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## Partial purification and characteristics of phospholipase A<sub>2</sub> from pyloric ceca of starfish *Plazaster borealis*

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### Abstract

Phospholipase A activities in the pyloric ceca and viscera from six species of marine invertebrates were determined. A high activity of the enzyme was found in the pyloric ceca of the starfish *Plazaster borealis*. Phospholipase A<sub>2</sub> was partially purified from the pyloric ceca of the *P. borealis*. The enzyme was purified 12-fold from the crude enzyme solution with a yield of 5 %. The optimum pH and temperature of the enzyme were approximately 10.0 and 50°C, respectively, and the activity was enhanced by Ca<sup>2+</sup> at 1 mM or higher. The enzyme had no fatty acid specificity. Partially purified *P. borealis* phospholipase A<sub>2</sub> hydrolyzed phosphatidylcholine more effectively than phosphatidylethanolamine similar to other starfish phospholipase A<sub>2</sub>.

**Key words:** Marine invertebrate, Phosphatidylcholine, Phospholipase A<sub>2</sub>, *Plazaster borealis*, Polar group specificity, Pyloric cecum, Starfish

### 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 and the production of fatty acids and lysophospholipids. PLA<sub>2</sub> is widely distributed in the tissues of various organisms and is classified into extracellular and intracellular types (Dennis, 1997). Extracellular PLA<sub>2</sub> is abundant in mammalian pancreas and snake venom, and the enzymatic properties and amino acid sequences have been well characterized (Dennis, 1983; Arni and Ward, 1996).

On the other hand, there are few studies on PLA<sub>2</sub> present in digestive glands of marine invertebrates. Recently, we have found high phospholipase A (PLA) activities in crude enzyme fractions from the pyloric ceca of the starfish *Solaster paxillatus* and *Asterina pectinifera* and succeeded in isolating a PLA<sub>2</sub> (Kishimura and Hayashi, 1998; Kishimura and Hayashi, 1999a; Kishimura and Hayashi, 1999b). The starfish PLA<sub>2</sub> exhibited similar enzymatic properties to mammalian pancreatic PLA<sub>2</sub> in terms of Ca<sup>2+</sup> requirement, fatty acid specificity and optimum pH. However, the specific activity of *A. pectinifera* PLA<sub>2</sub> was markedly higher than that of commercially available PLA<sub>2</sub> from porcine pancreas. In addition, *A. pectinifera* PLA<sub>2</sub> showed distinct substrate specificity hydrolyzing phosphatidylcholine more efficiently than phosphatidylethanolamine, whereas the porcine PLA<sub>2</sub> hydrolyzed them almost

equally. Recently, we have reported the complete amino acid sequence of *A. pectinifera* PLA<sub>2</sub> and its structural characteristics, compared with the porcine pancreatic PLA<sub>2</sub> (Kishimura et al., 2000a; Kishimura et al., 2000b). *A. pectinifera* PLA<sub>2</sub> consisted of 137 amino acids with a Mr 15,300 and contained 14 Cys residues at positions that have been shown to be involved in the formation of intramolecular disulfide bonds in the porcine pancreatic PLA<sub>2</sub>. The amino acid sequences of the putative active site and Ca<sup>2+</sup>-binding loop of *A. pectinifera* PLA<sub>2</sub> showed a moderately high homology to those of porcine pancreatic PLA<sub>2</sub>. However, in the alignment of the amino acid sequences of *A. pectinifera* PLA<sub>2</sub> and porcine pancreatic PLA<sub>2</sub>, two amino acid deletions in the pancreatic loop region, and sixteen insertions and three deletions in the  $\beta$ -wing region were required to maximize the sequence homology. In the previous study, we constructed a bacterial expression system for *A. pectinifera* PLA<sub>2</sub>, and determined some biochemical properties of the recombinant PLA<sub>2</sub> (Kishimura et al., 2001). Although N-terminal Ser in the native *A. pectinifera* PLA<sub>2</sub> was replaced by Ala in the recombinant PLA<sub>2</sub>, the recombinant PLA<sub>2</sub> showed essentially the same properties as those of the native PLA<sub>2</sub> with respect to specific activity, substrate specificity, optimum pH and temperature, and Ca<sup>2+</sup> requirement.

As noted above, PLA<sub>2</sub> from the pyloric ceca of the starfish *A. pectinifera* has functional and structural

properties different from those of mammalian pancreatic PLA<sub>2</sub>. In this study, to clarify whether the properties of *A. pectinifera* PLA<sub>2</sub> are common among asteroids, we determined PLA activities in pyloric ceca and viscera from six species of marine invertebrates and partially purified PLA<sub>2</sub> from the pyloric ceca of the starfish *Plazaster borealis*, and examined the characteristics of this enzyme.

## Materials and Methods

### Materials

The starfish (*P. borealis*, *Solaster borealis* and *Aphelasterias japonica*), sea urchin (*Strongylocentrotus franciscanus*), shellfish (*Neptunea arthritica* and *Patinopecten yessoensis*) and squid (*Todarodes pacificus*) were caught off Hakodate, Hokkaido Prefecture, Japan and were stored at -20°C until use. Porcine pancreatic PLA<sub>2</sub> was purchased from Sigma (St. Louis, MO, USA) and Amano Pharmaceutical Co. (Nagoya, Japan). Egg yolk phosphatidylcholine (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).

### Preparation of crude enzyme solution

The crude enzyme solution was prepared from the pyloric ceca and viscera of the invertebrates by the method of Kishimura and Hayashi (1999a).

### Partial purification of starfish PLA<sub>2</sub>

PLA<sub>2</sub> was partially purified from the crude enzyme extracted from the defatted powder of the pyloric ceca of *P. borealis* using sequential column chromatography: gel filtration on Sephacryl S-200, diethylaminoethyl (DEAE)-cellulose anion-exchange column chromatography, and gel filtration on Sephadex G-50 (Kishimura and Hayashi, 1999b).

### Lipid extraction and analysis

The extraction of tissue lipids and lipid analysis by thin-layer chromatography (TLC), preparative TLC, TLC-frame ionization detection method (TLC/FID), and gas-liquid chromatography (GLC) were performed as described by Hayashi (1989) and Hayashi and Kishimura (1996).

PC and phosphatidylethanolamine (PE) were prepared from the total lipids of the squid mantle muscle using preparative TLC with chloroform-methanol-acetic acid-water (55:17:3:2, v/v/v/v) as a developing solvent.

### PLA<sub>2</sub> activity assay

PLA<sub>2</sub> activity was measured as described by Kishimura and Hayashi (1999b). One unit of enzyme activity was defined as the micrograms of PC hydrolyzed per min.

Positional specificity, fatty acid specificity, and polar group specificity were analyzed by the method of Kishimura and Hayashi (1999b).

### Protein determination

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

## Results

### PLA activity in viscera of several marine invertebrates

We compared PLA activities in the pyloric ceca and viscera from six species of marine invertebrates. As shown in Table 1, maximum PLA activity was detected in *P. borealis*, followed by *S. borealis*. Low PLA activities were detected in *S. franciscanus*, *N. arthritica* and *P. yessoensis*, and *A. japonica*.

### Properties of *P. borealis* partially purified PLA<sub>2</sub>

Because a relatively high activity was detected in the pyloric ceca of *P. borealis*, PLA<sub>2</sub> was partially purified from the crude enzyme extracted from the defatted powder of the pyloric ceca of the starfish (Table 2). The enzyme, which was purified 12-fold from the crude enzyme solution with a yield of 5 %, included several proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The positional specificity of the partially purified *P. borealis* PLA<sub>2</sub> was examined using POPC. The enzyme mainly released oleic acid from POPC similar to the porcine pancreatic PLA<sub>2</sub> (Table 3). Figure 1 shows the pH and temperature dependence of the *P. borealis* PLA<sub>2</sub>. The enzyme hydrolyzed egg yolk PC effectively at an alkaline pH with an optimum activity at about pH 10.0 (Fig. 1a), and an optimum temperature at about 50°C (Fig. 1b). Figure 2 shows the effects of CaCl<sub>2</sub> on *P. borealis* PLA<sub>2</sub> activities. The enzyme was activated by Ca<sup>2+</sup> at 1 mM or higher. The fatty acid specificity of the *P. borealis* PLA<sub>2</sub> was examined using egg yolk PC as a substrate. The composition of fatty acids released from the substrate by the *P. borealis* PLA<sub>2</sub> was similar to that released by the porcine pancreatic PLA<sub>2</sub> (Table 4). The polar group specificity of the *P. borealis* PLA<sub>2</sub> was examined using squid PC and PE. The *P. borealis* PLA<sub>2</sub> hydrolyzed PC more effectively than PE (Fig. 3).

Table 1. Phospholipase A activities in pyloric ceca and viscera of several marine invertebrates

	Organ	Activity (U/g powder)* <sup>1</sup>	Specific activity (U/mg)
Starfish			
<i>Plazaster borealis</i>	Pyloric cecum	2,000	17
<i>Solaster borealis</i>	Pyloric cecum	1,900	14
<i>Aphelasterias japonica</i>	Pyloric cecum	210	1.8
Sea urchin			
<i>Strongylocentrotus franciscanus</i>	Viscera	120	2.0
Shellfish			
<i>Neptunea arthritica</i>	Viscera	110	1.9
<i>Patinopecten yessoensis</i>	Hepatopancreas	70	0.6
Starfish			
<i>Asterina pectinifera</i> * <sup>2</sup>	Pyloric cecum	540,000	1,400
<i>Coscinasterias acutispina</i> * <sup>3</sup>	Pyloric cecum	5,400	17
<i>Solaster paxillatus</i> * <sup>2</sup>	Pyloric cecum	1,000	12
<i>Distolasterias nippon</i> * <sup>2</sup>	Pyloric cecum	70	0.5
<i>Asterias amurensis</i> * <sup>2</sup>	Pyloric cecum	27	0.5

\*<sup>1</sup>One unit (U) of activity was determined as the microgram of phosphatidylcholine hydrolyzed per minute.

\*<sup>2</sup>Kishimura and Hayashi (1999a).

\*<sup>3</sup>Koyama et al. (2001).

 Table 2. Purification of phospholipase A<sub>2</sub> of *P. borealis*

Purification step	Protein (mg)	Total activity (× 10 <sup>3</sup> U)*	Specific activity (U/mg)	Purity (fold)	Yield (%)
Crude enzyme solution	23,500	438	19	1	100
Sephacryl S-200	4,400	78	18	1	18
DEAE-Cellulose	600	38	63	3	9
Sephadex G-50	103	24	230	12	5

\*One unit of activity was defined as the microgram of phosphatidylcholine hydrolyzed per minute.

DEAE : diethylaminoethyl

 Table 3. Composition of fatty acids released from POPC by the partially purified phospholipase A<sub>2</sub> of *P. borealis*

Fatty acid	(wt%)* <sup>1</sup>	
	Starfish enzyme	Porcine enzyme* <sup>2</sup>
16:0	15.3	15.4
18:1n-9	84.7	84.6

\*<sup>1</sup>Weight % relative to total fatty acid.

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

## Discussion

The PLA activities in the pyloric ceca and viscera from six species of marine invertebrates were compared

with those in the pyloric ceca from five species of starfish previously reported (Kishimura and Hayashi, 1999a; Koyama et al., 2001). As shown in Table 1, the PLA activity in *A. pectinifera* was extremely high. High PLA activities were detected in *P. borealis*, *Coscinasterias acutispina*, *S. borealis* and *Solaster paxillatus*. Low PLA activities were detected in *A. japonica*, *Distolasterias nippon* and *Asterias amurensis*, *S. franciscanus*, and *N. arthritica* and *P. yessoensis*.

PLA<sub>2</sub> from the pyloric ceca of *P. borealis* was partially purified. The partially purified enzyme released mainly oleic acid from POPC. The enzyme had an optimum alkaline pH of about 10.0, and was activated by Ca<sup>2+</sup> at 1 mM or higher. These properties of the enzyme were similar to those of the mammalian pancreatic PLA<sub>2</sub> (Dennis, 1983; Arni and Ward, 1996) and

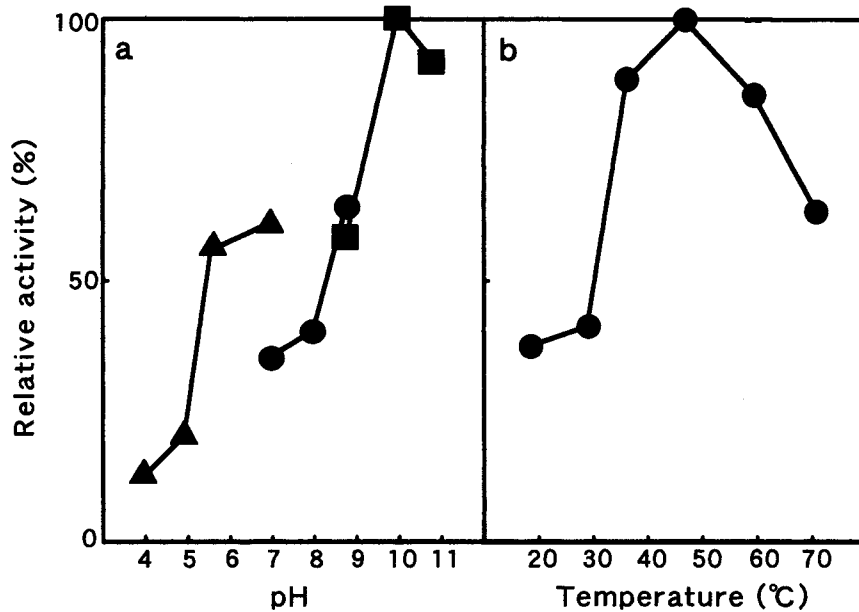


Fig. 1. Effects of pH and temperature on the activity of phospholipase A<sub>2</sub> of *P. borealis*. a: Reaction mixture containing 21  $\mu$ g of starfish phospholipase A<sub>2</sub>, 100  $\mu$ g of egg yolk PC, 2.7 mM sodium deoxycholate, and 5 mM CaCl<sub>2</sub> in a total volume of 130  $\mu$ l was incubated at 37°C for 10 min. The buffers used were 50 mM acetic acid-sodium acetate from pH 4.0 to 7.0 (▲), 50 mM Tris-HCl from pH 7.0 to 9.0 (●), and 50 mM glycine-NaOH from pH 9.0 to 11.0 (■). b: Reaction mixture containing 21  $\mu$ g of starfish phospholipase A<sub>2</sub>, 100  $\mu$ g of egg yolk PC, 2.7 mM sodium deoxycholate, 5 mM CaCl<sub>2</sub>, and 50 mM Tris-HCl (pH 8.5) in a total volume of 130  $\mu$ l was incubated at various temperatures for 10 min.

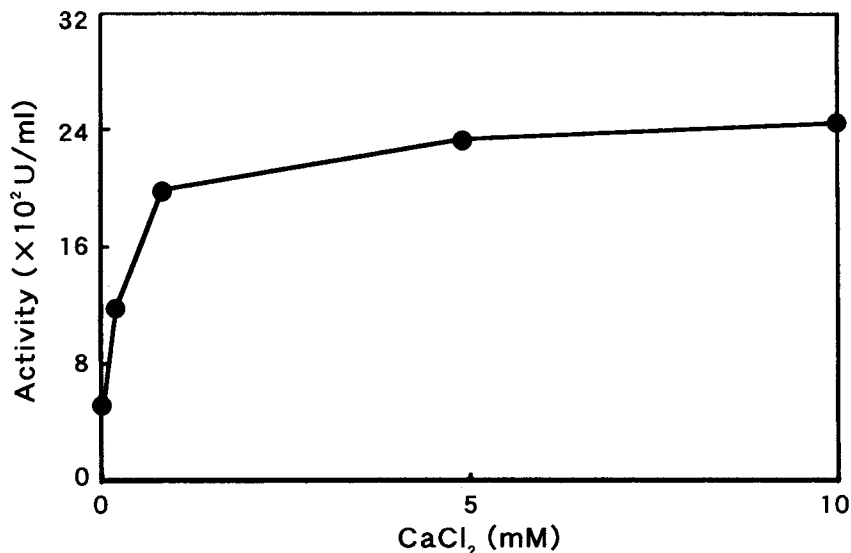


Fig. 2. Effects of Ca<sup>2+</sup> on activity of phospholipase A<sub>2</sub> of *P. borealis*. Reaction mixture containing 21  $\mu$ g starfish phospholipase A<sub>2</sub>, 100  $\mu$ g egg yolk PC, 2.7 mM sodium deoxycholate, 50 mM Tris-HCl buffer (pH 8.5), and various concentrations of CaCl<sub>2</sub> in a total volume of 130  $\mu$ l was incubated at 37°C for 10 min.

other starfish PLA<sub>2</sub> (Kishimura and Hayashi, 1998; Kishimura and Hayashi, 1999b; Koyama et al., 2001). Furthermore, the enzyme hydrolyzed fatty acid ester bond exclusively at the glycerol-*sn*-2 position of PC regardless of the chain length and degree of unsaturation, similar to the mammalian pancreatic PLA<sub>2</sub> (De

Haas et al., 1968) and other starfish PLA<sub>2</sub> (Kishimura and Hayashi, 1998; Kishimura and Hayashi, 1999b; Koyama et al., 2001). However, the optimum temperature (about 50°C) of the *P. borealis* PLA<sub>2</sub> in this study was higher than that of *S. paxillatus* PLA<sub>2</sub> (about 40°C) (Kishimura and Hayashi, 1998), but lower than that of

Table 4. Composition of fatty acids released from PC by the partially purified phospholipase A<sub>2</sub> of *P. borealis* (wt%)\*<sup>1</sup>

Fatty acid	Egg yolk PC	
	Starfish enzyme	Porcine enzyme* <sup>2</sup>
16:0	2.8	2.9
18:0	1.1	1.0
18:1n-9	55.9	57.6
18:1n-7	1.9	1.8
18:2n-6	26.9	24.2
20:4n-6	6.6	5.6
22:6n-3	1.1	1.6
Others* <sup>3</sup>	3.7	5.3

\*<sup>1</sup>Weight % relative to total fatty acid.

\*<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.

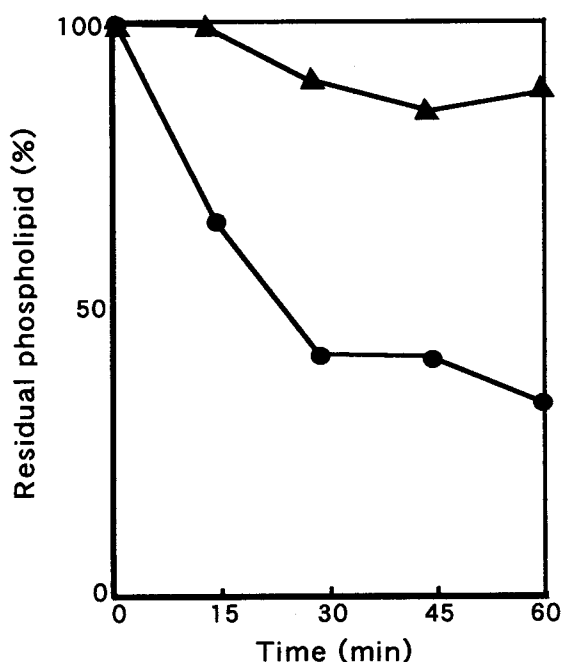


Fig. 3. Time course of phosphatidylcholine and phosphatidylethanolamine hydrolyses by phospholipase A<sub>2</sub> of *P. borealis*. Reaction mixture containing 21 μg starfish phospholipase A<sub>2</sub>, 100 μg PC (●) or PE (▲) prepared from squid mantle muscle, 2.7 mM sodium deoxycholate, 5 mM CaCl<sub>2</sub>, and 50 mM Tris-HCl buffer (pH 8.5) in a total volume of 130 μl was incubated at 37°C for various periods.

*C. acutispina* PLA<sub>2</sub> (about 60°C) (Koyama et al., 2001).

Although the mammalian pancreatic PLA<sub>2</sub> hydrolyzed PC almost equally to PE (De Haas et al., 1968; Hara et al., 1991), the snake venom PLA<sub>2</sub> hydrolyzed

PC more effectively than PE (Ibrahim et al., 1964). Moreover mammalian nonpancreatic extracellular PLA<sub>2</sub> hydrolyzed PE more effectively than PC (Chang et al., 1987; Hara et al., 1989; Mizushima et al., 1989). In this study, *P. borealis* PLA<sub>2</sub> hydrolyzed PC more effectively than PE similar to the snake venom PLA<sub>2</sub>. 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 provided an intermediate conformation between the wild type porcine PLA<sub>2</sub> and the snake venom PLA<sub>2</sub>, and enhanced the catalytic activity on zwitterionic substrates. In the alignment of the amino acid sequences of *A. pectinifera* PLA<sub>2</sub> and the porcine pancreatic PLA<sub>2</sub>, two amino acid deletions in the pancreatic loop region were required to maximize the sequence homology (Kishimura et al., 2000a; Kishimura et al., 2000b). Therefore, possibly the primary structure of *P. borealis* PLA<sub>2</sub> purified in this study also differed from that of the mammalian pancreatic PLA<sub>2</sub> at the region corresponding to the pancreatic loop.

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