Purification and Characterization of Human Pancreatic Phospholipase A₂

Jarkko U. Eskola, Timo J. Nevalainen, and Heikki J. Aho

We purified human pancreatic phospholipase A₂ from postmortem pancreatic tissue by elution of the semi-purified enzyme on CM-Sephadex C-25 with a linear NaCl gradient at pH 6.0. The enzyme appeared as a single polypeptide chain with an isoelectric point of 9.2 ± 0.1. The relative molecular mass of the enzyme was estimated to be 15800 ± 1000 by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. The enzyme is resistant to heating and to a 25 g/L concentration of sodium dodecyl sulfate. It is inhibited by Ca²⁺ ions in the presence of ovoalbumin and deoxycholate. By immunohistochemical methods we showed the enzyme to be localized in the apical zymogen granule portion of pancreatic acinar cells.

Additional Keyphrases: cellular localization, relative molecular mass, stability, isoelectric point, pH optimum

Mammalian phospholipase A₂ (phosphatidic 2-acetylhyd- drase, EC 3.1.1.4) has been isolated in the pure form from porcine (1), ox (2), sheep (2), and horse (3) pancreas, and from rat pancreatic juice (4). The first purification study with use of postmortem human pancreas was that of Magee et al. (5). Its β-specificity was confirmed by Van Deneen et al. (6). The presence of the inactive precursor of phospholipase A₂ was first demonstrated in porcine pancreas by De Haas et al. (7) and later also in human pancreatic juice by Figarella et al. (8). Recently, human pancreatic phospholipase A₂ has been purified and characterized from pancreatic juice (9, 10). However, human pancreatic juice is very difficult to obtain in reasonably large quantities.

Because it was our aim to measure pancreatic phospholipase A₂ immunoreactivity in human serum (11), we purified phospholipase A₂ from human pancreatic tissue. The purpose of the present study was to characterize human secretory phospholipase A₂ purified from cadaver pancreas.

Materials and Methods

Pancreatic Tissue

Normal human pancreatic tissue was obtained at autopsy within 24 h of death. The material was used either immediately or after storage at −20 °C for one to two weeks.

Procedures

Purification of phospholipase A₂. The enzyme was purified according to the method described by Dutilh et al. (2) for ox and sheep pancreatic phospholipase A₂, with minor modifications. Briefly, it was as follows. Connective tissue, blood vessels, and fat were removed, and the tissue was homogenized immediately in ice-cold 0.15 mol/L NaCl solution, after which the homogenate was heated at 70 °C at pH 4.0 for 3 min. After rapid cooling to 5 °C the suspension was centrifuged (3500 × g, 5 min, 0 °C) and filtered through filter paper at 4 °C. The filtrate was stored at −20 °C until used for further purification.

The filtrate was concentrated by ammonium sulfate fractionation at pH 7.0. The proteins that precipitated between 0.35 and 0.75 saturation were isolated and dissolved in a minimal amount of distilled water. This concentrated solution was dialyzed against distilled water, then against acetate buffer (50 mmol/L, pH 6.0), and applied to a CM-Sephadex C-25 column equilibrated with the same buffer. The column was first washed free of anionic proteins, then eluted with a linear NaCl gradient. Fractions containing phospholipase A₂ activity were pooled, dialyzed against distilled water, and lyophilized. The protein content was measured from the absorbance at 280 nm, with crystalline bovine serum albumin as the standard.

Assay of phospholipase A₂ activity. Phospholipase A₂ activity was measured by potentiometric titration (TTT 80 Radiometer autotitrator) at 40 °C with 0.01–0.1 mol/L NaOH, with egg-yolk emulsion as the substrate as described by De Haas et al. (1) and modified by Figarella et al. (8).

During the purification procedure, phospholipase A₂ activity was measured in 10 mmol/L glycine–NaOH buffer at pH 9.0 containing 2 mmol of CaCl₂, 1.3 mmol of Triton X-100 surfactant, 6 mmol of deoxycholate, and 5.5 mmol of lecithin per liter.

The unit of activity is defined as the uptake of alkali in micro-equivalents per minute per milligram of protein.

Evaluation of phospholipase A₂ activity by thin-layer chromatography. Fifty micrograms of purified human pancreatic phospholipase A₂ was incubated in 500 μL of substrate solution containing, per liter, 10 mmol of l-α-phosphatidylcholine (Sigma no. P-5763), 1 mmol of CaCl₂, 6 mmol of deoxycholate, and 0.1 mol of glycine, at pH 8.0 and 40 °C, for 30 min. The enzyme (500 μL) was omitted from the control, which was treated in the same way as the reaction mixture. The hydrolysis was terminated by adding 560 μL of chloroform and 560 μL of methanol. The mixture was vortex-mixed and centrifuged for 5 min at 1000 × g. The organic phase was removed and evaporated under nitrogen, and the residue was taken up in 50 μL of chloroform and spotted on a silica gel plate (Silica gel G, layer thickness 0.25 mm, Merck). The developing solvent mixture was chloroform/methanol/water (65/25/4 by vol). The developed and dried plate was stained by exposure to iodine vapor. Linoleic acid (Sigma), l-α-lyso phosphatidylcholine (Sigma), and lecithin (Merck) were used as the standards.

Polyacrylamide gel electrophoresis. Electrophoresis was carried out as described by O'Farrell (12) or on Pharmacia polyacrylamide gradient gel PAA 4/30 in 2 g/L sodium dodecyl sulfate (SDS) according to the manufacturer's instructions. The Pharmacia "Low Molecular Weight Electrophoresis Calibration Kit" was used to prepare the calibration curve. The kit contained the following proteins: rabbit muscle phosphorylase b (M₀, 94 000), bovine serum albumin (67 000), egg-white ovalbumin (43 000), bovine erythrocyte carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100), and bovine milk β-lactalbumin (14 400). Porcine insulin (M₀, 5700; Nordisk Insulin Laboratorium, Copenhagen) was also used in some cases. Gels were stained with

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Coomassie Brilliant Blue 250 (Sigma) or PAGE Blue 83 (BDH Chemicals). The presence of carbohydrate was investigated by periodic acid/Schiff staining of polyacrylamide gels after electrophoresis (13).

Preparation of antiserum. Antiserum against human pancreatic phospholipase A₂ was raised in rabbits. The enzyme (0.3 mg) was homogeneously suspended in 1 mL of isotonic saline and 2 mL of Freund's complete adjuvant (Difco, Detroit, MI) and injected subcutaneously into rabbits at three-week intervals. The antiserum was monitored by Ouchterlony double diffusion. Sera were collected 10 days after booster injections.

Time-resolved fluoroimmunoassay. A sensitive time-resolved immunofluorometric assay was developed for the measurement of human pancreatic phospholipase A₂ immunoreactivity. Details of this method are presented in the accompanying paper (11).

Isoelectric focusing. For isoelectric focusing on agarose gel (Agarose IEF, Pharmacia Fine Chemicals) we used the Pharmacia flat-bed apparatus. The pH gradient was made with carrier ampholyte in the pH range from 9 to 11 (Ampholine carrier ampholyte, 200 g/kg; LKB Instruments Inc.) and in the pH range from 6.5 to 9 (Pharmalyte carrier ampholyte, Pharmacia Fine Chemicals). The pH gradient profile was determined by using the Pharmacia pH Calibration Kit and by measuring the pH of 10 sliced-gel fractions extracted with 1 mL of distilled water. Phospholipase A₂ was identified from thirty 0.3-cm-wide gel fractions by fluoroimmunoassay (11). The enzyme was extracted from the gel with Tris HCl buffer (50 mmol/L, pH 7.4) containing 9 g of NaCl and 0.5 g of NaN₃ per liter. One part of the gel was stained with PAGE Blue 83.

"High-performance" ion-exchange chromatography. The instrument (Water Associates Inc.) we used consisted of the following components: Model 450 variable-wavelength detector (280 nm), Model 660 solvent programmer, Model 6000A solvent delivery systems, HP 3388A integrator terminal, and an LKB 2133 Ultra-Pac TSK 535 CM column, 7.5 × 150 mm. The eluent contained 0.1 mol of sodium acetate (pH 5.1) and 0.1 mol of NaCl per liter. A gradient from 0.1 to 1.0 mol/L NaCl in sodium acetate (0.1 mol/L, pH 5.1) was used. The flow rate was 0.8 mL/min. Samples were dissolved in the eluent buffer, injected in 20-μL batches, and collected in 0.8-mL fractions.

Immunolocalization in pancreatic tissue. Phospholipase A₂ immunoreactivity was localized in formalin-fixed, paraffin-embedded normal human pancreas by use of the rabbit anti-phospholipase A₂ serum prepared as described above and by the peroxidase-anti-peroxidase (PAP) technique of Sternberger (14). The staining procedures are detailed elsewhere (15).

Results

Purification of Phospholipase A₂

Human pancreatic phospholipase A₂ was obtained in pure form after CM-Sephadex chromatography. As shown in Figure 1, all phospholipase A₂ activity was accounted for by one symmetrical peak. Table 1 summarizes the analytic recoveries and specific activities at the different steps of purification.

Criteria of Homogeneity

With or without reduction, the enzyme behaved like a homogeneous protein during SDS-polyacrylamide gel electrophoresis (Figure 2). No carbohydrate was detected by periodic acid/Schiff staining of polyacrylamide gels after electrophoresis. The enzyme preparation was also analyzed by ion-exchange chromatography. Only one major peak was seen, which contained all the immunoreactivity as measured by time-resolved fluoroimmunoassay (Figure 3).

Effect of Reduction on the Electrophoretic Mobility of Phospholipase A₂

The mobility of phospholipase A₂ on SDS-polyacrylamide gel electrophoresis changed dramatically when the protein was reduced. As can be seen in Figure 2, without reduction the protein moves slower, and after reduction faster, than
Table 1. Purification of Phospholipase A₂ from Human Pancreas*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein, mg</th>
<th>Total acty, units</th>
<th>Recovery, %</th>
<th>Specific acty</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat treatment</td>
<td>52000</td>
<td>7644</td>
<td>100</td>
<td>0.15</td>
<td>1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1130</td>
<td>3910</td>
<td>51</td>
<td>3.46</td>
<td>24</td>
</tr>
<tr>
<td>CM-Sephadex C-25</td>
<td>70</td>
<td>3730</td>
<td>49</td>
<td>53.3</td>
<td>383</td>
</tr>
<tr>
<td>Lyophilization</td>
<td>30</td>
<td>730</td>
<td>23</td>
<td>57.8</td>
<td>393</td>
</tr>
</tbody>
</table>

* Eleven cadaver pancreases (total weight, 561 g) were used as the starting material. Enzyme activity was measured in 10 mmol/L glycine–NaOH buffer at pH 9.0 containing, per liter, 2 mmol of CaCl₂, 1.3 mmol of Triton X-100, 6 mmol of deoxycholate, and 5.5 mmol of lecithin.

the soybean trypsin inhibitor. The difference in mobility seems to depend on the failure of 25 g/L SDS to denature the protein under normal experimental conditions. Prolonged heating at 100 °C, however, gradually denatured the protein and changed its mobility. Because no other protein bands appeared, the enzyme seems to be composed of only one polypeptide chain.

Phospholipase A₂ Activity

Phospholipase A₂ activity was measured by the "pH-stat" titration method. Almost fourfold higher specific activity was found in the assay system when purified egg lecithin was replaced by egg-yolk emulsion. The enzyme activity was linearly proportional to the amount of added enzyme in both assay systems. When purified egg-yolk phosphatidylcholine was hydrolyzed by our enzyme and the hydrolysis products were analyzed by thin-layer chromatography on silica gel, it was evident that the enzyme hydrolyzed phosphatidylcholine into fatty acids and lysophosphatidylcholine.

The effect of pH on phospholipase A₂ activity was examined by pH-stat titration on egg-yolk emulsion. The optimum pH was 8.5.

The effect of CaCl₂ on the enzyme activity was examined by pH-stat titration of egg-yolk emulsion containing 6 mmol of deoxycholate per liter. At low concentrations, CaCl₂ seems to activate phospholipase A₂; activation was maximum at 1 mmol of CaCl₂ per liter. Further addition of CaCl₂ inhibits the enzyme activity: at 10 mmol of CaCl₂ per liter, only 26% of the maximum enzymatic activity was present. In the presence of 2 mmol of EDTA or 50 mmol of phosphate per liter, the enzyme activity was completely inhibited.

Relative Molecular Mass (Mₑ) Isoelectric Point

After repeated trials we found that human pancreatic phospholipase A₂ could not be stained on agarose gel after isoelectric focusing. Fixing and staining procedures recommended by Pharmacia did not denature the protein, and it diffused out of the gel. Therefore we measured phospholipase A₂ from sliced gel fractions after focusing. The isoelectric point was graphically estimated to be 9.2 ± 0.1 (Figure 4).

The molecular mass of the enzyme was estimated to be about 15 800 ± 1000 Da, based on SDS-polyacrylamide gel electrophoresis (Figure 5). Without reduction, the apparent molecular mass was 21 000 ± 1000 Da.

Immunolocalization in Pancreatic Tissue

Antiserum was raised in rabbits against the purified enzyme. Using this antiserum and the PAP technique, the enzyme was shown to be located in normal human pancreas in the apical zymogen granule portion of acinar cells (Figure 6). The centroacinar cells, ductal epithelium, blood-vessel walls, connective tissue, nerves, and islets of Langerhans were not stained. Ductal secretory material was weakly positive. Control serum taken before immunization gave negative staining.

Discussion

In the present investigation, phospholipase A₂ was purified from human postmortem pancreas. The recovery after purification on CM-Sephadex C-25 was 49%, which is similar to that for previous purification procedures of mammali-
an pancreatic tissues (2, 7) and human pancreatic juice (9). No difference was observed in phospholipase A₂ activity between samples extracted from fresh and frozen-stored tissue.

Pancreatic phospholipase A₂ is very resistant to heating and denaturing agents (1, 15, 16). Human pancreatic phospholipase A₂ tolerates boiling in the presence of 25 g/L SDS for at least 10 min, and even boiling for 45 min in the presence of 25 g/L SDS only partly denatures the enzyme, as shown by SDS-polyacrylamide gel electrophoresis.

In good agreement with earlier observations (5, 9, 17–19), human pancreatic phospholipase A₂ was found to be inhibited by Ca²⁺ ions in the assay system containing ovolecthin and deoxycholate. This is the reverse of (e.g.) porcine pancreatic phospholipase A₂, which is highly stimulated by the simultaneous presence of Ca²⁺ and deoxycholate (1).

The optimum pH of human pancreatic phospholipase A₂ was about 8.5, which is similar to that reported earlier (5, 9, 17, 18). The relative molecular mass of human pancreatic phospholipase A₂ and of phospholipase A₂ have been estimated earlier by SDS-polyacrylamide gel electrophoresis to be 14 500 and 14 000 ± 1000, respectively (9). The relative molecular mass of human zymogen as calculated from amino acid composition is 13 634 (9). In the present study, the molecular mass of human pancreatic phospholipase A₂ was estimated to be 15 800 ± 1000 Da by SDS-polyacrylamide gel electrophoresis.

We showed by thin-layer chromatography that the purified enzyme hydrolyzes lecithin to lysolecithin and fatty acids. The present enzyme resembles that purified by Magee et al. (5), which has been shown to be β-specific (6). Further, as shown immunohistochemically, the enzyme is present only in the apical zymogen granule portion of acinar cells. This enzyme is obviously the secretory pancreatic phospholipase A₂, which has been purified and characterized from human pancreatic juice by Grataroli et al. (9).

Human pancreatic phospholipase A₂ is characterized by its unusually high isoelectric point. Dutilh et al. (2) reported isoelectric points of 6.4 and 7.6 for ox, 6.0 for sheep, and 6.3 for pig pancreatic phospholipases A₂. Grataroli et al. (9) reported pl 8.2 for the human pancreatic pro phospholipase A₂. Our result, pl 9.2 ± 0.1 for the active enzyme, is in good agreement with the observation made on porcine phospholipase A₂ (7). In the pig, the change in isoelectric point from 6.5 to 7.4 upon conversion of thezymogen into phospholipase A₂ indicates that the activation peptide is acidic (7). The isoelectric points of a number of other phospholipases are also very high. The venoms of Vipera berus, Agkistrodon halys blomhoffii, and Vipera russelli reportedly have isoelectric points of 9.2 (20), 10.0 (21), and 9.9 (22), respectively. The isoelectric point of phospholipase A₂ purified from rabbit inflammatory exudate is >10.5 (23). The isoelectric point of 8.2-bungarotoxin (Mr, 21 000), which consists of two peptide chains (Mr, 13 000 and 7000), is about 5.5 (24).

A specific antiserum was raised in the rabbit against purified human pancreatic phospholipase A₂ in the present study. The use of the antiserum for the quantitation of phospholipase A₂ immunoreactivity in human sera by time-resolved fluoroimmunoassay is described in the accompanying paper.

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References

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**Fig. 5.** Relative molecular mass determination of human pancreatic phospholipase A₂ by SDS-polyacrylamide gel electrophoresis: a typical experiment

Electrophoresis was on Pharmacia PAA 4.30 gradient gel (8 × 8 × 0.27 cm) in 2 g/L SDS. Electrophoresis buffer: 40 mmol of Tris, 20 mmol of sodium acetate, pH 7.4, with 2 mmol of EDTA and 2 g of SDS per liter. Sample buffer: 10 mmol of Tris HCl, pH 6.0, 1 mmol of EDTA, with 25 g of SDS, per liter, with or without 50 g of dibromochloropetetol. Sample heated in the sample buffer at 100 °C for 5 min. Electrophoresis conditions: pre-electrophoresis 70 V for 1 h, run-in 300 V for 10 min, run 150 V for 2.5 h. PLA₂, phospholipase A₂

**Fig. 6.** Immunoreactive phospholipase A₂ in the apical portion of acinar cells in normal human pancreatic tissue (×640)

Peroxidase–antiperoxidase method