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Isolation of phospholipase A_2 from soybean (*Glycine max*) seeds The study of its enzymatic properties

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Abstract

A protein with phospholipase A_2 (PLA_2) activity was isolated from soybean (*Glycine max*) seeds. The affinity column chromatography techniques by using Cibacron Blue and immobilized substrate onto Eupergit C, were important steps in the purification protocol. The electrophoretic mobility showed by the purified enzyme, agreed with a molecular mass of 14 kDa. The PLA_2 activity against liposomes was determined by measurement of apparent absorbance changes at 340 nm. Also, high performance thin layer chromatography (HPTLC) analysis confirmed selective hydrolysis at the *sn*-2 position of soybean phospholipids. PLA_2 exhibited millimolar calcium dependence and had slightly alkaline pH optimum. The enzyme was stimulated by auxins and completely inactivated by ammonium sulfate. Furthermore, it was irreversibly inactivated by *p*-bromo-phenacyl bromide, the specific inhibitor of secretory PLA_2s. In addition, *Glycine max* PLA_2 showed high stability against heat treatment and organic solvents. The enzyme showed activity toward multilamellar vesicles of soybean phospholipids, with a $V_{max} = 950$ U/mg and a $K_M = 0.78$ mM. The hyperbolic behavior observed was coherent with a *hopping* mode of action, one of the two characteristic interfacial mechanisms of PLA_2s. All these data agree with the expected properties for a secretory PLA_2 being the first soybean enzyme of this type. The new PLA_2 developed lipolytic activity on a water in oil microemulsion reaction system, which is suitable for lysophospholipid production. These lysoderivatives are valuable biosurfactants for food and pharmaceutical industries.

Keywords: Phospholipase A_2; Soybean; Purification; Properties

1. Introduction

Phospholipase A_2 (PLA_2, phosphatide 2-acyl-hydrolase, EC 3.1.1.4) is a lipolytic enzyme that specifically hydrolyzes glycerophospholipids at the *sn*-2 position to yield free fatty acids and lysophospholipids. The phospholipase A_2 superfamily consists of a broad range of lipolytic enzymes from different sources [1]. The industrial applications of phospholipases are described and recently reviewed [2]. The enzymatic method is the only way to obtain *sn*-2 lysoderivatives from phospholipids because the bioconversion reaction is stereospecific. Such lysoderivatives are powerful biosurfactants with wide application field in food and pharmaceutical industries [3].

In plants, the products of PLA_2 activity stimulate several membrane enzymes and have been proposed to serve as second messengers in signal transduction. Also, the interaction with pathogen elicitors and the subsequent host defense response involve the activation of PLA_2 [4]. The first PLA_2 from plants purified until homogeneity and completely characterized is an enzyme present in developed elm seed (*Ulmus glabra*) in 1998 [5]. Based on sequence data and biological properties, plant PLA_2s are classified into two groups: the low molecular weight secretory PLA_2s (sPLA_2s) with histidine active site, known as group XI; and the patatin-like PLA_2s (serine active site) [6]. One of the principal differences between PLA_2 from different sources is related with their action on substrates at organized lipid–water interfaces. An explanation about this may be found in the diversity of the natural environments in which these enzymes function [7]. Nevertheless, little is known about the enzymatic properties of plant PLA_2s. At present, no PLA_2 enzyme have been purified and studied from soybean seeds, an abundant biomass that

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could serve as a PLA_2 source for biotechnological purposes. Therefore, the aim of this work was to study the biochemical properties and also to explore the technological potential of soybean (*Glycine max*) PLA_2.

2. Material and methods

Soybean seeds from 2004 harvest were provided by INTA Experimental Agricultural Station (Marcos Juárez, Argentina). Eupergit® C was a gift from Pharma Polymers Röhm Gmbh, Darmstadt Germany. Sephadex® G-50 was from Pharmacia. Cibacron Brilliant Blue FN-G was a gift from Ciba Especialidades Químicas S.A. Argentina. A commercial preparation of soybean lecithin (SL) was used as phospholipid source: this contained phosphatidylcholine (PC 33.4%), phosphatidylethanolamine (PE 28.6%), phosphatidylinositol (PI 29.6%) and phosphatidic acid (PA 8.3%) [8]. For microemulsion assays, pure phosphatidylcholine obtained by ethanol extraction from SL, was used [9]; *sn*-2 lysophosphatidylcholine was obtained from Sigma.

2.1. Assay of PLA_2 activity

A spectrophotometric method was used for PLA_2 activity assays. Measurements at λ 340 nm were carried out by using a Hewlett–Packard HP 8452 diode array spectrophotometer and running the kinetic software HP 89,532 K Multicell Kinetic UV–vis. The assay medium contained 0.4 mg of SL dispersed in 2 mL tris–HCl buffer pH 8 and 3 mM $CaCl_2$. Eluates (0.5 mL) from the affinity chromatography purification step were added resulting in a protein concentration between 0.15 mg/mL and 0.25 mg/mL at the reaction medium. The PLA_2 lytic action reduced the size of large initial phospholipid aggregates (multilamellar vesicles) by forming micelles, smaller structures arranged by lysophospholipids. As a consequence, the apparent 340 nm absorbance gradually decreased as hydrolysis advanced. In this method, PLA_2 activity (*U*) is defined as the amount of protein that produces a change of 10^{-3} of absorbance units in 1 min [10]. Also, in order to confirm hydrolysis reaction at the *sn*-2 position, the PLA_2 activity was measured by high performance thin layer chromatography (HPTLC). Briefly, the enzyme extract was incubated with the medium for 15 min at 40 °C and after this, an aliquot of 0.5 mL was extracted with a chloroform–methanol 1–1 mixture. A sample of 10 μL was run together with lysophosphatidylcholine (Sigma) as standard in a HPTLC Desaga horizontal chamber using $CHCl_3$–MeOH–H_2O 65–25–4 as elution solvent system [8].

2.2. Isolation of PLA_2 from soybean seeds

Soybean seeds were soaked overnight and disrupted by aqueous grinding in a homogenizer device. The slurry was diluted by distilled water (30%) and the homogenate was heated at 95 °C for 5 min to eliminate proteases and then centrifuged at 3500 rpm for 10 min to separate the insoluble material. The supernatant was treated with hexane to remove lipids and also phospholipids as endogen substrates. It was followed by addition of frozen acetone, maintaining the medium below 0 °C for 30 min to promote protein precipitation. The medium was decanted, the insolubilized proteins were redissolved in tris–HCl buffer pH 7.0 and the sample was centrifuged at 12,000 g. The supernatant was acidified (pH 4.4) with diluted HCl solution and centrifuged at 3500 rpm for 10 min. to obtain a clarified extract that showed high PLA_2 activity.

The clarified extract was applied to an affinity column containing Cibacron Brilliant Blue FN-G immobilized on Eupergit C. To prepare this adsorbent system, Eupergit C (2 g) was place in contact with Cibacron Brilliant Blue FN-G (10 mg) in borate buffer pH 8.2 (8 mL) for 24 h. The binding of Cibacron Blue to Eupergit C was evaluated by the difference in 636 nm absorbance, between the original solution of dye and the resulting supernatant from fixation process. The solid was vacuum filtered, washed with distilled water and 2 M NaCl solution, and the non-reacted oxirane groups of Eupergit were blocked with tris–HCl 5% pH 8.5 for 16 h [11]. The Eupergit C–Cibacron Brilliant Blue FN-G complex was packed in a 20 cm × 1.3 cm column, giving a 5.5 cm bed height [12]. It was carried out in three stages elution, by applying 0.01 M, 0.05 M and 0.1 M KCl.

For substrate affinity chromatography, phospholipid aggregates were prepared by dispersing SL (2 mg) in 10 mL borate buffer pH 8.2. This dispersion was mixed with Eupergit C and treated in the same manner as was described for Cibacron coupling. A column containing the SL-Eupergit adsorbent was loaded with a pool of fractions obtained from Cibacron affinity chromatography. Elution was carried out by a phospholipid dispersion of the same concentration as that used for adsorbent system preparation.

Fractions collected from the Cibacron Blue (CB)–Eupergit affinity column were further purified by gel filtration chromatography. The pooled fractions were previously concentrated on Amicon 1000 kDa cell from 10 mL to 1 mL. Sephadex G-50 was packed in a 140 cm × 1.5 cm column, giving a 120 cm bed height. Fractions of 1.2 mL were collected by using a Bio-Rad® fraction collector device.

2.3. SDS–PAGE assays

Protein fractions from CB–Eupergit affinity chromatography were concentrated by ultrafiltration in an Amicon 1000 kDa MWCO cell. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with 7.5% and 15% (w/v) acrylamide gels by using a vertical LKB 2297 Bio-Rad electrophoresis equipment. The electrolytic buffer was: 25 mM tris, 192 mM glycine and 0.1% SDS at pH 8.3. The composition of the sample buffer was: 0.1 M tris–HCl, 10% glycerol, 2% SDS, 5% 2-mercapto-ethanol and 0.008% bromophenol blue. Molecular weight protein markers (14–90 kDa) were run simultaneously for each electrophoresis gel. The protein bands were visualized by 0.2% Coomasie Brilliant Blue R-250 in 40% methanol and 10% acetic acid. In some cases, when it was necessary silver staining was used. The following analytical grade reactives and solutions were used: methanol, acetic acid, formaldehyde, 50% (v/v) ethanol, 0.05% (v/v) $Na_2S_2O_3{\cdot}5H_2O$, 0.2% (w/v) $AgNO_3$ in 0.75% (v/v) formaldehyde, 6% (w/v) Na_2CO_3 in 0.05% formaldehyde and 0.004% $Na_2S_2O_3{\cdot}5H_2O$ [13].

2.4. Microemulsion assays

Soybean lecithin is a phospholipid mixture (see above) and since only phosphatidylcholine is well soluble in alcohols, such a mixture was not useful to make microemulsions that include butanol or other medium chain alcohols. For this reason, PC was obtained from SL (20 g) by ethanol extraction (90 mL) [9]. The microemulsion system was prepared by adding butanol (3 g) to soybean PC (6 g) with magnetic stirring until a homogeneous medium was formed. The aqueous phase (1.6 mL) included the enzyme and 30 mM calcium activator. Therefore, the ternary system had the following composition: PC–butanol–water 56.6–28.3–15.1%. The progress of the hydrolysis reaction at 38 °C was followed by a pH-Stat technique (pH 8.0) using 0.5 M ethanolamine in butanol solution as titrator and a Hach digital microtitrator device [23].

3. Results and discussion

3.1. Purification of PLA_2

PLA_2 activity was obtained by measuring the apparent 340 nm absorbance (see Section 2). Nevertheless, the *sn*-2 hydrolysis was confirmed by HPTLC using lysophosphatidylcholine Sigma as standard, not detecting hydrolysis at the *sn*-1 position. Several purification techniques were suitable because of the extreme stability showed by *Glycine max* PLA_2. Heating at 95 °C, precipitation with acetone and hexane treatment are extremely harsh conditions that denature most enzymes. However, such purification steps were very useful in this case, in agreement with the knowledge about the robustness of secretories PLA_2 [5]. Also, the precipitation of homogenates at pH 4.4 was an effective path, since it allowed the obtainment of completely clarified extracts. Furthermore, it is known that the isoelectric point of majority soybean proteins is near to pH 4.4 and in principle only small globular proteins would

Table 1
Purification of soybean PLA_2: effect of substrate affinity chromatography

	Protein			Enzyme				
Purification step	Vol. (ml)	Conc. (mg/ml)	Total mass (mg)	Activity (U/ml)	Spec. act. (U/mg)	Total act. (U)	Yield (%)	Purif. factor
Centrifugation 12,000 × *g*	300	33	9900	2	0.06	600	–	1
Supernatant pH 4.4	250	2.1	513	144	70.4	36,060	100	1173
CB affinity chrom.	45	0.78	35.1	154	198	6,950	19.3	3300
Liposome rechrom.	1.5	0.056	0.084	22.3	398	33.5	0.09	6637

Protein concentration (mg/mL) was obtained by application of Layne equation, measuring 280 nm and 260 nm absorbance. The PLA_2 activity (*U*) is the amount of protein that produces a change of 10^{-3} AU/min.

remain in solution [14]. High PLA_2 activity value in supernatants from acid precipitation and a great purification factor compared with 12,000 × *g* centrifugation stage, are showing the purification effectiveness (see Table 1). Also, the strong increase in total activity (*U*) in an early stage of purification represented a recovery largely greater than 100%. This suggests that the crude extract contained an inhibitor that could itself be precipitated or dragged out by protein aggregation. This was also observed when a higher speed centrifugation (111,000 × *g*) was applied after the 12,000 × *g* centrifugation step, although no precipitation at pH 4.4 was carried out. This fact was systematically observed in all the experiments and was still more important when acid precipitation was performed (see Table 1). Probably, a large protein molecule acting as an inhibitor is interfering the *Glycine max* PLA_2 activity against multilamellar vesicles.

To perform affinity column chromatography, the triazine dye Cibacron Blue was coupled to Eupergit C support. From the known reactive properties of Eupergit C, the ligand fixation would carry out through nucleophilic groups such as amines. These groups bind by soft conditions to highly reactive oxiranes attached to polymer matrix [15]. The fixation of Cibacron Blue was almost complete at the experimental conditions, as it was confirmed by comparing absorbance measurements (λ 636 nm) from the original dye solution and the final supernatant. Neither neutral nor slightly alkaline solutions removed the dye from the matrix during column operation. It is known that the affinity between Cibacron and proteins is based on ionic and hydrophobic combined interactions [16] and that PLA_2 reversibly binds the Cibacron Blue dye with a $K_d = 2.10^{-6}$ M [27]. In our case, a stepwise elution by increasing the ionic strength from 0.01 M to 0.1 M KCl was an adequate experimental condition for purifying soybean PLA_2.

The major portion of non-desired proteins was eluted from the load and wash steps of the affinity column chromatography. None PLA_2 activity was recovered by elution with 10 mM KCl, but five or six active fractions (1.5 mL each one) were collected from subsequent elution with 50 mM KCl. Further elution with higher ionic strength didn't lead to other PLA_2 active fractions. Elution profiles from different samples showed good reproducibility (Fig. 1).

3.1.1. *Affinity substrate chromatography*

Immobilized liposomes were the stationary phase in substrate affinity chromatography [17]. In principle, it is not a specific adsorbent, since other enzymes could interact with bilayers. Nevertheless, in the case of phospholipases, the adsorption to interfaces is conditioning the proper behavior of the enzyme on catalysis [7]. Twelve milliliters of pooled fractions from Cibacron affinity chromatography were loaded onto LS-Eupergit C column. Neither an increase of ionic strength by step elution with KCl, nor elution by sodium sulfate [18], was successful. The elution by substrate (0.26 mM SL) allowed to collect five fractions (1.5 mL each one) with high PLA_2 specific activity (see Table 1). Fraction number 27 had the highest specific activity (398 U/mg) value, corresponding to a purification factor of 6637. Similar performance was obtained applying rechromatography CB–Eupergit, suggesting that no further purification could be achieved. The SDS–PAGE silver revealed gel of fraction number 27 showed the presence of only two bands: one of a mobility of 14.2 kDa (calculated from R_f proteins values, log molecular weight = *k*) and also other band of about 26 kDa. The mobility of 14.2 kDa was also found in fraction number 13 (the second more important specific PLA_2 activity, 142 U/mg), together a band of about 18 kDa. Since only the protein of 14.2 kDa was present in both fractions, the PLA_2 activity must be related with this mobility. The elution strategy by using liposomes was based on a *hopping* mode of PLA_2 action suggested by the kinetic behavior [7], as it was discussed below (kinetic parameters paragraph and Fig. 2). By this assumption, a relatively high value of K_d allows the enzyme to be desorbed from the interface during the elution [19].

A pool of fractions obtained from CB–Eupergit chromatography column was further purified by gel filtration using Sephadex G-50. The following elution profile was obtained (Fig. 3).

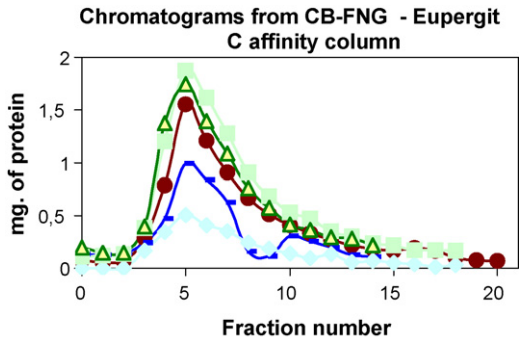

Fig. 1. Elution profiles from CB–Eupergit affinity column. Plots refer to independent experiments with different sample seeds and different protein mass loaded.

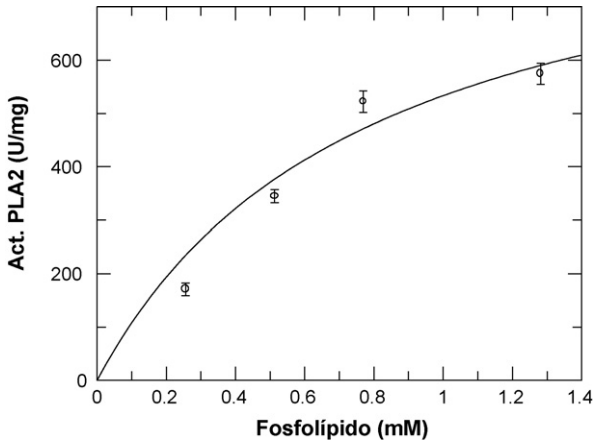

Fig. 2. Kinetics parameters of PLA_2. V_{max} = 950 U/mg; K_M = 0.78 mM.

SDS–PAGE analysis of fractions 9, 11 and 13 (Fig. 3) having PLA_2 activity, showed a gel enriched on proteins of 14–15 kDa accompanying principally by bands of 22–23 kDa relative mobility (Fig. 4).

3.2. SDS–PAGE electrophoresis and estimation of molecular mass

SDS–PAGE analysis shows (Fig. 4) electrophoretic gels obtained from Cibacron Blue affinity chromatography fractions and Sephadex G-50 exclusion chromatography. In order to estimate the molecular mass, SDS–PAGE was run together against molecular weight markers (MWM) obtaining a relative mobility of 14 kDa. The affinity lane shows a rich zone corresponding to about 14–15 kDa molecular sizes and also several other bands. After Sephadex G-50 column, a clear band of 14 kDa appears in lanes corresponding to fractions 9, 11 and 13.

3.3. Characterization of PLA_2 activity

Physical characterization study from SDS–PAGE electrophoresis (Fig. 4) showed that the protein under research had an approximate molecular mass of 14 kDa.

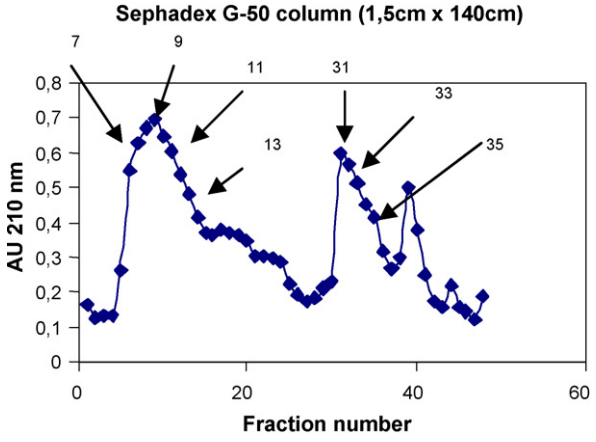

Fig. 3. Chromatogram obtained from Sephadex G-50 column. The marked fractions were run in SDS–PAGE electrophoresis (see Fig. 4).

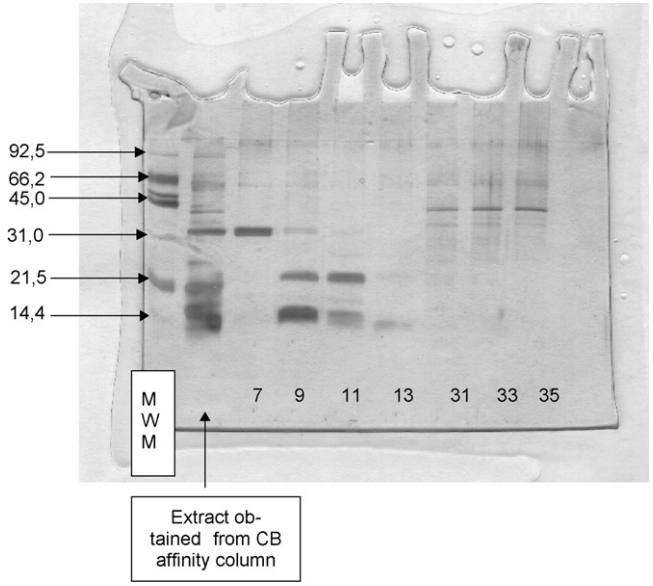

Fig. 4. SDS–PAGE analysis of a fraction collected from affinity column, and subsequently Sephadex G-50 gel filtration applied to the same sample (see chromatogram from Fig. 3). The first lane on the left shows the molecular weight markers (MWM).

PLA_2 *Glycine max* showed maximum activity at pH 7.8–8.0 (Fig. 5). Considering the molecular mechanism, a nitrogen atom of histidine should have the electron pair free to activate and orientate the water molecule to the subsequent nucleophilic attack on the hydrolysis reaction [20]. Therefore, PLA_2s having histidine group at the active site need slightly alkaline pH to arrange the catalytic center.

The temperature for an optimum activity was in the range 38–40 °C. Probably, the enzyme achieve a more favorable interaction state when the topography of the phospholipid interface have irregularities. It is known that near the phase transition temperature, both the gel and liquid crystalline phases co-exist and a large increase in packing defects is found at the interface [21].

Activity was linearly dependent on the enzyme concentration until 0.40 mg/mL. Nevertheless, in this work, PLA_2 was used in a concentration range of 0.15–0.25 mg/mL in the reaction medium.

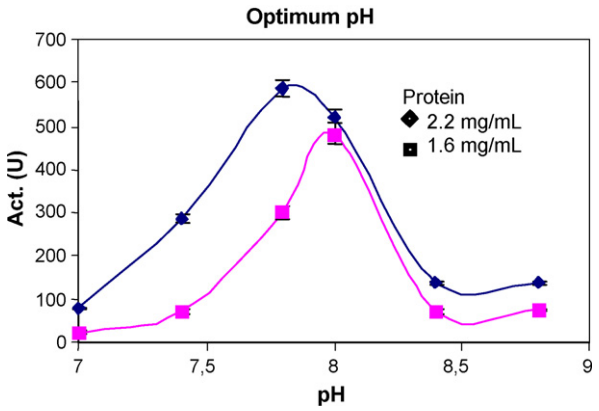

Fig. 5. pH dependence of PLA_2 activity.

3.4. Specific biochemical properties of PLA_2

PLA_2 *Glycine max* had millimolar calcium requirement and showed maximum activity by addition 3 mM calcium in the reaction medium. In order to avoid interference of endogenous calcium, EDTA solution was added. In order to stabilize the oxyanion at the intermediate transition state of the hydrolysis mechanism, it is known that PLA_2s have aspartate groups located at the calcium loop. These groups participate on cation coupling [20].

Bromo-phenacyl bromide (BPB) is a specific inhibitor of PLA_2 that covalently links to histidine groups at the active site of PLA_2 [22]. Complete inhibition was observed when 100 μL of BPB 100 mM was added to 0.5 mL of active PLA_2 fractions, while the addition of 20 μL only showed partial inhibition. The reaction was pH dependent. At pH below 6, BPB didn't affect the activity of the enzyme. This mean, a histidine group with pK 6.1 is involved. The inactivation was developed by keeping the enzyme solution in buffer pH 8 during 6 h. The results are reported in Fig. 6.

Auxins are phytohormones derived from indol-acetic-acid (IAA) and it is known that PLA_2 is a mediator in plant growing induced by these compounds [28]. In this work, the enzyme from affinity chromatography step, showed the highest stimulation (80%) when 4 mM auxin was present. No stimulation was observed when auxins were added in micromolar concentration. Similar results were obtained assaying PLA_2 from porcine pancreas (Novo). Although in the biological medium the auxin concentration is present at a micromolar order, the stimulation could be a consequence of a local accumulation of auxin at the protein interaction site.

As it is current for most proteins, the addition of ammonium sulfate (70% saturation) promoted soybean protein precipitation from affinity chromatography fractions. But, in this case, no PLA_2 activity was detected after salt treatment. Probably, at high concentration, the sulfate anion couples cationic sites of the enzyme located at the substrate union site. The binding site of $sPLA_2$ is formed by cationic residues, mainly lysine groups, that anchoring the enzyme to the anionic vesicle surface [18].

3.5. Kinetics parameters

The enzyme assays and activity measurements were carried out according to Section 2. The enzyme kinetic parameters (maximum velocity, V_{max} and Michaelis constant, K_M) were obtained (Fig. 2) by applying GraFit for Windows program.

Absorbance 340 nm was linearly dependent on substrate concentration until 1.6 mM SL phospholipid. The hyperbolic behavior (Fig. 2) is indicating that PLA_2 acts in the *hopping* or jumping mode [7], since the activity level was sensitive to the bulk substrate concentration. In principle, in the *hopping* mode, PLA_2 is able to hydrolyze all the phospholipid present at the aggregate developing a highly efficient hydrolytic process. Otherwise, in a *scooting* mode of action, no Michaelian behavior could be observed, because at catalytic concentrations, PLA_2 remains adsorbed during the catalytic cycle and could only hydrolyzes the outer phospholipid monolayer. Further increase in phospholipid concentration not enhances the enzyme activity in the *scooting* mode [7]. From the maximum velocity (950 U/mg) and Michaelis constant (0.78 mM) it can be calculated the performance constant or catalytic efficiency: V_{max}/K_M. In the experimental conditions, the catalytic efficiency for PLA_2 *Glycine max* was 1217 U/(mg mmol).

3.6. Extent of hydrolysis

It was used a 2% SL aqueous dispersion, working at 40 °C. The initial pH was 8.5 and it was kept during the overall reaction by adding 0.5 N NaOH. The reaction was followed by a pH-Stat method and started by adding calcium ion (Fig. 7).

After 4 h, 60 μmol of fatty acid was generated. This amount represents a hydrolysis degree of 4.7%. This relatively poor performance of PLA_2 could be related with the strong tendency of lecithin to form highly rigid films known as liquid crystal structures [23].

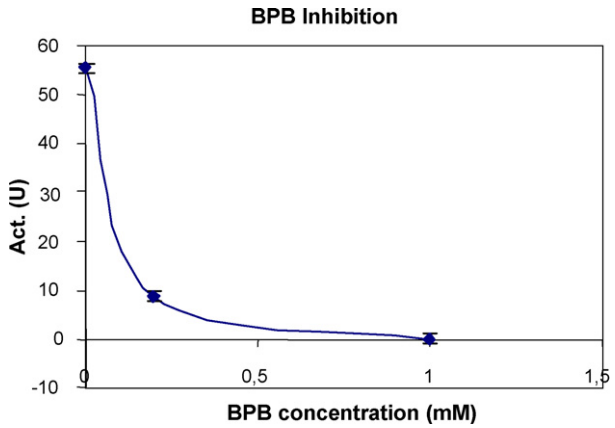

Fig. 6. PLA_2 activity vs. BPB concentration.

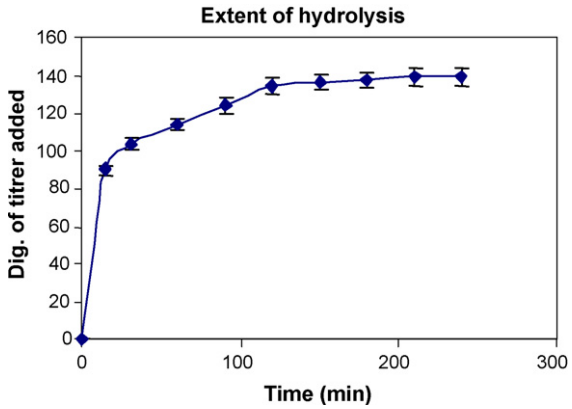

Fig. 7. 0.5 N NaOH added to the mixture in the extent experiment. One digit is equivalent to 1.25 μL. Control assay without enzyme was carried out.

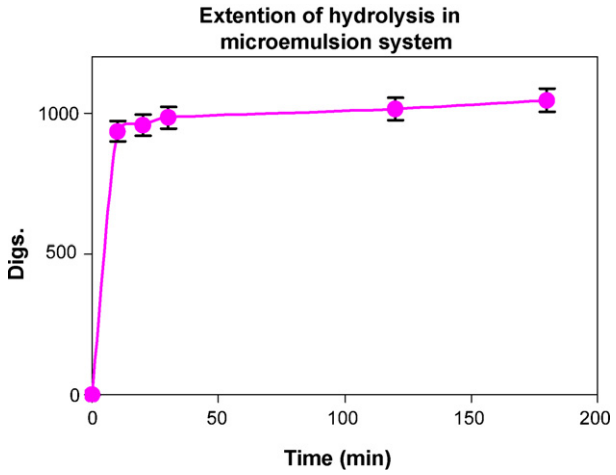

Fig. 8. Extent of hydrolysis in microemulsion.

3.7. Microemulsion assays

Soybean PC was mixed with butanol and water to obtain a ternary microemulsion system as it was described in Section 2. This system means water in oil (w/o) emulsion type, since water proportion is lower than organic phase. In this work, butanol was used because it gives an extended isotropic zone and consequently allows to use a wide phospholipid concentration range [23,24].

The hydrolysis reaction was followed by pH-Stat (Fig. 8).

A total of 1.3 mL (1045 digits) of 0.5 N ethanolamine butanol solution was consumed after 3 h of reaction, giving 8.5% hydrolysis level. This percentage was calculated on the basis of FFA milliequivalents. Almost all hydrolysis reaction take place within 10 min elapsed, since a hydrolysis level of 8% was reached at this time. Taking into account the high initial phospholipid concentration (PC–butanol–water 56.6–28.3–15.1%), that reaction level represented an important mass of lysophospholipid in the final product. It is known that in the case of reverse micelles, the inclusion of medium chain alcohols such as butanol in the outer layer (between fatty acid chains) enhance its curvature, forming smaller supramolecular structures [24]. Moreover, the polar region of phospholipids becomes exposed to hydrolysis by PLA_2 molecules entrapped within the aqueous core of reverse micelles. This experimental design could be defined as a micro reaction system, which allowed PLA_2 to work in a more favorable reaction ambient [25]. Conceptually, catalysis on w/o microemulsion could be compared with enzyme action in the crowded environments of living cells, where biological macromolecules occupy a significant fraction of the medium. In this condition, there is a high probability of an effective molecular interaction. The enzyme confinement designs could optimize the biocatalytic performance by mimic the *in vivo* conditions [26].

4. Conclusions

In this work, we identified and partially purified an enzyme from soybean seeds having PLA_2 activity. In our knowledge, it is the first description of a secretory PLA_2 from soybean (*Glycine max*) seeds. Affinity chromatography on Cibacron Blue and substrate affinity chromatography were crucial steps of the purification protocol, allowing PLA_2 to be purified about 6600 times. The biocatalyst showed high heat stability, was resistant to low pH and organic solvents but was inactivated by ammonium sulfate. The enzyme was complete and irreversibly inactivated by bromo-phenacyl bromide, the specific histidine PLA_2 inhibitor. Also, the millimolar calcium requirement, a molecular mass estimated in 14 kDa by SDS–PAGE and its catalytic properties are consistent with a $sPLA_2$ enzyme. The ability of *Glycine max* PLA_2 to work in the more efficient *hopping* mode of action resembles the characteristic behavior of known animal or snake venoms $sPLA_2$s. Such interfacial behavior and the performance developed in w/o microemulsion systems, indicate that PLA_2 from soybean could be successfully applied on hydrolytic processes for lysophospholipid production. Also, this work will yield new information that could increase the knowledge about PLA_2 action in plant tissues.

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