

PmAV, a novel gene involved in virus resistance of shrimp *Penaeus monodon*¹

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Abstract Diseases caused by viruses especially by white spot syndrome virus (WSSV) are the greatest challenge to worldwide shrimp aquaculture. The innate immunity of shrimp has attracted extensive attention, but no factor involved in the virus resistance has been reported. Here we report for the first time the identification of an antiviral gene from shrimp *Penaeus monodon*. A differential cDNA (designated as *PmAV*) cloned from virus-resistant shrimp *P. monodon* by differential display (DD) was found to have an open reading frame (ORF) encoding a 170 amino acid peptide with a C-type lectin-like domain (CTLDD). The *PmAV* gene was expressed in *Escherichia coli* and the protein was purified. Recombinant PmAV protein displayed a strong antiviral activity in inhibiting virus-induced cytopathic effect in fish cell in vitro. Moreover, native PmAV protein was isolated from shrimp hemolymph by immuno-affinity chromatography and confirmed by Western blot. No agglutination activity was observed both in recombinant and native PmAV protein. Immunohistological study showed that PmAV protein was located mainly in the cytoplasm, and not bound to the shrimp WSSV. It implies that the antiviral mechanism of PmAV protein is not by inhibiting the attachment of virus to target host cell. The discovery of *PmAV* gene might provide a clue to elucidate the innate immunity of marine invertebrates and would be helpful to shrimp viral disease control.

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Key words: Antiviral gene; C-type lectin domain; Differential display; Shrimp

1. Introduction

Shrimp is one of the most important species in aquaculture. During the last decade, the worldwide shrimp culture was greatly puzzled by diseases caused by viruses particularly by white spot syndrome virus (WSSV) and suffered significant economic losses [1,2]. Due to the extreme virulence of WSSV and a wide host range covering almost all crustaceans [3,4], it is difficult to prevent and inhibit the spread of the virus. People also noticed that although most of the WSSV-infected shrimps died, a few of them still survived [2]. There-

fore it is interesting to find out the immune factors responsible for the shrimp resistance against WSSV. Like other invertebrates, shrimp lacks specific immunity, and its disease resistance relies on its innate defense system including a series of humoral and cellular immune factors [5], so it is regarded as an appropriate species for studying the innate immunity. Nowadays it becomes clear that the innate immune system plays an important role in host defense reaction [6].

In recent years, some exciting progresses at the molecular level were made on shrimp innate immunity, such as proPO activating system [7–9] and antimicrobial peptides [10–12]. However, they all aimed at bacteria, fungi or parasites rather than viruses. Previous studies showed the existence of non-protein antiviral substance in crustaceans [13], but so far little is known about the possible innate antiviral factor generated by the interaction between host cell and virus, and neither antiviral gene nor antiviral protein has been characterized from crustaceans.

In this paper, mRNA differential display (DD) technique [14] was adopted to compare the virus-sensitive and virus-resistant shrimps *Penaeus monodon* and the first shrimp antiviral gene *PmAV* was identified. The PmAV protein might play an important role in the defense mechanism against viruses.

2. Materials and methods

2.1. Shrimps

Normal (virus-sensitive) and virus-resistant shrimps *P. monodon* were collected from shrimp ponds in Longhai, Fujian, China. We found in a pond while most shrimps died in the outbreak of WSSV, there were still a few shrimps that survived. The activities of immune factors, such as phenoloxidase (PO) [15], lysozyme [16], hemolysin [17] and hemagglutinin [18], were evidently higher in these survived shrimps than those in normal shrimps (data not shown), therefore they were considered as virus-resistant shrimps. Shrimps in the neighbor pond, which were originated from the same group of larvae, normally grew up under the same culture conditions but were not infected by virus, were collected as normal shrimps. WSSV was not found by polymerase chain reaction (PCR) [19] in both normal and virus-resistant shrimps. Live shrimps were immediately frozen in liquid nitrogen and stored at -70°C after collection.

2.2. Fluorescent DD of normal and virus-resistant shrimps

Total RNA was isolated respectively from hepatopancreases of two kinds of shrimps mentioned above by the acid guanidine-phenol-chloroform (AGPC) method [20], and then treated by DNase I (Stratagene). Reverse transcription (RT) and DD-PCR were performed in duplicate by FluoroDD Kit (Beckman-Coulter) as described in the manufacturer's manual. Superscript II enzyme (Gibco-BRL) was used for RT. GenomylRS instrument (Beckman-Coulter) was used for electrophoresis, fluorescence scanning and band analysis.

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¹ The nucleotide sequence reported in this paper has been submitted to GenBank with accession number AY302750.

2.3. Northern blot and sequence analysis of differential cDNA

Differential cDNA band was recovered from gel, then reamplified and labeled by digoxin (Dig). Total RNA (15 µg) from hepatopancreases of two kinds of shrimps were dotted respectively on positive-charged nylon membrane (Boehringer Mannheim), and hybridized to the Dig-labeled probe. Dig-labeling, hybridization and detection were performed using the kit from Boehringer Mannheim. Differential cDNA was sequenced, and then analyzed by BLAST on NCBI web-site.

2.4. cDNA library screening

After the isolation of hepatopancreas mRNA from virus-resistant shrimp by PolyATtract System1000 kit (Promega), a cDNA library was constructed using λ ZAP Express cDNA synthesis and Gigapack-III Gold cloning kit (Stratagene) according to the manufacturer's protocol. Subsequently the library was screened with Dig-labeled probe. Positive plaques were picked out, and then converted into phagemids by in vivo excision as described in the manual. Relatively long inserted fragments from several selected phagemids were sequenced.

2.5. Recombinant expression of PmAV cDNA, protein purification and refolding

An open reading frame (ORF) of PmAV cDNA was amplified from library with 5' primer TAGTGCATGCATATGCGTCATACAATCC-TA and 3' primer CTGTCTCGAGCTATGTGCTGCTTTCACA. *SphI* and *XhoI* sites were involved in the two primers, respectively (as shown in italic letters). The PCR fragment was inserted into the thio-redoxin-fused expression vector pThioHisC (Invitrogen). For the convenience of protein purification by metal (Ni) affinity chromatography, this vector was modified with six times histidine ahead of the stop codon. Recombinant plasmid was transformed into *Escherichia coli* XL1-Blue (Stratagene), and confirmed by sequencing. The expressed product was detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

The PmAV protein that appeared in inclusion body was purified with Ni-nitrilotriacetic acid (NTA) agarose (Qiagen) under denaturing (8 M urea) conditions as described by the manufacturer. Purified protein was refolded by gradient dialysis (urea concentration in the sample was reduced step by step, from 8 to 0 M). The induced control protein (named as TH) from the vector pThioHisC only was also purified with Ni-NTA agarose under native conditions.

2.6. Antiviral assays of the PmAV protein

The antiviral activity of the recombinant PmAV protein was monitored by the inhibition of SGIV (grouper iridovirus, originally isolated from diseased brown-spotted grouper *E. tauvina* [21])-induced cytopathic effect (CPE) in GP cells (grouper embryo cells from *E. tauvina*). Cells were seeded in 96-well plates at 10^5 cells/well in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum, 0.116 M NaCl, 100 IU/ml penicillin and 100 µg/ml streptomycin and incubated at 25°C [21]. After serial dilution, 10 µl of various concentrations of the PmAV protein or TH protein were mixed with equal volumes of virus at a multiplicity of infection (MOI, the average number of virus particles that infect a single cell) of 0.02. When the cells were confluent, the cell monolayers were infected with the mixtures of protein and virus, or virus only as control. After a 30 min adsorption period, the media were added, and the plates were incubated at 25°C. Each treatment was repeated four times. When the control culture showed complete destruction (usually 3 days after virus inoculation), the number of viable cells was determined by staining of the cells with 1% crystal violet in ethanol [22]. Following the 2–3 min staining period, excess dye was removed by washing the cell monolayer three times with phosphate-buffered saline (PBS). The concentration of the PmAV protein required to inhibit virus-induced CPE to 50% of the virus-infected control (without the PmAV protein) was estimated as the 50% effective concentration (EC_{50}) [22,23].

Cytotoxicity of the PmAV protein was evaluated as CC_{50} [22,23], which corresponds to the concentration required to reduce the viable cells to 50% of the control (without the PmAV protein). When seeded cells were confluent, the media were replaced by the media containing different concentrations of the PmAV protein. 7 days later, the number of viable cells was determined by staining of the cells with 1% crystal violet in ethanol.

2.7. Immuno-affinity chromatography and Western blot

Antibody against the recombinant PmAV protein was prepared by immunizing mouse according to the conventional method [24]. IgG was isolated from the antiserum by protein A Sepharose (Pharmacia), and then titered by enzyme-linked immunosorbent assay (ELISA) to determine the optimal working concentration. IgG was coupled to CNBr-activated Sepharose 4B (Pharmacia) to prepare the antibody affinity column as described by the manufacturer, then the column was equilibrated with 50 mM Tris–HCl (pH 8) containing 100 mM NaCl. Serum from healthy shrimps was loaded, and then the column was washed with enough 50 mM Tris–HCl (pH 8) containing 100 mM NaCl and eluted with 0.1 M glycine (pH 3).

The eluate was analyzed by 14% SDS–PAGE, and then Western blot was performed with mouse anti-PmAV IgG according to the conventional method [24]. Goat anti-mouse IgG alkaline phosphatase conjugate (Promega) was used as the secondary antibody.

2.8. Immunohistological analysis

Healthy shrimps *P. monodon* were infected with WSSV as described [25]. At various stages postinfection (0, 2, 6, 12, 24, and 48 h), four specimens were selected at random. Their hepatopancreases were immediately fixed in formaldehyde/glutaraldehyde solution. Samples were embedded at low temperature in Lowicryl K4M resin (Polysciences), followed by sectioning (LKB-5 ultramicrotome) and immunoreaction with 10 nm gold-labeled goat anti-mouse IgG (Academy of Military Medical Sciences of China). Finally sections were double-stained and observed under an electron microscope (JEOL-CX100) at 100 kV.

2.9. Virus affinity chromatography

Intact WSSV viruses were purified from infected crayfish *Cambarus clarkia* (Xiamen, China) as described [26]. Virus samples were examined under electron microscope for purity. WSSV were coupled to CNBr-activated Sepharose 4B as above. The WSSV-coupled column was equilibrated with 0.1 M Tris–HCl (pH 8.0). Then recombinant PmAV protein was loaded. After washing with 10 times of bed volumes of 0.1 M Tris–HCl (pH 8.0), the column was eluted with elution buffer (0.1 M Tris–HCl, 1 M NaCl, pH 8.0).

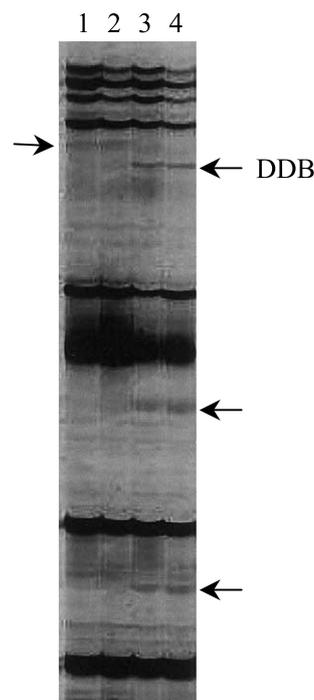


Fig. 1. A fraction of fluorescence scanning picture of DD-PCR products. PCR fragments were separated on a 5.6% high-resolution polyacrylamide gel under denaturing (urea) conditions. DD-PCR was carried out in duplicate. Lanes 1 and 2, DD-PCR products from normal shrimp; lanes 3 and 4, DD-PCR products from virus-resistant shrimp. The arrows indicate the differential bands, from which DDB was selected for further study in this paper.

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1   ACCCCCTCACTAAAGGGAACAAAAGCTGGAGCTCGCGCGCCTGCAGGTGCGACACTAGTGGGA
61  TCCAAAGAATTTCGGGCACGAGGCCACAAATGCGTCATACAATCCTAGTTTTCTTTCCCTC
1   M R H T I L V F L S L
121 GGTGTTGTTGGGTGGCTGTGGCAACATCATAACGAGAAAAGTGCCAATGATTCCAAGGCT
12  G V V G S A V A T S Y E K S A N D S K A
181 GTCTGCTATAGCCCCTATACTGCCATTGCGGATCGCTGTTTGTGTCGATCACCAGACA
32  V C Y S P Y T A I A D R C L F V D H Q T
241 GATGGAAGCTGGTATGACATGCGAGAGTACTGTAACTTATAAATGGAGACTTTCTCAAG
52  D G S W Y D M R E Y C N L I N G D F L K
301 CTGGATGACGCTAATCTCCTTACTGATATCGTTGAGTACATTACTTACCAAGTGGGTGTG
72  L D D A N L L T D I V E Y I T Y Q V G V
361 AACAGAGACTACTGGATCGGGGGAGTGACGAGAACCACGAGGGTCTTTGGCTGTGGAG
92  N R D Y W I G G S D E N H E G L W L W T
421 GACGGAATCTCATGCGGACAGGTGTTCCCTTGTGGTACCATTGCACCTCCATCTCTCAA
112 D G T L M R T G V P L W Y H C T S I S Q
481 CAACGATGGTGGCTCCTCAGAGAAGTGCCTGCATGCGCTGGGACTCATTTTACCAT
132 Q P D G G S S E N C A V M R W D S F Y H
541 ATCCATGATGTGCTTGTCTACACTTCTCGGTCTGTCAATTTGTGAAAGCAGGACACATTAA
152 I H D V S C Y T S R S V I C E S R T H *
601 TCTAACAAAGTTTTGGCCACAGAAAAGATATACAAATTGTTTTTTATTACAAATTTTCTTT
661 TATATCATATTGTGCCACAAGCCGAGATCATTGGCAAAGTACACATTAACCAATGATAT
721 CTATATACTATGTACATTGTTTATCACGCTGCCCGTGGCCACACTGCCTTATGCATTTA
781 ACTTTTCTAATCTGTCTGAAATCTTTTTTAGCTGTAACATTGCAAGAATAAATATTCTA
841 AAGGAAAAAAAAAAAAAAAAAAAA

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Fig. 2. Sequences of *PmAV* cDNA (863 bp) and deduced peptide (170 amino acids). Predicted ORF is from 88 to 597 bp (the start and stop codons are shown in bold). The differential fragment obtained by DD is from 447 to 863 bp. CTLD is located at the region from 33 to 166 amino acids.

3. Results

3.1. Fluorescent DD of normal and virus-resistant shrimp

After the electrophoresis and scanning of DD-PCR products, some differential bands between normal and virus-resistant shrimps were found (Fig. 1), but only parts of them were confirmed to be differentially expressed cDNA by Northern blot. BLAST analysis of sequences revealed that one of them (named as *DDB*, about 450 bp) was a possible virus resistance-related cDNA, therefore *DDB* was selected for further study. By Northern blot with Dig-*DDB* probe, hepatopancreas total RNA from virus-resistant shrimp displayed an obviously stronger signal than that from normal shrimp, showing that expression of *DDB* was upregulated in virus-resistant shrimp.

3.2. cDNA library screening and sequence analysis

Some positive plaques were obtained by screening the hepatopancreas cDNA library of virus-resistant shrimp with Dig-*DDB* probe. The whole cDNAs were sequenced. One of them contained an ORF (510 bp) encoding a 170 amino acid peptide with predicted molecular weight of 19.3 kDa. The peptide encoded by the cDNA (termed as *PmAV*) had no homologous sequence after searching in GenBank with BLAST. A C-type lectin-like domain (CTLD) was found and no signal peptide coding sequence existed (Fig. 2).

3.3. Expression of *PmAV* gene in *E. coli*

PmAV gene was inserted into vector pThioHisC and expressed (Fig. 3, lane 3). The *PmAV* protein in inclusion body was purified with Ni-NTA agarose under denaturing conditions. The purity was estimated to be higher than 95%

(Fig. 3, lane 4). After refolding by gradient dialysis, almost all the purified *PmAV* protein became soluble (Fig. 3, lane 5).

3.4. Antiviral assay of recombinant *PmAV* protein

As there is no suitable shrimp cell line for the function study of shrimp gene [27,28], fish cell (GP cell) was used in this investigation. After 3 days of virus inoculation, GP cells (Fig. 4A) were completely destructed by SGIV for virus only (Fig. 4B) as well as the mixture of SGIV and control protein

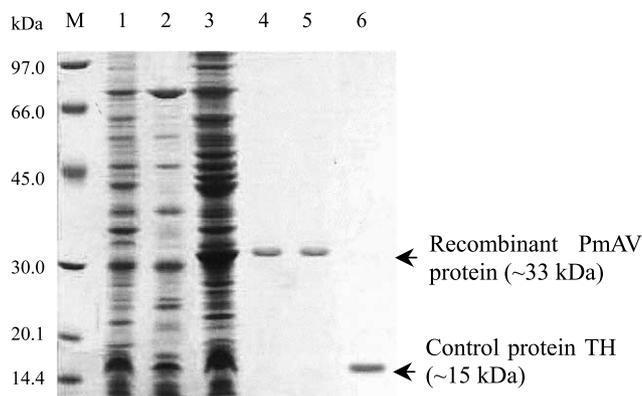


Fig. 3. 14% SDS-PAGE analysis of recombinant expressed and purified protein encoded by *PmAV* gene. Proteins were stained with Coomassie blue R-250. Lane M, low molecular weight marker (Pharmacia); lane 1, pThioHisC-XL₁-Blue, isopropyl thiogalactose (IPTG)-induced; lane 2, pThioHisC-PmAV-XL₁-Blue, non-induced; lane 3, pThioHis-PmAV-XL₁-Blue, IPTG-induced for 4 h; lane 4, affinity-purified recombinant *PmAV* protein; lane 5, refolded recombinant *PmAV* protein; lane 6, affinity-purified control protein TH.

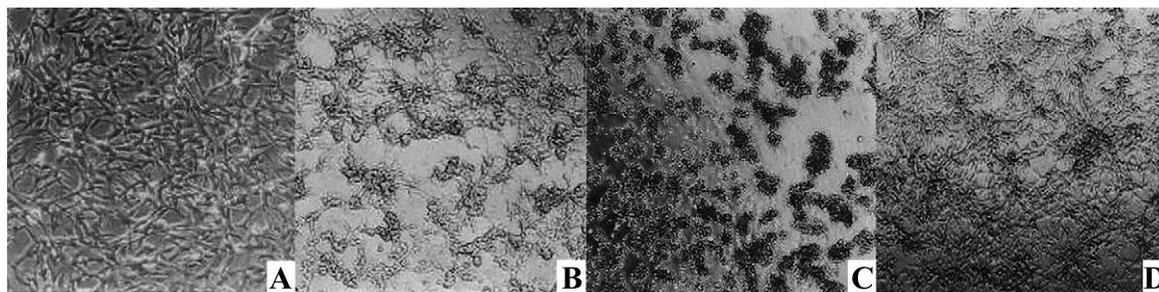


Fig. 4. Antiviral assay of recombinant PmAV protein. A: Normal GP cells. B: GP cells after 3 days of incubation with SGIV only. C: GP cells after 3 days of incubation with the mixture of SGIV and control protein TH (6.25 µg/ml). D: GP cells after 3 days of incubation with the mixture of SGIV and PmAV protein (6.25 µg/ml).

TH (Fig. 4C), while approximately 50% GP cells incubated with the mixture of SGIV and PmAV protein remained viable (Fig. 4D). EC_{50} was determined to be approximately 6.25 µg/ml. It was convinced that PmAV protein had non-specific antiviral activity.

On the other hand, the cytotoxicity of PmAV protein to the GP cell, evaluated as CC_{50} , was more than 200 µg/ml (data not shown), suggesting that PmAV protein had no cytotoxicity to tested cells.

3.5. Isolation of the native PmAV protein

An approximate 28 kDa protein was isolated from shrimp serum by immuno-affinity chromatography (Fig. 5A). The same result was obtained by Western blot with the anti-PmAV IgG (Fig. 5B), suggesting that this 28 kDa protein should be the native PmAV protein in shrimp *P. monodon*. Nevertheless, we have described previously that PmAV cDNA encodes a 170 amino acid peptide with a predicted molecular weight of 19.3 kDa, obviously there is a disagreement in molecular mass between the deduced peptide and native protein. Sequence analysis of the deduced peptide predicted that the protein may contain sites for phosphorylation, glycosylation and alkylation. This type of discrepancy between the calculated molecular mass and the apparent molecular mass on an SDS gel is quite common among proteins that are heavily modified.

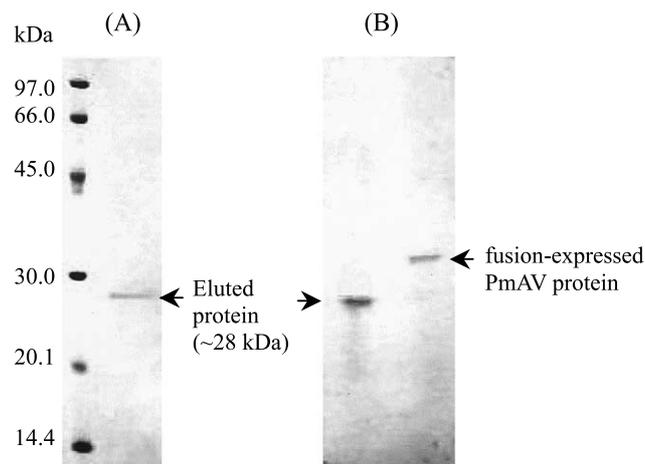


Fig. 5. Isolation and Western blot of the native PmAV protein. A: 14% SDS-PAGE of the eluate from antibody affinity column, silver staining. B: Western blot of the eluate.

3.6. Immunohistological analysis and virus affinity chromatography

Many gold particles were observed in the cytoplasm of hepatopancreas at 12 and 24 h postinfection with WSSV, but not in the organelles, the cell nucleus and extracellular matrix (Fig. 6A). WSSV was found in the nucleus at 24 h postinfection, but no gold particles were observed on or near WSSV (Fig. 6B). This result suggested that the PmAV protein didn't directly interact with virus when it performed the antiviral function.

Even detected by silver staining, PmAV protein was found flowing through the virus-coupled column without binding to WSSV.

4. Discussion

Virus infections are common among invertebrates, but little is known about the ability of invertebrates to deal with viral infections. So it is essential to study the virus-related genes and their functions involved in the innate immunity and disease resistance of invertebrates. Although the shrimp genome project is unable to be launched entirely now, a number of sequences of shrimp genes were determined by various methods [29–31]. Some of them were supposed to be immunity-related, but it is difficult to confirm their functions due to lack of shrimp cell culture system. So far, the immune mechanism of shrimp is not clear yet.

In this report, we cloned, expressed and identified an antiviral gene PmAV from shrimp *P. monodon* for the first time.

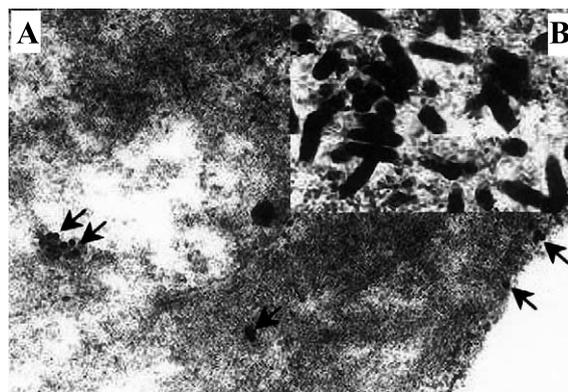


Fig. 6. Immunohistological analysis of the PmAV protein in shrimp hepatopancreas. A: The cytoplasm (24 h postinfection with WSSV, 40,000×), arrows indicating gold particles. B: WSSV in the nucleus, no gold particle (24 h postinfection, 20,000×)

The PmAV protein contains a CTLD. As we know, C-type animal lectin exists widely in vertebrates and invertebrates, and it represents an important recognition mechanism for oligosaccharides at cell surfaces [32]. All C-type lectins contain a carbohydrate recognition domain (CRD) which mediates sugar binding with Ca^{2+} [33]. Currently more than 100 human proteins containing CTLDs have been reported, and among them approximately half were supposed to function as C-type CRDs. Many CTLDs bind to protein ligands rather than sugars, and only some of these binding interactions are Ca^{2+} -dependent [34]. They are considered to be developed from original CTLDs by divergent evolution. Many molecules containing CTLD are related to immunity, such as C-type lectin, coagulation factor binding protein, IgE Fc receptor and NK cell receptor [32].

Our results showed that neither recombinant PmAV protein nor native PmAV protein had agglutination activity (data not shown), suggesting that *PmAV* doesn't encode a lectin. Since the PmAV protein has antiviral activity, does its CTLD recognize and bind to shrimp virus? As no gold particle adhered to WSSV in immunohistological observation, and no PmAV protein bound to WSSV when flowing through the virus affinity column, we assumed that the PmAV protein might act as an intermediate rather than a recognition factor during the antiviral reaction. Further works are needed to reveal the antiviral mechanism of *PmAV* in detail.

So far, neither antiviral gene nor antiviral protein from shrimp was reported, and almost all studies on molecular biology of innate immunity of shrimps aimed at bacteria, fungi, parasites, etc. The *PmAV* gene is the first antiviral gene from shrimp reported, and it might be an important innate host defense factor. It would provide us a clue to elucidate the innate immunity and to control diseases of marine invertebrates.

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