Title	Isolation and characteristics of phospholipase A2 from the pyloric ceca of the starfish <i>Asterina pectinifera</i>
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Citation	Comparative Biochemistry and Physiology Part B Biochemistry and Molecular Biology, 124(4), 483-488 https://doi.org/10.1016/S0305-0491(99)00142-X
Issue Date	1999-12
Doc URL	http://hdl.handle.net/2115/16941
Type	article (author version)
File Information	CBPB124-4.pdf

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Phospholipase A₂ of starfish

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Isolation and characteristics of phospholipase A₂ from
the pyloric ceca of the starfish *Asterina pectinifera*

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Abstract

Phospholipase A₂ was purified from the pyloric ceca of the starfish *Asterina pectinifera*. The final enzyme preparation was nearly homogeneous in sodium dodecyl sulfate–polyacrylamide gel electrophoresis and its molecular weight was estimated as approximately 20,000. The optimum pH and temperature of the enzyme were at around pH 9.0 and 50 °C, respectively, and the activity was enhanced by sodium deoxycholate and 1 mM or higher concentration of Ca²⁺. The enzyme had no fatty acid specificity. Starfish phospholipase A₂ hydrolyzed phosphatidylcholine more effectively than phosphatidylethanolamine.

Keywords: *Asterina pectinifera*; Isolation; Marine invertebrate; Phosphatidylcholine; Phospholipase A₂; Polar group specificity; Pyloric cecum; Starfish.

1. Introduction

Phospholipase A₂ (PLA₂) (EC3.1.1.4) catalyzes the selective hydrolysis

of the *sn*-2-acyl group in 1,2-diacyl-*sn*-glycero-3-phospholipids. PLA₂ is widely distributed in the tissues of various organisms and is classified into extracellular and intracellular types. Extracellular PLA₂ is abundant in mammalian pancreas and snake venom and has been well characterized [1,4].

However, few studies exist on PLA₂ from the digestive gland of marine invertebrates. Vaskovsky and Suppes [23] determined the phospholipase A activity in the extracts of 46 species of marine invertebrates and showed that the majority of these species contain phospholipase A. Hasegawa and Sasaki [9] found a high activity of phospholipase A in homogenates of the visceral organs of the starfish *Asterina pectinifera* that was stored at -20 °C for several months. The highest activity was observed in the pyloric ceca followed by the cardiac stomach and gonads. They also showed that the phospholipase A activity consisted mainly of the activity of PLA₂ with minor of phospholipase A₁ (EC3.1.1.3.2). Okabe and Noma [20] investigated the properties of PLA₂ using freshly prepared homogenate of the pyloric ceca of *A. pectinifera*. The maximum activity of PLA₂ was about pH 8 and the activity was enhanced by adding Ca²⁺ and/or sodium deoxycholate. Further, Okabe *et al.* [21] partially purified PLA₂ from the pyloric ceca of *A. pectinifera* and showed that basic properties of the enzyme, such as Ca²⁺ requirement, optimum pH, and heat stability, were similar to those of mammalian pancreatic PLA₂. However, both molecular weights of 40,000 using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and 72,000 using gel filtration were about three

and five times higher than that of mammalian pancreatic PLA₂. Recently, we isolated PLA₂ from the pyloric ceca of the starfish *Solaster paxillatus* and described some of its characters [13]. The PLA₂ from *S. paxillatus* had a molecular weight of approximately 13,000 using SDS-PAGE.

In the previous study, we found remarkably high activity of phospholipase A in the crude enzyme solution extracted from delipidated powder of the pyloric ceca of *A. pectinifera* [14]. In this study, we purified PLA₂ from the pyloric ceca of *A. pectinifera* and examined the characteristics of this enzyme.

2. Materials and methods

2.1 Materials

The starfish *A. pectinifera*, scallop *Patinopecten yessoensis*, and squid *Todarodes pacificus* were caught off Hokkaido Prefecture, Japan, in March 1994, January 1996, and September 1997, respectively. These specimens were stored at -20 °C for several months. Porcine pancreatic PLA₂ were purchased from Sigma (St. Louis, MO, USA) and Amano Pharmaceutical Co. (Nagoya, Japan). Egg yolk and soybean PC were 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).

2.2 Preparation of crude enzyme solution

Crude enzyme solution was prepared by the same method as described by Kishimura and Hayashi [14]. Pyloric ceca of the starfish were homogenized in 4 vols of chloroform-methanol (2:1, v/v) for 10 min, and the homogenate was filtrated *in vacuo* on ADVANTEC No.2 filter paper. Similarly the residue was homogenized in 2 vols of chloroform-methanol (2:1, v/v) and 1.3 vols of acetone for 10 min, and then the residue was air-dried at room temperature. PLA₂ was extracted by stirring the delipidated powder for 3 h at 5 °C in 50 vols of 50 mM Tris-HCl buffer at pH 8.0. The extract was centrifuged at 10,000g for 10 min, and then the supernatant was concentrated by lyophilization into crude enzyme solution.

2.3 Purification of starfish PLA₂

PLA₂ was purified from the crude enzyme solution extracted from the delipidated powder of the pyloric ceca of *A. pectinifera* using sequential column chromatography: gel filtration on Sephacryl S-200, diethylaminoethyl-cellulose anion-exchange column chromatography, and gel filtration on Sephadex G-50 (Table 1). The final enzyme preparation, which was purified 99-fold from the crude enzyme solution in a yield of 20 %, had a high activity (119,000 units/mg) (Table 1). The enzyme was found to be nearly homogeneous using SDS-PAGE (Fig.1a),

although a minor component with brown pigment was detected at the front of gel in PAGE (Fig.1b).

2.4 Lipid extraction and analysis

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 [10] and Hayashi and Kishimura [11].

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

2.5 Assay for PLA_2 activity

Ninety μ l of the aqueous medium containing a final concentration of 5 mM $CaCl_2$, 2.7 mM sodium deoxycholate, and 50 mM Tris-HCl buffer (pH 8.5) was pipetted into a test tube. Ten μ l of substrate solution containing 100 μ g of egg yolk PC dissolved in benzene-ethanol (1:1, v/v) was added, and the mixture was mixed vortically for 30 sec. Then 30 μ l of the enzyme solution was added to initiate the reaction. The mixture was incubated at 37 °C for 30 min, and the reaction was ended by adding 650 μ l of chloroform-methanol (2:1, v/v). The chloroform extract was concentrated by evaporation and the compositions of the

reaction products were analyzed using TLC and TLC/FID with a developing solvent of chloroform-methanol-acetic acid-water (55:17:3:2, v/v/v/v) and chloroform-methanol-acetic acid-water (55:17:6.5:2.5, v/v/v/v), respectively. One unit of enzyme activity was defined as the number of μg of substrates hydrolyzed per min.

Effect of CaCl_2 on the activity of starfish PLA_2 was examined in a reaction mixture containing 17 ng of the enzyme, 100 μg of egg yolk PC, 2.7 mM sodium deoxycholate, 50 mM Tris-HCl (pH 8.5), and 0 to 10 mM of CaCl_2 . Effects of other divalent cation and ethylenediaminetetraacetic acid (EDTA) were examined in the same reaction mixture containing 10 mM metal chloride or 10 mM EDTA instead of CaCl_2 .

Effect of sodium deoxycholate on the activity of starfish PLA_2 was examined in a reaction mixture containing 17 ng of the enzyme, 100 μg of egg yolk PC, 5 mM CaCl_2 , 50 mM Tris-HCl (pH 8.5), and 0 to 6.8 mM of sodium deoxycholate.

2.6 Positional and fatty acid specificity analysis

POPC was used for the positional specificity analysis and scallop and/or squid PC and PE were used for the fatty acid specificity analysis. Fifteen mg each of above substrates were almost hydrolyzed at 37 °C by 9 μg (1,071 units) of starfish PLA_2 for 3 h, and by 1 mg (440 units) of porcine pancreatic PLA_2 (Amano Pharmaceutical Co.) for 12 h. The released fatty acids were separated using preparative TLC with a

developing solvent of hexane–diethyl ether–acetic acid (85:15:1, v/v/v) and the fatty acid compositions were analyzed by GLC.

2.7 Polar group specificity analysis

Soybean and/or scallop PC and PE (100 μ g each) were hydrolyzed by 17 ng of starfish PLA₂ and 4.5 μ g of porcine pancreatic PLA₂ (Amano Pharmaceutical Co.) at 37 °C for various times (0–30 min). The compositions of the reaction products were analyzed using TLC/FID.

2.8 PAGE

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 [16]. 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.

2.9 Protein determination

The protein concentration was determined by the method of Lowry *et al.* [17] using bovine serum albumin as a standard.

3. Results

3.1 Properties of starfish PLA₂

The positional specificity of the purified starfish PLA₂ was examined using POPC. The enzyme released mainly oleic acid from POPC similar to porcine pancreatic PLA₂ (Table 2). The molecular weight of starfish PLA₂ was estimated as approximately 20,000 using SDS-PAGE (Fig.1a). Fig.2 shows the pH and temperature dependence of starfish PLA₂. 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.

Table 3 shows the effect of CaCl₂ on the activity of starfish PLA₂. The enzyme was activated by 1 mM or higher concentration of Ca²⁺. The activity of starfish PLA₂ was stimulated most by adding Ca²⁺ followed by Mg²⁺ and Co²⁺, while it was strongly inhibited by adding Hg²⁺, Zn²⁺, and EDTA (Table 3). The activity of starfish PLA₂ was enhanced by adding sodium deoxycholate at an optimum activity of 2 to 4 mM (Fig.3).

The fatty acid specificity of starfish PLA₂ was examined using scallop and/or squid PC and PE as a substrate. The compositions of the fatty acids released from these substrates by starfish PLA₂ were similar to those released by porcine pancreatic PLA₂ (Table 4).

The polar group specificity of starfish PLA₂ was examined using soybean and/or scallop PC and PE. Starfish PLA₂ hydrolyzed PC more effectively than PE, unlike porcine pancreatic PLA₂, which hydrolyzed PC

almost equally to PE (Table 5).

4. Discussion

PLA₂ was purified from the crude enzyme solution prepared from the pyloric ceca of the starfish *A. pectinifera*. The purified enzyme released mainly oleic acid from POPC. The enzyme had an optimum alkaline pH of about 9.0, and was activated by sodium deoxycholate and 1 mM or higher concentration of Ca²⁺. These properties of the enzyme were similar to those of mammalian pancreatic PLA₂ [4,1] and other starfish PLA₂ [21,13]. Further, the enzyme hydrolyzed the fatty acid ester bond exclusively at the glycerol-*sn*-2 position of PC and PE regardless of chain length and degree of unsaturation, similar to mammalian pancreatic PLA₂ [3] and *S. paxillatus* PLA₂ [13]. However, the optimum temperature (about 50 °C) of *A. pectinifera* PLA₂ in this study was higher than that of other starfish PLA₂ (about 40 °C) [21,13]. *A. pectinifera* PLA₂ in this study showed a molecular weight of about 20,000 on SDS-PAGE, which was larger than those of mammalian pancreatic PLA₂ (about 14,000) [5,22, 6,24,19] and *S. paxillatus* PLA₂ (about 13,000) [13], but smaller than that of *A. pectinifera* PLA₂ (about 40,000) of the study of Okabe *et al.* [21]. In a previous study, we compared the phospholipase A activity in the crude enzyme solutions of the pyloric ceca of four species of starfish (*A. pectinifera*, *S. paxillatus*, *Distolasterias nippon*, and *Asterias amurensis*) [14]. It was found that the phospholipase A

activity of *A. pectinifera* was markedly higher than that of the other species. In this study, the activity of purified PLA₂ (119,000 units/mg) of *A. pectinifera* was also higher than that of *S. paxillatus* PLA₂ (26 units/mg) [13] and the commercially available PLA₂ from porcine pancreas (Sigma) (4,300 units/mg) measured using the same assay system.

Mammalian pancreatic PLA₂ is secreted as a precursor and is activated by tryptic hydrolysis [25]. In a previous study, we found that the crude enzyme solution prepared from fresh samples of *A. pectinifera* showed a high phospholipase A activity (1,427 units/mg) similar to that from samples frozen at -20 °C for 35 or 46 months (1,214 and 1,655 units/mg, respectively) [14]. Whether the precursor of starfish PLA₂ exists remains unknown.

Mammalian pancreatic PLA₂ hydrolyzed PC almost equally to PE [3,8], and snake venom PLA₂ hydrolyzed PC more effectively than PE [12]. Also mammalian nonpancreatic extracellular PLA₂ hydrolyzed PE more effectively than PC [2,7,18]. In this study, starfish PLA₂ hydrolyzed PC more effectively than PE similar to snake venom PLA₂. Kuipers *et al.* [15] reported that a recombinant porcine pancreatic PLA₂ mutant with a deletion of the pancreatic loop at positions 62–66 gave an intermediate conformation between wild type porcine PLA₂ and snake venom PLA₂, and enhanced the catalytic activity on zwitterionic substrates. Therefore, possibly the primary structure of starfish PLA₂ in this study differed from that of mammalian pancreatic PLA₂ at the corresponding region to the pancreatic loop.

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(captions to figures)

Fig.1. Electrophoresis of the purified phospholipase A_2 of the starfish *Asterina pectinifera*. a: Electrophoresis was performed using a 0.1 % SDS-14 % polyacrylamide slab gel. Lane 1 contains starfish phospholipase A_2 . Lane 2 contains protein standards; bovine pancreatic

trypsinogen (molecular weight : 24,000), bovine milk β -lactoglobulin (18,400), and egg white lysozyme (14,300). b: Electrophoresis of starfish phospholipase A_2 was performed using a 12.5 % polyacrylamide slab gel at pH 8.9. Lane 1 contains starfish phospholipase A_2 .

Fig.2. Effects of pH and temperature on the activity of phospholipase A_2 of the starfish *Asterina pectinifera*. a: Reaction mixture containing 17 ng of starfish phospholipase A_2 , 100 μ g of egg yolk phosphatidylcholine, 2.7 mM sodium deoxycholate, and 5 mM $CaCl_2$ in a total volume of 130 μ l was incubated at 37 °C for 30 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 17 ng of starfish phospholipase A_2 , 100 μ g of egg yolk phosphatidylcholine, 2.7 mM sodium deoxycholate, 5 mM $CaCl_2$, and 50 mM Tris–HCl (pH 8.5) in a total volume of 130 μ l was incubated at various temperatures for 30 min.

Fig.3. Effect of sodium deoxycholate on the activity of phospholipase A_2 of the starfish *Asterina pectinifera*. Reaction mixture containing 17 ng of starfish phospholipase A_2 , 100 μ g of egg yolk phosphatidylcholine, 5 mM $CaCl_2$, 50 mM Tris–HCl (pH 8.5), and various concentration of sodium deoxycholate in a total volume of 130 μ l was incubated at 37 °C for 30 min.

Purification of PLA2 of the starfish *Asterina pectinifera*

Purification step	Protein (mg)	Total activity (units) $\times 10^3$ *	Specific activity (units/mg) $\times 10^3$	Purity (fold)	Yield (%)
Crude enzyme	774	892	1.2	1	100
Sephacryl S-200	254	609	2.4	2	68
DEAE-Cellulose	34	263	7.7	6	29
Sephadex G-50	3.3	180	55	46	20
Sephadex G-50	1.5	178	119	99	20

*One unit of activity was defined as the number of μg of phosphatidylcholines hydrolyzed per min.

Table 2

Composition of fatty acids released from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine by phospholipase A₂ of the starfish *Asterina pectinifera* and porcine pancreas (wt%)*¹

Fatty acid	Starfish enzyme	Porcine enzyme* ²
16:0	16.2	15.4
18:1n-9	83.8	84.6

*¹Wt% to total fatty acids.

*²Phospholipase A₂ from porcine pancreas (Amano Pharmaceutical Co.)

Table 3

Effects of metal ions and EDTA on the activity of phospholipase A₂ of the starfish *Asterina pectinifera*

Compound	Final concentration (mM)	Relative activity (%)*
Non	0	100
CaCl ₂	0.1	125
	1	604
	5	1,080
	10	1,496
	10	601
MgCl ₂	10	229
CoCl ₂	10	18
HgCl ₂	10	4
ZnCl ₂	10	11
EDTA	10	

*The activities were expressed as percentage of that with absence of metal ion, respectively.
 EDTA: ethylenediaminetetraacetic acid.

Table 4

Composition of fatty acids released from phosphatidylcholine and phosphatidylethanolamine by phospholipase A₂ of the starfish *Asterina pectinifera* and porcine pancreas (wt%)*¹

Fatty acid	PC from scallop* ²		PE from scallop* ²		PC from squid* ²		PE from squid* ²	
	Starfish enzyme	Porcine enzyme* ³	Starfish enzyme	Porcine enzyme	Starfish enzyme	Porcine enzyme	Starfish enzyme	Porcine enzyme
14:0	2.6	3.0	0.2	0.2	tr	tr	tr	tr
16:0	8.8	8.0	1.6	1.5	2.9	1.6	2.0	2.3
16:1n-7	1.1	1.1	0.2	0.1	0.3	0.2	0.1	0.1
18:0	1.6	1.7	1.2	1.2	1.0	1.1	1.2	1.7
18:1n-9	5.1	5.3	0.7	0.9	1.2	1.3	0.5	0.5
18:1n-7	4.2	4.0	1.4	1.3	0.5	0.4	0.4	0.4
18:2n-6	1.0	0.9	0.1	0.1	0.2	0.1	0.1	0.1
20:1n-11	0.2	0.3	3.3	3.5	0.3	0.6	0.1	0.1
20:1n-9	0.1	0.2	8.5	8.1	0.1	0.1	0.4	0.4
20:1n-7	0.4	0.4	10.5	11.4	nd	nd	nd	nd
20:4n-6	5.5	5.4	6.1	6.0	1.5	1.5	7.0	6.6
20:5n-3	35.7	35.2	39.6	39.7	13.5	14.3	42.3	40.8
21:5n-3	1.3	1.2	0.6	0.5	0.2	0.2	0.3	0.1
22:5n-3	1.6	1.7	0.5	1.4	0.3	0.2	0.5	0.5
22:6n-3	26.8	27.2	21.8	21.3	76.8	77.3	44.4	45.7
Others* ⁴	4.0	4.4	3.7	3.8	1.2	1.1	0.7	0.7

*¹Wt% to total fatty acids.

*²Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were prepared from scallop adductor muscle or squid mantle muscle.

*³Phospholipase A₂ from porcine pancreas (Amano Pharmaceutical Co.).

*⁴Consisted of minor (less than 1.0 %) and unknown compounds.

Abbreviations: tr, trace (less than 0.05 %); nd, not detected.

Table 5

Time-course of hydrolysis of phosphatidylcholine and phosphatidylethanolamine by phospholipase A₂ of the starfish *Asterina pectinifera* and porcine pancreas

Time (min)	Residual phospholipid (%) ^{*1}							
	PC from soybean ^{*2}		PE from soybean ^{*2}		PC from scallop ^{*2}		PE from scallop ^{*2}	
	Starfish enzyme	Porcine enzyme ^{*3}	Starfish enzyme	Porcine enzyme	Starfish enzyme	Porcine enzyme	Starfish enzyme	Porcine enzyme
0	100	100	100	100	100	100	100	100
15	67	58	101	61	60	72	99	48
30	42	53	85	56	37	31	95	26

^{*1}The residual phospholipid were expressed as percentage of that at 0 min, respectively.

^{*2}Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were prepared from soybean lecithin or scallop adductor muscle.

^{*3}Phospholipase A₂ from porcine pancreas (Amano Pharmaceutical Co.).

Fig. 1

Fig. 2

Fig. 3