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Cloning and functional expression of secreted phospholipases A_2 from *Bothrops diporus* (Yarará Chica)

Pablo Javier Yunes Quartino, José Luis Barra, Gerardo Daniel Fidelio *

Centro de Investigaciones en Química Biológica de Córdoba (CIQUIBIC, UNC–CONICET), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba. Haya de la Torre y Medina Allende, Ciudad Universitaria, X5000HUA Córdoba, Argentina

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ABSTRACT

Bothrops diporus is a very common viper in Argentina. At present, no complete sequence of secreted phospholipase A_2 (sPLA$_2$) from this snake has been reported. We have cloned two sPLA$_2$ isoenzymes as well as a putative sPLA$_2$-like myotoxin from venom gland. The two sPLA$_2$ were expressed as inclusion bodies in *Escherichia coli* with an N-terminal tag of ubiquitin. After *in vitro* renaturation and cleavage step, using an ubiquitin specific peptidase, the recombinants exhibited sPLA$_2$ activity when analyzed by means of Langmuir dilauroylphosphatidylcholine monolayers as substrate. Both enzymes have a similar surface pressure-activity profile when compared with non-recombinant purified isoforms. To our knowledge, this is the first time that analysis of optimal lateral pressure of substrate monolayers by using the surface barostat technique is performed on recombinant sPLA$_2$s.

1. Introduction

Venoms from *Viperiade* family of snakes (vipers) are complex mixtures of components, many of which are enzymes [1,2]. A ubiquitous class of enzyme in these venoms is secreted phospholipases A_2 (sPLA$_2$, EC 3.1.1.4), which is usually present as many isoforms in an individual [3]. sPLA$_2$ catalyzes the hydrolysis of the *sn*2-acyl ester bond of phospholipids releasing a free fatty acid and a lysophospholipid. sPLA$_2$s are compact, disulfide-rich proteins, requiring Ca^{2+} for hydrolytic activity [3,4]. Furthermore, some authors have shown sPLA$_2$s to have additional activities, including neuro and/or myotoxicity which persist even after the catalytic center is impaired [5–8], and may display target or organ specificity [3].

We have previously isolated three acidic sPLA$_2$ isoforms from a venom pool of the *Bothrops diporus* (former *Bothrops neuwiedii diporus*) by using conventional purification procedures [5,9]. The hydrolytic profile using DLPC monolayers as organized substrate showed *a striking difference in their behavior*. One of them, denoted as isoenzyme P-3 in Fig. 1, had an optimal activity of hydrolysis in monolayers at about 25–27 mN m^{-1}, whereas the others, denoted as P-1 and P-2, had an optimum around 14 mN m^{-1} [9]. In comparison, the N-terminal sequences were almost identical (Fig. 1). Thus, the sensing of substrate organization by these proteins could be a fine tuned property coded by subtle differences in primary sequence. To address this question the full sequence data is essential. We set out to clone and obtain a reproducible protocol of expression of the isoforms in their native primary sequence. As a same specimen may translate many isoenzymes [3], we overcame this problem by adapting a recombinant expression protocol for the cloned enzymes avoiding any additional residues in mature protein. The aim of this work was to gain insight into the sPLA$_2$s structure fine-tuning regarding to the optimal lateral pressure of hydrolysis.

2. Materials and methods

2.1. Materials

Taq (GoTaq), Pfu, MMLV polymerases as well as other DNA modifying enzymes were from Promega (USA). Glycerol, $CaCl_2$ and NaCl were from Merck (Argentina). $NiSO_4$ from Tetrahedron (Argentina). Other reagents were from Sigma Chem. CO. (USA).

2.2. Total RNA isolation and reverse transcription

Snakes were milked and after three days they were sacrificed. Heads were cooled on ice for 15 min and tissue was removed from venom glands. RNA isolation was according to Ref. [10]. Tissue was homogenized and mixed with ice-cold RNAgents® Total RNA Isolation System (Promega, USA) using an Ultra-Turrax. Successive steps were essentially as described in RNAgents brochure using

Abbreviations: DLPC, dilauroylphosphatidylcholine; IMAC, immobilized metal affinity chromatography; UTR, un-translated region.

* Corresponding author.
E-mail address: gfidelio@mail.fcq.unc.edu.ar (G.D. Fidelio).

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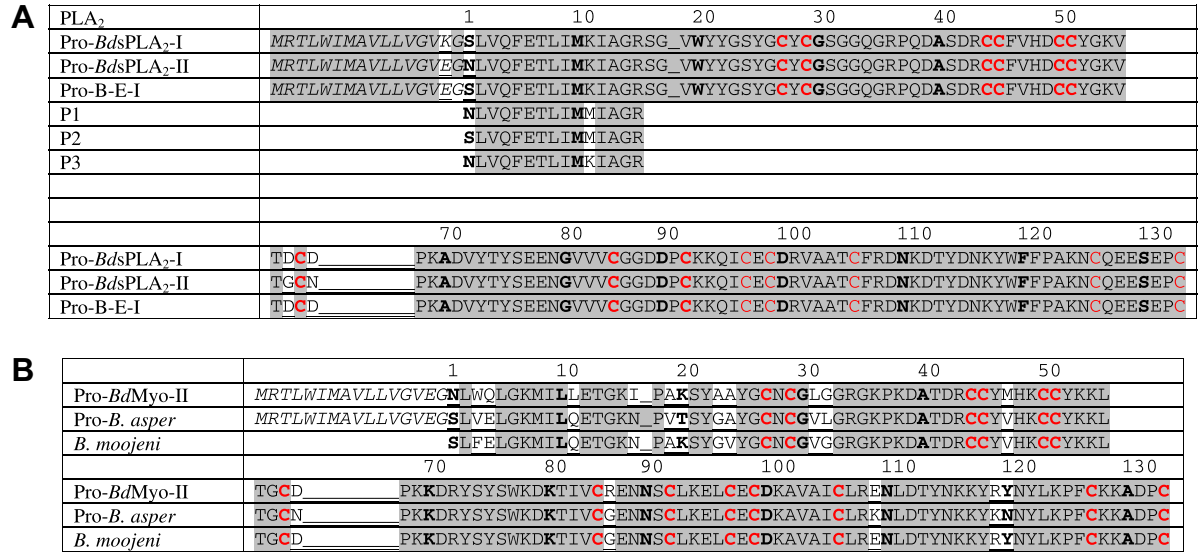

Fig. 1. Deduced aminoacid sequences of representative clones. All of these were replicated in independent reverse transcription-PCR amplifications. Putative signal peptides are in italics. Spaces are introduced for numbering purposes. Bold typeface is used every ten residues. A, *Pro-Bds*PLA$_2$-I and *Pro-Bds*PLA$_2$-II are the deduced aminoacid sequences of sPLA$_2$s cloned in this work (GenBank Acc.Nos JQ661499 and JQ661500 respectively). Mature sequence of sPLA$_2$ from *Bothrops erythromelas* (*B-E-I*, GenBank: DQ359953.1) is identical to *Bds*PLA$_2$-I. P1,P2 and P3 are N-terminal sequences from PLA$_2$s purified from venom [5]. B, Cloned putative PLA$_2$-like myotoxin *BdMyo-II*. For comparison myotoxins from *Bothrops asper* (Swiss-Prot: Q9PVE3.1) and *Bothrops moojeni* (Swiss-Prot: Q9I834.2.2) are included. Note the conservation of CYS (C, red) in all sequences and the change of ASP (D) to LYS (K) 49 in the case of the myotoxin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

diethylpyrocarbonate-treated and autoclaved milli-Q water. Solution containing total RNA was checked by electrophoresis in 1% agarose -TAE gel stained with ethidium bromide. Reverse transcription was carried out as described in the MMLV reverse transcriptase brochure protocol (Promega, USA) using Oligo-dT15 as primer and the RNA solution as template.

2.3. Primer design, PCR amplification, construction of expression plasmids and DNA sequencing

A database search using Blast-p [11] adjusted for short, nearly exact matches was performed on the server of NCBI (http://www.ncbi.nlm.nih.gov/). Query was the fifteen amino acid obtained [9], see Fig. 1. Most similar matches were used to expand the search for complete CDS or genes. Oligonucleotide primers were designed according to conserved residues. Forward (F1, F2 and F3) and reverse (R1) primers are in Table S1. F1 hybridizes to sequence of the signal peptide. F2 spans the last 17 nucleotides of the 5′UTR region and the first two codons of the signal peptide. F3 was used to check for genomic amplification and/or contamination with genomic DNA in RNA preparation (Table S1 and Fig. S1). R1 matches the 3′UTR region. Location of hybridization sites is related to the reference *T. gramineus* sPLA$_2$ gene, (GenBank D31780), Fig. 1A [12]. PCR was performed in 50 μL total volume according to GoTaq brochure instructions using cDNA as template (4 μL of reverse transcription reaction), with the exception of the mixed base primer, which was supplemented to a 15-fold greater concentration. PCR program consisted of a first step at 95 °C for 10 min, and 35 cycles at 94 °C for 45 s, annealing at 58 °C for 40 s, and extension at 72 °C for 60 s. PCR products were purified using the Wizard SV Gel & PCR CleanUP System (Promega, USA), and ligated to the pGEM-T easy cloning vector kit (Promega, USA). Ligations were transformed into *Escherichia coli* DH5α cells and plated onto LB- ampicillin-agar-IPTG-XGal medium. White colonies were selected and plasmid borne insert was subjected to dideoxy chain-termination sequencing (Macrogen Corp, USA) using T7 universal primers. Desired plasmids were propagated in *E. coli* DH5α, and extracted using an alkaline lysis method of minipreparation (Wizard® *Plus* SV Minipreps DNA Purification Systems, Promega, USA). Forward and reverse primers containing SacII and HindIII restriction sites (SIGMA Chem. CO., USA), respectively (Table S1), were used in a PCR with *Pfu* polymerase. Template was used either *Bds*PLA$_2$-I or *Bds*PLA$_2$-II (*Bothrops diporus* cloned sPLA$_2$ isoforms). PCR program was essentially the same as above, except that annealing step was performed at 60 °C for 30 s. PCR amplified fragments (Fig. S1B) were washed using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Subcloning into HindIII and SacII was carried out using standard restriction, gel purification and ligation procedures. Ligation mixtures were precipitated with absolute ethanol and washed with ethanol 70% and water before *E. coli* transformation. Clones were sequenced using T7 universal primers (University of Chicago Cancer Research Center-DNA Sequence Facility).

2.4. Bacterial cultures and induction

E. coli grown on LB agar plates [13] from frozen stocks and subcultured in LB liquid medium overnight at 37 °C shaking at 250 rpm. Growth and induction temperatures were 18, 30 and 37 °C. Transformation of *E. coli* BL21 (DE3) was done by electroporation. Plating was performed on LB-Agar-Ampicillin and grown overnight at 37 °C. Single colony was isolated and transferred to 50 mL of sterile LB-Ampicillin medium. Culture was orbitally shaken at 37 °C for 12–14 h. 50 mL of this culture was added to 1 L of LB-Ampicillin and shaken until D.O. ≈ 1.0 with 0.01 mM IPTG (4 h, 37 °C). Culture was centrifuged for 5 min at 3900*g* at room temperature and supernatant was discarded. Pellet was further processed or frozen at −75 °C. 6×H-specific deubiquitylating peptidase catalytic core 6×H-USP2-cc, kindly donated by An-Maree Catanzariti was expressed in *E.coli* BL21 (DE3) as described [14].

2.5. Solubilization of fusion protein from inclusion bodies and protein renaturation

Washing and solubilization of inclusion bodies were performed similarly as reported [15,16]. Briefly, bacterial pellet was

resuspended in 1/100 of original volume in sucrose 25% w/v, Tris HCl 50 mM pH 7.4. Homogenization was started by adding lysozyme (0.2 mg/ml), EDTA (10 mM), NaCl (100 mM) and ß-mercaptoethaonol (ß-ME, 10 mM), and incubated on ice for 1 h until the solution became viscous. Next, three freeze–thaw cycles were done in liquid air and water bath at 37 °C. DNAse I and RNAse A were added to a final concentration of 5 μg/mL, $MgCl_2$ 5 mM and incubated at room temperature. Solution was centrifuged 10 min at 12,000*g*, and the supernatant was collected. Pellet was washed with Tris ClH 50 mM, 1 mM EDTA, 10 mM ß-ME pH 8, then with the same buffer containing 2 M urea and Triton X-100 2% v/v. Washed inclusion bodies were dissolved by adding 6 M Gdn HCl, 50 mM Tris pH 8, and 10 mM DTT (or 100 mM ß-ME). The mixture was centrifuged at 14,100*g* and the supernatant collected and filtered through Amicon 100K filter (Millipore, USA). Dilution of reducing agent was performed by diafiltration through an Amicon 10K filter.

The original volume of solution, reduced by 90%, was restored by adding 6 M Gdn HCl. This procedure was repeated three times. Finally, solution was dialyzed for 2 days against urea 2 M, 0.2 g/L polyethyleneglycol 3350, EDTA 1 mM, Tris 50 mM pH 8 in an uncapped beaker at 4 °C. L-cysteine was added up to 10 mM after, during, or before incubation with peptidase.

2.6. Protein activation by cleavage with ubiquitin specific peptidase

Cleavage of N-terminal 6×H-Ub was done by incubating fusion protein solution in Tris 50 mM pH 7 or 8.5 at 4 °C, 25 °C or 37 °C with 6×H-USP2-cc peptidase. Mole relation peptidase:fusion protein varied from 1:100 to 1:20 (diluting Gdn HCl to 0.6 M). Incubation time varied from 3 h (at 37 °C) to 2 days (4 °C or 25 °C). Cleavage efficiency estimated by densitometry of 6×H-Ub band (10.7 kDa) in 15% SDS–PAGE. Band Densitometry analysis was performed by using commercial imaging software.

2.7. Affinity chromatography

Removal of histidine-tagged proteins after cleavage was done by means of IMAC chromatography. The nickel charged resin (Amersham, USA) and protein solution were equilibrated in NaCl 0.5 M, imidazole 100 mM, Tris 50 mM pH 7. Incubation with resin was performed on batch or column mode. Protein solution was dialyzed against water. The purification of 6×H-USP2-cc deubiquitylating peptidase was carried out as described [14].

2.8. Phospholipase A_2 activity assays

PLA_2 activity was followed by measuring the decrease in area of a dilauroylphosphatidylcholine (DLPC) monolayer at the air/water interface at constant lateral pressure (Π) using a surface barostat attachment as described [5,17–20] in a KSV-Minitrough system (KSV Instruments Ltd., Finland). Lipid monolayers were obtained by spreading onto the aqueous surface up to 15 μL from a 0.50 mM DLPC working solution dissolved in chloroform:methanol 2:1 (v/v). The subphase was 105 mM NaCl, 20 mM $CaCl_2$ and 10 mM tris, pH 8. All measurements were performed in a thermostatized room at 25 ± 1 °C. The reaction compartment was of cylindrical shape equipped with a small magnetic stirrer and the platinum foil for Wilhelmy balance. Reaction compartment connects to a lipid reservoir compartment throughout a narrow slit. DLPC molecular area was estimated by recording Π-A isotherms. Changes in area were recorded for a minimum of 10 min to account for lipid leakage before 0.3 μg of enzyme was injected into the subphase in the reaction compartment. Linear least square fitting was performed on the area *vs* time data of the last 5 min recorded before injection, and the last 5 min after injection. Lag times is defined as the interval of time between the injection and the intercept of the linear fit of activity obtained after injection.

3. Results

3.1. Cloning of PLA_2 from Bothrops diporus venom glands

The difference between the two most differing cloned *B. diporus* isoenzymes is only four residues, three of them in the mature sequence of the protein (Fig. 1A). We denote the sequence starting with Ser as *Bds*PLA_2-I (JQ661499) and as *Bds*PLA_2-II (JQ661500) the second one with Asn at the beginning. Both have acidic character (theoretical isoelectric point of 4.66 and 4.90, respectively) and very similar to other venom PLA_2s grouped as II in the PLA_2 superfamily [4]. The sequence of *Bds*PLA_2-I is identical to the acidic *B-E-I* PLA_2, from *B. erythromelas* (DQ359953) [21]. These putative sequences have 76% position identity with respect to *Crotalus atrox* $sPLA_2$ (AF269131) belonging to group IIA [4,22,23]. $sPLA_2$s from this group have seven disulfide bridges, a size in between 13 and 15 kDa and the 51–132 unique disulfide pair (numbering after alignment with bovine pancreatic $sPLA_2$). Both sequences have the typical His48/Asp49 pair, Asp 99 within the Asp-CysCys-HisAsp-CysTyr active site, and the TyrGlyCys-CysGgly-GlyGly Ca^{+2} binding loop domain. It is also a characteristic of this group's enzymes the extension ProCys at the C-terminus.

We also obtained the full open reading frame of a putative basic myotoxin, *Bd*Myo-II (JQ661501), a $sPLA_2$-like PLA_2 like molecule with the typical presence of Lys at position 49 instead of Asp [6] (Fig. 1B). The deduced putative myotoxin is basic with a theoretical isoelectric point of 9.10 and 78% of identity to the last 41 residues of the N-terminal sequence of the myotoxin *Bneu I* reported by Geoghegan et al. [24]. Our *Bd*Myo-II sequence has also the same cysteine distribution as *Bds*PLA_2 isoenzymes (Fig. 2B). We found the highest aminoacid identity (87.7%) after a BLAST-p search with myotoxins from *B. asper* and *B. moojeni* (Fig. 2B).

3.2. Expression constructs cleavage and *Bds*PLA_2 renaturation

We chose *Bds*PLA_2-I to begin studies on heterologous expression because its identity in mature sequence to B-E-I-PLA_2 from venom of *B. erythromelas*, (Fig. 1A, [21]). Expression of the insert sub-cloned into pET15b plasmid with ubiquitin motif (pHUE) produced a fusion protein containing six histidines (6H), a thrombin recognition site, a methionine, ubiquitin, and the PLA_2. This construct is termed here as pHUE-*Bds*PLA_2-I (Figs. S1C and S2A). The 6×His tag in the fusion protein allows protein purification under the denaturing condition used to dissolve inclusion bodies. In addition, the 6×H-USP2-cc deubiquitylating peptidase was chosen for its specificity and because it provided a simple and robust way for cleavage and further separation by IMAC. The adduct is found in inclusion bodies and migrates at expected molecular weight (Fig. S2A). PLA_2 *activity is only detected* after incubation with deubiquitylating peptidase (Fig. S2B). An optimum of yield of 50% of cleavage was obtained in 0.6 M Gdn HCl. After incubation, 6×His- tagged peptidase and the N-terminal ubiquitin tag were removed by a run in IMAC (Fig. S2). Identical procedures was used to obtain recombinant *Bds*PLA_2-II.

3.3. Recombinant phospholipase A_2 activity

As one of the aims of this work was to explore the relation of sequence/structure of $sPLA_2$ with its ability to sense differences *in lipid packing*, we routinely checked the activity on both recombinants by using DLPC monolayers at different lateral pressures. Lipid monolayers at constant lateral pressure (surface barostat)

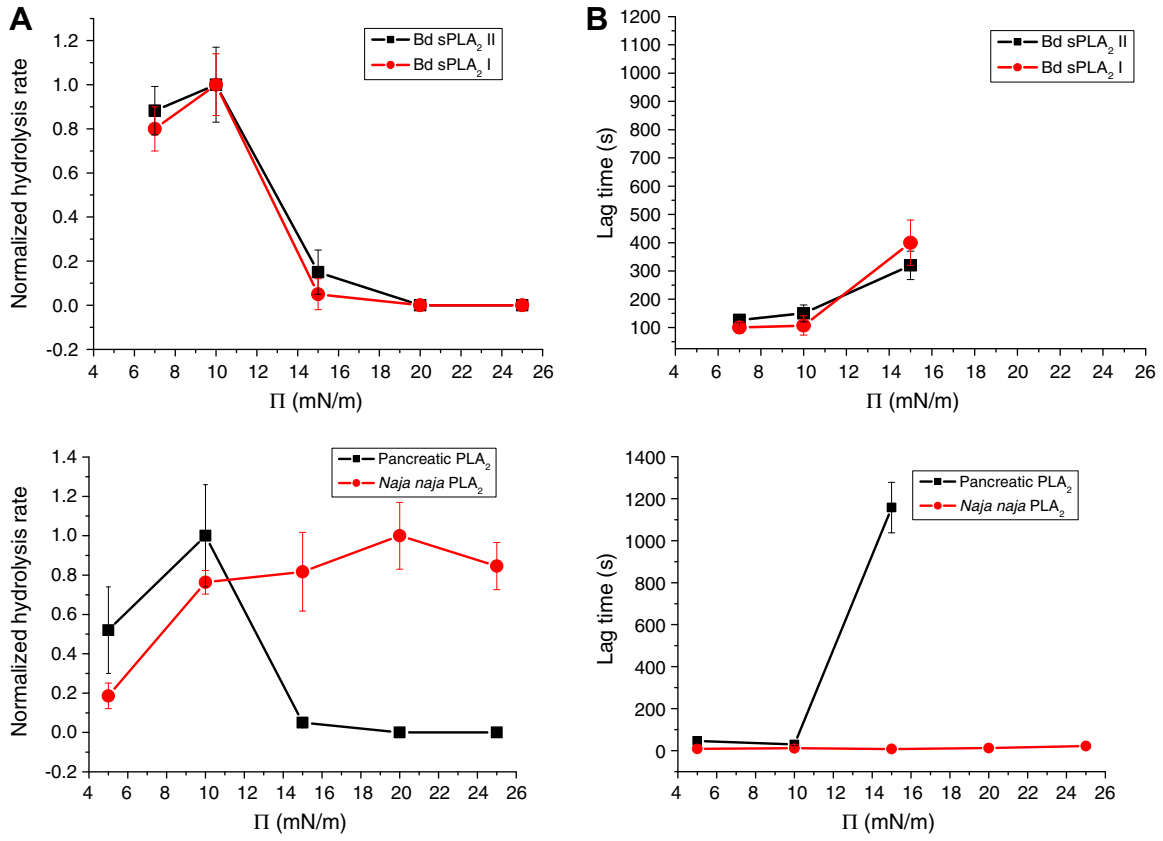

Fig. 2. Hydrolysis of DLPC monolayers by sPLA$_2$s. Subphase composition was NaCl 105 mM, Tris 50 mM, $CaCl_2$ 20 mM, at 25.0 ± 0.4 °C with constant stirring. Values are within a S.E.M of ±10%. A, Normalized hydrolysis rates vs. lateral pressure Π (mN/m) of DLPC monolayers. Each curve is normalized taken its maximum rate as 1. B, Lag times (s) *vs* lateral pressure Π (mN/m). Upper panel, recombinant *Bothrops* sPLA$_2$. Lower panel, *Naja naja* or porcine pancreatic sPLA$_2$. For lateral pressures where no changes in area could be recorded lag times are not defined. Points are shown as mean ± SD (n = 3).

allow the study of phospholipase activity on organized substrate at a desired lateral packing [5,20]. We measured the hydrolysis rates (Fig. 2A) and lag times (Fig. 2B) at 25 °C on DLPC monolayers at 5, 10, 15, 20, 25 mN m^{-1}. For comparison, we constructed the same profile for Cobra (*Naja naja*) venom and porcine (*Sus scrofa*) pancreatic sPLA$_2$s. Recombinant isoenzymes required Ca^{2+} for activity and the reaction was abolished by adding equimolar amounts of EDTA. Optimal lateral pressure of both recombinant sPLA$_2$ from *B. diporus* resembles those of venom purified sPLA$_2$s denoted as P-1 or P-2 (Fig. 1). For this type of enzymes, in general, lag time *vs* Π followed an inverse relationship between hydrolysis rates *vs* Π profile (Fig. 2) acquiring higher hydrolysis constant rates at shorter lag times. (The construct 6H-U- *Bds*PLA$_2$-I neither USP2cc peptidase had no phospholipase activity).

4. Discussion

Enzymes that "attack" lipid organized surfaces are subtly modulated by the membrane composition, packing and its physical state [25–28]. Previously , we found differences in the optimum of lateral surface pressure-activity profile for sPLA$_2$ isoenzymes purified from venom denoted as P1, P2 and P3 [5,9]. Amino acid composition, and N-terminal sequence were very similar, but P3 had an optimum of lateral pressure hydrolysis far larger than P1 and P2.

By assuming homology with known PLA$_2$ genes even for geographically distant viper species such as *T. gramineus*, we showed it was possible to design oligonucleotides suitable for cloning PLA$_2$ and a myotoxin. This "lucky strike" was supported by evidence of an unusual conservation of UTR regions flanking DNA coding sequence of sPLA$_2$ [12]. With this approach, we cloned putative sPLA$_2$s isoforms differing in four or less residues, and a putative Lys49 myotoxin from *B. diporus* venom glands.

After several attempts of expressing the histidine tagged construct (by using pET15b vector) and checking for activity, we confirmed other laboratories' observations that for this type of secreted PLA$_2$ a native N-terminus is essential for full enzymatic activity [29–31]. If the starting translation Met residue is not removed there exists the possibility of structure-distorting effects [32,33] and a 90% activity loss [34]. In our case, we found no detectable DLPC monolayer hydrolysis of the 6×His-ubiquitin-PLA$_2$ fusion. Some researches turned to the substitution of the first enzyme's residue, such as Asn for Gly [35], to allow for excision of Met by methionyl-aminopeptidase. This *E. coli* aminopeptidase reduces its efficiency if the adjacent residue to initial methionine has a bulky side chain, such as Asn [36]. Because it is likely that Asn residue was key for high packing hydrolysis (P3 isoform) we designed a system that allowed us to have the native N-terminus.

We resorted by using a system employing an ubiquitin motif that it is efficiently removed by a specific ubiquitin peptidase requiring no extra amino acid in the C-terminus of recognition sequence [14]. In addition the ubiquitin specific peptidase itself is fusioned to a 6×His tag (6×His-USP2cc) which it enabled the posterior removal from *Bds*PLA$_2$-I and *Bds*PLA$_2$-II solutions using the same nickel affinity procedure.

Our recombinant isoenzymes resemble the profile found for P1 and P2 purified from venom. We could not reproduce the profile of P3, which showed a markedly higher cut-off pressure. *Bds*PLA-II was thought to be the candidate to reproduce P3 profile since it

has the same N terminal sequence. Control values obtained for porcine pancreatic and cobra venom phospholipases in the surface pressure profile show the same tendency as those published by van Demel et al. [26].

One major concern is the possibility that recombinant expression process itself would render active PLA_2s with lower lateral pressure optima than their natural counterparts. This has been shown to certainly happen with staphylococcal lipases [37]. From a perusal searching of the literature for studies of hydrolysis rate *vs* Π of *recombinant* $sPLA_2$, we certainly did not find any report that actually measured this property.

In conclusion, this is the first report of heterologous production of an active phospholipase A_2 from *B. diporus* by using Ubiquitin/USP2cc system in *E. coli* and the construction of monolayer hydrolytic profile of recombinant enzymes. Our results should be taken into account since lipolytic *recombinant* enzymes may differ in the interfacial behavior compared with those obtained by conventional methods. The molecular details by which lipid packing can modulate $sPLA_2$ activity remain to be decoded.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.051.

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