Purification and characterization of a phospholipase A$_2$-IIA from common stingray (*Dasyatis pastinaca)* intestine

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A phospholipase A$_2$ belonging to IIA group secretory PLA$_2$ was isolated and purified to homogeneity from the intestine of common stingray (*Dasyatis pastinaca*) using acidic treatment (pH 1.5) and ammonium sulphate precipitation methods combined with single-column ion-exchange chromatography. The purified enzyme was found to be a glycosylated monomeric protein with a molecular mass of about 14 kDa. The stingray sPLA$_2$-IIA had optimum activity at 45°C, unlike known mammalian PLA$_2$-IIAs, which show optimum activity at 37°C. The purified enzyme exhibited a specific activity of 290 U/mg at optimal conditions (pH 9.5 and 45°C) in the presence of 6 mM NaDC and 8 mM CaCl$_2$ with egg yolk as substrate. The NH$_2$-terminal sequence of the enzyme and some protein fragments obtained from its tryptic digestion were also determined. All sequences obtained were similar to those of sPLA$_2$-IIA. The enzyme also showed good stability in the presence of organic solvents, acidic and alkaline pH media and high temperature conditions. Thus, the purified enzyme exhibited a number of unique and promising properties, making it a potential possible candidate for future applications in the treatment of phospholipid-rich industrial effluents and synthesis of useful preparations for the food production and processing industry.

Keywords: Marine phospholipase A$_2$, Characterization, Sequence, Stability, Organic solvent, Stingray, *Dasyatis pastinaca*

Phospholipases A$_2$ (PLA$_2$) are a large family of related enzymes that are commonly classified into groups I-XIV, based on several criteria, including the ability to catalyze the hydrolysis of the *sn*-2 ester bond of glycerophospholipids, completeness of the protein sequence, presence of homologous enzymes and gain of active splice variants[1-]. Based on broader structural differences, however, PLA$_2$ have generally been divided into four different classes, namely secretory PLA$_2$ (sPLA$_2$), cytosolic PLA$_2$ (cPLA$_2$), Ca^{2+}-independent PLA$_2$ (iPLA$_2$) and platelet activating factor-acetylhydrolase (PAF-AH)[4-6].

sPLA$_2$, the oldest class of PLA$_2$ are represented by groups I-III, V and IX-XIV with group II incorporating most members (Groups II A-F)[7]. They are ubiquitous in nature and have originally been characterized in snake and bee venom[4]. The secretory class of PLA$_2$ include low molecular mass (13-18 kDa), disulfide-rich, Ca^{2+}-dependent and phospholipid-selective enzymes with different polar heads and fatty acid tails. They share a common mechanism for cleaving the *sn*-2 ester bond of phospholipids involving a catalytic histidine, but show a different pattern of expression among the different tissues[8,9].

The sPLA$_2$ are often reported to provide the first line of anti-microbial defense against bacteria and other pathogens in the body. These enzymes have shown the anti-inflammatory and antibacterial properties[10-17]. Among the sPLA$_2$s so far investigated, group IIA represents a special type that possesses a significant anti-inflammatory and anti-bacterial potential that help regulate the synthesis of arachidonic acid and lysophospholipids[10,18-19]. In fact, the structure of human group IIA sPLA$_2$ is unusual because these types of proteins have a highly cationic nature, with a great number of positively charged residues (arginine and lysine) spread all over their surfaces which provides a molecular explanation for their well-established physiological anti-bacterial activities[20].

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Abbreviations: ChPLA$_2$-IB, chicken phospholipase A$_2$ group IB; ChPLA$_2$-IIA, chicken phospholipase A$_2$ group IIA; hPLA$_2$-IIA, human phospholipase A$_2$ group IIA; PLA$_2$, phospholipase A$_2$; PNGaseF, peptide-N-gycosidase F; sPLA$_2$, secretory phospholipase A$_2$; SPLA$_2$, stingray phospholipase A$_2$; SPLA$_2$-IB, stingray phospholipase A$_2$ group IB; SPLA$_2$-IIA, stingray phospholipase A$_2$ group IIA.

A wide array of sPLA$_2$-IIA species have been identified and characterized from several mammalian sources[21-23], as well as birds[24-25]. However, the data currently available on sPLA$_2$-IIA from marine origin are lacking, although the aquatic world offers a wide variety of biomass, which may be a source of novel enzymes. It is reported that fish viscera, a biomass that is often discarded either as waste or low value by-product and that generates troublesome waste disposal and environmental problems can be employed as a rich source for the recovery and/or discovery of attractive enzymes[26]. In particular, the viscera of certain vertebrate fish species having digestive systems similar to mammals, such as cartilaginous fish[26] might constitute a rich source for the production of novel sPLA$_2$-IIA species.

Recently, the purification and biochemical characterization of a novel hepatopancreatic PLA$_2$ with digestive and toxic activities has been reported from *Hexaplex trunculus*[27]. A thermoactive digestive PLA$_2$ has also been purified from the hepatopancreas of *Carcinus mediterraneus* crabs[28]. In a more recent work, we purified a pancreatic PLA$_2$ (SPLA$_2$-IB) from the common stingray (*Dasyatis pastinaca*, a marine cartilaginous fish) whose attractive potential has prompted further research[29-30]. In this study, we have further investigated the biochemical and structural properties of marine non-digestive PLA$_2$ and compared them with known mammalian and bird PLA$_2$ to gain further insights into the mode of action with regard to phospholipids. This study also reports for the first time the purification and biochemical characterization of a novel SPLA$_2$-IIA secreted from the intestine of common stingray.

Materials and Methods
Intestine collection

The common stingray (*Dasyatis pastinaca*) samples used in the study were collected from a local fish market (Riyadh, Saudi Arabia). The samples were packed in polyethylene bags, placed in ice and transported to the laboratory within 2 h after collection. Immediately after removal of internal organs, the most distal intestine was opened on an ice-cold glass plate. The content of each ileal segment was flushed twice with 100 ml of 0.15 M NaCl and cleaned of adherent mesentery.

Purification of SPLA2-IIA

The stingray intestine mucosa (80 g) was suspended in 120 ml of buffer A (50 mM Tris-HCl buffer, pH 8, containing 0.05% Triton X-100, 20 mM CaCl$_2$, and 150 mM NaCl) and grounded mechanically twice for 30 s using the Waring Blendor system. The mixture was stirred with a magnetic bar for 45 min at room temperature and centrifuged for 30 min at 12,000 rpm. The supernatant contained 8U PLA$_2$/g of intestine tissue.

Acidic treatment

To inactivate the proteins from the pancreatic juice secretions that empty into the intestine, the pH of supernatant was brought to 1.5 by adding 6 N HCl under gentle stirring at 0°C and subsequent incubation for 5 min. After centrifugation (30 min at 12,000 rpm), the clear supernatant was adjusted to pH 7 using 6 N NaOH and centrifuged for 20 min at 12,000 rpm to obtain a clear supernatant that contained approximately 80% of the starting PLA$_2$ activity.

Ammonium sulfate precipitation

The treated supernatant (110 ml, 518 U) was subjected to 70% saturation with solid ammonium sulphate under stirring conditions and maintained for 45 min at 4°C. After centrifugation (30 min at 12,000 rpm), the precipitated PLA$_2$ was resuspended in 10 ml of buffer A. Insoluble material was removed by centrifugation for 10 min at 12,000 rpm. Approximately 67% of the starting amount of PLA$_2$ was recovered. The resulting sample was then dialyzed over night against buffer B (50 mM Tris-HCl buffer, pH 8, containing 0.05% Triton X-100 and 20 mM CaCl$_2$).

Cation-exchange chromatography

The dialyzed sample was chromatographed on to a 2 cm × 20 cm column of CM-Sephadex, equilibrated with buffer B. Non-bound proteins were removed by rinsing the column with 0.3 M NaCl of buffer B. The adsorbed proteins were eluted with a linear gradient of NaCl (0.3 to 0.7 M).

Biochemical characterization
Determination of PLA$_2$-IIA activity and qualitative analysis of reaction products

The SPLA$_2$-IIA activity was measured titrimetrically at pH 9.5 and at 45°C with a pH-stat using a crude egg-yolk emulsion as a substrate in the presence of 6 mM sodium deoxycholate (NaDC) and 8 mM CaCl$_2$[31]. Several assays were performed with sodium taurodeoxycholate (NaTDC). One unit of PLA$_2$-IIA activity was defined as 1 μmole of fatty

acid liberated under standard conditions. Protein concentration was determined as by Bradford method using BSA ($E^{1\%}_{1\ cm} = 6.7$) as a reference standard[32].

In order to determine the acyl chain specificity, the enzyme was incubated at 45ºC in the presence of fine suspension of 1-stearoyl-2-oleoyl-3-sn-glycerophosphorylcholine. The suspension was then resuspended in 50 mM Tris-HCl, 10 mM $CaCl_2$, 4 mM NaTDC (pH 9.5) and sonicated. The composition of hydrolysis product was investigated by thin-layer chromatography (TLC) on silica gel plates, activated previously at 60°C for 30 min. The developing solvent was a mixture of chloroform/methanol/ammonia (60:35:5, v/v/v). The lipid spots were visualized with iodine vapour.

The spots corresponding to free fatty acids (FFA) were scraped, pooled, methylated and finally analyzed by gas chromatograph coupled with mass spectrometer (GC/MS) (QP2010, Shimadzu) equipped with a flame ionisation detector. Helium was used as a carrier gas. The temperature of column oven, injection ports and detector was maintained at 110, 250 and 285 °C, respectively. The sample (2 μl) was injected into the capillary column (30.0 m length, 0.25 mm internal diameter and 0.25 μm film thickness, Phenomenax). Chromatographic peaks were identified by comparing the retention times with those of known standards.

Effect of temperature and pH on SPLA-IIA stability

To check the thermal stability of SPLA$_2$-IIA, successive assays were performed wherein the homogeneous enzyme was incubated for different time intervals at 70°C. The pH stability of the enzyme was investigated at room temperature during 30 min using the following buffers: 50 mM sodium acetate buffer (pH 4-6), 50 mM potassium phosphate buffer (pH 6-8), and 50 mM Tris-HCl buffer (pH 7-11). Residual activity was measured for each incubation assay after centrifugation under optimal conditions.

Effect of organic solvents

To check the stability of SPLA$_2$-IIA in the presence of water-miscible organic solvents, successive assays were performed, wherein the purified enzyme was incubated for 2 h at 25°C, with 50% methanol, 50% ethanol, 50% 2-propanol, 50% acetonitrile or 50% acetone. Residual activity was measured for each incubation assay after centrifugation under optimal conditions.

Oligosaccharide content and enzymatic analysis of N-glycosylation

The presence of glycan chains in the purified protein was checked by the anthrone-sulfuric acid method using glucose as a standard[33]. One milliliter of pure SPLA$_2$-IIA (1 mg/ml in Tris-HCl buffer, pH 8) was mixed with 4 ml of distilled water in a screw-cap culture tube. The solution was then ice-cooled and 10 ml of cold freshly prepared anthrone reagent (0.2 g in 100 ml conc. H_2SO_4) were added. Thereafter, the solution was mixed and a marble lid was placed on top of the tube to prevent evaporation. The solution was subjected to incubation for 16 min in a boiling water bath and ice-cooled for 2-3 min and then for 5-10 min at room temperature. The absorbance was read at 620 nm against a reagent blank. The rate of glycosylation was calculated on the basis of percentage by weight.

Enzymatic analysis of N-glycosylation of purified SPLA$_2$-IIA was performed using the enzymatic protein deglycosylation kit (Sigma) according to the manufacturer's instructions. Briefly, 10 μg of purified enzyme was denatured with 10× glycoprotein denaturing buffer (NEB) at 100°C for 10 min. Then, 10× G7 reaction buffer, 10× NP-40 and 500 U of peptide-N-gycosidase F (PNGaseF) were added and the final volume brought to 20 μl using MilliQ water. Digestion was performed at 37°C for 1 h and stopped by the addition of 5× non-reducing Laemmli buffer. Result was analyzed by reducing SDS-PAGE using the method of Laemmli[34] and subsequent Coomassie brilliant blue staining.

Alkylation of cysteine residues

The alkylation of the cysteine residues of the enzyme was performed as described previously[35]. In brief, a volume of 100 picomoles of the enzyme were dissolved in 1 ml of 10 mM Tris-HCl (pH 8) and then denatured in 185 μl of 8 M guanidine hydrochloride, 65 μl of 1 M Tris-HCl, 4 mM EDTA (pH 8.5) and 80 mM DTT for 30 min at 60°C. S-Pyridylethylation of cysteine residues of protein was performed by adding 4 μl of vinyl pyridine and incubation at 25°C for 3 h. The modified enzyme was then dialyzed against water for NH$_2$-terminal sequencing.

Limited proteolysis

SPLA$_2$-IIA (1 mg) was dissolved in 1 ml of 50 mM Tris-HCl buffer, (pH 8.5) without benzamidine. The enzyme solution was digested at 30°C with trypsin having trypsin/SPLA$_2$-IIA molar ratio of 0.1. Samples (50 μl) were withdrawn from the incubation mixture

at various times (4, 8, 12 and 24 h) to assess the electrophoretic profile. The reaction was stopped by addition of benzamidine (4 mM final conc.).

Amino acid sequencing

For NH_2-terminal sequencing, the purified enzyme and fragments obtained after trypsin digestion were blotted (60 min, 50 mA, 4°C) on to a polyvinylidene difluoride (PVDF) membrane (Applied Biosystems, ProBlotTM) in 20 mM CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) buffer (pH 11) containing 10% methanol using a mini trans-blot cell (BioRad, Hercules, USA). The NH_2-terminal sequences were determined by automated Edman's degradation using an Applied Biosystems Protein Sequencer Procise 492 equipped with 140 C HPLC system (Roissy, France)[36].

Results and Discussion
Purification of SPLA$_2$-IIA

The SPLA$_2$-IIA was purified using acidic treatment, ammonium sulphate precipitation and cation-exchange chromatography. The SPLA$_2$-IIA activity emerged in a single peak (Fig. 1A) at 0.5 M NaCl. The active fractions of this peak were pooled and analyzed on SDS-PAGE under reducing conditions (Fig. 1B). The data clearly showed that SPLA$_2$-IIA was purified to homogeneity and had an apparent molecular mass of 14 kDa, as revealed by SDS-PAGE. This result was consistent with the

molecular mass determined under native conditions using gel filtration on HPLC column Bio-sil SEC-125 (300 mm × 7.8 mm) (data not shown). Taken together, these findings suggested that SPLA$_2$-IIA was a monomeric protein, similar to previously reported chicken PLA$_2$-IIA (ChPLA$_2$-IIA)[24] and mammal PLA$_2$-IIA[21-23] with a molecular mass of about 14 kDa. SPLA$_2$-IIA was lyophilized and conserved at -20°C.

The purification flow sheet given in Table 1 shows that specific activity of purified enzyme reached 290 U/mg, when egg yolk emulsion was used as a substrate at pH 9.5 and 45°C and in the presence of 6 mM NaDC and 8 mM CaCl$_2$.

The findings indicated that stingray intestine gave a high purification yield (54%) of SPLA$_2$-IIA, which was five-times higher than that of ChPLA$_2$-IIA[24]. Furthermore, SPLA$_2$-IIA purified after only one chromatographic step showed higher specific activity than that displayed by the ChPLA$_2$-IIA (160 U/mg) in egg yolk emulsion[24]. Interestingly, and unlike those from mammal and bird origin, the marine PLA$_2$ reported in this work was a glycosylated form of PLA$_2$ with a carbohydrate content of 2.5% (data not shown)[21-24].

The glycosylation of purified PLA$_2$-IIA was also checked with PNGaseF, which cleaves all N-linked glycans. Figure 1C shows that SPLA$_2$-IB which is a non-glycosylated control[29], was resistant
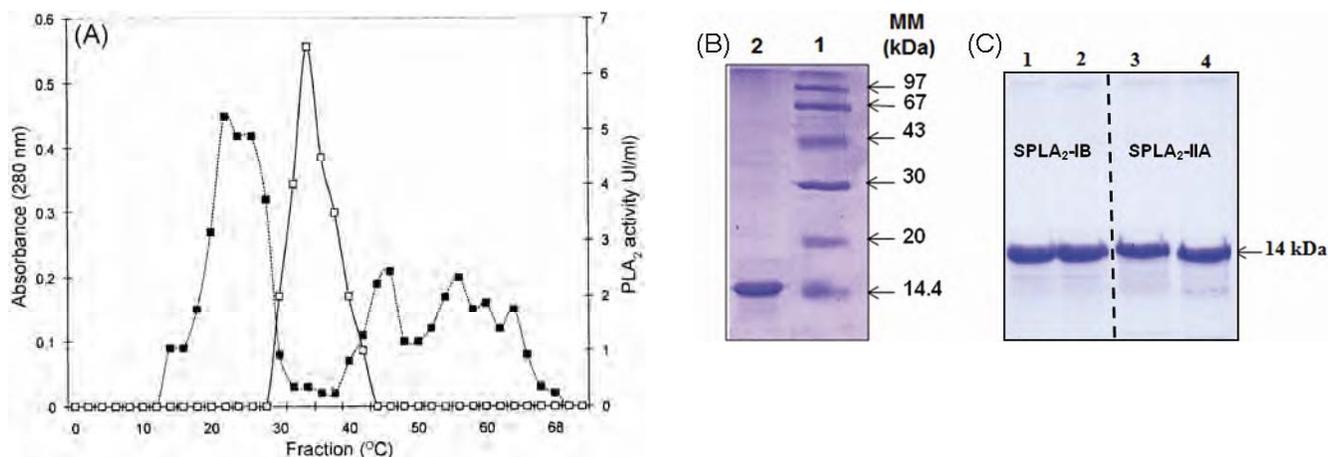

Fig. 1—Purification of SPLA$_2$-IIA [(**A**): Chromatography of stingray PLA$_2$-IIA on CM-Sephadex. The column (2 cm x 20 cm) was equilibrated with 50 mM Tris-HCl buffer, pH 8, containing 0.05% Triton X-100 and 20 mM CaCl$_2$ (buffer B); a linear salt gradient (0.3 to 0.7 M NaCl) in buffer A was applied to the column; gradient chamber 75 ml; 2 ml fraction; flow rate, 25 ml/h. Active fractions (30 to 39) were collected; (**B**): SDS-PAGE (15%) of pure SPLA$_2$-IIA. Lane 1, molecular mass markers (Pharmacia); and lane 2, 10 g of SPLA$_2$-IIA eluted from CM-Sephadex. The gel was stained with Comassie blue to reveal proteins; and (**C**): Enzymatic assessment of N-glycosylation SPLA$_2$-IIA and SPLA$_2$-IB. Aliquots of pure enzymes (10 µg) were denatured and incubated under denaturing conditions in the absence or presence of PNGase F. SDS-PAGE (15%) was performed under reducing conditions. Lane 1, untreated SPLA$_2$-IB; lane 2, treated SPLA$_2$-IB; lane 3, untreated SPLA$_2$-IIA; and lane 4, treated SPLA$_2$-IIA]

Table 1—Flow sheet of stingray PLA$_2$-IIA purification

Purification step	Total activity[a] (units)	Protein[b] (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
Extraction	640	7150	0.089	100	1
Acidic treatment (NH$_4$)$_2$SO$_4$	518	370	1.4	81	15.7
Precipitation (25-75 %)	430	50	8.6	67	96.6
CM-Sephadex	350	1.2	290	54.7	3258.4

[a]1 Unit: μmole of fatty acid released per min using yolk egg emulsion as a substrate in the presence of 6 mM NaDC and 8 mM CaCl$_2$.

[b]Proteins were estimated using the Bradford method[32]. The experiments were conducted three-times.

to PNGaseF. In contrast, the molecular mass of the treated SPLA$_2$-IIA (lane 4) band in the lane with theuntreated sample (lane 3) was slightly reduced, which might reflect a minimal amount of N-glycosylation.

Properties of SPLA$_2$-IIA
Positional specificity

It is well-known that specific PLAs hydrolyze only one bond ester at the *sn-1* or *sn-2* positions of phospholipids, with the concomitant production of lysophospholipid and free fatty acids[2]. In order to check positional specificity towards the ester bonds of phospholipids, the purified enzyme was incubated with 1-stearoyl-2-oleoyl-3-snglycerophosphorylcholine (Sigma) as substrate. Analysis of fatty acids obtained after the hydrolysis by GC-MS (Fig. 2) showed the presence of a main peak corresponding to methyl oleate C18:1 (90%). No free stearic acid was detected after TLC and GC-MS analysis. These results confirmed that purified enzyme belonged to the PLA$_2$ family (E.C. 3.1.1.4) and was able to hydrolyze only the *sn*-2 position of phospholipids.

Ca^{2+} dependence

The presence of Ca^{2+} is essential for both the catalysis and binding of enzymes to the substrate[37-39]. In order to investigate the effect of Ca^{2+} on the enzyme activity, the variation in hydrolysis rates of egg yolk phospholipids by the SPLA$_2$-IIA was investigated in the presence of different Ca^{2+} concentrations (Fig. 3A). The result showed no PLA$_2$ activity in the absence of Ca^{2+} and the presence of chelator, such as EDTA or EGTA (10 mM). In the absence of calcium chelators, the specific activity of SPLA$_2$-IIA was increased and reached a maximum (290 U.mg^{-1}) at 8 mM CaCl$_2$ (Fig. 3A). Similar results[29] were previously reported for the SPLA$_2$-IB (Fig. 3A). In agreement with previous findings with mammals and bird pancreatic PLA$_2$[21-24], the present study also showed that marine PLA$_2$-IIA required the presence of Ca^{2+} to trigger the hydrolysis of phospholipids emulsion.
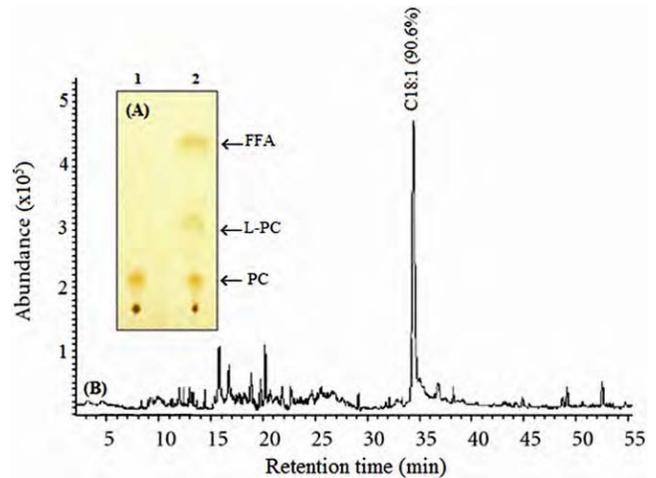

Fig. 2—(A): TLC analysis of the hydrolysis products of 1-stearoyl-2-oleoyl-3-snglycerophosphorylcholine by SPLA$_2$-IIA. [Lane 1, 1-stearoyl-2-oleoyl-3-snglycerophosphorylcholine before hydrolysis; and lane 2, 1-stearoyl-2-oleoyl-3 snglycero-phosphorylcholine after hydrolysis by SPLA$_2$-IIA]; and (B) GC–MS chromatogram of the free fatty acids liberated after hydrolysis of 2-oleoyl-1-stearoyl-3-sn-glycerophosphorylcholine by SPLA$_2$-IIA. The percent of methyl oleoyate C18:1 (90.6%) was marked]

Bile salts dependence

In order to investigate the effect of bile salts on the enzyme activity, the hydrolysis rate of egg yolk phospholipids by SPLA$_2$-IIA was assayed in the presence of various bile salt concentrations at pH 9.5 and 45°C. As shown in Figs 3B and C, NaDC and NaTDC were specifically required for the enzyme activity and the maximum activity was observed in the presence of 4 mM NaTDC or 6 mM NaDC.

Bile salts particularly in micellar form are tensioactive agents that ensure the dispersion of hydrolysis products, thus increasing the hydrolysis rate. Earlier, it has been reported that micellar forms of the substrate are hydrolyzed by PLA$_2$ at a much higher rate than molecularly dispersed substrates[40]. These observations also find support in several
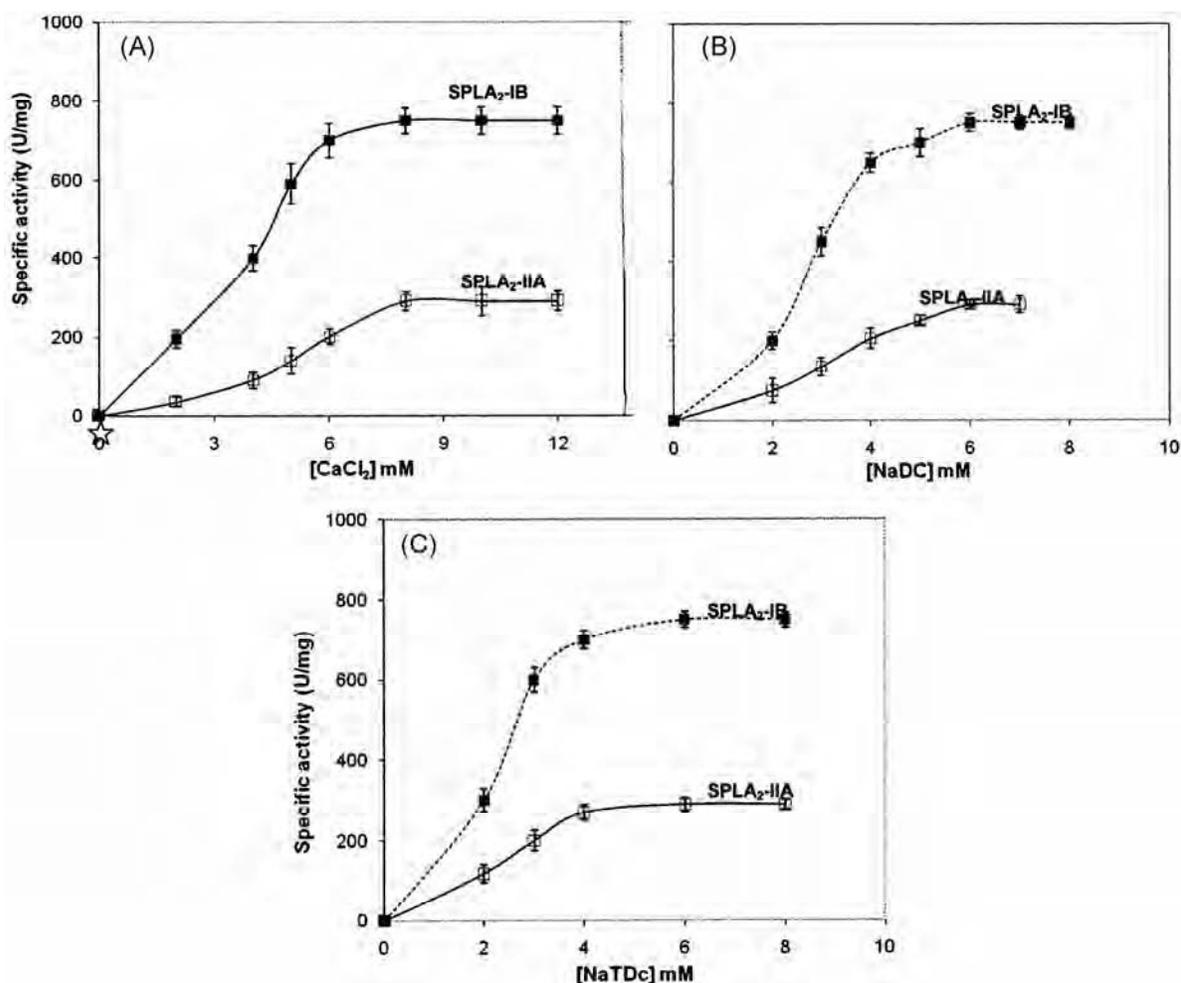
Fig. 3—Effect of Ca^{2+} conc. on SPLA$_2$-IIA and SPLA$_2$-IB activities [(**A**). Enzyme activity was measured at various concentrations of Ca^{2+} using egg yolk emulsion as a substrate at pH 9.5 and 45°C in the presence of 4 mM NaTDC. The star symbol indicates the absence of phospholipase activity checked in the absence of CaCl$_2$ and in the presence of 10 mM EDTA. (**B**) Effect of increasing conc. of NaDC (**B**) and NaTDC (**C**) on SPLA$_2$-IIA and SPLA$_2$-IB activities. PLA$_2$ activity was measured using egg yolk emulsion as a substrate under standard conditions. SPLA$_2$-IIA: (open square); SPLA$_2$-IB: (black square). Data shown are means ± SD (n = 3)]

findings previously reported on mammal and bird intestinal PLA$_2$[21-24]. Similar results were previously reported with SPLA$_2$-IB[29] which required 4 mM of NaTDC to reach maximum activity (Fig. 3C). In contrast, recently it has been reported that pancreatic chicken PLA$_2$-IB is active in the absence of NaTDC and shows optimal activity virtually independent of NaTDC[41].

Effect of pH and temperature on SPLA$_2$-IIA activity and stability

Thermophilic PLA$_2$ often show high thermostability, excellent activity at elevated temperatures and good resistance to chemical denaturation, which make them ideal for industrial and chemical processes, where relatively high reaction temperatures and/or organic solvents are employed[42,43]. However,

there is a need for search for better forms of PLA$_2$ to meet strict requirements of the current industrial applications and processes, such as good stability and activity in harsh conditions, including temperature and pH extremes and the presence of organic solvents.

The activity profile of purified SPLA$_2$-IIA was examined by assaying at various temperatures ranging from 20 to 55°C using homogeneous egg yolk emulsion as a substrate (Fig. 4A). The maximal activity by SPLA-IIA was recorded at 45°C In contrast, known intestinal[22-24] and pancreatic PLA$_2$, such as SPLA$_2$-IB[29] (Fig. 4A) have shown maximal activity at 37°C. SPLA$_2$-IIA activity increased significantly (five-fold) when the temperature was increased from 30 to 45°C. As SPLA$_2$-IIA maintained
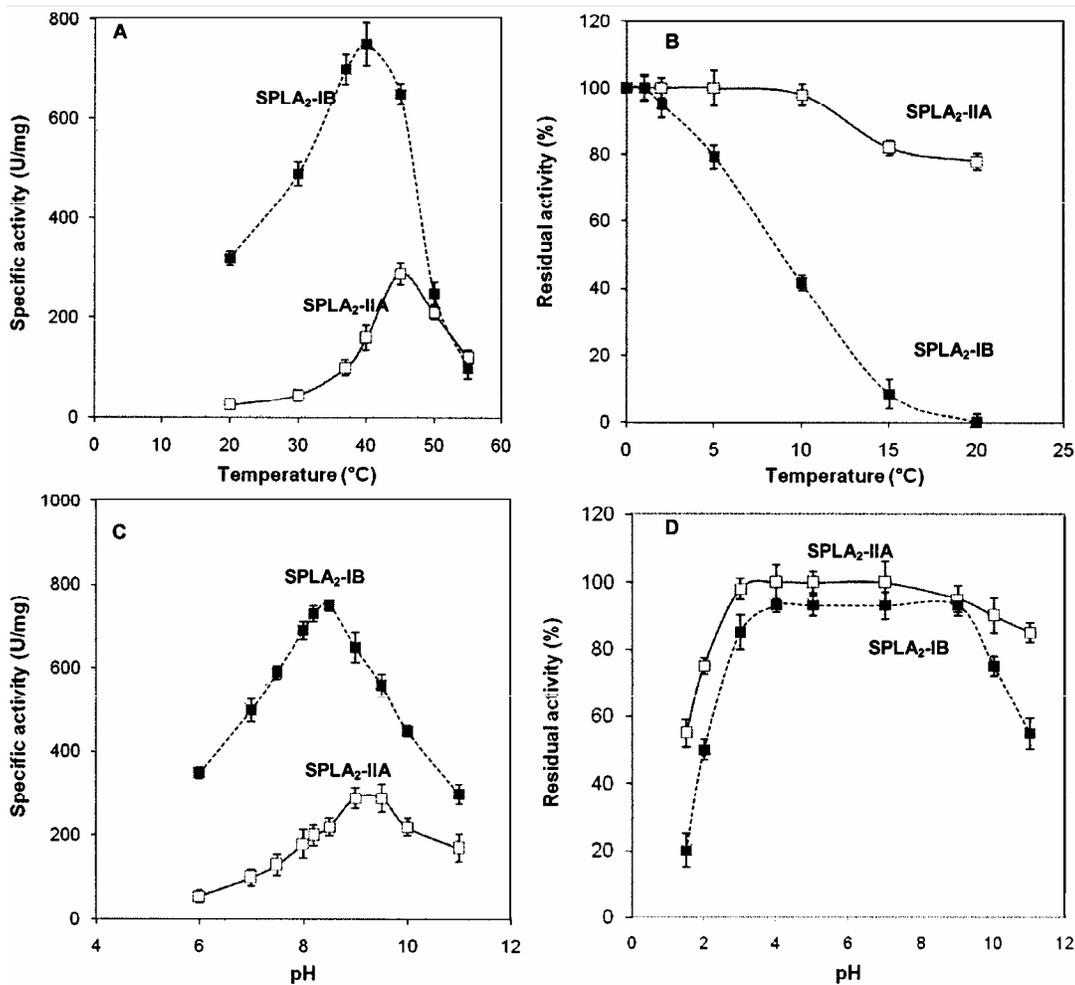
Fig. 4—Effect of temperature on SPLA$_2$-IIA and SPLA$_2$-IB activity and stability [Enzyme activity was assayed at various temperatures using egg yolk emulsion as a substrate in the presence of 8 mM Ca^{2+} and 6 mM NaDC (**A**) and at standard conditions after incubation at 70°C at different time intervals (**B**). Effects of pH on SPLA$_2$-IIA and SPLA$_2$-IB activity (**C**) and stability (**D**). Enzyme activity was assayed at various pH using egg yolk emulsion as a substrate in the presence of 8 mM Ca^{2+} and 6 mM NaDC (**C**) and under standard conditions after incubation at different pH (**D**). Data shown are means ± SD (n = 3). SPLA$_2$-IIA: (open square); SPLA$_2$-IB: (black square)]

its activity at relatively high temperatures (45-55°C), it can be considered as a potential candidate for application as a detergent additive for the synthesis or modification of industrial phospholipids[42].

The thermostability of SPLA$_2$-IIA was also investigated by measuring the residual activity after incubation of the enzyme at 70°C in Tris-HCl buffer (pH 8) at different time intervals (Fig. 4B). The SPLA$_2$-IIA maintained about 80% of its activity after 20 min of incubation at 70ºC, while SPLA$_2$-IB lost its complete activity when incubated under the same conditions (Fig. 4B). As was previously established for mammal and bird PLA$_2$-IIA[22-24], the PLA$_2$ purified in the present study was found to tolerate incubation at high temperature for long time

intervals (20 min at 70°C). However, SPLA$_2$-IIA was found to be more resistant to temperature than pancreatic SPLA$_2$[29].

The pH activity profile of purified SPLA$_2$-IIA is shown in Fig. 4C. The pH-optimum of the enzyme activity was slightly higher than that of mammal, bird and marine pancreatic PLA$_2$ [29,40,44]. The maximal activity of the enzyme was found at pH 9.5 and 45°C using egg yolk as substrate in the presence of 8 mM Ca^{2+} and 6 mM NaDC (Fig. 4C). The SPLA$_2$-IB, on the other hand, exhibited maximal activity at pH 8.5 under the same assay conditions[29] (Fig. 4C).

The pH stability (Fig. 4D) profile showed that SPLA$_2$-IIA was active over a broad range of pH between 2 and 11 during 15 min of incubation.

The purified SPLA$_2$-IIA maintained more than 70% of its activity when incubated at pH 2 (Fig. 4D). In contrast, pancreatic SPLA$_2$[29] is not stable at pH values less than 3. Earlier, several forms of rat[21], porcine[22], human[23] and chicken[24] intestinal PLA$_2$ have been previously reported to be stable at low pH values (pH 2-3). A number of pancreatic PLA$_2$ have been found to be less stable at low pH, such as turkey PLA$_2$-IB[20], ChPLA$_2$-IB[40], and marine snail[27] and crab[28] digestive PLAs, losing their complete activity when incubated at pH values of less than 5 for few minutes.

Long-term stability

The activity of SPLA$_2$-IIA, stored at room temperature or in the refrigerator did not drop below 85% and 95% of the initial values after 120 days, respectively (Fig. 5). Furthermore, the activity remained surprisingly stable up to 40 weeks, although SPLA$_2$-IIA was not maintained in stability-enhancing media, such as supplements of Ca^{2+}, glycerol, or ammonium sulphate, but only in plain demineralized water. These findings were, in fact, similar to those recently reported for the SPLA$_2$-IB[29] (Fig. 5).

Effect of organic solvents

Organic solvents have been found to be advantageous in various industrial enzymatic processes and their use can increase the solubility of non-polar substrates, increase the thermal stability of enzymes, decrease water-dependent side reactions, or eliminate microbial contamination[43]. The PLA$_2$ obtained in present study, like the pancreatic one[29] showed high stability in the presence of water-miscible organic solvents. It retained almost 100% of its activity after incubation for 2 h at 25°C with 50% methanol, ethanol, 2-propanol, acetonitrile and acetone (Table 2). Addition of 50% ethanol or acetonitrile to purified SPLA$_2$-IIA caused immediate increase of 23% and 15% in activity as compared to the controls, respectively (Table 2).

Tryptic cleavage of enzyme and NH$_2$-terminal sequences analysis

Purified SPLA$_2$-IIA was denatured, reduced and alkylated as described in 'Materials and Methods' section and also dialyzed against distilled water. The NH$_2$-terminal sequencing of SPLA$_2$-IIA allowed unambiguously the identification of 15 residues of the pure enzyme. The same 15 residues were obtained when the purified SPLA$_2$-IIA was transferred without alkylation on to a PVDF membrane. Table 3 shows the NH$_2$-terminal sequence of SPLA$_2$-IIA
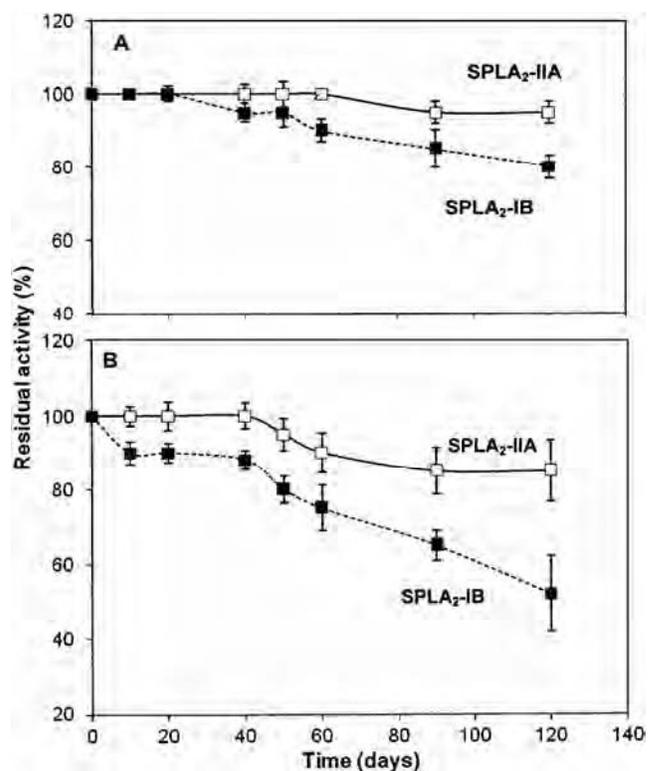

Fig. 5—Long-term stability of SPLA$_2$-IIA and SPLA$_2$-IB stored in the refrigerator (5°C) and at room temperature (25°C) [Assays were carried out under standard conditions as described in the text of the paper. SPLA$_2$-IIA: (open square); SPLA$_2$-IB: (black square). Data shown are means ± SD (n = 3)]

Table 2—Stability of SPLA$_2$-IIA and SPLA$_2$-IB in organic solvents
[Values represent the means of three replicates]

Organic solvent	Relative activity % (1 h)		Relative activity % (2 h)	
	SPLA$_2$-IB	SPLA$_2$-IIA	SPLA$_2$-IB	SPLA$_2$-IIA
Control	100 ± 0.5	100 ± 0.1	100 ± 2.4	100 ± 0.6
Acetone	95 ± 2.5	99 ± 0.5	92 ± 2.2	97 ± 1.7
Acetonitrile	105 ± 1.5	115 ± 4.5	105 ± 2.3	110 ± 1.9
Methanol	98 ± 2.4	97 ± 3.2	95 ± 1.9	96 ± 2.1
Ethanol	116 ± 2.7	123 ± 1.1	100 ± 3.3	115 ± 1.4
2-Propanol	97 ± 3.1	100 ± 0.6	93 ± 3.6	97 ± 0.3

Pure SPLA$_2$-IIA and-IB was incubated in each organic solvent (50%) at 25°C for 1 h and 2 h.

Table 3—Alignment of the NH$_2$-terminal sequences of stingray PLA$_2$-IIA and protein fragments isolated after tryptic proteolysis of SPLA$_2$-IIA with human PLA$_2$-IIA (hPPLA$_2$-IIA)[24]

[Italic and bold symbols of residues indicate homologous and different amino acids, respectively. The Ca^{2+} loop (C24–G30) is bold-shaded. Stars indicate amino-acid residues of the catalytic dyad (H64-D65). The arrows indicate the tryptic cleavage site of SPLA$_2$-IIA]

SPLA$_2$-IIA: NLVQF GLMIK LTTGK

hPLA$_2$-IIA: NLV*N*F **HR**MIK LTTGK

T1: EAALS YGFYG CHCGI GGKGA P

↓

hPLA$_2$-IIA: K EAALS YGFYG CHCGV GGRGS P

**

T2: GSPKD ATDRC CVVHDC CYKSL EK

↓

hPLA$_2$-IIA: R GSPKD ATDRC CV**T**HDC CYK**RL** EK

Fig. 6—SDS-gel electrophoresis (20%) analysis of tryptic cleavage of SPLA$_2$-IIA as a function of time [The gel was stained with Coomassie blue to reveal proteins. Lane 1, SPLA$_2$-IIA; lane 2, after incubation of SPLA$_2$-IIA with trypsin for 8 h; lane 3, after incubation of SPLA$_2$-IIA with trypsin for 24 h; and lane 4, molecular mass markers]

and the human PLA$_2$-IIA (hPLA$_2$-IIA)[24]. The NH$_2$-terminal sequence of PLA$_2$ obtained in present study exhibited a high level of identity (80%) with mammal PLA$_2$-IIA.

To gain insights into the marine PLA$_2$ primary structure, we performed a limited proteolysis experiment on SPLA$_2$-IIA using trypsin. Incubation of SPLA$_2$-IIA with trypsin at 30°C and using a protease/PLA$_2$ molar ratio of 0.1 generated two major fragments: T1 (~12 kDa) and T2 (~5 kDa) (Fig. 6). These fragments were transferred on to a PVDF membrane and their NH$_2$-terminal amino acids were sequenced (Table 3). The results are given in Table 3 together with the corresponding sequences from the hPLA$_2$-IIA. The NH$_2$-terminal sequencing showed that the larger 12 kDa fragment (T1) containing the Ca^{2+} loop (YGCHCGIGG) corresponded to an NH$_2$-terminal truncated form of SPLA$_2$, starting at residue E16. The NH$_2$-terminal truncated SPLA$_2$ form (~12 kDa) was thus generated from cleavage by trypsin of the K15-E16 bond of the enzyme. The sequencing of 5 kDa fragment (T2) showed that it contained the dipeptide H64-D65, which constituted the active site of mammalian and bird PLA$_2$[4, 25]. Based on its molecular mass (~5 kDa), this band was obtained from an N- and C-terminal truncations of the SPLA$_2$-IIA (14 kDa).

Conclusion

A new PLA$_2$ was purified from the intestine of a cartilaginous fish, namely the common stingray (*Dasyatis pastinaca*). This enzyme exhibited several attractive features,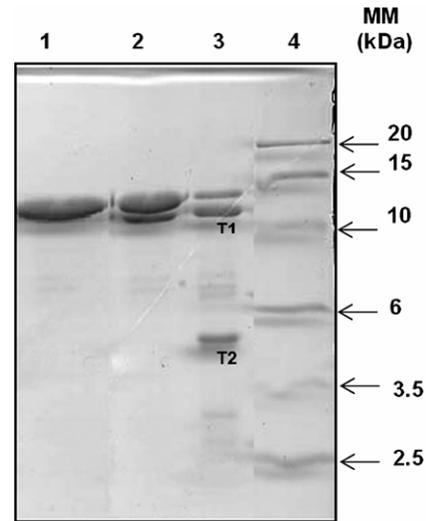 making it a potential candidate for various industrial applications and processes. Stability of SPLA$_2$-IIA in the presence of organic solvents and its tolerance to high temperatures, basic and acidic pH, might open new opportunities for the treatment of phospholipid-rich industrial effluents, the production of efficient inter-esterification substances in food processing industry, or the synthesis of cost-effective chemical compounds for the food production industry. However, further studies are needed to better establish the relation of structure–functions of the marine PLA$_2$-IIA.

Acknowledgements

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