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Characteristics of phospholipase A₂ mutant of the starfish
Asterina pectinifera

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Abstract

Site-directed mutagenesis study of phospholipase A₂ (PLA₂) from the pyloric ceca of starfish *Asterina pectinifera* was used to probe the relationship between polar-group specificity and structure of the pancreatic loop region. The sequence of the cDNA encoding the starfish PLA₂ was exchanged by the oligonucleotide-directed dual amber-long and accurate polymerase chain reaction method to insert Lys residue between Cys-62 and Gly-63. The modified cDNA was inserted into the expression plasmid pET-16b, and PLA₂ mutant was expressed in *Escherichia coli* OrigamiTM B (DE3) by induction with isopropyl-beta-D(-)-thiogalactopyranoside. The starfish PLA₂ mutant showed essentially the same properties as the starfish native PLA₂ with respect to substrate positional specificity, optimum pH, optimum temperature, Ca²⁺ requirement, and sodium deoxycholate requirement. However, the specific activity of the starfish PLA₂ mutant for egg yolk PC (950 U/mg) was extremely lower than that of native PLA₂ (119,000 U/mg), whereas near to that of porcine pancreatic PLA₂ (4,300 U/mg). Moreover, the ratio of specific activity of the PLA₂ mutant for phosphatidylcholine to phosphatidylethanolamine (98 times) was highly lower than that of native PLA₂ (2,650 times), but similar to that of porcine pancreatic PLA₂ (25 times). Therefore, it was suggested that the charge and structure of pancreatic loop region of the starfish PLA₂ might carry out important role on polar-group specificity.

Keywords: *Asterina pectinifera*; Starfish; Phospholipase A₂; Pancreatic loop; Mutant; 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 and produces free fatty acids and lysophospholipids. PLA₂ is widely distributed in the tissues of various organisms and consists of both extracellular- and intracellular-type enzymes [1]. Extracellular-type PLA₂ is abundant in mammalian pancreas and snake venom, and the enzymatic properties and amino acid sequences have been well characterized [2, 3]. Thus far, the molecular mechanism of catalytic action of the PLA₂ has been investigated on the basis of three-dimensional structure [2, 3].

On the other hand, few studies exist on PLA₂ from the digestive gland of marine invertebrates. Recently, we found remarkably high PLA₂ activity in the crude enzyme solution extracted from delipidated powder of the pyloric ceca of *Asterina pectinifera* [4]. Then we isolated PLA₂ from the pyloric ceca of the starfish *A. pectinifera*, and studied its enzymatic properties comparing with those of mammalian pancreatic PLA₂ [5]. The specific activity of the starfish PLA₂ for Egg yolk phosphatidylcholine (PC) was about 30 times higher than that of the commercially available PLA₂ from porcine pancreas. In addition, the starfish PLA₂ hydrolyzes PC more efficiently than phosphatidylethanolamine (PE) like snake venom PLA₂ but not mammalian pancreatic PLA₂. These facts suggest that the starfish PLA₂ possesses some different features in primary and higher order structure from the mammalian pancreatic PLA₂. Previously, Kuipers et al. [6] 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 up to 16 times on PC substrate. They deliberated that the Lys-62 is probably important for the interaction of porcine pancreatic PLA₂ with negatively

charged mixed micelles of bile salts and PC. Therefore, it was thought that the primary structure of starfish PLA₂ possibly differed from that of mammalian pancreatic PLA₂ at the corresponding region to the pancreatic loop. In fact, the amino acid sequence of the starfish PLA₂ (Fig. 1) showed some distinct features from mammalian PLA₂, e.g. two amino acids deletion in pancreatic loop region and thirteen amino acids insertion in beta-wing region when aligned with the sequence of the mammalian pancreatic PLA₂ [7, 8]. Thus, we considered that the above sequential differences might cause for the specific properties of the starfish PLA₂. In the previous study, we have succeeded to construct bacterial expression system for the starfish PLA₂ and found that basic properties of the recombinant PLA₂ were essentially the same as those of the starfish native PLA₂ [9]. 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 pancreatic loop and beta-wing regions of the starfish PLA₂.

In the present study, site-directed mutagenesis study was used to probe the relationship between polar-group specificity and structure of the pancreatic loop region of the starfish PLA₂.

2. Materials and methods

2.1. Materials

The "cDNA 1", fully encoding the starfish PLA₂ protein [8], was used for site-directed mutagenesis. Plasmid pET-16b and host strain, *E. coli* OrigamiTM B (DE3) were purchased from Novagen (Madison, WI, USA). Mutan-Super Express Km Kit and restriction endonucleases were purchased from TaKaRa (Kyoto, Japan). Egg yolk PC,

dipalmitoyl-PC, dipalmitoyl-PE 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). Sephadex G-50 was purchased from Pharmacia Biotech (Uppsala, Sweden). Diethylaminoethyl (DEAE)-cellulose was purchased from Whatman (Maidston, England). Porcine pancreatic PLA₂ were purchased from Sigma (St. Louis, MO, USA) and Amano Pharmaceutical Co. (Nagoya, Japan).

2.2. Lipid analysis

Thin-layer chromatography (TLC), preparative TLC, TLC with flame ionization detector (TLC/FID), and gas-liquid chromatography (GLC) were performed as described by Hayashi [10] and Hayashi and Kishimura [11].

2.3. Assay for PLA₂ activity

Ninety μ l of the aqueous medium containing a final concentration of 5 mM CaCl₂, 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, dipalmitoyl-PC, or dipalmitoyl-PE 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 qualitatively analyzed using TLC with a developing solvent of chloroform-methanol-acetic acid-water (55:17:3:2, v/v/v/v)

and quantitatively analyzed using TLC/FID with a developing solvent of chloroform-methanol-acetic acid-water (55:17:6.5:2.5, v/v/v/v) and hexane-diethyl ether (80:20, v/v). One unit of enzyme activity was defined as the number of μ g of substrates hydrolyzed per min.

POPC was used for the positional specificity analysis. Fifteen mg of POPC were almost hydrolyzed at 37 °C by 1mg (950 units) of starfish PLA₂ mutant for 3 h and by 1mg (440 units) of porcine pancreatic PLA₂ (Amano Pharmaceutical Co.) for 12 h, respectively. 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.

Effect of CaCl₂ on the activity of starfish PLA₂ mutant was examined in a reaction mixture containing 2 μ g of the enzyme, 100 μ g of egg yolk PC, 2.7 mM sodium deoxycholate, 50 mM Tris-HCl (pH 8.5), and 0 to 10 mM of CaCl₂. 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 CaCl₂.

Effect of sodium deoxycholate on the activity of starfish PLA₂ mutant was examined in a reaction mixture containing 2 μ g of the enzyme, 100 μ g of egg yolk PC, 5 mM CaCl₂, 50 mM Tris-HCl (pH 8.5), and 0 to 6.8 mM of sodium deoxycholate.

2.4. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 0.1 % SDS-15 % polyacrylamide slab-gel by the method of Laemmli [12]. Native PAGE was carried out using a 12.5 % polyacrylamide slab-gel with a 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 the background of the gel was destained with 7 % acetic acid.

2.5. Nucleotide sequencing

The nucleotide sequence was determined with ABI PRISM™ Dye Terminator Cycle Sequencing kit (Perkin Elmer-ABI (Foster City, CA, USA)) using a model 373A DNA sequencer (Perkin Elmer-ABI (Foster City, CA, USA)).

2.6. Protein determination

The protein concentration was determined by the method of Lowry et al. [13] using bovine serum albumin as a standard.

3. Results

3.1. Construction and purification of the starfish PLA₂ mutant

We constructed expression vector to make a mutant been inserted Lys residue between Cys-62 and Gly-63 in pancreatic loop region of the starfish PLA₂ (Fig. 2). The sequence of the “cDNA 1” encoding the starfish PLA₂ was exchanged by the oligonucleotide-directed dual amber-long and accurate polymerase chain reaction method. The oligonucleotide used for the construction of expression plasmid of the starfish PLA₂ mutant was 5'-GCGGAGGCCGACTGCAAAGGTTCTTGGGACCCC-3'. The underlined bases indicate the location of mutation. As shown Fig. 3, it was inserted a codon (AAG)

corresponding to Lys residue at positions 186-188 of the “cDNA 1”. The modified “cDNA 1” was subcloned into pET-16b plasmid for protein expression. The PLA₂ mutant protein was expressed in *E. coli* Origami™ B (DE3) by induction with IPTG. The PLA₂ mutant 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). The renatured PLA₂ mutant was purified by subsequent column chromatographies on DEAE-cellulose (DE-52) and Sephadex G-50. As shown in Fig. 4, purified PLA₂ mutant showed a single band on both SDS-PAGE and native PAGE.

3.2. Enzymatic properties of the starfish PLA₂ mutant

The positional specificity of the purified starfish PLA₂ mutant was examined using POPC. The enzyme released mainly oleic acid from POPC like the starfish native PLA₂ and porcine pancreatic PLA₂. The enzyme hydrolyzed egg yolk PC effectively at alkaline pHs with an optimum at around pH 9.0, and optimum temperature was observed at around 50 °C. The starfish PLA₂ mutant was activated by 1 mM or higher concentrations of Ca²⁺. The activity of the starfish PLA₂ mutant was stimulated most by adding Ca²⁺ followed by Mg²⁺ and Co²⁺, while it was strongly inhibited by adding Hg²⁺, Zn²⁺, and EDTA. The activity of the starfish PLA₂ mutant was enhanced by adding sodium deoxycholate at an optimum activity of 2 to 4 mM. The specific activity of the starfish PLA₂ mutant for egg yolk PC (950 U/mg) was extremely lower than that of native PLA₂ (119,000 U/mg), whereas near to that of porcine pancreatic PLA₂ (4,300 U/mg). As shown in Table 1, the ratio of specific activity of the starfish PLA₂ mutant for dipalmitoyl-PC to dipalmitoyl-PE (98 times) was highly lower than that of starfish native PLA₂ (2,650 times), but similar to that of porcine pancreatic PLA₂ (25 times).

4. Discussion

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 [2, 3]. 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 [14]. Recently, mutagenesis studies indicated that the Lys-62 and Arg-53 of porcine pancreatic PLA₂ and Lys-53 and Lys-56 of bovine pancreatic PLA₂ are involved in their specificities for polar-group of phospholipid presumably by electrostatically repelling cationic polar-group of phospholipid [15-18]. Noel et al. [16] reported that site-directed mutagenesis studies of bovine pancreatic PLA₂ showed replacement of surface residue Lys-56 by a neutral or hydrophobic amino acid residue resulted significant change in the function of the enzyme. The k_{cat} for PC micelles increased 3-4 fold for K56M, K56I, and K56F and ca. 2-fold for K56N and K56T but did not change for K56R. Also the mutation had not only perturbed the conformation of the side chain of Met-56 locally but also caused conformational changes in the neighboring loop (residue 60-70), resulting in the formation of a hydrophobic pocket by residues Met-56, Tyr-52 and Tyr-69. These results suggested that the side chain of residue 56 has significant influence on the interaction of PLA₂ with polar-group of phospholipid molecule. Moreover, 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 [17, 18]. As

shown in Fig.1, 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). 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 in mammalian pancreatic PLA₂, predicted to participate in substrate binding and interfacial interaction, were deleted or substituted for neutral and negatively charged residues in the starfish PLA₂ (Figs. 1 and 2). These facts imply that the substrate binding and interfacial binding mode of the starfish PLA₂ was somehow different from that of mammalian pancreatic PLA₂.

In the present study, site-directed mutagenesis study was used to probe the relationship between polar-group specificity and structure of the pancreatic loop region of the starfish PLA₂. The sequence of the cDNA encoding the starfish PLA₂ was exchanged to insert Lys residue between Cys-62 and Gly-63. The starfish PLA₂ mutant showed essentially the same properties as the starfish native PLA₂ with respect to substrate positional specificity, optimum pH, optimum temperature, Ca²⁺ requirement, and sodium deoxycholate requirement. However, the specific activity of the starfish PLA₂ mutant for egg yolk PC (950 U/mg) was extremely lower than that of native PLA₂ (119,000 U/mg), whereas near to that of porcine pancreatic PLA₂ (4,300 U/mg). Moreover, the ratio of specific activity of the starfish PLA₂ mutant for dipalmitoyl-PC to dipalmitoyl-PE (98 times) was highly lower than that of native PLA₂ (2,650 times), but similar to that of porcine pancreatic PLA₂ (25 times). Therefore, it was suggested that the charge and structure of pancreatic loop region of the starfish PLA₂ might carry out important role on polar-group specificity.

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(captions to figures)

Fig. 1. Alignment of the amino acid sequences of the starfish PLA₂ and porcine pancreatic PLA₂. Dashes indicate deletions introduced for maximizing the sequence similarity. The locations of the pancreatic loop and beta-wing are shown with solid bars based on the crystallographic studies of bovine pancreatic and *Crotalus atrox* venom PLA₂s. [2, 19] Starfish, *A. pectinifera* PLA₂. [6, 7]; Porcine, porcine pancreatic PLA₂. [20]

Fig. 2. Comparison of amino acid sequences of PLA₂s. Sixteen amino acid residues in pancreatic loop region (residue 54-69) are shown. Starfish, starfish PLA₂; Mutant, starfish PLA₂ mutant; Porcine; porcine pancreatic PLA₂; Bovine, bovine pancreatic PLA₂. Key residues are shown in shaded.

Fig. 3. The nucleotide and deduced amino acid sequence of the cDNA of the starfish PLA₂ mutant. The deduced amino acid sequence and the residue numbers are shown below the codons. The single-letter amino acid code is used. Numbers in the right margin refer to the last nucleotide in each row. Annealing site of mutation-primer is underlined.

Fig. 4. Electrophoresis of the purified starfish PLA₂ mutant. a: SDS-PAGE. Lane 1 contains starfish PLA₂ mutant. 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: native PAGE. Lane 1 contains starfish PLA₂ mutant.

Fig.1

	10	20	30	40	50	
Starfish	SVYQFGKFIS		CYGGAGFFDGLDYNGYGCYCGYGGKGTPLDDTDRCCLVHI			
Porcine	ALWQFRSMIK		C-AIPGSHPLMDFNNYGCYCGLGSGTVPDELDRCCETHDI			
	60	70	80	90	100	
	GKATAEADC	-GSWD	PYII	VDYE	QTTDASGN	---VIKCKKAADYSWSTNPECREFM
	RD	AKNLD	SCKFL	VDN	PYTESYS	-----CSNTEITC-----NSKNNACEAFI
		pancreatic loop		β -wing		
	110	120	130			
	CECDRAGAQCFAEK	RPTYNQAYESYD	-KDSC			
	CNCDRNAAICFS	-KAP	-YNKEHKNLDTKKYC			

Fig.2

	54	60	69	
Starfish		GKATAEAD	G	GSWD-P
Mutant		GKATAEAD	K	GSWD-P
Porcine		RD	AKNLDS	KFLVDNP
Bovine		KQ	AKLDS	KVLVDNP

Fig.3

TCAGTTTACCAGTTCCGCAAGTTCATTTTCGTGCTATGGTG 40
S V Y Q F G K F I S C Y G
1 10

GTGCTGGGTTTTTTCGATGGGTTGGACTACAACGGCTATGG 80
G A G F F D G L D Y N G Y G
20

GTGTTACTGCGGCTACGGAGGCAAAGGAACACCGTTGGAT 120
C Y C G Y G G K G T P L D
30 40

GACACCGACAGATGCTGTCTAGTGACAGATAACTGTTACG 160
D T D R C C L V H D N C Y
50

GCAAAGCTACCGCGGAGGCCGACTGCAAGGGTTCTTGGGA 200
G K A T A E A D C K G S W D
60

CCCCTACATCATAGTTTACGACTATGAACAAACCACTGAT 240
P Y I I V Y D Y E Q T T D
70 80

GCGTCTGGAAACTGTGTCAATGCAAGAAAGCGGCCG 280
A S G N C V I K C K K A A
90

ACTATTCTTGGTATTCTACCAATCCCGAATGCAGAGAGTT 320
D Y S W Y S T N P E C R E F
100

CATGTGCGAATGTGACCGCGCGGGGGCGCAGTGCTTCGCT 360
M C E C D R A G A Q C F A
110 120

GAAAAGCGCCCAACGTACAACCAAGCTTACGAGTCCG 400
E K R P T Y N Q A Y E S Y
130

ACAAGGATTCATGC 414
D K D S C

Fig.4

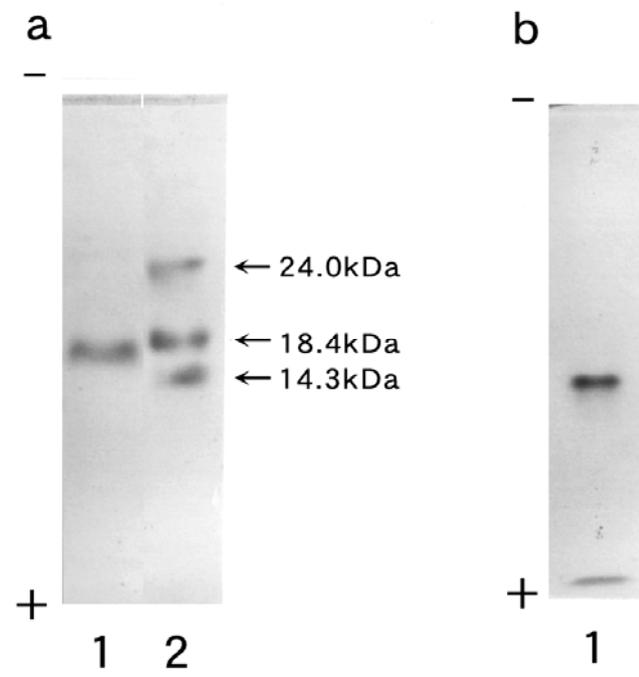


Table 1

Table 1
Specific activities of the starfish PLA_2 mutant for dipalmitoyl-PC and dipalmitoyl-PE

	Specific activity (U/mg)		Ratio of specific activity for dipalmitoyl-PC to dipalmitoyl-PE
	dipalmitoyl-PC	dipalmitoyl-PE	
Starfish native PLA_2	26,000	9.8	2,653
Starfish PLA_2 mutant	391	4.0	98
Porcine PLA_2 ^{*1}	368	14.8	25

*1 PLA_2 from porcine pancreas (Amano Pharmaceutical Co.).

