Purification and biochemical characterization of phospholipase A2 from dromedy pancreas

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Abstract
Dromedary pancreatic PLA2 (DrPLA2) was purified from delipidated pancreases. Pure protein was obtained after heat and acidic treatment (70 °C; pH 3.0), precipitation by ammonium sulphate and ethanol respectively, followed by sequential column chromatographies on Sephadex G-50, MonoS Sepharose, MonoQ Sepharose and C-8 reverse phase high pressure liquid chromatography. Purified DrPLA2, which is not glycosylated protein, was found to be monomeric protein with a molecular mass of 13748.55 Da. A specific activity of 600 U/mg for purified DrPLA2 was measured at optimal conditions (pH 8.0 and 37 °C) in the presence of 3 mM NaTDC and 7 mM CaCl2 using PC as substrate. The sequence of the first fourteen amino-acid residues at the N-terminal extremity of DrPLA2 was determined by automatic Edman degradation. One single sequence was obtained and shows a close similarity with all other known pancreatic secreted phospholipases A2.

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1. Introduction
Phospholipases A2 (PLA2, EC 3.1.1.4) catalyse specifically the cleavage of the fatty acid residue at the sn-2 position of phospholipids that liberate free fatty acid and lysophospholipid. Several new PLA2 have been identified. The diversity of these enzymes called for the reevaluation of PLA2 classification scheme that had been used for many years [1]. PLA2 were classified mainly into three groups: (i) cytosolic PLA2 (cPLA2); (ii) Ca2+-independent intracellular PLA2 (iPLA2) and (iii) Ca2+-dependent secreted PLA2 (sPLA2). There is also a PLA2 class which hydrolyses the platelet-activating factor (PAF) and the oxidized lipids, called PAF acetylhydrolases (PAF-AH) [1,2].
To date, there are 11 forms of mammal sPLA2 classified into groups IB, IIA, IIC, IID, IIE, IIF, V, X, III, XIIA and XIIB according to their origin, sequence similarity and molecular mass as well as substrate specificity [3,4]. sPLA2 exhibit tissue and species specific expression which suggests that their cellular behaviours and functions are different. It is well established that some sPLA2 participate in a variety of pathological processes by releasing arachidonic acid from membrane phospholipids, leading to the production of various types of proinflammatory lipid mediators such as prostaglandins, thromboxanes and leukotrienes [5]. The sPLA2 do not manifest significant fatty acid selectivity in vitro. They are Ca2+ dependent lipolytic enzymes with a conserved Ca2+ binding loop and His–Asp dyad at the catalytic site [3,4]. Moreover, these enzymes are low-molecular mass proteins (~14 kDa) containing 6–8 conserved disulfide bridges with a rigid tertiary structure, which confers both stability against proteolysis and resistance to denaturation [1,2]. Only sPLA2-IB and –X, have an N-terminal prepropeptide and the proteolytic cleavage of this prepropeptide is the regulatory step for the generation of an active enzyme [6,7].

sPLA2-IB was found in large amounts in the pancreas and its principal function is the digestion of dietary lipids [8]. This
enzyme is secreted as an inactive zymogen and it is activated after pentapeptide bond cleavage of the proform by trypsin into the mature form [8]. More recently, this enzyme was identified and cloned in other tissues such as lung, spleen, kidney and ovary [9,10], and it has now been proposed to be involved in various physiological and pathophysiological responses such as cell proliferation [11], cell contraction [12,13], lipid mediator release [14], acute lung injury [15] and endotoxic shock [16].

sPLA2-IB have been isolated from pancreases of various species [17–21]. The activation mechanism [22] and amino acid sequence of these enzymes [23,24] is known and extensive studies on the mechanism of action of these enzymes have been reported [25–28].

In our laboratory, some assays to purify the turkey PLA2 (TPP) were done. The pure enzyme on SDS-PAGE analysis was found totally inactive and the enzymatic properties were realized with the partially purified TPP [29].

The determination of the phospholipase content of pancreases from all species was performed in a homogenate prepared in a Waring Blender (2 × 30 s) with 10 ml per gram of fresh tissue, of 10 mM Tris–HCl, 150 mM NaCl, pH 8.5 at room temperature. After centrifugation at 12,000 rpm during 20 min, the amount of enzyme was estimated on an aliquot of the supernatant using PC emulsion as substrate in the presence of 3 mM NaTDC and 7 mM CaCl₂. The phospholipase activity was measured titrimetrically at pH 8.0 and at 37 °C using a pH-stat. One phospholipase unit corresponds to one μmole of fatty acid released per min using PC emulsion as substrate in the presence of 3 mM NaTDC and 7 mM CaCl₂. For each species, the activity represents the average ± standard error mean of five assays obtained from three different pancreases.

In the present study, we report the purification to homogeneity of the active sPLA2 from the dromedary pancreas. This mammalian phospholipase A2 was characterized with respect to its biochemical properties.

2. Material and methods

2.1. Materials

Trypsin (treated with l-1-tosylamino 2-phenylethyl chloromethylketrone), tributyrin (99%; puriss), benzamidine were from Fluka (Buchs, Switzerland), bovine serum albumine (BSA), sodium deoxycholate (NaDC), sodium taurodeoxycholate (NaTDC), triton X-100 (TX-100) were from Sigma Chemical (St. Louis, 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, MonoQ were from Pharmacia (Uppsala, Sweden); protein sequencer Procise 492 equipped with 140 C HPLC system provided from Applied Biosystems (Roissy, France); C-8 reverse-phase eurospher 100 column (250 mm × 4.6 mm) from Knauer (Germany); pH-stat was from Metrohm (Herisau, Switzerland).

2.2. Pancreas collections

Pancreases from dromedary were collected immediately after slaughter and kept at −20 °C. Pancreases were collected from a local slaughterhouse (Sfax, Tunisia).

2.3. Delipidation of pancreases

After decongelation, pancreases were cut into small pieces (1–2 cm²) and delipidated according to the method described previously [30]. After delipidation, about 15 g of delipidated powder of pancreas were obtained from 100 g of fresh tissue.

2.4. Determination of lipase activity

The lipase activity was measured titrimetrically at pH 8.5 and at 37 °C with a pH-stat, under the standard assay conditions described previously, using tributyrin (0.25 ml) in 30 ml of 2.5 mM Tris–HCl, 5 mM CaCl₂ pH 8.5
as substrate. One lipase unit corresponds to 1 μmole of fatty acid liberated per min.

2.5. Determination of phospholipase activity

The PLA2 activity was measured titrimetrically at pH 8.0 and at 37 °C with a pH-stat, under the standard assay conditions described previously [1], using PC or egg yolk emulsions as substrate in the presence of 3 mM NaTDC and 7 mM CaCl2. Some assays were performed with NaDC. One unit of phospholipase activity was defined as 1 μmole of fatty acid liberated under standard conditions.

2.6. Determination of protein concentration

Protein concentration was determined as described by Bradford et al. [32] using BSA (ε1%1cm=6.7) as reference.

2.7. Oligosaccharides content

The presence of glycan chains in the purified enzyme was checked by anthrone-sulfuric acid method using glucose as a standard [33].

Fig. 2. RP-HPLC and SDS-PAGE (15%) of DrPLA2. (A) RP-HPLC on a eurospher 100, C-8 column, elution was performed at room temperature within 30 min using a gradient from 0 to 90% solvent B at a flow rate of 0.6 ml/min. [solvent A, water/trifluoroacetic acid (1000:1, v/v)] and solvent B, acetonitrile]. The effluent was monitored at 220 nm. The gradient is indicated by the dotted line. (B) SDS-PAGE (15%) of pure DrPLA2 of RP-HPLC, lane a, 10 μg of DrPLA2 eluted from RP-HPLC, lane b, 15 μg of DrPLA2 obtained after MonoQ chromatography. lane c, 20 μg of DrPLA2 obtained after MonoS chromatography. Lane d, molecular mass markers (Pharmacia). The gel was stained with Coomassie blue.
2.8. Alkylation of Cys residues

The alkylation of Cys residues of phospholipase was realized as the technique of Okazaki [34]. 100 pmol of DrPLA2 in 1 ml of 10 mM Tris–HCl, pH 8.2 were 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 during 30 min at 60 °C. S-Pyridylethylation of cysteine residues of protein was performed by adding 4 μl of vinyl pyridine during 3 h at 25 °C. The modified enzyme was dialysed against water for N-terminal sequencing.

2.9. Analytical methods

Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed by the method of Laemmli [35]. The proteins were stained with Coomassie brilliant blue. The molecular mass of purified PLA2 was determined by MALDI-TOF (matrix assisted laser desorption ionization-time of flight).

2.10. Amino acid sequencing

The N-terminal sequence was determined by automated Edman’s degradation, using an Applied Biosystems Protein Sequencer Procise 492 equipped with 140 C HPLC system [36].

3. Results and discussion

3.1. Level of expression of DrPLA2 activity

In order to compare the level of dromedary PLA2 activity with other species, the rate of hydrolysis of PC by bovine, sheep, chicken, and turkey pancreases were measured under the same conditions. Results reported in Table 1 show that the pancreases tested secreted various levels of PLA2 activity. The highest level was observed with turkey (200 U/g). Dromedary presented only 20 U/g in its pancreas.

3.2. Purification of DrPLA2

48 g of delipidated dromedary pancreas were suspended in 600 ml of buffer A: 0.01 M Tris–HCl, 0.15 mM NaCl, pH 8.5 and ground mechanically twice for 30 s at room temperature using the Waring Blendor system. Then, the mixture was stirred with a magnetic bar for 20 min at room temperature and centrifuged during 30 min at 12,000 rpm. The total PLA2 activity obtained (1720 U) did not increase when trypsin was added at different ratios to the phospholipase A2 solution. It can be concluded that the endogenous trypsin is sufficient to achieve the PLA2 activation (data not shown).

3.2.1. Heat and acidic treatment

As has been established for many pancreatic PLA2 [17–22], dromedary PLA2 present in the homogenate can tolerate the incubation at pH 3.0 and at 70 °C. The previous PLA2 solution was incubated 3 min at 70 °C. After rapid cooling, insoluble denatured proteins were removed by centrifugation during 30 min at 12,000 rpm. Afterwards the pH of the supernatant was brought to 3.0 by adding 6N HCl under gentle stirring at 0 °C. After centrifugation (30 min at 12,000 rpm), the clear supernatant was adjusted to pH 7.0 with 3 N NaOH. The recovery of PLA2 activity was of about 85%.

3.2.2. Ammonium sulphate precipitation

The treated supernatant (500 ml, 1470 U) was brought to 75% saturation with solid ammonium sulphate under stirring conditions and maintained during 45 min at 4 °C. After centrifugation (30 min at 12,000 rpm), the precipitated PLA2 was resuspended in 10 ml of buffer A containing 2 mM benzamidine. Insoluble material was removed by centrifugation during 10 min at 12,000 rpm. Approximately 64% of the starting amount of PLA2 was recovered.

3.2.3. Ethanol fractionation

An equal volume of pure ethanol solution was added to the supernatant (11 ml, 1100 U) at 0 °C. Precipitated proteins were removed by centrifugation and the supernatant was added slowly with four times its volume of ethanol to bring the alcohol concentration to 90% (v/v) at 0 °C. After centrifugation for 30 min at 12,000 rpm the ethanol precipitated PLA2, which contains about 47% of the enzyme starting amount, was solubilized in 10 mM acetate buffer pH 5.0 containing 0.05% triton X-100 and 2 mM benzamidine (buffer B). The obtained sample was subjected to chromatography purification.

3.2.4. Filtration on Sephadex G-50

The sample containing PLA2 (9 ml, 800 U) was filtered through a column (95 cm × 2.6 cm) of sephadex G-50 equilibrated with buffer B. Elution of proteins was performed with the same buffer at 50 ml/h. The fractions containing the PLA2 activity eluted between 1.4 and 2 void volumes were pooled together (Fig. 1A).

3.2.5. Cation exchange chromatography

The pooled fractions containing PLA2 activity of Sephadex G-50 column were applied to a MonoS column (2.6 cm × 20 cm) equilibrated with buffer B. Non fixed proteins were washed out with buffer B. The elution of the adsorbed proteins was then

<table>
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<th>Table 2 Flow sheet of the DrPLA2 purification</th>
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<td>RP-HPLC</td>
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a 1 Unit: μmole of fatty acid released per min using PC emulsion as substrate in the presence of 3 mM NaTDC and in the presence of 7 mM CaCl2.

b Proteins were estimated by Bradford method [32]. The experiments were conducted three times.
performed with a linear gradient of NaCl (0 to 0.4 M). As shown in the elution diagram, dromedary PLA2 activity emerged in a single peak (Fig. 1B) at 0.15 M NaCl. The fractions of this peak were pooled and dialysed against 10 mM Tris–HCl buffer pH 8.5 containing 10 mM NaCl and 2 mM benzamidine (buffer C). The recovery of PLA2 from MonoS column was of about 30% of the starting amount of the enzyme.

3.2.6. MonoQ chromatography

A Mono-Q column (1.5 cm×10 cm) was prepared and equilibrated with buffer C. The dialysed sample from MonoS purification was poured into this column and non fixed proteins were removed with the equilibrium buffer. Then, linear salt gradient with a total volume of 150 ml, 0 to 0.25 M NaCl was applied. Fractions containing DrPLA2 activity were eluted at 0.1 M NaCl, pooled and lyophilized. At this stage of purification, the enzyme presented a specific activity of 290 U/mg. We obtained 350 UT of phospholipase A2 with a recovery of 70% at this stage which was not the case of the TPP partially purified in our laboratory. In deed, a very poor yield was obtained during the elution of the PLA2 enzyme from DEAE cellulose. This difference was explained by the fact that TPP strongly interacts with the chromatography supports [29].

3.2.7. RP-HPLC C-8 column

Twenty units of lyophilized sample from Mono-Q column were applied to RP-HPLC eurosuper 100, C-8 column (250 mm×4.6 mm). PLA2 activity was detected in a fraction eluted at 65% acetonitrile as a single peak (Fig. 2A) and the overall recovery of the enzyme activity was 11.63% of the starting amount. In contrast to TPP, the purified DrPLA2 was found to be stable in the presence of the hydrophobic solvent.

The purification flow sheet is given in Table 2 and shows that the specific activity of pure dromedary PLA2 reached 600 U/mg using PC emulsion as substrate at pH 8.0 and at 37 °C in the presence 3 mM NaTDC and 7 mM CaCl2.

The results of SDS-PAGE analysis of the purified DrPLA2 are given in Fig. 2B. As can be seen, the purified DrPLA2 exhibited one band corresponding to an apparent molecular mass of about 14 kDa. Mass spectrometry analysis indicated that DrPLA2 has a molecular mass of 13748.55 Da (data not shown). Pure PLA2 was lyophilized and conserved at −20 °C.

Glycan chains in pure PLA2, analyzed as described in Materials and methods, indicate that the DrPLA2 is not a glycosylated molecule (data not shown). Similar result was obtained with all purified pancreatic PLA2.

3.3. Enzymatic properties of the purified DrPLA2

3.3.1. Ca2+ dependence

It is well established that Ca2+ is essential for both, catalysis and enzyme binding to the substrate [37,38]. In order to investigate the effect of Ca2+ on DrPLA2 activity, we studied the variation of hydrolysis rates of PC emulsion by pure DrPLA2 in presence of
various Ca\(^{2+}\) concentrations. Our results showed that no PLA2 activity can be detected in the absence of Ca\(^{2+}\) and in the presence of 10 mM EDTA or EGTA when using PC or egg yolk emulsion as substrate. In the absence of chelators, the specific activity of purified PLA2 increases to reach 600 U/mg at 7 mM CaCl\(_2\) (Fig. 3). Similar results were obtained with other pancreatic PLA2 like porcine, bovine, turkey and horse [21,36,39,40].

3.3.2. Bile salts dependence

Several studies have provided evidence that the bile salts are tensioactive agents ensuring in micellar form, the dispersion of the products of hydrolysis [21,41]. In the same direction, De Haas et al., reported that micellar forms of the substrate were hydrolysed at a much higher rate than substrates molecularly dispersed by PLA2 [39]. In this study, we measured the DrPLA2 activity at pH 8.0 and at 37 °C using PC emulsion as a substrate in the presence of increasing concentrations of bile salts. As shown in Fig. 4, sodium taurodeoxycholate (NaTDC) (Fig. 4A) and sodium deoxycholate NaDC (Fig. 4B) were specifically required for DrPLA2 activity. The maximal phospholipase activity was observed in the presence of 3 mM NaTDC or 6 mM NaDC. These observations corroborate with previous findings with porcine pancreatic PLA2 [39].

3.3.3. Effects of pH and temperature on DrPLA2 activity

As shown in Fig. 5 the maximal activity of DrPLA2 was measured at pH 8.0 (Fig. 5A) and at 37 °C (Fig. 5B) using PC emulsion as a substrate in the presence of 7 mM Ca\(^{2+}\) and 3 mM NaTDC. The pH-optimum for dromedary PLA2 activity was similar to those of PLA2 described so far [17–21,36]. The purified enzyme was found to be stable between pH 3.0 and 9.0. In contrast to TPP, pure DrPLA2 maintained about 75% of its activity after 5 min incubation at 70 °C (data not shown). However, TPP was found to lose its full activity when incubated at pH lesser than 5. Comparable results were obtained using egg yolk emulsion as substrate in the presence of 7 mM Ca\(^{2+}\) and 3 mM NaTDC.

3.3.4. N-terminal sequence of DrPLA2

Purified DrPLA2 was alkylated as described in Material and methods and dialysed against distilled water. The NH\(_2\)-terminal sequencing of the DrPLA2 allowed unambiguously the identification of the first fourteen residues of pure enzyme. Table 3 shows the N-terminal sequence of DrPLA2, compared to those of bovine, pig, horse, human and turkey. Pure DrPLA2 exhibits a high degree of homology with both mammalian and avian pancreatic PLA2.

4. Conclusion

DrPLA2 was purified to homogeneity from delipidated pancreases. The pure enzyme was not a glycosylated protein with a molecular mass of 13748.55 Da. The maximal PLA2 activity was at pH 8.0 and at 37 °C. Pure dromedary pancreatic
PLA2 maintained of about 75% of its activity after incubation for 5 min at 70 °C and it was not stable at a pH lesser than 3. Moreover, bile salts and Ca\(^{2+}\) were required for pure PLA2 for 5 min at 70 °C and it was not stable at a pH lesser than 3.

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