Phospholipase A₂ in human ascitic fluid

Purification, characterization and immunochemical detection

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A phospholipase A₂ (PLA₂, EC 3.1.1.4) was purified from human cell-free ascitic fluid (a-PLA₂) by ion-exchange chromatography and h.p.l.c. on a reverse-phase column to apparent homogeneity. The enzyme had an $M_r$ of approx. 10000 as determined by SDS/PAGE. Polyclonal antibodies raised in a rabbit were specific to a-PLA₂, as judged by immunoblotting. A time-resolved fluoroimmunoassay (TR-FIA) for measuring the concentration of a-PLA₂ in various body fluids was developed. The detection limit of the assay was about 6 ng/ml. The antisera did not cross-react with pancreatic secretory phospholipase A₂ as measured by TR-FIA. The enzyme content was studied in various samples, including normal human serum, buffy-coat leucocytes, synovial fluid, and pancreas and spleen homogenates.

INTRODUCTION

High levels of soluble phospholipase A₂ (PLA₂) have been detected at sites of inflammation in numerous animal models. ARDS (the adult respiratory-distress syndrome), acute pancreatitis, septic shock and inflammatory arthritis have been associated with elevated levels of endogenous secretory PLA₂ (Weiss et al., 1978; Nevalainen, 1980; Vadas, 1984).

Several studies have examined soluble PLA₂s from polymorphonuclear leucocytes.Soluble enzymes stored in specific granules of polymorphs and lysosomes of phagocytes are released into the extracellular space in response to phagocytic, antigenic or chemoattractant stimuli (Vadas & Hay, 1980; Traynor & Authii, 1981; Lanni & Becker, 1983; Balsinde, et al., 1988). PLA₂ provides substrates for the synthesis of potent mediators of inflammation, including prostaglandins, leukotrienes and the platelet-activating factor (Pruzanski & Vadas, 1991). PLA₂ is thus a key enzyme in the pathogenesis of inflammatory disease. PLA₂ purified from rabbit granulocytes has been localized by immunocytochemical methods in rabbit phagocytes (Namba et al., 1983). The physical state of PLA₂s stored in lysosomal granules (soluble or membrane-bound) still remains unclear (Franson et al., 1974, 1977; Alonso et al., 1986; Gonzalbes-Buritica et al., 1989a,b). Adsorption of soluble PLA₂ to phospholipid membranes complicates the interpretation of subcellular localization (Vadas & Pruzanski, 1986). It has been reported that the enzyme becomes membrane-associated upon cell activation, which facilitates the hydrolysis of membrane-bound substrates (Channon & Leslie, 1990). Comparison of the structural and functional properties of PLA₂s purified from polymorphonuclear leucocytes, cell-free ascitic fluid and serum shows that all three enzymes are very closely related, which suggests that serum may be the source of PLA₂ in inflammatory exudate (Wright et al., 1990; Franson et al., 1974, 1978). PLA₂ has also been isolated from human synovial fluid and platelets, and the enzyme is identical in both sources (Stefanski et al., 1986; Hara et al., 1989; Seilhamer et al., 1989).

The purpose of the present study was to isolate human ascitic-fluid PLA₂ (a-PLA₂). A polyclonal (rabbit) antiserum was also prepared, and a time-resolved fluoroimmunoassay (TR-FIA) was developed for measuring the content of a-PLA₂ in various body fluids.

EXPERIMENTAL

Materials

Dextran T 500, CM-Sepharose CL 6B and activated CH-Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden). $[^14]C$oleic acid (57 mCi/mmol) and L-$\alpha$-dipalmitoyl-[2-palmitoyl-9,10-3H(n)]phosphatidylcholine (50 Ci/mmol) were from NEN (Boston, MA, U.S.A.), and 1-[14C]palmitoyl-$\alpha$-lyso-3-phosphatidylcholine (59 mCi/mmol) was from Amersham International (Amersham, Bucks, U.K.). Freund's adjuvant and fatty-acid-free BSA were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Calibration kits for determination of $M_r$ values of proteins were from Pharmacia. Assay buffer and Eu-chelate for TR-FIA measurements were from Wallac (Turku, Finland), and microtitre plates were from Eflab (Helsinki, Finland).

Ascitic fluid was collected from hospitalized patients suffering from ovarian carcinoma and peri toneal carcinoma. Human pancreatic phospholipase A₂ (p-PLA₂) was purified as described by Eskola et al. (1983a).

Purification of PLA₂ from human ascitic fluid

Ion-exchange chromatography of cell-free ascitic fluid was performed on a CM-Sepharose CL 6B column (2.4 cm x 40 cm) equilibrated with 2.5 mM-Tris/HCl, pH 7.4, containing 0.15 M-NaCl. Anionic proteins were eluted with the same buffer, and PLA₂ was eluted with buffered 2 M-NaCl. The elution was monitored by the $A_{280}$. PLA₂-containing fractions were pooled, trifluoroacetic acid (TFA) was added to 0.1% (v/v), and the fractions were subjected to h.p.l.c. on a reverse-phase column (Aqua pore Octyl RP 300; 4.6 mm x 220 mm; Brownlee Laboratory, Santa Clara, CA, U.S.A.). The column was equilibrated with 0.1% TFA, and PLA₂ was eluted with increasing amounts of 95% (v/v) acetonitrile in 0.1% TFA (solv. B), from 0 to 15% of soln. B during 5 min and from 15 to 50% of soln. B during 90 min at a flow rate of 1 ml/min. PLA₂ activities were eluted at about 30% concentrations of soln. B.

The h.p.l.c. system consisted of two Kontron model T414 LC pumps, a Kontron M 800 mixer (Kontron, London, U.K.) and a Rheodyne sampler injector (model 7126; Rhodyne Inc., Catati, CA, U.S.A.). The program for the gradient elution was controlled by a Kontron model 200 analytical programmer.

Abbreviations used: PLA₂, phospholipase A₂; a-PLA₂, ascitic-fluid PLA₂; p-PLA₂, pancreatic secretory PLA₂; TFA, trifluoroacetic acid; TR-FIA, time-resolved fluoroimmunoassay.
PLA₂ assays

Catalytic activity was measured by using either *Escherichia coli* (K12 strain) labelled with [14C]oleic acid (Patriarca et al., 1972) or a mixture of 2-[3H]palmitoyl-phosphatidylcholine and unlabelled dipalmitoyl-phosphatidylcholine as a substrate (Schädlitch et al., 1987). 1-[14C]Palmitoyl-lyso-phosphatidylcholine served as a substrate in the assay for lysophospholipase activity. *E. coli* was labelled as described by Patriarca et al. (1972) and used in the routine method for the determination of PLA₂ activity in the chromatographic steps. The reaction mixture consisted of 100 μl of autoclaved [14C]-labelled *E. coli* suspension (5000 c.p.m.) in 100 mM-Tris/HCl (pH 7) buffer containing 1 mM-CaCl₂ and 10 μl of sample to be measured. To determine the pH-dependence of activity, 100 mM-acetate (pH 3.0–6.0), -Tris/HCl (pH 7–9) and -glucose/NaOH (pH 9–10) buffers were used. The incubation time was 30 min and temperature 37 °C. The reaction was terminated by adding 200 μl of 2 M-HCl, followed by 200 μl of BSA solution (20 mg/ml). After cooling at 4 °C for 30 min, the samples were centrifuged at 9000 g for 5 min. The supernatant was mixed with 4 ml of Optiphase Hisafe II scintillation liquid (Wallac), and radioactivity was measured in a liquid-scintillation counter (1209 Rackbeta; Wallac). Enzyme activities were corrected for non-enzymic hydrolysis and expressed as percentages of the total *E. coli* lipid radioactivity.

Immunoreactive PLA₂ was measured by TR-FIA. Microtitre plates were coated overnight with affinity-purified antiserum raised against a-PLA₂ (10 μg/ml; 20 μl/well). The coating solution was prepared by incubating the antiserum with 3 vol. of HCl/water (154 μl of 11.6 M-HCl in 50 ml of water) for 5 min and diluted to 10 μg/ml with 50 mM-Tris/HCl buffer (pH 7.75)/0.15 mM-NaCl/0.05% NaN₃. After six washes, a-PLA₂ standard or unknown sample (25 μl) was added with 175 μl of assay buffer. After incubation and washing, 200 μl (1 μg/ml) of affinity-purified antiserum labelled with Eu-chelate, as described by Eskola et al. (1983b), was added and plates were incubated for 1 h. Fluorescence was measured in an Arcus fluorimeter (Wallac). The concentration of p-PLA₂ was measured by TR-FIA as described by Eskola et al. (1983b).

Preparation of antiserum

A New Zealand White rabbit was obtained from the Froxfield Farms (Hants., U.K.) and immunized 5 times subcutaneously at 4-week intervals with 100 μg of purified a-PLA₂ in Freund’s adjuvant. Serum was collected 2 weeks after the last booster injection. The antiserum was affinity-purified with a-PLA₂ coupled to activated CM-Sepharose 4B (Pharmacia) in accordance with the manufacturer’s instructions. Immunoglobulins specific to a-PLA₂ were eluted with 7 M-NaSCN in 50 mM-phosphate buffer, pH 7.3.

Electrophoresis and immunoblotting

Electrophoresis was performed with the Pharmacia PhastSystem with PhastGel gradient polyacrylamide gels (8–25%) for immunoblotting experiments and PhastGel High Density gels for estimation of *Mₚ*. Protein bands were made visible by Coomassie Blue staining. Proteins were transferred to nitrocellulose filters (Millipore, Molsheim, France) with the PhastSystem semi-dry immunoblotting device in accordance with the manufacturer’s directions. The Vectastain ABC-kit (Vector Laboratories Inc., Burlingame, CA, USA) was used for immunostaining of the nitrocellulose filters.

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951), with BSA as a standard.

RESULTS

Purification and characterization of human ascitic-fluid PLA₂

As the first purification step, ascitic fluid was applied to a CM-Sepharose CL 6B column for ion-exchange chromatography. More than 95% of the total protein and part of the PLA₂ activity (26%) and immunoreactivity (2.5%) were eluted in the flow-through fraction. A single peak of PLA₂ activity was eluted with buffered 2 M-NaCl (Fig. 1). The PLA₂-containing pool was further purified on a reverse-phase column h.p.l.c. The PLA₂ activity was eluted as a single peak at approx. 30% acetonitrile concentration (Fig. 2).

The h.p.l.c. fraction was judged to be homogeneous. The apparent *Mₚ* of the enzyme was estimated to be 10000 by SDS/PAGE (Fig. 3a) and by h.p.l.c. on a TSK G 3000 gel-filtration column (results not shown). The catalytic activity of the purified a-PLA₂ was optimal between pH 6 and 9. The enzyme was Ca²⁺-dependent (optimum at 1 mM-CaCl₂). No activity was detected in the presence of 1 mM-EDTA. The positional specificity of the enzyme was tested by using 1-[14C]palmitoyl-L-lysophosphatidylcholine as a substrate. Radioactive fatty acids were not released during the incubation.

The activity of the product increased to 260-fold that of the starting material. The final yield was about 300 μg of a-PLA₂, representing a recovery about 15% of total PLA₂ activity (Table 1).

Characterization of antibody

The antibody raised against a-PLA₂ was tested for specificity by TR-FIA and immunoblotting. Positive reaction on immunoblotting of ascitic fluid was found with several protein bands.
Table 1. Purification of human ascitic PLA₂

Purification was started with 15 ml of ascitic fluid. Catalytic activity of PLA₂ was measured by using a mixture of 2-[³H]palmitoyl-phosphatidylcholine and unlabelled dipalmitoyl-phosphatidylcholine as substrate as described by Schädlich et al. (1987).

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (nmol of substrate hydrolysed/ min)</th>
<th>Recovery (%)</th>
<th>Specific activity (nmol of substrate hydrolysed/ min per mg of protein)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascitic fluid</td>
<td>555</td>
<td>76.6</td>
<td>100</td>
<td>0.138</td>
<td>1</td>
</tr>
<tr>
<td>CM-Sepharose CL 6B</td>
<td>0.613</td>
<td>12.7</td>
<td>16.6</td>
<td>14.2</td>
<td>102</td>
</tr>
<tr>
<td>H.p.l.c.</td>
<td>0.323</td>
<td>11.6</td>
<td>15.2</td>
<td>36.1</td>
<td>260</td>
</tr>
</tbody>
</table>

DISCUSSION

The present results show that PLA₂ in the ascitic fluid of patients suffering from ovarian carcinoma and peritoneal carcinosis is a cationic protein. It is somewhat smaller than other PLA₂s of inflammatory exudates, M₉₁₀₀₀₀. PLA₂ subunits in some snake venoms have similar M₉ values to a-PLA₂ (Cate &
Table 2. Determination of the contents of a-PLA$_2$ and p-PLA$_2$ in body fluids from various sources

Tissue homogenates were prepared as described in the Experimental section. Protein concentrations of pancreas, spleen and leucocyte homogenates were 26, 17 and 0.15 mg/ml respectively. Concentrations of a-PLA$_2$ and p-PLA$_2$ were measured by TR-FIA as described in the Experimental section.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Conc. of PLA$_2$ as determined by TR-FIA (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a-PLA$_2$</td>
</tr>
<tr>
<td>Plasma (n = 9)</td>
<td>42.040 (s.d. 23.310)</td>
</tr>
<tr>
<td>Serum (n = 17)</td>
<td>68.170 (s.d. 24.000)</td>
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<tr>
<td>Ascitic fluid (n = 1)</td>
<td>49.650</td>
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<tr>
<td>Synovial fluid (n = 6)</td>
<td>23.765 (s.d. 15.330)</td>
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<td>Pancreatic juice (n = 1)</td>
<td>14.3</td>
</tr>
<tr>
<td>Pancreatic homogenate (n = 1)</td>
<td>11.4</td>
</tr>
<tr>
<td>Spleen homogenate (n = 1)</td>
<td>2.3</td>
</tr>
<tr>
<td>Leucocyte homogenate</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* Eskola et al. (1983b).

Bieber, 1978; Kondo et al., 1982). The purified enzyme does not hydrolyse 1-acyl-lysocephospholipid. In the presence of 2-[H]-palmitoyl-phosphatidylcholine radioactivity was recovered as non-esterified fatty acids. The a-PLA$_2$, therefore, seemed specific for the fatty acid at position 2. The enzyme was purified 260-fold to apparent homogeneity as judged by SDS/PAGE. Characteristics of the purified enzyme (pH optimum, Ca$^{2+}$ requirement) are similar to other non-pancreatic PLA$_2$s purified previously (Franson et al., 1978; Forst et al., 1986; Chang et al., 1987).

We investigated the distribution and the content of a-PLA$_2$ in body fluids from various sources by TR-FIA. The enzyme was not found in homogenates of pancreas or spleen, but the concentrations found in normal human serum, ascitic fluid and synovial fluid were relatively high. The concentration of a-PLA$_2$ in normal human serum is approx. 68 µg/ml (the present work), whereas that of p-PLA$_2$ is 6.5 ng/ml (Eskola et al., 1983b). Murakami et al. (1990) determined the concentration of the platelet-type PLA$_2$ by an enzyme immunoassy in rat serum to be about 1 µg/ml. Even in acute pancreatitis the concentration of p-PLA$_2$ increases, on average, only to 50 ng/ml (Nevalainen et al., 1985). Obviously, the source of a-PLA$_2$ (unknown at present) must be quite abundant.

The polyclonal antisera raised against a-PLA$_2$ showed specific reactivity towards its antigen, as determined by immunoblotting. Purified a-PLA$_2$ gave a single band in the immunoblotting. Several bands were found by immunoblotting in cell-free ascitic fluid. Two major bands were of M, 10000 and around 67000, and there were three minor bands, of M, 30000, 43000 and over 94000. These bands did not appear after immunostaining with pre-immune serum or with antisera previously incubated with an excess of antigen. One possibility is that these proteins are isoenzymes of a-PLA$_2$. Another explanation is that PLA$_2$ molecules aggregate in the absence of high salt concentrations (Franson et al., 1978). High-M, PLA$_2$s have been purified from a macrophage cell line (Leslie et al., 1988) and from human and sheep platelets (Apitz-Castro et al., 1979; Loeb & Guss, 1986). PLA$_2$ may also be associated with high-M, proteins present in ascitic fluid.

Several mammalian non-pancreatic PLA$_2$s have been purified. However, it is at present unclear which enzymes are relevant in inflammatory diseases. Inflammatory exudate contains at least two distinct PLA$_2$ activities: membrane-associated and soluble. They differ in their substrate specificity (Gonzales-Buritica et al., 1989a,b; Selhamer et al., 1989). The presence of various forms of PLA$_2$ in inflammatory exudates raises the possibility that each may have a different function in the inflammatory process. Their cellular sources remain still uncertain. It has been suggested that platelets may be the source of PLA$_2$ in synovial fluid and inflammatory exudate (Kramer et al., 1989). However, there is evidence that human platelets do not secrete PLA$_2$ upon stimulation (Harra et al., 1989). We have determined the concentration of a-PLA$_2$ in both plasma and serum samples from the same healthy individuals and found no difference. This result indicates that a-PLA$_2$ is not released by platelets. It is well known that PLA$_2$ is present in rabbit and human polymorphonuclear leucocytes (Elsbach et al., 1979; Forst et al., 1986; Mäkäräinen et al., 1986; Wright et al., 1990). On the other hand, the PLA$_2$ activity of inflammatory exudate does not correlate with white-cell count (Pruzzanski et al., 1985). However, recently Loezer et al. (1990) reported a positive correlation between arachidonic acid release and white-cell count in synovial fluid of patients with rheumatoid arthritis. Wright et al. (1990) suggest that polymorphonuclear leucocytes might be a minor additional source of inflammatory-fluid PLA$_2$. Our results also show that PLA$_2$ released from leucocytes represents less than 1% of a-PLA$_2$ present in ascitic fluid or serum.

We have ascertained in the present paper that the PLA$_2$ found in the ascitic fluid of patients suffering from ovarian carcinoma and peritoneal carcinoma is not of pancreatic origin.

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REFERENCES

Ascitic-fluid phospholipase A₂


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