



Title	cDNA cloning and sequencing of phospholipase A2 from the pyloric ceca of the starfish <i>Asterina pectinifera</i>
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cDNA Cloning and Sequencing of Phospholipase A₂
from the Pyloric Ceca of the Starfish *Asterina*
pectinifera

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Abbreviations: PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Abstract

Three cDNA from the pyloric ceca of the starfish *Asterina pectinifera*, (namely, cDNA 1, 2, and 3), encoding phospholipase A₂ (PLA₂), were isolated and sequenced. These cDNAs were composed of 415 bp with an open reading frame of 414 bp at nucleotide positions 1 to 414, which encodes 138 amino acids including N-terminal Met derived from the PCR primer. The amino acid sequence deduced from the cDNA 1 was completely consistent with the sequence determined with the starfish PLA₂ protein, while those deduced from cDNA 2 and cDNA 3 differed at one and twelve amino acid residual positions, respectively, from the sequence of the PLA₂ protein, suggesting the presence of multiple forms in the starfish PLA₂. All of the sequences deduced from cDNA 1, 2, and 3 required two amino acid deletions in pancreatic loop region, and sixteen insertions and three deletions in β -wing region when aligned with the sequence of mammalian pancreatic PLA₂. In phylogenetic tree, the starfish PLA₂ should be classified into an independent group, but hardly to the established groups I A and I B. The characteristic structure in the pancreatic loop and β -wing regions may account for the specific properties of the starfish PLA₂, e.g., the higher activity and characteristic substrate specificity compared with mammalian pancreatic PLA₂.

Keywords: cDNA cloning; Group I ; Isoforms; Pancreatic loop; Phospholipase A₂; Phylogenetic tree; Starfish; β -wing

1. Introduction

Phospholipase A₂ (PLA₂; EC 3.1.1.4) is the enzyme that 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 consists of both extracellular- and intracellular-type enzymes (Dennis, 1997). Extracellular-type PLA₂ is abundant in mammalian pancreas and snake venom, and the enzymatic properties and amino acid sequences have been well characterized (Arni and Ward, 1996; Dennis, 1983). Thus far, the molecular mechanism of catalytic action of the PLA₂ has been investigated on the basis of three-dimensional structure (Arni and Ward, 1996; Dennis, 1983).

On the other hand, there appear to be a few studies on the digestive gland PLA₂ from marine invertebrates. In 1975, Okabe et al. partially purified PLA₂ from the pyloric ceca of the starfish *Asterina pectinifera*, and later, we prepared PLA₂-like enzyme from the starfish *Solaster paxillatus* (Kishimura and Hayashi, 1998). The basic properties of these enzymes such as Ca²⁺ requirement, optimum pH, and heat stability were similar to those of the mammalian pancreatic PLA₂. However, detailed properties and primary structures of both the purified enzymes remained to be investigated.

Recently, we isolated PLA₂ from the pyloric ceca of the starfish *A. pectinifera*, and studied its enzymatic properties comparing with those of mammalian pancreatic PLA₂ (Kishimura and Hayashi, 1999). The specific activity of the starfish PLA₂ for phosphatidylcholine was about 30 times higher than that of the commercially available PLA₂ from porcine pancreas (Sigma). In addition, the starfish PLA₂ hydrolyzes phosphatidylcholine

more efficiently than phosphatidylethanolamine like a snake venom PLA₂ but not a mammalian pancreatic PLA₂. These facts suggest that the starfish PLA₂ possesses some different features in primary and/or higher order structure from the mammalian pancreatic PLA₂. In fact, the amino acid sequence of the starfish PLA₂ showed some distinct features from mammalian PLA₂, e.g., two amino acid deletions in pancreatic loop region, and sixteen insertions and three deletions in β -wing region when aligned with the sequence of the mammalian pancreatic PLA₂ (Kishimura et al., 2000). Thus, we considered that the above sequential differences might cause for the specific properties of the starfish PLA₂. Accordingly, the studies utilizing recombinant DNA techniques have been expected to provide great advantages for further investigation of the structure-function relationships of the starfish PLA₂.

In the present paper, we describe the cloning and sequencing of the cDNAs encoding the *A. pectinifera* PLA₂.

2. Materials and methods

2.1 Materials

The starfish *A. pectinifera* was collected from the tideland at Usujiri near Hakodate in Hokkaido Prefecture, Japan, in January 1998. Cloning vector, pBluescript II KS(+) and host strain, *Escherichia coli* XL1-Blue were purchased from Stratagene (La Jolla, CA). AMV reverse transcriptase XL,

TaKaRa Taq™, T4 DNA polymerase, and restriction endonucleases were purchased from TaKaRa (Kyoto, Japan).

2.2 PCR and cDNA sequencing

Pyloric ceca (7.5g) were dissected from living starfishes and the total RNA was extracted by guanidium thiocyanate method described in the standard protocol (Maniatis et al., 1982). Poly (A)⁺RNA was isolated from the total RNA with Oligotex-dT30 (TaKaRa, Kyoto, Japan). The first strand cDNA was synthesized with random hexanucleotide primers and reverse transcriptase, and the cDNA for the PLA₂ was amplified by PCR with mixed oligonucleotide primers designed on the basis of the N- and C-terminal amino acid sequences of the starfish PLA₂ protein (Fig. 1). The PCR products were subcloned to pBluescript II KS(+) plasmid vector for sequencing. The nucleotide sequence of the cDNA was determined with a dye terminator cycle sequencing kit (Perkin Elmer–Applied Biosystems (Foster City, CA)) using a model 373A DNA sequencer (Perkin Elmer–Applied Biosystems (Foster City, CA)).

3. Results and discussion

3.1 cDNA clones for the starfish PLA₂

cDNAs of approximately 400 bp estimated by agarose gel electrophoresis

were obtained by PCR with a set of primers shown in Fig. 1. The amplified cDNAs were blunted by T4 DNA polymerase reaction and subcloned to an *Sma* I site of pBluescript II KS(+). By determination of nucleotide sequences of 8 independent clones obtained three species of cDNAs (cDNA 1, 2, and 3) were found to encode the PLA₂, i.e., one, three, and four clones contained the cDNA 1, cDNA 2, and cDNA 3, respectively. The nucleotide and deduced amino acid sequences of cDNA 1, 2, and 3 are shown in Fig. 2. The cDNA 1, 2, and 3 were all composed of 415 bp with an open reading frame of 414 bp at nucleotide positions 1 to 414, which encode 138 amino acids including N-terminal Met derived from the "ATG" in the PCR primer. Pancreatic PLA₂ protein generally contains signal- and pro-sequences before maturation, however, in the present study, with the aim to bacterially express the starfish PLA₂ in mature form, we added "ATG" as a translational initiation codon to 5'-terminus of the forward primer. At present, it is not clear whether or not the starfish PLA₂ has signal- and pro-peptides in premature form.

The amino acid sequence deduced from cDNA 1, namely, PLA₂ 1, is completely consistent with the sequence determined previously with the PLA₂ protein (see Fig. 2) (Kishimura et al., 2000). While, the sequences deduced from cDNA 2 and cDNA 3 (termed PLA₂ 2 and PLA₂ 3, respectively) differed in one position (amino acid number 35) and twelve positions (numbers 32, 35, 55, 57, 65, 71, 80, 81, 87, 113, 116, and 120), respectively, from the sequence of the PLA₂ protein, suggesting the presence of multiple forms in the starfish PLA₂. Although PLA₂ 1 and PLA₂ 2 differ in one amino acid position, cDNA 1 and cDNA 2 differ in seven

nucleotide positions. Therefore, it seems that these differences are not due to the sequencing problem. On the other hand, separation and sequence determination of the isoforms were not achieved in the previous study (Kishimura and Hayashi, 1999). Since the PLA₂ 2 and PLA₂ 3 proteins can be expressed with the cDNAs in the appropriate host vector system, we will show their enzymatic activities elsewhere.

3.2 Comparison of the amino acid sequences of various PLA₂s

The amino acid sequences of the starfish PLA₂ 1, 2, and 3, were aligned with those of porcine pancreatic PLA₂ (group I B type) (Puijk et al., 1977), snake venom PLA₂s from elapinae (*Naja naja atra*, group I A type) (Chang et al., 1997), crotalinae (*Crotalus atrox*, group II A type) (Randolph and Henrikson, 1982), viperinae (*Bitis gabonica*, group II B type) (Botes and Viljoen, 1974), and rat brain PLA₂ (group II C type) (Chen et al., 1994) (Fig. 3). The amino acid residues 26–53 of the starfish PLA₂ 1, 2, and 3 showed fairly high sequence homology (75%) to the corresponding region of the group I and II type PLA₂s, and the residues involved in the catalytic network (His-49, Asp-111, Tyr-53, and Tyr-72) and the Ca²⁺-binding site (Tyr-29, Gly-31, Gly-33, and Asp-50) of the group I and II PLA₂s were completely conserved in the starfish PLA₂ 1, 2, and 3 (Fig. 3) (Arni and Ward, 1996; Renetseder et al., 1985). These data implies that the catalytic mechanism of the starfish PLA₂ is essentially the same as those of the group I and II type PLA₂s. Further, the starfish PLA₂ 1, 2, and 3 conserved the 14 Cys residues at the appropriate positions which were

involved in the intramolecular disulfide bonds in the group I type PLA₂ (Fig. 3) (Dennis, 1983). Accordingly, the starfish PLA₂ can be classified into the group I type. On the other hand, the homology calculated with whole sequences between the starfish PLA₂ 1, 2, and 3 and the other animal PLA₂s was relatively low (36–48%) since the high sequence divergency exists in the amino acid residues 54–107 including the pancreatic loop and β -wing regions (Fig. 3) (Arni and Ward, 1996; Renetseder et al., 1985). The starfish PLA₂ 1, 2, and 3 possess the pancreatic loop-like sequence in the residues 63–66, however, two amino acid deletions were required in the residues 62(+1) and 66(+1) to align with the sequence of the porcine pancreatic PLA₂. In addition, two insertions of each of eight residues (residues 76–83 and 89–96) and deletion of three residues (residues 84(+1)–84(+3)) were required for the starfish PLA₂ 1, 2, and 3 to align with the β -wing region of the porcine PLA₂. It has been reported that the PLA₂ of the groups I and II possesses N-terminal about ten residues forming the short amphiphilic helix (Arni and Ward, 1996). However, the secondary structure of the starfish PLA₂ predicted using SOPMA program (Geourjon and Deleage, 1995) showed to form extended strand structure in the N-terminal region (Fig. 4). Moreover, it was predicted that the starfish PLA₂ has an insertion of a long α -helix in the corresponding region to the β -wing of porcine pancreatic PLA₂ (Fig. 4). Therefore, we consider that the characteristic structures in these regions relate to the specific properties of the starfish PLA₂, such as the higher activity and characteristic substrate specificity comparing with those of the mammalian pancreatic PLA₂.

3.3 Phylogenetic relationship between the starfish PLA₂ and other PLA₂s

In order to clarify the molecular evolutionary relationship between the starfish PLA₂ and the group I and II type PLA₂s, we made a phylogenetic tree using CLUSTAL W program (Thompson et al., 1994). As shown in Fig. 5, the starfish PLA₂ 1, 2, and 3 are hardly placed in either group I A or I B. Therefore, the starfish PLA₂ should be classified into a new type of group I PLA₂. The occurrence of a new PLA₂ group has also been suggested by McIntosh et al. with the PLA₂ from the venom of marine snail *Conus magus* (McIntosh et al., 1995). The enzyme has been classified into the group IX PLA₂ since it is comprised of the two polypeptide chains and shows little sequence homology to other PLA₂s (Dennis, 1997). On the other hand, Shiomi et al. purified two PLA₂s from the venom of crown-of-thorns starfish *Acanthaster planci* and determined the N-terminal 62 amino acid sequences (Shiomi et al., 1998). Although the sequences of the pancreatic loop and β -wing regions of these enzymes have not been determined, they identified the enzymes as the group I type PLA₂ since these enzymes possess Cys-11 and elapid loop but without Cys-51. In a previous study, we reported that the molecular weight of *S. paxillatus* PLA₂ was slightly lower than that of *A. pectinifera* PLA₂ (approx. 13,000 on SDS-PAGE for *S. paxillatus* PLA₂ vs. 15,300 for *A. pectinifera* PLA₂) (Kishimura and Hayashi, 1998; Kishimura et al., 2000). In addition, the specific activity of *S. paxillatus* PLA₂ was not as high as that of *A. pectinifera* PLA₂ (26 units/mg for *S. paxillatus* PLA₂ vs. 119,000 units/mg for *A. pectinifera* PLA₂)

(Kishimura and Hayashi, 1998; Kishimura and Hayashi, 1999). Thus, it is unclear whether or not *S. paxillatus* PLA₂ should be classified into a new type of group I PLA₂ like *A. pectinifera* PLA₂. Therefore, at present, *A. pectinifera* PLA₂ seems to be the only enzyme belonging to a new type of group I PLA₂ among the starfish and other marine invertebrates.

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(Legends for figures)

Fig. 1. Oligonucleotide primers used for the amplification of DNAs by PCR.

The primers were designed based on the amino acid sequence of the

starfish PLA₂ protein (Kishimura et al., 2000). Upper and lower rows show the nucleotide and amino acid sequences, respectively. The single-letter amino acid code is used. F, forward primer synthesized based on the amino acid sequence of residues 1–5 in the starfish PLA₂ protein along with 5'-terminal "ATG" as a translational initiation codon. R, reverse primer corresponding to the residues 132–137 in the starfish PLA₂ protein.

Fig. 2. The nucleotide and deduced amino acid sequences of the starfish PLA₂ cDNAs.

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 sites of PCR-primers, F and R (see in Fig. 1), are underlined. Amino acid residues different from those of the starfish PLA₂ protein (Kishimura et al., 2000)

are boxed. a, cDNA 1*¹; b, cDNA 2*²; c, cDNA 3*³.

*¹Accession No. AB022278 in DDBJ.

*²Accession No. AB032266 in DDBJ.

*³Accession No. AB032267 in DDBJ.

Fig. 3. Alignment of the deduced amino acid sequences of the starfish PLA₂s with the sequences of group I and II PLA₂s.

Residues identical in all the PLA₂s in this figure are boxed. Dashes indicate deletions introduced for maximizing the sequence similarity. The location of the active site, Ca²⁺-binding loop, elapid and pancreatic loop, and β-wing region are shown with solid bars based on the crystallographic studies of bovine pancreatic and *Crotalus atrox* venom PLA₂s (Arni and Ward, 1996; Renetseder et al., 1985). The positions of deleted amino acids in pancreatic loop and β-wing regions of the starfish PLA₂s are

represented as 62(+1), 66(+1), 84(+1), 84(+2), and 84(+3). Starfish 1, 2, and 3, *A. pectinifera* PLA₂s from cDNAs 1, 2, and 3, respectively (present paper); Snake (I A), *Naja naja atra* venom PLA₂ (Chang et al., 1997); Porcine (I B), porcine pancreatic PLA₂ (Puijk et al., 1977); Snake (II A), *C. atrox* venom PLA₂ (Randolph and Heinrikson, 1982); Snake (II B), *Bitis gabonica* venom PLA₂ (Botes and Viljoen, 1974); Rat (II C), Rat brain PLA₂ (Chen et al., 1994).

Fig. 4. Predicted secondary structure of the starfish PLA₂.

Secondary structures of the starfish PLA₂ and porcine pancreatic PLA₂ were predicted using SOPMA program (Geourjon and Deleage, 1995). Dashes indicate deletions introduced for maximizing the structure similarity. The location of the active site, Ca²⁺-binding loop, elapid and pancreatic loop, and β -wing region are shown with solid bars based on the crystallographic studies of bovine pancreatic and *Crotalus atrox* venom PLA₂s (Arni and Ward, 1996; Renetseder et al., 1985). Starfish 1, predicted secondary structure of the starfish PLA₂ 1 (present paper); Porcine (I B), predicted secondary structure of porcine pancreatic PLA₂ (Puijk et al., 1977); h, α -helix; e, extended strand; t, β -turn; c, random coil.

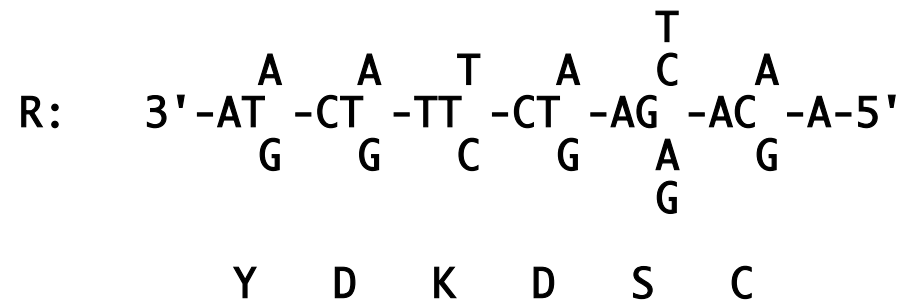
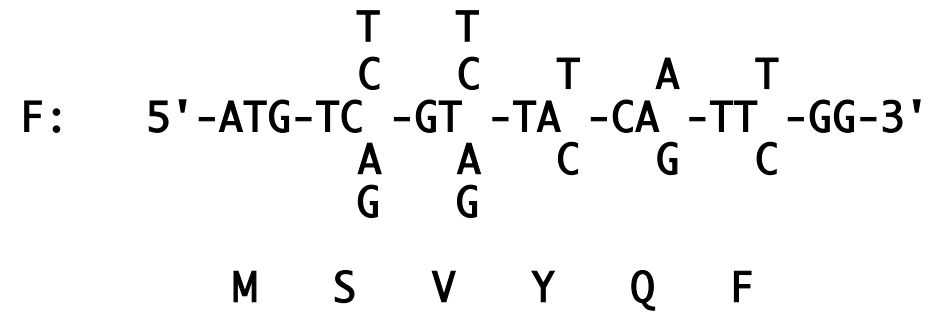
Fig. 5. Radial rootless phylogenetic tree of PLA₂s.

The phylogenetic tree was made using the programs of CLUSTAL W (Thompson et al., 1994) and TreeView (Page, 1996). The branch length represents the evolutionary distance between the proteins. PLA₂s belonging to the same group are boxed: group I A (O.s.scu, *Oxyuranus s. scutellatus* venom (Lind and Eaker, 1982); L.sem, *Laticauda semifasciata* venom (Takasaki et al., 1988); E.sch, *Enhydrina schistosa* venom (Lind and Eaker, 1981); N.s.scu, *Notechis scutatus scutatus* venom (Lind and Eaker, 1980); P.aus, *Pseudechis australis* venom (Nishida et al., 1985); B.mul, *Bungarus multicinctus* venom (Kondo et al., 1981); N.n.atr, *Naja naja atra* venom (Chang et al., 1997); N.mel, *Naja melanoleuca* venom (Joubert, 1975a); N.m.mos, *Naja mossanbica mossanbica* venom (Joubert, 1977); N.nig, *Naja nigricollis* venom (Chwetzoff et al., 1989); H.hae, *Hemachatus*

haemachatus venom (Joubert, 1975b)), group I B (Por, porcine pancreas (Puijk et al., 1977); Bov, bovine pancreas (Fleer et al., 1978); Hor, horse pancreas (Evenberg et al., 1977); Hum, human pancreas (Verheij et al., 1983); Dog, dog pancreas (Ohara et al., 1986); Rat, rat pancreas (Ohara et al., 1986); Red, red sea bream hepatopancreas*), group II A (C.ada, *Crotalus adamanteus* venom (Heinrikson et al., 1977); C.atr, *Crotalus atrox* venom (Randolph and Heinrikson, 1982); T.oki, *Trimeresurus okinavensis* venom (Joubert and Haylett, 1981); T.flu, *Trimeresurus flavoviridis* venom (Oda et al., 1990); A.h.blo, *Agkistrodon halys blomhoffii* venom (Tomoo et al., 1989); Hum.s, human synovial fluid (Kramer et al., 1989); Rat.p, rat platelet (Hayakawa et al., 1988)), group II B (B.gab, *Bitis gabonica* venom (Botes and Viljoen, 1974); B.cau, *Bitis caudalis* venom (Viljoen et al., 1982)), and group II C (Rat.b, rat brain (Chen et al., 1994)). Star1, 2, and 3, *A. pectinifera* PLA₂ 1, 2, and 3 from cDNA 1, 2, and 3, respectively (present paper).

*Accession No. AB009286 in DDBJ.

Fig. 1



H. Kishimura

cDNA cloning of starfish phospholipase A₂

a

ATGTCAGTTTACCAGTTCGGCAAGTTCATTTTCGTGCTATG
ATGTCGTCTATCAGTTCGGCAAGTTCATTTTCGTGCTATG

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

GTGGTGCTGGGTTTTTCGATGGGTTGGACTACAACGGCTA
 GTGGTGCTGGGTTTTTCGATGGGTTGGACTACAACGGCTA
 G G A G F F D G L D Y N G Y
 G Y

20

TGGGTGTTACTGCGGCTACGGAGGCAAAGGAACACCGTTG
 TGGGTGTTACTGCGGCTTAGGAGGCCAAGGAACACCGTTG
 G C Y C G Y G G K G T P L
 P L

30

GATGACACCGACAGATGCTGTCTAGTGACGATAACTGTT
 GATGACACCGACAGATGCTGTCTAGTGACGATAACTGTT
 D D T D R C C L V H D N C

C
 40
 50

50

ACGGCAAAGCTACCGCGGAGGCCGACTGCGGTTCTTGGA
 ACGGCAGAGCTGCCGCGAGGCCGACTGCGGTTCCCTGGA
 Y G K A T A E A D C G S W D
 L D

60

CCCCTACATCATAGTTTACGACTATGAACAAACCACTGAT
 CCCGTACATCATTATTTACGACTATGAACAAACCACTGAT
 P Y I I V Y D Y E Q T T D
 T D

70

GCGTCTGGAAACTGTGTCATCAAATGCAAGAAAGCGGCCG

b

ATGTCGTGTTTACCAGTTCGGCAAGTTCATTTTCGTGCTATG

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

GGGGTGCGGGTTTCTTCGATGGGTTGGACTACAACGGCTA
 80
 G G A G F F D G L D Y N G Y

20

TGGTTGTTACTGCGGCTACGGAGGCCAAGGAACACCGTTG
 120
 G C Y C G Y G G Q G T P L

30

GATGACACCGACAGATGCTGTCTAGTGACGATAACTGTT
 160
 D D T D R C C L V H D N C

40

50

ACGGCAAAGCTACCGCGGAGGCCGACTGCGGTTCTTGGA
 200
 Y G K A T A E A D C G S W D

60

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

70

GCGTCTGGAAACTGTGTCATCAAATGCAAGAAAGCGGCCG

c

S V Y Q F G K F I S
 1

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

G C Y C G L G G Q G T
 30

D D T D R C C L V H D N
 40

Y G R A A A E A D C G S
 60

P Y I I I Y D Y E Q T
 70

CAGGCTGGAACTGTGTCATCCAATGCAAAAAGCGGCCG

280

A S G N C V I K C K K A A

A S G N C V I K C K K A A

Q A G N C V I Q C K K

A A

80

90

80

90

80

90

ACTATTCTTGGTATTCTACCAATCCCGAATGCAGAGAGTT

ACTATTCTTGGTATTCTACCAATCCCGAATGCAGAGAGTT

320

ACTATTCTTGGTATTCTACCAATCCCGAATGCAGAGAGTT

D Y S W Y S T N P E C R E F

D Y S W Y S T N P E C R

E F

100

100

100

CATGTGCGAATGTGACCGCGGGGGCGCAGTGCTTCGCT

CATGTGCGAATGTGACCGCGGGGGCGCAGTGCTTCGCT

360

CATGTGCGAATGTGACCGCGAGGGGGCGAAGTGCTTCGCT

M C E C D R A G A Q C F A

M C E C D R E G A K C

M C E C D R A G A Q C F A

F A

110

110

110

GAAAAGCGCCCAACGTACAACCAAGCTTACGAGTCATACG

GAAAAGCGCCCAACGTACAACCAAGCTTACGAGTCCTATG

400

GACAAGCGCCCAACGTACAACCAAGCTTACGAGTCCTACG

E K R P T Y N Q A Y E S Y

D K R P T Y N Q A Y E

E K R P T Y N Q A Y E S Y

S Y

120

130

120

130

120

130

ACAAGATTTCATGCT

ACAAAGATTTCCTGCT

ACAAAGATTTCATGCT

415

D K D S C

D K D S C

D K D S C

Fig.4

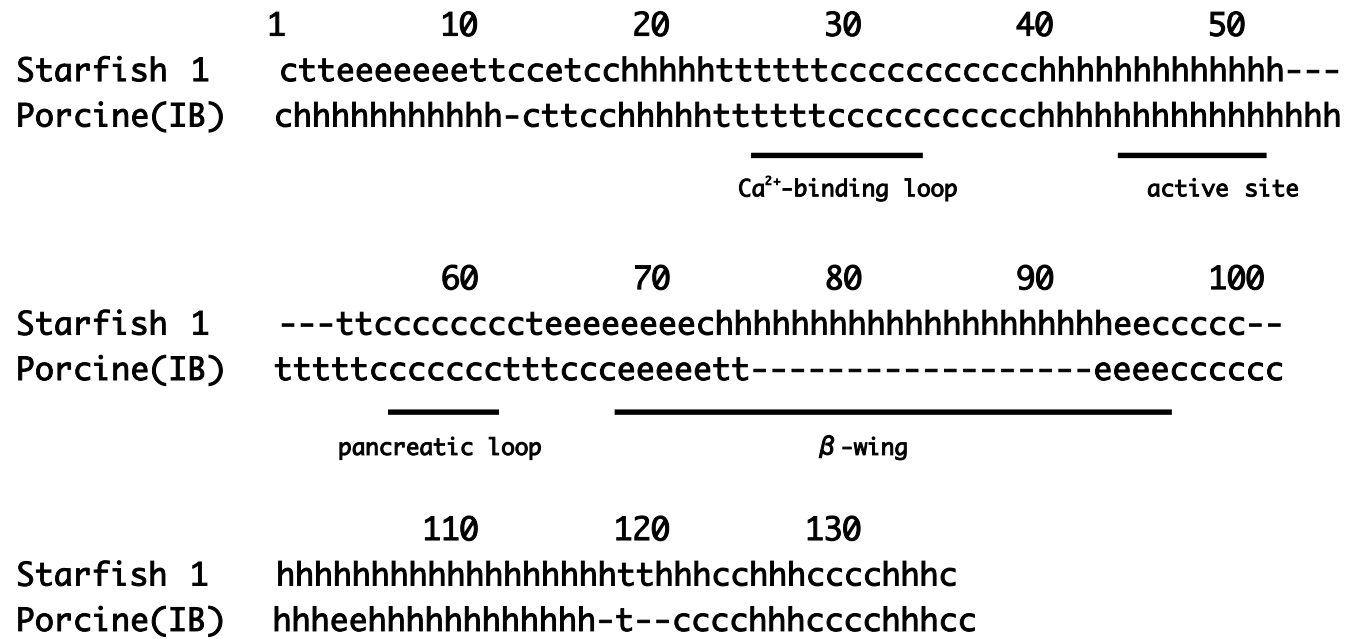


Fig.5

