Characteristics of phospholipase A_2 mutant of the starfish *Asterina pectinifera*

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Abstract

Site-directed mutagenesis study of phospholipase A_2 (PLA_2) 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 PLA_2 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 PLA_2 mutant was expressed in *Escherichia coli* Origami^{TM} B (DE3) by induction with isopropyl-beta-D(-)-thiogalactopyranoside. The starfish PLA_2 mutant showed essentially the same properties as the starfish native PLA_2 with respect to substrate positional specificity, optimum pH, optimum temperature, Ca^{2+} requirement, and sodium deoxycholate requirement. However, the specific activity of the starfish PLA_2 mutant for egg yolk PC (950 U/mg) was extremely lower than that of native PLA_2 (119,000 U/mg), whereas near to that of porcine pancreatic PLA_2 (4,300 U/mg). Moreover, the ratio of specific activity of the PLA_2 mutant for phosphatidylcholine to phosphatidylethanolamine (98 times) was highly lower than that of native PLA_2 (2,650 times), but similar to that of porcine pancreatic PLA_2 (25 times). Therefore, it was suggested that the charge and structure of pancreatic loop region of the starfish PLA_2 might carry out important role on polar-group specificity.

Keywords: Asterina pectinifera; Starfish; Phospholipase A_2; Pancreatic loop; Mutant; Polar-group specificity

1. Introduction

Phospholipase A_2 (PLA_2) (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. PLA_2 is widely distributed in the tissues of various organisms and consists of both extracellular- and intracellular-type enzymes [1]. Extracellular-type PLA_2 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 PLA_2 has been investigated on the basis of three-dimensional structure [2, 3].

On the other hand, few studies exist on PLA_2 from the digestive gland of marine invertebrates. Recently, we found remarkably high PLA_2 activity in the crude enzyme solution extracted from delipidated powder of the pyloric ceca of *Asterina pectinifera* [4]. Then we isolated PLA_2 from the pyloric ceca of the starfish *A. pectinifera*, and studied its enzymatic properties comparing with those of mammalian pancreatic PLA_2 [5]. The specific activity of the starfish PLA_2 for Egg yolk phosphatidylcholine (PC) was about 30 times higher than that of the commercially available PLA_2 from porcine pancreas. In addition, the starfish PLA_2 hydrolyzes PC more efficiently than phosphatidylethanolamine (PE) like snake venom PLA_2 but not mammalian pancreatic PLA_2. These facts suggest that the starfish PLA_2 possesses some different features in primary and higher order structure from the mammalian pancreatic PLA_2. Previously, Kuipers et al. [6] reported that a recombinant porcine pancreatic PLA_2 mutant with a deletion of the pancreatic loop at positions 62-66 gave an intermediate conformation between wild type porcine PLA_2 and snake venom PLA_2, 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 PLA_2 with negatively

charged mixed micelles of bile salts and PC. Therefore, it was thought that the primary structure of starfish PLA_2 possibly differed from that of mammalian pancreatic PLA_2 at the corresponding region to the pancreatic loop. In fact, the amino acid sequence of the starfish PLA_2 (Fig. 1) showed some distinct features from mammalian PLA_2, 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 PLA_2 [7, 8]. Thus, we considered that the above sequential differences might cause for the specific properties of the starfish PLA_2. In the previous study, we have succeeded to construct bacterial expression system for the starfish PLA_2 and found that basic properties of the recombinant PLA_2 were essentially the same as those of the starfish native PLA_2 [9]. The recombinant starfish PLA_2 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 PLA_2.

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 PLA_2.

2. Materials and methods

2.1. Materials

The "cDNA 1", fully encoding the starfish PLA_2 protein [8], was used for site-directed mutagenesis. Plasmid pET-16b and host strain, *E. coli* Origami™ 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 PLA_2 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 frame 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 PLA_2 activity

Ninety μl 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 μl of substrate solution containing 100 μg 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 μl of the enzyme solution was added to initiate the reaction. The mixture was incubated at 37 ℃ for 30 min, and the reaction was ended by adding 650 μl 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 μg of substrates hydrolyzed per min.

POPC was used for the positional specificity analysis. Fifteen mg of POPC were almost hydrolyzed at 37 ℃ by 1mg (950 units) of starfish PLA_2 mutant for 3 h and by 1mg (440 units) of porcine pancreatic PLA_2 (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 $CaCl_2$ on the activity of starfish PLA_2 mutant was examined in a reaction mixture containing 2 μg 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 mutant was examined in a reaction mixture containing 2 μg 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.

2.4. 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^{TM} 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 PLA_2 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 PLA_2 (Fig. 2). The sequence of the "cDNA 1" encoding the starfish PLA_2 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 PLA_2 mutant was 5'-GCGGAGGCCGACTGCAAGGGTTCTTGGGACCCC-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 PLA_2 mutant protein was expressed in *E. coli* Origami^{TM} B (DE3) by induction with IPTG. The PLA_2 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 PLA_2 mutant was purified by subsequent column chromatographies on DEAE-cellulose (DE-52) and Sephadex G-50. As shown in Fig. 4, purified PLA_2 mutant showed a single band on both SDS-PAGE and native PAGE.

3.2. Enzymatic properties of the starfish PLA_2 mutant

The positional specificity of the purified starfish PLA_2 mutant was examined using POPC. The enzyme released mainly oleic acid from POPC like the starfish native PLA_2 and porcine pancreatic PLA_2. 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 ℃. The starfish PLA_2 mutant was activated by 1 mM or higher concentrations of Ca^{2+}. The activity of the starfish PLA_2 mutant was stimulated most by adding Ca^{2+} followed by Mg^{2+} and Co^{2+}, while it was strongly inhibited by adding Hg^{2+}, Zn^{2+}, and EDTA. The activity of the starfish PLA_2 mutant was enhanced by adding sodium deoxycholate at an optimum activity of 2 to 4 mM. The specific activity of the starfish PLA_2 mutant for egg yolk PC (950 U/mg) was extremely lower than that of native PLA_2 (119,000 U/mg), whereas near to that of porcine pancreatic PLA_2 (4,300 U/mg). As shown in Table 1, the ratio of specific activity of the starfish PLA_2 mutant for dipalmitoyl-PC to dipalmitoyl-PE (98 times) was highly lower than that of starfish native PLA_2 (2,650 times), but similar to that of porcine pancreatic PLA_2 (25 times).

4. Discussion

Since PLA_2 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 PLA_2 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 PLA_2 and Lys-53 and Lys-56 of bovine pancreatic PLA_2 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 PLA_2 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 PLA_2 with polar-group of phospholipid molecule. Moreover, studies of mammalian pancreatic PLA_2 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 PLA_2 completely conserved the residues which are critical for forming the catalytic network (His-49, Asp-111, Tyr-53, and Tyr-72) and Ca^{2+}-binding loop (Tyr-29, Gly-31, Gly-33, and Asp-50). Therefore, we consider that the starfish PLA_2 may function through a similar mechanism of those of mammalian pancreatic PLA_2. However, most of the above positively charged residues in mammalian pancreatic PLA_2, predicted to participate in substrate binding and interfacial interaction, were deleted or substituted for neutral and negatively charged residues in the starfish PLA_2 (Figs. 1 and 2). These facts imply that the substrate binding and interfacial binding mode of the starfish PLA_2 was somehow different from that of mammalian pancreatic PLA_2.

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 PLA_2. The sequence of the cDNA encoding the starfish PLA_2 was exchanged to insert Lys residue between Cys-62 and Gly-63. The starfish PLA_2 mutant showed essentially the same properties as the starfish native PLA_2 with respect to substrate positional specificity, optimum pH, optimum temperature, Ca^{2+} requirement, and sodium deoxycholate requirement. However, the specific activity of the starfish PLA_2 mutant for egg yolk PC (950 U/mg) was extremely lower than that of native PLA_2 (119,000 U/mg), whereas near to that of porcine pancreatic PLA_2 (4,300 U/mg). Moreover, the ratio of specific activity of the starfish PLA_2 mutant for dipalmitoyl-PC to dipalmitoyl-PE (98 times) was highly lower than that of native PLA_2 (2,650 times), but similar to that of porcine pancreatic PLA_2 (25 times). Therefore, it was suggested that the charge and structure of pancreatic loop region of the starfish PLA_2 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 PLA_2 and porcine pancreatic PLA_2. 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 PLA_2s. [2, 19] Starfish, *A. pectinifera* PLA_2. [6, 7]; Porcine, porcine pancreatic PLA_2. [20]

Fig. 2. Comparison of amino acid sequences of PLA_2s. Sixteen amino acid residues in pancreatic loop region (residue 54-69) are shown. Starfish, starfish PLA_2; Mutant, starfish PLA_2 mutant; Porcine; porcine pancreatic PLA_2; Bovine, bovine pancreatic PLA_2. Key residues are shown in shaded.

Fig. 3. The nucleotide and deduced amino acid sequence of the cDNA of the starfish PLA_2 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 PLA_2 mutant. a: SDS-PAGE. Lane 1 contains starfish PLA_2 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 PLA_2 mutant.

Fig.1

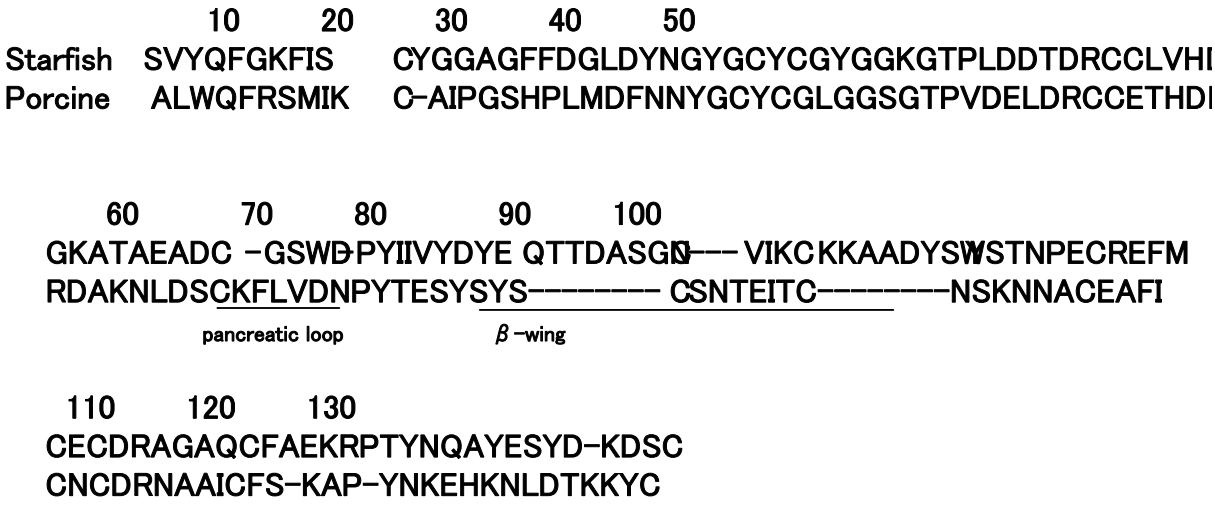

Fig.2

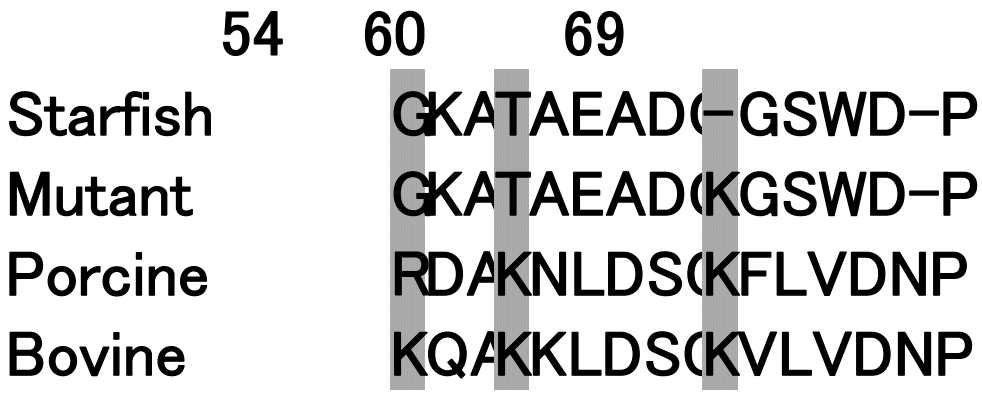
54 60 69
Starfish GKATAEADG-GSWD-P
Mutant GKATAEADGKGSWD-P
Porcine RDAKNLDSGKFLVDNP
Bovine KQAKKLDSGKVLVDNP

Fig.3


TCAGTTTACCAGTTCGGCAAGTTCATTTCGTGCTATGGTG 40
S V Y Q F G K F I S C Y G
1 10
GTGCTGGGTTTTTCGATGGGTTGGACTACAACGGCTATGG 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
GACACCGACAGATGCTGTCTAGTGCACGATAACTGTTACG 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
GCGTCTGGAAACTGTGTCATCAAATGCAAGAAAGCGGCCG 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
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ACAAGGATTCATGC 414
D K D S C

Fig.4

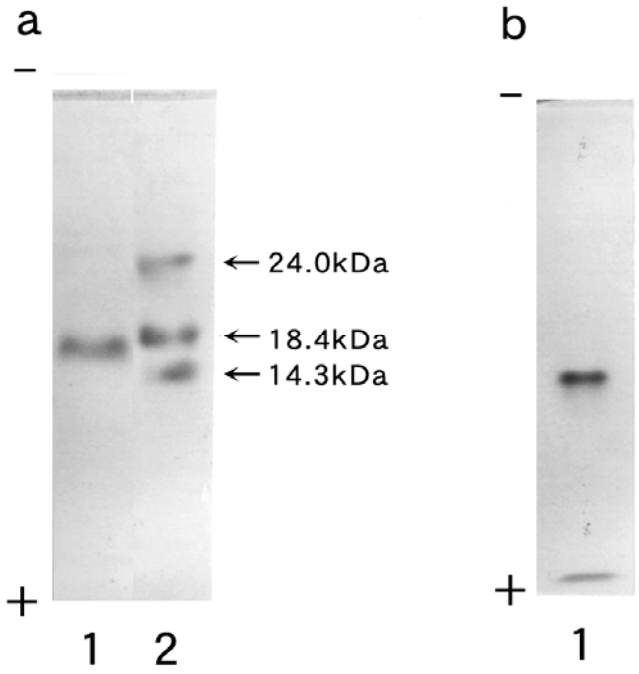

Table 1

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.).