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 antiserum 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 & Authi, 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; Gonzales-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). [¹⁴C]Oleic acid (57 mCi/mmol) and L- α -dipalmitoyl-[2-palmitoyl-9,10-³H(n)]phosphatidylcholine (50 Ci/mmol) were from NEN (Boston, MA, U.S.A.), and 1-[¹⁴C]palmitoyl-L-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 peritoneal carcinosis. 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 × 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 A₂₈₀. 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 (Aquapore Octyl RP 300; 4.6 mm × 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 (soln. 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; Rheodyne 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 (Schädlich *et al.*, 1987). 1-[¹⁴C]Palmitoyl-lysophosphatidylcholine 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 -glycine/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; 200 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 M-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 fluorometer (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 CH-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 Phast-System with PhastGel gradient polyacrylamide gels (8–25 %) for immunoblotting experiments and PhastGel High Density gels for estimation of *M_r*. 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.

Fig. 1. Ion-exchange chromatography of ascitic fluid

Elution profiles of protein (A_{280} , ○) and the catalytic activity of PLA₂ (●) from the CM-Sepharose CL 6B column. The volume of each fraction is 3.5 ml. PLA₂ was eluted in fractions 79–84. PLA₂ activity is expressed as percentage of the total *E. coli* lipid radioactivity.

Preparation of tissue homogenates

The homogenates of human pancreas and spleen were prepared by a method described for the purification of human p-PLA₂ (Eskola *et al.*, 1983a).

Leucocytes were separated from 40 ml of buffy coat, representing 450 ml of blood. Erythrocytes were sedimented with 1.3 % (w/v) dextran at room temperature. The leucocyte-rich supernatant was centrifuged at 300 *g* for 10 min. Isolated leucocytes were disrupted as described by Balsinde *et al.* (1988).

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 *M_r* 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-Ca²⁺). 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-lyso-3-phosphatidylcholine 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.

Fig. 2. Purification of PLA₂ by h.p.l.c.
Elution profiles of protein (○) and PLA₂ activity (●) from the reverse-phase column on h.p.l.c. The volume of each fraction is 2 ml. The broken line indicates the concentration gradient of eluent B from 0 to 50% in 95 min. PLA₂ was eluted in fractions 18–24.

Fig. 4. Standard curve for determination of a-PLA₂ by TR-FIA
Purified a-PLA₂ was used as a standard. The assay was performed as described in the Experimental section.

Fig. 3. SDS/PAGE (a) and immunoblotting (b) of a-PLA₂ and ascitic fluid
(a) SDS/PAGE was carried out as described in the Experimental section. Lane 1, *M_r* standards (14400–94000); lane 2, ascitic fluid; lane 3, a-PLA₂ after h.p.l.c.; lane 4, *M_r* standards (2500–17000). Migration of *M_r* standard proteins is indicated in the left and right margins. (b) Detection of a-PLA₂ by immunoblotting performed as described in the Experimental section. Lane 1, ascitic fluid; lane 2, a-PLA₂.

with purified a-PLA₂ (Fig. 3). It did not cross-react with PLA₂ in human pancreas or spleen homogenates as measured by TR-FIA (Table 2).

Ascitic PLA₂ content in body fluids as determined by TR-FIA

A sandwich TR-FIA method was developed for measurement of the a-PLA₂ concentration. A typical curve is shown in Fig. 4. The minimal amount of a-PLA₂ detectable with this method is approx. 6 ng/ml. The concentrations of a-PLA₂ in fluids from several sources as determined by TR-FIA are given in Table 2. The concentration of a-PLA₂ in normal human serum increased after repeated freezing and thawing. The PLA₂ content of fresh serum was 19 µg/ml on average, and that of frozen and thawed serum was 68 µg/ml. No difference was found in the content of a-PLA₂ between plasma and serum samples from the same healthy individuals. The concentration of a-PLA₂ in leucocyte homogenate was about 5 ng/ml. Leucocytes from 450 ml of blood gave in total about 40 ng of a-PLA₂.

DISCUSSION

The present results show that PLA₂ in the ascitic fluid of patients suffering from ovarian carcinoma and peritoneal carcinoma is a cationic protein. It is somewhat smaller than other PLA₂s of inflammatory exudates, *M_r* 10000. PLA₂ subunits in some snake venoms have similar *M_r* values to a-PLA₂ (Cate &

The antiserum revealed two major bands of apparent *M_r* around 67000 and 10000 and three minor bands of *M_r* over 94000, about 43000 and 30000. The antiserum detected a single protein band

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).

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

Table 2. Determination of the contents of a-PLA₂ and p-PLA₂ 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-PLA₂ and p-PLA₂ were measured by TR-FIA as described in the Experimental section.

Sample	Concn. of PLA ₂ as determined by TR-FIA (ng/ml)	
	a-PLA ₂	p-PLA ₂
Plasma (n = 9)	42040 (s.d. 23310)	2.2 (s.d. 0.8)
Serum (n = 17)	68170 (s.d. 24000)	6.5 (s.d. 2.0)*
Ascitic fluid (n = 1)	49650	2.9
Synovial fluid (n = 6)	23765 (s.d. 15330)	2.2 (s.d. 0.8)
Pancreatic juice (n = 1)	14.3	6590
Pancreatic homogenate (n = 1)	11.4	54385
Spleen homogenate (n = 1)	2.3	3.8
Leucocyte homogenate	5.0	—

* Eskola *et al.* (1983b).

Bieber, 1978; Kondo *et al.*, 1982). The purified enzyme does not hydrolyse 1-acyl-lysophospholipid. In the presence of 2-[³H]-palmitoyl-phosphatidylcholine radioactivity was recovered as non-esterified fatty acids. The a-PLA₂ 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, Ca²⁺ requirement) are similar to other non-pancreatic PLA₂s purified previously (Franson *et al.*, 1978; Forst *et al.*, 1986; Chang *et al.*, 1987).

We investigated the distribution and the content of a-PLA₂ 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-PLA₂ in normal human serum is approx. 68 µg/ml (the present work), whereas that of p-PLA₂ is 6.5 ng/ml (Eskola *et al.*, 1983b). Murakami *et al.* (1990) determined the concentration of the platelet-type PLA₂ by an enzyme immunoassay in rat serum to be about 1 µg/ml. Even in acute pancreatitis the concentration of p-PLA₂ increases, on average, only to 50 ng/ml (Nevalainen *et al.*, 1985). Obviously, the source of a-PLA₂ (unknown at present) must be quite abundant.

The polyclonal antiserum raised against a-PLA₂ showed specific reactivity towards its antigen, as determined by immunoblotting. Purified a-PLA₂ gave a single band in the immunoblotting. Several bands were found by immunoblotting in cell-free ascitic fluid. Two major bands were of *M_r* 10000 and around 67000, and there were three minor bands, of *M_r* 30000, 43000 and over 94000. These bands did not appear after immunostaining with pre-immune serum or with antiserum previously incubated with an excess of antigen. One possibility is that these proteins are isoenzymes of a-PLA₂. Another explanation is that PLA₂ molecules aggregate in the absence of high salt concentrations (Franson *et al.*, 1978). High-*M_r* PLA₂s 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). PLA₂ may also be associated with high-*M_r* proteins present in ascitic fluid.

Several mammalian non-pancreatic PLA₂s have been purified. However, it is at present unclear which enzymes are relevant in inflammatory diseases. Inflammatory exudate contains at least two distinct PLA₂ activities: membrane-associated and soluble. They differ in their substrate specificity (Gonzales-Buritica *et al.*, 1989a,b; Seilhamer *et al.*, 1989). The presence of various forms

of PLA₂ 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 PLA₂ in synovial fluid and inflammatory exudate (Kramer *et al.*, 1989). However, there is evidence that human platelets do not secrete PLA₂ upon stimulation (Hara *et al.*, 1989). We have determined the concentration of a-PLA₂ in both plasma and serum samples from the same healthy individuals and found no difference. This result indicates that a-PLA₂ is not released by platelets. It is well known that PLA₂ is present in rabbit and human polymorphonuclear leucocytes (Elsbach *et al.*, 1979; Forst *et al.*, 1986; Mäki & Franson, 1986; Wright *et al.*, 1990). On the other hand, the PLA₂ activity of inflammatory exudate does not correlate with white-cell count (Pruzanski *et al.*, 1985). However, recently Loeser *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 PLA₂. Our results also show that PLA₂ released from leucocytes represents less than 1% of a-PLA₂ present in ascitic fluid or serum.

We have ascertained in the present paper that the PLA₂ found in the ascitic fluid of patients suffering from ovarian carcinoma and peritoneal carcinosis is not of pancreatic origin.

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