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Characteristics of Cytosolic Calcium-Independent Phospholipase A_2 Isolated from Rat Liver

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A calcium-independent phospholipase A_2 ($iPLA_2$) was identified from the cytosolic fraction of rat liver cells. On gel filtration chromatography, the $iPLA_2$ activity was eluted as broad peaks of 150 to 500 kDa. The enzyme was maximally active at pH 7.5, retained 75% of its original activity after heating at 50°C for 5 h, and was inhibited by Ca^{2+}, Mg^{2+}, and Zn^{2+} ions, but was not affected by Na^+ and K^+ ions. The enzymatic activity was increased up to 150% by 1 to 4 mM DTT and was inhibited up to 25% by 0.1 to 1 mM PMSF. The $iPLA_2$ activity had preference for the head group of phospholipids, where phosphatidylethanolamine was preferred to phosphatidylcholine. The results suggest that the $iPLA_2$ may be a novel enzyme distinct from the previously reported $iPLA_2$s.

Keywords: Ca^{2+}-independent PLA_2, Enzyme activity, Rat liver.

Introduction

Phospholipase A_2 (PLA_2, EC 3.1.1.4) is a large superfamily of distinct enzymes (Dennis, 1997) which play important roles in cellular processes involving phospholipid digestion and metabolism, such as inflammation and signal transduction (Dennis *et al.*, 1991). PLA_2 hydrolyzes the acyl ester bond at the sn-2 position of membrane phospholipids and produces free fatty acids and lysophospholipids, the precursors of potent lipid mediators such as leukotrienes, prostaglandins, and platelet activating factor (Samuelsson *et al.*, 1987; Sirois, 1987; Sturk *et al.*, 1989; Dennis, 1994; Tischfield, 1997). Consequently, PLA_2 activation has been postulated to contribute to the pathophysiology of inflammation and tissue injury in various disease states.

Based on the biochemical properties and structural features, the PLA_2 superfamily proteins can be classified into three main kinds: the Ca^{2+}-dependent secretory enzymes ($sPLA_2$), the Ca^{2+}-dependent cytosolic enzymes ($cPLA_2$), and the Ca^{2+}-independent cytosolic enzymes ($iPLA_2$) (Dennis, 1997). $sPLA_2$s are characterized by their small sizes of 13 to 18 kDa, high disulfide content, and conserved three-dimensional structures. These enzymes require millimolar concentrations of Ca^{2+} for their maximal activity and do not have specificity for the fatty acid in the sn-2 position of phospholipids (Heinrikson *et al.*, 1977; Dennis, 1983; Renetseder *et al.*, 1985). $cPLA_2$s have apparent molecular weights of 70 to 100 kDa (Bennett *et al.*, 1990; Kim and Bonventre, 1993), require submicromolar levels of Ca^{2+} for activation (Kim *et al.*, 1991), and preferentially hydrolyze the arachidonic acid at the sn-2 position of phospholipids (Clark *et al.*, 1990).

$iPLA_2$s do not require Ca^{2+} for their maximal activities. $iPLA_2$ enzymes have been shown to be widely distributed and ubiquitously expressed in most mammalian tissues, underlining their potential importance in cellular functions (Ackermann and Dennis, 1995; Balsinde and Dennis, 1997). Only a few $iPLA_2$s have been purified and characterized from mammalian tissues, and the structures and mechanisms of $iPLA_2$s are still largely unknown. In this contribution, we have identified an $iPLA_2$ from rat liver and characterized its properties.

Materials and Methods

Chemicals DEAE-Sephacel, Sephacryl S-300, and Heparin Sepharose CL-6B were purchased from Pharmacia (Uppsala, Sweden). Arachidonic acid, dithiothreitol (DTT), phenylmethylsulphonyl fluoride (PMSF), and fatty acid-free

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bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). 1-Acyl-2-[1-^{14}C]-arachidonyl-sn-glycerol-3-phosphatidylethanolamine (55 mCi/mmol, 2-AA-PE), and 1-stearoyl-2-[1-^{14}C]-arachidonyl-sn-glycerol-3-phosphatidylcholine (55 mCi/mmol) were purchased from Amersham (Buckinlghamshire, UK). L-1-palmitoyl-2-[1-^{14}C]-arachidonyl-sn-glycerol-3-phosphatidylcholine (55 mCi/mmol) was purchased from New England Nuclear (Boston, USA).

PLA_2 activity assay Enzymatic activity was assayed using 2-AA-PE unless otherwise specified. Liposomes were prepared by sonication as previously described (Kim *et al.*, 1991; Huh *et al.*, 1998). The reaction mixture (200 μl final volume) contained 75 mM Tris·HCl (pH 7.5), 4 mM EGTA, 1 mg/ml fatty acid-free BSA, 0.5 nmol 2-AA-PE (60,500 dpm). The reactions were carried out at 37°C for 60 min and stopped by adding 600 μl of methanol:chloroform (2:1 v/v), 200 μl of chloroform, and 66 μl of distilled water. The lipid was extracted by the method of Bligh and Dyer (1959) and the organic solvent was evaporated using a Speed Vac evaporator (Savant, Holbrook, USA). The lipid was resuspended in chloroform, supplemented with cold arachidonic acid, and separated on a TLC plate (silica gel F254; Merck, Darmstadt, Germany) using petroleum ether:ethyl ether:acetic acid (80:20:1, v/v/v) as the developing solvent. Arachidonic acid spots were visualized by I_2 spray, cut out, and the radioactivity was counted with a liquid scintillation counter.

Partial purification of cytosolic Ca^{2+}-independent PLA_2 All purification steps were performed at 4°C. Rat liver (100 g) was minced into small pieces with sharp surgical scissors and blood was removed by washing with homogenization buffer (50 mM Tris·HCl, pH 7.4, 150 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA) containing 0.15 M sucrose. It was then homogenized in 4 vol of ice-cold homogenation buffer using a Polytron homogenizer. The homogenate was centrifuged at 20,000 × *g* for 30 min and the supernatant was subsequently centrifuged at 100,000 × *g* for 60 min to separate the cytosolic and microsomal fractions. The supernatant (cytosol) was filtered through glass wool and concentrated by ultrafiltration using an Amicon (Beverly, USA) YM-30 membrane. The cytosol was loaded onto a DEAE-Sephacel column (5 × 30 cm) pre-equlibrated with buffer A (50 mM Tris·HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol) and eluted with a linear salt gradient from 0 to 0.5 M NaCl in buffer A at a flow rate of 2 ml/min. The active fractions were combined, concentrated by ultrafiltration, and loaded onto a Heparin Sepharose CL-6B column (2.5 × 20 cm) pre-equlibrated with buffer B (25 mM Tris·HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 0.1 M NaCl, 10% glycerol). The column was eluted with a linear salt gradient from 0.1 to 1.5 M NaCl in buffer B at a flow rate of 0.5 ml/min. The active fractions were pooled, concentrated, and loaded onto a Sephacryl S-300 column (2.5 × 100 cm) pre-equlibrated with buffer C (25 mM Tris·HCl, pH 7.7, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 10% glycerol). The column was eluted with buffer C at a flow rate of 0.6 ml/min. The active fractions were pooled and frozen at −80°C until use.

Molecular weight estimation and determination of protein concentration Molecular weight estimation of $iPLA_2$ was carried out by Sephacryl S-300 column chromatography using apoferritin (443 kDa), amylase (200 kDa), alcohol dehydrogenase (150 kDa), and BSA (66 kDa) as molecular weight standards. Protein concentration was determined by the methods of Bradford (1976) with BSA as the standard.

Results

Partial purification of $iPLA_2$ from rat liver When the cytosolic proteins were separated by DEAE-Sephacel column chromatography with a salt gradient, most of the $iPLA_2$ activity was eluted as a single peak between 0.2 and 0.3 M NaCl (Fig. 1A). Fractions with major activity were pooled, concentrated, and loaded onto a Heparin Sepharose CL-6B column. The $iPLA_2$ activity was detected in the flow-through fractions (Fig. 1B), indicating that $iPLA_2$ did not bind to the Heparin Sepharose CL-6B column. The active fractions were pooled, concentrated, and loaded onto a Sephacryl S-300 column. Four peaks of $iPLA_2$ activity appeared as a broad peak of 150 to 500 kDa (Fig. 1C). Figure 1D shows the effects of Ca^{2+} and EGTA on the $iPLA_2$ activity of the four peak fractions. All showed $iPLA_2$ activity in the presence of EGTA, and all were inhibited by 4 mM Ca^{2+} ion. Peak IV was used for characterization of the enzymatic properties.

Characterization of $iPLA_2$ from rat liver

Optimum pH: The optimum pH for the partially purified $iPLA_2$ was estimated to be pH 7.5 (Fig. 2).

Thermal stability: The temperature effect on the $iPLA_2$ activity was determined by measuring the activity after incubation for 10 min at various temperatures. After incubation at temperatures between 20 and 50°C, the enzyme was fully active, but it dramatically lost activity at 60°C, and no activity remained if incubated at 70°C (Fig. 3A). The thermal stability of the enzyme at 50°C was determined at various times after incubation. The activity increased to 150% after incubation for 30 min, and 75% of the initial activity remained after 5 h incubation (Fig. 3B).

Effects of metal ions: Effects of various metal ions on the $iPLA_2$ activity were examined in the presence of 1 mM metal ions. Na^+ and K^+ ions had no effects. The enzyme was partially inhibited by Ca^{2+} and Mg^{2+} ions, and completely inhibited by Zn^{2+} ion (Table 1).

Figure 4 shows the dependence of the $iPLA_2$ activity on Ca^{2+} and Zn^{2+} ions. The enzyme activity was similar in the absence of and at low Ca^{2+} concentrations of between 10^{-7} and 10^{-4} M; however, the activity decreased to 68% of its original activity at high Ca^{2+} concentrations above 1 mM (Fig. 4A). The enzyme activity was highly sensitive to the concentration of Zn^{2+} ion. The activity decreased with increasing Zn^{2+} concentration and dropped to zero at concentrations above 1 mM (Fig. 4B).

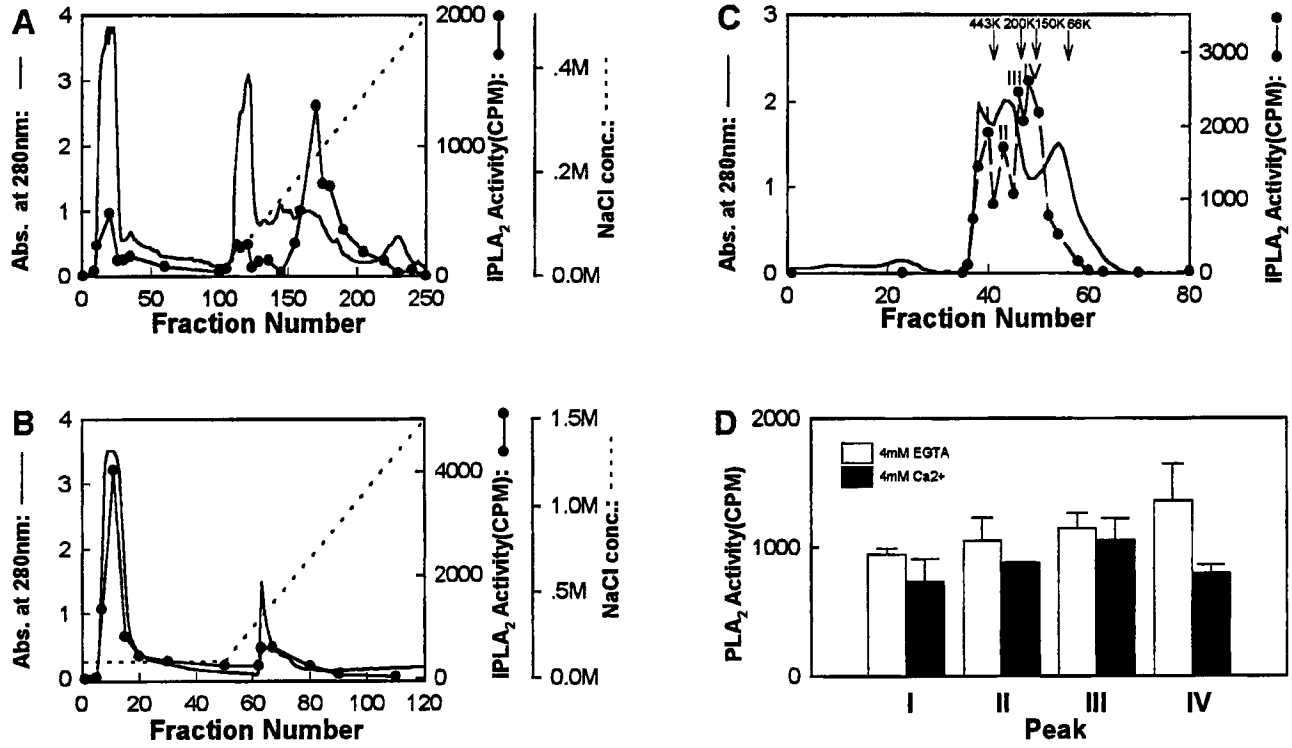

Fig. 1. Column chromatography profiles for purification of $iPLA_2$ from rat liver. A. DEAE-Sephacel ion-exchange, B. Heparin-Sepharose CL-6B, and C. Sephacryl S-300 column chromatography profiles. The enzyme activity in each fraction was assayed using 1-acyl-2-arachidonoyl-phosphatidylethanolamine (2-AA-PE) as the substrate. Composition of the assay mixtures was as in Materials and Methods. D. Effect of Ca^{2+} on the PLA_2 activity of peak fractions obtained from Sephacryl S-300 chromatography. PLA_2 activity was assayed in the presence of 4 mM EGTA or 4 mM Ca^{2+}. The data shown are the average of three experiments.

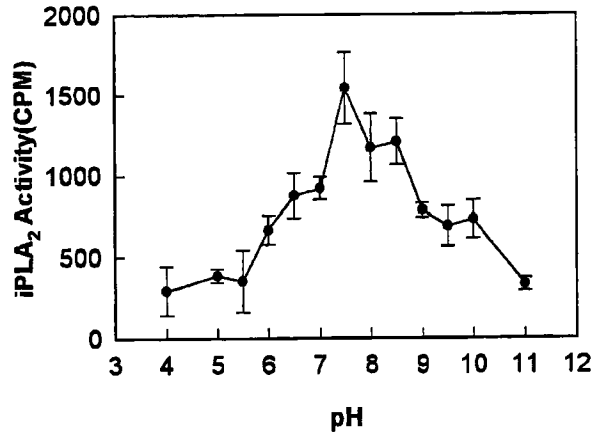

Fig. 2. Effects of pH on $iPLA_2$ activity. The peak IV fraction obtained from Sephacryl S-300 chromatography was assayed using the reaction mixture containing 4 mM EGTA, 1 mg/ml fatty acid-free BSA, and 0.5 nmol 2-AA-PE in 75 mM Tris·HCl buffers at various pH values. Non-specific hydrolysis at each pH was undetectable.

Effect of DTT and PMSF: The enzyme activity decreased to 25% as the concentration of PMSF increased from 0.1 to 1 mM and increased up to 150% by 1 to 4 mM DTT (Fig. 5).

Table 1. Effects of various metal ions on the $iPLA_2$ activity.

Metal ions	Relative activity (%)
EDTA	100.0 ± 2.4
NaCl	93.1 ± 0.6
KCl	104.0 ± 0.4
$CaCl_2$	64.5 ± 4.1
$MgCl_2$	78.4 ± 11.2
$ZnCl_2$	0.0

The peak IV fraction from Sephacryl S-300 chromatography was assayed with the indicated metal ions. Concentration of metal ions was 1 mM in all cases.

Substrate specificity and kinetic parameters: The substrate specificity of the enzyme was analyzed by using different phospholipids (Table 2). Phosphatidylethanolamine (2-AA-PE) was hydrolyzed at about 5-fold the rate of phosphatidylcholine. At the sn-1 position, the enzyme preferred palmitoic acid rather than stearic acid.

The substrate kinetics of the enzyme were determined using 2-AA-PE as the substrate (Fig. 6). A Lineweaver-Burk plot gave an apparent K_m value of 45 μM and a V_{max} of about 160 pmol/min/mg.

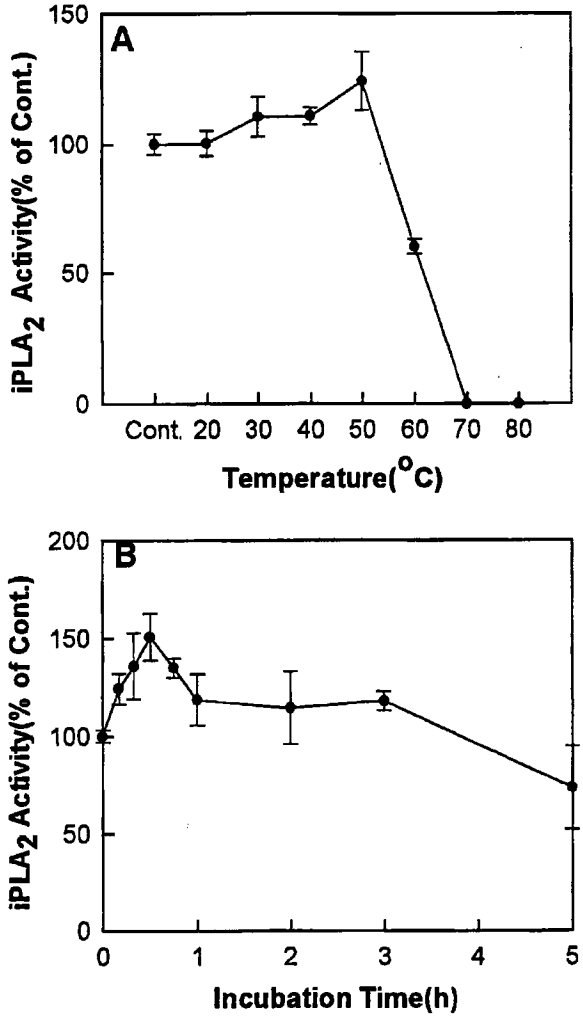

Fig. 3. Thermal stability of $iPLA_2$ activity. A. The peak IV fraction obtained from Sephacryl S-300 chromatography was incubated for 10 min at the indicated temperature and was then assayed for $iPLA_2$ activity. B. The peak IV fraction was incubated at 50°C for the indicated times and then assayed for $iPLA_2$ activity.

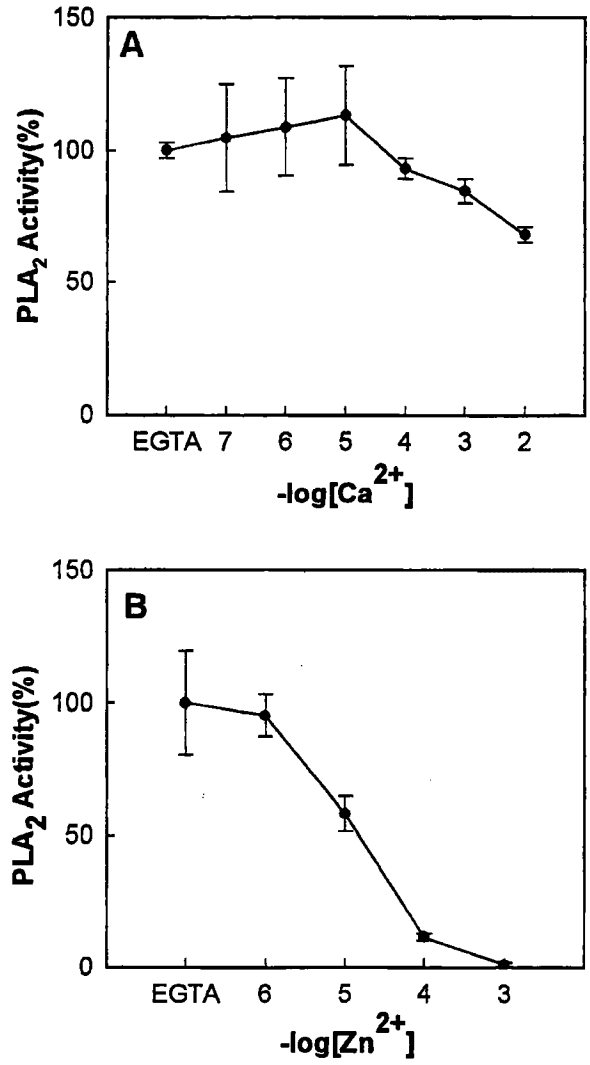

Fig. 4. Effects of Ca^{2+}(A) and Zn^{2+}(B) on $iPLA_2$ activity. The peak IV fraction obtained from Sephacryl S-300 chromatography was assayed with various concentrations of Ca^{2+} or Zn^{2+} at pH 7.5.

Table 2. Substrate specificity of the $iPLA_2$.

Substrate	Relative activity (%)
1-Palmitoyl-2-arachidonoyl-PC	20.8 ± 8.2
1-Stearoyl-2-arachidonoyl-PC	14.2 ± 1.2
1-Acyl-2-arachidonoyl-PE	100.0 ± 1.8

The peak IV fraction from Sephacryl S-300 chromatography was assayed with the indicated substrates.

Discussion

Although $iPLA_2$ activities have been found in various mammalian tissues, only a few have been purified and characterized. They have different molecular weights, substrate specificities, and optimum pHs, indicating the diversity of $iPLA_2$s. In this study, we identified a new form of $iPLA_2$ from a cytosolic fraction of rat liver. The $iPLA_2$ activity was eluted as a broad peak of 150 to 500 kDa from a gel filtration column. It was maximally active at pH 7.5 and thermally stable. The activity was highest in the absence of Ca^{2+}, partially inhibited by Ca^{2+} or Mg^{2+}, and strongly inhibited by Zn^{2+}. The enzyme was inhibited by PMSF but not by DTT.

When DEAE-Sephacel column chromatography was performed, the total $iPLA_2$ activity in the eluent decreased to 10% of the loaded activity (data not shown). A similar phenomenon has previously been observed during purification of $iPLA_2$ from rat brain using Phenyl Sepharose column chromatography (Yoshihara and

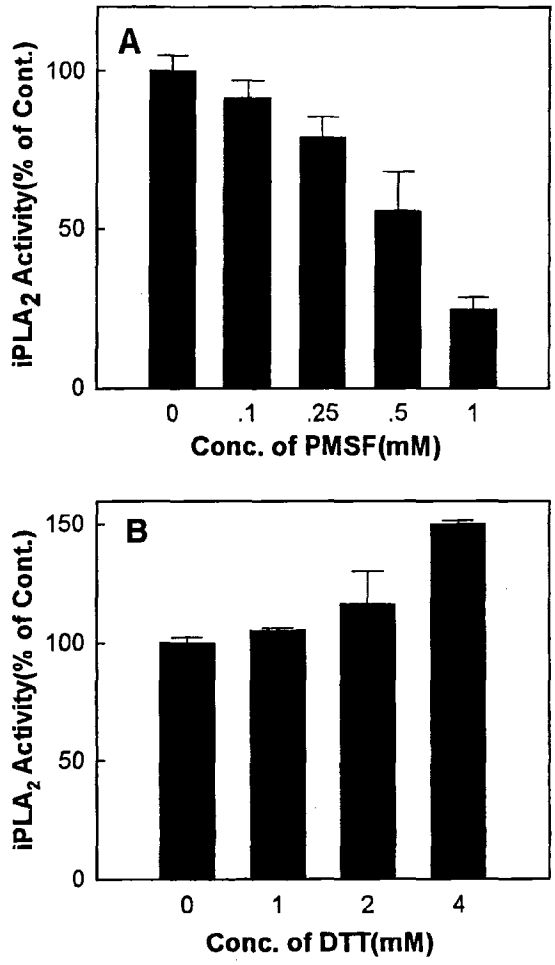

Fig. 5. Effects of PMSF (A) and DTT (B) on $iPLA_2$ activity. The peak IV fraction obtained from Sephacryl S-300 chromatography was assayed with various concentrations of PMSF or DTT at pH 7.5.

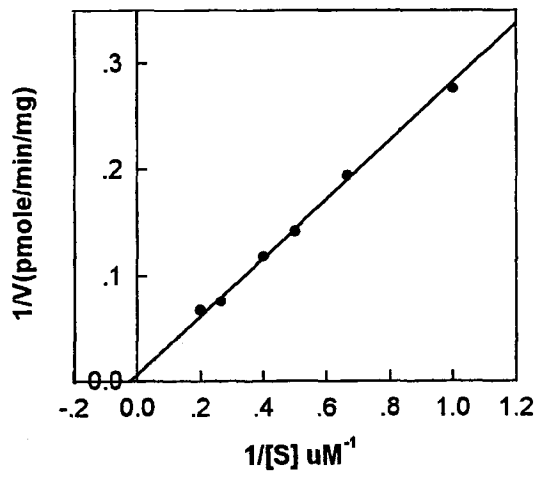

Fig. 6. Lineweaver-Burk plot of $iPLA_2$ activity. The peak IV fraction obtained from Sephacryl S-300 chromatography was assayed at various concentrations of 2-AA-PE.

Watanabe, 1990). The loss of activity was speculated to be due to the dissociation of activator or cofactor from the enzyme; however, the regulatory factors have not been identified to date. This may be the case with the $iPLA_2$ from this study since the $iPLA_2$ activity was partially restored by adding the other fractions with no $iPLA_2$ activity (Yoshihara and Watanabe, 1990).

The apparent size of 200 to 500 kDa (Fig. 1C) might not represent the true molecular weight and may arise due to self-association or association with other proteins. Recent reports have suggested that different molecular masses or multimeric forms of $iPLA_2$s exist in various cells. For example, a 40 kDa $iPLA_2$ from myocardial tissue and pancreatic islets was associated with a 400 kDa phosphofructokinase (Hazen and Gross, 1993; Ramanadham *et al.*, 1996), and a 80 kDa $iPLA_2$ from the P388D1 macrophage cell line was associated with a 330 kDa protein (Ackermann *et al.*, 1994). The $iPLA_2$ from CHO cells and pancreatic islets have been shown to possess eight ankyrin repeats, which may mediate self-aggregation or interaction with other proteins (Ma *et al.*, 1997; Tang *et al.*, 1997; Larsson *et al.*, 1998).

The inhibition of $iPLA_2$ by divalent ions is quite unexpected. In the case of Ca^{2+}-dependent $sPLA_2$, Zn^{2+} inhibits the enzyme by replacing Ca^{2+}, which is necessary for its activity (Mezna *et al.*, 1994). Since $iPLA_2$ does not require Ca^{2+} for the activity, the mechanism by which divalent ions inhibit $iPLA_2$ activity is intriguing and needs detailed investigation. It is important to know whether other $iPLA_2$s are also inhibited by divalent ions. It is worth mentioning that even though Ca^{2+} is not required for $iPLA_2$ activity there must in fact be Ca^{2+} binding site(s).

The dependence of the enzymatic activity on PMSF suggests that the $iPLA_2$ has a serine residue which is essential for the catalytic reaction. This is consistent with the reports that the $iPLA_2$ sequences deduced from the cDNA of CHO cells and human B-cells contain the GXSXG motif which is commonly found in other lipases (Tang *et al.*, 1997; Larsson *et al.*, 1998).

The $iPLA_2$ has characteristics that distinguish it from previously identified PLA_2s. It is different from $sPLA_2$ on the following points: high molecular weight, the independence of Ca^{2+}, insensitivity to DTT, inhibition by PMSF, and low affinity to Heparin-Sepharose resin. $cPLA_2$ has an alkaline pH optimum and requires micromolar concentrations of Ca^{2+} for activity.

Some biochemical properties of the $iPLA_2$ activity from rat liver, including insensitivity to DTT and Ca^{2+}-independence for activity, are found among other $iPLA_2$s. Myocardial $iPLA_2$ has a molecular weight of 40 kDa, an optimum pH of 6.4, and plasmalogen selectivity (Hazen *et al.*, 1990). The $iPLA_2$ from P388D1 macrophage has a molecular weight of 80 kDa and an optimum pH of 7.5 (Ackermann *et al.*, 1994). Both of these appeared to be associated with high molecular weight complexes. The

rabbit kidney $iPLA_2$ has a molecular weight of 28 kDa, an optimum pH of 8.0, and plasmalogen selectivity (Portilla and Dai, 1996). Recently, 58 kDa and 15 kDa acidic-pH optimum $iPLA_2$s were purified from rat brain (Thomson and Clark, 1995) and lung (Wang *et al.*, 1994), respectively. A high molecular weight $iPLA_2$, 200 to 500 kDa, has been partially purified from rat brain (Yoshihara and Watanabe, 1990). This enzyme displayed Ca^{2+}-concentration dependence similar to the rat liver $iPLA_2$ in this study. However, the $iPLA_2$ from rat brain has an alkaline pH optimum: the enzyme activity was four times higher at pH 9.0 than at pH 7.5. The $iPLA_2$ activity from this study was lower at pH 9.0 than at pH 7.5.

In conclusion, we have identified an $iPLA_2$ from rat liver. Characterization of its properties revealed that this enzyme may be a novel one.

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