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Research paper

Kinetic characterization, optimum conditions for catalysis and substrate preference of secretory phospholipase A_2 from *Glycine max* in model membrane systems

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ABSTRACT

Two secretory phospholipase A_2 ($sPLA_2$s) from *Glycine max*, $GmsPLA_2$-IXA-1 and $GmsPLA_2$-XIB-2, have been purified as recombinant proteins and the activity was evaluated in order to obtain the optimum conditions for catalysis using mixed micelles and lipid monolayers as substrate. Both $sPLA_2$s showed a maximum enzyme activity at pH 7 and a requirement of Ca^{2+} in the micromolar range. These parameters were similar to those found for animal $sPLA_2$s but a surprising optimum temperature for catalysis at 60 °C was observed. The effect of negative interfacial charges on the hydrolysis of organized substrates was evaluated through initial rate measurements using short chain phospholipids with different head groups. The enzymes showed subtle differences in the specificity for phospholipids with different head groups (DLPC, DLPG, DLPE, DLPA) in presence or absence of NaCl. Both recombinant enzymes showed lower activity toward anionic phospholipids and a preference for the zwitterionic ones. The values of the apparent kinetic parameters (V_{max} and K_M) demonstrated that these enzymes have more affinity for phosphatidylcholine compared with phosphatidylglycerol, in contrast with the results observed for pancreatic $sPLA_2$. A hopping mode of catalysis was proposed for the action of these $sPLA_2$ on mixed phospholipid/triton micelles. On the other hand, Langmuir-monolayers assays indicated an optimum lateral surface pressure for activity in between 13 and 16 mN/m for both recombinant enzymes.

1. Introduction

The secretory phospholipases A_2 (EC 3.1.1.4) are a family of ubiquitous soluble enzymes widespread in nature with a distinctive high capability to interact with lipid interfaces and catalyze the stereospecific cleavage of the *sn*-2 position of membrane diacylglycerophospholipids liberating 1-acyl-2-lysophospholipids and free fatty acids as products [1,2]. In a previous work, we reported the existence of five putative $sPLA_2$s enzymes within soybean *Glycine max*, denoted as $GmsPLA_2$-XIA-1,$GmsPLA_2$-XIA-2,$GmsPLA_2$-XIB-1,$GmsPLA_2$-XIB-2 and $GmsPLA_2$-XIB-3 [3].

Pancreatic phospholipase A_2 was the first mammalian $sPLA_2$ identified in pancreatic juices that plays a central role in the digestion of dietary phospholipids [4]. However, non-pancreatic $sPLA_2$s have been found in bee [5] and snake venoms [6,7], fungi [8] and in plants. Many $sPLA_2$s from plants have been purified and characterized [3,9–13], crystal structure of some of them were elucidated [14,15] and cellular function examined [12,16]. Despite more plant $sPLA_2$s enzymes have been studied within the last few years, still further investigation is needed in comparison to the huge data reported for animal's $sPLA_2$s. In this context, we further characterized two $sPLA_2$s isoforms present in *G. max*, since they could be potentially used in industrial applications as biocatalysts and lysophospholipids are stronger bioemulsifiers than lecithin [17,18]. At present, despite it would satisfy food regulation requirements such as Kosher and Halal, none report of plant $sPLA_2$s for such application has been done. However, $sPLA_2$s from microbes were also accepted, but they are poorly selective giving secondary products since they have PLA_1 and lipase activity [17]. Furthermore, in the case of soybean, the enzyme and the industrial substrate arise from the same raw source.

$sPLA_2$ enzymes from plants shared common molecular structural features such as: two conserved central α–helices containing the

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catalytic pair His–Asp and a hydrogen-bonding network connecting the interfacial binding site, the catalytic and the calcium binding sites. They contain 12 cysteins which can lead to 6 disulphide bonds, high resistance to denaturation, μM to mM calcium requirement and belong to groups XIA and XIB of the PLA_2 superfamily [19].

Since $sPLA_2$s act on lipid interfaces, their kinetic characterization is not trivial. $sPLA_2$s are interfacial enzymes that operate at an organized interface such as lipid aggregates in contact with the aqueous phase. They have very poor activity on monomeric substrates but become highly activated when the substrate is organized in lipid monolayers, micelles or liposomes [20]. Moreover, $sPLA_2$s hydrolyze substrates faster when they are organized in micelles than in the bilayer form of substrate aggregates. Furthermore, the charge, molecular dimensions of the phospholipid and the presence of surfactants in the micelle are important. The kinetic of these water-soluble enzymes is controlled not only by the substrate and enzyme concentration but also by the organization and dynamics of the interface where the catalysis takes place. The increased enzyme activity observed when phospholipids are present above their CMC implies that phospholipases have an interfacial binding surface and that interfacial activation will precede normal catalysis [21,22].

The catalytic turnover is determined by the kinetics at the interface and the binding/desorption equilibrium. Therefore, the hydrolysis of the organized substrate can occur in two distinct modes: i) in the *scooting* mode of catalysis, the enzyme does not leave the surface of the vesicle to which it is bound and, ii) in the pure *hopping* mode, the binding and the desorption of the bound enzyme occur during each catalytic turnover cycle [23].

There have been some reports in the literature regarding to $sPLA_2$ activity on different substrates and environments. Even more, it has been demonstrated that the surface charge on the lipid aggregates is an important factor in the rate of hydrolysis for some phospholipase A_2 [24]. Several kinetics studies on pancreatic as well as snake venoms and plants phospholipases has been reported. Usually, lipid with the zwitterionic phosphocholine head group have been employed as substrates in single component systems (short-chain) [21,25] or mixed with uncharged detergents (long-chain) [26–28]. Moreover, the activity is generally increased when the lipid substrate forms mixed micelles in presence of detergents [29,30]. The effect of enzyme immobilization on the $sPLA_2$ kinetics was also reported [31]. Description and kinetics properties of $sPLA_2$ from plants have been more frequent in their recombinant counterpart after an appropriate expression, purification and folding protocols [3,10,11,27,32] compared with their equivalent found in animals $sPLA_2$s. The reason of this is due to the relative high amounts of the latter proteins found in their respective natural sources (venoms and pancreatic juice) and, therefore it allows an efficient purification. However, few studies using purified plant enzymes were reported from seeds of elm [9] and of soybean [33].

The aim of the present work was to study the enzymatic properties of the novel and recently produced by heterologous expression in *E. coli* and purified $sPLA_2$ enzyme from soybean (*G. max*) called $GmsPLA_2$-XIB-2 comparatively with the previously reported $GmsPLA_2$-XIA-1 [3]. Both enzymes were expressed without *N*-terminal extension identical to the wild type protein sequence [3], since any alteration or extension of the *N*-terminal in $sPLA_2$ can drastically modify its catalytic properties [34,35]. Both enzymes were selected since they belong to different subgroups (XIA y XIB) within the superfamily of PLA_2. They have differences in their molecular weights, sequences and isoelectric points that might be affecting the optimum conditions for catalysis and/or substrate preference. So, $sPLA_2$ activity was evaluated to obtain the optimum conditions for catalysis as calcium ion requirement, pH and optimum temperature. The apparent kinetic parameters of both enzymes were analyzed using mixed micelles of phospholipid substrates with different head groups. Also, substrate preference was evaluated to determine a possible correlation between activity and the net charge on the substrate aggregates.

Moreover, to know the influence of substrate lipid packing on both $GmsPLA_2$s activities the Langmuir-lipid monolayers assays were performed at different surface pressure. To prevent any changes in the physicochemical properties of the lipid aggregate during the course of the lipid hydrolysis, the lipid monolayer technique in the "zero order" regime is quite suitable to study the catalytic activity at the air–water interface since the surface pressure is kept constant during the reaction [36].

2. Materials and methods

2.1. Materials

Pancreatic PLA_2 and Triton X-100 were purchased from Sigma Chem. Co. (St. Louis, MO, USA), Isopropyl-l-thio-β-D-galactopyranoside (IPTG) and ampicillin from Promega (Madison, WI, USA). Ni-NTA chelating sepharose was purchased from GE Healthcare, USA. The substrates used in this studies were 1,2-dilauroyl-*sn*-glycero-3-phosphate (DLPA), 1,2dilauroyl-*sn*-glycero-3-phosphoethanolamine (DLPE), 1,2 dilauroyl-sn-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt) (DLPG) and 1,2 dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), all purchased from AVANTI polar Lipids Inc. (Birmingham, AL, USA). All other reagents were of the highest analytical grade.

2.2. Cloning of $GmsPLA_2$-XIB-2

PCR amplification of the mature sequence of $GmsPLA_2$-XIB-2 gene was performed using specific primers designed as mentioned in Ref. [3] and using the full-length cDNA clone "JCVI-FLGm-7G8" (Plant Genomics, J. Craig Venter Institute Genbank BT091171) as template. For this purpose, PCR was carried out using 50 ng of template and GoTaq DNA polymerase (Promega,WI, USA), for 33 cycles of 5 min at 95 °C for initial denaturation, 94 °C 1 min for denaturation, 1 min with gradient annealing temperatures, 10 cycles at 48 °C and 25 cycles at 55 °C, based on Tm value of the primers and 1 min at 72 °C for polymerization, using a forward primer 5′-tccCCGCGGTGGA**GCG**AACATAGGAGCTGAAACC-3′ containing an alanine instead of leucine downstream the ubiquitin protein (see supplementary data S1) and a reverse primer 5′-aggCTATGGCTTGTGGAGAACTCTT-3′. The amplified product was analyzed and separated on 1% agarose gel, excised and eluted using Promega Wizard SV Gel and PCR Clean-UpSystem (Promega, WI, USA), cloned into pGEM-Teasy vector (Promega, WI, USA) and used to transform into DH5α *E.coli*. After selecting positive clones according to the manufacturer's protocol, plasmid DNA was purified from the positive clone with The Wizard® SV 96 Plasmid DNA Purification System (Promega, WI, USA). The constructed plasmid was digested using SacII (Promega, WI, USA) and Hind III (Promega, WI, USA.) restriction enzymes, and the fragment coding for the Ub-$GmsPLA_2$-XIB-2 was subsequently purified from 1% agarose gel as mentioned above and subcloned into the expression vector pHUE and the resulting plasmid transformed *E. coli* BL21(DE). Plasmid DNA was purified after selecting positive clones using ZR Plasmid Miniprep™ – Classic (ZYMO RESEARCH, CA, USA). The correctness of the nucleotide sequence of the construct Ub-$GmsPLA_2$-XIB-2-pHUE was verified by DNA sequencing by 3730XL automated DNA Sequencer (Applied Biosystems, University of Chicago Cancer Research Center-DNA sequencing facility). The full sequence of the Ub-$GmsPLA_2$-XIB-2-pHUE construct is shown in Fig. S1 of supplementary data.

2.3. Expression, purification and folding of the protein

Expression of the recombinant proteins (Poly-His-Ub-GmsPLA$_2$-XIA-1 and Poly-His-Ub-GmsPLA$_2$-XIB-2) in *E. coli* BL21 strain (DE3), isolation and folding were carried out following identical procedures as mentioned in Ref. [3]. The purity of mature GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2 was proven to be of 95% by SDS-PAGE and the correct molecular weights were corroborated by mass spectrometry (MALDI-TOF–TOF Applied Biosystems 4800 Plus). The correctness of the *N*-terminal sequence was determined by automated Edman's degradation, using an Applied Biosystems automated protein Sequencer 477A equipped online with 130A HPLC system provided by a 477A automated protein sequencer (Applied Biosystems, LANAIS PROEM, Laboratorio Nacional de Servicios en Péptidos y Proteínas. Estudios por Espectrometría de Masa, UBA, CONICET, Buenos Aires, Argentina).

2.4. Protein determination

To estimate the relative molecular mass of the protein, electrophoresis on 15% polyacrylamide slab gels under reducing conditions was performed following the Laemmli's method [37]. Proteins were visualized with Coomassie brillant blue R-250 staining. SDS-PAGE gels were scanned with a flat-bed scanner, and digital images were imported and quantified using Photoshop software (Adobe Photoshop CS). The intensities of bands were compared according to their grayscale using bovine serum albumin as standard according to (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/).

2.5. sPLA$_2$ activity measurements

2.5.1. Assay for sPLA$_2$ activity in mixed micelles

PLA$_2$ activities were determined using lipid substrate/Triton X-100 mixed micelles (1:4, mole ratio) following the fatty acids liberated during the reaction by using the adapted NEFA-HR kit from WAKO (WAKO Pure Chemical Industries, Japan) as mentioned previously [3]. The activity was quantified by determining the amount of fatty acids released from DLPC according to [38]. The assay mix was composed of 5 mM lipid substrate, 20 mM Triton X-100, 10 mM $CaCl_2$, and 10 mM Tris–HCl pH 8 in a total reaction volume of 30 μl. All assays were performed at 37 °C. The reaction was initiated by the addition of purified enzyme from a stock solution. Substrate preparation was done by dissolving appropriate amounts of lipid in 1 ml of chloroform/methanol (2:1, v/v) and dried with N_2 and subsequent overnight vacuum. The lipid film was dissolved in 10 mM Tris–HCl pH 8 containing 20 mM Triton X-100 and 10 mM $CaCl_2$ by vortexing for 15 min at 40 °C. The reaction was stopped by addition of 6 μL of 0.5 M EDTA. Initial rates were determined from the released fatty acids as described above, according to the manufacture instructions, with a sample reagent ratio of 36 μl of sample, 125 μl of reagent A plus 62 μl of reagent B. The absorbance of the final colorimetric complex was measured at 550 nm using oleic acid as standard (for details see http://www.wakodiagnostics.com/r_nefa.html and http://www.wakodiagnostics.com/pi/pi_hr_seriese_nefa-hr%282%29.pdf). PLA$_2$ activities were linearly dependent in the range of the enzyme concentration currently used in the assays. All experiments were run at least in duplicate. Uncleaved fusion protein showed no activity in all system assayed (data not shown).

2.5.2. Initial rates measurements

In order to define the range of initial velocity (v_o), the enzyme was incubated for 1–40 min at 37 °C with DLPC (5 mM final concentration) and free fatty acid liberated with the time of incubation were quantified using the NEFA-HR Kit as mentioned. Enzymes activities were linearly dependent on enzyme concentration up to 0.13 μM and 0.23 μM for GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2 respectively. All experiments were done at least in duplicate.

2.5.3. Determination of the optimum conditions for catalysis

In order to define the *optimum pH* of enzyme activity, different buffers at a final concentration of 25 mM were used to prepare mixed micelles of DLPC/Triton X-100 (1:4), Mes buffer (pH 5 and 6), Trizma-base buffer (pH 7 and 8) and Glycine-NaOH buffer (pH 9). Enzymes (2.66 μg/mL of GmsPLA$_2$-XIA-1 and 1.07 μg/mL of GmsPLA$_2$-XIB-2 respectively) were incubated with the mixed micelles in the corresponding buffer solutions containing 10 mM $CaCl_2$ during 5 min at 37 °C and then assayed as mentioned.

For the determination of the *optimum temperature*, the enzymes were incubated with mixed micelles of DLPC/Triton X-100, prepared as previously described, varying the temperature between 20 °C and 90 °C for 5 min in 10 mM of Tris–HCl buffer pH 8.5, containing 10 mM $CaCl_2$.

We determined the effect of calcium on GmsPLA$_2$-XIA-1stability by measuring the activity at 60 °C in the presence or the absence of the cation. The enzyme (1.75 μg/mL) activity was assayed at 60 °C at three different conditions incubated with mixed micelles of DLPC:Triton X-100 in buffer Tris–HCl 10 mM, pH 8.5: *i)* with calcium 10 mM to measure the activity of the enzyme (as control); *ii)* pre-incubated with EDTA 10 mM to chelate the calcium ions present in the sample mixture during 1 min at room temperature before heating at 60 °C and incubating with the substrate at 60 °C and, *iii*) enzyme pretreated with EDTA 10 mM (as *ii*) but adding a new excess of 10 mM $CaCl_2$ during 5 min before to incubate with the substrate mixture at 60 °C, to see if activity is restored. Then, activity was measured as described above.

For the determination of the *optimum requirement of calcium*, the enzymes were incubated with the mixed micelles of DLPC/Triton X-100 (ratio 1:4) in Tris–HCl buffer, pH 8.0, containing different $CaCl_2$ concentration (from 0.1 mM to 4 mM) for 5 min at 37 °C. 6 μl of EDTA 0.5 M was added to the enzyme mixture before the assay. The free fatty acids liberated in all the assays were quantified by using the NEFA-HR Kit as described above. Activity was expressed as $\mu mol.min^{-1}.mg^{-1}$ of protein or namoles liberated in the assay. All the experiments were done at least in duplicate.

2.5.4. Determination of kinetics constants

For the determination of the kinetics constants, initial reaction rates (v_o) were determined as described above at substrate concentrations in the range from 0 to 6 mM and from 0 to 18 mM for GmsPLA$_2$-XIA-1 (1.5 μg/mL) for DLPC and DLPG, respectively, and from 0 to 4 mM for GmsPLA$_2$-XIB-2 (2.66 μg/mL) in both DLPC and DLPG substrates, in buffer Tris–HCl 10 mM, pH 8, containing 100 mM NaCl and 5 mM $CaCl_2$ during 5 min at 37 °C. Activities were expressed as μmol of released free fatty acid min^{-1} mg^{-1} of enzyme. Apparent kinetics parameters K_M and V_{max} were obtained by plotting initial rates versus substrate concentration and subsequent non-linear regression using the Michaelis–Menten formalism (by using Sigma Plot 10.0 as fitting program). The concentration of enzymes apply in these experiments were 1.5 μg/mL and 2.66 μg/mL for GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2 respectively. The –catalytic efficiency for each enzyme was determined by the relation between V_{max} and K_M [39]. All experiments were done in duplicate, and the reported results were the mean values ± S.D.

2.5.5. Determination of substrate preference

To elucidate the substrate preference for both enzymes, substrates with the same chain length but with different head groups (DLPE, DLPC, DLPG and DLPA) were used to prepare substrate/Triton X-100 mixed micelles in buffer Tris–HCl, pH 8, containing 10 mM

$CaCl_2$. At lipid (DLPC-DLPG-DLPA): Triton X-100 ratio 1:4, the mixtures were observed as clear solutions. PE/Triton mixture showed evidence of turbidity, so we decrease the lipid to detergent ratio up to 1:8 to obtain a clear solution. Two conditions were assayed: with 100 mM NaCl and in absent of high salt. Enzyme concentrations were the same as mentioned above for the determination of the kinetic constants. All experiments were done in duplicate.

To calculate the values showed in Table 2, the results obtained at 5 min of reaction were normalized with respect to the highest value (the highest values were obtained with DLPC).

An assay with different percentage of DLPG (from 5 to 40 %) added to the mixed micelles of DLPC/TritonX-100 (1:4) at a final lipid concentration of 1 mM was also performed. Curves of activity *vs.* % DLPG added were plotted. The assay was also realized using pancreatic PLA_2 (1.5 μg/mL) for comparison. Enzyme concentrations were 1.5 μg/mL and 1.77 μg/mL for $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2, respectively.

2.5.6. Determination of the interfacial mode of action

For the determination of mode of catalysis carried out by the enzyme at the interface, the activity of $GmsPLA_2$-XIA-1 (final concentration 1.75ug/ml) was evaluated using two different concentrations of the DLPC substrate in a sequential way. The activity at 0.25 mM of DLPC (mixed micelles) was monitored up to 10 min; then, an aliquot of 0.75 mM DLPC was added to reach 1 mM of final concentration of substrate in the reaction tube and subsequent activity was measured. The buffer used in this assay was Tris–HCl 10 mM pH 8, containing 10 mM $CaCl_2$. The enzyme/micelle mole ratio at 1 mM of final substrate concentration ratio is 1:232.

2.6. PLA_2 activity using lipid monolayers in the zero order trough regime

Phospholipase activity was followed by measuring the decrease in area of DLPC monolayer at air/water interface at constant surface pressure (π) using a surface barostat (zero order trough [36]) The equipment was a mono-film-meter (Mayer Feintchnik, Gottingen, Germany) in which the original circular trough was replaced by one home-made specially designed trough [40]. Continuous monitoring was done with a double channel X-Y-Y recorder (Yokogawa Corp. Japan) coupled to the surface balance. Monolayers of pure DLPC films were prepared under true zero-order kinetic conditions by spreading pure substrate onto the reservoir and reaction compartments filled with buffer. As PLA_2 activity is detected by a positive movement of the barrier of the reservoir compartment substrate is continuously supplied from the reaction compartment. The products from DLPC degradation are immediately desorbed into the subphase under continuous stirring. After DLPC spreading (2–10 μl from 1 mM stock lipid solution dissolved in chloroform:methanol 2:1, v/v), were allowed 5 min for solvent evaporation and the films were compressed to the desire initial surface pressure which in turn it was maintained constant under the zero order system.

The desired amount of enzyme (in no more than 50 μl) was injected into the subphase of the reaction compartment (17 or 35 ml), made of 10 mM Tris–HCl buffer pH 8.0 containing 20 mM $CaCl_2$ and 100 mM NaCl, under continuous magnetic stirring at 25 ± 1 °C. An adjacent compartment (connected to the reaction compartment through a narrow and shallow slit) serves as a substrate reservoir in order to maintain constant surface pressure during the enzymatic reaction. Surface pressure (π) as a function of time (t) curves, which reflects the kinetics of monolayer hydrolysis, were used to calculate the enzymes activities at different surface pressures. The enzyme activity was calculated from changes with time course of the reaction in the surface area occupied by the substrate [36,41] and was expressed in molecules.cm^{-2}.min^{-1}. The enzymatic activity of $GmsPLA_2$s against DLPC monolayers can be determined by continuously measuring the decrease of the film area as a function of time at a desired constant surface pressure (π) using the surface barostat from the corresponding slope of the linear portion of the time–course curve. Lag times are taken by extrapolation of linear part to the abscissa at zero substrate degradation. All experiments were done in duplicate, and the results were mean values ± SD.

The assays of lag time and dependence of the enzymes activities with the enzyme concentration employed were measured at a constant surface pressure of 13 mN/m.

The enzyme concentrations used in the assay to determine the optimum surface pressure were 0.12 μg/mL and 0.145 μg/mL for $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2, respectively. For the curve of activity *vs*. concentration of substrate, the enzyme concentrations used were from 0.026 μg/mL to 2.5 μg/mL for $GmsPLA_2$-XIA-1 and from 0.073 μg/mL to 0.145 μg/mL for $GmsPLA_2$-XIB-2.

3. Results and discussion

3.1. Recombinant expression and purification of $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2

Recombinant $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2 were obtained by expression of the modified PHUE system in BL21 *E. coli* which allows to express the protein as histidine-tagged-ubiquitin-PLA_2 fusion. The solubilization from inclusion bodies was performed in Gdn.HCl 6 M and further purification by Nickel affinity Chromatography after digestion by deubiquitinase (USP2cc) as described [3,40]. To optimize the chance of obtaining correct refolding of the protein $GmsPLA_2$-XIB-2, the Leu amino acid immediately following the final Gly residue located at the carboxy-terminal of ubiquitin (Ub) was mutated to an Ala, as recommended in Ref. [34]. The purification steps was followed by using the indirect screening assay of $sPLA_2$ activity by the appearance of halos in plates of agar-eggPC-blood as mentioned in Ref. [3], using renaturation buffer as a negative control, pancreatic PLA_2 as positive control and the fusion protein to demonstrate that the proteins are inactive when the ubiquitin is ahead the $sPLA_2$ of interest (results not shown).

The purified recombinant $GmsPLA_2$s ($GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2) showed unique bands of approximately 14 kDa in SDS-PAGE (Supplementary data S2) and were used for analyzing the enzyme.

Table 1
Apparent constants for both $sPLA_2$s from *Glycine max* when substrate was DLPC and DLPG in mixed micelles containing 100 mM NaCl.

$GmsPLA_2$-XIA-1				$GmsPLA_2$-XIB-2		
Substrate	K_M	V_{MAx}	V_{MAx}/K_M	K_M	V_{MAx}	V_{MAx}/K_M
DLPC	0.23 ± 0.05	10.21 ± 0.06	1479.5	0.07 ± 0.06	19.71 ± 1.40	9376.3
DLPG	17.98 ± 4.20	13.87 ± 1.87	25.7	1.13 ± 0.35	6.69 ± 0.89	197.3

Units: K_M, mM; V_{MAx}, μmol min^{-1} mg^{-1}; V_{MAx}/K_M, min^{-1} mg.

Table 2
Influence of salt in *Glycine max* $sPLA_2$ activity.

	0 mM NaCl				100 mM NaCl			
	$GmsPLA_2$-XIA-1		$GmsPLA_2$-XIB-2		$GmsPLA_2$-XIA-1		$GmsPLA_2$-XIB-2	
Substrate	nmol liberated	%	nmol liberated	%	nmol liberated	%	nmol liberated	%
DLPC	1.43 ± 0.03	100	7.30 ± 0.64	100	3.26 ± 0.05	100	10.44 ± 0.45	100
DLPE	1.38 ± 0.03	96.5	4.95 ± 0.01	67.8	1.59 ± 0.07	48.8	6.53 ± 0.64	62.6
DLPG	0.0	0.0	3.29 ± 0,68	45.1	1.23 ± 0.08	37.7	3.45 ± 0.05	33.1
DLPA	0.13 ± 0.02	9.1	0.0	0.0	0.33 ± 0.02	10.1	0.13 ± 0.03	1.2

Product liberated expressed in nmol at 5 min of reaction against different substrates in presence and absence of NaCl. The percentages are related to each maximum of products generated for both enzymes in each condition tested.

3.2. N-terminal sequence of $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2

The N-terminal sequencing of the first ten residues of purified enzymes of $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2 allows the verification of the N-terminal of the sequences in order to ensure that there are no extra amino acids at this position that could have consequences on the activity, as reported for some recombinant $sPLA_2$s [34,35]. Fig. S3 of supplementary data shows the identified N-terminal sequence in the complete amino acid sequence of both $GmsPLA_2$s. Mass spectrometry analysis indicated that $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2 have molecular mass of 12,363.7 kDa and 13,852.1 kDa, respectively. These data are consistent with the theoretical molecular weight calculated from the sequences that are 12,374.20 Da and 13,859.80 Da, respectively.

3.3. Catalytic properties of the recombinant $GmsPLA_2$s

The catalytic properties of $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2 were investigated with respect to time, pH, and calcium ion requirement, in order to characterize the optimum conditions for its enzyme activity.

Fig. 1A shows the free fatty acids liberated *vs.* time. From these curves we estimate the initial rates at 5 min of hydrolysis progress.

The *pH optimum* was at around pH 7 for both enzymes (Fig. 1B), when using mixed micelles of DLPC:Triton X-100 as substrate in presence of calcium 10 mM. These results are very similar to those found for other plant or animal $sPLA_2$s. The $sPLA_2$s from *Nicotiana tabaccum* and elm have optimum pH in the range of 8–10 and 8–9 respectively [1,11]. In *Arabidopsis* the optimum pH ranges for the activities are pH 6–11, 6–7, 7–9, and 8–9 for AtsPLA2-α, −β, −γ,

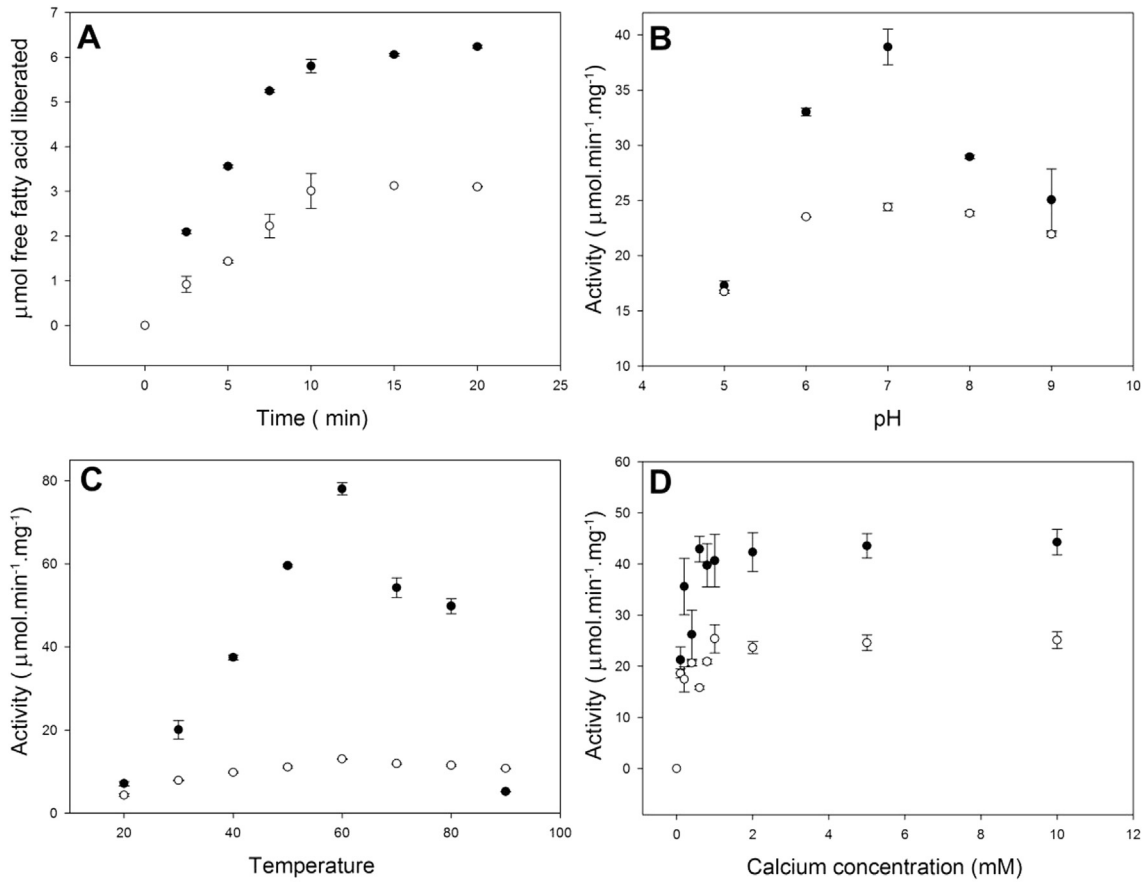

Fig. 1. Activity of $sPLA_2$ from *Glycine max* on mixed micelles. A) The time course of hydrolysis on mixed micelles of DLPC:Triton X-100 (ratio 1:4) by $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2. B) pH dependence of $GmsPLA_2$s enzymes. C) Optimum temperature for catalysis of the $GmsPLA_2$s enzymes. D) Hydrolytic activity of $GmsPLA_2$s enzymes as a function of Ca^{2+} ion concentration. $GmsPLA_2$-XIA-1, empty circles (○); GmsPLA2-XIB-2, filled circles (●).

and δ respectively [42]. Nevertheless, a similar situation was found for almost all the sPLA$_2$s found either in plants or animals. The optimum pH for pancreatic sPLA$_2$ is reported to be 8 [43,44] similar to that reported for bee venom [45]. For human non-pancreatic PLA$_2$ optimum pH is in between 8 and 10 [46]. However, it should be mentioned that different substrates have been used to determine optimum pH for the different sPLA$_2$s reported in the literature.

Both enzymes appeared to be very stable when increasing the temperature in presence of calcium, with an optimum at around 60 °C. However, the dependence of enzyme activity on temperature was higher for GmsPLA$_2$-XIB-2 than with GmsPLA$_2$-XIA-1. This demonstrates that these enzymes are highly resistant to temperature denaturation due in part to the disulfide bridges that are postulated to be involved in the stability of sPLA$_2$s [47,48].

The *optimum concentrations of calcium* ion for activity of GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2 are in the micromolar range and are saturating above 0.5 mM using mixed micelles as substrates (Fig. 1D). This micromolar calcium requirement is unusual for sPLA$_2$s enzymes that mostly posses millimolar requirement [19]. Moreover, the same behavior was observed for the activities of AtsPLA2-β, −γ and −δ [42]. It is important to highlight that these enzymes exhibit no activity in absence of calcium. In contrast, for AtsPLA2-α the activity augmented as the calcium concentration increased up to 10 mM and for elm sPLA$_2$ exhibited an optimal activity in between 10 and 15 mM $CaCl_2$ [9,27,42]. However, to achieve 50% of maximal activity a concentration of 0.5 mM was sufficient, at least, for these latter two enzymes. The maximal activity for sPLA$_2$ from *N. tabaccum* was detected above 1 mM calcium. This behavior is similar to that observed for the most animal sPLA$_2$s, which require millimolar concentrations of Ca^{2+} and have no activity in the absence of this cation [19,44].

3.4. Kinetic parameters for GmsPLA$_2$s

The initial rate of hydrolysis on mixed micelles of DLPC or DLPG, in Tris–HCl buffer (pH 8.5) containing 5 mM $CaCl_2$ and 100 mM NaCl, by GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2 as function of their concentration showed a Michaelis–Menten behavior. A concentration of 5 mM $CaCl_2$ (well above of the optimum found for our enzymes, see Fig. 1D) instead of the 10 mM routinely used in order to prevent turbidity in samples containing DLPG when high concentration of the cation.

Activity of GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2 towards DLPC/Triton X-100 mixed micelles (1:4, mole ratio), showed an apparent K_M of 0.23 mM and 0.07 mM and a V_{max} of 10.2 μmol min^{-1} mg^{-1} and 19.7 μmol min^{-1} mg^{-1}, respectively, (see Fig. 2 and Table 1). When substrate used was DLPG in the mixed micelles, the apparent K_M and V_{max} for both enzymes were 17.9 mM and 13.9 μmol min^{-1} mg $^{-1}$ for GmsPLA$_2$-XIA-1 and 1.1 mM and 6.7 μmol min^{-1} mg $^{-1}$ for GmsPLA$_2$-XIB-2.

Apparent K_M values obtained for GmsPLA$_2$s using short chain phospholipids as substrates were less different when compared with those obtained for sPLA$_2$s from animals [49] or for other plants [27], although a different substrate systems were used instead. K_M values of animal PLA$_2$s fall in between 0.18 and 3.2 mM for 1,2-dioctanoyl-sn-glycero-3-phosphocholine and 0.12 mM for sPLA$_2$-α from *Arabidopsis thaliana* [27].

The catalytic efficiencies calculated as the relation of V_{max} with respect to K_M (V_{max}/K_M, *c.f.* [39])are reported in Table 1.

3.5. Phospholipid head group specificity of GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2

Changing the phospholipid polar head group can produce significant changes in the characteristics of the interface affecting the

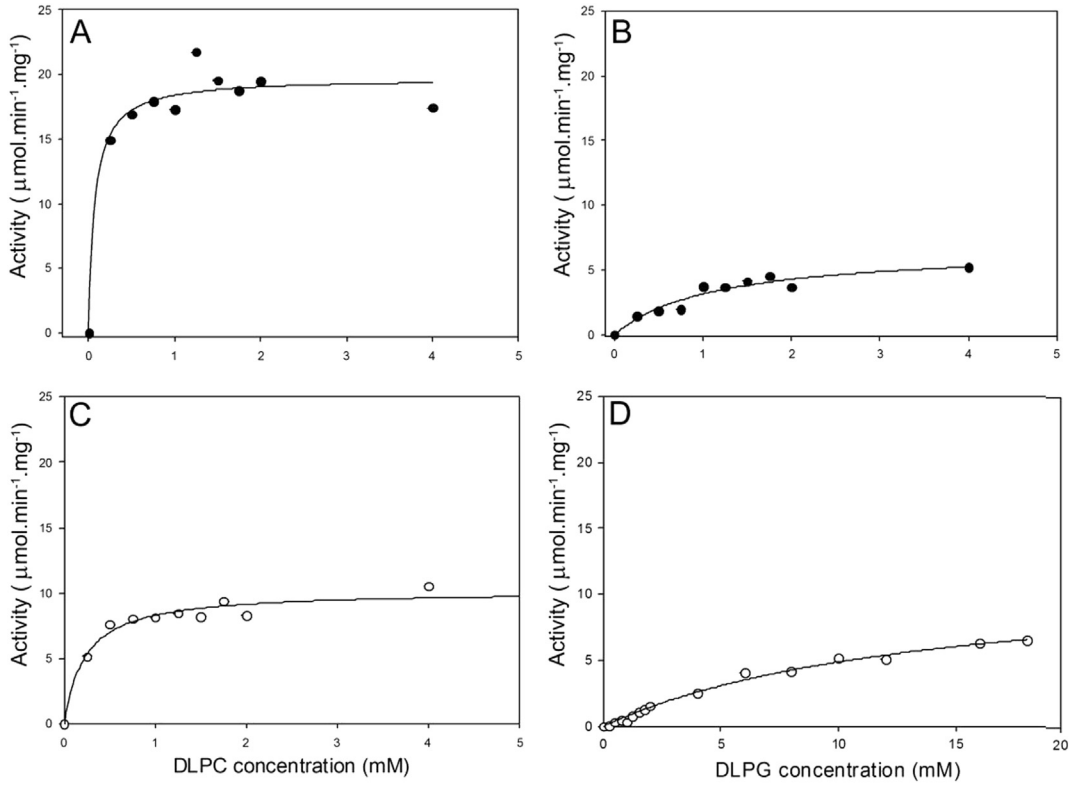

Fig. 2. Kinetics of GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2 on mixed micelles. Dependence of the initial rate of hydrolysis with substrate concentration in presence of NaCl 100 mM. A, B) Initial rate *vs.* substrate concentration for GmsPLA$_2$-XIB-2 and C, D) Initial rates *vs.* substrate concentration for GmsPLA$_2$-XIA-1. Substrate lipid:Triton-X100 (ratio 1:4).

activity of the enzyme. Striking differences in enzyme activity when hydrolyzing different phospholipids could be due to either a substrate recognition difference in the active site or rather to changes that may operate in the physical characteristics of the surface. The use of nonionic detergent on mixed micelles as organized membrane systems offers the advantage to present the phospholipid substrate in a uniform or homogeneous matrix [50]. The effect of the head group charge of the phospholipid substrate on GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2 hydrolysis was examined by measuring the initial rates of enzyme activity using DLPC, DLPG, DLPE and DLPA mixed with TritonX-100, (see Fig. 3).

Measurements were done in presence and absence of NaCl. The activity for GmsPLA$_2$-XIA-1in absent of high salt environment shows a preference for zwitterionic PC and PE compared with anionic phospholipids such as PG and PA (Fig. 3A and Table 2). In presence of 100 mM NaCl a clear preference of enzyme for PC is observed, but PE and PG are also accepted as well (Fig. 3B). Almost the same behavior is noticed for GmsPLA$_2$-XIB-2, since this enzyme prefers PC either in presence or absence of salt.

For another phospholipids, in presence of high salt concentration (Fig. 3B), GmsPLA$_2$-XIB-2 showed a slightly preference of PE over PG but this preference is abolished in absence of NaCl. When the substrate was PA, in all the cases, the activity was very poor or close to zero. The behavior found for GmsPLA$_2$-XIB-2 activity profile in presence of NaCl was similar to that reported for AtsPLA2-α [27]. Moreover, the catalytic efficiency was higher when the substrate used in the assay was DLPC (Table 2).

Independently of the presence or not of salt in the medium, both enzymes, GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2, showed a markedly preference for PC, indicating that this group of enzymes has a different behavior than that found for pancreatic PLA$_2$ [24,51–53]. A much higher selectivity for zwitterionic PC could be linked to the biological function of these enzymes, because of PC is one of the most abundant phospholipid in plant plasma membranes together with PE [27]. These results were comparable with those sPLA$_2$ obtained for other plants species: *Nicotiana tabacum* was shown to prefer PC over PG and PE in mixed micelles with sodium cholate [11]; in *Arabidopsis thaliana*, AtsPLA$_2$-α and −β showed a slightly preference of PE over PC when using mixed micelles with triton-X100 [42].

On the other hand, AtsPLA$_2$−γ and AtsPLA2 -δ exhibited a preference for PE over PC similar to those found for animal sPLA$_2$s from groups IB, II, III, and XII that were found to act on PE, PG, and PS over the preference to PC. In contrast, animal sPLA$_2$s belonging to groups V or X, similar to AtsPLA2-α and -β, can hydrolyze both PE and PC [28,29].

Mansfeld et al. have reported that the addition of anionic phospholipid enhances the initial reaction rates of plants sPLA$_2$ against zwitterionic phospholipid substrate [27]. So, the activity of GmsPLA$_2$-XIA-1 and GmsPLA$_2$-XIB-2 toward mixed micelles of DLPC was tested with the addition of increased percentages of DLPG. A decrease in the activity was observed as the proportion of anionic DLPG was increased in the mixed micelle (Fig. 4). As control, we compare the effect of anionic lipids on pancreatic sPLA$_2$, which it was reported to be more active in rich PG mixed micelles (Fig. 4), [24]. In agreement with the general behavior observed, comparatively, pancreatic sPLA$_2$ is more active against anionic phospholipids compared with plant sPLA$_2$s in keeping with results reported by others [51–53].

3.6. Effect of calcium on thermal stability of GmsPLA$_2$s

The presence of calcium ion was important to keep the stability of *Glycine max*s PLA$_2$ upon heating. If the enzyme is heated at 60 °C in absence of calcium or in presence of EDTA, the activity was completely lost, even when the calcium ion is restored in the medium after heating pretreatment (Table 3). As it was previously suggested for us, by using fluorescence experiments and molecular dynamics, the presence of calcium ion has a clear action on GmsPLA$_2$-XIA-1to preserve the overall folding of the protein and therefore the correct conformation for lipolysis [3].

3.7. Mode of catalysis

To determine if the GmsPLA$_2$-XIA-1 PLA$_2$ enzyme act on mixed micelles in the hopping (jumping from one micelle to another) or scooting (mostly adsorbed to micelle surface) mode, initial rate curves at different concentration of substrate (DLPC) in mixed micelles was evaluated (Fig. 5). Punctually, at 10 min of the reaction assay, the lower substrate concentration was increased from 0.25 mM to 1 mM and a jump in the activity level was observed, indicating that the enzyme was able to “see” the increment of bulk substrate concentration. The fact that the rate of hydrolysis depends on the bulk substrate concentration is suggesting a hopping mode mechanism by which the enzyme binds to the interface reversibly [20,54].

3.8. Assay of PLA$_2$ activity on Langmuir lipid monolayers at air water/interface

Due to physicochemical properties of the lipid aggregates that are changing during the course of the hydrolysis reaction and can

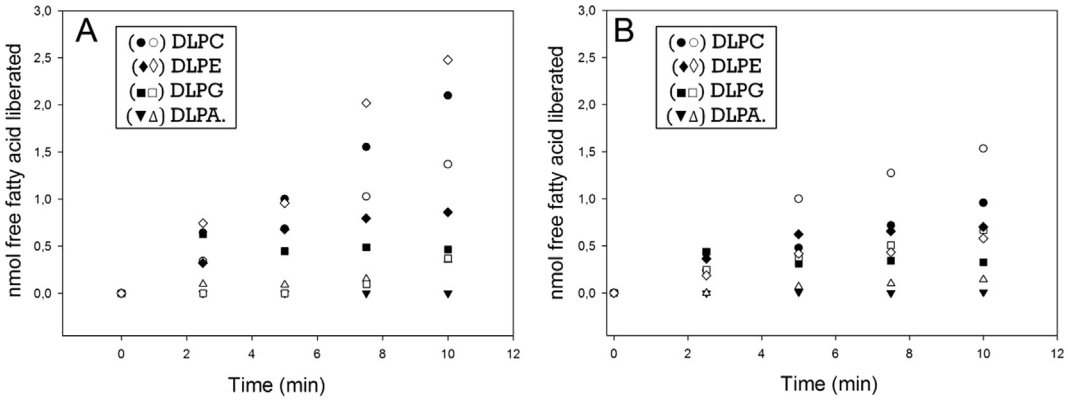

Fig. 3. Phospholipid polar head group specificity of *Glycine max* sPLA$_2$. Time course of hydrolysis on lipid substrate with different head group in absence A) or presence B) of NaCl 100 mM, by GmsPLA$_2$-XIA-1(empty symbols) and GmsPLA$_2$-XIB-2 (filled symbols) on mixed micelles of DLPC:Triton X-100 (ratio 1:4). (● ○) DLPC, (◇ ◆) DLPE, (■ □) DLPG and (▼ Δ) DLPA.

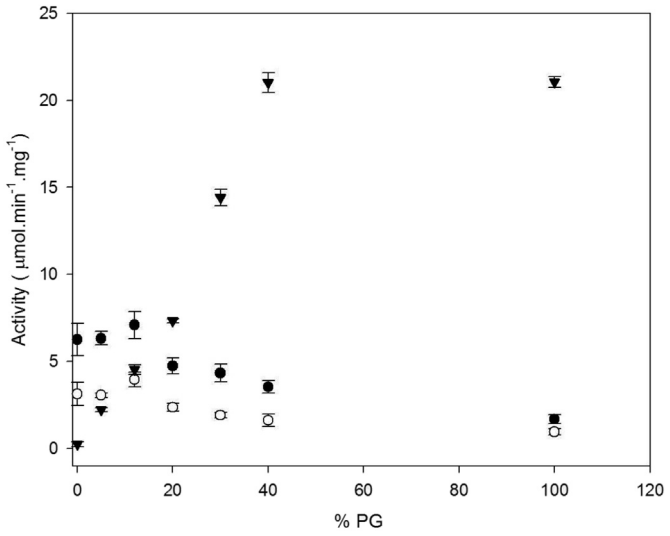

Fig. 4. Effect of DLPG on *Glycine max* $sPLA_2$s activity in mixed DLPC/Triton micelles. Comparative behavior of pancreatic PLA_2 (▼) with GmsPLA2-XIA-1 (○) and $GmsPLA_2$-XIB-2 (●) in mixed micelles of DLPC:Triton X-100 (ratio 1:4) with different percentages of DLPG.

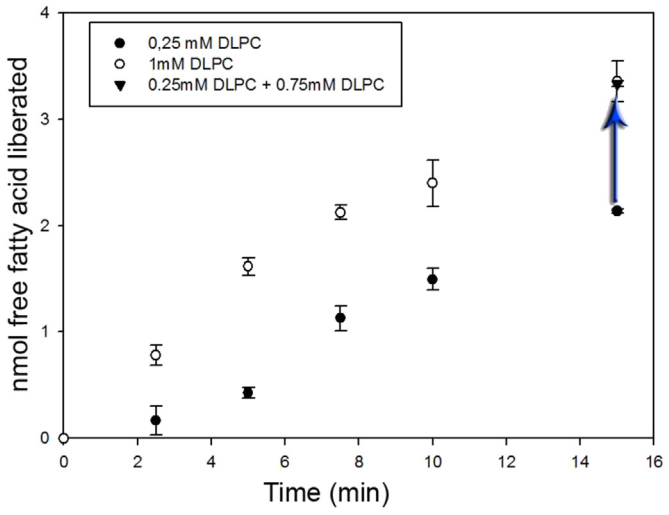

Fig. 5. Study of the mode of catalysis of $GmsPLA_2$-XIA-1. The arrow shows the jump in the activity measured at 15 min of time-course, after addition of 0.75 mM of DLPC substrate to the previous 0.25 mM to complete a final concentration of 1 mM of DLPC substrate (mixed micelles of lipid:Triton X-100, ratio 1:4) at 10 min of reaction time.

disturb the protein-lipid interaction, we decided to use the monolayer technique to study the catalytic activity at the air–water interface when the surface pressure was kept constant in the reaction course and using medium (C12) chain phospholipids (DLPC) as substrate. Since the products of the lipolysis are water soluble and do not accumulate at the interface, changes in the properties of the lipid interface do not occur (monolayer surface barostat or zero order kinetics, see Ref. [36]).

To our knowledge, it is the first time a plant $sPLA_2$ activity was studied using lipid monolayers. Both $sPLA_2$ activities were measured on monolayers of pure DLPC in the range in between 11 and 18 mN/m using the surface barostat to keep constant the interfacial quality during the reaction (Fig. 6). The activity profile assayed at different surface pressures (π) shows an optimum for activity at 16 mN/m and 13 mN/m for $GmsPLA_2$-XIA-1 and $GmsPLA_2$-XIB-2 respectively (Fig. 6A). The hydrolytic rates of $GmsPLA_2$-XIA-1 are very slow at lower or higher surface pressures than those assayed (data not shown). This decrease in activity at both extreme of surface pressure tested could be indicative of subtle modulation for this type of lipolytic enzyme with lateral packing of substrate. However, it is known that the presence of products in the subphase will lead to rearrangement of the membrane and cause much more effect on the hydrolytic reaction. The results observed were different in contrast with other animal $sPLA_2$s [55] or pancreatic $sPLA_2$ [53], and clearly show that these enzymes can be potentially active depending on the substrate organization and the quality of the interface.

The lag times as a function of surface pressure have the same behavior for both enzymes, being minimum at 13 mN/m (Fig. 6B).

$GmsPLA_2$s activity *vs.* enzyme concentration measurements using monomolecular films of pure DLPC, set at a constant lateral pressure of 13 mN/m (minimum lag times), shows a lineal dependence on enzyme concentration (Fig. 7A). As expected, lag times have the same behavior and decrease as the enzyme concentration increase, suggesting that more enzyme molecules reach the

Table 3
Effect of calcium on preserving activity of GmsPLA2 upon heating.

Condition assay	Activity (µmol min^{-1} mg^{-1})
Calcium 5 mM at 60 °C	35.2 ± 2.2
Without calcium at 60 °C	0.0
EDTA + Calcium 10 mM at 60 °C	0.0

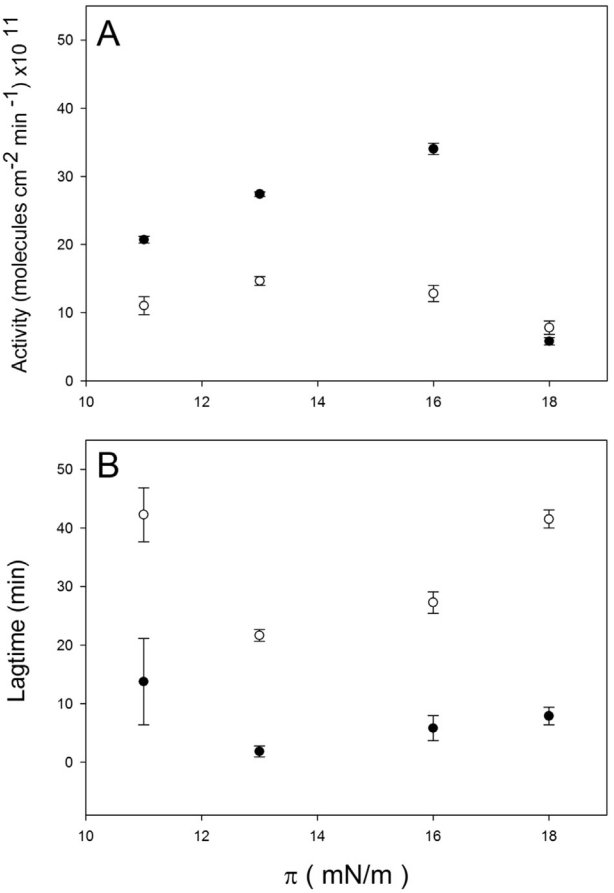

Fig. 6. Variation of *Glycine max* $sPLA_2$ activityas function of lateral surface pressure (π). $GmsPLA_2$s activities set at different surface pressure (A) with their respective lag times (B). $GmsPLA_2$-XIA-1, empty symbols (○); GmsPLA2-XIB-2, filled symbols (●).

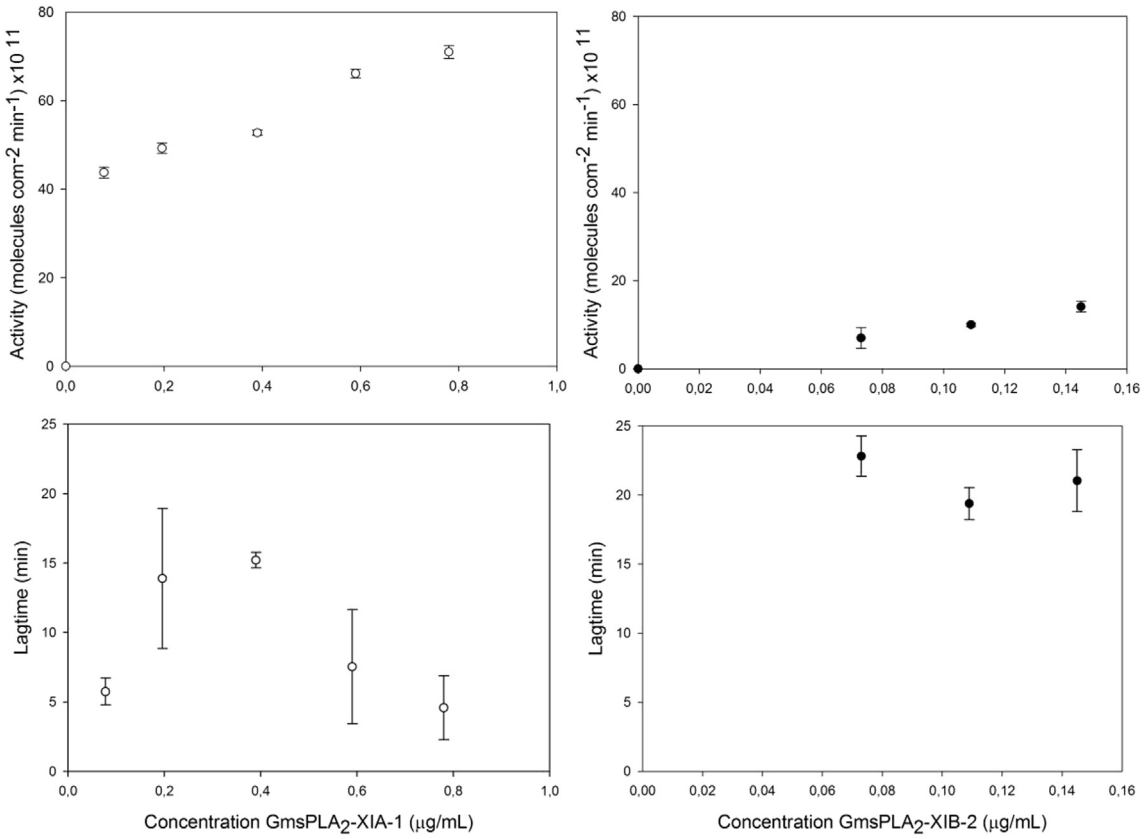

Fig. 7. *Glycine max* $sPLA_2$ activity into DLPC monolayers. Influence of enzyme concentration on $GmsPLA_2$s activities (upper part) with their respective lag times (lower part), measured in the zero order Langmuir trough covered by lipid monolayers of DLPC. Recombinant enzyme: $GmsPLA_2$-XIA-1, empty symbols (○) and GmsPLA2-XIB-2, filled symbols (●); (see text for definitions and details).

interface with a higher activity of the enzyme to hydrolyze substrate (Fig. 7B).

Both $GmsPLA_2$ enzymes displayed an optimum at 13–16 mN/m, a rather medium surface pressure, similar to that found for pancreatic $sPLA_2$ [56], if it is compared with the equivalent surface pressure estimated for phospholipids packed in bilayers calculated to be more than 31 mN/m by using theoretical approach [57]; or indirectly whereby lipolytic enzymes that are able to hydrolyze phospholipids of intact erythrocyte membranes hydrolyze lipid monolayers at above 31 mN/m [58]. The secretrory nature of $sPLA_2$ in plants has been elegantly tested checking the subcellular localization of *Arabidopsis* PLA_2s with constructs of *Ats*PLA_2s-green fluorescent protein fusions introduced into onion epidermal cells indicating that *Ats*PLA_2s are secreted and accumulated mainly in the interstices of the extracellular space [10]. On the other hand, the expression levels of *Ats*PLA_2s in *A. thaliana* is rather low and depends on the secreted isoforms in the different parts of the plants [42]. All these facts are indicating that the $sPLA_2$ isoforms in plants may have subtle differences in their action once they are secreted and reached the target interface or membrane domains compatible with their surface pressure profile.

4. Conclusions

In a recent work [3] we characterized five putative secretory phospholipase A_2 enzymes present in *G. max* ($GmsPLA_2$). One of them, $GmsPLA_2$-XIA-1, has been cloned, expressed and purified, and full activity was demonstrated [3]. In the present study, we have focused on the similarities and differences of this enzyme compared with enzymatic activity of the recently purified $sPLA_2$ from *G. max* $GmsPLA_2$-XIB-2, particularly in the optimum conditions for catalysis and substrate preference. $GmsPLA_2$-XIB-2 enzyme showed to encode a functional enzyme since hydrolyzes phospholipid substrate when offered as Triton/lipid mixed micelles, as it was previously reported for $GmsPLA_2$-XIA-1 [3]. Both recombinant enzymes are able to hydrolyze lipid monolayer and showed to be sensitive to the lateral packing of substrate. It is the first time that two plant $sPLA_2$s behavior is studied by using Langmuir monolayer as organized substrate. The different surface pressure profiles observed for the activity, confirmed that the behavior depends on the lipid organization at the interface.

The results obtained in the present work, allow us to conclude that optimal conditions for activity of these novel enzymes resemble that found for $sPLA_2$s from other plants or animals, except the optimum temperature, that showed to be higher.

The rates of hydrolysis for both enzymes are higher when the substrate are zwitterionic and diminish when the substrate phospholipid is negatively charged. Both enzymes showed head group specificity to phosphatidylcholine rather than phosphatidylglycerol. This fact may be functionally important when these secretory enzymes reach the target interface.

All this results suggest that neutral phospholipid increased the affinity of $GmsPLA_2$s in mixed Triton/substrate micelles since both enzymes showed a reduced apparent K_M value for PC compared with that obtained for PG. There was also an increase in the apparent V_{max} showing that the head group type is affecting the catalytic step. All these catalytic properties are consistent with $sPLA_2$s enzymes. Moreover, phosphatidylglycerol act as an inhibitor when mixed with phosphatidylcholine since a decrease in enzyme rates towards mixed ternary micelles are observed, contrary to the

well defined behavior reported for pancreatic PLA_2 that is more active when acidic substrate or amphiphilic negatively charged non substrate is present at the interface [20,53].

Another novel and kinetically important feature is that these are the first recombinant plant $sPLA_2$s that show a tendency to act in the hopping mode of hydrolysis since, in conditions of initial rates, the activity is sensitive to the enhance of substrate concentration and $GmsPLA_2$ is capable to hydrolyze the new bulk substrate added to the initial conditions of the assay by favoring the adsorption due to changes in the micelle/enzyme ratio (Michaelis–Menten behavior).

Since animal and plant $sPLA_2$s acts on similar substrates but in different membrane environments and with different biological consequences, remarkable differences in substrate specificity may be expected when a comparison is done.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2014.10.016.

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