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Thesis for the Degree of Master of Science

Molecular cloning and immunological characterization of annexin isolated from rockfish gill monogenean, *Microcotyle sebastis*

by

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Department of Fish Pathology

The Graduate School

Pukyong National University

Feb. 2007

Molecular cloning and immunological characterization of annexin isolated from rockfish gill monogenean, *Microcotyle sebastis*

조피볼락 아가미흡충 *Microcotyle sebastis*에서 분리한 annexin의 분자·면역학적 특성

Advisor: Prof. Ki Hong Kim

by

Seung-Hyuk Choi

A thesis submitted in partial fulfillment of the requirements

for the degree of

Master of science

in the Department Fish Pathology, Graduate School,

Pukyong National University

Feb. 2007

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Molecular cloning and immunological characterization of annexin isolated from rockfish gill monogenean, *Microcotyle sebastis*

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Abstract

In this study, we have cloned the full cDNA of an annexin gene from *Microcotyle sebastis*, for the first time in monogeneans, and preliminarily analyzed the possibility of the annexin (MsANX) as an antigen for the development of recombinant subunit vaccines against *M. sebastis*. The cDNA of MsANX comprises 1,238 bp with a 68 bp 5' untranslated region, an open reading frame of 1062 bp, and a 108 bp 3' untranslated region. The recombinant MsANX bound phosphatidylserine vesicles in the presence of Ca^{2+}, whereas no MsANX was precipitated in the absence of free Ca^{2+}, indicating that MsANX is a functional annexin. And the recombinant MsANX has anticoagulant acticity. In Western blot analysis, the sera of 2-year-old adult rock fish experienced heavy infection by *M. sebastis* specifically bound to the recombinant MsANX. However, sera collected from fingerlings that were not infected with *M. sebastis* did not bind to the MsANX. The specific humoral immune response against MsANX in rockfish, which were naturally infected with *M. sebastis*, suggests that MsANX has a possibility to be used

as a subunit vaccine antigen. It remains to be investigated whether immunization of naive fish with the recombinant MsANX can induce protective immune responses against *M. sebastis* infection.

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Introduction

The farming of rockfish, *Sebastes schlegeli*, has rapidly increased during the past decade, and has become an important marine cultured fish in Korea. In 2005, the annual production of black rockfish in Korea was 28,548 metric ton (2005 Statistics, Ministry of Maritime Affairs & Fisheries, Korea). One problem associated with rockfish farming in Korea is infestation with the gill monogenean parasite *Microcotyle sebastis*, which causes extensive mortalities of juvenile rockfish every year. High cumulative mortalities of juvenile rockfish at summer by heavy infestation with *M. sebastis* occurred frequently in many farms not published.

Most polyopisthocotyleans have not been reported to be overtly pathogenic (Paperna, 1987; Thoney and Hargis, 1991.) but mortalities were described in various cultured fish species (Kubota and Takakuwa, 1963; Silan et al., 1985; Faisal and Imam, 1990.). Polyopisthocotylean monogeneans are sanguinivorous (Llewellyn, 1954; Halton and Jennings, 1965). A small amount of blood is ingested by each worm, but mass infection could remove a significant amount of blood. Thoney (1986) suggested that heavy infection of polyopisthocotyleans on individual host can be pathogenic and cause mortalities.

The ESTs represent the largest amounts of information possible per seqneuced base and the vision of rapid identification of all human genes led to the development of several commercially financed data banks with EST sequences.

ESTs have also been great resources from genomic mapping (Boguski and Schuler, 1995; Hudson *et al.*, 1995; Schuler *et al.*, 1996). The potential use of ESTs for the discovery of *Caenorhabditis elegans* genes has previous

been shown (Waterston R *et al.*, 1995).

It has been known that ESTs from parasite account for only about 1% of almost five million ESTs in the dbEST division of Genebank. The greatest effort so far has been made in *Caenorhabditis elegans* (nematode) (Waterston R *et al.*, 1995), *Schistosoma mansoni* (blood fluke) (Tanaka M *et al.*, 1995), *Toxoplasma gondii* (Ajioka JW *et al.*, 1998), *Trypanosoma cruzi* (Verdun RE *et al.*,1998), *Tetrahymena thermophila* (Fillinqham *et al.*, 2002).

The ectoparasitic, blood-sucking polyopisthocotylean *Microcotyle sebastis* is a major parasitic disease agent of net-pen-farmed rockfish *Sebastes schlegeli* in Korea. Recently, praziquantel has been used successfully to control *M. sebastis* infection (Kim & Cho 2000, Kim et al. 2001), but this has certain drawbacks such as environmental and residue problems, high cost, and the possibility of resistant populations induction by long-term use. As an alternative to chemical treatments, vaccines would provide a way to overcome most of the shortcomings associated with the use of chemicals. Since the first reports of acquired protection in fish against monogenean reinfections (Jahn & Kuhn 1932, Nigrelli & Breder 1934) were made, several studies have reported on the acquired partial protection of fish against monogeneans by artificial infection-treatment-reinfection scheme, or active immunization (Scott & Robinson 1984, Scott 1985, Buchmann 1993, Slotved & Buchmann 1993, Bondad-Reantaso et al. 1995, Richards & Chubb 1996, Kim et al. 2000, Rubio-Godoy et al. 2003). Since monogeneans cannot be mass-cultured in vitro, the identification of protective antigens and the mass-production of vaccine antigens would be inevitable to develop effective and commercially available vaccines. However, to our knowledge, there is no available information on the subunit vaccines against monogenean diseases.

Annexins are a large family of calcium-dependent phospholipid-binding proteins widely distributed in eukaryotes and plants. Proteins of this family

have been implicated in a wide range of important biological processes, such as membrane trafficking and fusion, anticoagulation, inhibition of phospholipase A2 and interaction with cytoskeletal proteins. Some annexins are expressed in a growth-dependent manner and are substrates for protein kinase C (PKC), suggesting that annexin might be involved in cell proliferation and differentiation (Raynal P *et al.*, 1994; Gerke V *et al.*, 1997; Dubois T *et al.*,1996). However, since most of the experiments were carried out in vitro, the real physiological role of annexins in vivo remains unclear. A typical annexin is characterized by two distinct features: (1) biologically, the protein binds acidic phospholipids such as phospatidylserine (PS) in the presence of calcium; (2) structurally, the protein is formed by a core domain of 4 or 8 homologous repeats and an N-terminal tail. Each repeat is composed of 70-80 amino acids and contains a canonical motif "M-K/R-GX-G-T (38 residues)-D/E" defined as type II calciumbinding site (Huber R *et al.*, 1996). The core domains are conserved and distinguish annexins from other calcium-binding proteins. In contrast, the N-terminal tails are diverse in sequence and length, ranging from 11 to 196 residues. Most of the post-translational modification such as phosphorylation occurs in this region. Therefore the tail is thought to be the regulatory domain and has been used to divide the annexins into different subfamilies. At least 31 subfamilies in different species have been classified (Morgan RO *et al.*, 1999).

Recently, it has been shown that annexins can be used as antigens for vaccines against some mammalian parasites such as *Giardia lamblia* (Weiland et al. 2003) and *Cysticercus cellulosae* (Sun et al. 2000, Wu et al. 2000).

In the present study, we have cloned full cDNA of an annexin from *M. sebastis* cDNA library, and produced it as a recombinant protein to characterize its biological functions including phopholipid vesicle binding, anticoagulant activity , for the first time in monogeneans, and preliminarily

analyzed the possibility of the annexin as an antigen for the development of recombinant subunit vaccines against *M. sebastis*.

Table. 1. The number of parasites ESTs deposited in the dbEST.

(as of september, 2006)

Species	dbEST
Caenorhabditis elegans (nematode)	346,064
Schistosoma mansoni (blood fluke)	158,841
Toxoplasma gondii	125,741
Schistosoma japonicum (blood fluke)	97,526
Tetrahymena thermophila	56,543
Schmidtea mediterranea	45,755
Ascaris suum (pig roundworm)	40,704
Eimeria tenella	34,035
Brugia malayi (parasitic nematode)	26,215
Neospora caninum	25,064
Meloidogyne hapla	24,452
Heterodera glycines (soybean cyst nematode)	24,438
Trichomonas vaginalis	22,488
Plasmodium vivax	22,238
Haemonchus contortus	21,967
Lutzomyia longipalpis	21,069
Euglena gracilis	20,426
Entamoeba histolytica	20,404
Caenorhabditis remanei	20,292
Plasmodium falciparum	20,176

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MATERIALS AND METHODS

1. Fish

Netpen-reared juvenile rockfish *Sebastes schlegeli* (average body weight [BW] 20 g) were obtained from a local rockfish farm in Tongyoung, Korea.

2. Parasites

M. sebastis were isolated from the infected rockfish *Sebastes schlegeli*, which were obtained from the fish farm located in Tongyoung, Korea. *M. sebastis* were isolated from the tissues of gill, of infected rockfish *Sebastes schlegeli*.

3. *M.sebastis* cDNA Library construction and analysis

3.1 *M. sebastis* cDNA library construction.

The isolated 300 worms were washed 2 times with phosphate-buffered saline (PBS), and total RNAs were extracted using the Tri Reagent (Sigma). From total RNA, the poly $(A)^+$ RNAs were purified using biotin-labeled oligo-$d(T)_{20}$ and streptavidin-coated magnetic particles (Promega) according to the manufacturer's instructions. 5 micrograms of poly $(A)^+$ RNA was used as template for cDNA synthesis. All the procedures for cDNA library construction including cDNA synthesis and vector ligation were performed following the protocol of the Creator™ SMART™ cDNA library construction Kit (Clontech).

3.2 Sequense and Bioinformatics analysis

The nucleotide and predicted peptide sequenses were analyzed BioEdit and BLAST program in the non-redundant of National Center for Biotechnology Infomation(NCBI BLAST).

4. Full sequencing of *M. sebastis* annexin (MsANX) cDNA.

Of the expressed sequence tag (EST) clones in the cDNA library, a clone showed higher similarity (e-value of $4E^{-39}$) with *Schistosoma japonicum* annexin sequences (GenBank accession no. AAW25344) based on the BLASTx search against NCBI GenBank. To determine full cDNAsequence of the selected clone,5'- and 3'- RACE were performed separately using the SMART^{TM} RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. Universal primers and gene specific primers (Table. 2.). Primers used in the RACE PCR were designed from partial sequences obtained by library and their nucleotide sequences were shown in Table 3. The amplified products were purified using spin column (Qiagen) and cloned into pGEM-T easy vector (Promega). The recombinant clones of correct size were selected, and the inserts were sequenced using ABI377 automatic sequencer (Applied Biosystems).

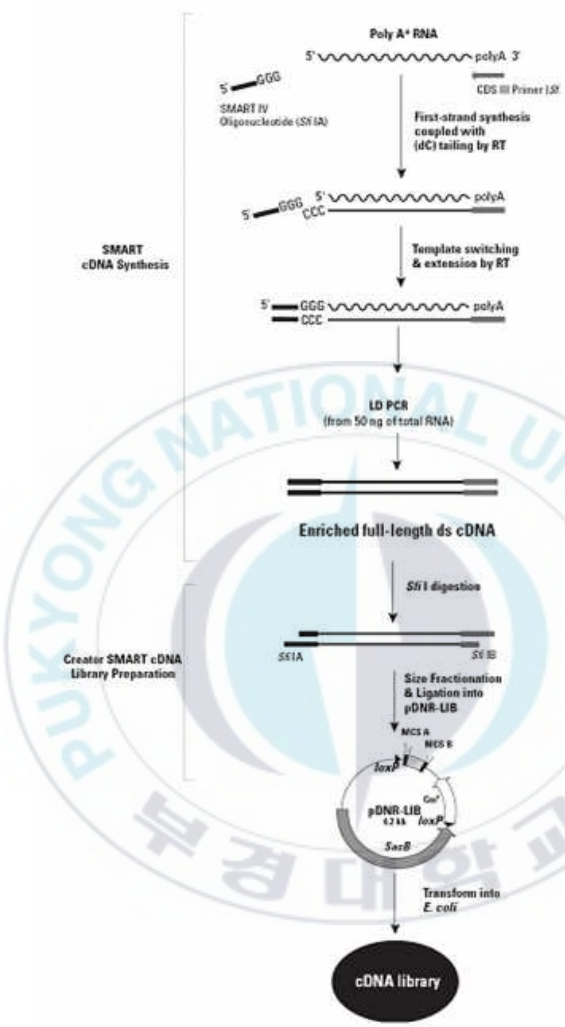

Fig. 1. Creator™ SMART™ libraries allow you to transfer gene into multiple expression systems quickly and inexpensively.

Table. 2. The sequences of primers used in RT-PCR

Primer sets		Primer sequence (5'-3')
up stream	1	CCCTATACGATGTTTGTGATGCTGAAT
	2	AGATTAGTTCAAGATGACGCAAGAA
down stream	1	CATATAACAAAGGGCATGTCTGTA
	2	AATCCAGCCATTGTCGATCGTATCAA
CDS Primer	FORWARD	AAGCAGTGGTATCAACGCAGAGT
	REVERSE	ATTCTAGAGGCCGAGGCGGCCGACATG

Table. 3. Specific primer sets used for ANNEXIN detection and construction of recombinant protein expression vector

Primer sets		Primer sequence (5'-3')
ANNEXIN ORF	Forward	ATGTCGAGGCCGGGATTCTCTGTGT
	Reverse	CTTCTGGCCCTGTGCGGTTGTTAG
expression vector primer	Forward	**GGATCC**ATGTCGAGGCCGGGATTCTCTGTGT
	Reverse	**AAGCTT**CTTCTGGCCCTGTGCGGTTGTTAG

5. Phylogenetic analysis

The protein sequenses of annexin and other annexin family obtained Gene-Bank were aligned using CLUSTAL W version 1.8 (Thompson et al., 1994) as included in BioEdit(Hall, 1999), with default parameters and phylogenetic analysis carried out using MEGA version 3.0(Kumar et al.,2000). A phylogenetic tree was constructed by neighbor-joining methods.

The compared genebank numbers were displaed in table. 4.

6. Predicted structure analysis of annexin

Predicted structure of annexin was analyzed by using SWISS-3D MODEL software program (http://swiss. model.expasy.org/SWISS-MODEL.html).

7. Production of recombinant protein and purification

7.1. Construction of recombinant protein expression vector

Sequenced plasmids of annexin were digested with appropriate restriction enzymes (BamH 1 and Not I) and inserted into pET28a vector (Novagen, USA). The recombinant plasmids were prepared by transforming *Escherichia coli* BL21 (DE3) competent cells.

7.2. Expression and purification of recombinant annexin

The expression of recombinant annexin was then induced by 1mM IPTG (isopropyl-β-D-thiogalactoside). The insoluble MsANX protein was dissolved using denaturation buffer (8 M urea, 5 mM imidazole, 0.5M NaCl, 20mM Tris, 1%Triton X100, pH 7.9), purified using a Ni-NTA His-Bind ® Resin (Novagen, USA) open column, and refolded in binding buffer (5 mM

imidazole, 5 mM imidazole, 0.5M NaCl, 20mM Tris, pH 7.9). Protein purity was monitored by SDS-polyacrylamide gel electrophoresis, stained with Coomassie Brilliant Blue, and the protein concentration was determined using the BCA protein assay (Sigma).

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Table. 4. The amino acid sequence identities and similarities *M.sebastis* annexin and human annexin family and other parasite annexin

sequence for comparison	identity (%)	similarity (%)	GenBank accession
Human annexin A1	34%	51%	P04083
Human annexin A2	30%	50%	P07255
Human annexin A3	32%	53%	P12429
Human annexin A4	33%	54%	P09525
Human annexin A5	31%	51%	P08758
Human annexin A6	31%	54%	P08133
Human annexin A7	34%	51%	P20073
Human annexin A8	29%	49%	P13928
Human annexin A9	30%	52%	O76027
Human annexin A10	31%	49%	Q9UJ72
Human annexin A11	31%	53%	P50995
Human annexin A13	35%	57%	P27216
annexin famaily protein1 [Caenorhabditis elegans]	36%	60%	AAB52702
annexin famaily protein4 [Caenorhabditis elegans]	35%	58%	AAB42365
annexin B2[Taenia solium]	37%	59%	AAY17503
annexin B3[Taenia solium]	35%	58%	AAY27744
annexin XX1 [Giardia lambilia]	34%	52%	XP768617
annexin XX1 [Giardia intestinalis]	33%	53%	CAB86987
annexin homolog [Giardia intestinalis]	33%	52%	T18517
annexin [Schistosoma mansoni]	37%	61%	AAC79802
annexin [Schistosoma japonicum]	39%	63%	AAW25344

8. Antibody production

8.1. Production of Ab (antibody) from rat against recombinant annexin protein

Specific-pathogen-free male Wistar rats (4 weeks old) were used for the immunization experiment. Three rats were immunized by intraperitoneal injection of 0.15 ml of His-tagged MsANX (120 μg) emulsified with an equal volume of Freund'sadjuvant (FCA, Sigma), and boosted with the injection of a same dose of the protein emulsified with Freund'sadjuvant (FIA, Sigma) two weeks later. Three rats of the control group were intraperitoneally injected with PBS and FCA mixture at the first injection, and boosted with PBS and FIA mixture. On 2 weeks post-boost immunization, all rats were bled to obtain serum. These sera were stored at -80℃ until analysis.

8.2 Production of rabbit polyclonal antibody against rockfish Igs

8.2.1. Purification of rockfish serum Ig using protein A affinity chromatography

Rockfish Ig was purified with protein A beads (Sigma, USA), using a slight modification of the method described by Bollag et al.(1996). Briefly, protein A beads were packed in a disposable polystyrene column (Bio-Rad, USA) according to manufacturer's instructions and washed with binding buffer (10 mM Tris, 1.25 M NaCl, 20 mM $CaCl_2$, 0.02% (w/v) NaN_3, pH 7.4). Fish sera (approximately 30 mg ml^{-1}) was mixed with an equal volume of binding buffer and applied to the packed column. Unbound proteins were removed by washing with 150 ml of binding buffer, and the Ig-protein A was colleted by washing with 1 M glycine-HCl (pH 2.5). The eluate was collected in an Eppendorf tube containing 0.1 ml of neutralising buffer (1 M Tris-HCl, pH

7.4), and the protein content was determined according to the method of Bradford(1976). The Ig-containing eluted fractions were desalted and concentrated by centrifugation with phosphate-buffered saline (PBS: 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.2) in Amicon Ultra-4 (Millipore, USA).

8.2.2. Production of rabbit polyclonal antibody against rockfish Igs

Polyclonal antibody against the protein A-purified rockfish serum Ig was produced in rabbit ascites according to the method described by Overkamp et al.(1976). Briefly, a single rabbit (Hyochang Science, Inc., Korea) was intraperitoneally (IP) injected with a mixture of protein A affinity purified rockfish serum Ig (50 μg ml^{-1}) and Freund's complete adjuvant (Gibco BRL, USA) (1:9) (Solution A) followed 15 days later by injection with an emulsion containing Freund's complete adjuvant plus purified serum Ig and Pristane (Sigma, USA) (1:2.5) (Solution B). Seven and 14 days after the third immunisation, the rabbit was injected with solution A. One week after the final boost, ascitic fluid was collected from the abdominal cavity of the rabbit.

9. Phospholipid vesicle binding activity

Phospholipid vesicles of phosphatidylserine (PS Sigma) were prepared accoring to Boustead et al. (1988). The PS vesicles (final concentration, 1mg ml^{-1}) were suspended in 400㎕ of a solution containing 20 mM100mM KCl, 2 mM $MgCl_2$, and 1 mM EGTA, pH 7.4. This suspension was mixed with 100 ㎕ of a solution containing 10 ㎍ MsANX in 20 mM Tris/HCl, 10 mM EGTA,

pH 7.5. Free Ca^{2+} was adjusted to different concentrations (0.1-1mM) by adding aliquots from a 1 M $CaCl_2$ stock solution. After 40 min incubation at room temperature, the bound (pellet) and unbound (supernatant) proteins were separated by 15000 *g* centrifugation for 10 min. The pellets were washed three times with 500 ㎕ of the incubation buffer and once with 1 ml of a 4:8:3 (v/v/v) mixture of chloroform/methanol/water to remove the phospholipids. All the samples were analyzed using 10% (w/v) SDS-PAGE, and visualized by Coomassie staining.

10. Anticoagulant assay

Anticoagulant activity of the recombinant annexin was assayed using modified APTT as previously described J. Romisch(1990), with Tris-.HCl buffer, recombinant annexin.

Nine parts of blood was drawn by venipuncture into one part of 3.2% sodium citrate from healthy volunteers and pooled. The blood was separately cenrtifuged at 1,600g for 10 min to obtain platelet poor plasma(PPP). All coagulation assays were performed with three individual replicates using the ACL 7000 coagulyzer and diagnostic kit, and mean values were taken. As following the instruction manual of each diagnostic kit, normal citrated PPP(80㎕) was iccubated with the anticoagulant solution(20㎕) for 3 min at 37℃. For activated partial thromboplastin time(APTT) assay, 100㎕ of APTT reagent was added to the above mixure and incubated at 37℃ for 3 min and cloting time was recorded following the addition of 100㎕ of 20mM $CaCl_2$.

11. Immunogenicity

11. 1. Location of annexin in parasite

11. 1. 1. Preparation of parasite ES and crude

M. sebastis isolated carefully from the gill of infected juvenile rockfish were washed 3 times with filtered sea water containing 200 units/ml of penicillin G (Sigma) and 200 units/ml of streptomycin (Sigma) by centirifugation at 1,000g for 5 min. About 200 worms were incubated in filtered sea water at 20℃ for 24 h. To collect ES products, the incubation medium was centrifuged at 1000 g for 10 min at 4℃ and supernatant was concentrated using Centricon-10 (AMICON) with a size exclusion of 10 kDa. To collect crude worm extracts, *M. sebastis* in distilled water containing 0.2% (v/v) TritonX-100 were sonicated at 30 sec interval for 1.5 min on ice. After centrifugation at 1000 g for 5 min at 4℃, the supernatant was used as the worm crude extracts.

11. 1. 2. Western blotting

For Western blots, ES and crude extractswere added per lane to a 10% SDS-PAGE gel, run and blotted to a nitrocellulose membrane. The membrane was blocked with blocking solution (3% bovine serum albumin in TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 2 h at room temperature (RT), washed with TTBS (0.05% Tween 20 in TBS, pH 7.5) and incubated with either rat antisera against MsANX or rat control sera for 2 h at RT. The membranes were washed three times with TTBS and incubated with alkaline phophatase conjugated goat anti-rat IgG (1:2000, Santa Cruz Biotechnology) for 1.5 h at RT. After washing off unbound secondary antibody, the specific antigen-bound antibody was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoly phosphate (NBT-BCIP) substrate buffer (Sigma).

11. 2. Antiboby presence in the infected rock fish serum

The recombinant MsANX protein was added per lane to a 10% SDS-PAGE gel, run and blotted to a nitrocellulose membrane. Blots were either incubated with infected rockfish (2 year-old) serum(1:500) or with uninfected rockfish (fingerling) serum (1:500). After three washes with TTBS, the blots were incubated with 1:2000 a rabbit polyclonal antiserum against rockfish IgM for 90 min at RT. The membranes were washed three times with TTBS and incubated with alkaline phophatase conjugated goat anti-rabbit IgG (1:2,000, Santa Cruz Biotechnology) for 1.5 h at RT. The remaining procedures were identical to the above mentioned method.

12. Statistical analysis

Blood coagulation data data was analyzed by the Student's *t*-test and the chi-square test, respectively. Significant differences were determined at $P<0.05$.

RESULTS

EST sequencing and similarity search of ESTs

A total of 476 clones were single-pass sequenced from the 5'-end, resulting in the characterization of cDNA clones that were longer than 100 bp after elimination of vector sequence. The number of clones sequenced from the subtracted cDNA library, the average size of inserts are given in Table. 5. The average insert size was estimated to be 412 ± 200 bp by PCR amplification of inserts from 100 randomly selected clones. The nucleotide and predicted peptide sequenses were analyzed BioEdit and BLAST program in the non-redundant of National Center for Biotechnology Infomation(NCBI BLAST).

Gene annotation procedures and homology searches of the sequenced ESTs have been locally done by BLASTX for amino acid similarity comparisons. The ESTs with significant similarities ($E < 1 \times e^{-4}$) to known proteins were evaluated to determine if the significant similarities were caused by simple amino acid matches (Table. 5.). Of the 476 clones, 258(54.2 %) were identified as orthologues of known genes from parasites and other organisms. The remaining 153 (21.6 %) clones could not be identified by similarity comparisons ($E \geq 1 \times e^{-4}$). The fact that the majority of EST clones could be identified by similarity comparisons suggests that high-quality EST analysis is an efficient way for gene annotation in less-well studied species.

Functional categorization of *M.sebastis* ESTs is shown in Fig. 2. cDNA of *M.sebastis* consisted of 10 functional groups and the highest group of ESTs is a Regulatory & signal proteins (13%). Best matched organisms of *M.sebastis* ESTs is shown in Fig. 3. cDNA of *M.sebastis* was highest

Schistosoma and *Caenorhabditis elegans* of ESTs.

Table. 5. General characteristics of *M. sebastis* ESTs.

Total cDNA sequenced[a]	476
Average insert size[b]	412 ± 200 bp
ESTs with E value ≧ 1 x e^{-4} (matched)	176 (37 %)
ESTs with E value ≦ 1 x e^{-4} (unknown)	300 (63 %)

a Length of sequence used for comparison after editing.

b The average insert size was calculated for 100 randomly cDNA clones.

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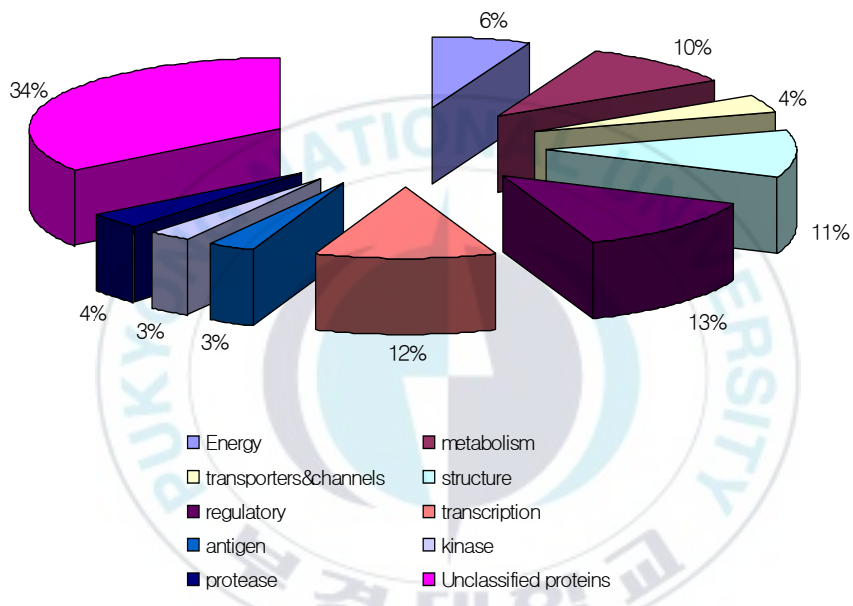

Fig. 2. Functional categorization of *M.sebastis* ESTs.

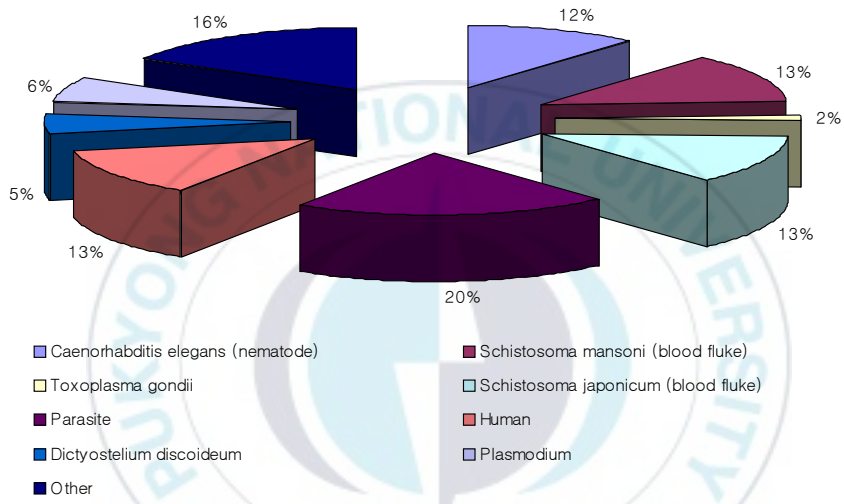

Fig. 3. Clustering of *M.sebastis* ESTs based on putative best matched organisms.

Isolation and analysis of annexin of *M.sebastis*

For rapid identification of differentially expressed genes, the recently described Clontech SMART^{TM} RACE cDNA Amplification Kit (Diatchenko *et al.*, 1996). Total RNA was extracted from M.sebastise. The M.sebastise annexin gene consisting of 1238 bp with 69 bp of 5'-UTR, and had open reading frame that could encode 354 amino acid and 108 bp of 3'-UPR. The 3'-UPR contains single typical polyadenylation signal (AATAAA) (cDNA sequence 1167-1173) and ATTTA sequence motif (1211-1216) (Fig. 5).

This sequence had a putative 3 amino acid signal peptide,(Fig. 6) followed by 354 amino acid residues namely identified as putative mature proteins with a predicted size of 40 kDa. Other parasite such as Schistosoma mansoni, Caenorhabditis elegans and mammalian annexin family proteins have signal peptide.

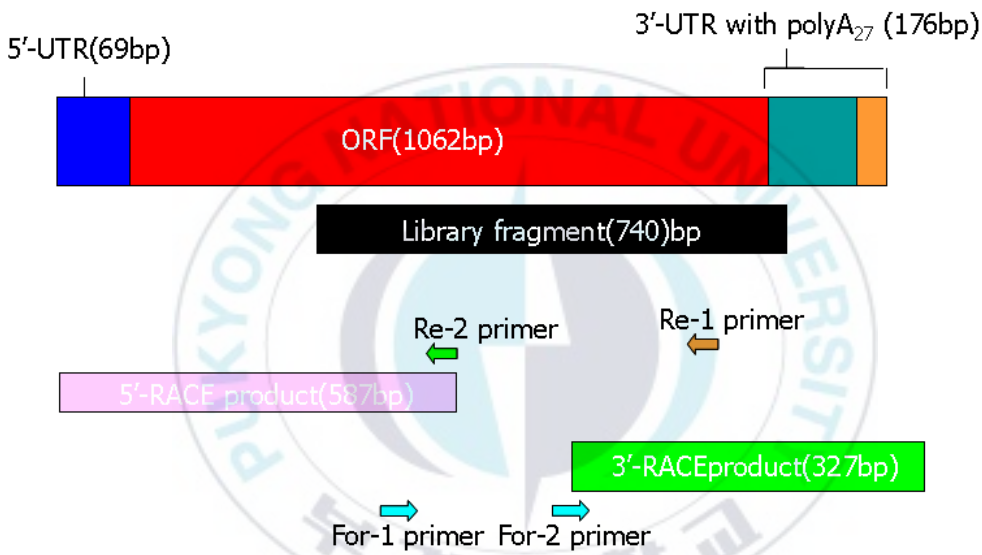

Fig. 4. The RACE-PCR strategy for *M.sebastis* annexin.

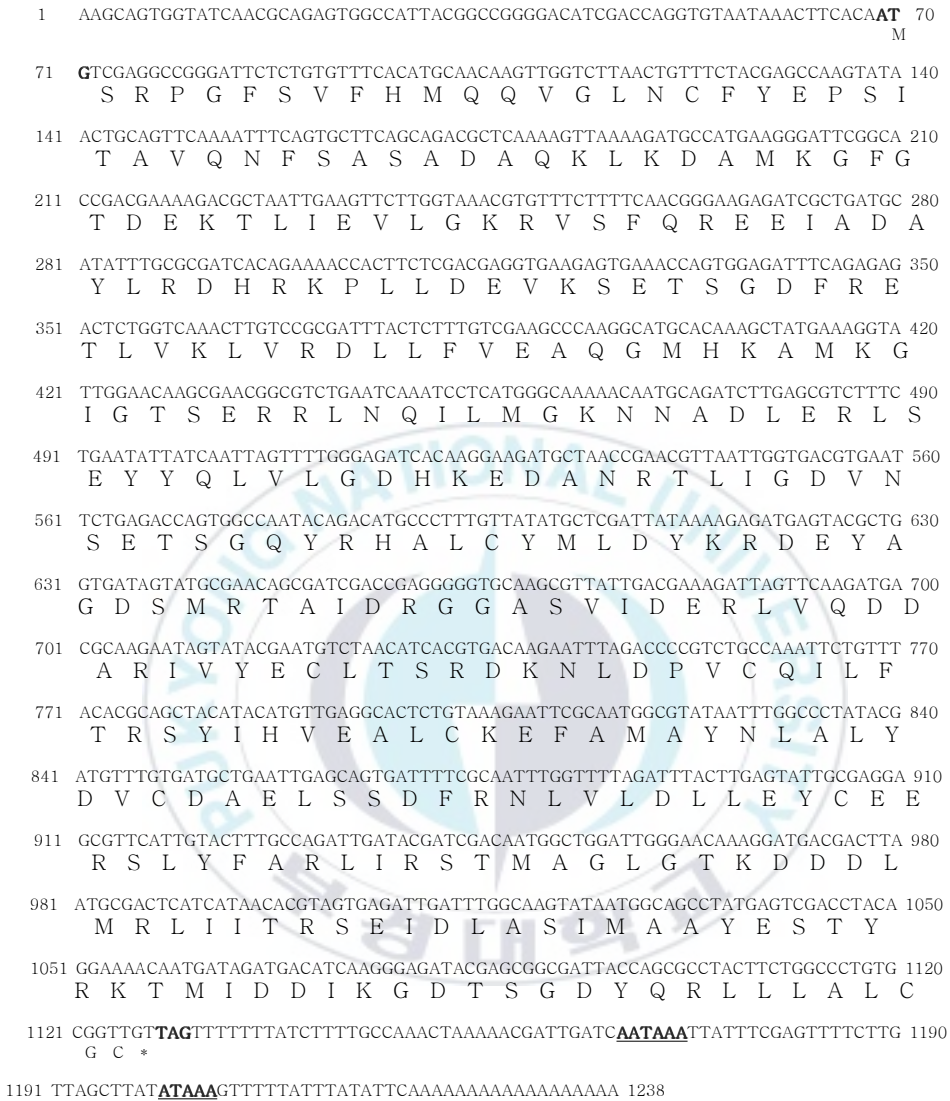

Fig. 5. Nucleic and deduced amino acid sequence of *M.sebastis* annexin. Nucleotide numbers, starting from methionine (M) initiation code, are presented on the right side. The stop codon (TAG) is represented with an asterisk (*). The conventioal poly adenylation signal (ATTAAA) is shown as underscore and in bold. One mRNA instability motif (ATTTA) in 3-UTR are shown as a capital and underlined.

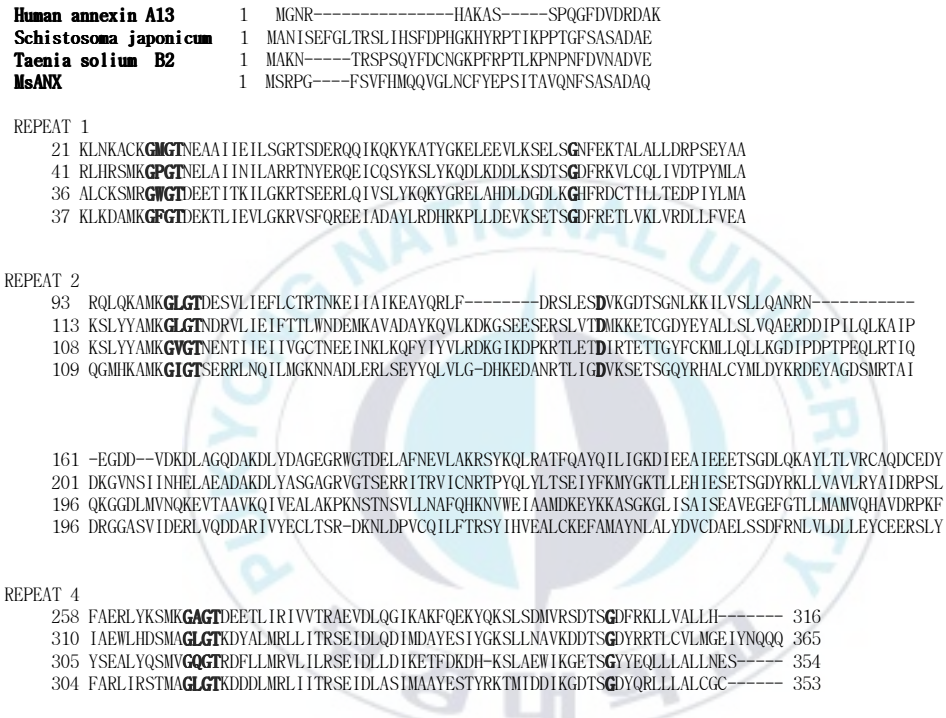

Fig. 6. The present MsANX had the type 2 motif in the repeat 1, 2, and 4. It was 'type 2' motif for binding calcium ions with the sequence 'GxGT-[38 residues]-D/E'

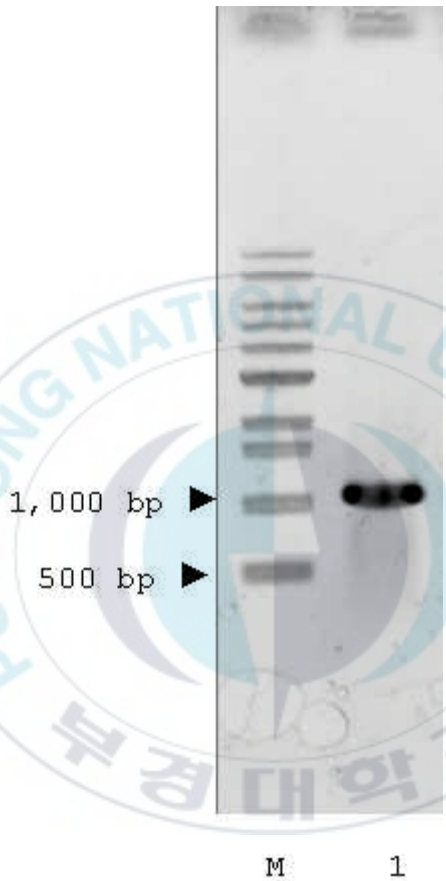

Fig. 7. PCR product of annexin gene was visualized. Lane M: indicates 1kb ladder. Lane 1: The size of annexin gene is 1,062bp,

Comparison of amino acid sequence and phylogenetic analysis

A molecular phylogenetic analysis was constructed further analyze the evolutionary relationship among known human and knew parasite annexin protein family by using previous neighbor-joining method (Saitou and Nei, 1987). As shown in Table. 4, annexin [*Schistosoma* japonicum] was the highest value (39%) to among other parasite annexin proteins. When compared to human annexin , they formed a cluster with Human annexin A13 maintaining a high bootstrap value (Fig. 8).

Prediction of 3D model of annexin

Predicted structure of *M.sebastis* annexin protein was performed by using SWISS-3D MODEL software program. The annexin of *M.sebastis* has annexin repeat domain.(39-103, 112-183, 293-353) The molecular structure expected of *M.sebastis* annexin protein were shown in Fig. 9. and respectively. Red and blue and green and orange color indicate the annexin repeat.

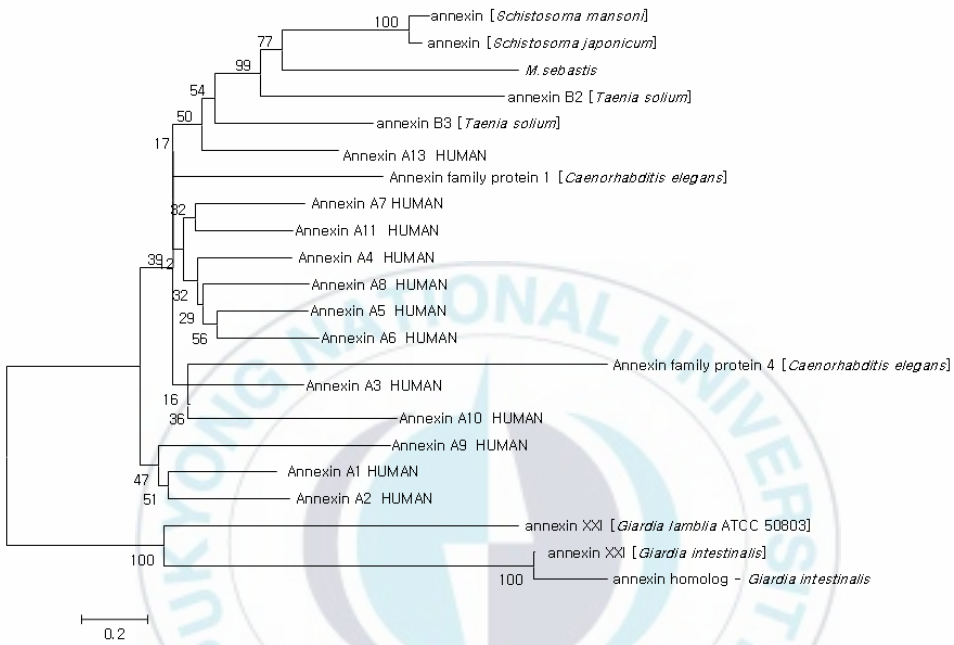

Fig. 8. Phylogenetic analysis of *M.sebastis* annexin protein with annexin protein family of in mammalian species and *Schistosoma mansoni*, *Caenorhabditis elegans*. Phjylogenetic analysis was done by neighbor-joining (NJ) method using Phylip software. The numbers indicate the bootstrap confidence values obtained for each node after 1000 replications.

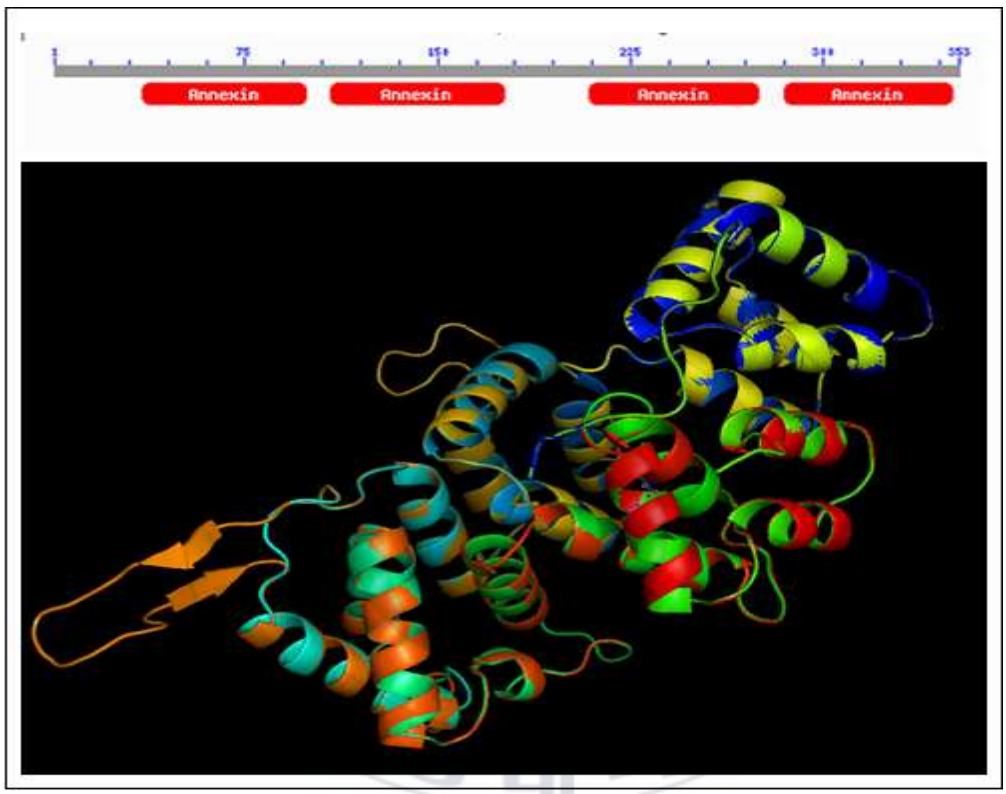

Fig. 9. Predicted protein structure of annexin. 3D predicted structure was performed by SWISS-3D MODEL software.

Recombinant protein production and purification

Annexin genes of *M.sabastis* were amplified from total RNA of parasire of *M.sabastis* of by RT-PCR (Fig. 7.). The recombinant proteins were successfully expressed using *E. coli* BL21(DE3). The recombinant annexin were purified as His-tagged proteins and were analyzed using SDS-PAGE (Fig. 10.). The molecular weights of the annexin were approximately 40 kDa .

Phospholipid vesicle binding activity

To find out whether the purified annexin exhibited an annexin-typical interaction pattern with phospholipids, we tested for Ca^{2+} dependent binding to multilamellar phosphatidyl serine-containing vesicles. As shown in Fig. 11, the protein did indeed associate with the vesicle pellet in the presence of 0.1mM Ca^{2}. (lane 3 vs. lane 4), 1mM Ca^{2}. (lane 5 vs. lane 6) whereas no annexin was precipitated in the absence of free Ca^{2}. (lane 1 vs. lane 2).

Purification of Ig from rockfish serum

Protein A affinity chromatography was able to purify approximately 0.8 mg $ml^{-1}$of Ig from about 30 mg ml^{-1} rockfish serum. SDS-PAGE under reducing conditions revealed that the purified serum Ig consisted of two bands of approximately 70 and 26 kDa, with no notable non-specific proteins bands(Fig. 12.).

Anticoagulant coagulation assay

Prolongation of APTT by purified anticoagulant recombinant annexin .(Fig. 13.) All coagulantion assays were performed with three individual replicates using the ACL® 7000 Coagulation analyzer and diagnostic kit, and mean values were taken as described herein. Samples incubated for 3 min at 37℃ cotain the normal citrated PPP(80㎕) and purified recombinant annexin (20㎕, 300ug/mL)

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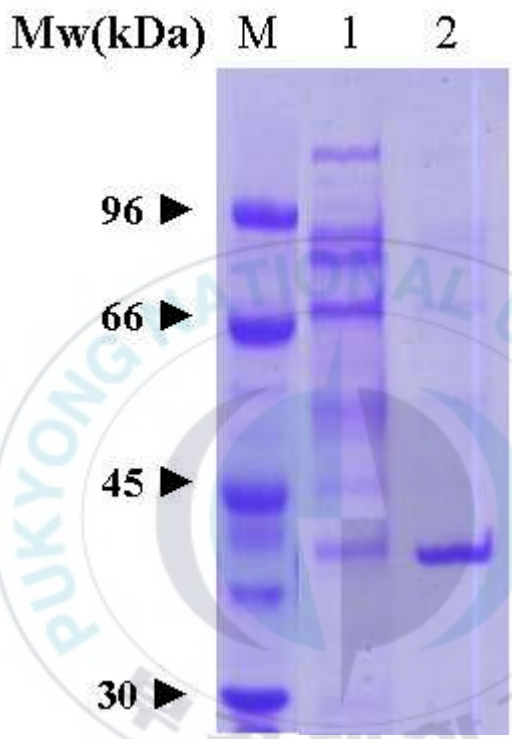

Fig. 10. SDS-PAGE analysis for purification of induced annexin. M: protein molecular marker, Lane 1: Recombinant annexin protein is approximately 40 kDa.

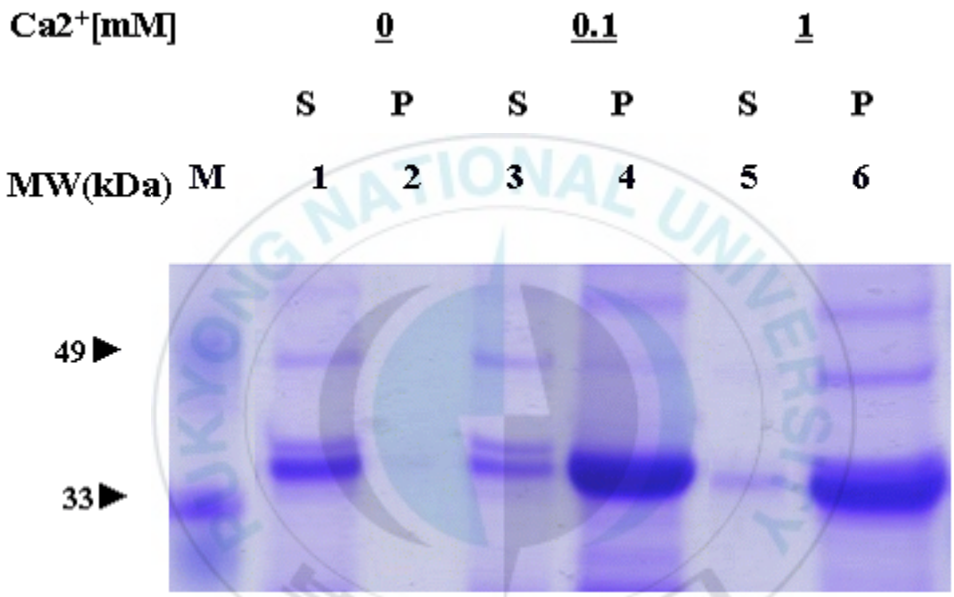

Fig. 11. Association of ANEXIN with multilamellar phosphatidyl serine-containing vesicles. Lane M, molecular mass markers; lanes 1 and 2, absence of free Ca^{2+}. lanes 3 and 4 , 0.1 mM free Ca^{2+}. lanes 5 and 6 , 1 mM free Ca^{2+}. . S, supernatant; P, pellet.

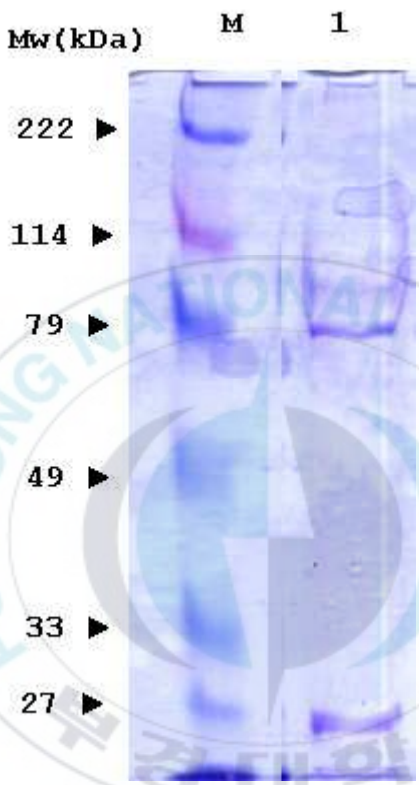

Fig. 12. SDS-PAGE analysis of rockfish serum Ig purified by protein A. The serum proteins were resolved by 10% SDS-PAGE and visualised by CBB G-250 staining. Lane 1, purified rockfish serum Ig

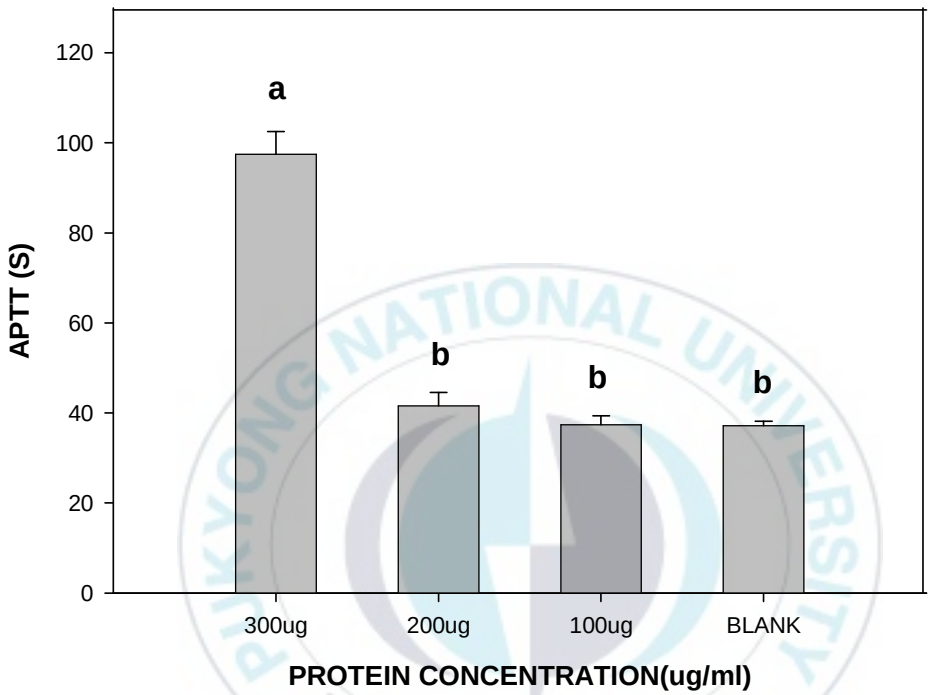

Fig. 13. Prolongation of APTT by purified anticoagulant recombinant annexin . All coagulantion assays were performed with three individual replicates using the ACL® 7000 Coagulation analyzer and diagnostic kit, and mean values were taken as described herein. Samples incubated for 3 min at 37℃ cotain the normal citrated PPP(80㎕) and purified recombinant annexin (20㎕, 300ug/mL)

Location of annexin in parasite

The rat antiserum against the recombinant MsANX bound to a protein corresponding to the MsANX in 40MW when worm crude extracts were used as antigens (Fig. 14).

Antiboby presence in the infected rock fish serum

The sera of 2-year-old adult rock fish experienced heavy infection by *M. sebastis* specifically bound to the recombinant MsANX (Fig. 15) In contrast, sera collected from fingerlings that were not infected with *M. sebastis* did not bind to the MsANX.

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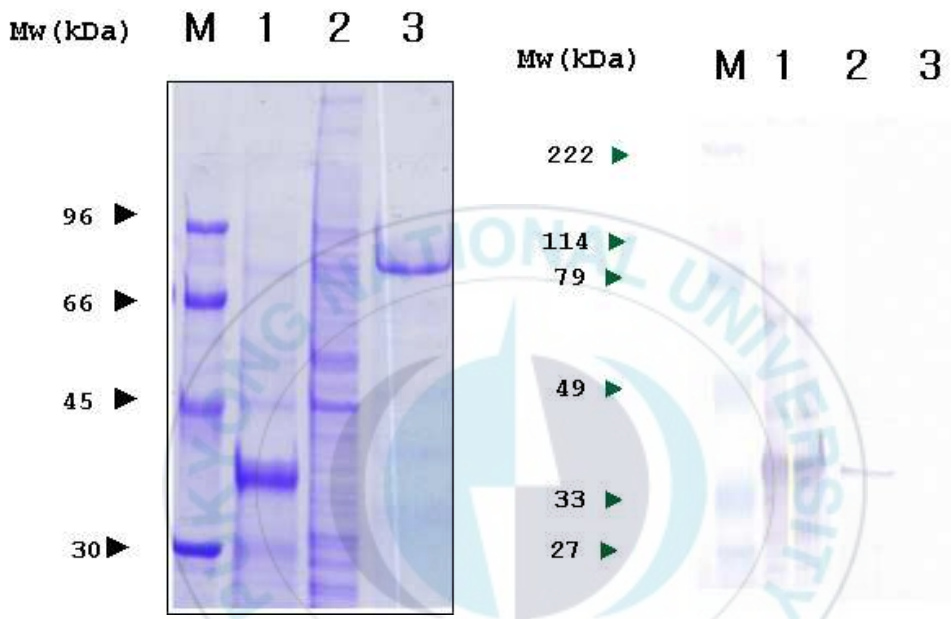

Fig. 14. Location of annexin in parasite. Left figure; SDS-PAGE gel was *M.sebastis* of ES and crude and recombinant annexin: Right figure; Western blot of the transferred proteins onto a nitrocellulose membrane. M prestained protein marker (Pierce) : Lane1 recombinant annexin : Lane 2 *M.sebastis* of crude : Lane 3 *M.sebastis* of ES

Fig. 15. Antiboby presence in the infected rock fish serum. M : prestained protein marker (Pierce), Lane 1, 2; purified recombinant annexin. The primary antibody in Lane 1, was infected rock fish serum, in Lane 2 was non-infected rock fish serum. The secondary antibody was rabbit polyclonal antibody against rockfish Igs

Discussion

In the present results, A total of 476 random clones from *M.sebastis* of rock fish subtracted cDNA libraries were partially sequenced.

In the present study, an annexin gene of *Microcotyle sebastis* was cloned for the first time from monogeneans. Structurally, all annexins share a core domain made up of four similar repeats, each approximately 70 amino acids long. Each repeat is made up of five α helices and usually contains a characteristic 'type 2' motif for binding calcium ions with the sequence 'GxGT-[38 residues]-D/E' (Moss & Morgan 2004). The present MsANX also had the type 2 motif in the repeat 1, 2, and 4. In the present study, Ca^{2+}-dependent binding of the recombinant MsANX to phosphatidyl serine vesicles indicatesthat the present recombinant MsANX is a functional annexin.

In the present Western blot analysis using antisera against the recombinant MsANX, the band of MsANX was not detected from the proteins secreted during in vitro maintenance of the worms but detected from the worm crude extracts. Generally, blood-sucking ectoparasites inject their enzymes that can act as anticoagulant or other functions into host blood before feeding blood. In this study, adult fish experienced heavy infection had antibodies against the annexin of *M. sebastis* suggesting that the MsANX would be secreted specifically when the worms penetrate their head part into fish blood vessel to feed blood, and the MsANX introduced into fish blood would elicit specific humoral immune responses.

It is frequently observed that the infection intensity of *M. sebastis* in rockfish cultured in the epizootic area of *M. sebastis* is negatively correlated with fish age (not published). This suggests that exposure of fish to repeated

infection with *M. sebastis*results in the development of immunity to further infection. To design potent subunit vaccines, it is necessary to identify those antigens that are recognized as nonself by the immune systems of hosts during infection. In the present study, the specific humoral immune response against MsANX in rockfish, which were naturally infected with *M. sebastis*, suggests that MsANX has a possibility to be used as a subunit vaccine antigen. Immunization of fish with the recombinant MsANX and the protective efficacy against *M. sebastis* infection should be further investigated.

조피볼락 아가미흡충 *Microcotyle sebastis*에서 분리한 annexin의 분자·면역학적 특성

최 승 혁

부경대학교 대학원 어병학과

Summary

조피볼락 아가미흡충 (*Microcotyle sebastis*)에 대한 재조합백신개발을 위해 충의 cDNA library를 제작하여 항원 후보 유전자를 선정하였다. 제작된 cDNA library로부터 476개의 clone을 선별하여 염기서열을 분석하였고 NCBI의 BLAST X program을 사용하여 단백질에 대한 상동성 조사를 실시하였다. 분석된 유전자 중에서 annexin으로 추정된 유전자에 대하여, 5'-과 3'- RACE를 실시하여 full-length cDNA를 얻었다. annexin의 방어항원으로서의 가능성을 분석하기 위해 full ORF annexin을 재조합 단백질로 발현, 정제하였으며 이 단백질로 annexin의 분자 면역학적 특성을 알아보았다. 분석결과로부터 본 연구에서 제작한 재조합 annexin은 일반적인 annexin 단백질의 특성인 phospholipid vesicle에 결합하는 능력 및 anticoagulant acticity를 가지고 있음을 알 수 있었다. 또한 Western blot 결과 아가미흡충에 감염된 조피볼락의 혈청은 이 annexin과 binding 하는 반면 아가미흡충에 전혀 감염되지 않은 조피볼락의 혈청은 annexin에 대해 반응이 없는 것으로 보아 이번 연구에서 밝힌 아가미흡충의 annexin이 추후 아가미흡충증의 재조합 백신 개발에 유용하게 사용될 수 있을 것으로 여겨진다.

감사의 글

지난 2년간의 석사생활을 한 편의 논문으로 끝내기에는 아쉬움이 많이 남습니다. 하지만 저를 진심으로 아껴주시고, 이끌어 주신 많은 분들이 계셨기에 부족하지만 이러한 결실을 얻을 수 있었습니다. 주위 분들에게 많은 도움을 받다 보니 감사해야 될 분들이 많습니다.

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함께 동고동락하며 석사를 함께 마치는 혜경이누나, 수진이, 큰 힘이 되어준 성돈이형, 정현이에게도 진심으로 감사의 말을 전합니다. 진정으로 크나큰 힘이 되어준 윤임와 유미에게도 감사의 말씀을 전합니다
못난 저에게 항상 격려해주시고 아껴주시는 할머니, 할아버지, 고모, 삼촌들, 사촌형들, 마지막으로 저에게 언제나 뒤에서 크나큰 힘이 되고 있는 아버지, 유미에게도 이 자리를 빌려 고맙다는 말과 함께 사랑한다는 말을 전하고 싶습니다.

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