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Characterization of phospholipase A₂ from the pyloric ceca of two species
of starfish *Coscinasterias acutispina* and *Plazaster borealis*

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Abstract

Phospholipase A (PLA) activities in the pyloric ceca and viscera from seven species of marine invertebrates (four starfish, one sea urchin, and two shellfish) were determined. Relatively high PLA specific activities were found in the pyloric ceca of two species of starfish (*Coscinasterias acutispina* and *Plazaster borealis*). Phospholipase A₂s (PLA₂s) were partially purified from the pyloric ceca of the starfish *C. acutispina* PLA₂ (C-PLA₂) and *P. borealis* PLA₂ (P-PLA₂). The C-PLA₂ and P-PLA₂ were mainly released oleic acid from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine. Temperature optima of the C-PLA₂ and P-PLA₂ were at around 60 °C and 50 °C, respectively, and pH optima of the C-PLA₂ and P-PLA₂ were both at around pH 10.0. The activities of the C-PLA₂ and P-PLA₂ were enhanced by sodium deoxycholate and 1 mM or higher concentration of Ca²⁺. The C-PLA₂ and P-PLA₂ did not show the fatty acid specificity for hydrolysis of phosphatidylcholine. Unlike porcine pancreatic PLA₂, the C-PLA₂ and P-PLA₂ hydrolyzed phosphatidylcholine more effectively than phosphatidylethanolamine.

Keywords: Phospholipase A₂, *Coscinasterias acutispina*, *Plazaster borealis*, Starfish, Marine invertebrate, Pyloric cecum, Polar group specificity

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 tissues of various organisms and is classified into extracellular and intracellular types. Extracellular PLA₂ is abundant in mammalian pancreas and snake venom, and these enzymatic and structural characteristics have been well studied (Arni & Ward 1996; Dennis 1983).

On the other hand, the PLA₂s from mammalian pancreas and snake venom have been used as diagnostic biochemical reagents. Stoll (1996) employed snake venom PLA₂ 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 PLA₂. Additionally, commercial PLA₂ mainly produced from porcine pancreas is used for industrial processes in the food industry. Soy lysophosphatidylcholine (lysoPC), which is an excellent emulsifier for food, is prepared by porcine pancreatic PLA₂-catalyzed hydrolysis of soy phosphatidylcholine (PC) (Aoi, 1990). The emulsion with soy lysoPC is stable under various conditions at high temperature, acidic solution and/or 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 PLA₂ 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 processes. It also allows the extraction of PC and lysoPC as a valuable by-product for the fortification of other foods. Recently, it was shown that LDL modified by PLA₂ 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 PLA₂ contained in an extracorporeal shunt.

In contrast, few studies exist on the enzymology and application of PLA₂ from marine invertebrates. In the previous study, we isolated PLA₂ from the pyloric ceca of starfish (*Asterina pectinifera*), and compared its enzymatic properties with those of porcine pancreatic PLA₂ (Kishimura & Hayashi 1999b). The specific activity of the *A. pectinifera* PLA₂ for PC was about 30 times higher than that of the commercially available PLA₂ from porcine pancreas (Sigma). In addition, the *A. pectinifera* PLA₂ hydrolyzed PC more effectively than phosphatidylethanolamine (PE) like snake venom PLA₂ (Ibrahim et al., 1964) but not porcine pancreatic PLA₂.

In this study, we partially purified PLA₂s from the pyloric ceca of the starfish (*C. acutispina* and *P. borealis*) and examined the characteristics of these enzymes.

2. Materials and methods

2.1. Materials

The starfish (*C. acutispina*) was caught off Uozu, Toyama Prefecture, Japan and was stored at -20 °C until use. The starfish (*P. borealis*, *Solaster borealis*, and *Aphelasterias japonica*), sea urchin (*Strongylocentrotus franciscanus*), shellfish (*Neptunea arthritica* and *Patinopecten yessoensis*) and common squid (*Todarodes pacificus*) were caught off Hakodate, Hokkaido Prefecture, Japan and were stored at -20 °C until use. Porcine pancreatic PLA₂ was 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).

2.2. Preparation of crude enzyme solution

Crude enzyme solution was prepared from the pyloric ceca and viscera of the invertebrates by the same method as described by Kishimura & Hayashi (1999a). The pyloric ceca and viscera were homogenized in 4 volumes of chloroform-methanol (2:1, v/v) for 10 min, and the homogenates were filtrated *in vacuo* on ADVANTEC No.2 filter paper. Similarly the residues were homogenized in 2 volumes of chloroform-methanol (2:1, v/v) and 1.3 volumes of acetone for 10 min, and then the residues were air-dried at room temperature. PLA was extracted by stirring the defatted powder for 3 h at 5 °C in 50 volumes of 50 mM Tris-HCl buffer at pH 8.0. The extracts were centrifuged at 10,000g for 10 min, and then the supernatants were concentrated by lyophilization into crude enzyme solution.

2.3. Purification of starfish PLA₂

The crude enzyme solutions were applied on a column (3.9×44 cm) of Sephacryl S-200 pre-equilibrated with 50 mM Tris-HCl buffer (pH8.0) and PLA₂ was eluted with the same buffer. Main active fractions were concentrated by lyophilization and dialyzed against 10 mM Tris-HCl buffer (pH8.0). The dialyzates were applied on a column (1.1×18 cm) of DEAE-cellulose pre-equilibrated with 10 mM Tris-HCl buffer (pH8.0). PLA₂ was eluted with a linear gradient of NaCl from 0 to 0.5 M in 10 mM Tris-HCl buffer (pH8.0), and main active fractions were obtained. The fractions were dialyzed against 10 mM Tris-HCl buffer (pH8.0) and the dialyzates were concentrated by lyophilization. The concentrates were applied on a column (3.9×64 cm) of Sephadex G-50 pre-equilibrated with 10 mM Tris-HCl buffer (pH8.0) and PLA₂ was eluted with the

same buffer. Consequently, main active fractions were obtained. The final enzyme preparations were purified 63-fold (C-PLA₂) and 12-fold (P-PLA₂) from the crude enzyme solutions in a yield of 14 % and 5 %, respectively. The C-PLA₂ and P-PLA₂ included small amounts of several proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

2.4. Lipid extraction and analysis

The extraction of tissue lipids and lipid analyses 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 & Kishimura (1996).

PC and 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.

2.5. PLA₂ activity assay

PLA₂ activity was measured as described by Kishimura & Hayashi (1999b). One unit of enzyme activity was defined as one microgram of PC hydrolyzed per min.

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

2.6. Protein determination

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

3. Results

3.1. PLA activity in viscera of several marine invertebrates

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

3.2. Properties of *C. acutispina* and *P. borealis* partially purified PLA₂s

Because relatively high specific activities were detected in the pyloric ceca of the starfish *C. acutispina* and *P. borealis*, PLA₂s were partially purified from the crude enzymes of the *C. acutispina* (C-PLA₂) and *P. borealis* (P-PLA₂). The positional specificities of the C-PLA₂ and P-PLA₂ were examined using POPC. The C-PLA₂ and P-PLA₂ mainly released oleic acid from POPC similar to the porcine pancreatic PLA₂ (Table 2). Figure 1 shows the pH dependences of the C-PLA₂ and P-PLA₂. Both C-PLA₂ and P-PLA₂ hydrolyzed egg-yolk PC effectively at alkaline pH with an optimum activity at about pH 10.0 (Fig. 1). Figure 2 shows the temperature dependences of the C-PLA₂ and P-PLA₂. The optimum temperature of the C-PLA₂ and P-PLA₂ was at about 60 °C and 50 °C, respectively (Fig. 2). Figure 3 shows the effects of CaCl₂ on the C-PLA₂ and P-PLA₂ activities. The C-PLA₂ and P-PLA₂ were activated by Ca²⁺ at 1 mM or higher. The fatty acid specificities of the C-PLA₂ and P-PLA₂ were examined using egg yolk PC as a substrate. The compositions of fatty acids released from the substrate by the C-PLA₂ and

P-PLA₂ were similar to that released by the porcine pancreatic PLA₂ (Table 3). The polar-group specificities of the C-PLA₂ and P-PLA₂ were examined using squid PC and PE. The C-PLA₂ and P-PLA₂ hydrolyzed PC more effectively than PE (Fig. 4).

4. Discussion

The PLA activities in the pyloric ceca and viscera from seven species of marine invertebrates were compared with those in the pyloric ceca from four species of starfish previously reported (Kishimura & Hayashi, 1999a). As shown in Table 1, the PLA specific activity in *A. pectinifera* was extremely high. Relatively high PLA specific activities were detected in *C. acutispina*, *P. borealis*, *S. borealis*, and *Solaster paxillatus*. Low PLA activities were detected in *A. japonica*, *Distolasterias nippon*, *Asterias amurensis*, *S. franciscanus*, *N. arthritica*, and *P. yessoensis*.

PLA₂s from the pyloric ceca of *C. acutispina* (C-PLA₂) and *P. borealis* (P-PLA₂) were partially purified. The C-PLA₂ and P-PLA₂ released mainly oleic acid from POPC. The C-PLA₂ and P-PLA₂ had an optimum alkaline pH of about 10.0, and was activated by Ca²⁺ at 1 mM or higher. These properties of the C-PLA₂ and P-PLA₂ were similar to those of the mammalian pancreatic PLA₂s (Dennis, 1983; Arni & Ward, 1996) and other starfish PLA₂s (Kishimura & Hayashi, 1998; Kishimura & Hayashi, 1999b). Furthermore, the C-PLA₂ and P-PLA₂ 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 porcine pancreatic PLA₂ (De Haas et al., 1968) and other starfish PLA₂s (Kishimura & Hayashi, 1998; Kishimura & Hayashi, 1999b). However, the optimum temperature the C-PLA₂ (about 60 °C) and P-PLA₂ (about 50 °C) in this study was higher than that of *S. paxillatus* PLA₂ (about 40 °C) (Kishimura & Hayashi, 1998).

Although the mammalian pancreatic PLA₂s hydrolyzed PC almost equally to PE (De Haas et al., 1968; Hara et al., 1991), the snake venom PLA₂ hydrolyzed PC more effectively than PE (Ibrahim et al., 1964). Moreover mammalian nonpancreatic extracellular PLA₂s hydrolyzed PE more effectively than PC (Chang et al., 1987; Hara et al., 1989; Mizushima et al., 1989). In this study, the C-PLA₂ and P-PLA₂ hydrolyzed PC more effectively than PE similar to the snake venom PLA₂s. Kuipers et al. (1989) reported that the recombinant porcine pancreatic PLA₂ mutant with a deletion of the pancreatic loop at positions 62-66 provided an intermediate conformation between the wild-type porcine PLA₂ and the snake (*Crotalus atrox*) venom PLA₂, and enhanced the catalytic activity on zwitterionic substrates. Therefore, possibly the primary structure of the C-PLA₂ and P-PLA₂ differed from that of the porcine pancreatic PLA₂ at the region corresponding to the pancreatic loop.

In conclusion, the C-PLA₂ and P-PLA₂ hydrolyzed PC more effectively than PE unlike porcine pancreatic PLA₂, which hydrolyzed PC almost equally to PE. In addition, the specific activities of the C-PLA₂ (1,066 U/mg) and P-PLA₂ (230 U/mg) for PC were similar to that of commercially available PLA₂ (440 U/mg) from porcine pancreas (Amano Pharmaceutical Co.). These results suggest that the pyloric ceca of the starfish (*C. acutispina* and *P. borealis*) would be a potential source of PLA₂.

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(Figure Legends)

Fig.1. Effects of pH on the activity of phospholipase A₂s of the starfish (*C. acutispina* and *P. borealis*). The activities were determined in 50 mM buffer solutions [acetic acid-sodium acetate from pH 4.0 to 7.0 (▲), Tris-HCl from pH 7.0 to 9.0 (●), and glycine-NaOH from pH 9.0 to 11.0 (■)] at 37 °C for 10 min. a: *C. acutispina* phospholipase A₂; b: *P. borealis* phospholipase A₂.

Fig.2. Effects of temperature on the activity of phospholipase A₂s of the starfish (*C. acutispina* and *P. borealis*). The activities were determined in 50 mM Tris-HCl buffer (pH 8.5) at various temperatures for 10 min. a: *C. acutispina* phospholipase A₂; b: *P. borealis* phospholipase A₂.

Fig. 3. Effects of Ca²⁺ on activity of phospholipase A₂s of the starfish (*C. acutispina* and *P. borealis*). The activities were determined in 50 mM Tris-HCl buffer (pH 8.5) at various concentration of CaCl₂ at 37 °C for 10 min. a: *C. acutispina* phospholipase A₂; b: *P. borealis* phospholipase A₂.

Fig. 4. Time-course of phosphatidylcholine and phosphatidylethanolamine hydrolyses by phospholipase A₂s of the starfish (*C. acutispina* and *P. borealis*). Hydrolysis of squid

phosphatidylcholine (●) and phosphatidylethanolamine (▲) carried out in 50 mM Tris-HCl buffer (pH 8.5) at 37 °C for various periods. a: *C. acutispina* phospholipase A₂; b: *P. borealis* phospholipase A₂; c: porcine pancreatic phospholipase A₂ (Amano Pharmaceutical Co.).

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

	Organ	Activity (U ^a /g powder)	Specific activity (U/mg)
Starfish			
<i>Coscinasterias acutispina</i>	Pyloric cecum	5,400	17
<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> ^b	Pyloric cecum	540,000	1,400
<i>Solaster paxillatus</i> ^b	Pyloric cecum	1,000	12
<i>Distolasterias nippon</i> ^b	Pyloric cecum	70	0.5
<i>Asterias amurensis</i> ^b	Pyloric cecum	27	0.5

^a One unit (U) of activity was determined as one microgram of phosphatidylcholine hydrolyzed per minute.

^b Kishimura & Hayashi (1999a).

Table 2. Composition of fatty acids released from 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-phosphocholine by Phospholipase A₂ from the Pyloric Ceca of Starfish (*C. acutispina* and *P. borealis*) (%)

Fatty acid	<i>C. acutispina</i>	<i>P. borealis</i>	Porcine enzyme ^a
16:0	16.0	15.3	15.4
18:1n-9	84.0	84.7	84.6

^a Phospholipase A₂ from porcine pancreas (Amano Pharmaceutical Co.).

Table 3. Composition of fatty acids released from squid egg-yolk phosphatidylcholine by Phospholipase A₂ from the Pyloric Ceca of Starfish (*C. acutispina* and *P. borealis*)

(%)

Fatty Acid	<i>C. acutispina</i>	<i>P. borealis</i>	Porcine Enzyme ^a
16:0	2.3	2.8	2.9
18:0	0.7	1.1	1.0
18:1n-9	59.0	55.9	57.6
18:1n-7	2.0	1.9	1.8
18:2n-6	24.9	26.9	24.2
20:4n-6	5.5	6.6	5.6
22:6n-3	1.5	1.1	1.6
Others ^b	4.1	3.7	5.3

^a Phospholipase A₂ from porcine pancreas (Amano Pharmaceutical Co.).

^b Consisted of minor (less than 1.0 %) and unknown compounds.

Fig. 1

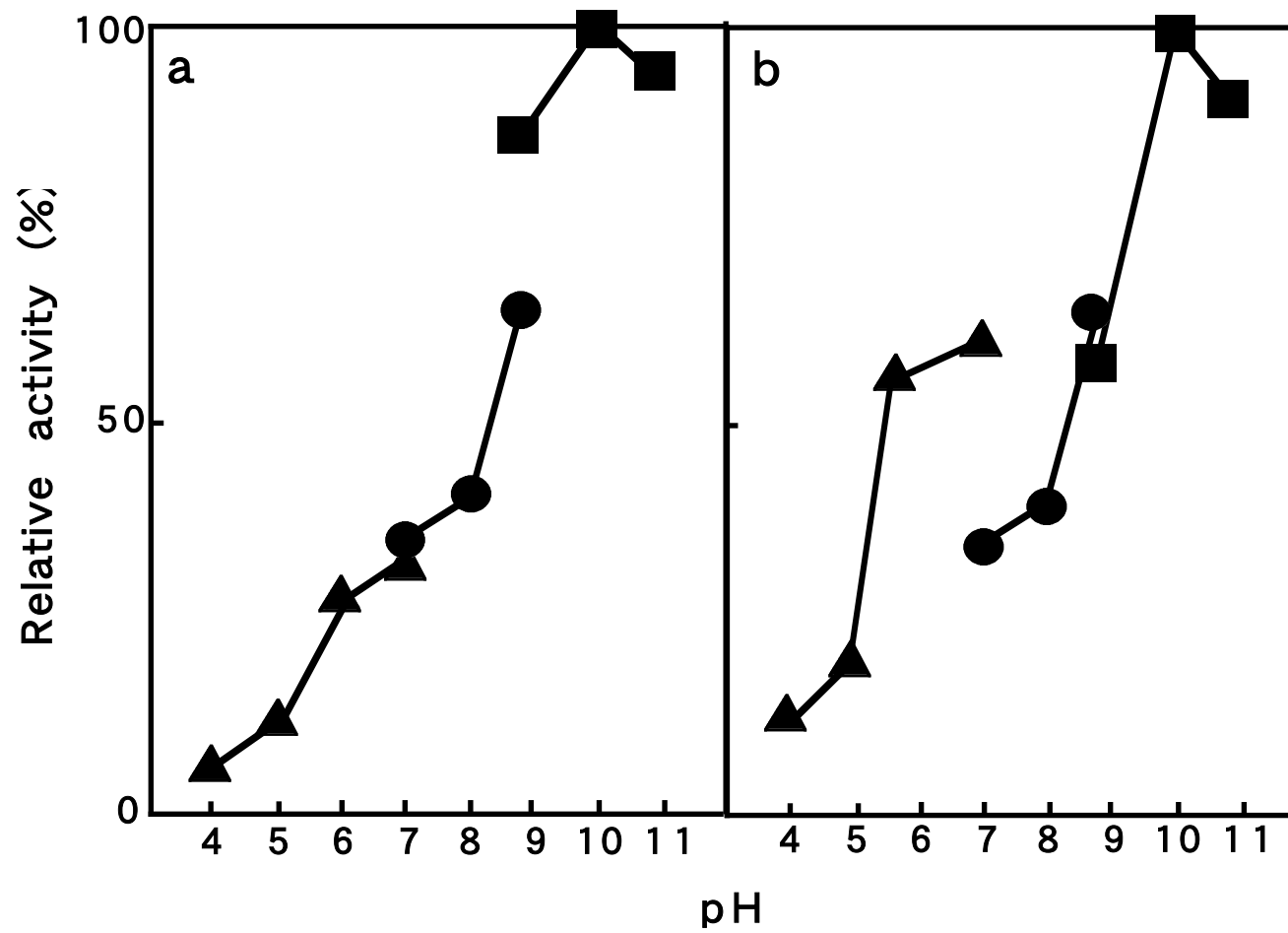


Fig. 2

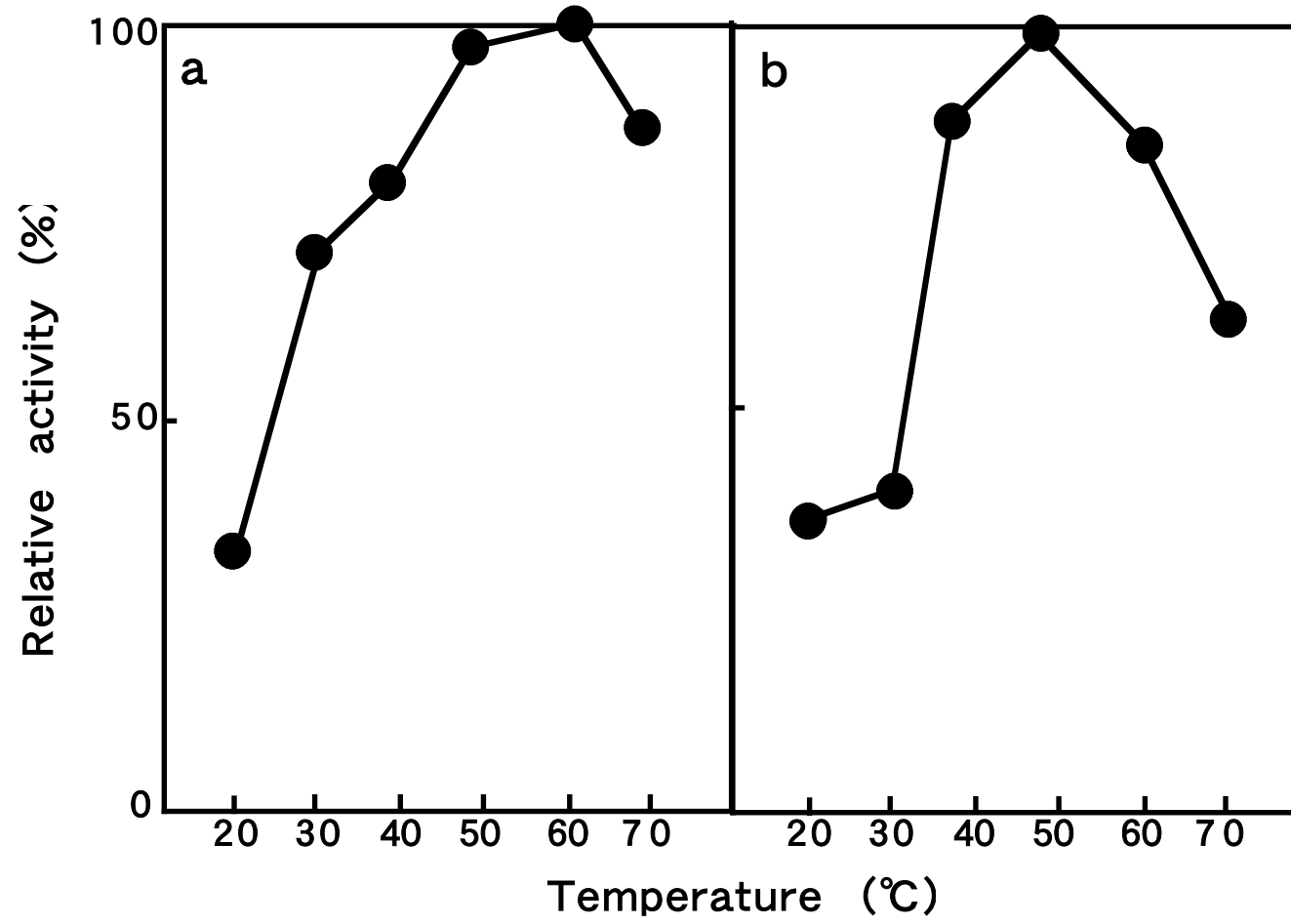


Fig. 3

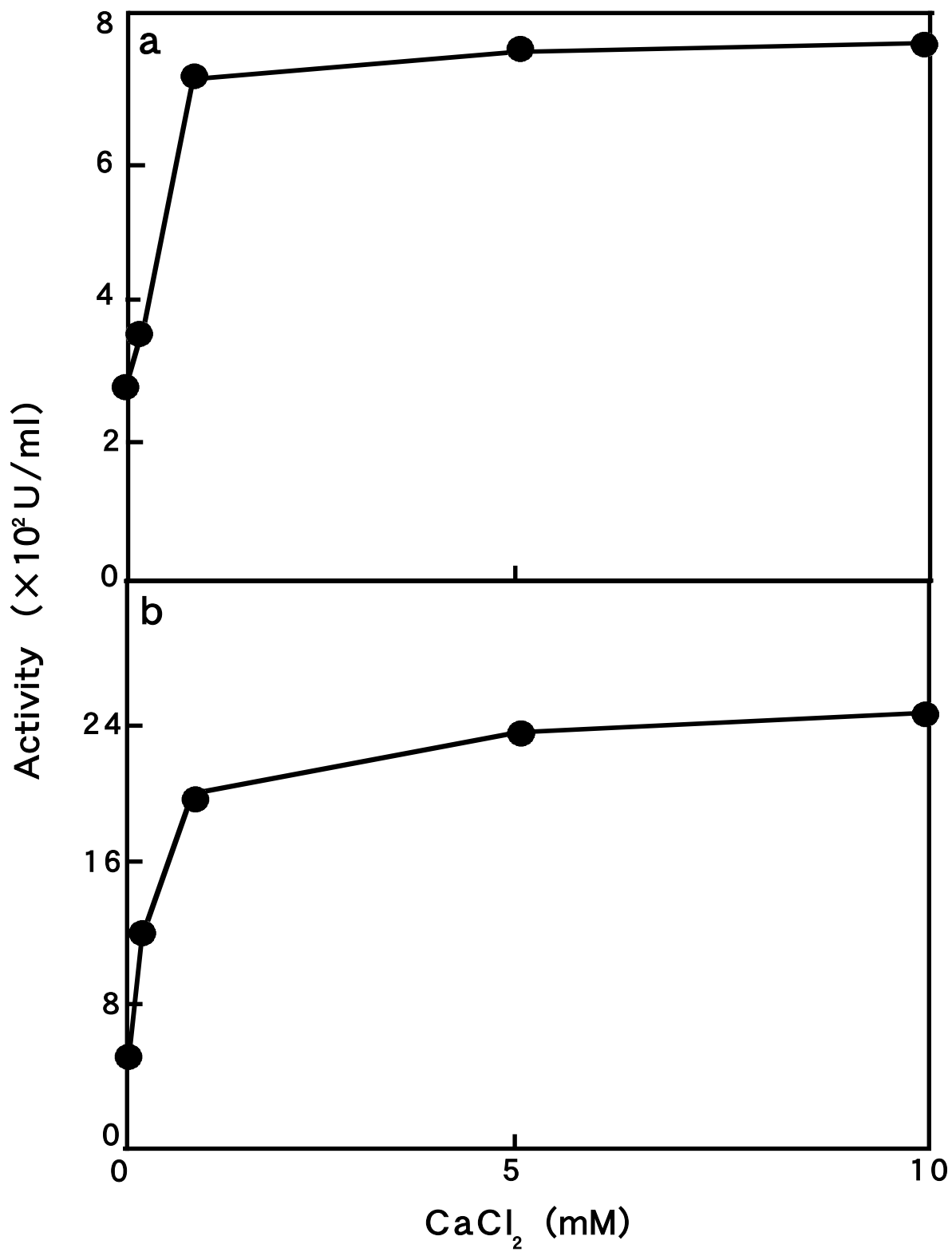


Fig. 4

