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Purification and biochemical characterization of a secreted group IIA chicken intestinal phospholipase A₂

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Abstract

Background: Secretory phospholipase A₂ group IIA (IIA PLA₂) is a protein shown to be highly expressed in the intestine of mammals. However, no study was reported in birds.

Results: Chicken intestinal group IIA phospholipase A₂ (ChPLA₂-IIA) was obtained after an acidic treatment (pH.3.0), precipitation by ammonium sulphate, followed by sequential column chromatographies on Sephadex G-50 and mono-S ion exchanger. The enzyme was found to be a monomeric protein with a molecular mass of around 14 kDa. The purified enzyme showed a substrate preference for phosphatidylethanolamine and phosphatidylglycerol, and didn't hydrolyse phosphatidylcholine. Under optimal assay conditions, in the presence of 10 mM NaTDC and 10 mM CaCl₂, a specific activity of 160 U.mg⁻¹ for purified ChPLA₂-IIA was measured using egg yolk as substrate. The fifteen NH₂-terminal amino acid residues of ChPLA₂-IIA were sequenced and showed a close homology with known intestinal secreted phospholipases A₂. The gene encoding the mature ChPLA₂-IIA was cloned and sequenced. To further investigate structure-activity relationship, a 3D model of ChPLA₂-IIA was built using the human intestinal phospholipase A₂ structure as template.

Conclusion: ChPLA₂-IIA was purified to homogeneity using only two chromatographic columns. Sequence analysis of the cloned cDNA indicates that the enzyme is highly basic with a pI of 9.0 and has a high degree of homology with mammalian intestinal PLA₂-IIA.

Background

Phospholipases A₂ (PLA₂s) hydrolyse the *sn*-2 bond of phospholipids resulting in the release of a fatty acid and lysophospholipid. Mammalian PLA₂s are classified in three broad categories of secreted PLA₂ (sPLA₂) and cytosolic PLA₂s which are either calcium dependent as to their catalytic activity, or calcium independent cytosolic PLA₂s [1]. Secreted PLA₂s are small molecular size proteins (14-19 kDa) with a rigid tertiary structure, having five to eight disulfide bonds that probably confer resistance to proteolysis and thermal denaturation [1,2] with a highly conserved catalytic site and a Ca²⁺-binding loop. These secreted PLA₂s were first detected in snake venom pancreatic juice and in tissues [3]. Secreted PLA₂s are also expressed in a number of cell types and present in various body fluids. They participate in the first line in

antimicrobial defence of the body against bacteria and other pathogens. The extensive literature on sPLA₂s in inflammatory diseases has been reviewed [4-11].

The gene of pancreatic PLA₂-IB was first isolated in 1986 [12] followed in 1989 by the cloning of non-pancreatic PLA₂-IIA from rheumatoid arthritic synovial fluid [13] and blood platelets [14]. Together with pancreatic PLA₂-IB, the sPLA₂-IIA are the best known and biochemically characterized enzymes. Novel sPLA₂ were identified in the 90th by screening nucleic acid data bases. Up to now, eleven sPLA₂s have been cloned: IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XIIA PLA₂s together with the XIIB PLA₂-like protein devoid of catalytic activity [1,15,16]. They express different catalytic and binding properties to natural phospholipids [17,18].

Earlier studies have localized sPLA₂-IIA in the intestine [19,20] and in the synovial fluid of patients with rheumatoid arthritis as well as sPLA₂ released from platelets [14,21]. The concentration levels of PLA₂-IIA

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increase in sera of patients suffering from severe acute inflammatory diseases, such as sepsis and bacterial infections [22,23] and acute pancreatitis [24]. The sPLA₂-IIA was originally localized in Paneth cells of the rat intestine [25,26] and later on in macrophages [27,28]. The two above mentioned cell types are both involved in the antibacterial response.

Later on it was demonstrated that the PLA₂-IIA from human and mouse, with high activity on phosphatidylglycerol and bearing cationic properties (pI > 9.0), are highly bactericidal against gram positive bacteria [29-33] by perturbing the anionic bacterial cell wall [34]. The bacteria digested by the intestinal PLA₂ do not necessarily have to be within the intestinal lumen. Some bacteria specifically invade the intestinal mucosa from the lamina propria, as it has been postulated to occur in Whipple's disease [35]. The human PLA₂-IIA enzyme shows low affinity for zwitterionic interfaces, and in the absence of interfacial binding mammalian membrane hydrolysis is not possible [36].

With the growing interest in chicken diseases, such as avian influenza, we recently focused on bird sPLA₂ to further gain some functional and pathological insights. Ben Bacha et al. [37] have biochemically characterized an active thermo stable PLA₂-IB from ostrich pancreas (OPLA₂). Another PLA₂-IB was also purified from chicken pancreas and biochemically characterized [38]. It was therefore of interest to further study some biochemical and structural properties of avian non-pancreatic PLA₂ to gain more insights into their mode of action on phospholipids and to compare their properties with those of mammalian PLA₂. We report here, the purification and some biochemical properties of a secreted PLA₂ from chicken intestine (ChPLA₂-IIA). This work reports also the cloning of the corresponding bird PLA₂ cDNA and the comparison of its deduced amino acid sequence with other known mammalian PLA₂. A molecular 3D model of ChPLA₂-IIA is also proposed to explain some biochemical differences of ChPLA₂-IIA with other intestinal and pancreatic PLA₂.

Results and discussion

Determination of phospholipase activity

The PLA₂ activity was measured titrimetrically at pH 9.0 and at 40°C with a pH-stat, under the standard assay conditions as described previously [39], using egg yolk (0.5% W/V) as substrate, in 30 ml of 150 mM NaCl, 10 mM NaTDC and 10 mM CaCl₂. The analysis of the purified egg yolk phospholipids by thin layer chromatography revealed the presence of two spots. The major one (90%) corresponds to the phosphatidylcholine (PC, or lecithin) and the second one (10%) phosphatidylethanolamine (PE). These two phospholipids were separated by adsorption chromatography on a silica gel column. Elution was performed using stepwise ratios of chloroform/methanol.

We incubated the purified PC or PE samples with ChPLA₂-IB or ChPLA₂-IIA respectively and a thin layer chromatography was performed as shown in Figure 1. As expected, ChPLA₂-IB hydrolyzes efficiently PC and PE since the spots of these two phospholipids totally disappeared and a spot of free fatty acid appeared. In contrast, ChPLA₂-IIA didn't hydrolyze PC even after a long incubation period. Interestingly, PE was totally hydrolyzed by ChPLA₂-IIA similarly to what observed with ChPLA₂-IB. These analytical results clearly indicate that the hydrolytic activity of ChPLA₂-IIA, measured with pH-stat using egg yolk as substrate, is mainly due to the hydrolysis of the PE fraction.

Purification of ChPLA₂-IIA

ChPLA₂-IIA was purified from the intestine mucosa using an acidic treatment, ammonium sulfate precipitation, followed by two chromatography steps on Sephadex G-50, and Mono-S Sepharose according to the procedure described in Material and Methods (Figure 2). The main steps of the purification procedures and flow sheet are summarized in Table 1. The specific activity of pure ChPLA₂-IIA reaches 160 U.mg⁻¹ when egg yolk was used as substrate, at pH 9.0 and at 40°C, in the presence of 10 mM NaTDC, and 10 mM CaCl₂. The ChPLA₂-IIA purification yield was about 10%. SDS-PAGE analysis of the purified ChPLA₂ IIA eluted from the Mono-S column show that the enzyme exhibited one homogenous band corresponding to an apparent molecular mass of about 14 kDa (Figure 2). The calculated molecular mass, using the amino acid sequence is 13616Da. Furthermore, the calculated isoelectric pH was equal to pH 9.01.

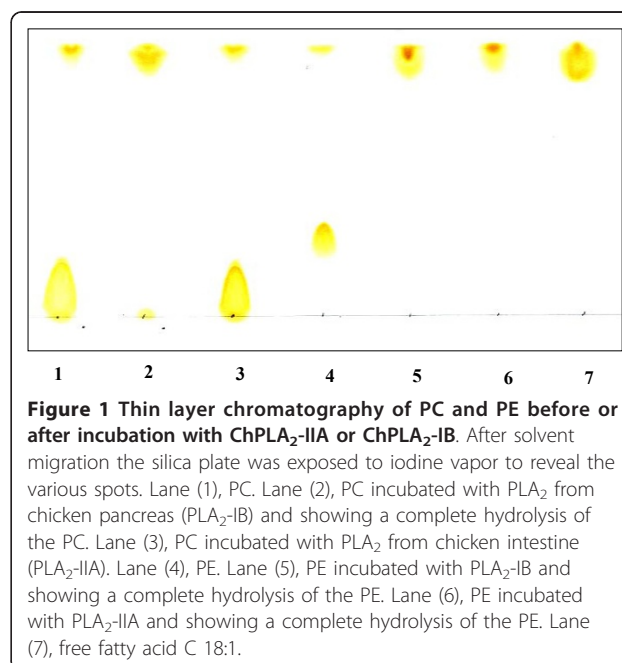


Figure 1 Thin layer chromatography of PC and PE before or after incubation with ChPLA₂-IIA or ChPLA₂-IB. After solvent migration the silica plate was exposed to iodine vapor to reveal the various spots. Lane (1), PC. Lane (2), PC incubated with PLA₂ from chicken pancreas (PLA₂-IB) and showing a complete hydrolysis of the PC. Lane (3), PC incubated with PLA₂ from chicken intestine (PLA₂-IIA). Lane (4), PE. Lane (5), PE incubated with PLA₂-IB and showing a complete hydrolysis of the PE. Lane (6), PE incubated with PLA₂-IIA and showing a complete hydrolysis of the PE. Lane (7), free fatty acid C 18:1.

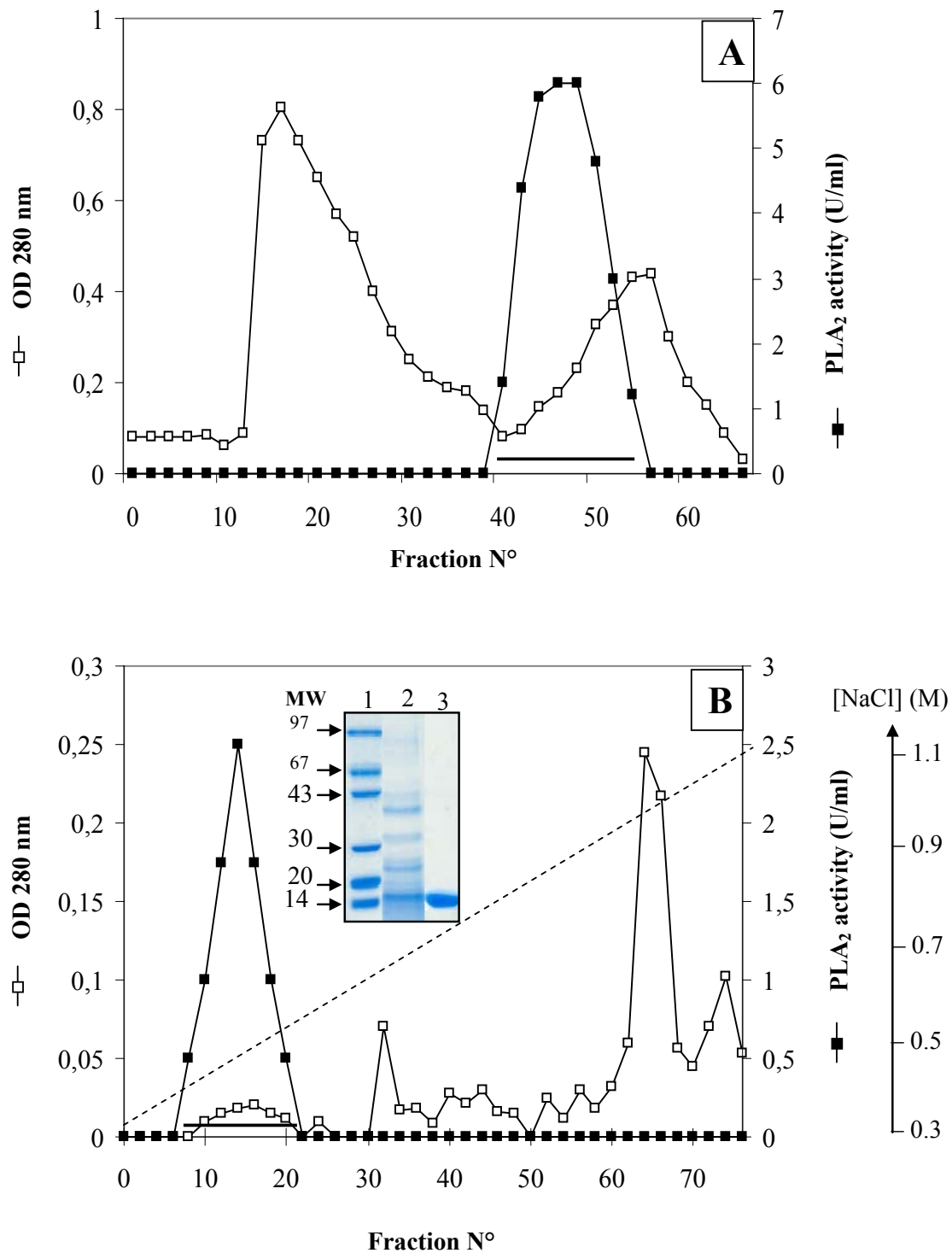


Figure 2 Purification of ChPLA₂-IIA. (A) Gel filtration chromatography of intestinal ChPLA₂-IIA on Sephadex G-50. The column (1.5 cm × 34 cm) equilibrated in 20 mM Tris-HCl buffer pH 8.0 containing 20 mM CaCl₂ and 2 mM benzamidine. Elution was performed with the same buffer at a flow rate of 40 ml.h⁻¹ and 3 ml samples were collected. ChPLA₂-IIA activity was measured as described in Material and methods section using egg yolk emulsion as substrate. The pooled fractions containing the PLA₂ activity were indicated by horizontal line. (B) Mono-S Sepharose chromatography. The column (5 cm × 2 cm) was equilibrated with 20 mM Tris HCl buffer pH 8.0 containing 20 mM CaCl₂ and 2 mM benzamidine; and then washed with the same buffer containing 0.3 M NaCl. Linear salt gradient (0.3 to 1 M NaCl, dotted line) was applied to the column; gradient chamber 75 ml; 2 ml fraction; flow rate, 40 ml/h. The pooled fractions containing the PLA₂ activity were indicated by horizontal line. SDS-PAGE (15%) analysis of pure ChPLA₂-IIA was inserted in Figure 2B. Lane 1, molecular mass markers (MM); Lane 2, 15 µg of proteins obtained after Sephadex G-50 chromatography; Lane 3, 15 µg of purified ChPLA₂-IIA, obtained after Mono-S chromatography.

Table 1 Flow sheet of chicken intestinal phospholipase A₂ purification

Purification Step	Total Activity (U) ^a	Total Protein (mg) ^b	Yield (%)	Specific activity (U/mg)	Purification Factor
Extraction (pH 8.5)	250	5520	100	0.045	1
Acidic treatment (pH3)	123	205.5	49	0.6	13.33
(NH ₄) ₂ SO ₄ Precipitation (60%)	108	108	43	1	22.22
Sephadex G-50	95	6.41	38	14.8	328.88
MonoS Sepharose	25	0.156	10	160	3555

a) 1 Unit: μmole of fatty acid released per min using egg yolk as substrate in the presence of 10 mM NaTDC and in the presence of 10 mM CaCl₂.

b) Proteins were estimated by Bradford method [30]. The experiments were conducted in triplicate.

Enzymatic properties of the purified ChPLA₂-IIA

Ca²⁺ dependence

It is well established that Ca²⁺ is essential for both, catalysis and enzyme binding to the substrate [40-42]. In order to investigate the effect of Ca²⁺ on ChPLA₂-IIA activity, we studied the variation of hydrolysis rates of egg yolk phospholipids by pure ChPLA₂-IIA in the presence of various Ca²⁺ concentrations (Figure 3A). Our results showed that no PLA₂ activity can be detected in the absence of Ca²⁺ and in the presence of 10 mM EDTA or EGTA. In the absence of calcium chelators, the specific activity of purified ChPLA₂ increases to reach 160 U.mg⁻¹ at 10 mM CaCl₂ (Figure 3A).

These observations corroborate previous findings with porcine [20], rat [19], and human [14] intestinal PLA₂. We previously reported that pancreatic chicken PLA₂-IB requires only 4 mM of CaCl₂ to reach its maximal activity (Figure 3A and [38]).

Bile salts dependence

Several studies have provided evidence that bile salts are tensioactive agents ensuring in their micellar form, the dispersion of the lipolytic products (of hydrolysis,) [43,44]. Along the same line, De Haas et al. reported that micellar forms of the substrate were hydrolysed at a much higher rate than substrates molecularly dispersed by PLA₂ [45]. In this study, we measured the ChPLA₂-IIA activity at pH 9.0 and at 40°C using egg yolk as substrate in the presence of increasing concentrations of bile salts. As shown in Figure 3B, NaTDC was required for ChPLA₂-IIA activity. ChPLA₂-IIA was poorly active at concentrations lower than 2 mM of NaTDC. This activity increases with increasing bile salts concentration and the maximal PLA₂ activity was measured in the presence of 10 mM NaTDC. In contrast, we have presently shown, confirming our previous work, that pancreatic chicken PLA₂-IB was found to be active in the absence of NaTDC and its optimal activity was found to be nearly independent of NaTDC (Figure 3B and [38]). One has to recall that in the assay using egg yolk emulsion as substrate, the

pancreatic PLA₂ hydrolyses both PC and PE whereas the intestinal enzyme hydrolyses only PE.

Effect of temperature on ChPLA₂-IIA activity and stability

Figure 4A shows that the maximal activity of ChPLA₂-IIA was measured at 40°C using egg yolk as substrate in the presence of 10 mM Ca²⁺ and 10 mM NaTDC. Unlike pancreatic ChPLA₂-IB [38] which is totally inactivated at high temperature, the ChPLA₂-IIA maintained about 60% of its activity after 15 min of incubation at 60°C (Figure 4B). Comparable results were obtained with mammalian intestinal PLA₂ from various species which show a good stability at high temperature [46,47].

Effects of pH on ChPLA₂-IIA activity and stability

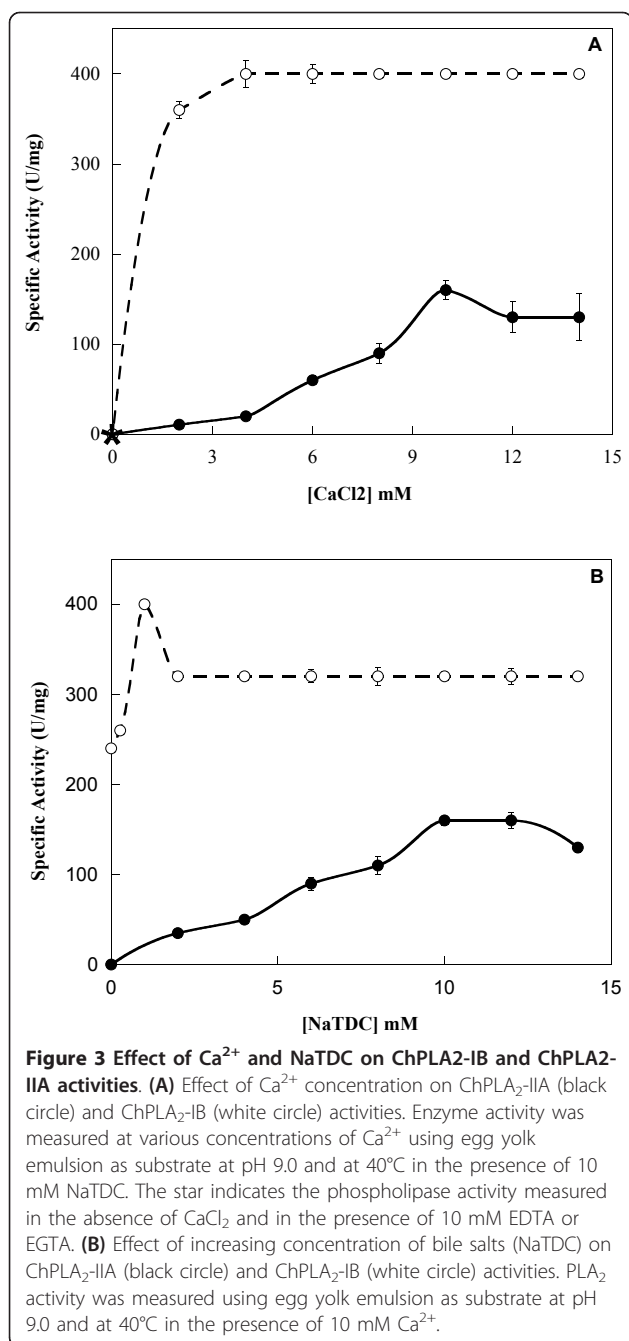
As shown in Figure 4C, the maximal activity of ChPLA₂-IIA was measured at pH 9.0 and at 40°C using egg yolk as substrate in the presence of 10 mM Ca²⁺ and 10 mM NaTDC. Similar results were obtained with ChPLA₂-IB and human intestinal PLA₂-IIA. However, the purified ChPLA₂-IIA was found to be stable between pH 3.0 and 10.0 (Figure 4D). In contrast to the ChPLA₂-IB, which was found to lose its activity when incubated at pH lower than 5 [38], pure ChPLA₂-IIA maintained about 50% of its activity after 15 min of incubation at pH 3.0 (see Figure 4D). It was also reported that porcine [20] rat [19] and human [14] intestinal PLA₂ are stable at low pH values as compared to ChPLA₂-IIA. Whereas, some pancreatic PLA₂-IB are very acid and thermo stable [37,48].

N-terminal sequence of ChPLA₂-IIA

The NH₂ terminal sequencing of the ChPLA₂-IIA allowed unambiguously the identification of the first fourteen residues of pure enzyme. Table 2 shows that the N-terminal sequence of ChPLA₂-IIA has identity at 80% with mouse, 53% with human, and 53% with porcine PLA₂-IIA respectively. Pure ChPLA₂-IIA exhibits a high degree of homology with mouse intestinal PLA₂-IIA.

Cloning and sequencing of the gene coding ChPLA₂-IIA

The cDNA encoding ChPLA₂-IIA was selectively amplified by RT-PCR from total mRNA extracted from



chicken intestine as described in material and methods. The corresponding DNA was ligated into pET 21a(+) vector and used for transformation into *E. coli* DH10B cells. Several clones are selected and some of them contained a recombinant plasmid with a 500 pb EcoRI/XhoI insert. The cDNA sequencing, confirmed that the PCR product corresponds to the gene encoding for the mature ChPLA₂-IIA (Figure 5). The deduced polypeptide sequence of ChPLA₂-IIA, corresponding to the mature protein, comprises 123 amino acids. ChPLA₂-IIA shares

45% of identity with human PLA₂-IIA and 51% of identity with mouse PLA₂-IIA. Residues of the catalytic diad are conserved in ChPLA₂-IIA. The 14 cystein residues involved in disulfide bridges in all known PLA₂ IIA are also conserved in ChPLA₂-IIA suggesting also the presence of 7 disulfide bridges in its 3D structure.

Homology modelling

To provide an insight into the biochemical properties, the structure model of the ChPLA₂-IIA was built, using the 3D structure of the hPLA₂ (PDB code: 1N28) as template. These two proteins shares 45% amino acid identity. The model of the ChPLA₂-IIA was then subjected to molecular mechanics optimization using CHARMM27 force-field. Energy minimization (geometry optimization) was performed until the gradient of 0.01 kcal/(Å.mol) was reached. The RMS deviations involving α -carbons between the initial and the optimized models was 0.95 Å. The Ramachandran plot statistics of the final ChPLA₂-IIA model and of the hPLA₂, determined using the PROCHECK program, showed that 97.2%, and 100% of the residues were either in the most favored or in the additional allowed regions, respectively.

Overall 3D structure model of ChPLA₂

As shown in Figure 6, the ChPLA₂-IIA has a globular shape with an α/β hydrolase fold stabilized by seven disulfide bridges similar to those present in all group IIA sPLA₂. The core structure consists of four α -helices (α A: Ile2-Thr13; α B: Ala17-Gly22, α C: Ala38-Ser55 and α D: Ser81-Thr103) and two short anti-parallel β -strands (β 1 : Phe67-Lys70 and β 2 : Gln73-Cys76) located between helix α C and α D. The catalytic Ca^{2+} ion is coordinated via oxygen atoms of His26 and Gly28 which belong to the Ca^{2+} -binding loop and the two oxygen atoms, O δ 1 and O δ 2 of Asp47. The ChPLA₂-IIA model showed that (O) Gly22, (O) Gly24, (O) Tyr111 and (O δ 1) Asn113 are located at an interaction distance of 2.2 Å with a second Ca^{2+} ion. However these residues correspond in ChPLA₂-IB to Asp24, Gly26, Leu118 and Lys120, respectively. No fourth amino acid was found in a favorable position to interact with this ion in ChPLA₂-IB due to the substitution of Asn113 (O δ 1) (in ChPLA₂-IIA) by Lys120 (in ChPLA₂-IB). This second Ca^{2+} ion may help the stabilization of the C-terminal (Tyr111 and Asn113) with the helix α B (Gly22, and Gly24) and furthermore the Ca^{2+} -binding loop.

Although ChPLA₂-IIA and ChPLA₂-IB share seven disulfide bridges, only six of them are structurally conserved. In ChPLA₂-IB, the disulfide bridge between Cys11 and Cys77 may stabilize the N-terminal α -helix α A with one of the two short strands of an anti-parallel β -sheet (β -wing) (β 2). However, in ChPLA₂-IIA, one

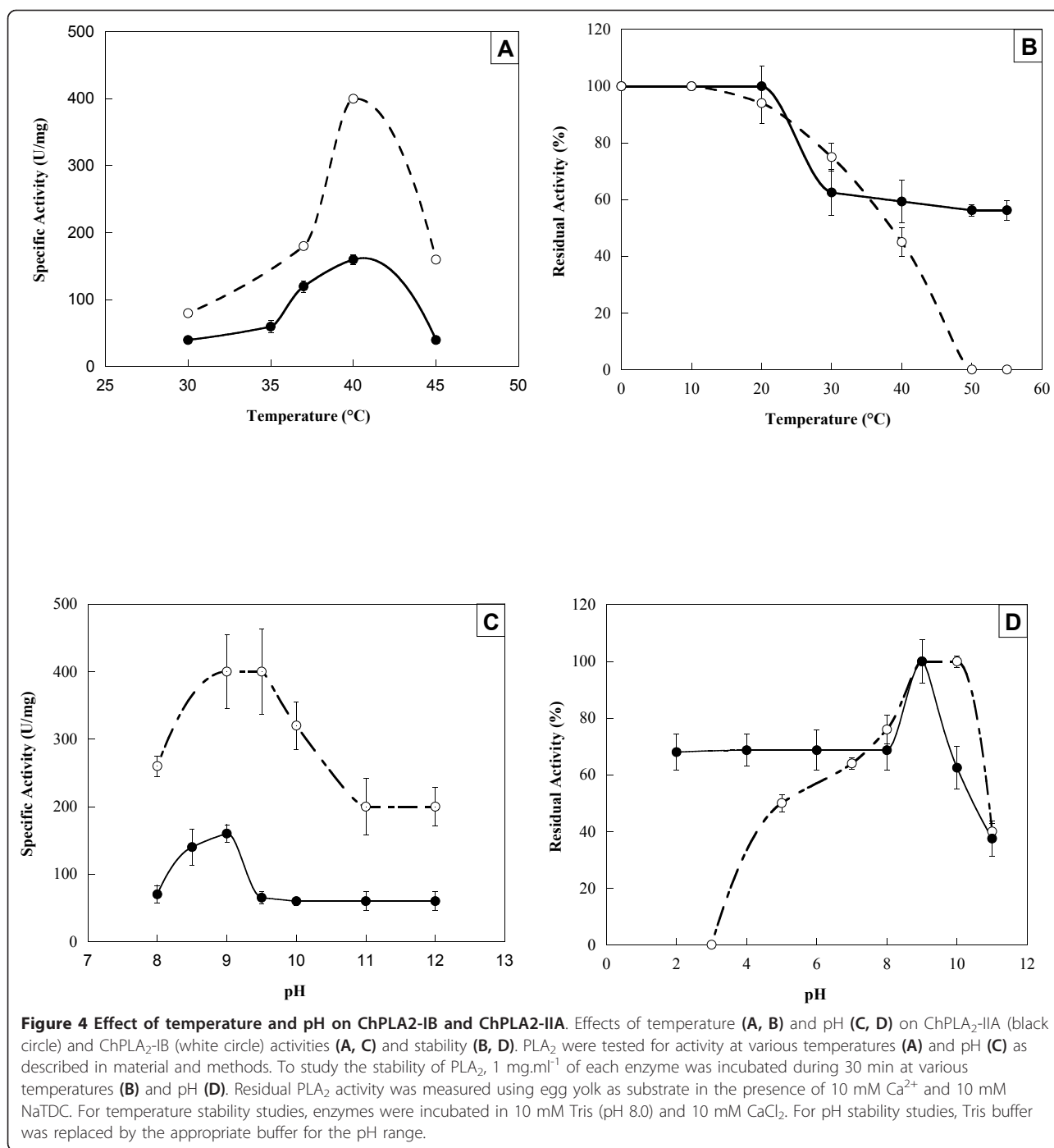


Table 2 Alignment of the N-terminal amino-acid sequence of ChPLA₂-IIA with mouse, human and porcine intestinal phospholipases

Chicken:	<i>N I A Q F G I M I K E K T G K</i>	(present work)
Mouse:	<i>N I A Q F G E M I R L K T G K</i>	[19]
Human:	<i>N L V N F H R M I K L T T G K</i>	[14]
Porcine:	<i>D L L N F R K M I K L K T G K</i>	[20]

Identical aminoacids are in bold, and homologous aminoacids are in italic.

new disulfide bridge is formed between Cys48 (belong to helix α C) and C-terminal Cys123.

To get more insight on the stabilization effect of the second calcium ion, molecular dynamics simulations were carried out (1 ns) at 330°K for the minimized models of the ChPLA₂-IIA in the presence and the absence of this ion. The RMSD value for the backbone atoms was used to understand the response behavior. The 2D-RMSD plot,

1	atg aag ctc ctg gtg ctc ctc gtc tgc ctt gca gtg ctg gcc ccc	45
1	M K L L V L L V C L A V L A P	15
46	gcg ggc tgc aac ata gct cag ttt ggt atc atg att aag gag aag	90
16	A G C N I A Q F G I M I K E K	30
91	act ggg aag cct gcg ctg tcc tac aat ggc tat ggc tgc cac tgc	135
31	T G K P A L S Y N G Y G C H C	45
136	ggc ctt ggg gga tcc aag tac ccg gtg gat gcg acc gac tgg tgc	180
46	G L G G S K Y P V D A T D W C	60
181	tgc cat gcc cac gac tgc tgc tac aag agg ttg tca tcc gtg acc	225
61	C H A H D C C Y K R L S S V T	75
	☆ ☆	
226	tgc gtt ccc cac ctt gcc act tac aaa ttc tcc atc aag aga ggc	270
76	C V P H L A T Y K F S I K R G	90
271	cag atc aca tgc gga tct gga agt tca tgc caa aga gct gct tgc	315
91	Q I T C G S G S S C Q R A A C	105
316	gag tgt gac aag aag gca gca gag tgt ttc aag agg aac ctc aga	360
106	E C D K K A A E C F K R N L R	120
361	act ttc aac aaa agc tac cag aat tat cta aac ttc aag tgc aag	405
121	T F N K S Y Q N Y L N F K C K	135
406	ggc tca cgt ccc tct tgc tag	426
136	G S R P S C	

Figure 5 Nucleotide sequence of the cDNA of ChPLA₂-IIA and the deduced amino acid sequence. Sequencing was performed in triplicate with three independent PCRs. The amino acid sequence obtained by N-terminal amino-acid sequence of the pure ChPLA₂-IIA is shown in italic. M1-C18 in bold, signal peptide; Y41-G49 in square, Ca²⁺ loop; and H64-D65 with stars, active site.

where the root mean square deviation of every conformation to all others of a simulation is shown, demonstrated that the conformational space sampled by ChPLA₂-IIA in the simulations was larger in the absence of Ca²⁺ ion (Figure 6).

Docking

The total accessible surface area of the ChPLA₂-IIA and ChPLA₂-IB were 6591 and 6522 Å², respectively. The positive charged surface of the ChPLA₂-IIA (876 Å², 12.6%) was higher than that of ChPLA₂-IB (440 Å², 6.7%). Furthermore, the negative charged surfaces of the ChPLA₂-IIA and ChPLA₂-IB were 69 Å², (1%) and 399 Å², (6.1%), respectively.

Analysis of PLA₂ structures bound to a substrate can help in understanding the interaction mode of the substrate once located in the catalytic pocket.

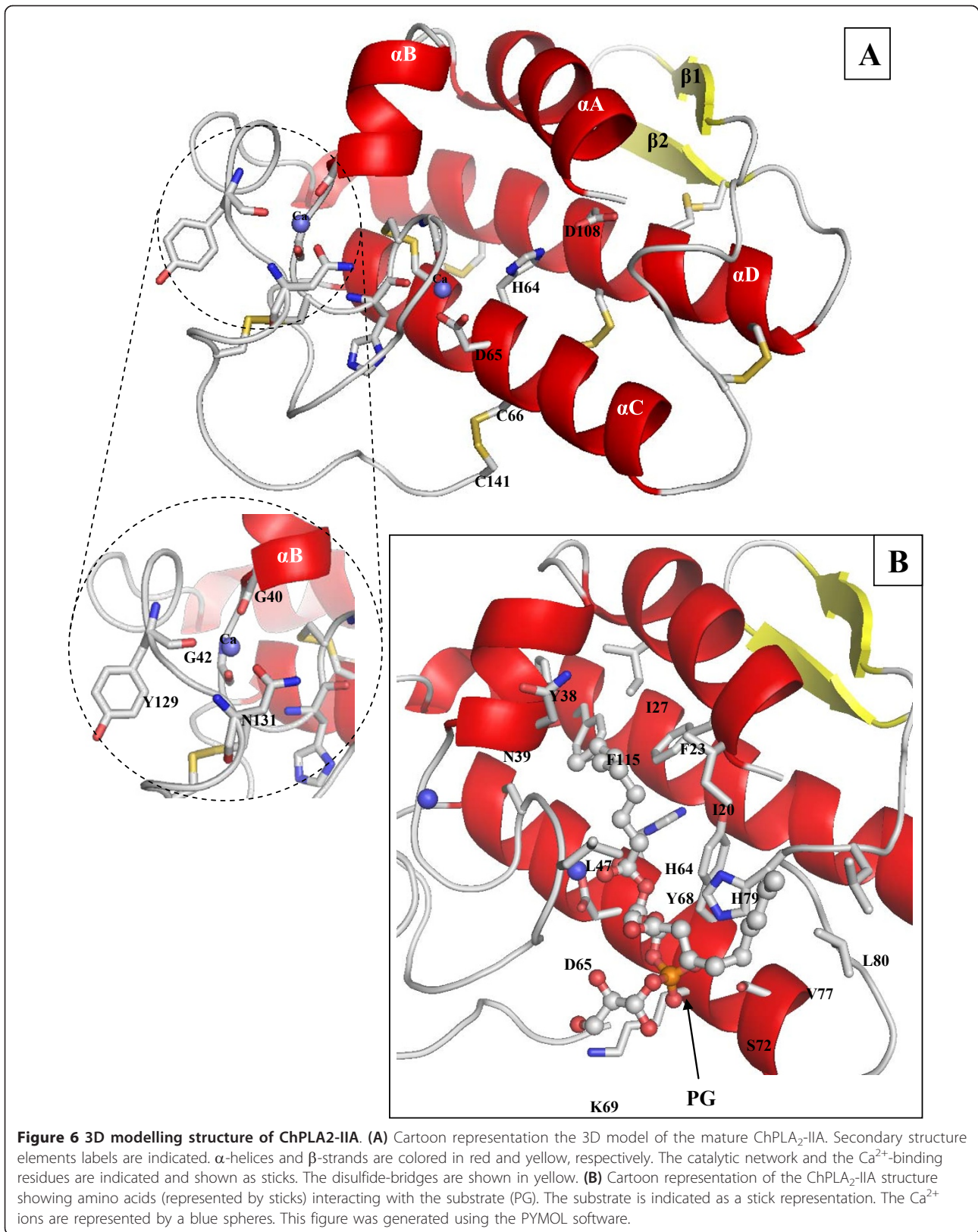
The docking of the substrate (PC, PE or PG) to the ChPLA₂-IIA and the ChPLA₂-IB protein was performed using the Molegro Virtual Docker v.4.0.2 software,

respectively (data not shown). According to the protein-substrate complexes, 13 or 12 residues are in van der Waals contact with the substrate in the case of the ChPLA₂-IIA and ChPLA₂-IB, respectively. In the case of the ChPLA₂-IIA, the total interaction energy between the protein and the target substrate is -134.8, -128.4 and -137.9 kJ/mol for PC, PE or PG respectively. In the case of the ChPLA₂-IB, the total interaction energy is -183.8, -142.3 and -192.2 kJ/mol for PC, PE or PG respectively. These above mentioned interaction energy may help to explain the lack of catalytic activity of the ChPLA₂-IIA on PC as substrate.

Materials and methods

Materials

Benzamidine was from Fluka (Buchs, Switzerland), bovine serum albumine (BSA), anhydrous magnesium sulfate, anhydrous sodium sulfate, potassium chloride, sodium chloride, taurodeoxycholic acid sodium salt (NaTDC) and PC were purchased from Sigma Chemical



(St. Louis, MO, USA); acrylamide and bis-acrylamide electrophoresis grade were from BDH (Poole, UK), marker proteins and supports of chromatography used for phospholipase purification: Sephadex G-50, MonoS sepharose, were from Pharmacia (Uppsala, Sweden); protein sequencer Procise 492 equipped with 140 C HPLC system provided from Applied Biosystems (Roissy, France); pH-stat was from Metrohm (Herisau, Switzerland).

All enzymes and reagents used in DNA manipulations were from Promega and Invitrogen (Paris, France). Oligonucleotides were synthesized by Invitrogen. *E. coli* strain DH10B was used as cloning host for the gene part encoding the mature phospholipase. PCR products were purified using the Wizard PCR Preps DNA purification System (Promega).

Thin-layer Silica Gel 60 plates (10 × 20 cm from Merck) were used for the separation of lipids. Lipid standards (α -L-oleic acid C 18:1) and silica gel 60 powders (70-230) were from Sigma-Aldrich (Steinheim, Germany). All solvents with HPLC grade were purchased from SDS (Peypin, France).

Lipid extraction, separation and analysis

Ten micrograms of purified ChPLA₂-IB and purified ChPLA₂-IIA were incubated respectively with 25 μ M of pure phosphatidylcholine (PC) and phosphatidylethanol (PE) dispersion in 1 ml buffer (10 mM Tris HCl, 10 mM NaTDC and 10 mM CaCl₂) at 37°C for 10 minutes. Lipolysis was stopped by adding 200 μ l of 1 M HCL and mixing vigorously with 5 ml of chloroform/methanol mixture (2:1, v/v) in a 15 ml glass tube with a Teflon-lined screw cap. Lipids were immediately extracted as follows: after separation phases, the lower organic phase was transferred to a 15-ml test tube and dried over anhydrous magnesium sulphate. Once MgSO₄ had precipitated, the clear organic phase was removed by centrifugation and stored at -20°C before TLC analysis.

To separate lipid classes, 1 to 50 μ l of lipid extracts or lipid standards at known concentrations were first spotted onto a thin-layer silica plate. The elution of the lipids was then performed in one step with a chloroform/methanol/water (65/25/4, v/v/v) solvent mixture. Following chromatography, the plates were dried at room temperature for 10 min then immediately placed in the iodine.

Enzyme samples

The intestine from chicken was collected from a local slaughterhouse (Sfax, Tunisia). Immediately after slaughter, the most distal intestine was opened on a glass plate on ice. The content of each ileal segment was flushed twice with 100 ml of 0.15 M NaCl. Adherent mesentery was removed as completely as possible.

Purification of ChPLA₂-IIA

Fifty grams of chicken intestine mucosa were suspended in 50 ml of buffer A (0.01 M Tris-HCl pH 8, 0.15 M NaCl, 0.02 M CaCl₂ and 4 mM Benzamidine), and ground mechanically twice for 30 s at room temperature using the Waring Blendor system. Then, the mixture was stirred in a cold room for 1 h and centrifuged during 30 min at 12.000 rpm. The total PLA₂ intestine activity obtained was 250 U.

Acidic treatment

To inactivate proteins from the pancreatic juice and secreted into the intestine, the supernatant was brought to pH 3.0 by adding 6 N HCl under gentle stirring at 0°C. Insoluble denatured proteins were removed by centrifugation during 30 min at 12.000 g and the clear supernatant was adjusted to pH 8.0 with 4 N NaOH. The recovery of PLA₂ activity was of about 50%.

Ammonium sulphate precipitation

The supernatant (55 ml, 123 U) was brought to 60% saturation with solid ammonium sulphate under stirring conditions and maintained during 45 min at 4°C. After centrifugation for 30 min at 12.000 g and at 4°C, the precipitated PLA₂ was resuspended in 2 ml of buffer A containing 2 mM benzamidine. Insoluble material was removed by centrifugation for 10 min at 24.000 g. The recovery of PLA₂ activity was about 45%.

Filtration on Sephadex G-50

The sample containing PLA₂ activity (4 ml, 108 U) was loaded on a column of sephadex G-50 (34 cm × 1.5 cm) equilibrated with buffer B (20 mM Tris-HCl, pH 8 and 20 mM CaCl₂). Elution of proteins was performed with the same buffer at 40 ml.h⁻¹. The fractions containing the PLA₂ activity eluted between 1.5 and 1.8 void volumes were pooled (Figure 2A).

Cation exchange chromatography

The pooled fractions of Sephadex G-50 column containing PLA₂ activity were poured into a Mono-S column (5 cm × 2 cm) equilibrated with buffer B. Under these conditions, the enzyme is adsorbed to the anionic support and the column was washed with 60 ml of the same buffer containing 0.3 M NaCl. ChPLA₂-IIA was eluted by a linear salt gradient (0.3-1 M) NaCl as shown in Figure 2B.

Analytical methods

Protein concentration was determined as described by Bradford et al. [49] using BSA ($\frac{1\%}{1\text{cm}} = 6.7$) as reference.

Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate

(SDS-PAGE) was performed by the method of Laemmli [50]. The proteins were stained with Coomassie brilliant blue.

Amino acid sequencing

The N-terminal sequence was determined by automated Edman's degradation, using an Applied Biosystems Protein Sequencer Procise 492 equipped with 140C HPLC system.

Bacterial strains, plasmids and media

E. coli strain DH10B was used as cloning host for the gene part encoding for the mature PLA₂. *E. coli* strain was grown in Luria-Bertani medium, supplemented with 100 µg.ml⁻¹ ampicilline whenever plasmid maintenance was required. The plasmid pET21a(+) (Novagen) was used as cloning vector.

cDNA synthesis and amplification

Total mRNAs were isolated from chicken intestine using the single step guanidine isothiocyanate/phenol/chloroform isolation method as described by Chomczynski and Sacchi [51]. ChPLA₂-IIA cDNA was obtained from total mRNAs by the reverse transcription procedure (Promega). First strand cDNAs were prepared using 10 µg of total mRNAs as template (heat-denaturated for 5 min at 70°C,) 200 U MMLV reverse transcriptase (Invitrogen), 20 pmol of each deoxynucleoside triphosphate, and 20 pmol of each primer: forward primer, 5'- GAA TTC ATG AAG CTC C TG GTG CTC CT C -3' and reverse primer, 5'- CT C GAG CTA GCA AGA GGG ACG TGA GCC -3'. The N-terminal primer was predicted, from the N-terminal sequence of the ChPLA₂; however the C-terminal primer was deduced from the genome of *Gallus gallus* (GenBank accession number: XP 424364). Reverse transcription was carried out in a total reaction volume of 20 µl for 5 min at room temperature and 60 min at 42°C. The cDNA/RNA heteroduplex was then denaturated at 70°C for 15 min and cooled on ice.

Cloning of the mature PLA₂ gene

Amplification of the specific ChPLA₂-IIA cDNA was carried out by PCR using the single strand cDNAs as template with the forward and reverse primers previously described. PCR was performed in a 0.2 ml Eppendorf tube with a Gene Amp[®] PCR System 2700. The PCR mixture contained 20 pmol of both primers, 20 pmol of each deoxynucleoside triphosphate, 5 U pfx polymerase and polymerisation buffer in final volume of 100 µl. The single strand cDNAs were directly used as template. The thermal profile involved 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 3 min. The PCR product (500 pb) was isolated and ligated into the EcoRI and XhoI linearised and dephosphorylated pET21a(+)

vector, according to the manufacturer's protocol (Promega). Protoplasts of *E. coli* DH10B were transformed with the ligation mixture. The resulting recombinant plasmid was named pChPLA₂. The presence of the appropriated insert was verified by restriction analysis. DNA sequences were elucidated by the dideoxynucleotide chain termination method according to a cycle sequencing protocol using thermosequenase (Amersham Pharmacia Biotech). The sequencing reactions were analysed with the DNA sequencer ABI PRISM 3100/3100-Avant Genetic Analyser (California, USA). It was performed three times, using the recombinant vector pChPLA₂ as template with T7 promoter primer and the T7 reverse primer (Invitrogen).

Software for infrastructure

The sequence alignment was performed with BioEdit Version 4.8.4 software. The Molecular Operating Environment 2008.10 (MOE) software was used for homology modelling, molecular dynamics and structures visualization. Models were stereochemically evaluated by the program PROCHECK. The Visualization was performed with PyMol version 0.99beta06. Figures were generated by PyMol program.

Homology Modelling

The 3-D coordinates of the human group IIA secreted PLA₂ (hPLA₂) (PDB code: 1N28) was extracted from the Protein Data Bank <http://www.rcsb.org>. The hPLA₂ structure was used as template to build a model of the ChPLA₂-IIA by using the structure-modelling program Molecular Operating Environment 2008.10. The model was then subjected to molecular mechanics optimization using CHARMM27 force field, until a gradient of 0.01 kcal/(Å.mol) was reached.

Docking

The 1,2-dioctylcarbamoylglycero-3-O-phosphatidylcholine (PC), 1,2-dioctylcarbamoylglycero-3-O-phosphatidylethanolamine (PE) and 1,2-dioctylcarbamoylglycero-3-O-phosphatidylglycerol (PG) were modelled by the Molecular Operating Environment 2008.10 (MOE) software using a *Simplified Molecular Input Line Entry Specification (SMILES)*. The Molegro Virtual Docker v.4.0.2 software was used for docking substrates, PC, PE or PG to the ChPLA₂-IIA and the ChPLA₂-IB protein, respectively.

The potential binding sites (also referred to as cavities or active sites) was being identified using the built-in cavity detection algorithm, molecular surface with 0.5 Å grid resolutions and 1.2 Å Probe size.

After preparation of the protein and the ligand, the docking was performed using MolDock Score function, MolDock SE search algorithm, 10 runs, energy minimization and optimize H-Bonds after docking, 2500

iterations and 500 steps Simplex Evolution to generate the best five poses scores which were visually analyzed. Only one of these poses correctly bind to the active site with a high score was used. The protein-substrate complex was then subjected to molecular mechanics optimization using CHARMM27 force field as described previously. The Score of the final protein-substrate complex was evaluated using the total interaction energy between the active site and the substrate.

Statistical analysis

All the results in figures and text are the average of at least three replicate experiments. They were statistically analyzed with SPSS software (version 100), using the Duncan test performed after analysis of variance (ANOVA).

Abbreviation

PLA₂: phospholipase A₂; sPLA₂: secreted PLA₂; ChPLA₂-IB: Group IB chicken pancreatic PLA₂; ChPLA₂-IIA : Group IIA chicken intestinal PLA₂; hPLA₂-IIA: Group IIA human PLA₂; NaTDC: taurodeoxycholic acid sodium salt; PC: phosphatidylcholine; PE: phosphatidylethanolamine; TLC: thin layer chromatography; ns: nanosecond. MMLV: Moloney murine leukemia virus.

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Authors' contributions

AK carried out all the studies, analyzed the data and drafted the manuscript. FF carried out modelling and structural analysis. YBA helped with the analysis of the data and to correct the manuscript. YG helped with the discussion of the data and the correction of the manuscript. SB participated in the study design and helped to draft the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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