bulk (Hofmann,
455 1963; Hofmann & Borgström, 1964; Pabois et al., 2021).

456 In the case of the GL/DPPC/pS liposomes in the absence of bile salts, they showed an average
457 diameter of 2990 nm that was much larger than the average diameter measured in the case of the
458 GL system (198 nm). The fact that their diameter was larger than 1 μm despite the filter used during
459 extrusion indicates that these objects were relatively stable, as they were able to deform during
460 extrusion without breaking. The appearance of a population of smaller objects upon addition of
461 NaTDC could reflect the re-organization induced by NaTDC molecules. Nevertheless, it seems
462 that the GL/DPPC/pS system remains stable even in the presence of bile salts, as the population of
463 larger droplets remained similar in size in the absence and presence of bile salts

464 **3.6. Interaction of gPLRP2 with galactolipid-based liposomes in the absence and presence of**
465 **bile salts**

466 As the adsorption capacities and enzymatic activity of gPLRP2 is highly dependent on the substrate
467 organization (Mateos-Diaz, Bakala N’Goma, et al., 2018; Mateos-Diaz, Sutto-Ortiz, et al., 2018),
468 the changes observed with liposomes following the addition of bile salts could most likely modify
469 the access of the enzyme to its substrate and its lipolytic activity.

470 The impact of bile salt on the galactolipase and phospholipase A1 activities of gPLRP2 was thus
471 assayed in “bulk conditions” using GL/DPPC/pS dispersed liposomes, this system having been
472 shown to be the most stable even in presence of bile salts. No significant hydrolysis activity on
473 GL/DPPC/pS liposomes could be detected in the absence of bile salts after 5 minutes of incubation
474 with gPLRP2 (supplementary material, Table S4). This result was in line with the previous study
475 by Mateos-Diaz et al. (2018), which has shown that gPLRP2 did not possess enzymatic activity on
476 DPPC liposomes in absence of bile salts. These results were however in disagreement with the
477 results obtained with monolayers of the same lipid mixture, on which the lipolytic activity of
478 gPLRP2 was detected (see figure 2 presented in section 3.3 - monolayer results). Nevertheless, the
479 surface pressure of the lipid monolayer at the air/water interface was optimum for the adsorption
480 and enzymatic activity of gPLRP2, which may explain the observed lipolysis under these
481 conditions. The organization of lipids into monolayers is indeed different from that of the bilayers
482 surrounding liposomes, and higher lateral pressure and packing of the latter systems could prevent
483 gPLRP2 from penetrating and degrading its substrate (Kergomard, Carrière, Paboeuf, Artzner, et
484 al., 2022).

485 However, when bile salts were added to GL/DPPC/pS liposomes, a lipolytic activity of gPLRP2
486 could be detected by TLC analysis of lipolysis products (Table 1). After 5-min incubation, around
487 74% wt. of MGDG and 55% wt. of DGDG were converted into MGMG and DGMG, respectively,
488 with the production of FFA. Given the differences in the substrate and lipolysis product

489 concentrations, it is likely that some MGMG and DGMG have been in turn converted into
490 monogalactosylglycerol (MGG) and digalactosylglycerol (DGG), respectively, by gPLRP2 but
491 these two compounds being water-soluble, they could not be extracted and revealed upon TLC
492 analysis of the organic phase (Sahaka, 2020). It should be noted that the quantification of DGMG
493 after 5 minutes digestion was hampered, given the fact that its retention factor was similar to that
494 of DPPC on the TLC plate. The galactolipase activity observed in the presence of bile salts could
495 be due to the adsorption of NaTDC at the liposome interface, decreasing the lateral pressure and
496 interfacial packing of polar lipids, as previously observed with DPPC domains (Pabois et al. 2019),
497 and thus creating more favorable conditions for the adsorption and activity of gPLRP2.
498 Nevertheless, it cannot be excluded that polar lipids from liposomes were gradually solubilized
499 into mixed micelles prior to their hydrolysis by gPLRP2. This latter hypothesis is supported by the
500 preference of gPLRP2 for micellar substrates (Mateos-Diaz, Bakala N’Goma, et al., 2018).
501 Moreover, the difficulty for gPLRP2 to access its substrate in liposomes was confirmed here when
502 GL/DPPC/pS liposomes were tested in the absence of bile salts.

503 In addition to the galactolipase activity of gPLRP2, the TLC analysis of lipolysis products also
504 revealed the phospholipase activity of gPLRP2 on the DPPC present in GL/DPPC/pS liposomes,
505 in the presence of bile salts. These results confirm the previous study by Mateos-Diaz et al. (2018)
506 which has shown that gPLRP2 was active on mixed bile salts/DPPC micelles, but not on DPPC
507 liposomes in the absence of bile salts. As in the case of galactolipid hydrolysis, two main
508 hypotheses can be raised about the mode of action of gPLRP2 on PL: a decrease in the packing of
509 the bilayer by bile salts that could promote gPLRP2 adsorption and activity, or the conversion of
510 liposomes into micelles containing DPPC.

511 Given the lack of activity of gPLRP2 on GL-based liposomes in the absence of bile salts, mixed
512 MGDG/DGDG/SQDG/PG liposomes mimicking the lipid composition of thylakoid membranes
513 were also tested in presence of bile salts. After 5 minutes incubation with gPLRP2 in presence of
514 4 mM NaTDC (Table 1), about 90% wt. of MGDG and 94% wt. of DGDG were hydrolyzed, while
515 FFA and MGDG were produced. More interestingly, gPLRP2 was also able to hydrolyze 91% of
516 the initial SQDG substrate (Table 1), emphasizing its action on all galactolipids (Andersson et al.,
517 1996). This result confirmed the ability of gPLRP2 to hydrolyze GL from liposomes in the presence
518 of bile salts.

519 The ability of gPLRP2 to hydrolyze galactolipid membranes even in the absence of bile salts was
520 however recently shown by FTIR on natural chloroplast membranes (Sahaka et al., 2023). This
521 result could be explained by the fact that these natural systems are more complex than the model
522 systems considered in this study, and naturally include negatively charged lipids, shown to enhance
523 the adsorption and extent of lipolysis on model monolayers, independently of the presence of bile
524 salts. Thus, pursuing this study by exploring the degradation of liposomes with more complex
525 compositions in the absence of bile salts, for example MGDG/DGDG/SQDG/PG, could provide
526 insight into the composition at which a galactolipid liposome can become a gPLRP2 substrate.

527 **3.7. Interfacial organization of GL and GL/DPPC/pS liposomes in presence of bile salts** 528 **obtained at T₀ and after 5 min of gPLRP2 digestion**

529 Lipids products obtained at T₀ and after 5 minutes incubation of GL or GL/DPPC/pS liposomes in
530 the presence of gPLRP2 and bile salts were extracted by Folch method and deposited at the
531 air/water interface at $\pi=7.2 \pm 0.1$ mN/m. We chose to deposit the lipids at this surface pressure in
532 order to approximate the organization of the substrates and digestion products of the interfacial
533 films obtained at the end of the monolayer digestion kinetics for the GL, GL/DPPC/pS, and

534 MGDG/DGDG/SQDG/PG systems, respectively (π between 5 to 8 mN/m). After stabilization of
535 the respective T_0 and $T_{5\text{min}}$ films, Langmuir-Blodgett samples were observed in AFM. For both
536 systems, the images obtained at T_0 and $T_{5\text{min}}$ are displayed in Figure 8. For the GL system, AFM
537 images obtained at T_0 revealed the presence of small condensed domains of $h_1=1.3 \pm 0.1$ nm height,
538 that could be attributed to the presence of some NaTDC adsorbed at the air/water interface. At
539 $T_{5\text{min}}$, flower-shape domains of $h_1=1.9 \pm 0.1$ nm in height were evidenced, similar to those obtained
540 after 2h hour kinetic digestion of GL monolayer by gPLRP2 at the air/water interface. These
541 domains were attributed to the generation of the FFA by lipolysis of MGDG and DGDG, in
542 agreement with their detection by TLC (Table 1).

543 For the GL/DPPC/pS system, the interface obtained at T_0 was clearly different from the one
544 obtained for the GL/DPPC/pS monolayer at 20 mN/m during the interfacial study (section 3.1), but
545 was similar to the one obtained two hours after injecting gPLRP2 in the subphase, when the surface
546 pressure reached $\pi=6.3$ mN/m (Figure 2B). Thus, the low surface pressure could explain the
547 fragmentation of the condensed phase domains, as previously observed, with two identified height
548 levels (h_1 and h_2) probably enriched in DPPC and pS, coexisting with a fluid phase probably
549 enriched in MGDG. Additionally, the inclusion of bile salts at the interface could have spaced out
550 the neighboring DPPC molecules, thus disordering their tight packing and the interfacial
551 organization (Chu et al., 2010). At $T_{5\text{min}}$, the highest domains became more numerous, probably
552 related to the generation of FFA, with a height $h_1=1.7 \pm 0.1$ nm. The organization of the interface
553 was similar to that obtained in the images at 2h kinetics after injection of gPLRP2 in the subphase
554 of GL/DPPC/pS monolayers, highlighting the galactolipase activity of gPLRP2 at the level of
555 heterogeneous liposomes in the presence of bile salts, but also at the level of heterogeneous
556 monolayers.

557 **OVERALL SUMMARY**

558 The adsorption and enzymatic activity of gPLRP2 was studied on GL-based substrates exhibiting
559 different supramolecular structures, and presenting or not phase heterogeneity. The galactolipase
560 activity of gPLRP2 was evidenced at the level of both homogeneous GL and heterogenous
561 GL/DPPC/pS monolayers, after a decrease in surface pressure that allowed reaching the optimum
562 range for gPLRP2 activity on substrate monolayers (Amara et al., 2013; Eydoux, Caro, et al., 2007;
563 Hjorth et al., 1993; Sias et al., 2004). The presence of charged lipids (SQDG, PG) at the interface
564 improved the adsorption capacities of the enzyme through the establishment of electrostatic
565 interactions between the substrate and the interfacial recognition site of the active site, resulting in
566 improved adsorption and enzymatic activity of gPLRP2. The optimal activity of gPLRP2 was
567 obtained at a surface pressure of 15 mN/m for homogeneous or heterogeneous systems, even if the
568 tighter packing of the heterogeneous monolayer has induced a lag phase period before the on-set
569 of the lipolysis.

570 However, no galactolipase activity could be detected on liposomes made with the same lipid
571 mixtures, confirming the previous finding that gPLRP2 does not interact with phospholipid (DPPC)
572 liposomes and does not display phospholipase A1 on this form of substrate. Therefore, galactolipid-
573 based liposomes are not equivalent to monolayers of the same lipids in terms of recognition by
574 gPLRP2. Since we have shown that gPLRP2 preferentially binds at boundaries between liquid and
575 condensed phases in monolayers, one can assume that lateral packing of lipid molecules and phase
576 heterogeneity are not the same in liposomes. gPLRP2 adsorption to heterogeneous monolayers
577 induces a decrease in surface pressure that further accelerates enzyme activity. This mechanism of
578 action seems to be impaired with liposomes in the absence of bile salts.

579 Nevertheless, both galactolipase and phospholipase A1 activities of gPLRP2 were detected when
580 heterogeneous GL/DPPC/pS liposomes were incubated in the presence of bile salts. Bile salt
581 adsorption onto the liposomes can accelerate enzyme activity by changing the interfacial properties
582 and this is probably one of the mechanisms by which gPLRP2 becomes active on liposomes.
583 However, knowing the micellar solubilization properties of bile salts on polar lipids and the
584 preference of gPLRP2 for micellar substrates, one can speculate that lipolysis of both GL and PL
585 rapidly proceeds through liposomes disruption and formation of mixed micelles onto which
586 gPLRP2 preferentially binds.

587 Monolayer studies with heterogeneous lipid films revealed that the presence of surfactants like bile
588 salts is not an absolute requirement to accelerate gPLRP2 activity on GL. It is now tempting to
589 investigate whether gPLRP2 can act directly on plant membranes.

590

591 **CONCLUSION**

592 The enzymatic activity of gPLRP2 was evidenced onto the galactolipid-based monolayers, with an
593 optimum activity in the range of 10 to 15 mN/m, in the absence of bile salts. The adsorption
594 capacity of gPLRP2 and the subsequent extent of lipolysis, however, was dependent on the
595 chemical composition, but also on the physical environment of the monolayer substrates. In bulk,
596 no enzymatic activity has been evidenced on GL-based liposomes in the absence of bile salts,
597 probably due to the high lateral pressure of the lipid bilayers. In the presence of NaTDC (4 mM),
598 however, gPLRP2 showed both high galactolipase and moderate phospholipase A1 activities on
599 liposomes, probably due to a decrease in packing and lateral pressure upon NaTDC adsorption, and
600 subsequent disruption of liposomes.

602 **Declaration of Competing Interest**

603 The authors declare that they have no known competing financial interests or personal relationships
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618

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803

804

805 **FIGURES**

806 Figure captions.

807 **Figure 1** – $5 \times 5 \mu\text{m}^2$ AFM images of A) GL, B) GL/DPPC/pS, and C) MGDG/DGDG/SQDG/PG
808 monolayers at 20 mN/m.

809 **Figure 2** – A) Kinetic evolution of surface pressure (π , mN/m, red circles) and ellipsometric angle
810 (Δ , °, blue triangle) upon the adsorption and kinetic activity of gPLRP2 (0.128 mg/L) onto GL
811 monolayer. B) AFM images of Langmuir-Blodgett samples after 1) 35 minutes and 2) 1 hour
812 kinetic of gPLRP2 adsorption onto GL monolayer, respectively.

813 **Figure 3** – Schematic representation of galactolipid lipolysis by PLRP2. MGDG –
814 monogalactosyldiacylglycerol, DGDG – digalactosyldiacylglycerol, FFA – Free fatty acid,
815 MGMG – monogalactosylmonoacylglycerol, DGMG – digalactosylmonoacylglycerol, MGG –
816 monogalactosylglycerol, DGG – digalactosylglycerol.

817 **Figure 4** – Kinetic evolution of the surface pressure (π , mN/m, red circle) and the ellipsometric
818 angle (Δ , °, blue triangle) over one hour after the injection of the inactive variant of gPLRP2 in the
819 subphase of the GL monolayer. B) $5 \times 5 \mu\text{m}^2$ images of the Langmuir-Blodgett sample obtained
820 after 1 hour kinetic.

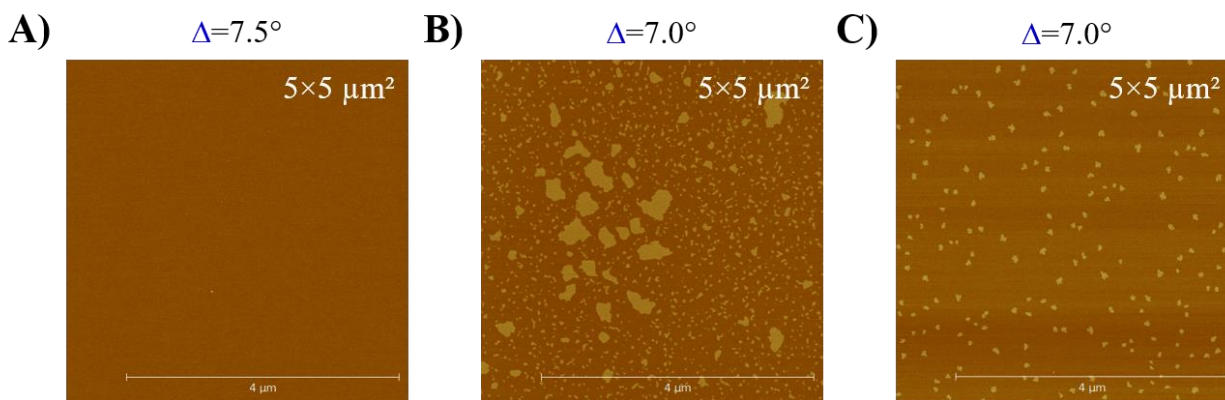
821 **Figure 5** – A) Kinetic evolution of surface pressure (π , mN/m, red circles) and ellipsometric angle
822 (Δ , °, blue triangle) upon the adsorption and kinetic activity of gPLRP2 (0.128 mg/L) onto
823 GL/DPPC/pS monolayer. B) AFM images of Langmuir-Blodgett samples after 1) 45 minutes and
824 2) 1h45 kinetic of gPLRP2 adsorption onto GL/DPPC/pS monolayer, respectively.

825 **Figure 6** – A) Evolution of the surface pressure (π , red, mN/m) and the ellipsometric angle (Δ ,
826 blue, °) upon the adsorption of gPLRP2 onto MGDG/DGDG/SQDG/PG (1h kinetic). B) AFM
827 images ($5 \times 5 \mu\text{m}^2$) of the interface of MGDG/DGDG/SQDG/PG (1h kinetic, $\pi=8.5$ mN/m, $\Delta=5.2^\circ$).

828 **Figure 7** – Typical evolution of the particle diameter distribution of A) GL, and B) GL/DPPC/pS
829 dispersed diluted liposomes (0.04%) in the absence and presence (4 mM NaTDC) of bile salts.
830 Results were obtained by DLS measurements.

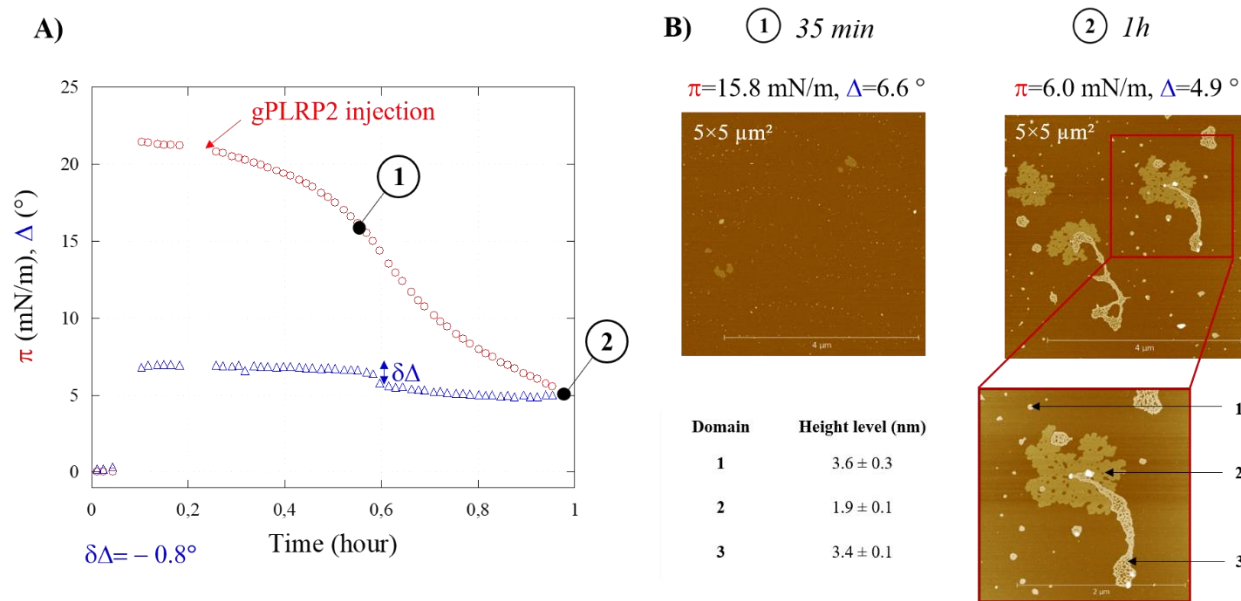
831 **Figure 8** – AFM images ($5 \times 5 \mu\text{m}^2$) of substrates and lipolysis products obtained at T5min and
 832 deposited at $7.2 \pm 0.1 \text{ mN/m}$ at the air/water interface of A) GL, and B) GL/DPPC/pS monolayers.
 833 For each identified domain, the mean height level was given in the table and was obtained as an
 834 average over three sections of the image. Lipophilic substrates and products were extracted using
 835 Folch method.

836 **Figure 1**



837

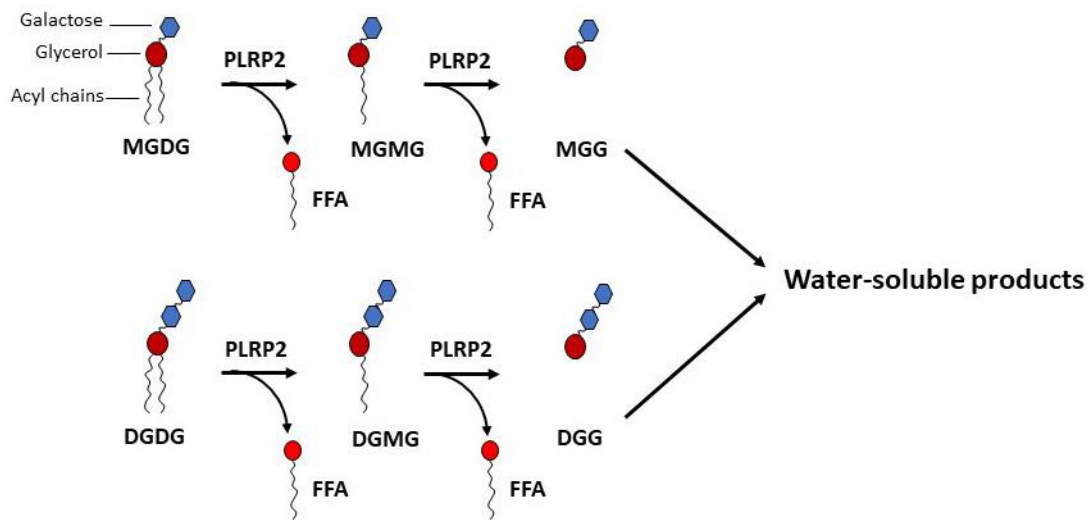
838 **Figure 2**



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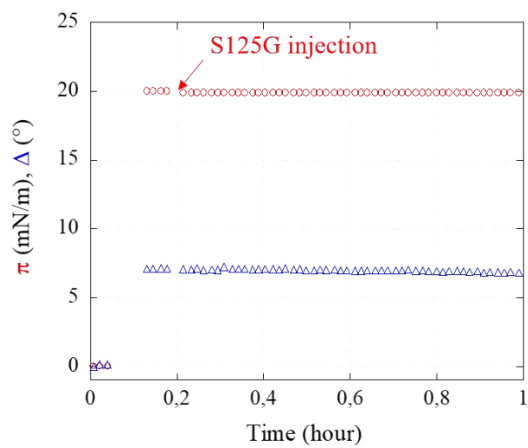
841 **Figure 3**



842

843 **Figure 4**

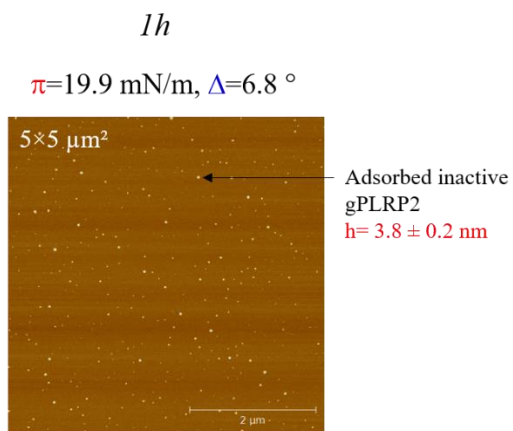
A)



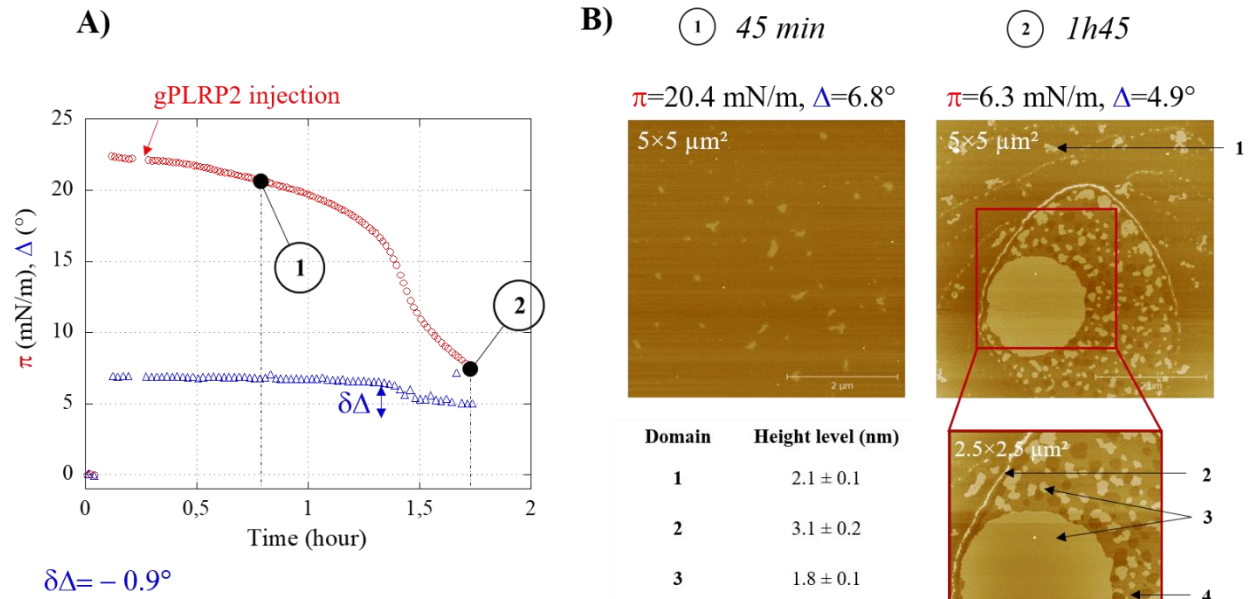
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B)

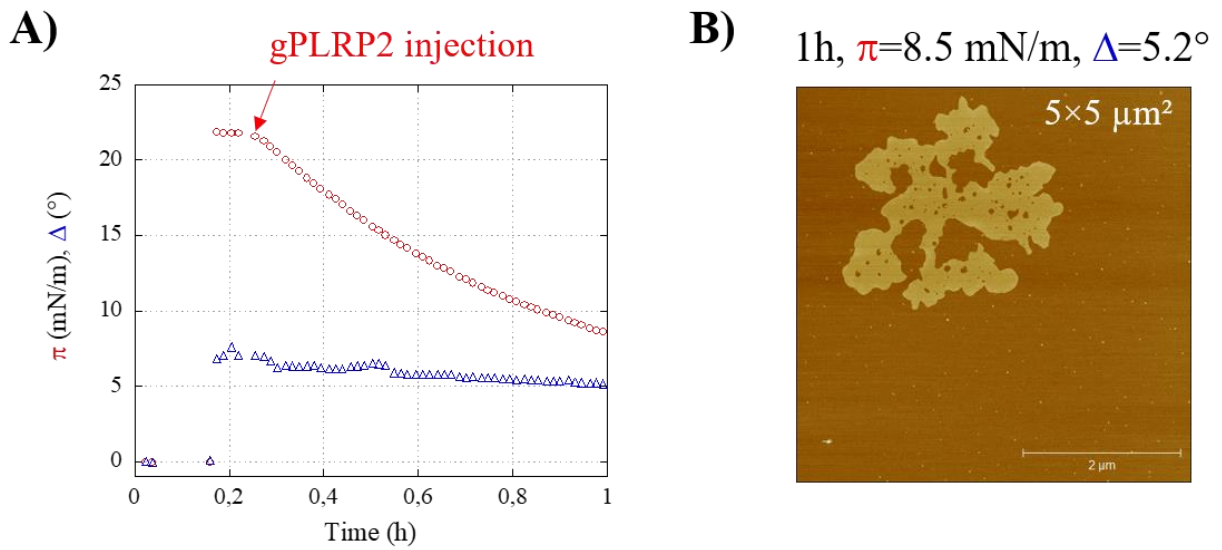


846 **Figure 5**



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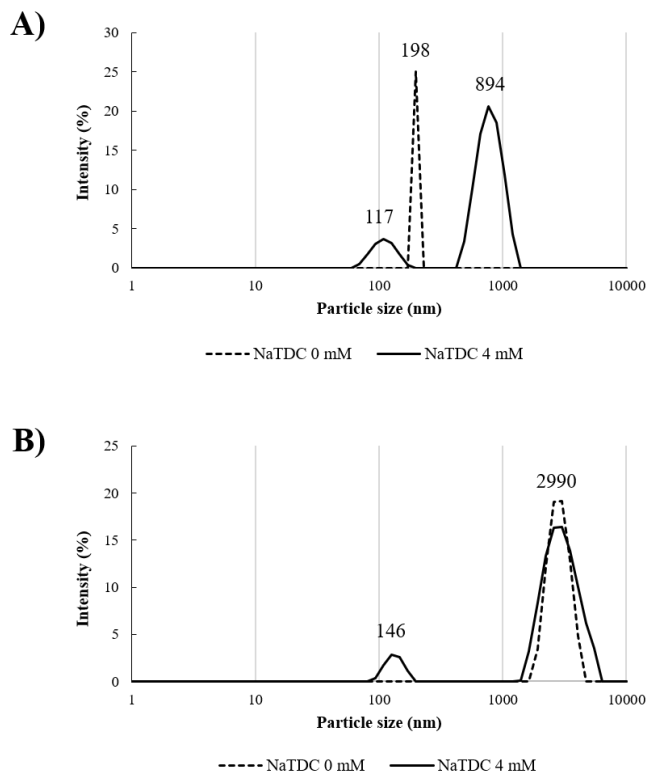
848 **Figure 6**



849

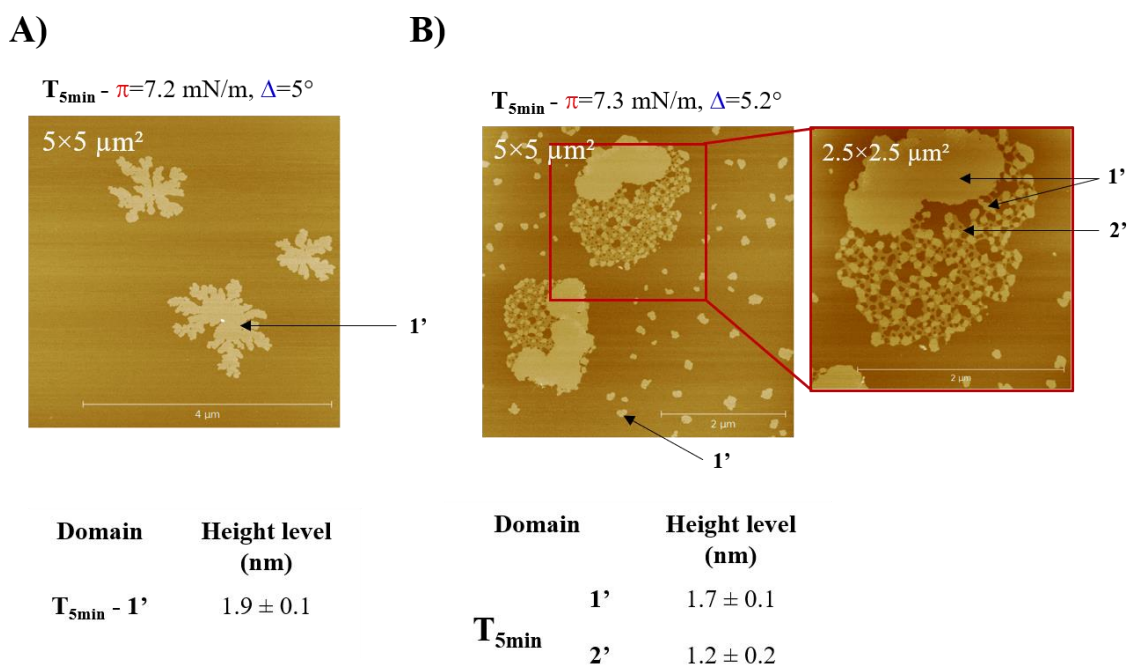
850

851 **Figure 7**



852

853 **Figure 8**



854

855 **TABLES**

856 **Table 1** – Quantitative determination of lipid classes composition obtained by TLC at T₀ and after
 857 5 minutes (T_{5min}) digestion by gPLRP2 of GL/DPPC/pS, GL, MGDG/DGDG/SQDG/PG
 858 liposomes. The reaction was performed at pH 7 in Tris HCl buffer containing 4 mM of NaTDC.
 859 Data are given in relative percentages of the total lipids.

Relative %	GL/DPPC/pS		GL		MGDG/DGDG/SQDG/PG	
	T ₀	T _{5min}	T ₀	T _{5min}	T ₀	T _{5min}
FFA	7.2 ± 1.1	31.1 ± 4.5	-	43.8 ± 7.5	-	54.3 ± 2.4
MGDG	40.3 ± 0.5	10.3 ± 3.0	48.2 ± 0.3	4.9 ± 1.5	65.4 ± 1.0	10.6 ± 0.1
MGMG	0.3 ± 0.1	20.0 ± 1.3	-	18.8 ± 2.2	-	31.3 ± 2.2
DGDG	33.9 ± 0.9	15.2 ± 0.6	51.8 ± 0.3	3.1 ± 0.8	34.6 ± 1.0	3.7 ± 0.1
DGMG	3.5 ± 2.7	3.8 ± 0.9	-	29.5 ± 3.5	<i>n.q.</i>	<i>n.q.</i>
DPPC	7.3 ± 3.7	10.1 ± 1.0	-	-	-	-
Lyso-PC	-	2.9 ± 1.6	-	-	-	-
pS	7.5 ± 0.7	6.4 ± 0.3	-	-	-	-
SQDG	-	-	-	-	24.7 ± 0.4	2.3 ± 0.3

**n.q. – non-quantifiable*

860

861

862 **Supplementary Material**

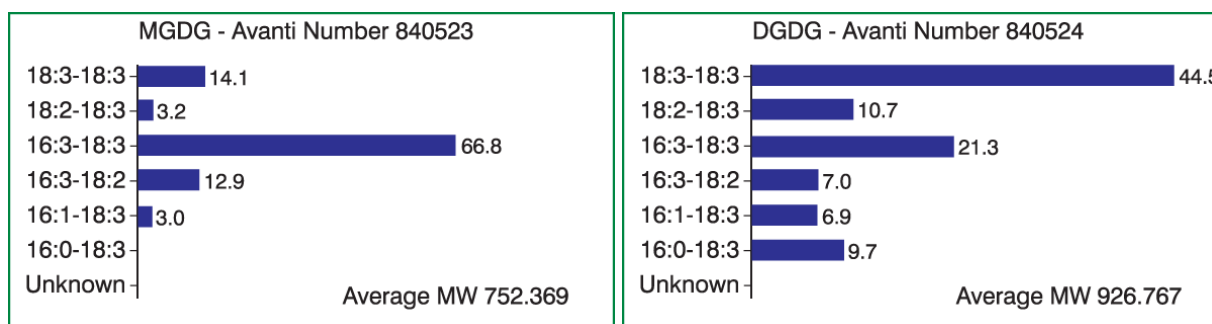
863 **Table S1 - Molar composition of mixed Langmuir monolayers used as model membranes**

Monolayer composition	
(1) GL	MGDG/DGDG 60:40 mol.mol ⁻¹
(2) GL/DPPC	MGDG/DGDG/DPPC 30:20:50 mol.mol ⁻¹ .mol ⁻¹
(3) GL/DPPC/pS	MGDG/DGDG/DPPC/pS* 27:18:45:10 mol.mol ⁻¹ .mol ⁻¹ .mol ⁻¹
<i>β-sitosterol, campesterol, brassicasterol 50:40:10 mol.mol⁻¹.mol⁻¹</i>	

864

865 **Figure S2 – Fatty acid distribution of MGDG and DGDG purchased from Avanti Polar Lipids**

866



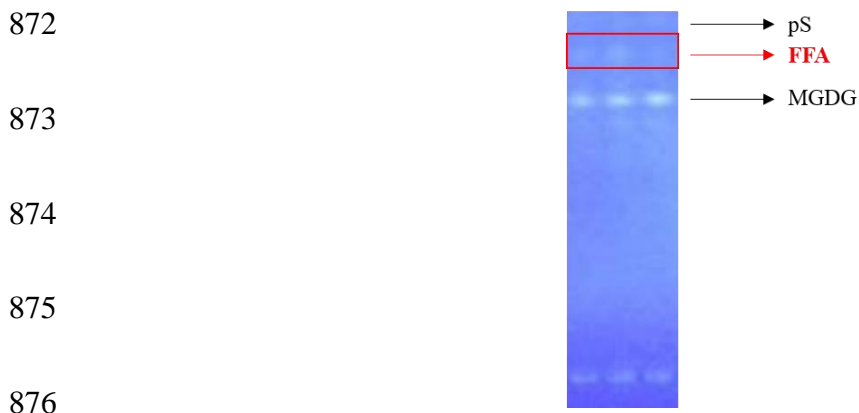
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869 **Figure S3 – Lipid classes composition obtained by TLC after 1h45 digestion by gPLRP2 of**

870 GL/DPPC/pS monolayer, showing the appearance of FFA at the interface. The reaction was

871 performed at pH 7 in Tris HCl buffer in the absence of NaTDC.



877 **Table S4** - Quantitative determination of lipid classes composition obtained by TLC at T_0 and after
 878 5 minutes (T_{5min}) digestion by gPLRP2 of GL/DPPC/pS liposomes **in absence of NaTDC**. The
 879 reaction was performed at pH 7 in Tris HCl buffer. Data are given in relative percentages of the
 880 total lipids.

Relative %	GL/DPPC/pS	
	T_0	T_{5min}
FFA	9.2 ± 3.1	4.9 ± 0.5
MGDG	41.2 ± 2.9	42.0 ± 1.0
MGMG	1.0 ± 1.4	0.5 ± 0.3
DGDG	35.8 ± 1.7	38.3 ± 2.3
DGMG	3.0 ± 0.5	4.9 ± 0.9
DPPC	2.0 ± 0.5	2.8 ± 0.4
LysoPC	-	-
pS	7.7 ± 0.6	6.5 ± 2.1

**n.q. – non-quantifiable*

881