



Title	Purification and properties of phospholipase A2 isozymes from pyloric ceca of the starfish ( <i>asterina pectinifera</i> )
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Citation	Journal of Food Biochemistry, 28(3), 181-194
Issue Date	2004-08
Doc URL	<a href="http://hdl.handle.net/2115/8339">http://hdl.handle.net/2115/8339</a>
Type	article (author version)
Note	The definitive version is available at <a href="http://www.blackwell-synergy.com">www.blackwell-synergy.com</a>
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Running title: STARFISH PHOSPHOLIPASE A<sub>2</sub> ISOZYMES

**PURIFICATION AND PROPERTIES OF PHOSPHOLIPASE A<sub>2</sub> ISOZYMES  
FROM PYLORIC CECA OF THE STARFISH (*ASTERINA PECTINIFERA*)**

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## ABSTRACT

*Phospholipase A<sub>2</sub> isozyme II (PLA<sub>2</sub> II) which showed different mobility on native PAGE from that of the PLA<sub>2</sub> isozyme I (PLA<sub>2</sub> I) isolated previously was purified from pyloric ceca of the starfish (Asterina pectinifera). The PLA<sub>2</sub> II was mainly released oleic acid from 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. N-terminal amino acid sequence of the PLA<sub>2</sub> II was SVYQF. Temperature and pH optimums of the PLA<sub>2</sub> II were at around 50 and pH 9.0, respectively, and the enzyme activity was enhanced by sodium deoxycholate and 1 mM or higher concentration of Ca<sup>2+</sup>. The PLA<sub>2</sub> II did not show the fatty acid specificity for hydrolysis of phosphatidylcholine (PC). Specific activity of the PLA<sub>2</sub> II was about 10 times higher than that of commercially available porcine pancreatic PLA<sub>2</sub>. The PLA<sub>2</sub> II hydrolyzed PC more effectively than phosphatidylethanolamine. These characteristics of the PLA<sub>2</sub> II were the same as those of the PLA<sub>2</sub> I.*

## INTRODUCTION

Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) (EC3.1.1.4) catalyzes the selective hydrolysis of the *sn*-2-acyl group in 1, 2-diacyl-*sn*-glycero-3-phospholipids. PLA<sub>2</sub> is widely distributed in tissues of various organisms and is classified into extracellular and intracellular types. Extracellular PLA<sub>2</sub> is abundant in mammalian pancreas and snake venom, and these enzymatic and structural characteristics have been well studied (Arni and Ward 1996; Dennis 1983).

On the other hand, the PLA<sub>2</sub>s from mammalian pancreas and snake venom have been used as biochemical reagent. Stoll (1996) employed snake venom PLA<sub>2</sub> to analyze the positional distribution of fatty acids in glycerophospholipids from guinea pig and pig cardiac membranes. Mine (1997) examined structural and functional changes of hen egg-yolk low density lipoproteins (LDL) as a result of modifying its phospholipids using porcine pancreatic PLA<sub>2</sub>. Additionally, commercial PLA<sub>2</sub> mainly produced from porcine pancreas is used for industrial processes in food industry. Soy lysophosphatidylcholine (lysoPC) which is an excellent emulsifier for food is prepared by porcine pancreatic PLA<sub>2</sub>-catalyzed hydrolysis of soy phosphatidylcholine (PC) (Aoi, 1990). The emulsion with soy lysoPC is stable in various conditions at high temperature, acidic solution and high salt concentration. Soy lysoPC is also a good solubilizer, and the interaction between soy lysoPC and protein is very strong. Dahlke *et al.* (1995) reported that PLA<sub>2</sub> is suitable for the enzymatic degumming of edible oils. The enzymatic degumming of crude edible oils reduces the amounts of acids, bases, and wastes during the refining process. It also allows the extraction of PC and lysoPC as a valuable byproduct for the fortification of other foods. Recently, it was shown that LDL modified by PLA<sub>2</sub> was removed from the circulation to the liver more rapidly than unmodified LDL (Labeque *et al.* 1993). Based on this finding, a novel therapy for hypercholesterolemia has been recently developed that utilizes immobilized PLA<sub>2</sub> contained in an extracorporeal shunt.

In contrast, few studies exist on enzymology and application of PLA<sub>2</sub> from marine invertebrates. In the previous study, we isolated PLA<sub>2</sub> isozyme I (PLA<sub>2</sub> I) from the pyloric ceca of starfish (*Asterina pectinifera*), and showed its enzymatic properties comparing with those of mammalian pancreatic PLA<sub>2</sub> (Kishimura and Hayashi 1999b). The specific activity of the starfish PLA<sub>2</sub> I for PC was about 30 times higher than that of the commercially available PLA<sub>2</sub> from porcine pancreas (Sigma). In addition, the starfish PLA<sub>2</sub> I hydrolyzed PC more effectively than phosphatidylethanolamine (PE) like snake venom PLA<sub>2</sub>, but not mammalian pancreatic PLA<sub>2</sub>.

In this study, we purified PLA<sub>2</sub> isozyme II (PLA<sub>2</sub> II) from the pyloric ceca of starfish (*A. pectinifera*) and examined the characteristics of the PLA<sub>2</sub> II.

## MATERIALS AND METHODS

Starfish (*A. pectinifera*) and squid (*Todarodes pacificus*) were caught off Sarufutsu and Hakodate, Hokkaido Prefecture, Japan, in April 2001 and September 2002, respectively. These specimens were stored at -20 °C for a few months. Porcine pancreatic PLA<sub>2</sub> were purchased from Sigma (St. Louis, MO, USA) and Amano Pharmaceutical Co. (Nagoya, Japan). Egg-yolk PC was purchased from Wako Pure Chemicals (Osaka, Japan). 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

Crude enzyme solution was prepared by the same method as described by Kishimura and Hayashi (1999a). The crude enzyme solution was applied on a column (3.9×44 cm) of Sephacryl S-200 pre-equilibrated with 50 mM Tris-HCl buffer (pH8.0) and PLA<sub>2</sub> was eluted with the same buffer. Main active fraction was concentrated by lyophilization and dialyzed against 10 mM Tris-HCl buffer (pH8.0). The dialyzate was applied on a column (1.1×18 cm) of DEAE-cellulose

pre-equilibrated with 10 mM Tris-HCl buffer (pH8.0). PLA<sub>2</sub> was eluted with a linear gradient of NaCl from 0 to 0.5 M in 10 mM Tris-HCl buffer (pH8.0), and a main active fraction was obtained. The fraction was dialyzed against 10 mM Tris-HCl buffer (pH8.0) and the dialyzate was concentrated by lyophilization. The concentrate was applied on a column (3.9×64 cm) of Sephadex G-50 pre-equilibrated with 10 mM Tris-HCl buffer (pH8.0) and PLA<sub>2</sub> was eluted with the same buffer. Consequently, two active fractions were obtained. One of the two fractions was concentrated and re-chromatographed with Sephadex G-50. The final enzyme preparation (PLA<sub>2</sub> II) was found to be nearly homogeneous using SDS-PAGE (Fig. 1a) and native PAGE (Fig. 1b). The PLA<sub>2</sub> II was purified 52-fold from the crude enzyme solution in a yield of 7 % (Table 1).

Extraction of tissue lipids and lipid analysis using thin-layer chromatography (TLC), preparative TLC, TLC-frame ionization detector method (TLC/FID), and gas-liquid chromatography (GLC) were performed as described by Hayashi (1989) and Hayashi and Kishimura (1996). PC and PE were prepared from the squid mantle muscle lipids, using preparative TLC with chloroform-methanol-acetic acid-water (55:17:3:2, v/v/v/v) as a developing solvent.

The PLA<sub>2</sub> activity was measured as described by Kishimura and Hayashi (1999b). One unit of enzyme activity was defined as the number of  $\mu\text{g}$  of the substrate hydrolyzed per min. Positional specificity and polar group specificity for PC and /or PE were analyzed by the method of Kishimura and Hayashi (1999b).

PAGE was carried out using a 12.5 % polyacrylamide slab-gel with a Tris-HCl buffer at pH 8.9. SDS-PAGE was carried out using a 0.1 % SDS-14 % polyacrylamide slab-gel by the method of Laemmli (1970). The gel was stained with 0.1 % Coomassie Brilliant Blue R-250 in 50 % methanol-7 % acetic acid and the background of the gel was destained with 7 % acetic acid.

To analyze the N-terminal sequence of the purified PLA<sub>2</sub>, the enzyme was electroblotted to polyvinylidenedifluoride (PVDF) membrane after SDS-PAGE. The amino acid sequence of the

enzyme was analyzed by using a protein sequencer, Procise 492 (Perkin Elmer, Foster City, CA, USA).

The protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

## RESULTS AND DISCUSSION

PLA<sub>2</sub> II which showed different mobility on native PAGE from the PLA<sub>2</sub> I previously isolated (Kishimura and Hayashi 1999b) was purified from the pyloric ceca of starfish *A. pectinifera*. Although the purified PLA<sub>2</sub> II showed the same molecular weight with PLA<sub>2</sub> I (about 20,000) on SDS-PAGE (Fig. 1a), the mobility of the starfish PLA<sub>2</sub> II on native PAGE was slightly greater than that of the starfish PLA<sub>2</sub> I (Fig. 1b). The positional specificity of the purified PLA<sub>2</sub> II was examined using POPC. The PLA<sub>2</sub> II released mainly oleic acid from POPC similar to the PLA<sub>2</sub> I and porcine pancreatic PLA<sub>2</sub> (Table 2).

The N-terminal amino acid sequence of the *A. pectinifera* PLA<sub>2</sub> II was determined after separation by SDS-PAGE and electroblotting to PVDF membrane. A sequence of five amino acid residues, SVYQF, was determined, indicating that the N-terminus of the PLA<sub>2</sub> II was unblocked. This sequence was completely consistent with that of the PLA<sub>2</sub> I (Kishimura *et al.* 2000). It was reported that the amino acid residues at positions 4 and 5 were Gln and Phe, respectively, in mammalian pancreatic PLA<sub>2</sub> and snake venom PLA<sub>2</sub> (Dennis 1983). These residues were conserved in the PLA<sub>2</sub> I and PLA<sub>2</sub> II.

The molecular weight of the *A. pectinifera* PLA<sub>2</sub> I and PLA<sub>2</sub> II was estimated as approximately 20,000 using SDS-PAGE, which was larger than those of mammalian pancreatic PLA<sub>2</sub> (about 14,000) (Fleer *et al.* 1991), red sea bream (*Pagrus major*) pyloric ceca PLA<sub>2</sub> (14,000)

(Iijima *et al.* 1997), and starfish (*Solaster paxillatus*) pyloric ceca PLA<sub>2</sub> (about 13,000) (Kishimura and Hayashi 1998). Mammalian pancreatic PLA<sub>2</sub> is secreted as a precursor and is activated by tryptic hydrolysis (Verheij and De Haas 1991). Previously, we found that the crude enzyme solution prepared from fresh pyloric ceca of the starfish (*A. pectinifera*) showed a high phospholipase A activity (1,427 U/mg) similar to those from samples frozen at -20 °C for 35 or 46 months (1,214 and 1,655 U/mg, respectively) (Kishimura and Hayashi 1999a). Existence of the precursor of starfish PLA<sub>2</sub> was unknown.

Fig. 2 shows the pH and temperature dependences of the *A. pectinifera* PLA<sub>2</sub> II. The enzyme hydrolyzed egg-yolk PC substrate effectively at alkaline pH with an optimum activity of about pH 9.0, and it had an optimum temperature at about 50 °C. The pH dependence of the PLA<sub>2</sub> II was similar to those of starfish (*A. pectinifera*) pyloric ceca PLA<sub>2</sub> I (Kishimura and Hayashi 1999b), mammalian pancreatic PLA<sub>2</sub> (Arni and Ward 1996; Dennis 1983), red sea bream pyloric ceca PLA<sub>2</sub> (Iijima *et al.* 1997), and starfish (*S. paxillatus*) pyloric ceca PLA<sub>2</sub> (Kishimura and Hayashi 1998). Although the optimum temperature of the PLA<sub>2</sub> II was the same as starfish (*A. pectinifera*) pyloric ceca PLA<sub>2</sub> I (Kishimura and Hayashi 1999b), it was higher than that of starfish (*S. paxillatus*) pyloric ceca PLA<sub>2</sub> (about 30 °C) (Kishimura and Hayashi 1998).

The activity of the *A. pectinifera* PLA<sub>2</sub> II was enhanced by adding sodium deoxycholate at an optimum activity of 2 to 3 mM (Fig. 3). Fig. 4 shows the effect of CaCl<sub>2</sub> on the activity of PLA<sub>2</sub> II. The PLA<sub>2</sub> II was activated by 1 mM or higher concentration of Ca<sup>2+</sup>. The activity of the PLA<sub>2</sub> II was stimulated most by adding Ca<sup>2+</sup> followed by Mg<sup>2+</sup> and Co<sup>2+</sup>, while it was strongly inhibited by adding Hg<sup>2+</sup>, Zn<sup>2+</sup>, and EDTA (Table 3). These properties of the PLA<sub>2</sub> II was the same as those of the PLA<sub>2</sub> I (Kishimura and Hayashi 1999b), mammalian pancreatic PLA<sub>2</sub> (Arni and Ward 1996; Dennis 1983), and red sea bream pyloric ceca PLA<sub>2</sub> (Iijima *et al.* 1997).

The fatty acid specificity of the *A. pectinifera* PLA<sub>2</sub> II was examined using squid PC as a substrate. The compositions of fatty acids released from the substrate by the PLA<sub>2</sub> II were similar



to those released by the *A. pectinifera* PLA<sub>2</sub> I (Kishimura and Hayashi 1999b) and porcine pancreatic PLA<sub>2</sub> (Table 4). The PLA<sub>2</sub> I and PLA<sub>2</sub> II hydrolyzed the fatty acid ester bond exclusively at the glycerol-*sn*-2 position of PC regardless of chain length and degree of unsaturation, similar to mammalian pancreatic PLA<sub>2</sub> (Arni and Ward 1996; Dennis 1983) and starfish (*S. paxillatus*) pyloric ceca PLA<sub>2</sub> (Kishimura and Hayashi 1998).

The polar group specificity of the *A. pectinifera* PLA<sub>2</sub> II was examined using squid PC and PE. The PLA<sub>2</sub> II hydrolyzed PC more effectively than PE like the *A. pectinifera* PLA<sub>2</sub> I (Kishimura and Hayashi 1999b), but not porcine pancreatic PLA<sub>2</sub>, which hydrolyzed PC almost equally to PE (Fig. 5). Mammalian pancreatic PLA<sub>2</sub> hydrolyzed PC almost equally to PE (De Haas *et al.* 1968), and snake venom PLA hydrolyzed PC more effectively than PE (Ibrahim *et al.* 1964). Also mammalian non-pancreatic extracellular PLA<sub>2</sub> hydrolyzed PE more effectively than PC (Hara *et al.* 1989). In this study, the PLA<sub>2</sub> II hydrolyzed PC more effectively than PE similar to the PLA<sub>2</sub> I (Kishimura and Hayashi 1999b) and snake venom PLA<sub>2</sub> (Ibrahim *et al.* 1964). Kuipers *et al.* (1989) reported that a recombinant porcine pancreatic PLA<sub>2</sub> mutant with a deletion of the pancreatic loop at positions 62-66 gave an intermediate conformation between wild type porcine PLA<sub>2</sub> and snake venom PLA<sub>2</sub>, and enhanced the catalytic activity on zwitterionic substrates. Therefore, possibly the primary structures of PLA<sub>2</sub> I and PLA<sub>2</sub> II from the pyloric ceca of starfish (*A. pectinifera*) differed from that of mammalian pancreatic PLA<sub>2</sub> at the corresponding region to the pancreatic loop.

The specific activities of the *A. pectinifera* PLA<sub>2</sub> I and PLA<sub>2</sub> II for egg-yolk PC were 119,000 U/mg (Kishimura and Hayashi 1999b) and 52,000 U/mg, respectively. These activities were 27 times and 12 times higher, respectively, than that of the reagent grade PLA<sub>2</sub> from porcine pancreas (Sigma) (4,300 U/mg) measured using the same assay system.

In conclusion, the PLA<sub>2</sub> isozymes (PLA<sub>2</sub> I and PLA<sub>2</sub> II) from the pyloric ceca of starfish (*A. pectinifera*) hydrolyzed PC more effectively than PE unlikely porcine pancreatic PLA<sub>2</sub>, which

hydrolyzed PC almost equally to PE. In addition, the specific activities of these isozymes for PC were 27 times (PLA<sub>2</sub> I) and 12 times (PLA<sub>2</sub> II) higher, respectively, than that of commercially available PLA<sub>2</sub> from porcine pancreas (Sigma). These results suggest that the pyloric ceca of the starfish (*A. pectinifera*) would be a potential source of PLA<sub>2</sub>.

## ACKNOWLEDGMENT

The authors wish to thank Mr. Y. Abe, the Center for Instrumental Analysis, Hokkaido University, for amino acid sequence analysis.

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(captions to figures)

FIG. 1. ELECTROPHORESIS OF PURIFIED PLA<sub>2</sub> AND PLA<sub>2</sub> FROM THE STARFISH *ASTERINA PECTINIFERA*.

a: Electrophoresis was performed using a 0.1 % SDS-14 % polyacrylamide slab-gel. Lane 1 contains *A. pectinifera* PLA<sub>2</sub>. Lane 2 contains *A. pectinifera* PLA<sub>2</sub>. Lane 3 contains protein standards; bovine pancreatic trypsinogen (molecular weight, 24,000), bovine milk -lactoglobulin (18,400), and egg-white lysozyme (14,300). b: Electrophoresis was performed using a 12.5 % polyacrylamide slab-gel at pH 8.9. Lane 1 contains *A. pectinifera* PLA<sub>2</sub>. Lane 2 contains *A. pectinifera* PLA<sub>2</sub>.

FIG. 2. EFFECTS OF PH AND TEMPERATURE ON THE ACTIVITY OF PLA<sub>2</sub> AND PLA<sub>2</sub> FROM THE STARFISH *ASTERINA PECTINIFERA*.

a: The activities were determined in 50 mM buffer solutions [acetic acid-sodium acetate (pH 4.0-7.0), Tris-HCl (pH 7.0-9.0), and glycine-NaOH (pH 9.0-11.0)] at 37 °C. b: The activities were determined at pH 8.5 and at various temperatures. *A. pectinifera* PLA<sub>2</sub>: closed symbol (Kishimura and Hayashi 1999b). *A. pectinifera* PLA<sub>2</sub>: open symbol.

FIG. 3. EFFECT OF SODIUM DEOXYCHOLATE ON THE ACTIVITY OF PLA<sub>2</sub> AND PLA<sub>2</sub> FROM THE STARFISH *ASTERINA PECTINIFERA*.

The activities were determined at pH 8.5 and at various concentration of sodium deoxycholate. *A. pectinifera* PLA<sub>2</sub>: closed symbol (Kishimura and Hayashi 1999b). *A. pectinifera* PLA<sub>2</sub>:

open symbol.

FIG. 4. EFFECT OF CALCIUM ION ON THE ACTIVITY OF PLA<sub>2</sub> AND PLA<sub>2</sub> FROM THE STARFISH *ASTERINA PECTINIFERA*.

The activities were determined at pH 8.5 and at various concentration of CaCl<sub>2</sub>. *A. pectinifera* PLA<sub>2</sub> : closed symbol (Kishimura and Hayashi 1999b). *A. pectinifera* PLA<sub>2</sub> : open symbol.

FIG. 5. TIME-COURSE OF HYDROLYSIS OF PHOSPHATIDYLCHOLINE AND PHOSPHATIDYLETHANOLAMINE WITH PLA<sub>2</sub> AND PLA<sub>2</sub> FROM THE STARFISH *ASTERINA PECTINIFERA* AND PORCINE PANCREATIC PLA<sub>2</sub>.

a: *A. pectinifera* PLA<sub>2</sub> ; closed symbol (Kishimura and Hayashi 1999b), *A. pectinifera* PLA<sub>2</sub> ; open symbol. b: porcine pancreatic PLA<sub>2</sub>. Circle: phosphatidylcholine. Triangle: phosphatidylethanolamine.

Fig. 1

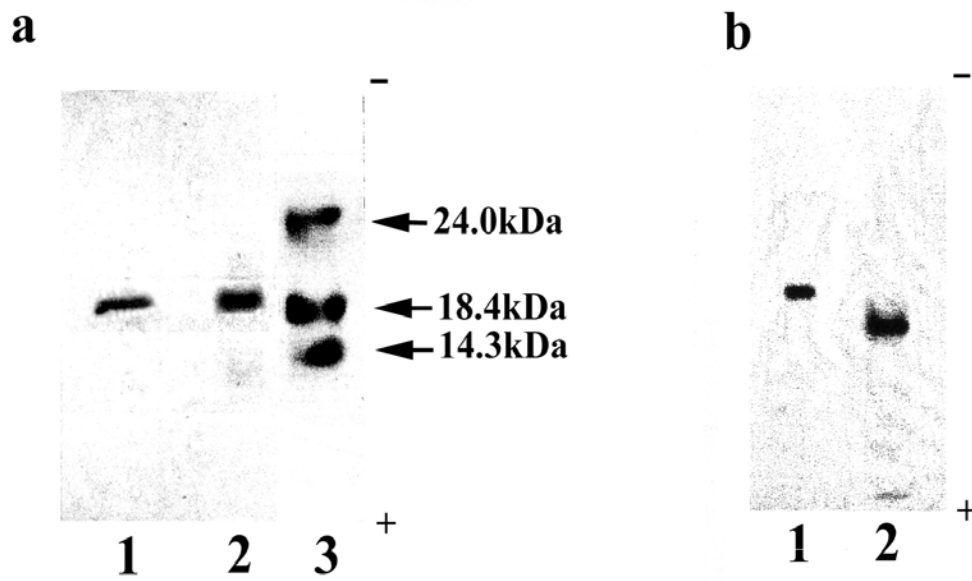


Fig.2

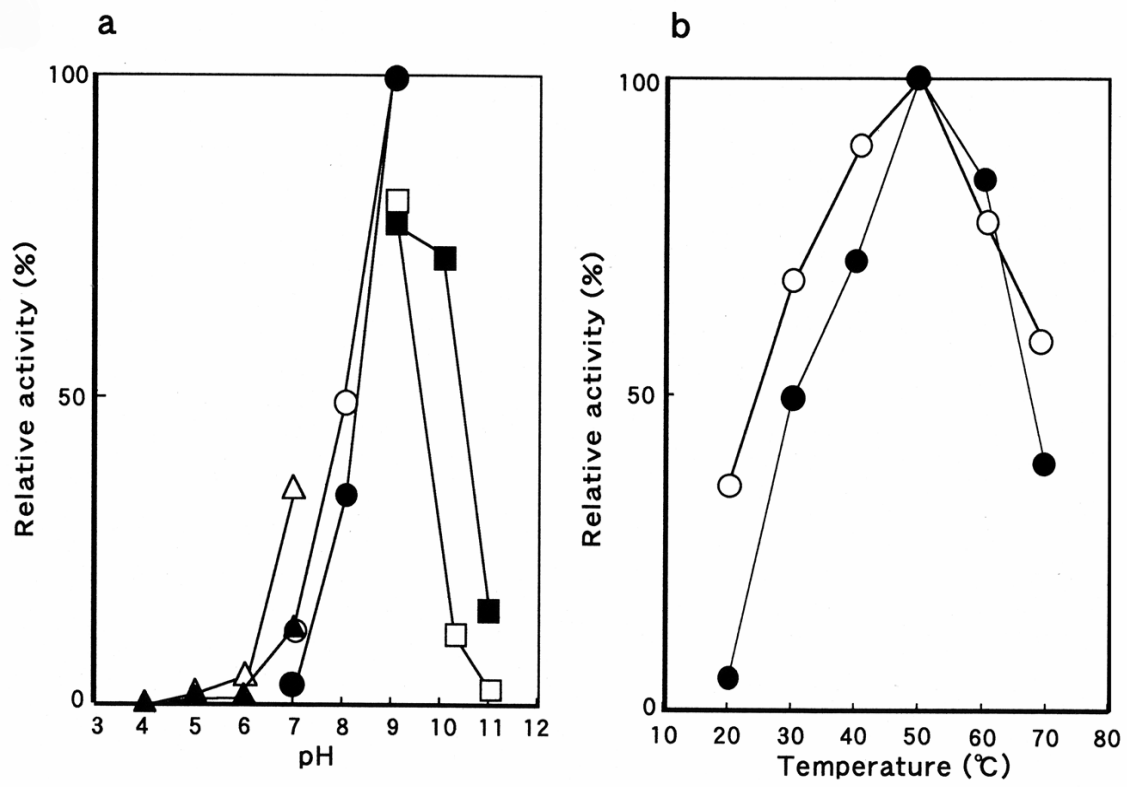




Fig.3

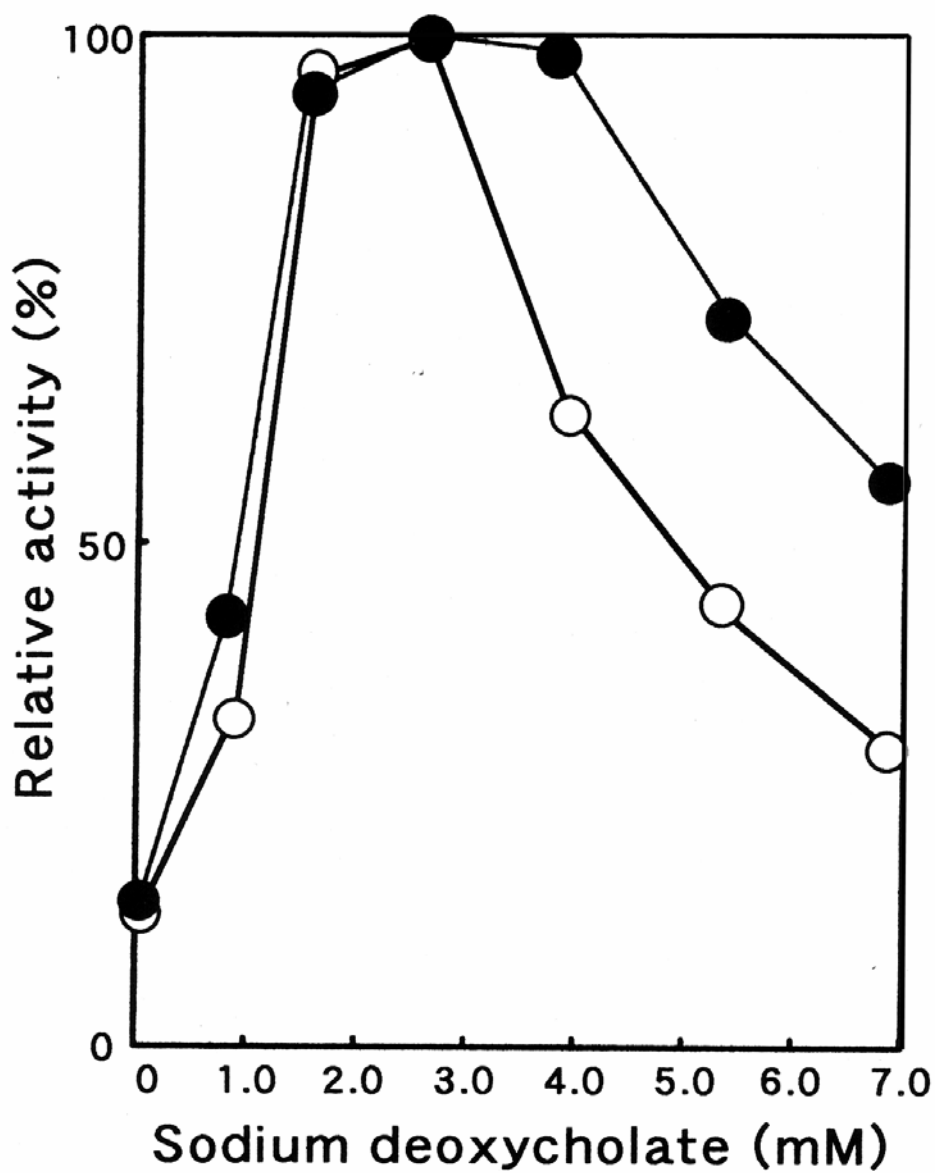


Fig. 4

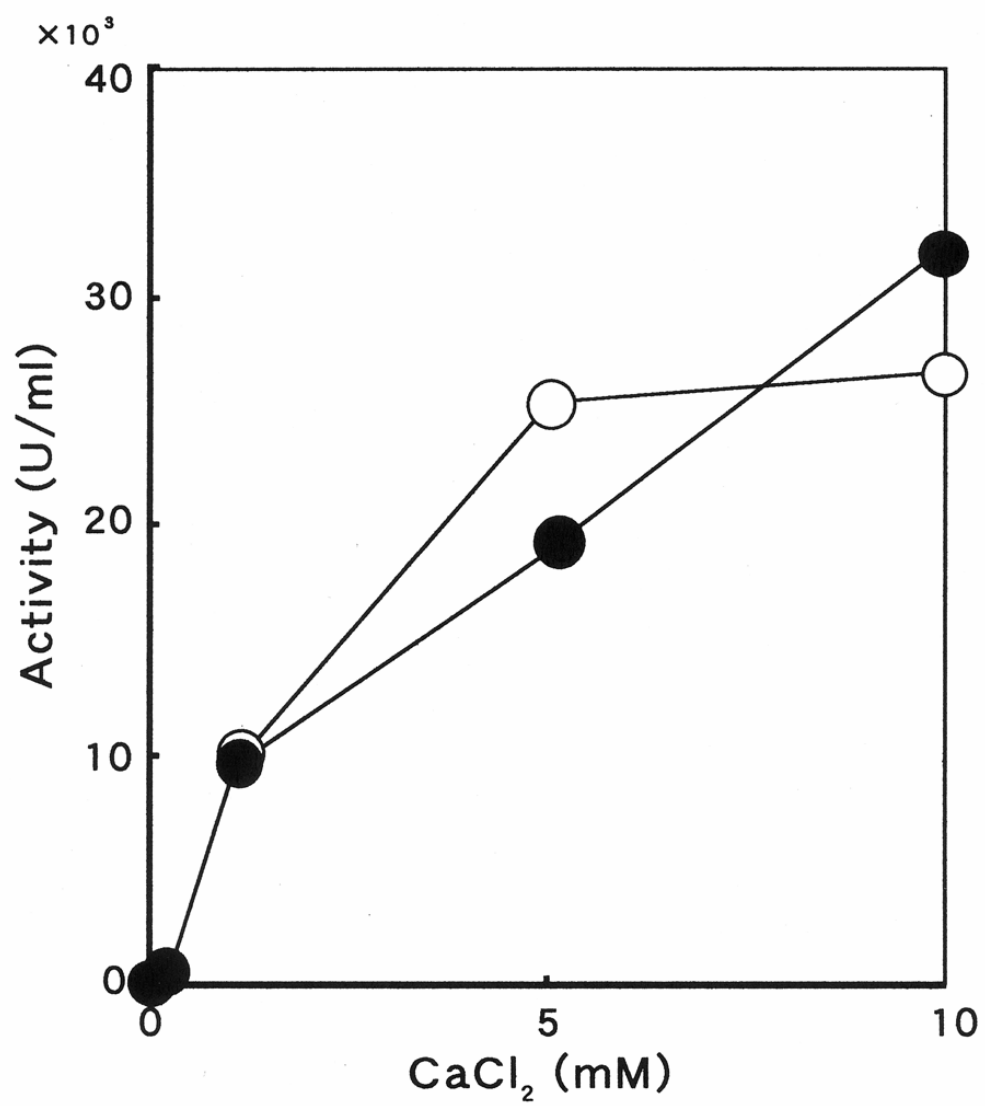


Fig. 5

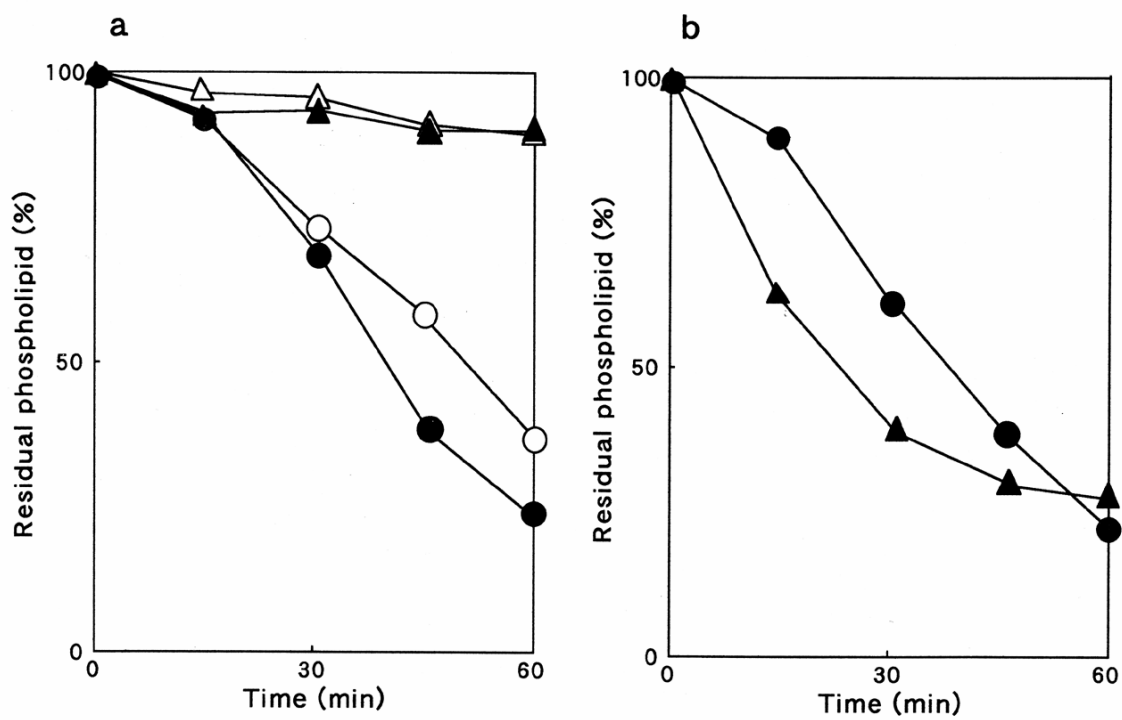


TABLE 1.  
PURIFICATION OF PLA2 FROM THE PYLORIC CECA OF STARFISH *ASTERINA PECTINIFERA*

Purification Step	Total Protein (mg)	Total Activity (U <sup>1</sup> ) × 10 <sup>3</sup>	Specific Activity (U/mg) × 10 <sup>3</sup>	Yield (%)	Purity (fold)
Crude Extract	1858	2141	1.2	100	1
Sephacryl S-200	610	1462	2.4	68	2
DEAE-Cellulose	82	631	7.7	29	6
Sephadex G-50 (1st)	9.9	196	20	9	17
Sephadex G-50 (2nd)	2.7	140	52	7	43

<sup>1</sup> One unit (U) of activity was defined as one  $\mu$ g of phosphatidylcholines hydrolyzed per min. DEAE - diethylaminoethyl.

TABLE 2.  
 COMPOSITION OF FATTY ACIDS RELEASED FROM 1-PALMITOYL-2-OLEOYL-*SN*-GLYCERO-3-  
 PHOSPHOCHOLINE BY PLA<sub>2</sub> AND PLA<sub>2</sub> FROM THE PYLORIC CECA OF STARFISH *ASTERINA PECTINIFERA* (%)

Fatty acid	PLA <sub>2</sub> <sup>1</sup>	PLA <sub>2</sub>	Porcine enzyme <sup>2</sup>
16:0	16.2	15.0	15.4
18:1n-9	83.8	85.0	84.6

<sup>1</sup> Kishimura and Hayashi (1999b).

<sup>2</sup> Phospholipase A<sub>2</sub> from porcine pancreas (Amano Pharmaceutical Co.).

TABLE 3.  
EFFECTS OF METAL IONS AND EDTA ON THE ACTIVITY OF PLA<sub>2</sub> AND PLA<sub>2</sub> FROM THE PYLORIC CECA  
OF STARFISH *ASTERINA PECTINIFERA*

Compound	Final Concentration (mM)	Relative Activity (%) <sup>1</sup>	
		PLA <sub>2</sub> <sup>2</sup>	PLA <sub>2</sub>
None	0	100	100
CaCl <sub>2</sub>	10	1,496	1,429
MgCl <sub>2</sub>	10	601	683
CoCl <sub>2</sub>	10	229	332
HgCl <sub>2</sub>	10	18	32
ZnCl <sub>2</sub>	10	4	13
EDTA	10	11	26

<sup>1</sup> The activities were expressed as percentage of that with absence of metal ion, respectively.

<sup>2</sup> Kishimura and Hayashi (1999b).

EDTA-ethylenediaminetetraacetic acid.

TABLE 4.  
COMPOSITION OF FATTY ACIDS RELEASED FROM SQUID MANTLE MUSCLE PHOSPHATIDYLCHOLINE BY PLA<sub>2</sub> AND PLA<sub>2</sub> FROM  
THE PYLORIC CECA OF STARFISH *ASTERINA PECTINIFERA* (%)

Fatty Acid	PLA <sub>2</sub> <sup>1</sup>	PLA <sub>2</sub>	Porcine Enzyme <sup>2</sup>
16:0	2.9	3.7	1.6
16:1n-9	0.3	0.3	0.2
16:1n-7	0.3	0.3	0.2
18:0	1.0	0.9	1.1
18:1n-9	1.2	1.6	1.3
18:1n-7	0.5	0.8	0.4
18:2n-6	0.2	0.1	0.1
18:4n-3	0.1	0.1	0.1
20:1n-11	0.3	0.6	0.6
20:1n-9	0.1	0.2	0.1
20:4n-6	1.5	1.6	1.5
20:5n-3	13.5	14.2	14.3
21:5n-3	0.2	0.3	0.2
22:5n-6	0.8	0.7	0.8
22:5n-3	0.3	0.3	0.2
22:6n-3	76.8	74.3	77.3
Others <sup>3</sup>	tr	tr	tr

<sup>1</sup> Kishimura and Hayashi (1999b).

<sup>2</sup> Phospholipase A<sub>2</sub> from porcine pancreas (Amano Pharmaceutical Co.).

<sup>3</sup> Consisted of minor (less than 1.0 %) and unknown compounds.

tr - trace (less than 0.05 %)