# Purification and characterization of human platelet phospholipase A2 which preferentially hydrolyzes an arachidonoyl residue

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A phospholipase A2 with an arachidonoyl residue preference was purified about 11 700-fold from human platelet soluble fraction to near homogeneity. The purified phospholipase A2 exhibited a molecular mass of about 90 kDa on SDS polyacrylamide gel electrophoresis and hydrolyzed phospholipids with an arachidonoyl residue more effectively than those with a linoleoyl residue. The catalytic activity of the purified enzyme detected with phosphatidylcholine as a substrate increased sharply between $3 \times 10^{-7}$ and $10^{-6}$ M free calcium ion. Thus, the 90-kDa phospholipase A2 is considered to be a novel enzyme, distinct from the 14-kDa one previously purified from human platelets. The 90-kDa phospholipase A2 may participate mainly in arachidonate metabolism of platelets.

Phospholipase A2; Human platelet; Calcium ion

## 1. INTRODUCTION

Intracellular phospholipase A2 has attracted considerable attention because it may liberate from membrane phospholipids an arachidonoyl residue, which can subsequently be metabolized to various bioactive lipid mediators, such as prostaglandins, thromboxane and leukotrienes [1]. The activation of phospholipase A2 has been suggested to represent a rate-limiting step in the whole process of lipid mediator synthesis, although the molecular basis of the regulatory mechanism is not fully understood at present [2]. We previously reported the detection in human platelet lysate of a phospholipase A2 activity which preferentially released an arachidonoyl residue [3]. From the acid extract of human platelet homogenate, Kramer et al. [4] have purified a 14-kDa phospholipase A2 whose properties were not distinguishable from those of the human synovial fluid enzyme [5]. Thus, the acid-extractable phospholipase A2 activity detected in human platelet must be attributed to the 14-kDa group

*Abbreviations:* GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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II phospholipase A2 that exhibits no preference for an arachidonoyl residue [4,5] and would essentially function in the extracellular space [6]. The present study was undertaken to purify human platelet phospholipase A2 without acid treatment. The characteristics of the human platelet phospholipase A2 that most probably functions intracellularly are discussed.

## 2. MATERIALS AND METHODS

### 2.1. Preparation of enzyme source

Platelet prepared for transfusion were used after the expiration date. Washing and disruption of human platelets were performed as described previously [7]. Platelet lysate in TCB buffer (129 mM NaCl, 10.9 mM sodium citrate, 8.9 mM NaHCO3, 1 mg/ml dextrose, 10 mM Tris-HCl pH 7.2, 2.8 mM KCl, 0.8 mM KH2PO4, 2 mM EGTA, 10 mM mercaptoethanol) at a concentration of $5 \times 10^9$ cells/ml was centrifuged at 105 000 × g for 60 min at 4°C. The resulting supernatant, with a protein concentration of about 14 mg/ml, was stored at -80°C.

### 2.2. Standard assay for phospholipase A2

1-Acyl-2-[1-14C]arachidonoyl-glycerophosphocholine (-GPC) (59.0 mCi/mmol), 1-acyl-2-[14C]linoleoyl-GPC (58.5 mCi/mmol), 1-acyl-2-[14C]arachidonoyl-glycerophosphoethanolamine (-GPE) (58.0 mCi/mmol), or 1-acyl-2-[14C]linoleoyl-GPE (59.0 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, UK and used as substrates. The standard incubation system (250 μl) for the assay of phospholipase A2 contained 100 mM Tris-HCl (pH 9.0), 4 mM CaCl2, 1 mg/ml bovine serum albumin and 0.5 nmol of radioactive substrate. The reaction was carried out at 37°C for 30 min. The reaction was stopped by adding 1.25 ml of Dole's reagent and the radioactive free fatty acid released was extracted using the method described previously [8].

### 2.3. Purification of phospholipase A2

STEP 1. The soluble fraction of human platelet homogenates (100

ml) was loaded onto a 2.5 × 20 cm DEAE-Sephacel column (Pharmacia, Sweden) pre-equilibrated in 50 mM Tris-HCl pH 7.5 (buffer A) containing 150 mM NaCl. The column was washed with buffer A and eluted with buffer A containing 600 mM NaCl.

STEP 2. The pooled phospholipase A₂ was loaded onto a 2.5 × 10 cm Butyl-Toyopearl column (Tosoh, Tokyo) pre-equilibrated in buffer A containing 600 mM NaCl. The column was washed thoroughly with buffer A containing 600 mM or 200 mM NaCl and then eluted with buffer A.

STEP 3. The pooled phospholipase A₂ was loaded onto a 7 × 150 mm HA-1000 Hydroxyapatite HPLC column (Tosoh, Tokyo) in 10 mM sodium phosphate buffer (pH 7.4). The protein was eluted with a linear gradient from 10 mM to 250 mM sodium phosphate buffer.

STEP 4. The active fraction was concentrated using a Centricon 10 (Amicon, USA) and loaded onto a Superose 12 (Pharmacia, Sweden) HPLC column. The protein was eluted with buffer A containing 150 mM NaCl.

## 3. RESULTS

### 3.1. Purification of an arachidonoyl-preferential phospholipase A₂

Using 1-acyl-2-[$^{14}$C]arachidonoyl-GPC as a substrate, the human platelet soluble phospholipase A₂ was purified by sequential use of column chromatographies on DEAE-Sephacel, Butyl-Toyopearl, HA-1000 Hydroxyapatite (HPLC), and Superose 12 (HPLC) (Fig. 1). In the appropriate fractions from each column chromatography, essentially a single peak with phospholipase A₂ activity was observed.

The specific activity of the final preparation was 259 nmol/min/mg protein and, starting from the soluble fraction of human platelet homogenates, the purification achieved was about 11 700-fold (Table I). These are the highest values so far reported for platelet non-group II phospholipase A₂ [9–11]. The yield was 4.1%. The final preparation yielded a single protein band on SDS polyacrylamide gel electrophoresis with a molecular mass of about 90 kDa (Fig. 2A). The appearance of the 90-kDa protein band in each fraction from the Superose gel filtration column paralleled that of the enzymatic activity (Figs 1D and 2B). Addition of mercaptoethanol did not change the apparent molecular mass (data not shown). Thus, the purifed phospholipase A₂ consisted of a single polypeptide with a molecular mass of about 90 kDa.
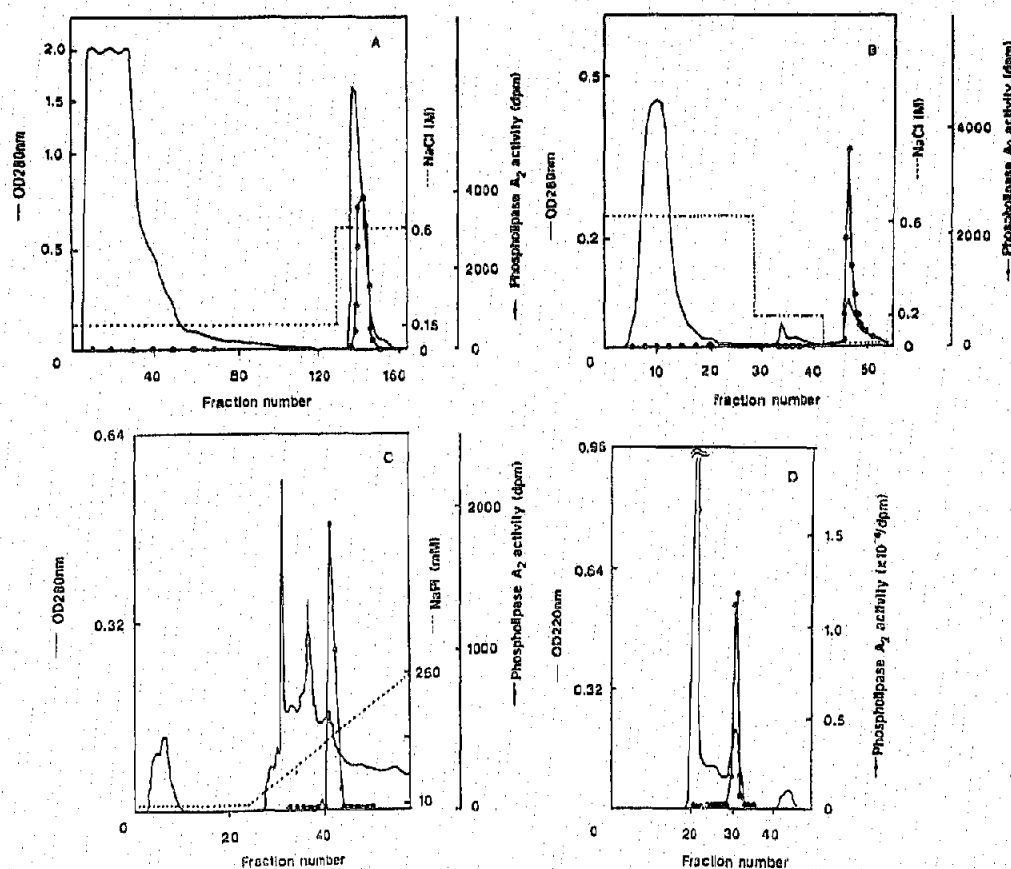


Fig. 1. Elution profiles of protein (———) and phospholipase A₂ activity (●) from DEAE Sephacel column chromatography (A), Butyl-Toyopearl hydrophobic chromatography (B), HA-1000 Hydroxyapatite HPLC (C), and Superose 12 gel filtration HPLC (D). Flow rate: 10 ml/h (A and B), 0.5 ml/min (C), 0.3 ml/min (D). Fraction volume: 6 ml (A), 3 ml (B), 0.5 ml (C), 0.3 ml (D). Aliquots used for phospholipase A₂ assay: 10 μl (A and B), 3 μl (C), 1 μl (D).

Table I

Summary of the purification of phospholipase $A_2$ from human platelet soluble fraction

| Step | Protein (mg) | Total activity (pmol/min) | Specific activity (pmol/min·mg) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| 100 000 × g supernatant | 1400 | 31 533 | 22 | 100 | 1 |
| DEAE-Sephacel | 57.7 | 38 470 | 666 | 121 | 33 |
| Butyl-Toyopearl | 1.37 | 18 332 | 13 381 | 58 | 660 |
| Hydroxyapatite | 0.08 | 3 657 | 45 721 | 11.6 | 2 078 |
| Superose 12 | 0.005 | 1 295 | 259 000 | 4.1 | 11 700 |

### 3.2. Enzymatic properties

When assayed using 1-acyl-2-[1-$^{14}$C]arachidonoyl-GPC as a substrate, the 90-kDa phospholipase $A_2$ was sensitive to a micromolar concentration of calcium ions (Fig. 3); an addition of 2 mM EGTA inhibited the hydrolyzing activity. No appreciable activity was observed at $3 \times 10^{-7}$ M free calcium concentration, and the activity increased sharply as the free calcium concentration was increased from $3 \times 10^{-7}$ to $10^{-6}$ M. At $10^{-6}$ M, almost full activity was observed. A similar calcium ion requirement was observed when 1-acyl-2-[1-$^{14}$C]arachidonoyl-GPE was hydrolyzed by

the 90-kDa phospholipase $A_2$ (data not shown). It should be noted that the human 14-kDa group II phospholipase $A_2$ required a higher concentration of calcium ions for the maximum activity [5].

The purified 90-kDa phospholipase $A_2$ hydrolyzed 1-acyl-2-[1-$^{14}$C]arachidonoyl-GPC more effectively than 1-acyl-2-[1-$^{14}$C]linoleoyl-GPC (Fig. 4A). A similar fatty acid preference was also observed when phosphatidylethanolamine (PE) was used as a substrate (Fig. 4B). The human 14-kDa group II phospholipase $A_2$ did not exhibit such a fatty acid preference [5].

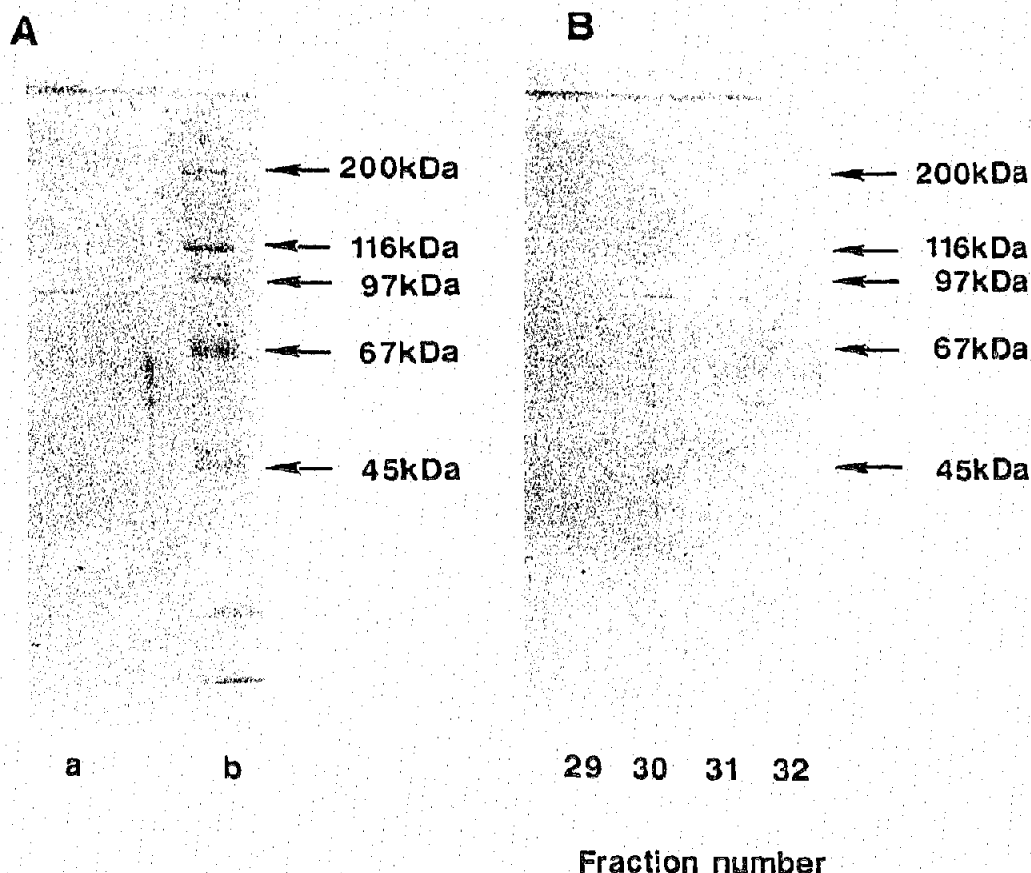Exposure of the purified 90-kDa phospholipase $A_2$ to



Fig. 2. SDS-polyacrylamide (10%) gel electrophoresis of the purified phospholipase $A_2$ (A) or of each fraction from Superose 12 HPLC (B). A. Lane a contains purified phospholipase $A_2$ (about 100 ng as protein). Lane b contains marker proteins: rabbit muscle myosin (molecular mass 200 kDa), E. coli β-galactosidase (116 kDa), rabbit muscle phosphorylase B (97 kDa), bovine serum albumin (67 kDa) and egg albumin (45 kDa). B. 10 μl of each fraction was applied.
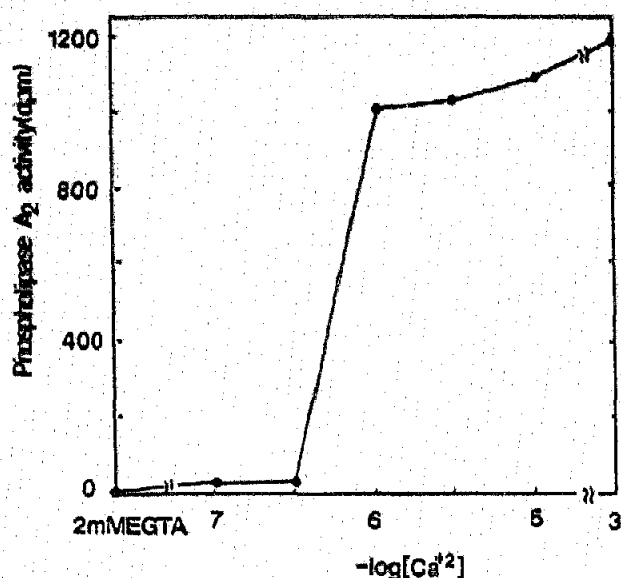
Fig. 3. Effect of calcium ions on the purified human platelet phospholipase $A_2$. The purified enzyme (6 ng) was incubated with 0.5 nmol of 1-acyl-2-[1-$^{14}$C]arachidonoyl-GPC in 100 mM Tris-HCl (pH 7.4) containing 2 mM EGTA and various concentrations of $CaCl_2$ in a total volume of 250 μl for 15 min at 37°C. The absolute concentration of free calcium was calculated as described [22]. Results are shown as means of three separate experiments.
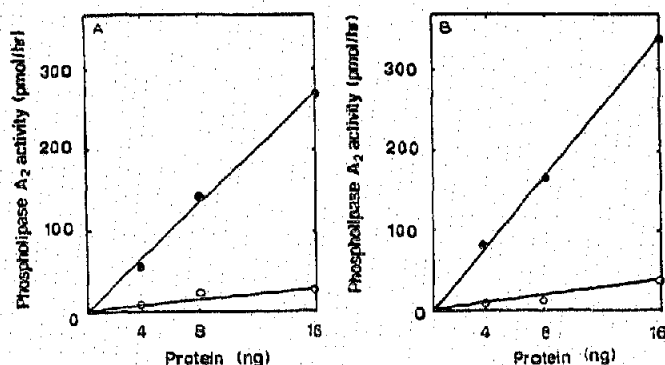


Fig. 4. Substrate specificity of purified human platelet phospholipase $A_2$. Activity was assayed with indicated amounts of purified enzyme under standard assay conditions except that a nmol of radioactive substrate was used. A, 1-acyl-2-[1-$^{14}$C]arachidonoyl-GPC (●), 1-acyl-2-[1-$^{14}$C]linoleoyl-GPC (○), B, 1-acyl-2-[1-$^{14}$C]arachidonoyl-GPE (●), 1-acyl-2-[1-$^{14}$C]linoleoyl-GPE (○).

acidic conditions caused irreversible loss of the activity; when incubated in 100 mM glycine-HCl buffer (pH 2) for 60 min at 4°C, neutralized, and then assayed, no enzymatic activity was detected.

## 4. DISCUSSION

From the soluble fraction of human platelet homogenates, we purified a phospholipase $A_2$ with a

molecular mass of about 90 kDa. It catalyzes the release of an arachidonoyl residue from the sn-2 position of phosphatidylcholine (PC) and PE more effectively than that of a linoleoyl residue. The catalytic activity of the purified 90-kDa enzyme increased sharply between $3 \times 10^{-7}$ and $10^{-6}$ M calcium ions. This calcium ion dependence roughly corresponds to the increase in free calcium ions induced upon stimulation of platelets [12]. These observations may indicate that the 90-kDa phospholipase $A_2$ participates in the stimulus-dependent release of arachidonate from either PC or PE.

Several investigators have detected and partially purified a phospholipase $A_2$ activity in acid-extracted fraction of human platelets [13,14]. In 1988, Kramer et al. [4] purified this acid-stable human platelet enzyme and found its molecular mass to be about 14 kDa. They concluded that its properties resembled those of the phospholipase $A_2$ purified from synovial fluid of patients with rheumatoid arthritis, suggesting that their enzyme can most probably be categorized as a mammalian group II phospholipase $A_2$. Antibodies raised against rat platelet secretory [15] and human synovial fluid [16] phospholipases $A_2$, both of which belong to the class of mammalian group II phospholipase $A_2$, failed to neutralize the purified 90-kDa phospholipase $A_2$ activity (data not shown). Thus, the 90-kDa enzyme distinctly differed from the mammalian group II enzyme. The findings that the purified 90-kDa phospholipase $A_2$ was labile under the acidic condition and that it exhibited a unique fatty acid preference and a high sensitivity to free calcium ion further strengthened this conclusion.

Besides the acid-extractable enzyme, the existence in human platelets of another type of phospholipase $A_2$ has been suggested by several authors [4,9,10,17,18]. For instance, a deoxycholate-solubilized activity was detected in human platelet homogenates which were prepared at neutral pH [9,10]. Judging from the subcellular distribution, the present 90-kDa enzyme with an arachidonoyl-residue preference would correspond to that activity.

We recently purified the 88-kDa phospholipase $A_2$ from rabbit platelet cytosolic fraction [19]. The human 90-kDa and rabbit 88-kDa enzymes exhibited very similar properties to each other. Like rat platelets [8], rabbit platelets contain a secretory 14-kDa group II phospholipase $A_2$ [20]. Since the group II phospholipase $A_2$ present in rabbit platelets is measurable without acid treatment, we previously detected two distinct phospholipase $A_2$ activities in the soluble fraction of rabbit platelets [21]. The data accumulated so far and discussed in part above are consistent with the conclusion that human platelets also bear at least two distinct phospholipase $A_2$; i.e. a secretory 14-kDa group II and intracellular 90-kDa ones. Indeed, when the human platelet homogenate was extracted

with an acidic solution, the phospholipase $A_2$ activity observed was completely neutralized with anti-human synovial fluid antibody, indicating that it was due to the mammalian group II enzyme (to be published). It should, however, be emphasized that no additional phospholipase $A_2$ activity was found during purification of the 90-kDa enzyme, suggesting that the human 14-kDa group II enzyme activity present in a crude platelet homogenate could not be detected under the conditions employed here. Some inhibitory factor(s) that is labile under acidic conditions might have prevented us from detecting it.

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