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Bacterial Expression and Characterization
of Starfish Phospholipase A₂

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Abstract

Phospholipase A₂ (PLA₂) from the pyloric ceca of the starfish *Asterina pectinifera* showed high specific activity and characteristic substrate specificity, compared with commercially available PLA₂ from porcine pancreas. To investigate enzymatic properties of the starfish PLA₂ in further detail, we constructed a bacterial expression system for the enzyme. The starfish PLA₂ cDNA isolated previously (Kishimura et al., 2000. Comp. Biochem. Physiol. 126B, 579–586) was inserted into the expression plasmid pET-16b and the PLA₂ protein was expressed in *Escherichia coli* BL21 (DE3) by induction with isopropyl-β-D (-) -thiogalactopyranoside. The recombinant PLA₂ produced as inclusion bodies was dissociated with 8 M urea and 10 mM 2-mercaptoethanol and renatured by dialyzing against 10 mM Tris-HCl buffer (pH 8.0). Renatured PLA₂ was purified by subsequent column chromatographies on DEAE-cellulose (DE-52) and Sephadex G-50. Although an N-terminal Ser in the native starfish PLA₂ was replaced by an Ala in the recombinant PLA₂, the recombinant enzyme showed essentially the same properties as did the native PLA₂ with respect to specific activity, substrate specificity, optimum pH and temperature, and Ca²⁺ requirement.

Key words: *Asterina pectinifera*; Bacterial expression; Group I ; Phospholipase A₂; Starfish; Substrate specificity; Surface loop; β-wing

1. Introduction

Phospholipase A₂ (PLA₂, EC 3.1.1.4) catalyzes the selective hydrolysis of the *sn*-2-acyl group in 1, 2-diacyl-*sn*-glycero-3-phospholipids and occurs in both intracellular and secreted forms. Secretory PLA₂s are low

molecular mass proteins (approx. 14 kDa) and classified into at least four groups based on their primary structures (Dennis, 1997). Among them, group I PLA₂s (mammalian pancreatic and elapinae and hydrophiinae snake venom PLA₂s) and group II PLA₂s (mammalian non-pancreatic and crotalinae and viperinae snake venom PLA₂s) have been studied extensively (Dennis, 1983; Arni and Ward, 1996). In contrast, there are few studies on PLA₂s from the digestive gland of marine invertebrates. Recently, we found high PLA₂ activity in crude enzyme fraction from pyloric ceca of the starfish *A. pectinifera* and succeeded in isolating a PLA₂ (Kishimura and Hayashi, 1999a and b). The starfish PLA₂ exhibited similar enzymatic properties to mammalian pancreatic PLA₂ in Ca²⁺ requirement, fatty acid specificity, and optimum pH and temperature. However, the specific activity of the starfish PLA₂ was remarkably higher than that of commercially available PLA₂ from porcine pancreas. In addition, the starfish PLA₂ showed distinct substrate specificity hydrolyzing phosphatidylcholine more efficiently than phosphatidylethanolamine, while the porcine PLA₂ hydrolyzed them almost equally. Recently, we reported the complete amino acid sequence of starfish PLA₂ and its structural characteristics, compared with porcine pancreatic PLA₂ (Kishimura et al., 2000a). The starfish PLA₂ consisted of 137 amino acids with Mr 15,300 and contained 14 Cys residues at positions that have been shown to be involved in intramolecular disulfide bonds in porcine pancreatic PLA₂. The amino acid sequences for putative active site and Ca²⁺-binding loop of the starfish PLA₂ showed fairly high homology to those of porcine pancreatic PLA₂. However, in the alignment of the amino acid sequence of the starfish PLA₂ to porcine pancreatic PLA₂, two amino

acid deletions in pancreatic loop region, and sixteen insertions and three deletions in β -wing region were required to maximize the sequence homology. In the previous paper, we described the cloning of three kinds of cDNA encoding the starfish PLA₂ protein (Kishimura et al., 2000b). These cDNAs namely "cDNA 1-3" in that paper consisted of 415 bp including an open reading frame of 414 bp at nucleotide positions 1 to 414. The deduced sequence from cDNA 1 was shown to be identical to that determined with the PLA₂ protein except for an initiation codon.

In the present study, we constructed a bacterial expression system for the starfish PLA₂ and determined some biochemical properties of the recombinant PLA₂. This enables us to investigate the structure-function relationship in the starfish PLA₂ through recombinant DNA approaches.

2. Materials and methods

2.1. Materials

The "cDNA 1", fully encoding the starfish PLA₂ protein (Kishimura et al., 2000b), was used for the construction of expression vector. Plasmid pET-16b and host strain, *E. coli* BL21 (DE3) were purchased from Novagen (Madison, WI, USA). DNA ligation kit ver. 2 and restriction endonucleases were purchased from TaKaRa (Kyoto, Japan). Egg yolk phosphatidylcholine and isopropyl- β -D (-) - thiogalactopyranoside (IPTG) 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. Lipid extraction and analysis

Extraction of tissue lipids, thin-layer chromatography (TLC), preparative TLC, TLC with flame ionization detector (TLC/FID), and gas-liquid chromatography were performed as described by Hayashi (1989) and Hayashi and Kishimura (1996).

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were prepared from the mantle muscle of squid, *Todarodes pacificus*, caught off Hakodate, Hokkaido Prefecture, Japan, in September 1997, using preparative TLC with chloroform-methanol-acetic acid-water (55:17:3:2, v/v/v/v) as a developing solvent.

2.3. Assay for PLA₂ activity

PLA₂ activity was measured as described by Kishimura and Hayashi (1999b). One unit of enzyme activity was defined as the number of micrograms of substrate hydrolyzed per minute.

Positional specificity and polar group specificity for PC and/or PE were analyzed by the method of Kishimura and Hayashi (1999b).

2.4. Other analytical procedures

Amino acid sequence of the starfish recombinant PLA₂ was analyzed by a 473A protein sequencer (Perkin Elmer-ABI, Foster City, CA, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 0.1 % SDS-14 % polyacrylamide slab-gel by the method of Laemmli (1970). PAGE under non-denaturing conditions was carried out using a 12.5 % polyacrylamide slab-gel with Tris-HCl buffer at pH 8.9. The gel was stained with 0.1 % coomassie brilliant blue R-250 in 50 % methanol-7 % acetic acid and destained with 7 % acetic acid.

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. Construction of expression vector for the starfish PLA₂

The 5'- and 3'-terminal nucleotide sequences of the cDNA 1 for the starfish PLA₂ were modified by polymerase chain reaction (PCR) with a set of primers (forward primer: 5'-gcagcccatggcagtttaccag-3'; reverse primer: 5'-agtggatccctcagcatgaatc-3'). By the PCR, an *Nco* I site which includes an "ATG" sequence being applicable to a translational start codon was introduced to 5'-terminus of the cDNA, while a stop codon "TGA" and a *Bam* HI site were introduced to 3'-terminal region (Fig. 1). The modified cDNA was digested with *Nco* I and *Bam* HI and ligated into an *Nco* I-*Bam* HI cloning site of pET-16b, thus resulting the recombinant PLA₂-pET-16b. As

shown in Fig. 1, "T" following "ATG" was substituted for "G" accompanied by the introduction of *Nco* I site in 5'-terminus of the cDNA. Therefore, the N-terminal Ser in the native starfish PLA₂ is expected to be substituted for Ala in the recombinant PLA₂ protein.

3.2. Expression of the starfish recombinant PLA₂

A single colony of *E. coli* BL21 (DE3) transformed with the recombinant PLA₂-pET-16b was inoculated to a 2 ml of Luria-Bertain's broth containing 50 μ g/ml ampicillin and cultivated at 37 °C for 4 h. Subsequently, IPTG was added to the culture in a final concentration of 1 mM and the cultivation was continued for another 4 h. At various time intervals, an aliquot of the culture (200 μ l) was pipetted out and centrifuged at 5,000 \times g for 5 min. The pellet was dissolved in 0.1 ml of a solution containing 10 M urea, 4 % SDS, 0.125 M Tris-HCl (pH 6.8) and 0.5 M 2-mercaptoethanol, and subjected to SDS-PAGE. As shown in Fig. 2, a protein with the same mobility as the native starfish PLA₂ was increasingly expressed by the induction with IPTG. The densitometric analysis showed that the expression level of the protein reached a maximum (approximately 15 % of total protein) in 2 h of the induction. To identify the expressed protein as the recombinant PLA₂, the N-terminal sequence of the protein was determined after separation by SDS-PAGE and electroblotting to PVDF membrane. A sequence of 20 amino acid residues, AVYQFGKFI SCYGGAGFFDG, was determined. This sequence is completely consistent with that expected from the nucleotide sequence of

the cDNA (see Fig. 1). Thus, the expressed protein was identified as the recombinant starfish PLA₂.

3.3. Large scale production and purification of the starfish recombinant PLA₂

The overnight culture of bacteria (150 ml) containing the starfish PLA₂-pET-16b plasmid was transferred to a 1000 ml culture and cultivated at 37 °C for 6 h, and the recombinant PLA₂ was expressed by induction with 1 mM IPTG for 2 h. The bacteria were harvested at 10,000×g for 5 min and lysed by repeated freeze and thaw in 40 ml of 50 mM Tris-HCl buffer (pH 8.0) containing 50 mM EDTA, 8 % sucrose, 0.5 % Triton X-100 and 0.5 mM phenylmethanesulfonyl fluoride. The lysate was centrifuged at 10,000×g for 5 min and the inclusion bodies precipitated were suspended in 40 ml of the same buffer. The suspension was sonicated and simultaneously passed through a needle at 5 °C for 10 min, then the inclusion bodies were dissolved in 8 M urea and 10 mM 2-mercaptoethanol and incubated at room temperature for 3 h. After centrifugation at 10,000 ×g for 10 min, the recombinant PLA₂ obtained in the supernatant was renatured by successive dialysis against 6 M, 4 M, 2 M, and 0 M urea containing 10 mM Tris-HCl buffer (pH 8.0) at 5 °C for three hours each. The renatured PLA₂ was lyophilized, dissolved in 10 mM Tris-HCl buffer (pH 8.0), and centrifuged at 10,000×g for 10 min. By this procedure, 184 mg of crude enzyme was obtained (Table 1).

The crude enzyme was applied to a column of DEAE-cellulose (1.1×

21 cm) preequilibrated with 10 mM Tris-HCl (pH 8.5). Proteins were eluted with a linear gradient of 0–0.4 M NaCl followed by a stepwise increase to 0.5 M NaCl. As shown in Fig. 3, the PLA₂ activity was detected mainly in A5 fraction eluted around 0.2–0.3 M NaCl. The A5 fraction was concentrated and applied to a column of Sephadex G-50 (3.9×64 cm) preequilibrated with 10 mM Tris-HCl (pH 8.0), and proteins were eluted with the same buffer. As shown in Fig. 4, the major activity was detected in B4 fraction and the protein contained in B4 fraction showed a single band on both SDS-PAGE and native PAGE. The recombinant PLA₂, which was purified 97-fold from the crude enzyme, had a high specific activity (87,000 U/mg) (Table 1).

3.4. Properties of the starfish recombinant PLA₂

The positional specificity of the purified recombinant PLA₂ was examined using POPC. The enzyme released mainly oleic acid from POPC like native starfish PLA₂ and porcine pancreatic PLA₂ (Table 2). Fig. 5 shows the pH and temperature dependence of the recombinant PLA₂. The enzyme hydrolyzed egg yolk PC effectively at alkaline pHs with an optimum at around pH 9.0 (Fig. 5a), and optimum temperature was observed at around 50 °C (Fig. 5b). The enzyme was activated by 1 mM or higher concentrations of Ca²⁺ (Fig. 6). The enzyme hydrolyzed squid mantle muscle PC more efficiently than PE (Fig. 7). These properties of the recombinant PLA₂ were essentially the same as those of the native starfish enzyme.

4. Discussion

Previously, bovine and porcine pancreatic PLA₂s were shown to be over-expressed in *E. coli* cells as proenzymes and matured by tryptic digestion (Geus et al., 1987; Deng et al., 1990). Recently, human pancreatic PLA₂ was expressed in *E. coli* cells as a mature form in high yield by using an expression vector involving the PLA₂ synthetic gene (Han et al., 1997). In the previous study, we cloned cDNAs for the starfish PLA₂ concomitantly introducing a translational initiation codon "ATG" to 5'-termini of the cDNAs (Kishimura et al., 2000b). These cDNAs, named cDNA 1-3, encodes the amino acid sequence of mature form, i.e., the form lacking signal peptide in the N-terminus, so that they can be bacterially expressed as a mature enzyme. In the present study, we constructed the expression vector for the mature form of the starfish PLA₂ using the cDNA 1 and pET-16b plasmid. Although an N-terminal Ser in the native starfish PLA₂ was replaced by Ala in the recombinant PLA₂, this enzyme showed essentially the same properties as those of the native PLA₂ with respect to specific activity, substrate specificity, optimum pH and temperature, and Ca²⁺ requirement.

Since PLA₂ exhibits enhanced activity towards lipids in lamellar and micellar aggregates both in membranes and other lipid-water interfaces, the reaction cycle has been considered to include the interfacial binding which is distinct from the binding of a phospholipid molecule to the active

site (Dennis, 1983; Arni and Ward, 1996). An earlier crystallographic study of bovine pancreatic PLA₂ predicted that interfacial binding surface is composed of the residues clustered in the N- and C-termini and several other residues (Dijkstra et al., 1981). Previous mutagenesis studies indicated that the Lys-62 or Arg-53 of porcine pancreatic PLA₂ and Lys-53 or Lys-56 of bovine pancreatic PLA₂ are involved in their specificities for anionic head group of phospholipids presumably by electrostatically repelling cationic head groups of zwitterionic phospholipids (Kuipers et al., 1989; Dua et al., 1995; Snitko et al., 1999). Recent studies of mammalian pancreatic PLA₂ indicated that Arg-6, Lys-10, and Lys-116 of porcine, Lys-10, Lys-56, and Lys-116 of bovine, and Arg-6, Lys-7, Lys-10, and Lys-116 of human enzymes were involved in electrostatic interactions with anionic interfaces (Noel et al., 1991; Lugtigheid et al., 1993). As reported previously, the starfish PLA₂ completely conserved the residues which are critical for forming the catalytic network (His-49, Asp-111, Tyr-53, and Tyr-72) and Ca²⁺-binding loop (Tyr-29, Gly-31, Gly-33, and Asp-50) (Kishimura et al., 2000a and b). Therefore, we consider that the starfish PLA₂ may function through a similar mechanism of those of mammalian pancreatic PLA₂. However, most of the above positively charged residues, predicted to participate in the interfacial interaction of mammalian pancreatic PLA₂ to substrates, were deleted or substituted for neutral and negatively charged residues in the starfish PLA₂. These facts imply that the interfacial binding mode of the starfish PLA₂ was somehow different from that of mammalian pancreatic PLA₂.

Snake venom PLA₂ often displays some pharmacological activities

such as neurotoxic, myotoxic, cardiotoxic, hemolytic, and anticoagulant activities in addition to the primary catalytic function (Arni and Ward, 1996). It was proposed that the anticoagulant and myotoxic sites were located around residues 54–77 and 89–97, respectively, which were near by β -wing (Kini and Evans, 1987). The neurotoxic site was suggested to be the region between residues 60–100 (Dufton and Hider, 1983). These regions were identified by means of sequence comparisons. The suggested neurotoxic region comprises the whole of the β -wing and its vicinity and overlaps with the postulated anticoagulant and myotoxic regions. In case of the starfish PLA₂, the sequence required about thirteen insertions in β -wing region to align with the sequences of group I and II PLA₂s. However, the functional significance of the β -wing region in the starfish PLA₂ remains to be elucidated.

In conclusion, we have succeeded to construct bacterial expression system for the starfish PLA₂ and found that basic properties of the recombinant PLA₂ are essentially the same as those of the native starfish PLA₂. The recombinant starfish PLA₂ together with various kinds of site-directed mutants will allow us to investigate the structure–function relationship with respect to the interfacial binding surface and β -wing region of the starfish PLA₂. The production of the starfish PLA₂ mutants and determination of more detailed properties such as Michaelis constant and the range of substrate specificity is now under way.

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Legends for figures

Fig. 1. Structure of recombinant plasmid constructed from the starfish PLA₂ cDNA 1 and pET-16b.

Shaded regions show the cloning sites of pET-16b. The 5'- and 3'-termini of the starfish PLA₂ cDNA 1 was modified as described in "Results" and the cDNA was ligated between the *Nco* I and *Bam* HI sites of pET-16b in frame. Transcriptional direction is indicated with a curved arrow. At the bottom of the figure, some parts of the nucleotide and deduced amino acid sequences are shown. The substituted amino acid at the N-terminus of

the starfish PLA₂ is boxed.

Fig. 2. Overexpression of the recombinant starfish PLA₂ by IPTG-induction.

E. coli BL21 (DE3) containing recombinant pET-16b was induced by 1 mM IPTG. Aliquots of the culture (200 μ l) were pipetted off at 1-4 h after the induction and the protein composition was analyzed by SDS-PAGE.

PLA₂, the native starfish PLA₂ prepared from the pyloric ceca of *A. pectinifera* (Mr 15,300).

Fig. 3. Purification of the recombinant starfish PLA₂ by DEAE-cellulose column chromatography.

The crude enzyme (184 mg) was applied to a column (1.1 \times 21 cm) of DEAE-cellulose preequilibrated with 10 mM Tris-HCl buffer (pH 8.5) and eluted with a linear gradient of 0-0.4 M NaCl followed by a stepwise increase to 0.5 M NaCl. Total volume of elution buffer was 500 ml and flow rate was 40 ml/h. Each fraction contained 3.0 ml. The solid line and dotted line in the lower panel indicate absorbance at 280 nm and NaCl concentration, respectively. Thin-layer chromatograms in the upper panel show the reaction products of A1-A6 fractions with egg yolk phosphatidylcholine. PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

Fig. 4. Purification of recombinant starfish PLA₂ by gel-filtration.

A5 fraction, the major active fraction in Fig. 3, was concentrated by

lyophilization and applied to a Sephadex G-50 column (3.9×64 cm) equilibrated with 10 mM Tris-HCl buffer (pH8.0) and eluted with the same buffer at a flow rate of 25 ml/h. Each fraction contained 2.5 ml. The solid line in the lower panel indicates absorbance at 230 nm, and inset photos "a" and "b" indicate electrophoretic patterns of B5 fraction on SDS-PAGE and native PAGE, respectively. Thin-layer chromatograms in the upper panel indicate reaction products of B1-B5 with egg yolk phosphatidylcholine. PC, phosphatidylcholine; LPC, lysophosphatidylcholine.

Fig. 5. Effects of pH and temperature on the activity of recombinant starfish PLA₂.

a, pH dependence of the recombinant PLA₂ activity. The reaction mixture contained 24 ng starfish recombinant PLA₂, 100 μg egg yolk phosphatidylcholine, 2.7 mM sodium deoxycholate, 5 mM CaCl₂, and 50 mM acetic acid-sodium acetate (▲), 50 mM Tris-HCl (●), or 50 mM glycine-NaOH (■) buffer in a total volume of 130 μl. The mixture was incubated at 37 °C for 30 min. b, temperature dependence of the activity. Reaction mixture containing 50 mM Tris-HCl buffer (pH 8.5) and the other constituents described above was incubated at various temperatures for 30 min.

Fig. 6. Effects of Ca²⁺ on the activity of recombinant starfish PLA₂.

Reaction mixture containing 24 ng starfish recombinant PLA₂, 100 μg egg yolk phosphatidylcholine, 2.7 mM sodium deoxycholate, 50 mM Tris-HCl buffer (pH 8.5), and various concentrations of CaCl₂ in a total

volume of 130 μ l was incubated at 37 °C for 30 min.

Fig. 7. Time-course of hydrolysis of phosphatidylcholine and phosphatidylethanolamine by recombinant starfish PLA₂.

Reaction mixture containing 24 ng recombinant starfish PLA₂, 100 μ g phosphatidylcholine (●) or phosphatidylethanolamine (▲) prepared from squid mantle muscle, 2.7 mM sodium deoxycholate, 5 mM CaCl₂, and 50 mM Tris-HCl buffer (pH 8.5) in a total volume of 130 μ l was incubated at 37 °C for various time periods.

Table 1
Purification of the starfish recombinant phospholipase A₂

	Protein (mg)	Total Activity ($\times 10^3$ U)*	Specific Activity ($\times 10^3$ U/mg)	Purity (fold)	Yield (%)
Crude Enzyme	184	174	0.9	1	100
DEAE-Cellulose	17	193	11	12	111
Sephadex G-50	2.3	200	87	97	115

* One unit of activity was defined as the number of micrograms of phosphatidylcholines hydrolyzed per minute.

DEAE, Diethylaminoethyl.

Table 2

Composition of fatty acids released from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine by the starfish recombinant phospholipase A_2 (%)

Fatty acid	Starfish recombinant enzyme	Starfish native enzyme*1	Porcine pancreatic enzyme*2
16:0	15.5	16.2	15.4
18:1n-9	84.5	83.8	84.6

*1 Phospholipase A_2 from the pyloric ceca of the starfish *A. pectinifera*.

*2 Phospholipase A_2 from porcine pancreas (Amano Pharmaceutical Co.).

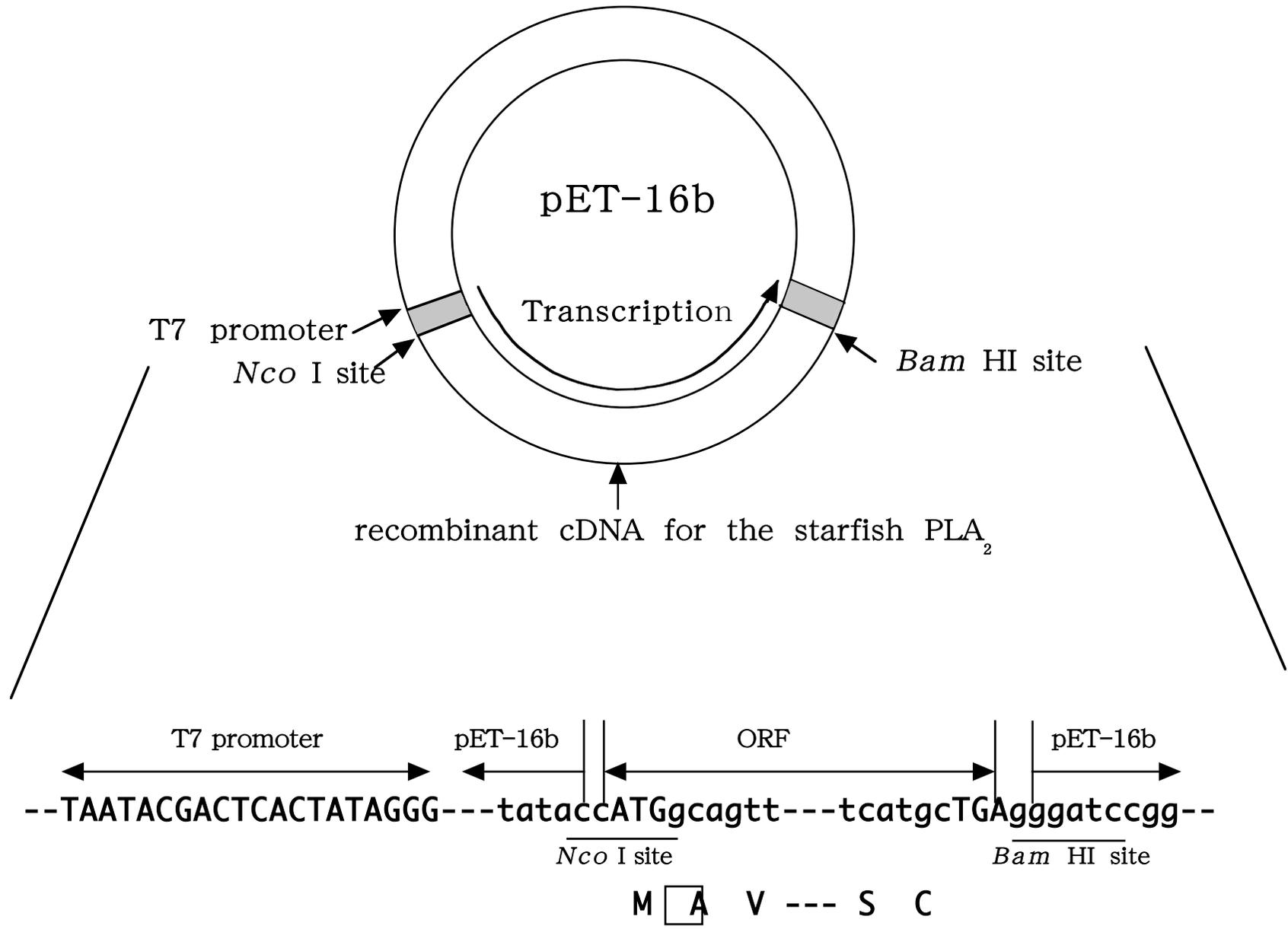
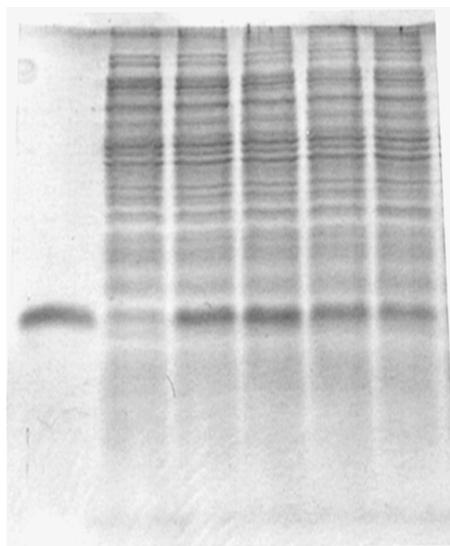


Fig.2



PLA₂ 0 1 2 3 4
Incubation time (h)

Fig.3

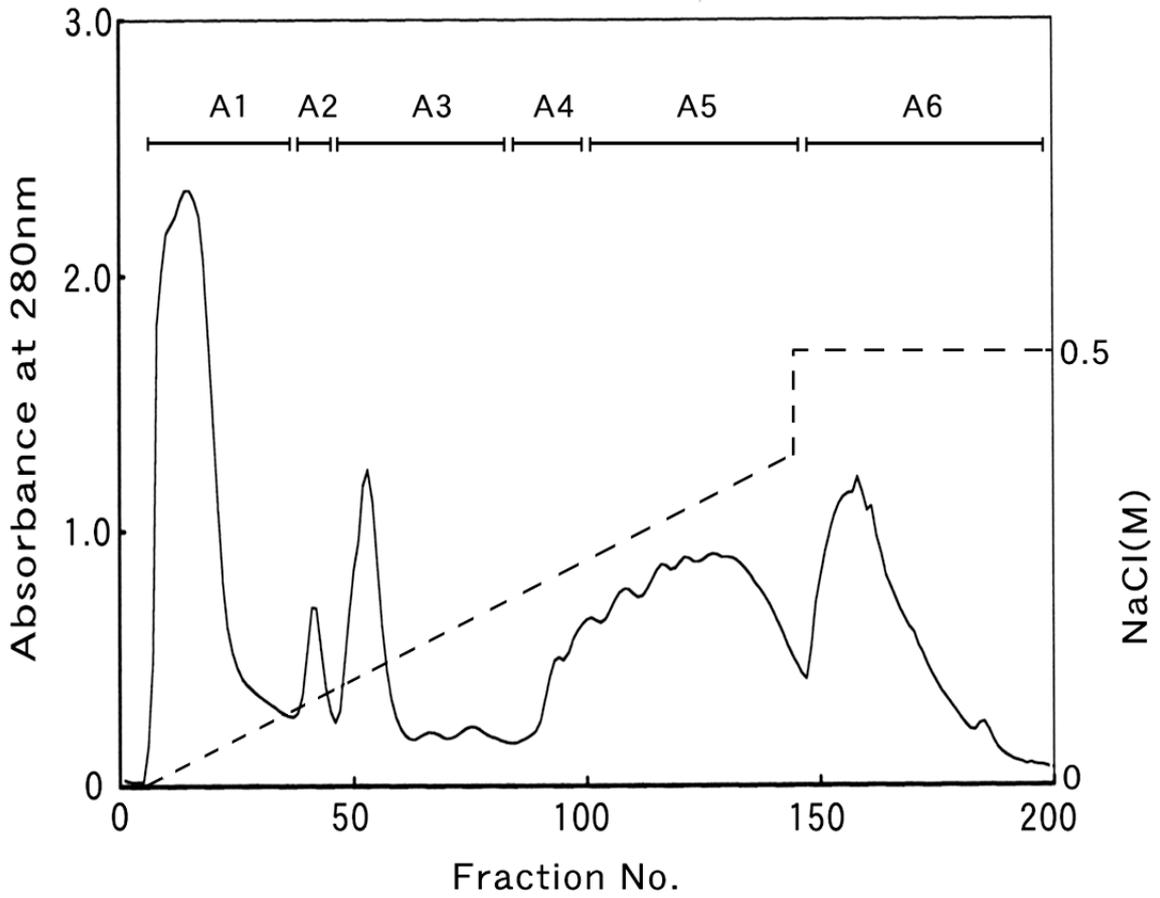
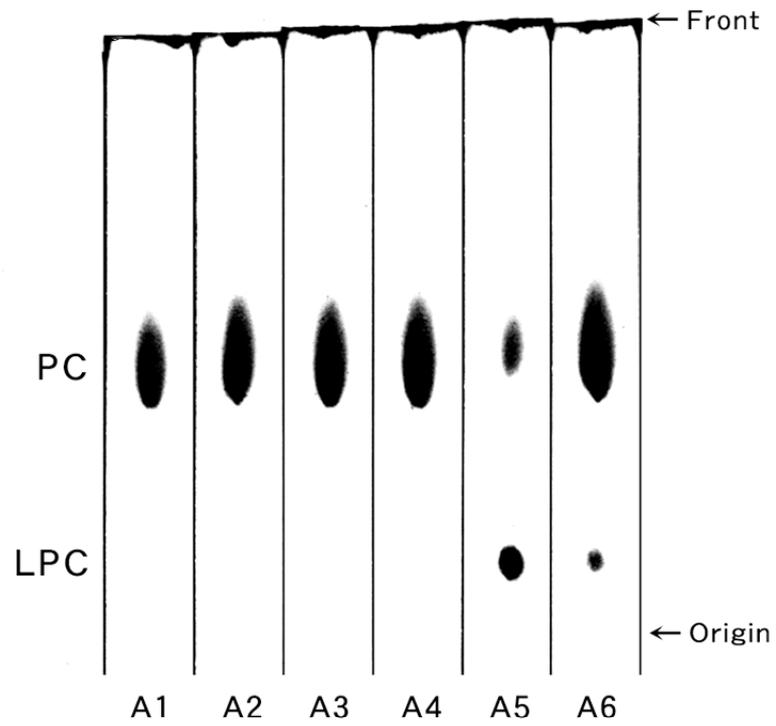
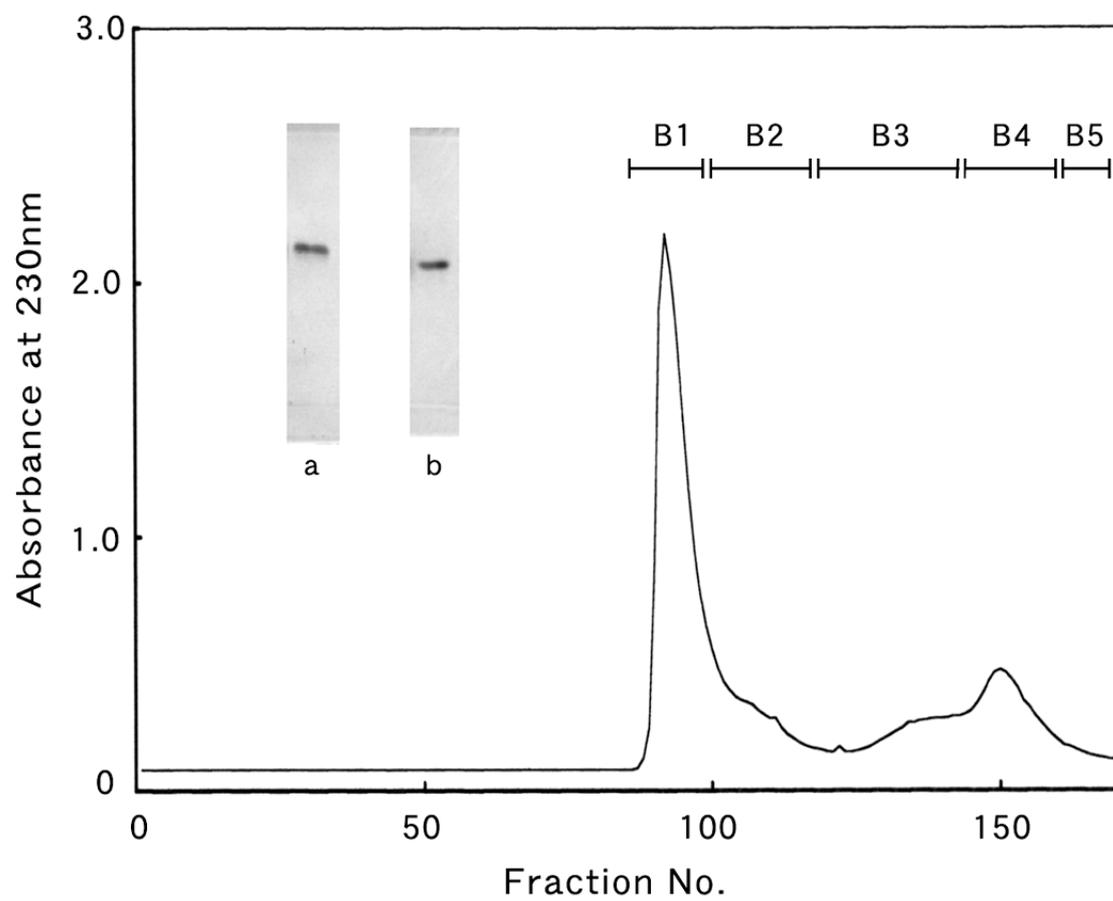
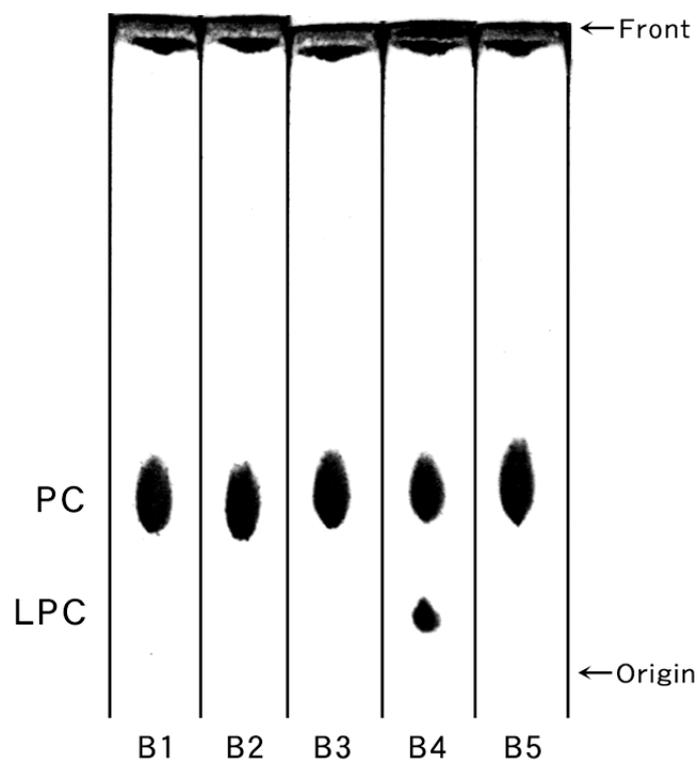


Fig.4



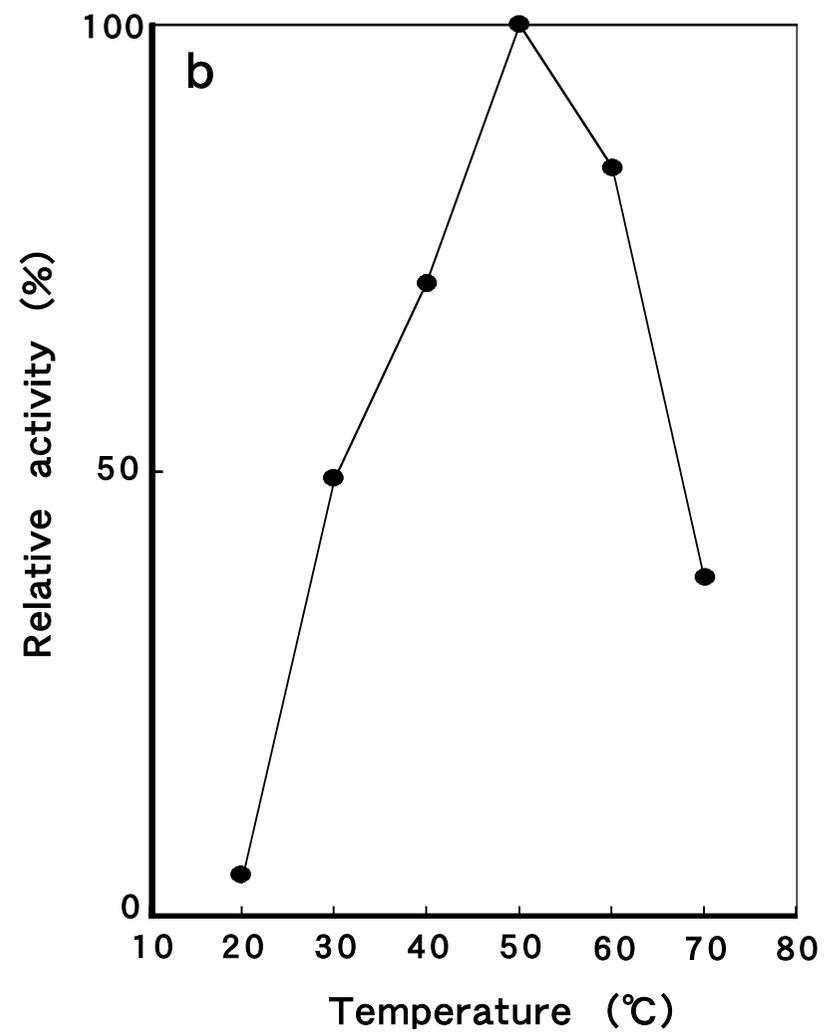
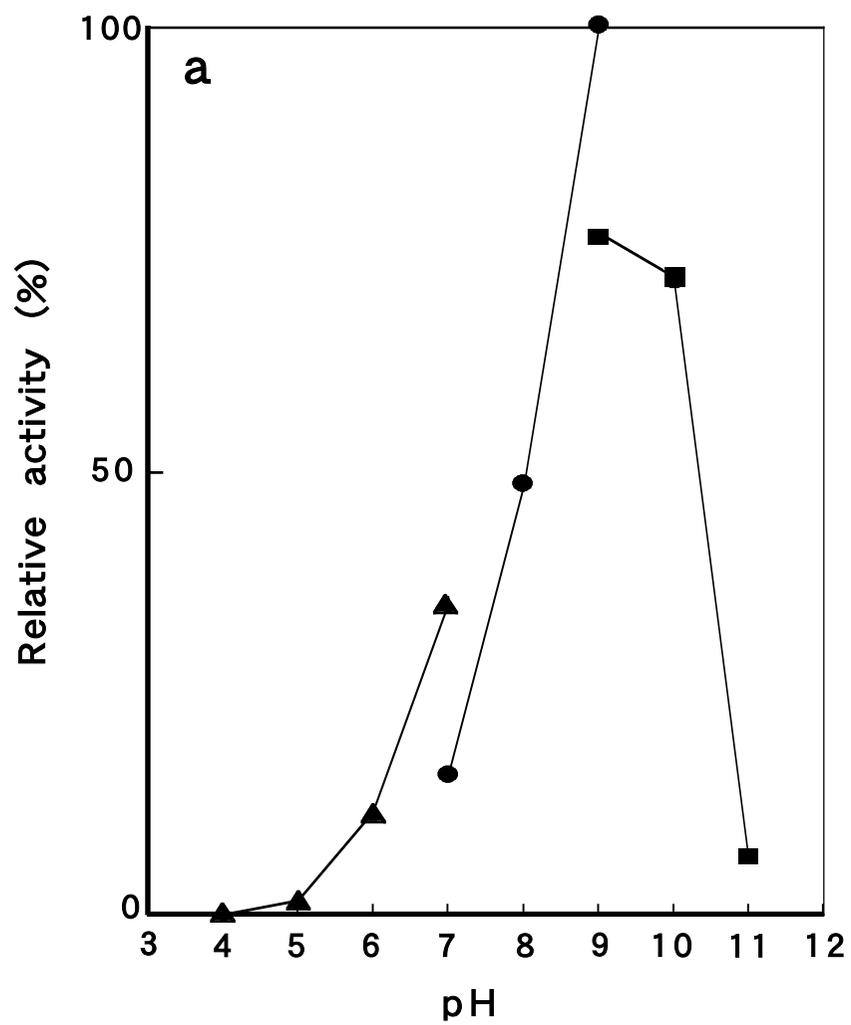


Fig.

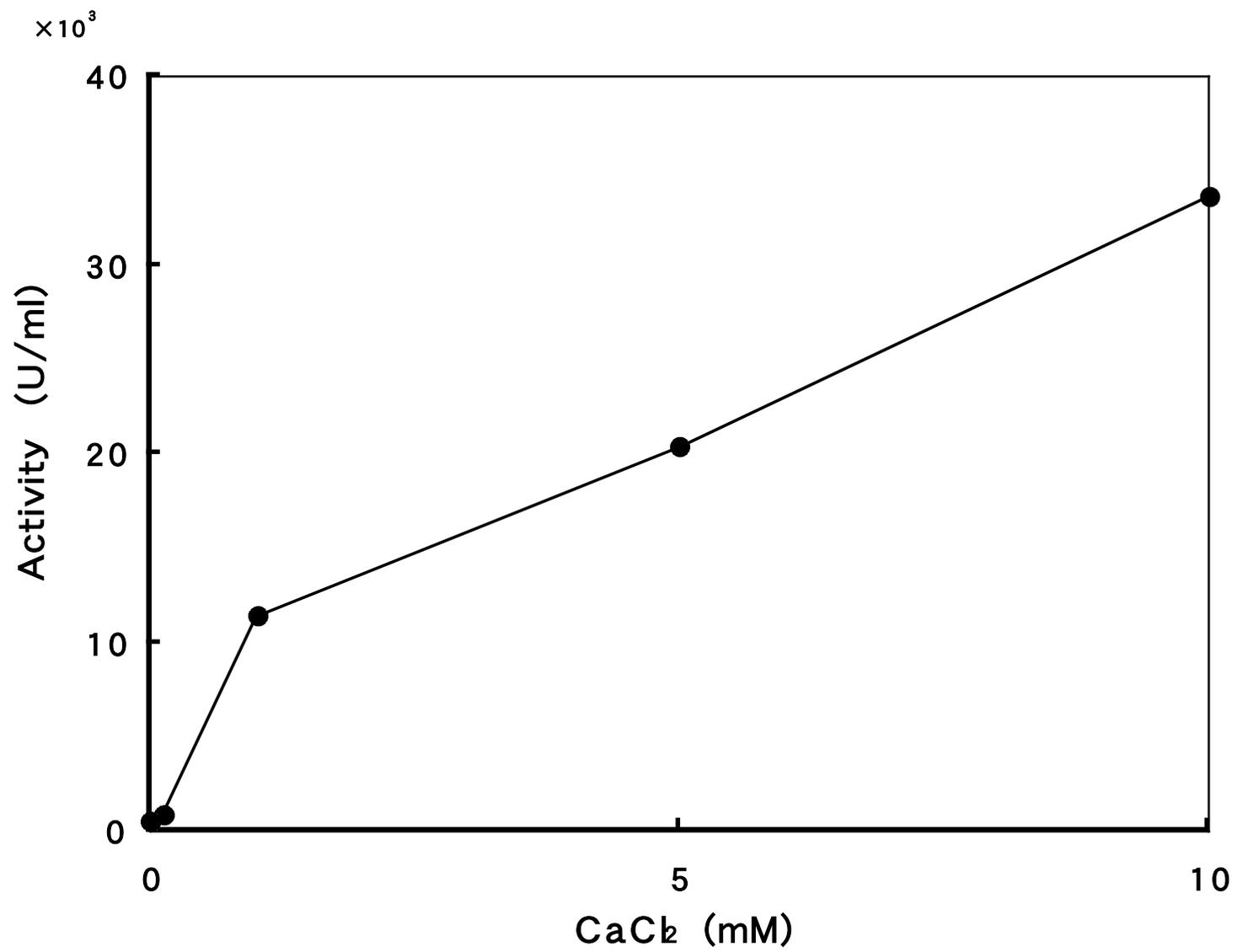


Fig.7

