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A constant area monolayer method to assess optimal lipid packing for lipolysis tested with several secreted phospholipase A_2

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ABSTRACT

We present an analysis of lipid monolayer hydrolysis at a constant area to assess the optimal lateral surface pressure value (Π_{opt}) and thus, the surface packing density of the lipid, at which the activity of a given lipolytic enzyme is maximal. This isochoric method consists of a measurement of the decrease down to zero of the Π_{opt} of phospholipid substrate monolayer due to continuous hydrolysis using only one reaction compartment. We performed the comparison of both approaches using several commercially available and literature-evaluated $sPLA_2$s. Also, we characterized for the first time the profile of hydrolysis of DLPC monolayers catalyzed by a $sPLA_2$ from *Streptomyces violaceoruber* and isoenzymes purified from *Bothrops diporus* venom. One of these viper venom enzymes is a new isoenzyme, partially sequenced by a mass spectrometry approach. We also included the basic myotoxin $sPLA_2$-III from *Bothrops asper*. Results obtained with the isochoric method and the standard isobaric one produced quite similar values of Π_{opt}, validating the proposal. In addition, we propose a new classification parameter, a lipolytic ratio of hydrolysis at two lateral pressures, 20 $mN \cdot m^{-1}$ and 10 $mN \cdot m^{-1}$, termed here as $LR_{20/10}$ index. This index differentiates quite well "high surface pressure" from "low surface pressure" $sPLA_2$s and, by extension; it can be used as a functional criterion for the quality of a certain enzyme. Also, this index could be added to the grouping systematic criteria for the superfamily proposed for phospholipase A_2.

1. Introduction

Secreted phospholipases A_2 ($sPLA_2$s) are small (13–15 kDa), highly disulfide-linked enzymes that hydrolyze the *sn-2* ester bond of glycero-phospholipids, requiring Ca^{2+} in the millimolar range for activity [1,2]. These enzymes show interfacial activation, i.e., their specific activity is several times greater when the substrate is organized as a multimolecular structure than when it is found as a monomer [3]. In contrast to micellar or vesicular lipid presentation, the monolayer status renders all the lipid substrate equally accessible to the enzyme, with the polar head groups towards the aqueous phase and the hydrocarbon chains towards the air [4]. Besides, the monolayer technique allows a suitable control of the substrate surface density (or surface pressure) under hydrolysis. Moreover, other parameters of the quality of the interface are easily monitored in the monolayer state, such as the difference in electric surface potential across the interface [4,5].

The mean molecular area (MMA) of a substance at the air/water interface is defined as the total monolayer area divided by the amount of molecules at the interface. Its reciprocal is the surface density N_S, which is directly related to the lateral surface pressure (Π). This parameter is computed as the surface tension difference from the pure liquid/air interface (an aqueous buffer solution in our case) and the same liquid interface covered with a monolayer of the substance of interest. The variation of Π with N_S or MMA gives a characteristic curve at constant T, known as Π-A isotherm, for any lipid mixture which forms stable insoluble monolayers in the time of measurement [6].

Among the most common in vitro features reported for any newly discovered $sPLA_2$, are the amino acid sequence, Ca^{2+} requirement, activity and stability dependency on pH, toxicity as in vivo effects, and enzyme preferences for lipid head-group when presented as micelles or vesicles [7–11]. Less frequently, data on activity vs. surface density of lipid substrate is also presented in the literature for some $sPLA_2$ [7,12–17], although rather scarcely for new $sPLA_2$. Regardless of

Abbreviations: ACN, acetonitrile; CHCA, hydroxycynnamic acid (α-cyano-4-hydroxycinnamic acid); DLPC, dilauroyl phosphatidylcholine; $LR_{20/10}$, lipolytic hydrolysis ratio taken at 20 and 10 $mN \cdot m^{-1}$ measured by either isochoric or isobaric mode; MALDI, matrix-assisted laser desorption ionization; MMA, mean molecular area; N_S, molecule surface density; TOF, time of flight; $sPLA_2$, secreted phospholipase A_2; Π_{opt}, optimal surface pressure for $sPLA_2$ lipid hydrolysis using pure DLPC as substrate; *N.n, Naja naja*; P.p, pig pancreas; *N.m, Naja mossambica mossambica*; Π_{cutoff}, maximal surface pressure of DLPC monolayer at which no activity of enzyme is observed; Π_i, the initial surface pressure of DLPC monolayer before $sPLA_2$ enzyme injection; TFA, trifluoroacetic acid; τ, lag time.

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the biological relevance of this parameter, it has been shown that there could be remarkable differences in the optimal lateral pressures (Π_{opt}) for activity between these enzymes as well as in the lag or induction time (τ) dependency on Π, which in some cases correlated with their ability to attack red cell membranes [18] or with the inhibition of the clotting process [16]. From our own measurements and from a perusal of the literature it is evident that Π_{opt} is very reproducible for a specific $sPLA_2$ and independent of the laboratory and trough geometry assayed. Therefore, we suggest that a numerical value that represents the hydrolysis rate of a specific neutral lipid, such as DLPC, be used as an additional property to the classification criteria proposed by the group of E. Dennis [1] and followed by many researchers. This property could be expressed as a ratio of hydrolysis rates at two Π, after enzyme concentration standardization.

The standard method used to evaluate Π_{opt} at constant Π employs the surface barostat described by Dervichian [4]. The key point for its use is that the hydrolysis products of the $sPLA_2$ catalyzed reaction are rapidly released from the monolayer into the aqueous phase producing a continuous decrease in Π. When the lipid film is laterally compressed to keep Π constant, i.e. by using the surface barostatic mode, the reaction progress is reflected in a continuous change in the monolayer surface area. This method was refined by Verger and De Haas with the introduction of a second compartment next to the one where the enzyme is injected, to act as a reservoir of substrate lipid [19]. Both compartments are interconnected by a shallow and narrow surface channel. By adjusting the monolayer area as the reaction proceeds, zero-order kinetics for substrate is obtained, which is reflected by a linear decrease of area vs. time after some induction or lag time. This approach is not restricted to phospholipases, e.g. lipase kinetic studies on monolayers are also reported since the beginning of these types of studies [20,21].

The main difference between the isobaric and isochoric methods is that with the latter one, i.e. at a constant area, the lipid packing is allowed to decrease by substrate hydrolysis and product desorption. In this paper, we demonstrate that by using 1,2 dilauroyl-*sn*-phosphatidylcholine (DLPC) as substrate in a isochoric measurement, the estimation of the Π_{opt} and the overall shape of the hydrolysis rate vs. Π curves for the enzymes tested are in complete agreement to those obtained by the barostatic technique within the experimental error. This is possible by following the full time course of the reaction until surface pressure drops to zero. We did this analysis for commercially available $sPLA_2$ from pig pancreas, for $sPLA_2$ from venoms of Indian cobra (*Naja naja*), Mozambique spitting cobra (*Naja mossambica mossambica*) and bee (*Apis mellifera*). We tested as well, non-commercial $sPLA_2$, which included one $sPLA_2$ from a prokaryote source, which had not been tested before (from *Streptomyces violaceoruber*), described in 2002 [22]; three $sPLA_2$s found in *Bothrops diporus* venom, two of which were recently cloned, expressed in *Escherichia coli* and renatured by us [17]; and the D49 basic myotoxin III $sPLA_2$ present in *Bothrops asper* venom, described to act synergistically with the K49 non-lipolytic myotoxin II [23,24].

2. Materials and methods

2.1. Reagents, solvents and enzyme sources

1,2-Dilauroyl-*sn*-glycero-3-phosphocholine (DLPC, 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine) was purchased from Avanti Polar Lipids Co. (USA) and used without further purification. Stock (4 mM) and working solutions (0.1 mM) were dissolved in chloroform using HPLC grade solvents (Merck, Argentina) and stored at −20 °C in Pyrex glass tubes (Corning), sealed with Teflon coated caps. Lipid solution handling and spreading onto aqueous phase were performed using Hamilton syringes.

Commercial venom phospholipases were from Sigma Chemical (USA). They were from Cobra species (*N. naja* and *N. mossambica mossambica*) and Bee (*A. mellifera*), catalogue number: P-6139, P-7778 and P-9279, respectively. The three isoforms from *B. diporus* venom were purified by using the chromatographic steps previously reported [13], denoted as *B. diporus* $sPLA_2$ I, II and III with accession numbers from UniProtKB/Swiss-Prot I2DAL4, I2DAL5 and C0HJP9 respectively. *B. asper* basic PLA_2 myotoxin III (accession number GenBank P20474) was essentially purified as described [23,24]. Non-venom $sPLA_2$ sources were from pig pancreas (Lecitase™ 10L, Novo Nordisk, Denmark) and bacterial suspension (LysoMax™, Danisco, Denmark). These last two were a kind gift from Dr. Ricardo Madoery. Enzyme concentration in storage stocks (in ammonium formate 10 mM, pH = 4.5) was estimated by densitometry of the 13–16 kDa band in a non-reducing SDS-PAGE gel stained with Coomassie Brilliant Blue (R250) using 4 points of a acetylated bovine serum albumin R3961 (Promega, USA) as a standard mass band.

LysoMax™ is a preparation containing $sPLA_2$ from *S. violaceoruber*. The $sPLA_2$ was purified as follows. 500 μL of LysoMax™ was loaded onto a Superdex™ 75 HR 10/30 column (Amersham Biosciences, Sweden), equilibrated with NaCl 0.5 M and Tris 10 mM pH = 8 for size exclusion chromatography. 1.5 mL samples were collected from void volume (8 mL) to 24 mL. The fractions 4, 5 and 6 showed the greatest activity. These fractions were then dialyzed overnight against water using 3 kDa dialysis tubing (Sigma, D-2272). After this, the mixture was centrifuged at 14,100 × *g* for 5′ to discard insoluble material. Supernatant was supplemented with Tris pH = 8 up to 10 mM. This solution was loaded into an anionic exchanger column, Mono Q 5/50 GL (GE Healthcare, Uppsala, Sweden), equilibrated with Tris 10 mM pH = 8. After elution of ten bed volumes no changes in UV or conductivity were observed. Then, a linear gradient with NaCl 0.5, M Tris 10 mM pH = 8 was started. Fraction 4 (1.5 mL) obtained at a conductivity of 5 mS/cm (25 °C) showed the highest lipolytic activity with no surface activity at the amounts tested. This fraction was used in the monolayer experiments.

2.2. Protein trypsinization and mass spectrometry analyses

For mass spectrometric sequence-analyses of the $sPLA_2$s, protein spots or bands obtained from Coomassie Brilliant Blue-stained acrylamide gels, were manually cut, de-stained, and in-gel digested with sequence grade trypsin (Promega) as described [25]. Briefly, proteolytic digestion was carried out by incubating the ACN-desiccated gel piece containing the protein band with trypsin in 50 mM ammonium bicarbonate, pH 8.3, overnight at 37 °C (enzyme–substrate ratios 1:10). Then, peptides were extracted from gels using two successive steps with aqueous 60% ACN containing 0.1% TFA, and stirring for 30 min. Finally, peptide solution was concentrated by vacuum drying and desalted using C18 reverse phase micro-columns (Omix®Tips, Varian). Elution from the C18 tips was carried out with CHCA matrix solution in aqueous 60% ACN containing 0.1% TFA onto the plate for MALDI-MS. In some cases, the free amines of the tryptic peptides were sulfonated using the Ettan™ CAF MALDI Sequencing Kit in order to simplify the spectra (mainly Y series) for manual sequencing (Fig. S1) and increase the efficiency of PSD fragmentation.

Mass spectra of peptide mixtures were acquired in a 4800 MALDI TOF/TOF instrument (Applied Biosystems) in positive ion reflector mode. Mass spectra were externally calibrated using a mixture of peptide standards (Applied Biosystems). MS/MS analyses of selected peptides were performed and manually sequenced.

Two proteins were also identified by database searching of measured peptide m/z values using the MASCOT program (Matrix Science http://www.matrixscience.com), and based on the following search parameters: database, NCBInr 20141130; monoisotopic mass tolerance, 0.05 Da; fragment mass tolerance, 0.3 Da; cysteine carbamidomethylation and one missed tryptic cleavage allowed. Taxonomy was restricted to bony vertebrates. Significant scores ($p < 0.05$) were used as criteria for positive protein identification.

Purity of *B. asper* $sPLA_2$-III was assessed by nano-electrospray mass spectrometer in a QTrap-3200 instrument (Applied Biosystems) as described [23].

2.3. PLA_2 activity measurements

The lipolytic activity of chromatographic fractions was firstly followed by a bulk assay with Triton:DLPC 9:1 substrate mixture with the same solution composition as the monolayer aqueous phase, quantifying released fatty acids by a colorimetric assay [26,27] using a commercial kit, NEFA HR (Wako, Japan). For selected fractions, the monolayer assay at a constant area described next was employed.

2.4. Lipid monolayers set up

To assure reproducibility all the following steps were performed. After each measurement, NaOH 2 M solution at 85–90 °C was poured into the trough and left for 5 min to inactivate any adsorbed protein. After this, the NaOH was removed and the trough was thoroughly washed with deionized water. Finally it was rinsed with ethanol and again with distilled water. Buffer was loaded into the trough and if necessary (barostatic mode) the recently washed barrier was put in place at a maximum area.

New DLPC working solution was checked by pig pancreatic $sPLA_2$ injected below a film set at a 20 $mN \cdot m^{-1}$ at final concentration of 7 nM. It was considered as a suitable solution if pressure drop detected was less than 1 $mN \cdot m^{-1}$ after 20/30 min of injection/reaction (see Supplemental data). The stability of DLPC monolayers was routinely checked by leakage either in isobaric or isochoric mode (see Fig. S4).

2.5. Trough and general monolayer assay descriptions

The trough was drilled in Teflon supported by aluminum (Fig. S2). It consisted of a circular reaction compartment (3.8 cm^2, 0.75 cm depth) with two orifices aside which allowed the enzyme injection with a pipette tip into the aqueous phase without touching the lipid monolayer. Connected to the reaction through a small surface channel (0.5 cm^2), there was a larger rectangular compartment (0.75 cm deep, 9.5 cm long and 3.3 cm wide, 31.2 cm^2), used as a lipid reservoir. Volume change after injection was compensated by removing the same amount of liquid after injection. When both compartments were used in conjunction under barostatic mode (one movable barrier), this served as a zero order trough as described in [28]. The aqueous solution composition was NaCl 100 mM, $CaCl_2$ 20 mM, and Tris 10 mM pH = 8 (25 °C). The temperature was kept constant at 25 °C ± 0.3 °C by placing the trough in a larger thermostated trough filled with water and in turn connected to an external thermo-circulating bath (Haake F3, Germany). The temperature probe was set in the reaction compartment.

The lipid working solution was prepared weekly by diluting the stock solution, and kept at room temperature during the assays to avoid water vapor condensation. All enzymatic assays and isotherms were performed under constant stirring with a magnetic bar of cylindrical shape (approximate volume of 50 μL). The solution composition was NaCl 100 mM, $CaCl_2$ 20 mM (or 20 mM EDTA), Tris 10 mM, pH = 8 (25 °C). DLPC isotherms (either with 20 mM Ca^{2+} or 20 mM EDTA), were indistinguishable at 25 °C. No surface impurity could be detected by compressing with a Teflon barrier (no increase in Π value). Π was measured with a platinum plate immersed 2–3 mm into the aqueous solution and hanging from a hook to an electrobalance (KSV layer builder, Biolin Scientifc Ab, Finland). Data sampling was 1 point per second. All data was integrated by a computer using the standard software provided by the manufacturer of the electrobalance.

DLPC (Avanti Polar Lipids, 850335P) 0.1 mM working solution in chloroform was spread onto the surface of the reaction compartment volume (2.5 mL) up to the desired initial lateral pressure (Πi). Alternatively, both compartments were employed (zero order trough configuration), with a total solution volume of 22.5 mL.

For the surface density calculation, a reference isotherm was constructed by a drop-wise spreading of the lipid onto the aqueous buffered solution, and taking the Π measurement after 2 min, under identical stirring and temperature conditions of the hydrolysis measurements. The data were fitted using the following equation, where N_s is the DLPC surface density at a given Π (Fig. S3).

$$N_S(\Pi) = \left(-2.8e^{-\Pi/112.5} - 1.04e^{-\Pi/0.11} + 3.84\right).10^{14} \text{molecules} \cdot \text{cm}^{-2}. \quad (1)$$

By multiplying N_s by the monolayer area, the total amount of molecules at a time was calculated from a Π measurement.

Other controls and consideration taken into account for the monolayer set-up are summarized in the Appendix (Table S1).

2.6. Barostatic mode and curve analysis

Teflon barrier position was controlled by the equipment (KSV layer builder). The parameters of constant Π mode were: gain control 1.0 and constant linear compression. After target parameters, forward barrier movement rate: 10 $mm \cdot min^{-1}$; backwards movement: 3 $mm \cdot min^{-1}$. The rate of area decrease was converted into hydrolysis rate by assuming that hydrolysis products were rapidly desorbed from the interface and taking into account the DLPC molecular area calculated from the compression Π-A isotherm in the same trough with stirring (Fig. S3). The rate reported was obtained in the linear portion of the curve. In barostatic mode, Π values had always a standard deviation of less than 0.1 $mN \cdot m^{-1}$, with continuous magnetic stirring.

After lipid spreading, the barostat was set on, and the changes of area were recorded at the desired Π. If no major change was seen in 10/15 min (slope within ± 0.15 $cm^2 \cdot h^{-1}$ in barostatic mode, less than 1% of total of lipid area covered), the enzyme was injected into the aqueous solution.

Barostatic kinetics were analyzed similarly to [19,13,14]. Briefly, the rate of hydrolysis was the slope ($cm^2 \cdot s^{-1}$ units) of the linear portion of the area vs. time curve after enzyme injection corrected by the slope prior injection (in the order of 1×10^{-4} $cm^2 \cdot s^{-1}$). This rate was converted into molecules $\cdot s^{-1}$ by multiplying it by the DLPC surface density in molecules $\cdot cm^{-2}$ using Eq. (1). Lag time was calculated as reported in [13,17,19]. Briefly, the data recorded for 10 min (t = −600 s) prior to enzyme injection (t = 0 s) were fitted with linear regression. The data after injection that showed a linear portion was also linearly fitted, using a minimum of 180 points (3 min measurement as a minimum) and checking the residual to be R ≥ 0.997. Then, the time of the intercept of both lines was taken as the lag time.

Calculation of hydrolyzed molecules was done assuming that all reaction products are desorbed from the interface. Jain and co-workers showed that this may not strictly be the case [29]. However, for comparative purposes the amount of product not desorbed was not considered.

2.7. Isochoric mode

At a constant area, known aliquots of DLPC 0.1 mM working solution in chloroform were spread onto the surface of the reaction compartment volume (2.5 mL) until the desired initial pressure was attained. The stability of DLPC film was satisfactory at least up to 25 $mN \cdot m^{-1}$ (Fig S4). The small reaction compartment was used to follow Π, enzyme injection, and temperature sensing. When the enzyme produced a very fast drop in Π, the left reservoir compartment was used as a lipid substrate but in isochoric mode. To determine the $LR_{20/10}$ ratio, the enzyme injection was performed at Π_i = 25 $mN \cdot m^{-1}$ for all enzymes at 7 nM final concentration.

Isochoric kinetics were analyzed as follows. The total amount of molecules at a given time (dashed lines in Fig. 1) was calculated by converting Π into surface density N_S using Eq. (1), and then multiplying it by the total monolayer area. Then, the molecules vs. time curve (Fig. 2A) were differentiated vs. time, sign reversed and smoothed using 25-point neighbor averaging (Fig. 2C). Finally, the hydrolysis rate vs. Π profile was built by plotting that differentiated curve (molecules per unit of time) against the corresponding Π at a given time (Fig. 2D), obtaining a bell shaped curve. To define a unique Π value at which the rate is maximal the portion of the non-smoothed curve of molecules vs. time around the inflection point was fitted using a 5th degree polynomial and 200 points were evaluated. After this, the derivative of this fitted curve was taken, and the time at which a minimum was found (Fig. 2B) defined the time of maximum rate, which corresponds to Π_{opt} (Table 1). Numerous assays were performed, varying Π_i and enzyme concentration.

Each condition was assayed at least by duplicate. Reported values are the mean of those values, and error bars represent data dispersion. All calculations and plots were performed using Microcal Origin 7.0 software.

3. Results

We performed barostatic and isochoric assays for two paradigmatic $sPLA_2$, one from pig pancreas and the other from Indian cobra venom (*N. naja*). This served us to test the similarity between the isochoric and the barostatic methods to obtain the Π of a DLPC monolayer at which the rate was maximal (Π_{opt}). When following Π vs. time at constant areas, after enzyme injection, a decreasing curve (Fig. 1) for all $sPLA_2$s and concentrations used was obtained. This was expected since the amount of protein used was tested to make negligible the measurable increase in Π for protein penetration/adsorption at the lipid interface. Virtually all substrates available were degraded when the reaction proceeded for sufficient time (from minutes to hours depending on Π_i and enzyme tested), evidenced by the Π approaching an almost zero value at the end of the reaction (Fig. 1A). By adding EDTA (up to 40 mM if 20 mM Ca^{2+} was present) to the aqueous phase, the rate of Π fall was abruptly decreased by more than 20 times (Fig. S4), as it was expected given the well known Ca^{2+} dependency of the catalytic mechanism proposed for $sPLA_2$ [30]. If EDTA is present in the subphase before enzyme injection the activity is markedly diminished (Fig. S4).

After converting the Π values to surface molecules (Fig. 1), the portion of the curve molecules vs. time near the inflection point was fitted with a 5th grade polynomial (Fig. 2A). When differentiating this fitted curve, the minimum was taken and its time was defined as that of the maximum rate of hydrolysis (Fig. 2B). This time value allowed us to obtain a corresponding Π_{opt} value using the Π vs. time curve. Alternatively this value could be assessed by simply plotting the opposite of the smoothed derivative of the molecules (rate of hydrolysis) vs. time (Fig. 2C). When changing the time axis by the corresponding Π values, one obtains the profile curve, typically bell-shaped (Fig. 2D). We tested this procedure using other equipment [31], with a trough of trapezoidal shape and 17 mL of volume, and we obtained basically the same results.

The choice of Π_i slightly affected the Π_{opt} at which the rate is maximal, since Π_i is always greater. When numerous time courses were recorded varying Π_i (Fig. 3A), it was evident that the increase of the Π at maximum rate depends on Π_i up to a value which was stable (Fig. 3B). This value was defined as Π_{opt} in the isochoric method, and was very close to that determined by the barostatic method within experimental error (Table 1). For a particular enzyme, there was a particular value of Π_i by which above it, the Π vs. time curves showed a *quasi* linear decrease before the sigmoid-like shape started to develop (see Fig. 1B, for *N. naja* curve Π vs. time, injection at Π_i 36.6 $mN \cdot m^{-1}$). The great asymmetry in the sigmoid-like shape shown for some of the Π vs. time curves, or the absence of a sigmoid shape (no obvious inflection point, with an abrupt decrease of Π) served as an indication that tested Π_i was well above or below of Π_{opt} respectively.

To obtain a sigmoid, and thus, the derived bell-shaped activity vs. Π curve, it is necessary that $\Pi_i > \Pi_{opt}$ in order to span most of those curves. Since Π_{opt} is unknown a priori, it is necessary to test at least two Π_i values. In our hands, injections at Π_i 15 and 25 $mN \cdot m^{-1}$ were usually enough to get a glimpse of the position of the Π_{opt}. This last value of Π_i was chosen to standardize the comparison between the $sPLA_2$s with a final concentration of 7 nM. In the case of the pig pancreatic and *S. violaceoruber* $sPLA_2$ the time required to develop all the curves was greater than 2 h. $sPLA_2$ from cobra species venoms showed an abrupt decrease when set at 25 $mN \cdot m^{-1}$ as well as the newly described isoform from *B. diporus* $sPLA_2$ III. To reduce the error induced by very few data points (given the short time it took for the enzymes to hydrolyze the samples) we also tested them using the second compartment (full trough). This produced a slower decrease since the amount of lipid was greater (almost ten times greater) with the same rate of hydrolysis. By doing so, a more precise estimation of the inflection point of the molecules vs. time curve can be achieved.

When injecting the pancreatic enzyme at high lateral pressures ($\Pi i = 25\ mN \cdot m - 1$), where Π decrease was very slow, the rate at Π_{opt} was 13 times lower than when determined using lower Π_i (15 $mN \cdot m^{-1}$) (Fig. 3A, for pig pancreatic curves). A major difference iEn both determinations is the time required to attain Π_{opt}, which is

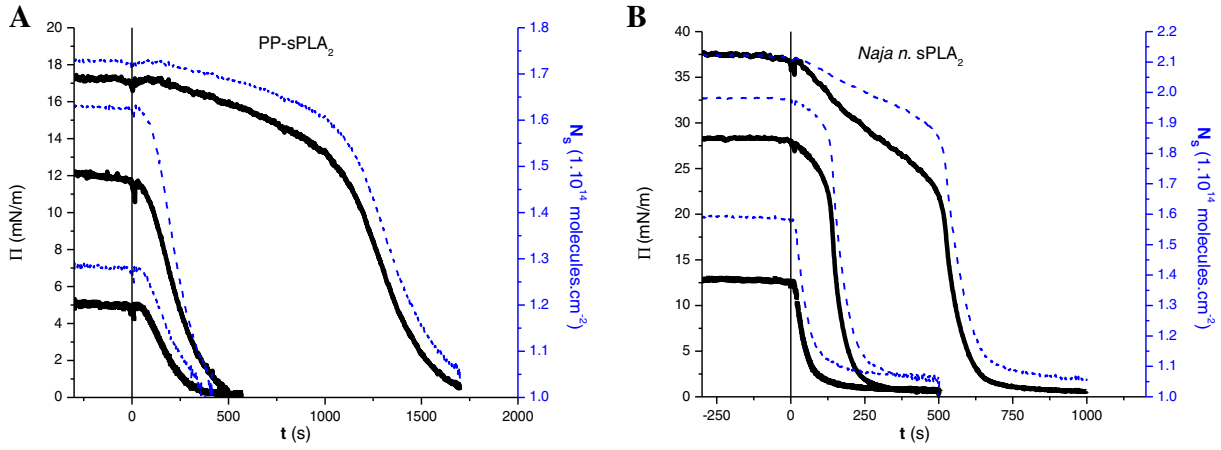

Fig. 1. Time course of hydrolysis reaction of DLPC monolayers at different initial lateral surface pressures. Enzyme injection defines t = 0 and Πi. Continuous lines are measured Π (left axis), discontinuous lines are the calculated surface density N_S of remaining DLPC molecules (right axis) at 25.0 ± 0.5 °C. Aqueous solution was 100 mM NaCl, 20 mM $CaCl_2$, 10 mM Tris, pH = 8.0, final enzyme concentration 7 nM. (A), $sPLA_2$ from pig pancreas. (B), $sPLA_2$ from Indian cobra (*Naja naja*) venom.

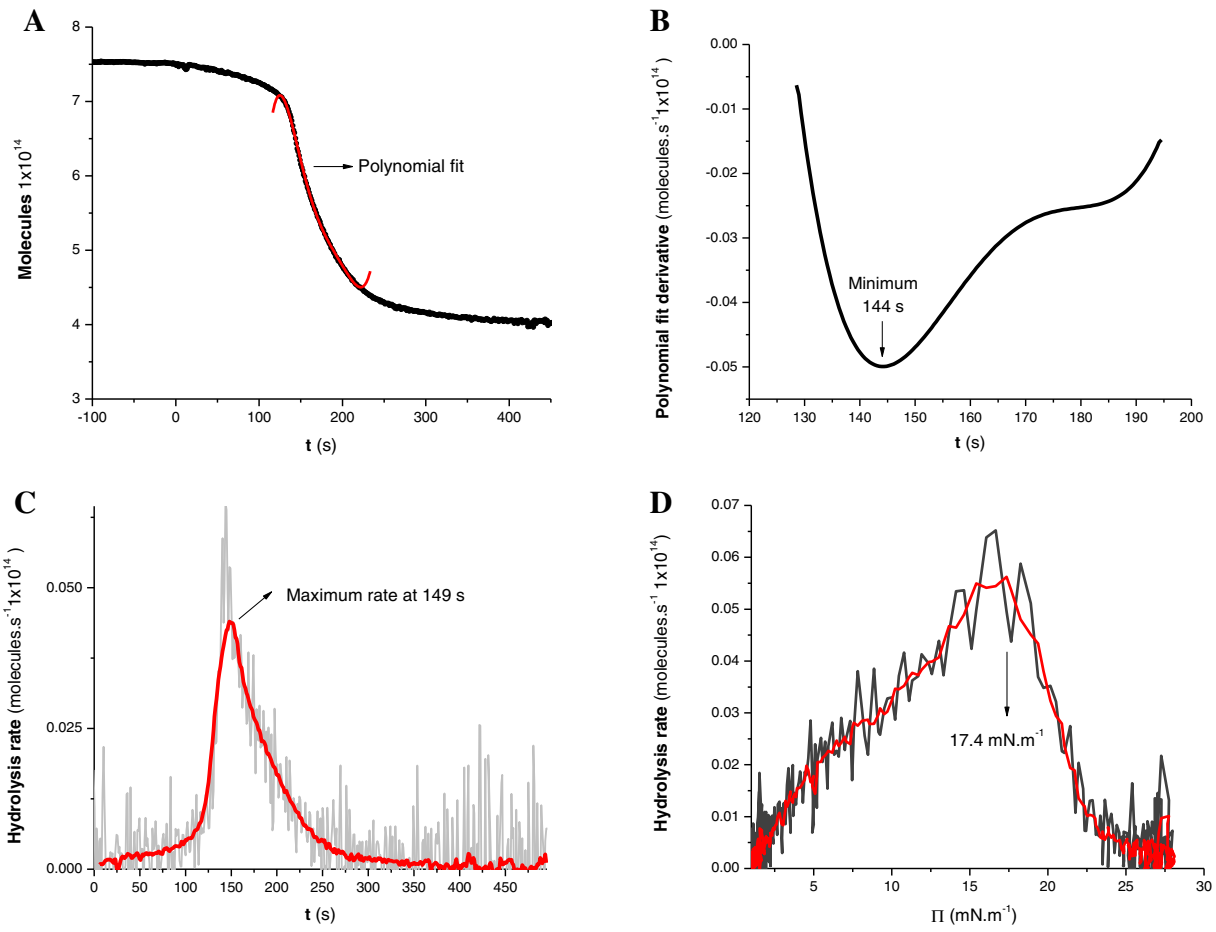

Fig. 2. Curve analysis to build the hydrolysis rate vs. Π curve from Π vs. time data. Example with $\Pi_i = 28\ mN \cdot m^{-1}$ for *Naja naja* $sPLA_2$ (Fig. 1B). (A), the molecules vs. t curve was obtained by multiplying the surface density vs. t (Fig. 1B) by the reaction compartment surface area. A polynomial fit of 5th grade and 200 points was performed to obtain a noise-free curve (red segment). (B), the derivative of the polynomial fit is shown with the time of optimal hydrolysis (curve minimum, arrow). (C), the raw data of molecules vs. time was differentiated (gray, background lines) to obtain a hydrolysis rate for every time, and the resultant curve was smoothed (red). (D), the hydrolysis rate was plotted against the corresponding Π for a given t, thus obtaining the hydrolysis rate vs. Π curve. In red it is shown the smoothed curve. The arrow shows the Π at which maximal rate was obtained (see Table 1 for values obtained at $\Pi_i = 25\ mN \cdot m^{-1}$).

more than 2 h for $\Pi i = 25\ mN \cdot m^{-1}$, and less than 10 min for $\Pi i = 15\ mN \cdot m - 1$ (Fig. 4B). This drop in rate at Π_{opt} was also verified under barostatic conditions. To do this, the enzyme was injected below DLPC at 25 mN·m − 1 (the area change is negligible) and incubated for a short period (10 min) or longer (60 min), before setting the barostat to 10 mN·m − 1, to allow for optimum hydrolysis to be displayed. There was a 10 fold drop in the barostatic rate after 60 min of incubation in relation to 10 min of incubation at 25 mN·m − 1. These additional experiments conducted us to conclude that there may be less amount of active enzyme after long time incubation, perhaps by denaturation aided by the basic solution or adsorption at the Teflon surface.

Table 1
Comparison between the isochoric and barostatic methods for obtaining Π_{opt} and $LR_{20/10}$.

$sPLA_2$	Π_{opt}[a] Isochoric	Π_{opt} Barostatic	$LR_{20/10}$ Isochoric	$LR_{20/10}$ Barostatic
S. violaceoruber[b]	8 ± 1	9 ± 1	0.08 ± 0.03	~0
Pig pancreas[c]	9.2 ± 0.6	9 ± 1	0.1 ± 0.02	~0
B. diporus $sPLA_2$-I	9.4 ± 0.7	11 ± 1	0.10 ± 0.02	~0
B. diporus $sPLA_2$-II	10 ± 0.8	12 ± 1	0.10 ± 0.02	~0
Bee venom $sPLA_2$	16 ± 1	18 ± 1	0.8 ± 0.2	1.1 ± 0.2
B. diporus $sPLA_2$-III	16 ± 1	20 ± 1	1.0 ± 0.3	1.3 ± 0.2
N. naja	16.5 ± 0.5	17 ± 1	1.1 ± 0.2	1.5 ± 0.2
N. m. mossambica	17.5 ± 0.5	18 ± 1	1.2 ± 0.2	1.6 ± 0.1
B. asper $sPLA_2$-III	17.8 ± 0.5	18 ± 1	1.3 ± 0.1	1.3 ± 0.2

[a] Π_{opt} values for the isochoric method were taken from $\Pi_i = 25\ mN \cdot m^{-1}$.
[b] Enzymes are tabulated from low to high Π_{opt} according to isochoric method.
[c] Total time until Π_{opt} was reached for the pig pancreas $sPLA_2$ at around 2 h.

In general, when normalized, the profiles obtained with the barostatic method were very similar to those obtained with the isochoric method (Fig. 4). As we standardize all the isochoric studies by setting the Π_i at $25\ mN \cdot m^{-1}$, there is a deviation for *N. naja* enzyme at higher surface pressure (above Π_{opt}) values near Π_i (Fig. 4A). Since it required some time for the enzyme to fully adsorb and the full catalysis begins. However, for this enzyme, if the Π_i is set at $30\ mN \cdot m^{-1}$ instead, the isochoric profile behaves similarly to that obtained for barostatic procedure.

In general, the absolute rates of hydrolysis at Π_{opt} were greater by a factor of 2 when using the barostatic technique compared with isochoric mode (for Πi 3 to 5 units above Π_{opt}). However, when using 10 times more total pancreatic enzyme (70 nM, $\Pi i = 13\ mN \cdot m^{-1}$, full trough), the rates at Π_{opt} ($9\ mN \cdot m^{-1}$) were the same using both approaches. This led us to the conclusion that for comparison of rates, description of the initial conditions of isobaric vs. isochoric should be as complete as possible, regarding the trough set up and enzyme amount used. These requirements are less stringent for comparative purposes and Π_{opt} estimation.

Another usual parameter that is presented in barostatic analysis articles is the lag time or induction time τ [15,16]. The τ dependencies on Π (barostatic method) for pig pancreas and *N. naja* enzymes showed very similar profiles from the time to the maximum rate vs. Π profiles (isochoric method, Fig. 4B). Both the lag time (barostatic mode) and

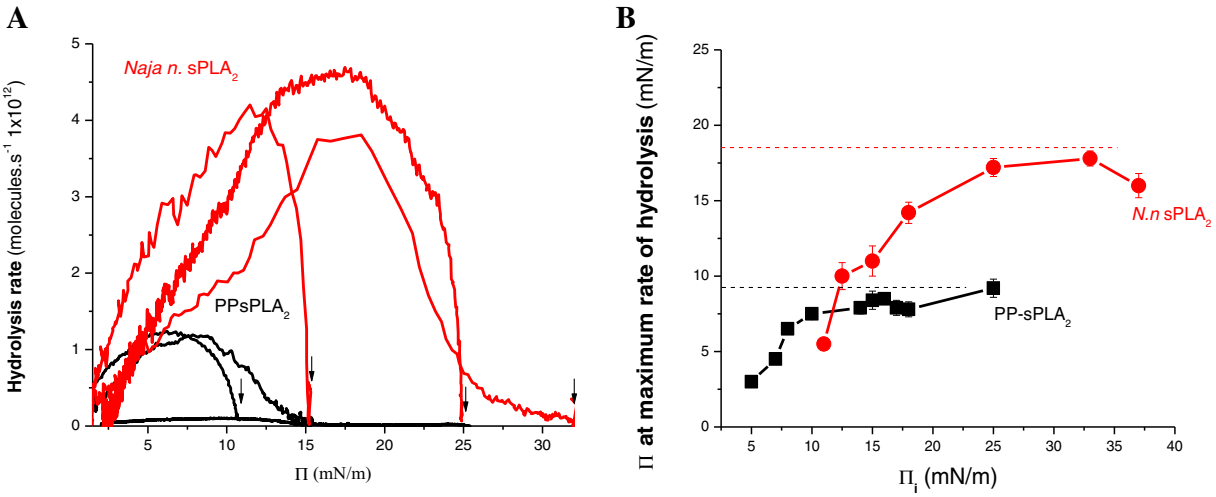

Fig. 3. Effect of different values of initial Π_i on hydrolysis in isochoric mode. (A), hydrolysis rate profiles. Each curve has different Π_i (greatest Π value for each curve, intersection with horizontal axis). Pig pancreas $sPLA_2$ hydrolysis (black curves) with Π_i 10, 15, and 25 $mN \cdot m^{-1}$ (arrows). The rate of the last one is notably reduced. *Naja naja* $sPLA_2$ (red curves) with Π_i 15, 25, and 33 $mN \cdot m^{-1}$ (arrows). Π_{opt} is defined as the maximum rate of hydrolysis obtained in isochoric mode when the initial surface pressure is set at $\Pi_i = 25\ mN \cdot m^{-1}$ and taken by a polynomial fit differentiation (see text for details). (B), dependency of maximum rate of hydrolysis vs. Π_i. Πi is the initial stabilized surface pressure of the DLPC monolayer before enzyme injection at a constant area (isochoric mode). The asymptotic value for both enzymes is the same as the Π_{opt} within experimental error (dashed lines). Measurements by duplicate, error bars show the interval between the two values.

also the time to optimum rate (isochoric mode) show an abrupt increase just above Π_{opt} for $sPLA_2$s (Fig. 4B).

We also evaluated the dependency of enzyme concentration on rate and Π_{opt} (Fig. S5). There were no drastic changes in Π_{opt}, however there was a slight tendency of Π_{opt} being greater as enzyme concentration increases (Fig. S5B). On the other hand, when testing concentrations below 1 nM, we observed that there is a small shift in the optima towards lower values. For instance, at 0.7 nM of *N. naja* $sPLA_2$ displays an optimum in either barostatic or isochoric mode of around 16 $mN \cdot m^{-1}$, instead of the 17.5 $mN \cdot m^{-1}$ at 7 nM (Table 1). At very low enzyme concentration the maximum becomes diffuse using the isochoric mode, and this requires to neighbor-average the curves with more points. To reduce error, we integrated the profiles (Fig S5A) and plotted against enzyme concentration (Fig S5C), there was a linear dependency above 2 nM.

All other enzyme preparations were analyzed using both approaches at the same enzyme concentration (7 nM) and Πi (25 $mN \cdot m^{-1}$). Results are summarized in Table 1. There are differences in the Π_{opt} between these $sPLA_2$. To quantify those differences and to aid in the enzyme classification we propose an index termed $LR_{20/10}$ which is the ratio of rates of hydrolysis of DLPC monolayers at 20 $mN \cdot m^{-1}$ vs. 10 $mN \cdot m^{-1}$. For instance, this index is below 0.1 for the pig pancreatic $sPLA_2$, and above 1 for the *N. naja* enzyme. Besides, the $LR_{20/10}$ ratio can be used as a measure of "true identity, similarity and equivalence" between recombinant and purified wild type $sPLA_2$, since it is known that even having the same primary sequence, there could be detectable differences in the lipolytic action between a native and a recombinant enzyme, as exemplified by a work with lipases [32].

We also analyzed the monolayer hydrolysis by three $sPLA_2$ purified from the venom of *B. diporus* (non-commercial). Two of them were identified as the previously cloned and recombinantly expressed ones [17]. These enzymes, *Bd* $sPLA_2$ II and I, behaved chromatographically as the ones P1 and P2 respectively, whose N terminal sequence of 15 aminoacids was determined by Edman degradation [13,14], but

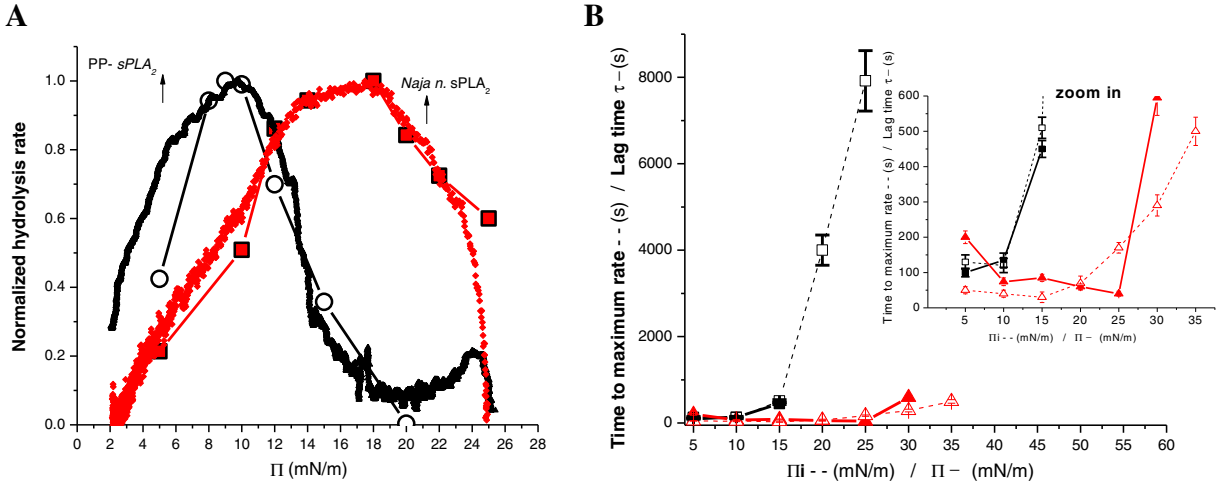

Fig. 4. Comparison between the barostatic and isochoric methods. (A), normalized hydrolysis rate vs. Π profiles: same assay conditions as described in Fig. 1 legend, profiles of enzymes from pig pancreas $sPLA_2$ (black) and *Naja naja venom* $sPLA_2$ (red), constructed at constant Π with the surface barostat (line-connected discontinuous plot) or at a constant area using the isochoric method (continuous scatter plot). The largest Π value of the isochoric curves (scatter plot) equals the Π_i. Non-normalized curves for different Π_i are shown in Fig. 4A. (B), lag time τ (constant Π, surface barostat) or the time required to reach the maximum rate (constant area, isochoric method). Relative error was approximately 10% for each measurement (duplicate and triplicate obtained at different days). Inset: zoom in.

differing in a single residue. In fact, *Bd* $sPLA_2$ II has the same N terminal sequence of P3 [13], although it was purified from the monomeric portion of the size exclusion chromatogram. We obtained 100% sequence coverage after tryptic digestion and PSD fragmentation analysis using the MASCOT server. We could not identify tryptic peptides compatible with the exact N-terminal sequence proposed for P1 and P2 previously [14], although the difference is minimal (M11K substitution). This was possibly an erroneous assignment of the earlier Edman sequencing process. The third isoenzyme isolated was a new $sPLA_2$ tentatively termed as P4 or *Bd* $sPLA_2$-III, and its N terminal tryptic peptide and two internal ones were de novo sequenced by a manual procedure using the mass spectrometry approach. *Bd* $sPLA_2$ III is clearly a "high Π-DLPC" enzyme (Table 1). To increase confidence of the N-terminal peptide sequence assignment, a sulfonation protocol was followed to aid in fragmentation and obtaining y series spectra. This step also allowed assigning a Q instead of K in the sequence, almost isobaric residues (Fig. S1). The resulting N-terminal sequence was NI/LWQFGR (Uniprot accession number C0HJP9); L residue is not discriminated from I by the methodology employed.

In our study we also included the myotoxin-III (*Ba* $sPLA_2$-III) a basic D49 $sPLA_2$ from *B. asper* venom, known to be cytotoxic either in vitro and in vitro and to act synergistically with the enzymatic inactive counterpart myotoxin K49 [23,24].

It is evident that the analyzed $sPLA_2$ can be roughly grouped into at least two kinds according to $LR_{20/10}$ index using DLPC as substrate. Those with $LR_{20/10} \leq 0.1$ (*Bd* $sPLA_2$-I and -II, pancreatic, *S. violaceoruber*), and the other ones with $LR_{20/10} \geq 1$ or higher, such as *Bd* III, and the ones from venoms of *N. naja*, *N. mossambica*, Bee and *Ba* $sPLA_2$-III from *B. asper* (see Table 1). This is a characteristic that could be used as an additional and functional criterion to distinguish between different isoforms.

4. Discussion

As lateral pressure is an organizational parameter that modulates the activity of lipolytic enzymes, the $LR_{20/10}$ index (defined as the ratio of hydrolysis rates of phospholipid monolayers measured at 20 and 10 $mN \cdot m^{-1}$) distinguishes enzymes that are more active at high surface pressure than those that best attack interfaces at low packing. The reasons for which we have chosen these two values of surface pressure to build the conceptual $LR_{20/10}$ index for $sPLA_2$s is based on the data of two paradigmatic enzymes widely tested by several laboratories and by us. At the lower edge, 10 $mN \cdot m^{-1}$ is near the Π_{opt} reported for one of the most studied enzyme: the pig pancreatic $sPLA_2$ [33]. The activity of pig pancreatic $sPLA_2$ is negligible at 20 $mN \cdot m^{-1}$, so the ratio $LR_{20/10}$ for this enzyme will be near zero and representative of "low pressure" $sPLA_2$ (see Figs. 3, 4 and Table 1). On other hand, $sPLA_2$ from *N. naja* venom (Cobra) has a Π_{opt} centered at 18–20 $mN \cdot m^{-1}$ with a rate almost twice as fast as the one displayed at 10 $mN \cdot m^{-1}$, as reported by Demel et al. [18] and corroborated by us (see Figs. 3 and 4). So, this enzyme has a higher $LR_{20/10}$ ratio (higher than 1) and it is representative of "high pressure" enzyme (Table 1).

The aim of our work was to show the similarity of the *barostatic* (activity measured by changes in area keeping constant the surface pressure) and *isochoric* (activity measured by changes in surface pressure keeping constant the surface area) methods to evaluate Π_{opt} and the $LR_{20/10}$ ratio for different $sPLA_2$ using phospholipid monolayers as a substrate. There has been work done showing the consistency in rates obtained at constant areas with constant Π with lipases acting on lipid monolayers [34]. We provided evidence for the same consistency between methods for different secreted phospholipases and suggested the $LR_{20/10}$ index as an easy access parameter to classify these types of lipolytic enzymes. We also presented data on the Π_{opt} for several enzymes.

Early reports on lipases and phospholipases acting on substrate monolayers were indeed performed at constant areas [34–36]. Usually, these isochoric kinetic experiments were recorded in range of time where linear relationships between changes either in lateral pressure (Π), surface potential (ΔV) or surface radioactivity were obtained [18,35,37,38]. In all those previous examples, workers used lipids whose reaction products did not leave the interface readily, or, if they did (radioactive phosphocholine for instance), the changes in Π were minimal. When the reaction products leave the interface producing a Π fall, it is possible to use the changes in Π as an indicator of the reaction progress and calculate the rate of hydrolysis [34]. The constant area approach was also employed to differentiate the effect of oxidized phospholipids in PLA_2 hydrolysis of monolayers [39], although there was no analysis of optimal surface pressure as frequently is the case when using the surface barostat.

It has been known for a long time that $sPLA_2$ may behave differently when exposed to a hydrolyzable lipid such as a short chain phosphatidyl-choline (PC) [18]. Their optimal pressure for hydrolysis (Π_{opt}) is markedly different as well as the lag times for each Π. These differences, at the same enzyme concentration, were ascribed to different penetration powers of the lipid monolayer by the phospholipases by some workers [16] but also lipid film composition through the concept of *interfacial water activity* [40].

Enzymes that have low or high Π_{opt} on PC can be distinguished regardless of the method employed when the hydrolysis products are soluble into the subphase using substrate monolayers. The rate of hydrolysis of a lipid at a certain Π is influenced by the amount of enzyme bound to the interface as well as by the catalytic efficiency of that enzyme [21].

The changes in area (or surface pressure) of pure DLPC monolayers upon $sPLA_2$ hydrolysis are attributed to product desorption only when the subphase is continuously stirred at pH above 7. This was early seen by Zografi et al. [41] using pig pancreatic $sPLA_2$ against C-8, C-10 and C-12 as substrates, concluding that the rate of product desorption is faster than hydrolysis. Besides, there were no major differences in Π_{opt} using different short chain length PCs, from 9 to 12 C, despite a variation of the mean molecular area, when tested against the same enzyme [41], with all of them having liquid-expanded character and with soluble hydrolysis products. With longer chain PCs there is an increment in the stiffness of the substrate and the remaining insoluble products at the interface avoids a full drop of Π. To overcome this some workers turned to add a fatty acid scavenger such as bovine serum albumin (BSA) in the subphase [12,42].

When using the barostatic technique, the area change is attributed to product desorption mainly. However it has been shown that not all products are rapidly desorbed [29], although it is clear that the remainder does not build up in a measurable fashion since speed is kept constant in the time range measured. When new substrate is added after hydrolysis, the Π_{opt} increases, presumably because of the remainder of hydrolysis products at the interface. We confirmed this observation using the barostatic technique and the pig pancreatic enzyme, by allowing the reaction to proceed for 10 min at 10 $mN \cdot m^{-1}$ and rapidly compressing thereafter. In this case, there was still activity at 30 $mN \cdot m^{-1}$ which is compatible with hydrolysis products remaining in the interface.

Surface barostatic technique is the gold standard for kinetic measuring of lipolytic enzymes [43] and it was reported that lipid monolayers is up to five orders of magnitude more sensitive compared with the rate of hydrolysis that occurred at the interface of lipids organized as small vesicles or nanocapsules [44]. However, surface barostatic method requires careful set up and constant controls, which take a great deal of time if one's intention is to search for the Π_{opt} region for a given substrate and enzyme. Lipid leakage throughout the compressing barrier is often found to be one of the major problems in setting up a monolayer experiment, together with slow, but sometimes meaningful diffusion of the enzyme to the reservoir compartment in the case of long time measurements. This concern is avoided by using the constant area approach since the lipid monolayer is constrained by the limit of the edges of the trough.

The advantages of the isochoric over the barostatic method are essentially the shorter times required to characterize hydrolytic profiles of rate vs. Π, Π_{opt} and $LR_{20/10}$, since, as stated previously [34], the variation of the rate could be followed in a continuous way in terms of Π which decreases progressively. For instance, four Π-points in the barostatic curve imply four times setting up the monolayer, cleaning the trough, and so on. The procedure we described could easily fit applications where movement of barriers or trough is not desirable such as microscopic methods following reaction in real time. We believe that our method aids to achieve a greater adoption of optimal lateral pressures for hydrolysis among the laboratory groups that work with lipolytic enzymes. The isochoric method can be employed in very small reaction compartments, the purified protein mass requirements to achieve ~5 nM bulk concentrations (~2–3 μg in 3 mL) are not usually hard to obtain and makes it ideal for comparative studies. There is commercial equipment to measure in small volumes that have a good precision and are able to sustain stirring, for example the DeltaPi® tensiometer from the Kibron manufacturer.

We believe that this convenient and simple experimental technique translates into lower cost of implementation and opens the way to a larger set of laboratories working on phospholipases and related enzymes to implement monolayer kinetic measurements. A hook electrobalance with mg resolution and a drilled circular Teflon trough are the only essential special requirements. Besides, the monolayer method in general is sensitive, and amenable to quantitative analysis giving access to distinguish experimental parameters between related enzymes. The index $LR_{20/10}$ could be a novel criterion for fine tuning enzyme classification.

Other applications of the monolayer method here described could be, for example, the assessment of interspecies venom variability [35], studies analyzing relationships between hydrolysis parameters and toxicity [23,24], or a rapid discernment between recombinant and natural occurring enzyme preparations [32].

For simplicity, availability and standardization, we propose DLPC (12 C) to be used for phospholipase characterization of the activity-Π profile. This is so not only because we have proved the equivalence between the well established surface barostatic mode with the simpler constant area mode using this lipid, but also because it is already widely used in monolayer studies. For instance it was recently revisited for testing subtle differences between the pro-enzyme and the mature form of $sPLA_2$ of group X [45]. Also, DLPC substrate was previously validated to study the modulation of phospholipase C using lipid monolayers with two step enzymatic system with an excess of lipase/colipase [46,47]. On the other hand, negative lipids, such as DLPG (dilauroyl phosphatidyl glycerol), are readily hydrolyzed by both paradigmatic enzymes (from cobra venom and pig pancreas), and the differences between them regarding optimum Π for hydrolysis are not as striking as when using the neutral DLPC.

The biological implications of optimum packing Π_{opt} for the hydrolysis of DLPC monolayers for distinct "high surface pressure" ($LR_{20/10} \geq 1$) from "low surface pressure" ($LR_{20/10} \leq 0.1$) enzymes still remain uncertain. However, there are some clear correlations with the ability of inhibiting the clotting process or hydrolysis of red cell membranes for the enzymes which have Π_{opt} higher than that observed for pancreatic $sPLA_2$ [16,18]. A priori, it might be speculated that $sPLA_2$s from venoms were favored by evolution with the ability to attack cell membranes (considering the red cell membrane as a general model of natural membrane). This property correlates with high Π_{opt} (high surface pressure enzyme) when compared with pig pancreatic $sPLA_2$ that is more suitable to hydrolyze lipids dispersed in micellar aggregates. However, some venom $sPLA_2$s such as those purified from *B. diporus* venom have a similar profile to that found for pig pancreatic $sPLA_2$ as it was previously shown [13,14] and confirmed by this work for *Bd* $sPLA_2$-I and II. So, not all of the isoenzymes present in a venom would show a LR20/10 ≥ 1. Subtle differences in the sequence from the same species o genus, as we have shown for purified enzymes from the *Bothrops* venom, conducted to different profiles. So, despite the divergence of its origin, *Bd* $sPLA_2$-I and II have a low $LR_{20/10}$ index, similar to that obtained for pancreas $sPLA_2$ (Table 1). It should be emphasized that $sPLA_2$s from the *Bothrops* genus belong to the group IIA, but pancreas $sPLA_2$ belongs to group IB (according to Six and Dennis PLA_2 classification [1]). Similarly, differences in the primary sequence found in phospholipases coming from the venom from *Viperidae* family (*Bd* $sPLA_2$-III and *Ba* $sPLA_2$-III, group IIA) compared with those coming from *Elapidae* family (cobra venom $sPLA_2$s, group IA) that were checked here all of them have a similar higher Π_{opt} (see Table 1). Furthermore, the profile activity obtained for *S. violaceoruber* is similar to that found for pig pancreas enzyme.

To our knowledge this is the first report of hydrolysis of monolayers of substrates by a $sPLA_2$ from prokaryotic origin. *S. violaceoruber* $sPLA_2$ has only two disulfide bridges and belongs to group XV [22]. So, we conclude that the sensitivity to lateral pressure is not exclusive to one group of PLA_2 family classification. The specificity for *sn-2* bond in the glycerol backbone of phospholipids exerted (and shared) by all $sPLA_2$s absolutely needs the well conserved calcium binding domain loop coordinating with the HD pair and tied with, at least, two disulfide bonds. On the other hand, the sensitivity of the enzyme relative to the lateral packing of lipids at the interface appears to be a global property of the molecule probably given by the *i-face* while conserved structurally, it is not highly conserved in sequence [48]. However, as the studies of activity of well purified and identified $sPLA_2$ free of isoforms (especially those coming from venoms) using adequate lipid monolayer equipment are rather scarce, it is not possible to propose a generalization yet.

In conclusion, our work demonstrates that isochoric hydrolysis of monolayers by $sPLA_2$ is an alternative to barostatic approach using DLPC as a substrate to obtain the optimum surface pressure of hydrolysis. For technical approach, a simple Langmuir monolayer apparatus equipped with a small trough is the only requirement. We propose the ratio of hydrolysis at 20/10 $mN \cdot m^{-1}$ (lipolytic ratio $LR_{20/10}$) as an effective index of functional characteristic for lipolytic enzymes.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2015.06.003.

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