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Deciphering the recombinant thermostable phosphatidylcholine-specific phospholipase C activity from *Bacillus thuringiensis:* biochemical and interfacial properties

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Abstract

A novel alkaline thermostable phosphatidylcholine-specific phospholipase C (PC-PLC$_{Bt}$) was expressed in *E. coli* system. Recombinant PC-PLC$_{Bt}$ (rPC-PLC$_{Bt}$) activity and thermostability were shown to be significantly dependent on the Zn^{2+}. The maximum rPC-PLC$_{Bt}$ catalytic activity was found to be 1372 U.mg^{-1} in the presence of 0.1 mM Zn^{2+} and at 60 °C using an Egg PC as substrate. The interfacial kinetic data show that nPC-PLC$_{Bt}$ and rPC-PLC$_{Bt}$ display similar substrate specificity on various phospholipid monolayers. The maximal rPC-PLC$_{Bt}$ activities were recorded, at decreasing order, on 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine (DLPE), 1,2-diacyl-*sn*-phosphoglycerol (PG), and 1,2-diacyl-*sn*-phosphoserine (PS) monolayers at interfacial surface pressures of 15, 25, 20, and 25 mN.m^{-1}, respectively. Such important penetrating power could be exploited for pharmacological purposes. The highest activities were recorded on the DLPC monolayer and shown to be 121.61 and 40.13 mmol.cm^{-2}.min^{-1}.M^{-1} for native and recombinant PC-PLC$_{Bt}$, respectively. Interestingly, compared to all known *Bacillus* PLCs, both PC-PLC$_{Bt}$ forms showed an exclusive capacity to hydrolyze the PG film with a more pronounced rate of hydrolysis for the native form with a specific activity of 58.29 mmol.cm^{-2}.min^{-1}.M^{-1}. Therefore, the high enzyme level production of about 14 mg.L^{-1}, the thermostability as well as the broad phospholipid specificity of PC-PLC$_{Bt}$ represents great potential in the crude oil refining industry.

Keywords: *B. thuringiensis* IL14; PC-PLC; thermostability; substrate specificity; interfacial properties; cytotoxic properties.

1.Introduction:

Phosphatidylcholine-phospholipase C (PC-PLC) is a lipolytic enzyme that preferentially hydrolyzes the PC, as a major substrate, as well as other kinds of phospholipids, into 1,2-diacylglycerol (1,2-DAG) and the correspondent phosphate monoesters [1]. Bacterial PC-PLC is the most promising enzyme for application in the enzymatic degumming of plant oils, considering its high activity, easy production, wide substrate spectrum, and approved safety [2, 3]. It offers the opportunity of oil enrichment with released DAG and contributes to the final oil yield [4]. Moreover, the microbial PLC appeared as a potentially useful model for mammals PC-PLC to study cellular signaling, lipid metabolism, blood coagulation, and eukaryotic cell membranes [5]. *Bacillus cereus* PLC (PLC_{Bc}) is the most studied among the bacterial PC-PLC. It is an extracellular monomeric zinc-dependent enzyme of 28.5 kDa synthesized as a 283 residues pre-proenzyme from which the 24-residue long signal peptide and the 14-residue long pro-peptide are cleaved to give a fully active enzyme [3]. Crystallographic [6, 7] and chemical modification [8] studies of the PC-PLC_{Bc} have provided an insight into the molecular architecture of this enzyme. By comparing with other metallo-PLC from *Clostridium perfringens* and *Clostridium bifermentans*, this enzyme presents only the N-terminal part 250 residues and truncated of the so-called C-terminal side, conferring sphingomyelin-hydrolyzing, lytic and hemolytic properties to these enzymes [9, 10]. Structurally, PC-PLC_{Bc} is composed of eight α-helices, forming a twisted barrel structure [6]. PC-PLC_{Bc} is an unusual protein with a trinuclear-zinc center playing a crucial role in the catalytic process, where nine residues (5 His, 2 Asp, 1 Glu, and 1 Trp) are known to be involved in the coordination of zinc ions [11]. Recently, an extracellular nPC-PLC_{Bt} has been purified in our laboratory [12]. Interestingly, this enzyme displayed the highest catalytic activity of about 6000 $U.mg^{-1}$ using egg PC as the substrate measured at 55-60 °C. nPC-PLC_{Bt} has also the capacity to retain its activity at high temperatures and in alkaline conditions and it

has an exclusive capacity to hydrolyze the phosphatidylglycerol (PG) headgroup comparing to the *Bacillus* PC-PLC group [13, 14]. These biochemical criteria suggested that PC-PLC$_{Bt}$ might be suitable for biotransformation in emulsifier production in food industries and as a potential catalyst in industrial oil degumming purposes for the edible oil industry which requires extreme temperatures exceeding the 60 °C. These attractive catalytic and biochemical capacities for industrial processes incite the over-expression of PC-PLC$_{Bt}$ to achieve enough protein amounts. Based on the available literature, several systems have been tried for *Bacillus* PLC gene expression. Among them, the *P. pastoris* system could yield large amounts of the active extracellular enzyme [15], although its cultivation period is relatively long[16]. PC-PLC was also overexpressed in *B. subtilis* using a novel expression system [17], but this latter used is due to licensing fees. Ravasi et al. in 2015 reported that high-level production of *B. cereus* PLC could be achieved by using the *C. glutamicum* system [18]**.** Nevertheless, the mature expression system of *E. coli* remains often the choice allowing a large protein quantity and a high-throughput structural analysis [19]**.** Although much is known about the function and the structure of the PC-PLC of *Bacillus* group, there remain numerous unanswered questions concerning its kinetic, substrate specificity and interfacial properties towards monomolecular phospholipid films at the air-water interface. The monolayer technique has many advantages. On one hand, it defines the substrate physicochemical state such as the molecular area *via* the interfacial surface pressure. On the other hand, this technique makes possible the investigation of the enzyme capacity to penetrate various phospholipid monolayers at various initial surface pressure [20]. Formerly, kinetic studies of PLC used monomolecular film of radiolabeled phospholipids [21]. However, such approach suffers from the DAG accumulation provoking a variation in the monolayer lipid composition and a decrease in the phospholipid surface density [22]. To avoid these constraints, an alternative monolayer procedure previously adopted for studying the interfacial behavior of

77 PLC activity from *C. perfringens* α toxin [22] was developed. This new approach consists of
78 rapid hydrolysis, by a lipase, of DAG molecules generated from the PLC action on
79 phospholipid films into fatty acid and 2-monoacylglycerol (2-MAG), which are then desorbed
80 from the lipid interface.
81 In the present investigation, a high-level expression of the rPC-PLC$_{Bt}$ in the *E. coli* system is
82 reported. Since the *Bacillus* PC-PLC interfacial behavior has not been investigated yet, we are
83 establishing a comparative monolayer study between the nPC-PLC$_{Bt}$ and the rPC-PLC$_{Bt}$
84 enzymes, based on a new coupled reaction between the PLC and a strict lipase unable to
85 hydrolyze phospholipid films. Furthermore, new insights into the affinity of PC-PLC$_{Bt}$ for
86 biomembrane are given, in correlation with its interfacial capacity to hydrolyze phospholipid
87 monolayers at high surface pressure.
88

2. Material and methods

2.1. Chemicals and lipids

Benzamidine was from Fluka (Buchs, Switzerland), tryptone, yeast extract, sodium deoxycholic acid (NaDOC), ethylene diamine tetra-acetic acid (EDTA), β-cyclodextrin (β-CD), and Egg PC (99% purity) were purchased from Sigma Aldrich (St. Quentin Fallavier, France). Isopropyl Thio-D-galactopyranoside (IPTG) was purchased from Boehringer. Marker proteins and Ni-NTA (nickel nitriloacetic acid) resin for affinity chromatography used for enzyme purification were obtained from Bio-Rad. DLPC, DLPE, PG, and PS were purchased from Avanti Polar Lipids. Pure lipid solutions were prepared by adding a volume of chloroform to a known mass of dry sample to obtain the desired concentration.

2.2. Bacterial strains and plasmids

E. coli strain Top 10 was used for cloning the gene encoding PC-PLC$_{Bt}$. *E. coli* strain BL21 (DE3) served as the expression host for the rPC-PLC$_{Bt}$ gene construct. The pGEM-T Easy and pET 28b (+) plasmids were used as cloning and expression vectors, respectively. *E. coli* strains were grown in Lysogeny broth (LB) medium, supplemented with 100 µg.mL^{-1} ampicillin or 100 µg.mL^{-1} kanamycin according to the used plasmid. Restriction endonucleases were from New England BioLabs, wizard PCR preps DNA purification system, and midi-prep kit were bought from Macherey-Nagel, and oligonucleotides were synthesized by Eurofins.

2.3. Proteins

nPC-PLC$_{Bt}$ was purified previously according to our described procedure [12]. The pure native enzyme consists of a single polypeptide chain with a molecular mass of about 28 kDa and specific activity of about 6000 U.mg^{-1} measured at 60 °C °C and pH 8 using pure egg PC as the substrate in presence of 0.5 mM Mg^{2+}. Recombinant *Serratia* sp. W3 lipase (rSmL) was overproduced in *E. coli* BL21 and purified in the laboratory as previously described [23].

Protein concentrations were determined routinely using the Bradford method [24] with dye reagent and bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed adopting the procedure of Laemmli [25].

2.4. Molecular cloning of rPC-PLC$_{Bt}$

The gene part encoding the mature PLC$_{Bt}$ was amplified by PCR from genomic DNA of *B. thuringiensis* IL14 using the following primers 5'-ATCG*CATATG*TGGTCTG CTGAAGATAAACAT-3' and 5'-TACG*GCGGCCGC*TTAACGATCTCCGTA CGTATC-3'. The primers were predicted from the N-terminal sequence of the purified nPC-PLC$_{Bt}$ [12] and the C-terminal sequence of the *Bacillus* PLC group. PCR reaction was performed in 50 µL reaction mixtures containing 25 pmol of each primer, 0.5 U of Q5®High-Fidelity DNA Polymerase with its adequate buffer (New England Biolabs), 2 mM dNTP mix, and 25 ng DNA as a template. The PCR protocol consisted of 30 cycles at 98°C (30 s), 40°C (30 s), 72°C (60 s) respectively and a final extension at 72°C for 10 min. The amplicon (700 bp) was first cloned into pGEM-T Easy, sequenced, excised with *NdeI* and *NotI*, then cloned into the expression pET-28b(+) vector (Invitrogen). The ligation reaction was set in the ratio of 1:1 (insert: vector). Competent *E. coli* Top10 cells were transformed with the ligation mixture and transformants were selected on LB plates supplemented with kanamycin at a final concentration of 100 µg.mL^{-1}. The presence of the appropriate insert was determined by colony PCR. The NCBI BLAST and FASTA programs were used for homology searches in the GenBank and Swiss-Prot databases.

2.5. rPC-PLC$_{Bt}$ expression and purification

The *E. coli* BL21 (DE3) strains to contain the different recombinant plasmids were grown at 37°C in a 50 mL LB medium containing 100 µg.mL^{-1} Kanamycin. 0.5 mM IPTG was added to the medium, upon reaching the exponential growth phase, and the cultures were incubated at 23°C for 12 h. Cells were harvested by centrifugation at 3500 × g for 10 min and washed

twice with buffer A (20 mM Tris–HCl, pH 8.5, 20 mM NaCl). Cells collected by centrifugation were resuspended in buffer A and were disrupted by sonication using an MSE Sonyprep 150 at full power; 15 s on 15 s off for 15 cycles at 4°C. Cell debris was then removed by centrifugation at 9500 × *g* for 20 min and the supernatant was assayed for PLC activity. The resulting sample was subjected to a Ni-NTA affinity chromatography substantially equilibrated with buffer A supplemented with 2 mM benzamidine and rPC-PLC$_{Bt}$ was then eluted with an imidazole gradient. The active fractions were then pooled, concentrated, and further purified using Sephadex G-75 column (2.5 cm×150 cm), pre-equilibrated with 25 mM Tris–HCl buffer (pH 8).

2.6. PC-PLC$_{Bt}$ assay

The PC-PLC$_{Bt}$ activity was checked titrimetrically at pH 8.5 and 55°C with a pH-stat using Egg PC as a substrate in the presence of 7 mM NaDOC [12]**.** Under standard conditions, one unit of the PLC activity corresponds to 1 µmol of PC liberated per minute.

2.7. Biochemical characterization of rPC-PLC$_{Bt}$

To check the effect of the recombination process on the biochemical characteristics of the rPC-PLC$_{Bt}$, this enzyme was characterized and compared with the native one. The effect of pH and temperature on the rPC-PLC$_{Bt}$ activity was determined by measuring continuously the PLC activity over the pH range of 7-10 and the temperature range of 20 to 70 °C using an egg PC emulsion. The enzyme pH stability was measured by incubating the enzyme in different pH values (3-12) using the corresponding buffers at a final concentration of 100 mM. The residual activity was determined after incubation at room temperature for 2 h. The thermal stability was determined by incubating the enzyme solution at different temperatures (20-60°C) and pH 8.5 for 1 h. The rPC-PLC$_{Bt}$ residual activity was determined after centrifugation to remove the denatured enzyme fraction under optimal assay conditions. The effect of

NaDOC and metal ions on enzyme activity was also checked at increasing concentrations under predicted optimal conditions using Egg PC as the substrate.

2.8. Monitoring reaction rates using monomolecular film technique

2.8.1. Monolayer measurements

Measurements were performed with KSV-2200 Baro-stat equipment (KSV-Helsinki). The principle of the method was described elsewhere [26]. A zero-order trough with two compartments, a reservoir compartment 14.8 × 24.9 cm in size, and a reaction compartment in which the enzymes were injected (total volume 130 mL; total surface area 120 cm^2) connected by a small surface channel was used in this study [27]. A thin platinum plate immersed in the surface of the aqueous phase is attached to an electro-microbalance for measuring the surface pressure (π). A mobile barrier, automatically driven by the barostat, moves over the reservoir compartment to maintain the surface pressure constant and to compensate for the substrate molecules removed from the lipidic film by enzyme hydrolysis. All experiments were performed at room temperature (25 °C). The subphase of the reaction compartment was continuously stirred with two magnetic stirrers (250 rpm) and the enzyme solution was injected with a Hamilton syringe only when the phospholipid film covered both compartments. The following buffer was used throughout the experiments: 10 mM Tris–HCl buffer, pH 8.0, 21 mM $CaCl_2$, 150 mM NaCl, 1 mM EDTA.

2.8.2. Two enzymatic steps to measure PC-PLC$_{Bt}$ activity on phospholipid monolayer

The enzymatic activities of the PC-PLC$_{Bt}$ were investigated with various phospholipids monolayers using a coupled assay as described by Moreau et al [22]. The principle of this assay is shown schematically in **Figure 1.** The assay involves coupling the PLC catalytic reaction, using a phospholipid monolayer as the first substrate, with the subsequent hydrolysis of the formed DAG by a lipase. This method requires a lipase that is not able to hydrolyze phospholipid monolayer such as rSmL according to our previous report [28].

The phospholipid solution was spread, with a Hamilton syringe, into a clean air/water interface. The monomolecular film was maintained at constant surface pressure. Initially, rSmL was added to the aqueous subphase and no activity was detected. When PC-PLC_{Bt} was injected into the reaction compartment, the reaction was started as reflected by the mobile barrier movement back and forth over the reservoir. PLC hydrolyzed the insoluble phospholipid films generating a stable 1,2-DAG film and water-soluble phosphocholine. The 1,2-DAG, in contrast to phospholipid film, was subsequently hydrolyzed by the rSmL ultimately producing the 2-MAG and free fatty acid. These enzyme-catalyzed reaction products were rapidly desorbed from the interface in the form of complexes with β-CD allowing a constant surface pressure. Specific activities were expressed as the number of moles of the substrate hydrolyzed per unit time and per unit surface ($mol.cm^{-2}.min^{-1}$) of the reaction compartment of the "zero-order" trough for an arbitrary PLC concentration of 1M.

2.9. Hemolysis assay

The hemolytic activity of the rPC-PLC_{Bt} was performed using the direct hemolytic test. Freshly collected blood human erythrocytes were immediately mixed with drops of heparin (5 $mg.mL^{-1}$), to prevent blood coagulation. In the first step, the suspension is centrifuged at 780 x *g* for 15 min to eliminate the serum and the obtained pellet undergoes three successive washes with 0.9% NaCl, each of which is followed by gentle centrifugation at 780 x *g* for 15 min. Recovered erythrocytes were suspended in phosphate-buffered saline (PBS pH 7.4) and centrifuged at 970 x *g* for 5 min at 4°C to obtain a pure suspension of erythrocytes. The supernatant and buffy coats were removed by gentle aspiration, and the above process was repeated two more times. Finally, pure erythrocytes were suspended in PBS to make 1% solution for the hemolytic assay and various concentrations of pure rPC-PLC_{Bt} were added to red blood cells suspension. The rPC-PLC/erythrocyte mixture was incubated at 37°C for 30 min and then centrifuged at 970 x *g* for 5 min at 4°C. The absorbance of the supernatants was

213 determined at 545 nm to measure the extent of red blood cell lysis and hemoglobin release. In
214 parallel, erythrocytes were incubated in PBS containing 1% Triton X-100 (100% hemolysis)
215 and PBS alone (0% hemolysis), which will serve as a positive and negative control,
216 respectively.

217 **2.10. Cell Culture**

218 Primary chondrocytes were obtained from newborn (4-6 days) SWISS mice by successive
219 enzymatic digestion of knee and femoral head cartilage [29]. Cells were cultured for 7 days in
220 Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v:v) FBS, penicillin
221 (100 $U.mL^{-1}$), and streptomycin (0.1 $mg.mL^{-1}$). The medium was changed every 2-3 days.
222 Non-tumor human prostatic cell line WPMY-1 was purchased from ATCC (Manassas, USA).
223 Human prostate cancer bone metastasis-derived cell line PC-3 was purchased from DSMZ
224 (Braunschweig, Germany). WPMY-1 cells were cultured in DMEM supplemented with 10 %
225 (v:v) FBS and antibiotics. The PC-3 cell line was cultured in Roswell Park Memorial Institute
226 (RPMI) medium supplemented with 10 % (v:v) FBS and antibiotics. Prostatic cell lines were
227 maintained in a humidified atmosphere (5% CO_2) at 37 °C and passaged twice a week. They
228 were weekly monitored by the following tests: morphology check by visual inspection,
229 growth curve analysis, and mycoplasma detection (Mycoplasma detection kit; InvivoGen,
230 California, USA). All experiments were started with low-passaged cells (<20 times).

231 **2.11 Cell viability and cytotoxicity assays**

232 The number of viable cells was determined using the MTT assay, which is based on the
233 reaction of a yellow tetrazolium salt with cellular dehydrogenases to form purple formazan
234 crystals [30]. Cells ($3x10^4$ cells/cm^2 for murine chondrocytes, $2x10^4$ cells/cm^2 for WPMY-1
235 cells and 10^4 cells/cm^2 for PC-3 cells, respectively) were seeded in 12-well plates. Cells were
236 treated a week later (chondrocytes) or after 72 h with increasing concentrations of rPC-PLC_{Bt}
237 (0.05-0.2 $mg.mL^{-1}$) during 72 h. MTT was then added at a final concentration of 0.125 g/L.

The plate was further incubated for 4 h at 37°C, after which the culture medium was removed, and the formed formazan crystals were dissolved in 1 mL of DMSO. After 30 min incubation, the absorbance of the plate was measured at 570 nm on an Infinite-M200 pro Plate reader (TECAN, Männedorf, Switzerland). Results were expressed as mean ± standard deviation of three independent experiments.

The cytotoxic effect of rPC-PLC$_{Bt}$ treatment was assessed using the Lactate Dehydrogenase (LDH) assay that relies on the detection of a cytosolic enzyme, the LDH, in the culture medium as an indicator of membrane disruption after cell death. Cells were seeded in 12-well plates and treated as described previously. LDH was detected in 50 µL of culture medium using an LDH Cytotoxicity Assay Kit (Sigma-Aldrich), as described by the manufacturer. Results were expressed as mean ± standard deviation of three independent experiments.

2.12. Modeling, Molecular dynamic (MD) simulation, and MM/PBSA calculation

As no crystal structures were available for PC-PLC from *Bacillus thuringiensis*, molecular modeling has been performed. Briefly, a BLAST search [31] performed against the Protein Data Bank [32] identified PDB ID 1AH7 [6], PC-PLC from *Bacillus cereus,* as a template (99.6% identity on 245 residues). The reconstruction of the three-dimensional (3D) models was carried out by substitution and reorientation of the substituted amino acid (S188P) using the COOT program [33]. After this step, the structure was energy minimized, computations being done in *vacua* by using the GROMOS96 43B1 parameters set [34]. Docking of a 1,2-diacyl-*sn*-phosphoglycerol (PG) into the PC-PLC$_{Bt}$ model structure was performed using AutoDockVina embedded in PyRx 0.8 [35, 36]. Briefly, the protein structure was prepared for docking by removing unwanted water molecules and bound ligands and by adding polar hydrogens atoms using Discovery Studio Visualizer. The same program was used to build the substrates molecules as PDB files and for energy minimization. PyRx was used for converting all molecules to AutoDock Ligand format (PDBQT). The three-dimensional grid box for

molecular docking simulation was obtained using Autodock tools. The Grid box was centered to cover the active site and all essential residues. The docking results were analyzed by comparing the binding interactions and binding energies between substrate molecules and the enzyme.

To give a better binding energy approximation of the PG/PLC and PC/PLC complexes, MM/PBSA approximation was realized using YASARA Structure. First, the phospholipid/PLC_{Bt} complex structure was filled with water and cell neutralization was performed. MD simulation was started at 303 K, with Amber14 force-field, and ran for 20 ns. Simulation snapshots were saved every 100 ps for MM/PBSA calculation. For each simulation snapshot, binding energy was calculated as the difference between the sum of potential and solvation energies of the separated compounds and the sum of potential and solvation energies of the complex. Because the entropy term from normal mode analysis is missing, we get almost 'MM/PBSA' via binding energy average from all snapshots.

3. Results and discussion

3.1. Molecular cloning and expression of rPC-PLC$_{Bt}$

The PCR product corresponding to the gene part encoding the mature PC-PLC$_{Bt}$ sequence length was 735 bp encoding 246 amino-acids including the first methionine inserted **(Figure S1)**. The catalytic triad active site residues E_4, Y_{56}, and F_{66} could be identified **(Figure S1).** The rPC-PLC$_{Bt}$ protein sequence shows an identity of 99 % with the PC-PLC$_{Bc}$ already reported and predicted [37-39] through comparison with the protein sequences of *Bacillus* group PLC produced and crystallized [6], in addition to the first methionine inserted, one substitution (S188P) is noted **(Figure S1)**. Expression of rPC-PLC$_{Bt}$ was performed in *E. coli* BL21 (DE3). The medium composition, the inducer concentration, and the induction temperature for optimum enzyme production by *E. coli* BL21 were optimized using the classical method 'one variable at a time'. In the light of the obtained results, we could conclude that by decreasing the incubation temperature to 23°C and IPTG concentration to 0.5 mM, the enzyme production was oriented towards the intracellular medium underactive form reaching 24000 U.L^{-1} of culture. The enzyme activity was observed to start soon after induction and reached its optimum after 12 h of induction; in addition, the results show that the LB medium is more adequate allowing the best level of activity. At 37 °C, rPC-PLC$_{Bt}$ was found to agglomerate in the inclusion bodies [40]. Based on the available literature the expression of PLC from bacillus can lead to two voices: intracellular expression in an inactive form, and extracellular expression in the native form [3]. *E. coli* host is often the choice for a large amount of inclusion bodies, which could only be converted into an active protein by a lengthy protocol with low yields [41]. In this report we have interestingly succeeded, by optimization of the induction conditions, to overexpress the mature PC-PLC from *Bacillus thuringiensis* in an active soluble form in the E.coli system, avoiding thus the tedious refolding steps.

3.2 Purification and biochemical characterization of rPC-PLC$_{Bt}$

The purification of rPC-PLC$_{Bt}$ was performed using a Ni-NTA affinity column. The protein was eluted by a linear imidazole gradient at around 50 mM, as an inactive form under the imidazole effect **(Figure 2A).** Protein fractions were collected, concentrated, and subsequently filtrated using Sephadex G-75 gel filtration column to get rid of any imidazole traces susceptible to influence the pure recombinant enzyme stability **(Figure 2B).** Active fractions analysis by SDS-PAGE reveals the presence of the rPC-PLC$_{Bt}$ as a pure protein band of about 28 kDa **(Figure 2B).** From 250 mL of culture medium, about 3.5 mg of pure rPC-PLC$_{Bt}$ were obtained with a specific activity of 1372 U.mg^{-1}. This protein amount has never been obtained from the wild *B. thuringiensis IL14* allowing only 0.83 mg of nPC-PLC$_{Bt}$ [12]. It should be noted that this rPC-PLC$_{Bt}$ amount is expected since it was previously reported that when the PC-PLC expression is controlled under the strong T7 promoter, a higher protein amount can be produced in this recombinant system than in *B. thuringiensis* [41]**.**

In our previous study [12], three metal ions (Mg^{2+}, $Ca^{2+,}$ and Zn^{2+}) were reported as nPC-PLC$_{Bt}$ activators. Thus, we tested the effect of these metal ions on the recombinant form activity under the same optimal conditions used for the wild-type form. From **Figure 3A**, we were able to confirm that Ca^{2+}, Zn^{2+} and Mg^{2+} are phospholipase activity stimulators and zinc ions are shown to be the most effective on the activity of rPC-PLC$_{Bt}$. The presence of only 0.1 mM of Zn^{2+} in the assay medium induces an increase of the rPC-PLC$_{Bt}$ activity 5 times as compared to twice for nPC-PLC$_{Bt}$ [12]. However, an excessive Zn^{2+} concentration in the assay medium begets a rollback in the phospholipase activity which comes in agreement with findings published by Lyu et al., 2016. The latter reported that although the metallo-character of PC-PLC$_{Bc}$, when expressed in *P. pastoris* X-33 its activity is inhibited beyond 5 mM Zn^{2+} [3]. Concerning the detergent effect, rPC-PLC$_{Bt}$ activity was assayed in the presence of increasing concentrations of NaDOC **(Figure 3B)**. As previously described for the native

form, rPC-PLC$_{Bt}$ is still always characterized as a surfactant enzyme since it showed the highest specific activity at 7 mM NaDOC and beyond this concentration, the NaDOC does not exert any inhibitory effect [12]. Indeed, as well explained [42, 43], this surfactant is involved in the solubilization of phospholipids or the DAG hydrolysis product. The effect of pH and temperature on the rPC-PLC$_{Bt}$ activity was investigated and our findings prove that the expression process kept the thermo-activity character of the native enzyme [12]. As it could be seen from **Figure 3C,** the rPC-PLC$_{Bt}$ displays its optimal hydrolytic activity of about 1372 U.mg^{-1} at 60 °C which is 7 times higher than when the enzyme is incubated at room temperature. Such characteristic is one of the most sought-after criteria, for an industrial application because the realization of enzymatic process at high temperature offers many advantages [44, 45]. Furthermore, rPC-PLC$_{Bt}$ activity was measured at 60 °C at different pH values using egg PC dispersion as substrate. The pH *vs* activity profile (**Figure 3D)** demonstrates that rPC-PLC$_{Bt}$ is active in a pH range from 7.5 to 9.5 with an optimum reached at pH 8. On both sides of this pH zone, the activity starts to decrease contrary to an overexpressed PC-PLC$_{Bc}$ in *P. pastoris* X-33 presenting the optimal activity under mildly acidic conditions (pH 4-5) [3]. Unlike published findings for nPC-PLC$_{Bt}$ [12], the thermal and pH stability of the rPC-PLC$_{Bt}$ were affected by the presence of zinc ions in the reaction medium. In fact, from the thermo-stability profile of the enzyme (**Figure 3E)**, we conclude that rPC-PLC$_{Bt}$ retained around 60% of its initial activity after incubation for 1 h at 65 °C in the presence of 0.1 mM zinc ions compared to only 35% of remaining activity in the absence of zinc ions. Furthermore, the rPC-PLC$_{Bt}$ shares the same stability profile as the native form [12] where rPC-PLC$_{Bt}$ keeps the full initial activity after incubating the enzyme at various pH values (from 6 to 8.5) for 1 h (**Figure 3F)**. However, on both sides of this range, the activity decreased slightly since rPC-PLC$_{Bt}$ retained 70% and 50% of its initial activity when incubating the enzyme at pH 5 and pH 10, respectively for 1h (**Figure 3F**). This weak

stability could be caught up by the presence of the zinc ions in the reaction medium, which can restore a stability profile like the native form. Therefore, when adding 0.1 mM Zn^{2+}, rPC-PLC_{Bt} keeps 70% and 50% of its initial activity at pH 4 and at pH 12, respectively (**Figure 3F**). The experiment was extended up to 3 hours and interestingly, the recombinant enzyme remains stable within a pH range of 6-8 where nearly 90% of residual activity was recorded. The presence of zinc ions allows more than 55% of rPC-PLC_{Bt} activity at pH 4 and 11 after 3 h. Consequently, it appears that the presence of zinc ions induces conformational changes which involve a significant modification in the secondary structure of PC-PLC_{Bt} leading to its stability and to the improvement of its catalytic properties. In that context, it was reported that the complexed Zn^{2+} forms a key bond with the E_{146} residue in the active site of PC-PLC_{Bc} indispensable for the stabilization of the secondary and tertiary conformation of the enzyme [40]. It appears that E_{146} in PC-PLC_{Bc} serves as an essential ligand for Zn^{2+}. Mutation at this position generates an enzyme with reduced thermostability which binds only two zinc ions rather than three compared to wild-type PC-PLC_{Bc} [46].

As it was mentioned above, by comparing the PC-PLC_{Bt} with the protein sequences of the *Bacillus* PLC group already produced and expressed, a substitution of S_{188} in PC-PLC_{Bc} by P_{188} in PC-PLC_{Bt} was noted. This finding led us to conceive the hypothesis that this mutation is at the origin of the major differences, including thermostability and thermoactivity, between the PC-PLC_{Bt} and that of *B. cereus*. The molecular model of PC-PLC_{Bt} (**Figure 4A**) shows that mutation S188P occurs in between the two helices αF and αG. Consequently, this proline, considering its particular geometric properties, may block the hinge (between residues 187 and 192) allowing the structure to "bend" in *B. cereus*. Another hinge (hinge 2 in **Figure 4A**), between helices αH1 and αH2, can be located at P218. These 2 hinges would therefore allow certain flexibility to this part of PC-PLC_{Bc} (helices αG and αH1) and considering long-distance interactions, even flexibility to helix αE (**Figure 4B**). Conversely, for thermostable

enzymes, an increase in rigidity is generally observed (notably due to an increased proline number). The S188P mutation, therefore, goes exactly in this direction since PC-PLC$_{Bt}$ is shown to be more thermostable than PC-PLC$_{Bc}$.

Even though it cannot be excluded that the structural bases responsible for thermostability and pH stability may be located elsewhere in the 3D structure of the enzyme. The rPC-PLC$_{Bt}$ used, due to a different amino-terminal extremity (presence of an extra methionine, **see Figure S1**), may adopt a different conformation and display different structural properties, especially concerning thermostability, and so "cancel" the consequences of the S188P mutation.

3.3. Kinetic measurements of rPC-PLC$_{Bt}$ activity at the monolayer interface: Influence of Film Pressure and Substrate Specificity

The DAG generated under PLC-catalyzed of phospholipid film was subsequently hydrolyzed by the rSmL (**Figure 1**). Initially, the addition of the rSmL underneath the phospholipid's monolayer was not followed by any detectable change in surface pressure. This indicates that no significant penetration of lipase and no hydrolysis of the phospholipid film by the rSmL were detected since phospholipids are not substrates for rSmL according to our previous report [28]. Ten minutes later, pure PC-PLC$_{Bt}$ was then injected and the enzymatic activity as reflected by the barrier movement of the barostat was recorded continuously at constant surface pressure. It should be noted that the PLC activity was measured as a function of the rSmL amount, ranging from 0 to 50 ng. When no lipase is added, no advancement of barostat barrier is registered then the hydrolysis rates were linear with the rSmL amount up to around 20 ng above which remained constant (data not shown). Therefore, in all subsequent experiments, a final rSmL amount of 40 ng was selected to measure the PC-PLC$_{Bt}$ activity on monolayers of four phospholipids (DLPC, DLPE, PG, and PS). Besides, when adding PC-PLC$_{Bt}$, enzymatic activity was not detected until after a certain time that corresponds to the

lag period indicative of a presteady state, according to the literature it corresponds to the time required for the diffusion of the enzyme from the subphase to the interface and its adsorption to the phospholipid monolayer [47, 48]**.** After the latency period, the surface pressure increases abruptly (data not shown) until a maximum level confirming the lag-burst behavior previously described by He and Li for PLC [49] and PLA_2 [50, 51]**.** According to BAM and PMIRRAS experiments, this sharp increase in surface pressure is due to the molecular reorganization of the hydrolysis product (DAG) [47, 52].

The rate of hydrolysis of phospholipid films by the native and the recombinant PC-PLC_{Bt} forms was plotted as a function of the surface pressure and results showed bell-shaped curves with the four phospholipids (DLPC, DLPE, PG, PS) tested as substrates **(Figure 5)**. By focusing on the surface pressure, whatever the native or the recombinant forms, a lack of PLC activity was noted at low surface pressures (below 10 $mN.m^{-1}$). This irreversible interfacial denaturation of the enzyme at low surface pressures (corresponding to high interfacial tension and energy levels) can be prevented by increasing the surface pressure (thus lowering the interfacial tension), either by adding amphiphiles such as bile salts or proteins or by compressing the monomolecular films mechanically [53, 54]. PLC activity increased continuously by surface pressure rise until reaching the highest enzyme activities on DLPC, DLPE, PG, and PS headgroups at surface pressures of 15 and 25, 20, and 25 $mN.m^{-1}$, respectively (**Figure 5**). However, the hydrolysis rates are negligible at surface pressure superior to 30 $mN.m^{-1}$. This low PC-PLC_{Bt} activity is due to a decrease of SmL adsorption to phospholipid monolayer above 31 $mN.m^{-1}$ as previously reported [23]. Similar results were found by Moreau et al [22], who reported that the *C. perferingens* PLC activity decreased sharply around a threshold surface pressure of about 17 $mN.m^{-1}$ using 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine films however no hydrolysis was detected when PE, PS, or PG films were used as substrates [22]. In addition,

even with varying hydrolysis efficacy, the enzymatic activity could be detected for all the substrates tested. It is worth noticing that heterologous expression did not affect the substrate specificity of the PC-PLC$_{Bt}$ since the nPC-PLC$_{Bt}$ hydrolyze more efficiently all the phospholipids compared to the recombinant enzyme (**Figure 5**). Indeed, at optimum surface pressures, the maximum specific activities of nPC-PLC$_{Bt}$ on DLPC, DLPE, PG, and PS headgroups were 125 (**Figure 5A**), 86 (**Figure 5B**), 58 (**Figure 5C**), and 30 mole.cm^{-2}.min^{-1}.mg^{-1} (**Figure 5D**), respectively, while the maximum specific activities of rPC-PLC$_{Bt}$ on the same substrates were shown to be 40 (**Figure 5A**), 30 (**Figure 5B**), 8.5 (**Figure 5C**) and 7.5 mole.cm^{-2}.min^{-1}.mg^{-1} (**Figure 5D**), respectively. According to our forecasts, we suggest that this difference is due to the addition of the first M residue start codon upstream of W residue in the recombinant construction comparing to the native form where the W residue was identified by the NH_2 terminal sequence as the first residue [12]. Indeed, it was published for the PC-PLC$_{Bc}$ [3] that the initial amino acid is widely considered as indispensable for enzyme activity. Indeed, this first W residue contributes to the coordination with the essential zinc ions [41, 55] and when it was exposed, a sharp increase in the activity is noted. Besides, this activity decrease could be explained by the fact that the recombinant enzyme wasn't properly folded in the *E. coli* system, as in the wild strain. Furthermore, the ability of both PC-PLC$_{Bt}$ forms to hydrolyze the PG **(Figure 5D)**, more pronounced for the native form, remains exclusive compared to all *Bacillus* PLC published [13, 56]. Because mutation S188P is located far from the catalytic site (**Figure 4B**) and does not appear to alter the overall tertiary structure of the enzyme (data not shown), exclusive capacity to hydrolyze the PG substrate must be due to subtle steric and/or electronic perturbations in the substrate-binding pocket. The altered dynamic motion of helices αE, αG, and αH1 due to P188 (**Figure 4B**) may induce conformational changes or differences in hydration network in the active site and so be responsible for enhanced affinity for PG headgroup in PC-PLC$_{Bt}$. Indeed, results from

MM/PBSA evaluation (**Figure 4C and 4D**) show that the mutation S188P seems to enhance affinity for PG as its binding energy is increased by almost 40% (difference of 236.75 kJ/mol between PC-PLC$_{Bc}$ and PC-PLC$_{Bt}$). On the contrary, the affinity for PC is not altered by the mutation since the difference concerning its binding energy is shown to be only 14.85 kJ/mol (corresponding to the energy of one hydrogen bond) between *B. cereus* and *B. thuringiensis* enzyme. The capacity of the PC-PLCs to efficiently hydrolyze PE and PC two of the most abundant phospholipids present in vegetable oils [57], the large amount of enzyme produced, and the significant stability at high temperatures present this new phospholipase in the hot spot for oil enzymatic degumming allowing a higher yield of oil, both by generating 1,2-diacylglycerol (DAG) from phospholipids and by releasing the trapped TAGs following the reduction of gums volume [18, 58].

3.4. Hemolytic activity and cytotoxic potential of rPC-PLC$_{Bt}$

Biomembranes provide a dynamic and complex environment for biological reactions [59]. In the erythrocyte membrane, PC and sphingomyelin-containing choline are mainly located in the outer monolayer while aminophospholipid, including PS and DLPE, are enriched in the cytoplasmic leaflet of the membrane [59]. Several studies highlighted evidence of a correlation between the kinetic properties of phospholipases and their efficient attack of biological membranes. For example, Saikia *et al*. [60] demonstrated the link between catalytic and erythrocytes membrane damaging activities of an acidic PLA2 purified from *Daboia russelli* venom. Moreover, marine snail digestive [61] and venom glands *Scorpio maurus* phospholipases [62] were found to exhibit hemolytic activity towards erythrocytes which could be related to their ability to interact with phospholipid monolayer at high surface pressure. In our report, given the prominent catalytic power of the PC-PLC$_{Bt}$ and the great abilities to bind and hydrolyze phospholipid monolayers at high surface pressure, we aimed to investigate the individual cytotoxic potential of pure rPC-PLC$_{Bt}$ on mammalian cells.

Initially, the hemolytic activity of the PC-PLC$_{Bt}$ was measured with various concentrations using rat erythrocytes as a model for membranes. As can be seen from **Figure 6A**, the rPC-PLC$_{Bt}$ exhibits a prominent direct hemolytic power since it was shown able to lyse all the erythrocytes at 200 µg.mL^{-1} after only 30 min at 37°C. Indeed regarding the hemolytic power, it is noted that there is a divergence in the bibliography between those who reported that the *bacillus* PC-PLC metalloenzyme provides the bacteria with a lecithinase activity but not a hemolytic activity and the induction of hemolytic power is due to an effective hemolytic complex, called cereolysin AB resulting of an enzymatic coupling between sphingomyelinase and PC-PLC [37, 63, 64] and those who published that the *B. cereus* and *C. perfringens* PLCs are talented by a high hemolytic power [65].

Considering this ability to disrupt biological membranes, PC-PLC$_{Bt}$ could exhibit a cytotoxic effect in mammalians cells. The lytic potential of rPC-PLC$_{Bt}$ was assessed by treating murine primary chondrocytes, human prostatic stromal myofibroblast cell line WPMY-1, and human prostatic metastatic cell line PC-3 with pure rPC-PLC$_{Bt}$ at different concentrations (50-200 µg.mL^{-1}) (**Figure 6B**). The cytotoxicity effect of the PLC is based on the measurement of lactate dehydrogenase (LDH) activity released by damaged cells after cell bursting to rPC-PLC$_{Bt}$ during 72 h. As indicated in Figure 8B, rPC-PLC$_{Bt}$ reduced viability and also induced cytotoxicity on cultured cells in a dose-dependent manner. A linear correlation was observed between cell viability and LDH activity. Overall, rPC-PLC$_{Bt}$ presented high cytotoxic effects on primary chondrocytes, WPMY-1, and PC-3 cells, with cell viability mostly decreasing varying between 60 and 70 % at the highest concentrations (**Figure 6B**). By comparing WPMY-1 with PC-3, it would seem that the decrease in the viability of PC-3 is greater than that of WPMY-1, in particular with the lowest concentration of 50 µg.mL^{-1} (**Figure 6B,** upper panel). For toxicity, an increase in LDH activity released by damaged cells was noted after the three doses injection for the different lines although the cytotoxic effect is more pronounced

502 in the PC-3 tumor cell line compared to its control line WPMY-1 since even at a reduced dose
503 of 50 μg.mL^{-1}, approximately 50% of lysed PC-3 tumor cells were detected against 30% for
504 the WPMY-1 cells (**Figure 6B**, bottom panel).
505 This bacterial rPC-PLC$_{Bt}$ great lytic power could be attributed to the DAG released
506 subsequent of the action of the rPC-PLC$_{Bt}$ on membrane phospholipids which disturbs firstly
507 the properties of the biophysical membrane, especially the fluidity, the charge, and the
508 permeability [66, 67], with a possibility of interaction with cytosolic proteins inducing spatial
509 reorganization signaling complexes leading to the affection of cellular processes [68]. Indeed
510 bacterial PLCs have been included among the virulence factors key to bacterial pathogenesis
511 [69] and evidence implicating the Zn^{2+} metalloPC-PLCs in host cell penetration, injury, and
512 lysis by microorganisms has been proved since the inactivation of the PLC-encoding gene
513 impairs the virulence of a pathogen [70-72].

Conclusions

Successful production of soluble active is reported when express the PC-PLC from *B. thuringiensis* in the *E coli* host avoiding thus time-consuming refolding step. The specific activity of the pure rPC-PLC$_{Bt}$ was around 1372 U.mg^{-1} measured at 60 °C on a PC emulsion in the presence of 0.1 mM Zn^{2+}. Purified phospholipase kept at almost, its biochemical properties with an improvement of the Zn^{2+} role required for catalysis and maintaining of the rPC-PLC$_{Bt}$ structure. The high expression yield and the excellent thermophilic properties are valuable features for this rPC-PLC$_{Bt}$ in industrial applications, especially for oil degumming carried out under extreme conditions and offering both economic and environmental benefits. A comparative kinetic study was investigated for the first time with various phospholipids monolayers using a coupled assay. The main conclusions indicate that both native and recombinant PC-PLC$_{Bt}$ forms can hydrolyze phospholipid monolayers with different efficiency and various optimal surface pressures ranging from 15 to 25 mN.m^{-1}. The DLPC is the major hydrolyzed substrate followed by the DLPE however the hydrolysis rate is low with PG and PS. The interesting capacity binding to phospholipid monolayers at high surface pressure is demonstrated correlated to the prominent direct hemolytic power and cytotoxic potential of this rPC-PLC on tumoral mammalians cells. New insights on the exact mechanistic aspects of this enzyme's cytotoxicity will be forthcoming soon and exploited for therapeutic and pharmacological purposes as a bactericidal and anti-tumor agent.

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745

746

747 **Figure captions**

748 **Figure 1:** Principle of the coupled assay for determining PC-PLC$_{Bt}$ activity using
749 phospholipid monolayers as substrates. rSmL was injected first into the subphase at a fixed
750 final amount of 40 ng and nPC-PLC$_{Bt}$ or rPC-PLC$_{Bt}$ was added 10 min later. The kinetic
751 experiments were recorded as described in Material and methods.

752 **Figure 2:** Purification of the rPC-PLC$_{Bt}$ **(A)** Chromatography of rPC-PLC$_{Bt}$ on a Ni-NTA
753 affinity column (5 cm × 1.5 cm). The column was equilibrated with buffer A (25 mM
754 Tris−HCl buffer at pH 8.5 supplemented with 25 mM NaCl and 2 mM benzamidine).
755 Unbound proteins were washed out with the same buffer A and the rPC-PLC$_{Bt}$ was eluted
756 with a linear gradient of imidazole from 0 to 0.2 M. The flow rate was set at 60 mL/h with a
757 fraction size of 1 mL. **(B)** Chromatography profile of rPC-PLC$_{Bt}$ from Sephadex G-75
758 column. The column (2.5 cm × 150 cm) was equilibrated with buffer A and the rPC-PLC$_{Bt}$
759 elution was performed with the same buffer at a rate of 12 mL/h and 3 mL fractions were
760 collected. The rPC-PLC$_{Bt}$ activity was measured as described in Materials and Methods using
761 egg PCas the substrate. Insert, SDS-PAGE (15% of acrylamide) analysis of rPC-PLC$_{Bt}$ at
762 final purification step (Sephadex G-75 column). The gel was stained with Coomassie blue to
763 reveal the proteins. **Lane 1**: molecular mass marker, **lane 2**: Purified enzyme fraction eluted
764 from G-75 column.

765 **Figure 3:** (**A**) Effect of metal ions on rPC-PLC$_{Bt}$ activity. Enzyme activity was measured at
766 increasing concentrations of Ca^{2+}, Mg^{2+}, and Zn^{2+} using PC as a substrate under optimal
767 conditions. The star indicates that the rPC-PLC$_{Bt}$ measured in the absence of ions and the
768 presence of 10 mM EDTA. **(B)** Effect of increasing concentration of bile salt (NaDOC) on
769 rPC-PLC$_{Bt}$ activity using the PC as the substrate.

770 Temperature and pH effects on rPC-PLC$_{Bt}$ and rPC-PLC$_{S188P}$ activity and stability.
771 Temperature **(C)** and pH **(D)** effects on enzyme activity were determined using PC as the

772 substrate under standard conditions. The thermo-stability profile **(E)** was determined by
773 incubation of the pure enzyme at different temperatures (30°C–65°C) for 1 h, and the residual
774 enzyme activity was measured under optimal conditions. The pH stability **(F)** was analyzed
775 after incubating the pure rPC-PLC$_{Bt}$ at various pH values (from 3 to 12) for 1 h and the
776 residual activity was determined under optimal conditions.
777 **Figure 4:** Molecular modeling, docking experiment and MM/PBSA simulations. **(A**) Ribbon
778 representation of PC-PLC$_{Bt}$ molecular model. P188 and P218 are depicted as sticks **(B)**
779 Docking of a 1,2-diacyl-*sn*-phosphoglycerol (PG) into the PC-PLC$_{Bt}$ model. The enzyme
780 backbone is depicted as a ribbon covered by a semitransparent Van der Walls surface, while
781 PG is depicted as sticks and zinc ion as a grey sphere. The hatched arrows materialize the
782 potential motions of the protein domains**. (C)** Docking of a 1,2-diacyl-*sn*-phosphocholine
783 (PC) and of a 1,2-diacyl-*sn*-phosphoglycerol (PG) into the PC-PLC$_{Bc}$ x-ray-structure or into
784 the PC-PLC$_{Bt}$ model. The enzymes are covered by a Van der Walls surface, while ligands are
785 depicted as sticks. Residues that show hot-spot interactions with PC during the simulations
786 are colored in blue while ones interacting with PG are in orange **(D)** MM/PBSA binding free
787 energy calculations for PC-PLC$_{Bc}$ and PC-PLC$_{Bt}$ with PC or PG ligands.
788 **Figure 5:** Variation with a surface pressure of nPC-PLC$_{Bt}$ and rPC-PLC$_{Bt}$ on various
789 phospholipids; DLPC **(A**), DLPE **(B),** PG **(C),** and PS **(D).** PLC activity is expressed as the
790 number of moles of substrates hydrolyzed by unit time and the unit surface of the reaction
791 compartment of the "zero-order" trough.
792 **Figure 6:** rPC-PLC$_{Bt}$ hemolytic and cytotoxic potential (**A)** Hemolytic activity of rPC-PLC$_{Bt}$:
793 Freshly prepared human erythrocytes were incubated with various enzyme concentrations of
794 rPC-PLC for 30 min at 37 °C. (**B**) Cell viability and cell toxicity of cultured cells (murine
795 primary chondrocytes, human prostatic cell lines WPMY-and PC-3) in the presence of 50,

100, and 200 μg.mL^{-1} of rPC-PLC$_{Bt}$ .Values are expressed as mean +SD for three different experiences realized in triplicate for in each group).

List of symbols and abbreviations

Bt : *Bacillus thurigiensis*

β-CD: β-cyclodextrin

DLPC: 1,2-dilauroyl-*sn*-glycero-3-phosphocholine

DLPE: 1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamine,

EDTA:Ethylene diamine tetra-acetic acid

IPTG: Isopropyl thio-D-galactopyranoside

MD: Molecular dynamic

NaDOC: Sodium deoxycholic acid,

PBS: phosphate-buffered saline

PC-PLC: Phosphatidylcholine-specific phospholipase C

rPC-PLC$_{Bt}$: Recombinant PC-PLC of *Bacillus thurigiensis*

nPC-PLC$_{Bt}$: Native PC-PLC of *Bacillus thurigiensis*

PG: 1,2-diacyl-*sn*-phosphoglycerol,

PS: 1,2-diacyl-*sn*-phosphoserine

1,2-DAG: 1,2-diacylglycerol

Figure 1

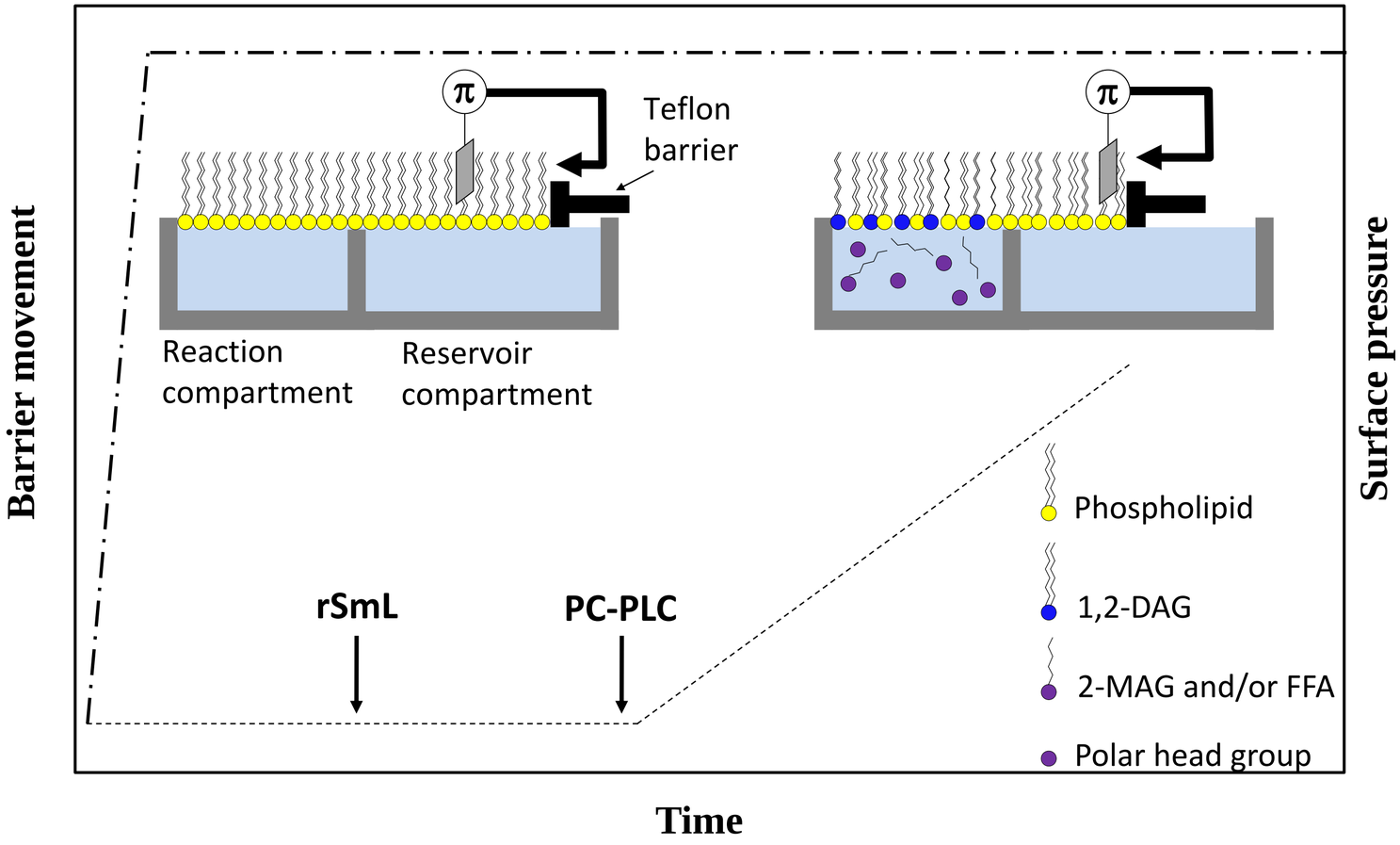
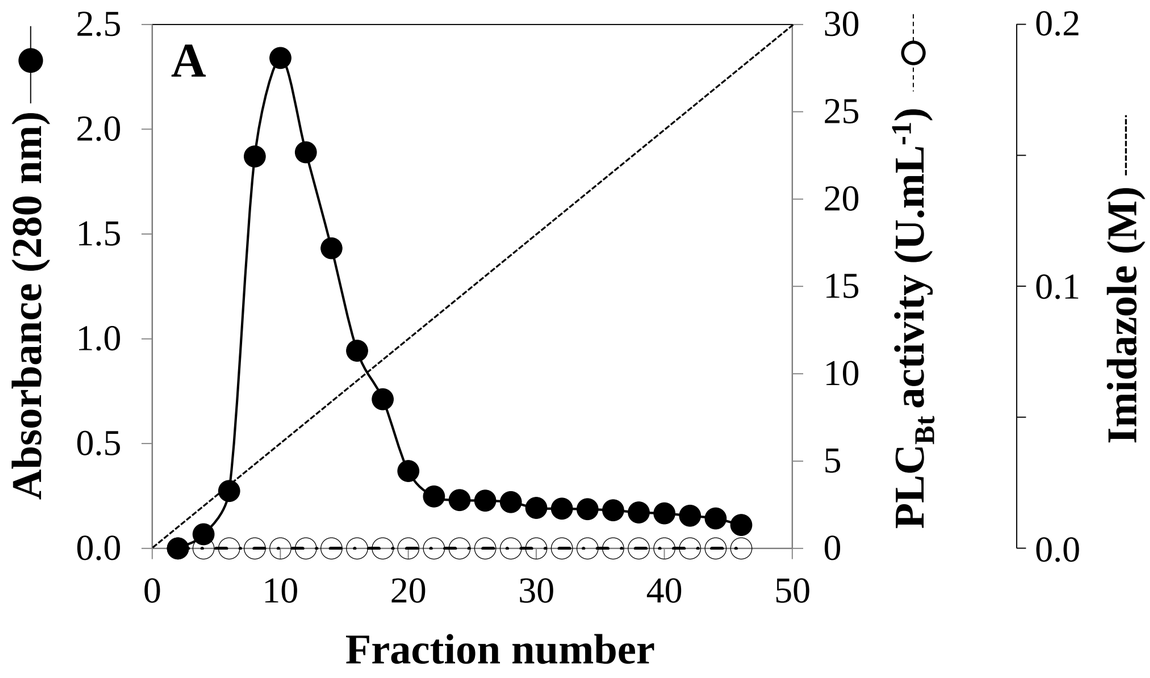

Barrier movement
Surface pressure
π
Teflon barrier
Reaction compartment
Reservoir compartment
rSmL
PC-PLC
Phospholipid
1,2-DAG
2-MAG and/or FFA
Polar head group
Time

Figure 2


A
Absorbance (280 nm)
PLC$_{Bt}$ activity (U.mL^{-1})
Imidazole (M)
Fraction number
0.0
0.5
1.0
1.5
2.0
2.5
0
5
10
15
20
25
30
0.0
0.1
0.2
0
10
20
30
40
50

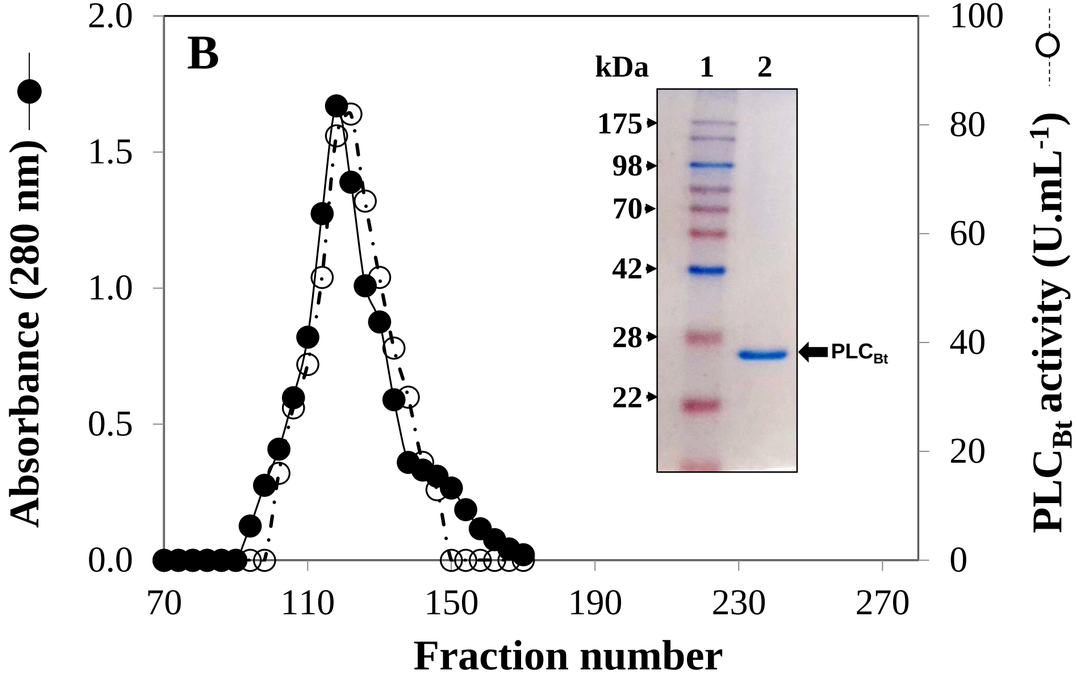
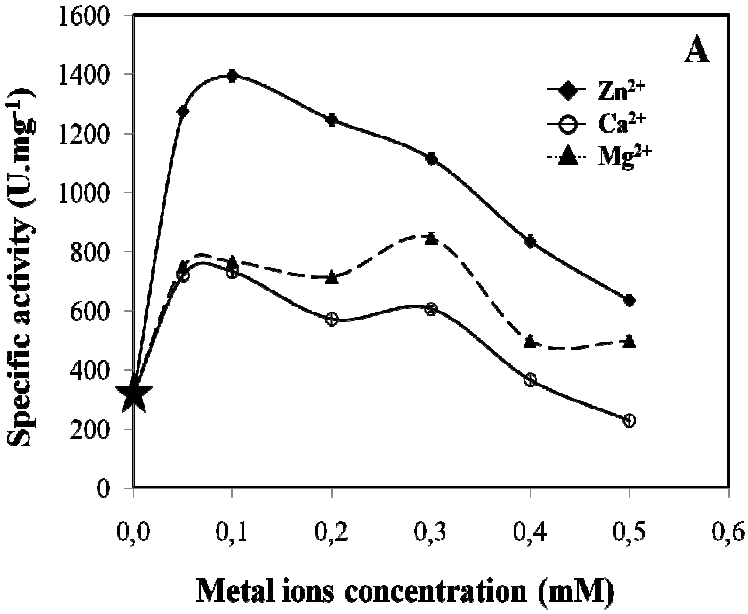
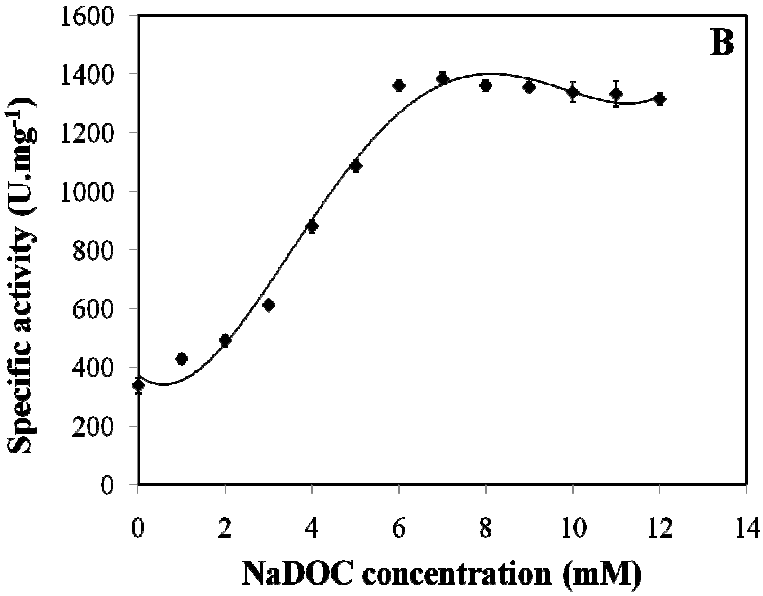
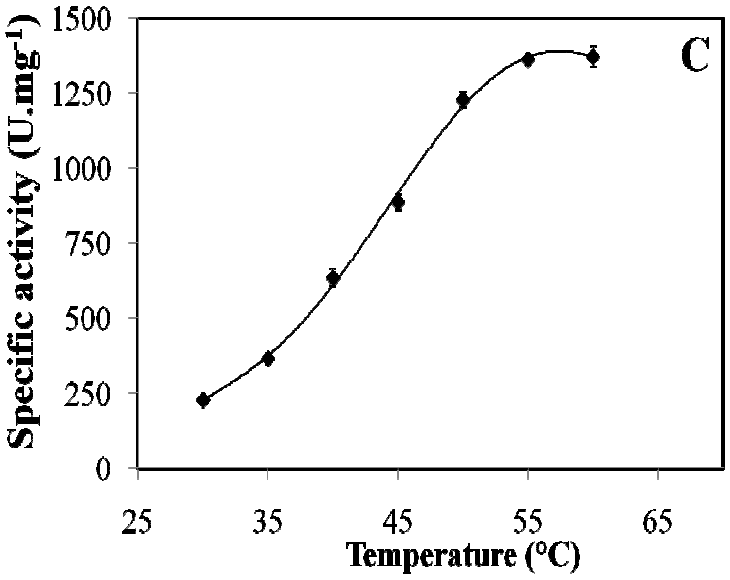
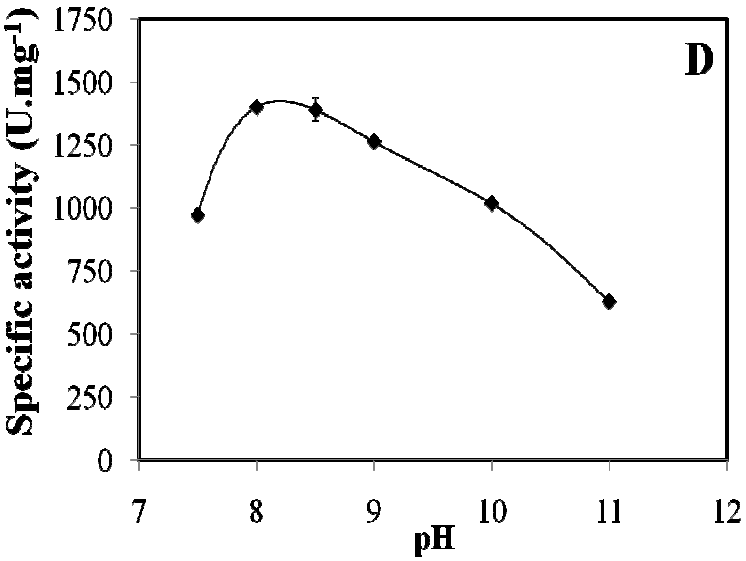

B
Absorbance (280 nm)
PLC$_{Bt}$ activity (U.mL^{-1})
Fraction number
kDa
1
2
175
98
70
42
28
22
PLC$_{Bt}$
0.0
0.5
1.0
1.5
2.0
0
20
40
60
80
100
70
110
150
190
230
270

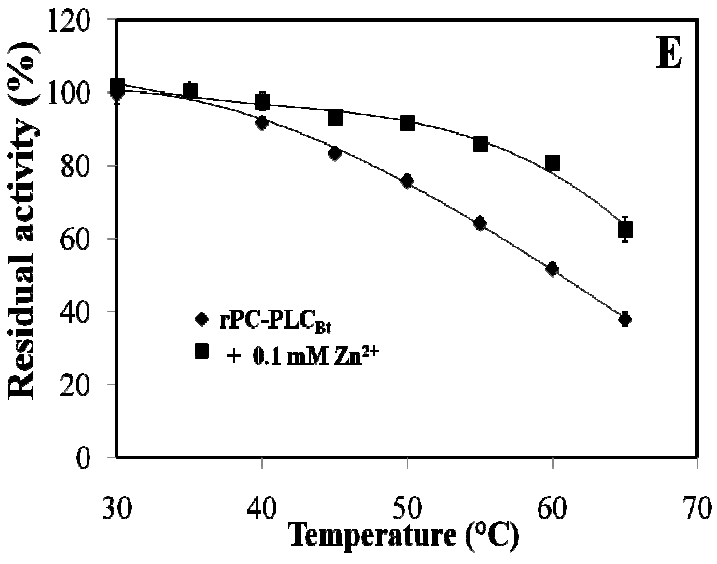
Figure 3


A
Specific activity (U.mg-1)
Zn2+
Ca2+
Mg2+
Metal ions concentration (mM)


B
Specific activity (U.mg-1)
NaDOC concentration (mM)


C
Specific activity (U.mg-1)
Temperature (°C)


D
Specific activity (U.mg-1)
pH


E
Residual activity (%)
rPC-PLCBt
+ 0.1 mM Zn2+
Temperature (°C)

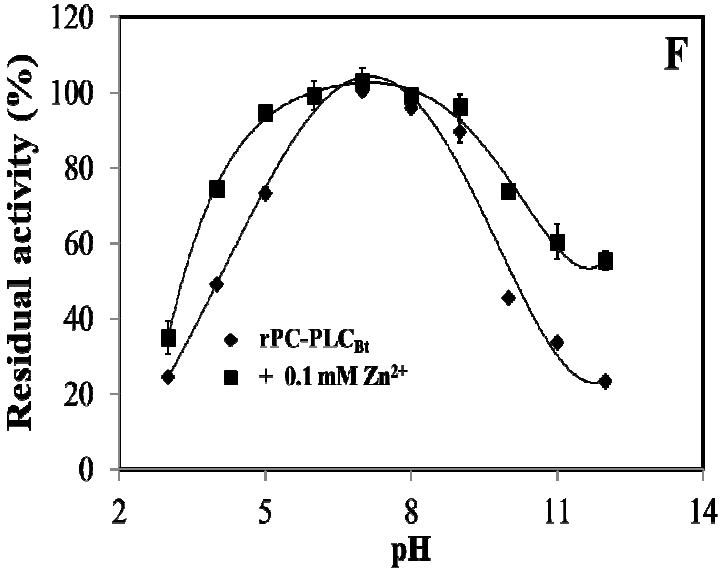

F
Residual activity (%)
rPC-PLCBt
+ 0.1 mM Zn2+
pH

Figure 4

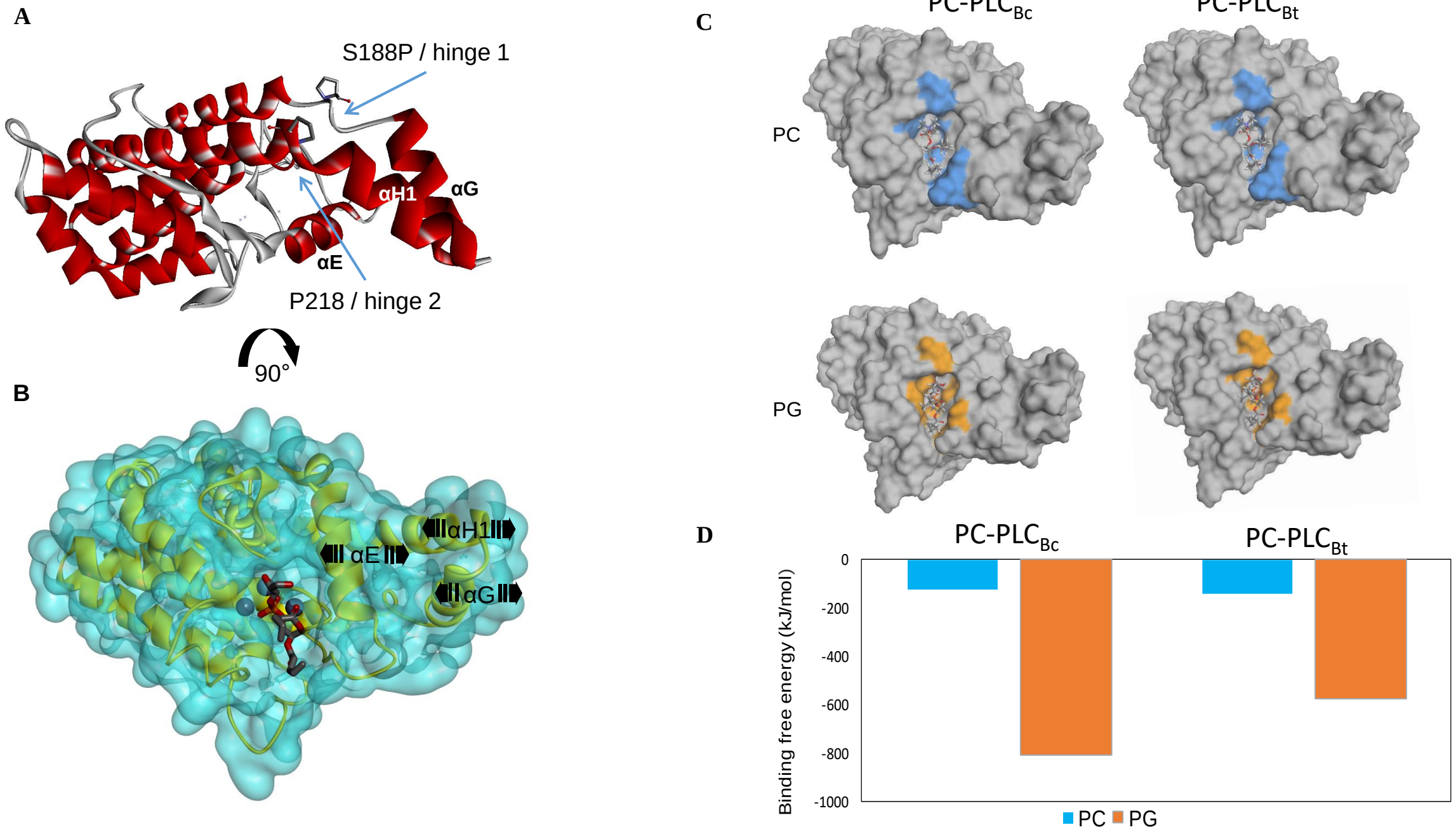
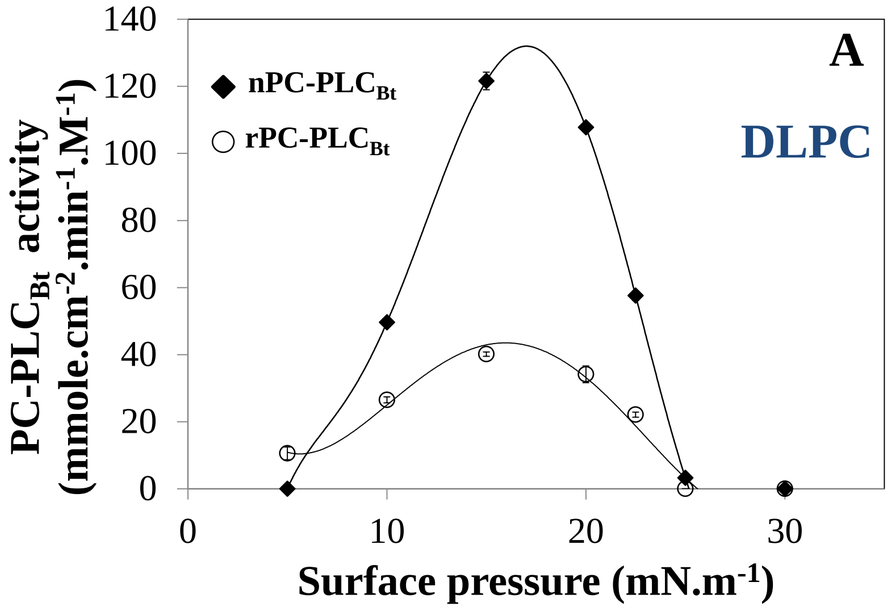
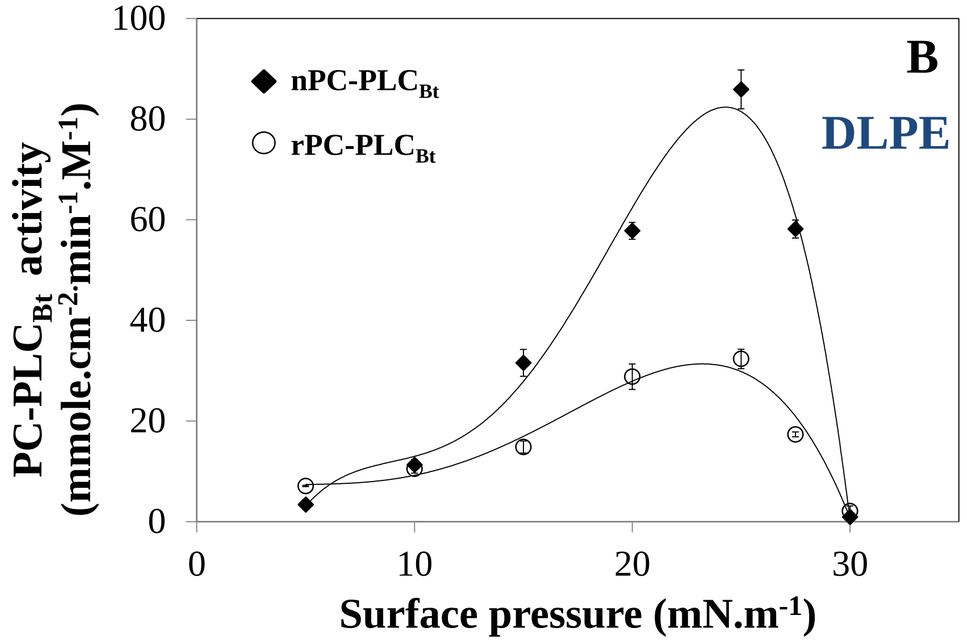
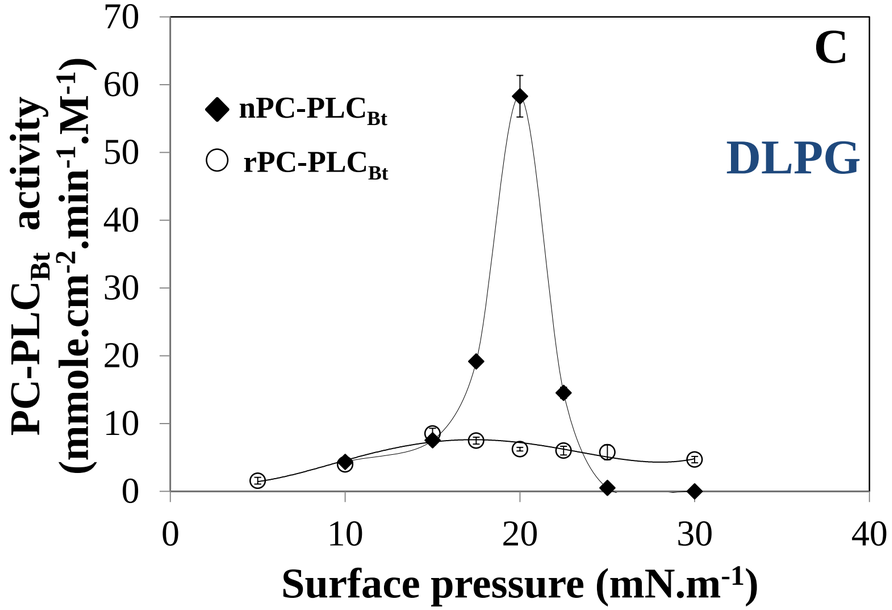

A
S188P / hinge 1
αH1
αG
αE
P218 / hinge 2
90°
B
αH1
αE
αG
C
PC-PLC$_{Bc}$
PC-PLC$_{Bt}$
PC
PG
D
PC-PLC$_{Bc}$
PC-PLC$_{Bt}$
Binding free energy (kJ/mol)
0
-200
-400
-600
-800
-1000
PC
PG

Figure 5


A
DLPC
nPC-PLC$_{Bt}$
rPC-PLC$_{Bt}$
PC-PLC$_{Bt}$ activity (mmole.cm^{-2}.min^{-1}.M^{-1})
Surface pressure (mN.m^{-1})
0
20
40
60
80
100
120
140
0
10
20
30


B
DLPE
nPC-PLC$_{Bt}$
rPC-PLC$_{Bt}$
PC-PLC$_{Bt}$ activity (mmole.cm^{-2}.min^{-1}.M^{-1})
Surface pressure (mN.m^{-1})
0
20
40
60
80
100
0
10
20
30


C
DLPG
nPC-PLC$_{Bt}$
rPC-PLC$_{Bt}$
PC-PLC$_{Bt}$ activity (mmole.cm^{-2}.min^{-1}.M^{-1})
Surface pressure (mN.m^{-1})
0
10
20
30
40
50
60
70
0
10
20
30
40

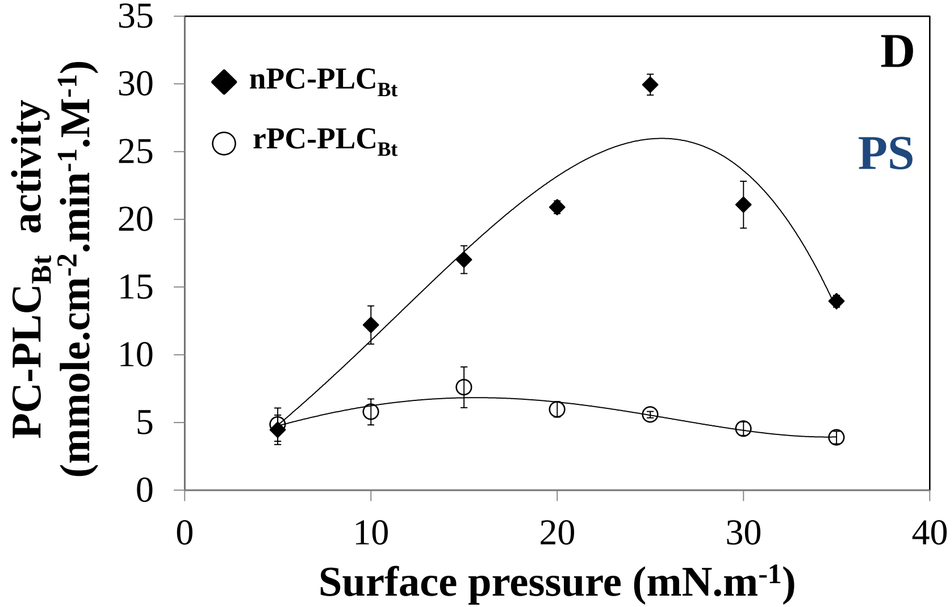
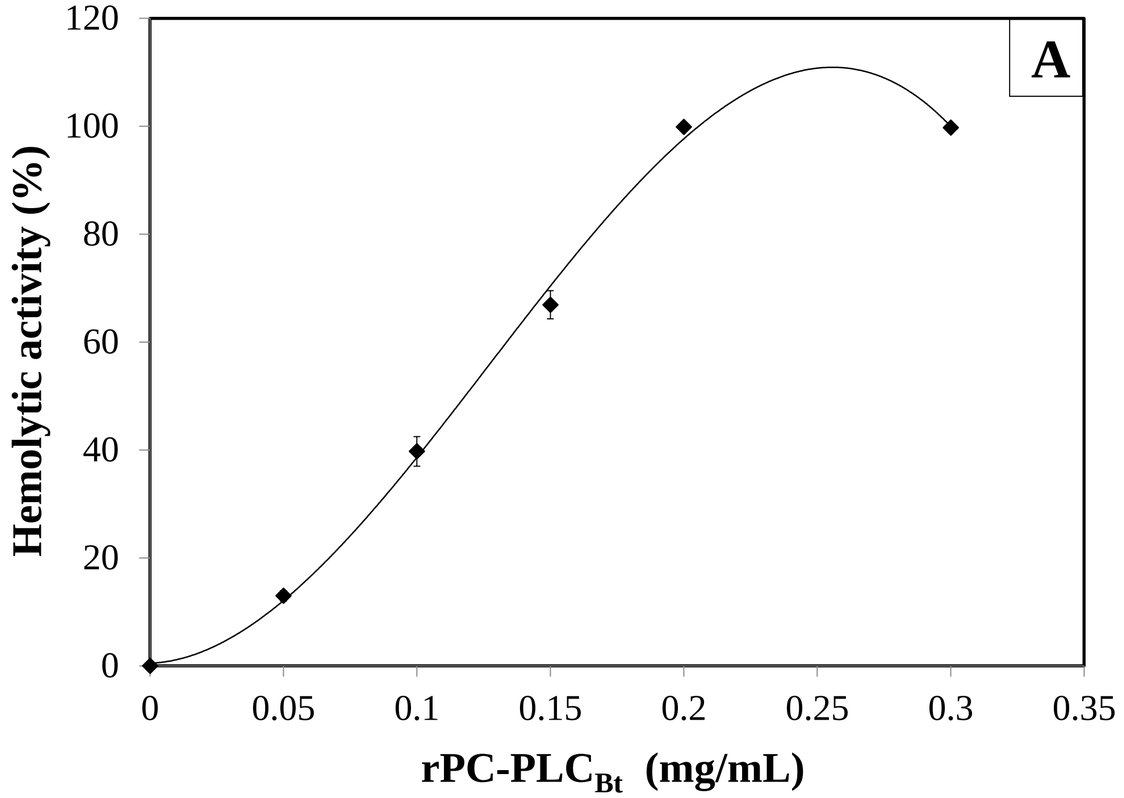
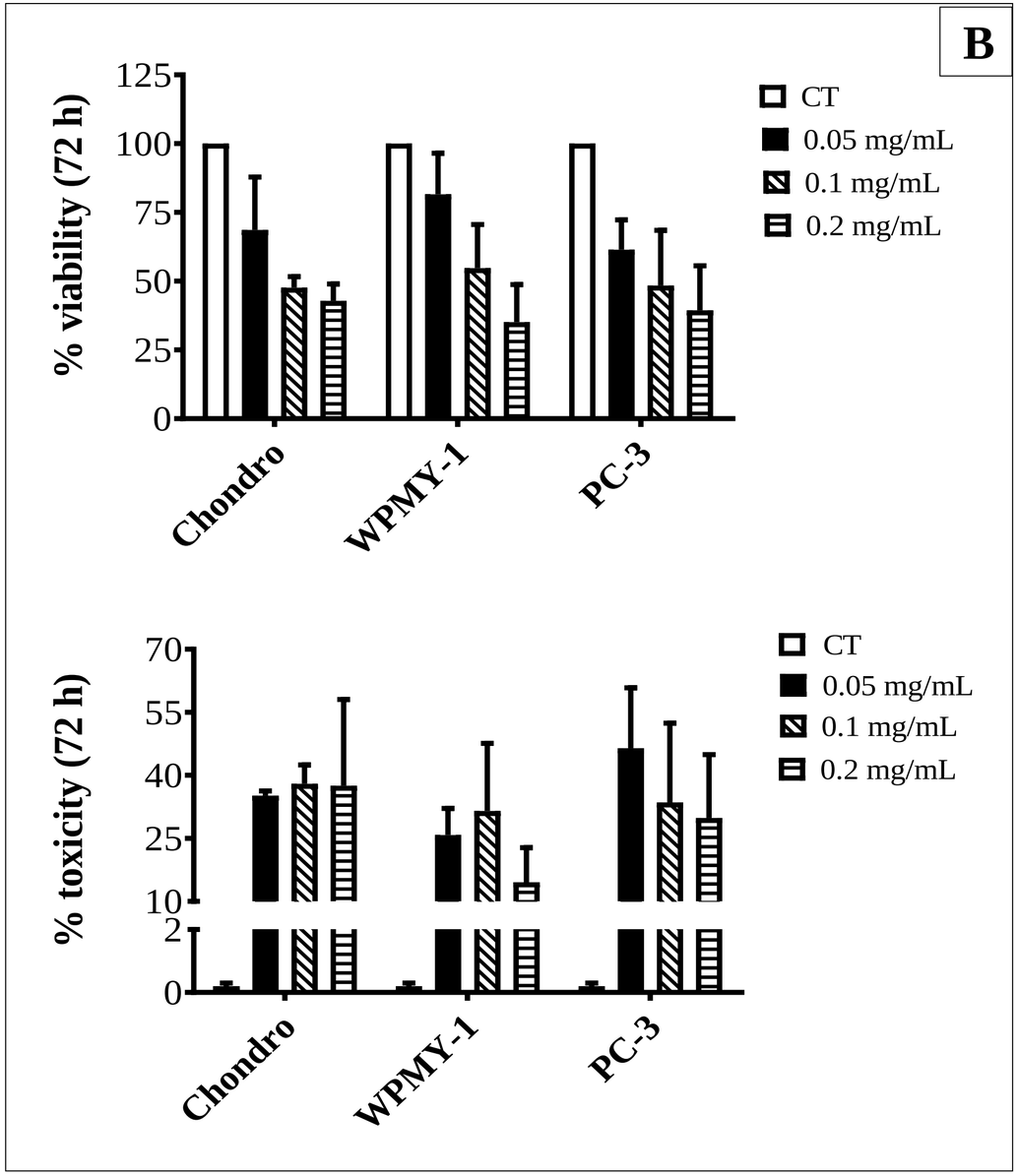

D
PS
nPC-PLC$_{Bt}$
rPC-PLC$_{Bt}$
PC-PLC$_{Bt}$ activity (mmole.cm^{-2}.min^{-1}.M^{-1})
Surface pressure (mN.m^{-1})
0
5
10
15
20
25
30
35
0
10
20
30
40

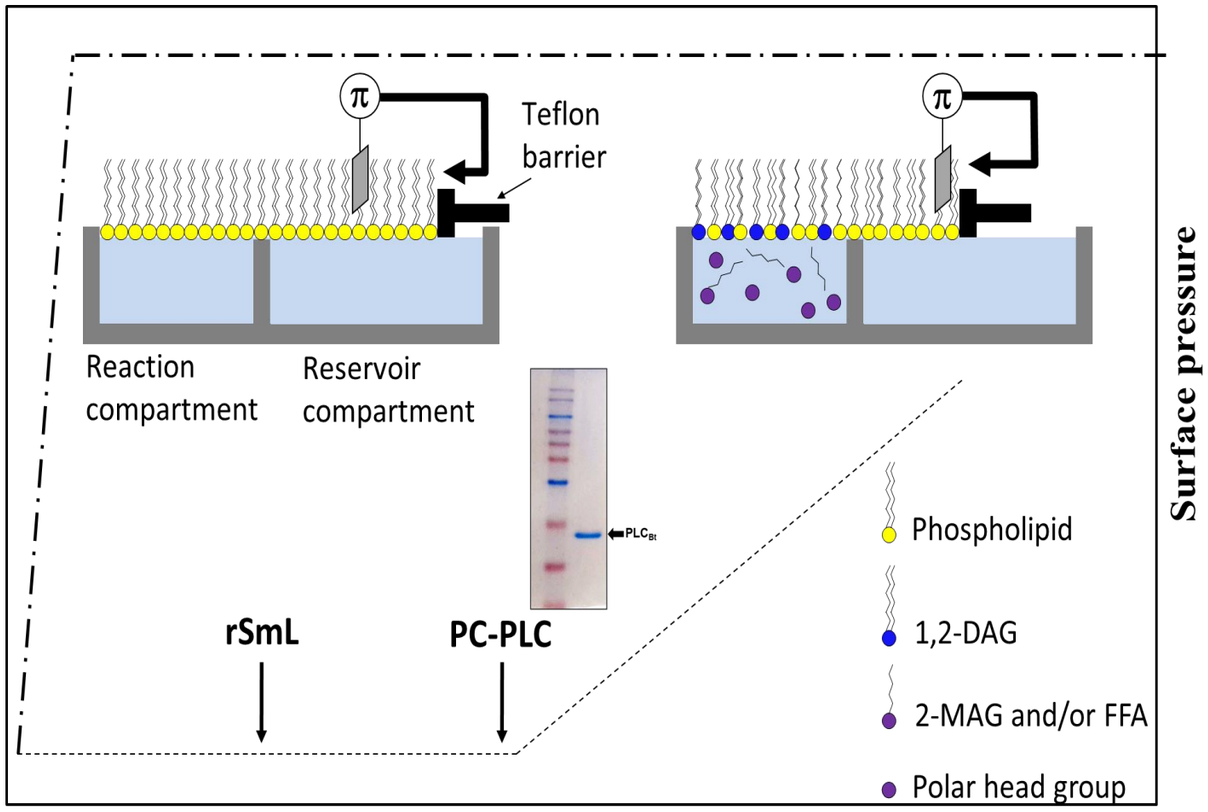
Figure 6


A
Hemolytic activity (%)
120
100
80
60
40
20
0
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
rPC-PLC$_{Bt}$ (mg/mL)


B
% viability (72 h)
125
100
75
50
25
0
CT
0.05 mg/mL
0.1 mg/mL
0.2 mg/mL
Chondro
WPMY-1
PC-3
% toxicity (72 h)
70
55
40
25
10
2
0
CT
0.05 mg/mL
0.1 mg/mL
0.2 mg/mL
Chondro
WPMY-1
PC-3

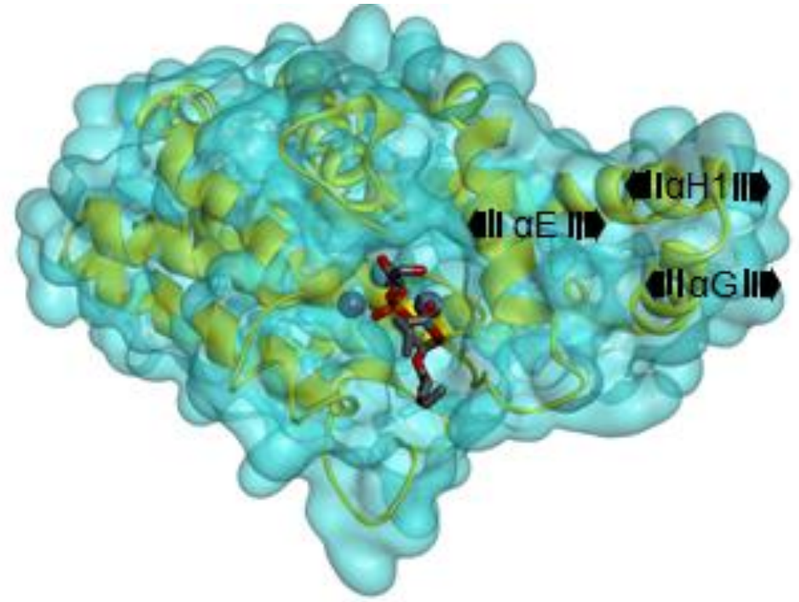
Graphical Abstract

1- Expression and interfacial properties of rPC-PLC$_{Bt}$ using monomolecular film technique



- ✓ *high expression yield and excellent thermophilic properties*
- ✓ *rPC-PLC$_{Bt}$ activity could be exploited for therapeutic and pharmacological purposes*
- ✓ *PC-PLC$_{Bt}$ represents a great potential in the crude oil refining industry.*

2- Modeling, Molecular dynamic (MD) simulation



3- cytotoxic potential of the rPC-PLCBt