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PURIFICATION AND SOME PROPERTIES OF A PHOSPHOLIPASE A_2 FROM BOVINE PLATELETS

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SUMMARY : An intracellular form of phospholipase A_2 was purified about 47,500-fold to near homogeneity from bovine platelets 100,000 × g supernatant by sequential use of column chromatographies on Heparin-Sepharose, DEAE-Sephacel, Butyl-Toyopearl, Sephacryl S-300, DEAE-5PW HPLC, TSK G 3000 SW HPLC and Mono Q FPLC. The final preparation showed a single band on SDS-polyacrylamide gel, and its molecular mass was estimated to be approximately 100,000 daltons. The purified PLA_2 showed maximal activity at alkaline pH(pH 9.0-10.0) and considerable activity at 0.3-1.0μM calcium concentration. It hydrolyzed phosphatidylcholine containing arachidonate at sn-2 position with high selectivity in comparison to linoleate.

It has been generally accepted that activated platelets release arachidonates, which are further metabolized to biologically active lipid mediators such as prostaglandins or thromboxanes by cyclooxygenase and 12-HPETE or 12-HETE by lipoxygenase (1-3). The arachidonate release is rate-limiting step of the production of the chemical mediators and is mainly performed by phospholipase A_2 (PLA_2) activation from membrane phospholipids in response to stimulation of a variety of cells (4,5). It has been, recently, reported that after stimulation of human platelet with thrombin or collagen, the molecular species of phosphatidylcholine(PC) and phosphatidylethanolamine(PE) containing arachidonate were selectively hydrolyzed with concomitant accumulation of the other product, lyso-phospholipid (6,7). Furthermore, in the recent studies on a variety of cells including platelets, intracellular forms of PLA_2 have been found in cytosol (8-13). In contrast to the class of enzymes designated mammalian 14 kDa group II PLA_2 (14,15), these enzymes showed high molecular weights and were optimally activated under micromolar calcium

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The abbreviations used are: EGTA, ethyleneglycol bis(β-aminoethyl ether) tetraacetic acid; EDTA, ethylenediaminetetraactic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; Tris, tris (hydroxymethyl) aminoethane; 12-HPETE, hydroperoxyeicosatetraenoic acid; 12-HETE, hydroxyeicosatraenoic acid; PLA_2, phospholipase A_2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography.

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concentration with high selectivity for arachidonate at sn-2 position. Although these observations suggested that PLA_2 recognizing an arachidonoyl residue is involved in the release of arachidonates on cell activation, little is known about the intracellular forms of the PLA_2. In the present study, an intracellular form of PLA_2 was purified to near homogeneity from bovine platelet soluble fraction and partially characterized.

MATERIALS AND METHODS

Chemicals : 1-Stearoyl-2-[1-^{14}C]arachidonoyl-glycerophosphocholine (-GPC) (55.9 mCi/mmol) and 1-palmitoyl-2-[1-^{14}C]linoleoyl-GPC (54 mCi/mmol) were purchased from the Radio-chemical Center, Amersham, UK. 1-Stearoyl-2-arachidonoyl-GPC and 1-palmitoyl-2-linoleoyl-GPC were obtained from Sigma, St. Louis. Heparin-Sepharose CL-6B and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals, Sweden. Butyl-Toyopearl 650M was obtained from Tosoh, Tokyo. All other chemicals were of the highest purity available from commercial sources.

Preparation of enzyme source : Blood was collected with anticoagulant solution (1.25% trisodium citrate and 2% dextrose) in the ratio of 1 part to 5 parts blood from three cattle (500-600 kg) in the local slaughterhouse. The blood was centrifuged for 10 min at 270 × g at room temperature to prepare platelet-rich plasma. The platelet was then spun down from the plasma by centrifugation at 1,200 × g for 10 min at 4°C. The pellet was gently suspended in 50 mM Tris-HCl (pH 7.5) buffer containing 0.1 M NaCl and 1 mM EDTA. The centrifugation and suspension was then repeated. The pellet was finally suspended in the same buffer at a final concentration of $1\text{-}2\times10^{10}$ platelets/ml. Each of the washed platelet suspension (80 ml) was sonicated at 4°C with a Branson sonifier Model 450 cell disruptor for 60 sec with duty cycle 50 at an output setting of 10 using 1 inch diameter horn. The resulting lysate was then centrifuged at 100,000 × g for 60 min. The supernatant was used as a source of cytoplasmic fraction for purification of PLA_2.

Standard assay for PLA_2 : Each substrate (300-350 nmol) was dried under nitrogen gas and suspended in 2 ml distilled water by sonication at 4°C with a Branson Sonifier Model 450 with 3 inch diameter Branson Cup Horn for 2 min with duty cycle 50 at an output setting of 10. The standard incubation system (200 μl) for assay of PLA_2 contained 75 mM Tris-HCl (pH 9.0), 5 mM $CaCl_2$, 1 mg/ml bovine serum albumin and 5 nmol of radioactive phospholipids (approximately 65,000 dpm). The reaction was carried out at 37°C for 5 min and was stopped by adding 1.25 ml of Dole's reagent (16). The radioactive free fatty acid released was extracted using the method described previously (13).

Purification of PLA_2 ; Step 1. Heparin-Sepharose Affinity Chromatography: 12 g protein of 100,000 × g supernatant (1 liter) was loaded on a Heparin-Sepharose column (2.5×13cm) pre-equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl and 1 mM EDTA. The column was washed with the same buffer until no UV absorbance at 280 nm was observed and eluted stepwisely with the same buffer containing 2.0 M NaCl at a flow rate of 0.8ml/min to obtain heparin-binding PLA_2 activity. The active pass-through fractions (1.6 liters) were pooled.

Step 2. DEAE-Sephacel Anion Exchange Chromatography: 0.9 g of protein was loaded on a DEAE-Sephacel column (5.0×32 cm) pre-equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and 1 mM EDTA and extensively washed with the starting buffer. The column was eluted stepwisely with 50 mM Tris-HCl (pH 7.5) containing 0.25 M NaCl and 1 mM EDTA at a flow rate of 3.3 ml/min. The active fractions were pooled.

Step 3. Butyl-Toyopearl Hydrophobic Chromatography: The active pool (210 ml) of protein 180 mg from step 2 was adjusted to 0.35 M NaCl and loaded onto a Butyl-Toyopearl column (2.5×20 cm) pre-equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.35 M NaCl and 1 mM EDTA. The column was extensively washed until no detectable protein was found in the washout fractions and eluted with 10 mM Glycine-NaOH (pH 11.5) containing 1 mM EDTA with a stepwise gradient at a flow rate of 0.8 ml/min. To avoid the loss of the activity by alkalization, each fraction was adjusted to pH 8.7 of final 100 mM Tris-HCl. The active fractions were pooled and concentrated to 2.2 ml with Centriprep 30 (Amicon, Danvers).

Step 4. Sephacryl S-300 Gel Filtration Chromatography: The concentrated active pool (45 mg) was loaded onto a Sephacryl S-300 column (1.5×48 cm) pre-equilibrated with 50 mM Tris-HCl (pH 8.7) containing 0.1 M NaCl and 1 mM EDTA and eluted with the same buffer at a flow rate of 0.6 ml/10 min. The active fractions were pooled.

Step 5. DEAE-5PW Anion Exchange HPLC: 12 mg of protein from the pooled fractions (step 4) was injected to a DEAE-5PW column (0.75×7.5 cm) pre-equilibrated with 50 mM Tris-HCl (pH 8.7) and washed until no detectable protein was observed. The protein was eluted for 50 min with a linear gradient from 0 to 0.8 M NaCl in starting buffer at a flow rate of 0.5 ml/min. The active fractions were pooled and concentrated to approximately 200 μl with Centricon 30 (Amicon, Danvers).

Step 6. TSK G 3000 SW Gel Filtration HPLC: The concentrated enzyme preparation (0.9mg) was injected into a TSK G 3000 SW column (0.75×60 cm) pre-equilibrated with 50 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. The protein was eluted with the same buffer at a flow rate of 0.3 ml/min. The active fractions were pooled. To estimate the apparent molecular mass, a series of molecular weight marker proteins (ferritin 440 kDa, catalase 232 kDa, aldolase 158 kDa, bovine serum albumin 67 kDa) was loaded onto the same column under the same condition.

Step 7. Mono Q Anion Exchange Chromatography: 150 μg of protein from step 6 was loaded onto an HR 5/5 Mono Q column pre-equilibrated with 30 mM Hepes-NaOH (pH 7.0) and washed with the same buffer. The protein was eluted for 50 min with a linear gradient from 0 to 0.8 M NaCl in the same buffer at a flow rate of 0.5 ml/min. The active fractions were pooled.

Analytical procedures : Protein concentration was measured with BCA Protein Assay Reagent (Pierce, Rockford) with bovine serum albumin as the standard. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (17) and the protein bands were stained with Coomassie brilliant blue.

RESULTS AND DISCUSSIONS

As shown in Fig. 1, when each fraction was examined for PLA_2 activity using 1-stearoyl-2[1-^{14}C] arachidonoyl-GPC (2-AA-PC) and 1-palmitoyl-2-[1-^{14}C]linoleoyl-GPC (2-LA-PC) as a substrate, respectively, the most of the activity was detected in the heparin-non-binding fractions with high selectivity for 2-AA-PC, whereas no appreciable activity was observed in the heparin-binding fractions with any substrate. It was ensured that the column was not overloaded by re-applying a part of the pool of the non-binding

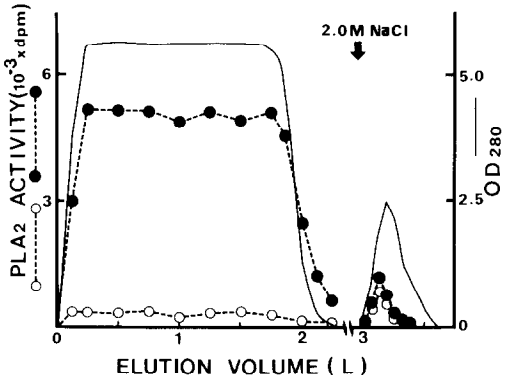


Fig. 1. Heparin-Sepharose chromatography of bovine platelets phospholipase A_2. 100,000×g supernatant of bovine platelets was applied to a previously equilibrated heparin-sepharose column. Aliquots of column eluates were incubated with 1-stearoyl-2-[1-^{14}C]arachidonoyl-GPC(●) and 1-palmitoyl-2-[1-^{14}C] linoleoyl-GPC (○) and released radiolabeled fatty acids were counted as described under "Materials and Methods". Solid line indicated the absorbance at 280 nm.

fraction to the same column. Although this phenomenon was very similar to that of human platelet which showed no appreciable activity in the heparin-binding fractions (unpublished observation), it was not consistent with that of rat (18) or rabbit (9) platelets which had an appreciable activity in heparin-binding fractions with both substrates. Kim et al. (10), recently, reported that when rabbit platelets were gently disrupted by nitrogen cavitation method, the heparin-non-binding activity was mainly recovered in the platelet cytosolic fraction. Thus, the possibility was indicated that the PLA_2 activity detected in the heparin-non-binding fractions in the present report may be localized in the cytosol of bovine platelets.

The active heparin-non-binding fractions were pooled and used for further purification. The purification profiles were shown in Fig. 2. The specific activity of the final preparation was estimated 3,800 nmol/min/mg protein. Starting from the heparin-non-binding fraction, the PLA_2 enzyme was purified 47,500-fold and the yield was 4.3% (Table I). The

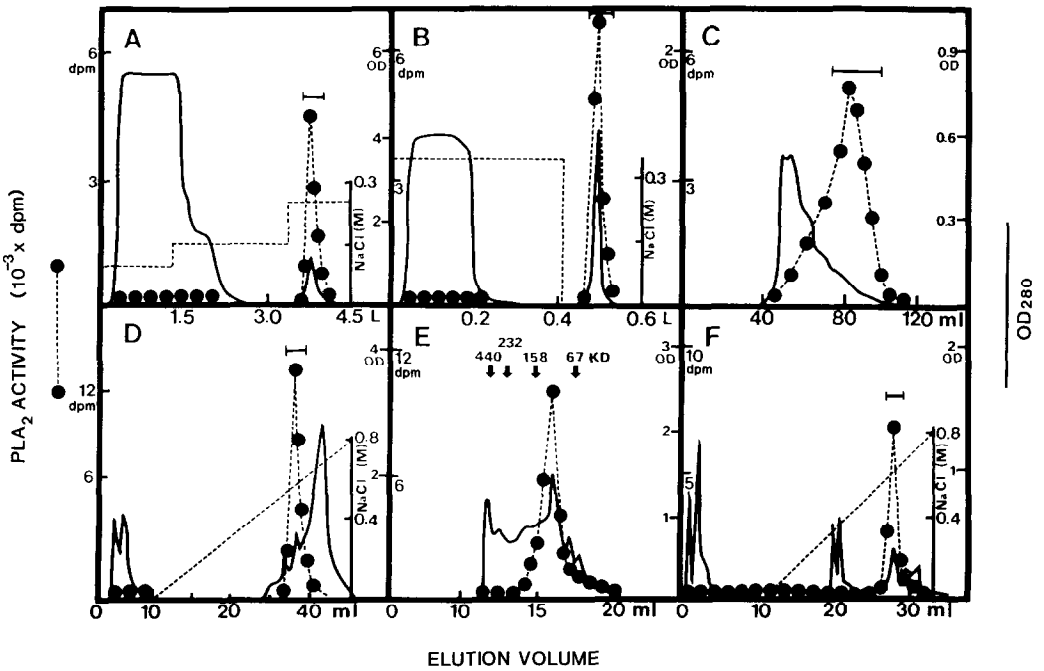


Fig. 2. Chromatographic profiles of a phospholipase A_2 from bovine platelets. A, DEAE-Sephacel chromatography; B. Butyl-Toyopearl chromatography; C, Sephacryl S-300 chromatography; D, DEAE-5PW HPLC; E, TSK G 3000 SW HPLC; F, Mono Q FPLC. All steps were carried out at 4°C except for the HPLCs, which were performed at room temperature. Aliquots of column eluates were assayed by counting radiolabeled fatty acid release from 1-stearoyl-2-[1-^{14}C]arachidonoyl-GPC(●) as described under "Materials and Methods". Solid line indicated the absorbance at 280 nm. Dashed line indicated NaCl concentration. Molecular weight markers (kDa) were indicated by arrowheads.

activity of the enzyme in this final step was very unstable. The loss of the activity could be overcome by immediately freezing at -80°C or by adding 1 mg/ml fatty acid-free bovine serum albumin. Anaysis of the active fractions from step 6 and step 7 by SDS-polyacrylamide gel electrophoresis under reducing conditions demonstrated the enrichment of estimated molecular mass of approximately 100 kDa (Fig. 3). The final preparation yielded a single protein band with 100 kDa molecular weight with Coomassie brilliant blue, which was apparently identical to that estimated on the basis of TSK G 3000 SW gel filtration; that is, it showed an apparent molecular mass of 100-110kDa (Fig. 2E).

Recent reports suggested the existence of multiple forms of intracellular PLA_2 in variety of cells (9,11-13,19-24) and some of the PLA_2 s have been purified to apparent homogeneity. For example, 30 kDa PLA_2 from sheep platelet cytosol(19), a cytosolic form of 70 kDa PLA_2 from RAW 264.7 murine macrophage cell line (12), 40 kDa PLA_2 from canine myocardial cytosol(23), 88 kDa PLA_2 from rabbit platelet cytosol(9) and 56 kDa PLA_2 from the cytosol of human monocytic leukemic U937 cells(13) have been reported, indicating that the PLA_2 s of different molecular mass exist in the different cells.

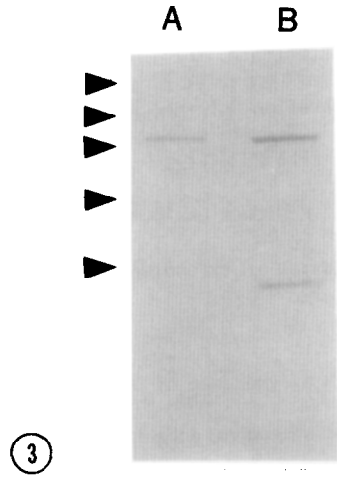
Specificity for acyl chain at sn-2 position was determined using 2-AA-PC and 2-LA-PC as a substrate, respectively. When 2-AA-PC was used as a substrate, the initial velocity of hydrolysis increased significantly with increasing concentration of the substrate, whereas no appreciable amount of hydrolysis was observed with 2-LA-PC (Fig. 4). The PLA_2 activity showing preferential hydrolysis of arachidonoyl residues has been also found in other cells(11-13). This indicates that these PLA_2 may exist in cytosol of a wide variety of cells.

The purified PLA_2 activity showed biphasical profile by increase of pH; that is, the first activation was observed at neutral pH 7.5 and the second at more than pH 9.0 (Fig. 5). The calcium requirement of the purified 100 kDa PLA_2 enzyme was determined. The 100 kDa PLA_2 hydrolyzed the substrate effectively in submicromolar concentration of calcium ion (Fig. 6); the enzyme activity was not detected in the presence of 1 mM EGTA and EDTA. Although the negligible PLA_2 activity was observed at 0.1 μM calcium ion, the activity increased sharply when the free calcium ion concentration increased from 0.1 μM to 1μM. The further elevation of calcium ion to 10 mM gradually increased the enzyme activity. The

Table I. Summary of phospholipase A_2 purification

Step	Protein (mg)	Total activity (nmol/min)	Yield (%)	Specific activity (nmol/mg/min)	Purification (fold)
100,000×g Supernatant	12,000	1,116		0.09	
Heparin-Sepharose	10,900	872	100	0.08	1
DEAE-Sephacel	180	484	55.5	2.69	34
Butyl-Toyopearl	45	296	33.9	6.58	82
Sephacryl S-300	12	212	24.3	17.8	221
DEAE-5PW	0.90	150	17.2	166.7	2,084
TSK G 3000 SW	0.15	100	11.5	667.7	8,346
Mono Q	0.01	38	4.3	3,800	47,500

observed calcium ion dependence corresponds to the increase in free calcium induced upon stimulation of platelets. Although the detailed mechanisms of the biphasic activation by pH or calcium ion are unknown, it is most likely that the 100 kDa enzyme purified in the present report may be activated under physiological conditions.



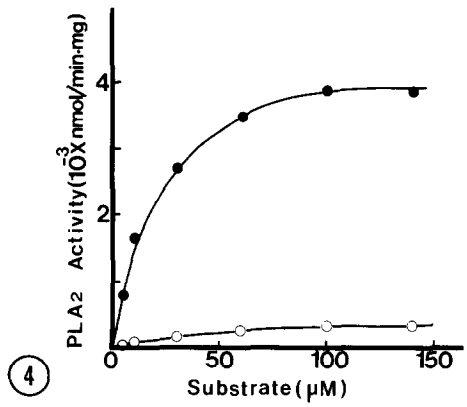


Fig. 3. SDS-PAGE analysis of bovine platelets phospholipase A_2. Aliquots from each of the final two steps were heated at 100℃ for 5 min in the presence of 100 mM 2-mercaptoethanol and 10% SDS, loaded onto a 10% polyacrylamide slab gel and visualized by Coomassie brilliant blue staining. Lane A, 1 μg of Mono Q FPLC active pool; Lane B, 3 μg of TSK G 3000 SW HPLC active pool. Arrows indicated molecular standards from top, myosin (200,000), β-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (67,000) and ovalbumin (45,000).

Fig. 4. Substrate depedence of the purified phospholipase A_2 activity. Activity was assayed with 20 ng of purified PLA_2 under standard assay conditions that the indicated concentrations of 1-stearoyl-2-[1-^{14}C] arachidonoyl-GPC (●) or 1-palmitoyl-2-[1-^{14}C] linoleoyl-GPC(○) were used as substrates.

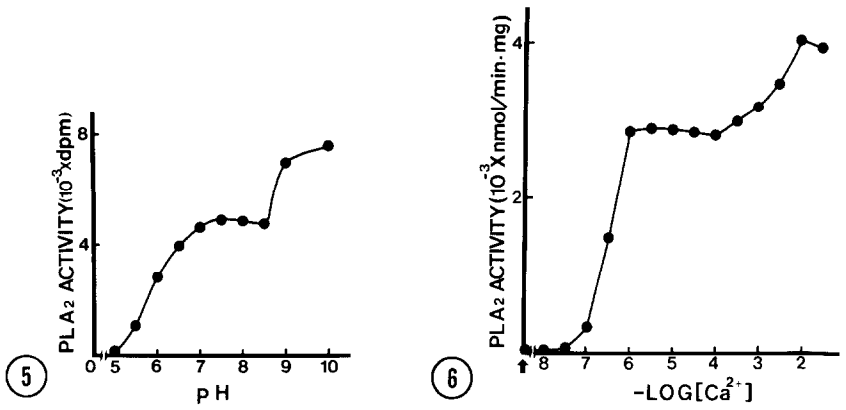


Fig. 5. The pH dependence of the purified phospholipase A_2 activity. A reaction mixture containing purified enzyme (4 ng), 5 mM $CaCl_2$, 1 mg/ml bovine serum albumin, and 2.5μM 1-stearoyl-2-[1-^{14}C] arachidonoyl-GPC was incubated for 5 min at 37°C in a total volume of 200μl. The buffer used was 75 mM Tris-maleate. Non-specific hydrolysis by each pH was not detected and results were shown as means of three separate experiments.

Fig. 6. Effect of calcium ion on the purified bovine platelet phospholipase A_2. The purified bovine platelet phospholipase A_2 (10 ng) was incubated with 100μM 1-stearoyl-2-[1-^{14}C]arachidonoyl-GPC(13,000 dpm/nmol) in 25 mM Tris HCl (pH 7.4) containing 1 mM EGTA and various concentrations of $CaCl_2$ in a total volume of 200 μl for 10 min at 37°C. The absolute concentration of free calcium was calculated using an equation based on the stability constant of the EGTA/$CaCl_2$ system described previously(25). Results were shown as means of three separate experiments. Arrow indicated 1 mM EDTA and 1 mM EGTA.

At present, it remains unclear why the intracellular forms of PLA_2 which have similar biochemical properties show differences in their molecular sizes from various mammalian species and different cells.

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