Purification of Turkey Pancreatic Phospholipase A2

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Turkey pancreatic phospholipase (TPP) has been purified from delipidated pancreases. The purification included ammonium sulfate fractionation, acidic (pH 5) treatment, followed by sequential column chromatographies on DEAE-cellulose, Sephadex G-75, and reverse phase high pressure liquid chromatography. The purified enzyme was found to be a monomeric protein with molecular mass of 14 kDa. The optimal activity was measured at pH 8 and 37°C using egg yolk emulsion as substrate. Our results show that the enzyme (TPP) was not stable for 1 h at 60°C, and that bile salt and Ca2+ were required for the expression of the purified enzyme. The sequence of the N-terminal amino acids of the purified enzyme shows a very close similarity between TPP and all other known pancreatic phospholipases.

Key words: pancreatic phospholipase; amino acids sequence; bile salt; Ca2+; egg yolk

Phospholipase A2 (PLA2) (EC 3.1.1.4) which hydrolyzes the 2-acyl ester bonds of 1,2-diacylglycerol-3-phospholipid, is intra or extracellularly produced by eukaryotic1) and prokaryotic cells.2) All secreted PLA2 require calcium for the expression of catalytic activity. These PLA2 have been grouped into three classes based on their primary structures.3) Class I enzymes are found in mammalian pancreatic juice and cobra venom. Class II enzymes are contained in synovial fluid and rattle snake venom. The PLA2 included in both classes have a molecular mass of 14 kDa and mutually have high similarities in their primary structure. A PLA2 contained in bee venom has a molecular mass of 12 kDa and is classified into Class III.4,5)

In this study, we describe the purification of the PLA2 from turkey pancreas (TPP). Our work aims to compare the pancreatic phospholipase turkey taken as a bird with the pancreatic phospholipases of mammals described up to now.

Material and Methods

Material. Benzamidine and tributyrin were from Fluka (Buchs, Switzerland); sodium deoxycholate and phosphatidylcholine (PC) were from Sigma Chemical (St. Louis, USA). Acrylamide and bisacrylamide were from BDH (Poole, UK). Marker proteins were provided by BioRad (Paris, France). Support of chromatography used for TPP purification: anion exchange chromatography DEAE-cellulose and Sephadex G-75 were from Pharmacia (Uppsala, Sweden). RP-HPLC column nucleosil (100-5-C18) was provided by Macherey-Nagel (Dueren, Germany). Stirred ultrafiltration cells were from Amicon (Beverly, USA). PVDF membrane and Protein Sequencer Procise 492/610A were provided from Applied Biosystems (Roissy, France). The transblott cell apparatus was from BioRad (Paris, France).

All chemicals used in HPLC were of analytical grade from Merck (Darmstadt, Germany) and from Applied Biosystems (Roissy, France). The pH stat

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Abbreviations: TPP, turkey pancreatic phospholipase; PLA2, pancreatic phospholipase A2; PVDF, polyvinylidene difluoride; RP-HPLC, reverse phase-high pressure liquid chromatography; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; DOC, sodium deoxycholate
was from Methrom (Herisan, Switzerland).

Pancreas collection. Turkey pancreases were collected immediately after death and kept frozen at 
−20°C. Pancreases of turkey and chicken or cattle, goat, and sheep were collected from a local firm
(STUPOUL) and in our local slaughter-house, respectively.

Delipidation of turkey pancreases. After defrosting, the pancreases were cut into small pieces
(1–2 cm²) and delipidated by the method described previously by Verger et al. After delipidation, 100 g of powder were obtained from 800 g of fresh tissue.

Lipase activity measurement. The lipase activity was measured titrimetrically at pH 8.5 and 37°C
using a pH stat (Metrohm, Switzerland), under the standard assay conditions described previously, using tributyrin (0.5 ml of tributyrin in 30 ml of 2.5 mM
Tris-HCl (pH 8.5), 100 mM NaCl, and 5 mM CaCl₂) or olive emulsion as substrate.

Phospholipase Activity measurement. The phospholipase A₂ activity was measured by potentiometric
titration with 0.1 M NaOH on a pH stat at 37°C and
pH 8 under the standard assay conditions described, using an aqueous emulsion of egg yolk in
the presence of 5 mM DOC and 7 mM Ca²⁺.

One unit of phospholipase activity was defined as
the activity that liberated one micromole of fatty acid
under these conditions.

Measurement of protein concentration. Protein
concentration was measured as described by
Bradford.

Analytical method. Analytical polyacrylamide gel
electrophoresis of protein in the presence of sodium
dodecyl sulfate was done by the method of Laemli,
samples for sequencing were electrophobotted
generously by Bergman and Jornvall, protein trans-
fer was done for 1 h 30 min at 1 mA/cm² and at room
temperature. The molecular mass of TPP was
estimated by SDS/PAGE.

Amino acid sequencing. The N-terminal part of
TPP was sequenced by automated Edmans degrada-
tion, using an Applied Biosystems Protein Sequencer
Procise 492/610A.

Results and Discussion

Level of TPP activity
In order to compare the level of TPP activity with
other species we measured under the same conditions
the rate of hydrolysis of egg yolk emulsion by sheep,
goat, cattle, and chicken pancreatic phospholipases

Table 1. Pancreas Level in Some Animals

<table>
<thead>
<tr>
<th>Species</th>
<th>U/g Fresh pancreases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey</td>
<td>150</td>
</tr>
<tr>
<td>Chicken</td>
<td>45</td>
</tr>
<tr>
<td>Cattle</td>
<td>12</td>
</tr>
<tr>
<td>Goat</td>
<td>7</td>
</tr>
<tr>
<td>Sheep</td>
<td>6</td>
</tr>
</tbody>
</table>

The measurement of the phospholipase content of pancreases from sever-
al species was performed in a homogenate prepared in a waring blender
(2 × 30 s) with 2 ml of 10M Tris-HCl, 1 mM benzamidine, pH 8 per gram of
fresh tissue. After centrifugation at 10000 rpm for 10 min, the amount of
enzyme was estimated on a sample of the supernatant using egg yolk emul-
sion as substrate in the presence of 7 mM Ca²⁺ and 5 mM DOC. The phos-
holphase activity was measured at pH 8 and 37°C using a pH stat. For each
animal, the experiment was done three times and no significant difference
was observed. The same results were obtained with a phosphatidyl choline
emulsion as substrate on the presence of 7 mM Ca²⁺ and 5 mM DOC.

U: One unit corresponds to 1 micromole of fatty acid liberated per
minute.

Fig. 1. Effects of Temperature on Enzyme Stability.

The homogenate was prepared as described in Table 1 and it
was incubated in 10 mM Tris-HCl, 1 mM benzamidine, pH 8, for
various times at 60°C. The remaining activity was measured
under the standard conditions. Sheep pancreatic phospholi-
pase; Cattle pancreatic phospholipase; Turkey pancreatic phospholipase.

(personal data). Results reported in Table 1 show
that turkey’s one had the highest level of phospholi-
pase activity.

Comparaison of heat stability
As shown in Fig. 1, a no significant difference in
enzyme thermal stability was observed between the
different species checked. The pancreatic enzymes of
turkey, sheep, and cattle were completely inactivated
by treatment for 1 h at 60°C. A similar result was
obtained with the partially purified TPP which was
eluted from Sephadex G-75 (data not shown).
**TPP purification**

Step 1: 100 g of delipidated powder of turkey pancreas were suspended in buffer A: (25 mM sodium acetate, 1 mM benzamidine, pH 6.4) and mixed mechanically twice for 30 s at room temperature using a waring blender system. The mixture was then stirred with a magnetic bar for 1 h at 4°C and then centrifuged for 20 min at 9000 rpm. The supernatant containing phospholipase activity (15000 U) was passed on to an anion exchange column (DEAE-cellulose). The TPP was not retained on the column gel. This step was used to eliminate the pigments from turkey pancreas.

Step 2: Ammonium sulfate fractionation. The supernatant fluid was mixed with solid (NH₄)₂SO₄ to reach 30% saturation. The resulting precipitate was removed by centrifugation at 9000 rpm for 30 min. The precipitate formed by the further addition of (NH₄)₂SO₄ to reach 60% saturation to the supernatant was collected and dissolved in buffer A. We obtained 12500 U.

Step 3: Acidic treatment. To inactivate the lipase, the supernatant was adjusted to pH 5 with 4 M HCl, and the insoluble material was removed by centrifugation (20 min at 9000 rpm). The supernatant was concentrated in an amicon cell to 45 ml.

Step 4: Filtration on Sephadex G-75. The supernatant obtained (4500 U) was passed on a Sephadex G-75 column (6 cm × 100 cm) using buffer A as the eluent at a flow rate of 60 ml/h. The fraction containing the TPP activity were pooled and lyophilized. We obtained 900 U.

Step 5: RP-HPLC on a nucleosil 100-5, C 18 column. Ten U of this phospholipase was put on a RP-HPLC nucleosil 100-5, C 18 column. A phospholipase activity was detected in a fraction eluted at 86% acetonitrile (data not shown). This fraction was re-injected under the same conditions in an RP-HPLC column. The chromatographic profile is presented in Fig. 2(A). The fraction containing the TPP was eluted at 90% of acetonitrile. As Fig. 2 shows, the peak of the TPP is very small. This is probably due to the poor yield of elution of the enzyme. Indeed, after injection of ten U of the pure TPP in RP-HPLC column (data not shown), we recovered less than one unit. This result suggests that the hydrophobic sites of the TPP interact strongly with the chromatographic support of the column C 18. Besides, the TPP is eluted only with one concentration, with acetonitrile of 90%. In the same way, a poor yield was obtained during the elution of the TPP of an exchanging column of ions (DEAE-cellulose) (data not shown). These results imply that the TPP strongly interacts with the chromatography supports. A very small peak of phospholipase A₂ from guinea pig pancreas was isolated by Tojo, H. et al., and a weak specific activity was found by these authors.

The results of SDS/PAGE analysis in Fig. 2(B) showed that the TPP had a single band corresponding to a molecular mass of 14 kDa. So the enzyme was found to be a monomeric protein like all phospholipases A₂ described so far.

The yield as well as the specific activity of the stages of purification are given in Table 2.

**Table 2. Summary of the Purification of Turkey Pancreatic Phospholipase A₂**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction</td>
<td>15000</td>
<td>48387</td>
<td>0.31</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation</td>
<td>14000</td>
<td>519</td>
<td>27</td>
<td>93</td>
<td>89</td>
</tr>
<tr>
<td>Acidic treatment</td>
<td>4500</td>
<td>80</td>
<td>56</td>
<td>36</td>
<td>179</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>900</td>
<td>5</td>
<td>167</td>
<td>20</td>
<td>539</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

**Effects of pH on TPP activity**

As shown in Fig. 3, the maximal enzyme activity was obtained at pH 8 when tested at 37°C using egg yolk emulsion as a substrate in the presence of 7 mM Ca²⁺ and 5 mM DOC.

**Effect of temperature on TPP activity**

The enzyme manifested its maximal activity at 37°C when it was tested between 25 and 45°C at pH 8.
Fig. 3. Effects of pH on TPP Activity.
The enzyme activity was tested at 37°C at various pHs using egg yolk emulsions as substrates in the presence of 7 mM Ca\textsuperscript{2+} and 5 mM DOC.

Fig. 4. Effects of the Concentration of Bilt Salt (DOC) and Ca\textsuperscript{2+} on PLA\textsubscript{2} Activity.
(A) Effects of the concentration of bilt salt (DOC) on PLA\textsubscript{2} activity. The enzyme activity was tested at 37°C at various concentrations of bile salt using egg yolk emulsions as the substrate in the presence of 7 mM Ca\textsuperscript{2+}. (B) Effects of the concentration of Ca\textsuperscript{2+} on PLA\textsubscript{2} activity. The enzyme activity was tested at 37°C at various concentrations of Ca\textsuperscript{2+} using egg yolk emulsions as the substrate in the presence of 5 mM DOC.

Table 3. The NH\textsubscript{2}-Terminal Amino Acids Sequence Alignment of Six Mammals PLA\textsubscript{2}.

<table>
<thead>
<tr>
<th>Species</th>
<th>NH\textsubscript{2}-terminal sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Turkey (this study)</td>
<td>A K E F R S M I K E T I P S E L</td>
</tr>
<tr>
<td>Pig (II)</td>
<td>A L Y F R S M I K E T P S E P L N</td>
</tr>
<tr>
<td>Cattle (I)</td>
<td>A L Y O F R S M I K E T I P S E P L L</td>
</tr>
<tr>
<td>Human (II)</td>
<td>A V Y O F R S M I K E T I G D S P E L</td>
</tr>
<tr>
<td>Rat (I)</td>
<td>A V Y O F R S M I K E T I G D S P E L R</td>
</tr>
<tr>
<td>Horse (II)</td>
<td>A V Y O F R S M I C T I P H S P Y L</td>
</tr>
</tbody>
</table>

DOC and Ca\textsuperscript{2+} were specially required for TPP activity.

de Haas et al.\textsuperscript{26} have reported that micellar solutions were hydrolyzed at a much higher rate than substrates molecularly dispersed by PLA\textsubscript{2}.\textsuperscript{27} As shown in Fig. 4(A), the maximal phospholipase activity of TPP was observed in the presence of 5 mM DOC. This result suggested that the bile salts prevent the accumulation of the products of hydrolysis on the water-phospholipids interface. This remark goes in the same direction as that of Evenberg et al.\textsuperscript{28} and Nieuwenhuizen et al.,\textsuperscript{29} who showed that the bile salts are tensioactive agents ensuring in micellar form, the dispersion of the products of hydrolysis. It was also shown that Ca\textsuperscript{2+} was specifically required for the phospholipase activity from porcine pancreases.\textsuperscript{26,28} In fact, Ca\textsuperscript{2+} is essential both for the binding of the substrate and for catalysis.\textsuperscript{12,21} In order to investigate the influence of Ca\textsuperscript{2+} on TPP activity, we measured the hydrolysis of egg yolk emulsion in the presence and absence of Ca\textsuperscript{2+}. A significant difference was observed when phospholipase activity was measured in the presence of 7 mM Ca\textsuperscript{2+} (Fig. 4(B)). These observations indicate that Ca\textsuperscript{2+} and bile salt were essential for TPP activity.

N-terminal sequence of TPP

The NH\textsubscript{2}-terminal sequencing of the blotted TPP allowed the unambiguous identification of the first twenty residues. Table 3 shows the alignment of the NH\textsubscript{2}-terminal of TPP with five mammalian PLA\textsubscript{2} derived respectively from Pig,\textsuperscript{32} Cattle,\textsuperscript{33} human,\textsuperscript{34} rat,\textsuperscript{32} and horse.\textsuperscript{32} TPP has a remarkable sequence homology with the other five mammalians PLA\textsubscript{2}.

Conclusion

TPP was isolated to electrophoretic purity from delipidated pancreases. The native enzyme is a monomer with a molecular mass of 14 kDa, and as with all mammals, PLA\textsubscript{2}. Ca\textsuperscript{2+}, and bile salts are essential for turkey pancreatic phospholipase activity. TPP has sequence homology with all mammal PLA\textsubscript{2}.

Acknowledgment

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References


