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Shrimp arginine kinase being a binding protein of WSSV envelope protein VP31*

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Abstract Viral entry into the host is the earliest stage of infection in the viral life cycle in which attachment proteins play a key role. VP31 (WSV340/WSSV396), an envelope protein of white spot syndrome virus (WSSV), contains an Arg-Gly-Asp (RGD) peptide domain known as a cellular attachment site. At present, the process of VP31 interacting with shrimp host cells has not been explored. Therefore, the VP31 gene was cloned into pET30a (+), expressed in *Escherichia coli* strain BL21 and purified with immobilized metal ion affinity chromatography. Four gill cellular proteins of shrimp (*Fenneropenaeus chinensis*) were pulled down by an affinity column coupled with recombinant VP31 (rVP31), and the amino acid sequences were identified with MALDI-TOF/TOF mass spectrometry. Hemocyanin, beta-actin, arginine kinase (AK), and an unknown protein were suggested as the putative VP31 receptor proteins. SDS-PAGE showed that AK is the predominant binding protein of VP31. An in vitro binding activity experiment indicated that recombinant AK's (rAK) binding activity with rVP31 is comparable to that with the same amount of WSSV. These results suggested that AK, as a member of the phosphagen kinase family, plays a role in WSSV infection. This is the first evidence showing that AK is a binding protein of VP31. Further studies on this topic will elucidate WSSV infection mechanism in the future.

Keyword: white spot syndrome virus (WSSV); VP31; arginine kinase; shrimp; binding protein

1 INTRODUCTION

White spot syndrome virus (WSSV) is one of the most serious pathogens in shrimp farming since its first outbreak 1990s. Shrimps suffer high-mortality rates of up to 100% within 3 to 10 days after WSSV infection (Lightner, 1996). WSSV causes huge losses to the shrimp industry worldwide; therefore, studies have been carried out in terms of morphology, pathology, genomics, and proteomics. WSSV is a large, enveloped rod-shaped virus with a long envelope extension (Huang et al., 1995; Sánchez-Paz, 2010). Whole genome sequencing of four different WSSV isolates from the mainland of China, Taiwan, Thailand and Korea showed that WSSV contains more than 500 putative open reading frames (ORFs) (Yang et al., 2001; van Hulten et al., 2001; Chen et al., 2002; Chai et al., 2013).

To explore the pathogenesis and functions of viral genes, many scientists have studied the viral proteins (Sánchez-Paz, 2010). The Arg-Gly-Asp (RGD) peptide is known as a cellular attachment site (Pierschbacher and Ruoslahti, 1984). Previous studies found that a WSSV envelope protein VP37 with a RGD motif and its receptor protein F_1ATP synthase beta subunit played important roles in WSSV infection (Liu et al., 2009; Liang et al., 2010). Studies on WSSV envelope proteins with RGD motifs and their cellular receptors may help to uncover the mechanism of

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WSSV entry into host cells. VP31 (WSV340/WSSV396) was identified and characterized as a WSSV envelope protein with a RGD motif, which plays an important role in cell adhesion (Tsai et al., 2004; Li et al., 2011).

In this study, we attempted to study the cellular proteins of the shrimp *Fenneropenaeus chinensis* that interact with recombinant VP31 protein (rVP31). Four putative binding proteins from shrimp gill protein preparation, including hemocyanin, beta-actin, arginine kinase (AK) and an unknown protein, were pulled down in an affinity column coated with rVP31. SDS-PAGE showed that AK is the predominant receptor of VP31. In vitro binding activity assays indicated that the recombinant AK (rAK) binding activity with rVP31 is comparable to that with the same amount of WSSV. These results prompt that AK is an essential protein involved in WSSV infection.

2 MATERIAL AND METHOD

2.1 Shrimp

Shrimp (*F. chinensis*), approximately 10 cm in body length, were bought from Nanshan market in Qingdao. The animals were maintained in seawater in 80-L tanks with aeration and were tested randomly for WSSV infection following the World Organisation for Animal Health (OIE) standard procedure (OIE, 2012) to prove a WSSV-free status. Gill cellular proteins and hemocytes were prepared from the shrimp.

2.2 Preparation of shrimp gill cellular proteins

Gill cellular proteins were collected from WSSV-free *F. chinensis* and prepared as described by Liang et al. (2005) with modifications. Briefly, shrimp gills were washed three times using homogenization buffer (1.5 mmol/L $MgCl_2$, 10 mmol/L Tris-HCl, 10 mmol/L NaCl, 0.5% NP-40, 2 mmol/L EDTA) and homogenized in 5× volume of homogenization buffer with add 1 tablet of protease inhibitor cocktail (Roche) in 50 mL of buffer. The gill homogenate was then sonicated for 10 min, and incubated with 1% Triton X-100 and 0.5% NP-40 for 1 h with gentle shaking. The lysate was centrifuged at 1 500×*g* for 15 min at 4°C. The supernatant was collected as the shrimp gill cellular protein preparation.

2.3 WSSV purification

The virus stored in our laboratory used in this study was isolated from WSSV infected *Penaeus monodon* shrimp from China. Crayfish bought from the market for agricultural products in Qingdao were injected with WSSV. The moribund crayfish were collected and dissected immediately to separate the tissues of cephalothoraxes and muscles for WSSV purification as previously described by Xie and Yang (2005). The purified WSSV was used in the binding assay.

2.4 Cloning, expression and purification of recombinant VP31

The WSSV VP31 gene was amplified from WSSV genomic DNA by PCR using a pair of specific primers (VP31F1: 5′-ATA CCA TGG GAA TGT CTA ATG GCG CAA CTA TAA-3′, VP31R1: 5′-TAT CTC GAG TTA CTC CTC CTT AAA AGC AGT G-3′) with the underlined recognition sequences of *Nco*I and *Xho*I (TaKaRa, Japan). The amplified product was cloned into expression vector pET30a (+) (Merck, Germany). The recombinant plasmid pET30a (+)-VP31 was transformed into chemically competent *Escherichia coli* strain BL21 (DE3) and selected on Luria-Bertani (LB) plates containing 34 μg/mL kanamycin. The inserted VP31 sequence was confirmed by colony PCR and sequencing. BL21-pET30a (+)-VP31 was cultured in LB broth with 50 μg/mL kanamycin at 37°C and induced by isopropyl-β-D-1-thiogalactopyranoside (IPTG). Recombinant VP31 (rVP31) with a 6-His tag was purified on a cobalt-based immobilized metal affinity chromatography (IMAC) resin (Clontech, Japan) and verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) (Fudan University, Shanghai, China). To obtain native rVP31, the purified protein was dialyzed against a buffer containing 50 mmol/L Tris-HCl pH 8.0, 50 mmol/L NaCl, 1 mmol/L EDTA, 10 g/L glycine, 10% glycerin, 0.615 g/L L-glutathione reduced and 0.123 g/L L-glutathione oxidized, first with 4 mol/L urea, then with 2 mol/L urea, and finally without urea. After protein refolding, rVP31 was dialyzed in a coupling buffer (0.5 mol/L NaCl, 0.1 mol/L $NaHCO_3$, pH 8.3) for 12 h and concentrated by centrifugal filter concentrators (Millipore, Ultra-15). A BCA Protein Assay Kit (Tiangen) was used to quantify the purified protein.

2.5 rVP31 binding with shrimp hemocytes

The purified rVP31 and bovine serum albumin (BSA) were separately labelled with fluorescein isothiocyanate (FITC, SIGMA) following the procedure described by Liu et al. (2009). Healthy

shrimp were swabbed with 75% ethanol and its haemolymph was taken from the heart using a 2-mL syringe containing 1 mL anticoagulant (3.8% sodium citrate). It was then centrifuged at 200×*g* for 10 min to separate the cells. The cell pellet was resuspended gently in 2×L15 medium (containing 15% FBS, 10 U/mL penicillin, 0.1 mg/mL streptomycin) at 10^7 cells/mL and dropped into 24-well cell cultures plates. After adherence for 2 h, the cells were washed three times with medium to remove non-adherent cells. The preparations containing 5 μg FITC-rVP31 or 5 μg FITC-BSA were added separately into different cellchambers and incubated at 28°C for 2 h. Unbound proteins were removed by phosphate-buffered saline (PBS) washing. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) for 5 min and extra DAPI was removed by PBS washing. Cells were observed under confocal microscope (Nikon A1, Japan).

2.6 Identification of binding proteins of rVP31 by affinity chromatography

Freeze-dried CNBr activated Sepharose 4B (GE Healthcare) powder was washed in 1 mmol/L HCl for 15 min. Sepharose 4B and concentrated rVP31 solution was mixed at 4°C overnight. Excess protein was washed away using coupling buffer. The remaining active groups were blocked in 0.1 mol/L Tris-HCl (pH 8.0) and then washed six times including three alternations of buffer 1 (0.5 mol/L NaCl, 0.1 mol/L Acetic acid, pH 4.0) and buffer 2 (0.5 mol/L NaCl, 0.1 mol/L Tris-HCl, pH 8.0). After washing in PBS (pH7.4), the coupled gel was stored in 20% ethanol at 4°C.

The coupled gel was equilibrated in equilibrium buffer (PBS containing 1% Triton X-100 and 0.5% NP-40). After equilibration, the gel was collected and mixed with the shrimp gill cellular protein preparation by rotating for 1 h at room temperature. The mixture was then transferred into the affinity chromatography column. The gel was washed with equilibrium buffer twice. Finally, an elution buffer (0.5 mol/L NaCl, 0.1 mol/L sodium acetate, pH 3.0) was added to elute the binding protein and then analyzed with SDS-PAGE. Four putative viral binding bands were cut off from SDS-PAGE and analyzed with MALDI-TOF (Fudan University, Shanghai, China).

2.7 Expression and purification of recombinant arginine kinase (rAK)

The *F. chinensis* cDNA was used to amplify part of the AK gene (GenBank accession number: AY661542; REGION: 24..1094) using a pair of specific primers (AKF1: 5'-CAT GGA ATT CAT GGC TGA CGC TGC TG-3', AKR1: 5'-GCT ACT CGA GTT ACA TCT CCT TCT CCA TCT TG-3'). The forward primer contained recognition sequences of *EcoR* I and the reverse primer contained recognition sequences of *Xho* I. The restriction enzyme recognition sites are underlined in the primers. The amplicon was inserted into the expression vector pET30a (+) and the recombinant AK (rAK) was expressed in *E. coli* BL21 (DE3). The inserted AK sequence was confirmed by colony PCR and sequencing. rAK was purified with IMAC and verified in MALDI-TOF/TOF-MS analysis (Fudan University, Shanghai, China). The Bradford method was used to quantify the purified protein. For the binding assay, the purified rAK was labeled with Digoxigenin-3-O-methylcarbonyl-ε-aminocaproicacid-N-hydroxysuccinimide ester (DIG-MAHE, Roche) as follows: DIG-MAHE was dissolved in dimethylsulfoxide (DMSO) to a final concentration of 20 mg/mL. The DIG-MAHE and rAK solutions were mixed in a ratio of 1:20 (V/V) and incubated for 2 h at room temperature. The extra DIG-MAHE was washed from the labeled rAK through a 5-mL HiTrap Desalting column (GE Healthcare).

2.8 Determination of binding activity of rVP31 with recombinant Arginine kinase

The binding activity was determined using the binding assay described by Liang et al. (2010) with some modifications. A Costar 96-well EIA/RIA Stripwell Plate was coated with rVP31 (2 μg, 4 μg) and purified WSSV(2 μg, 4 μg) in coating buffer ($NaHCO_3$, 0.293%, Na_2CO_3, 0.159%) at 4°C overnight; 2% skim milk was used as a negative control. After coating, the plate was washed with PBS containing 0.05% Triton X-100 (PBST) and then blocked with 5% skim milk in PBS (MPBS) for 2 h at room temperature. The plate was washed with PBST four times. DIG-MAHE labeled rAK diluted with MPBS and 100 μL (1.2 μg) was loaded into each well. After 2 h of incubation at room temperature and washing four times with PBST, 100 μL Anti-DIG-OPD (1:5 000, Roche) was loaded into each well and incubated at 37°C for 1 h. The wells were developed using the HRP substrate O-phenylenediamine for approximately 10 min. H_2SO_4 (2 mol/L) was used to stop the reaction. The plate was immediately read on a Tecan Safire Reader at 492 nm.

3 RESULT

3.1 Expression and purification of rVP31

The VP31 gene encoding a protein of 261 amino acids with a theoretical molecular mass of 30 kDa was inserted into the pET30a (+) vector and expressed in *E. coli* BL21 (DE3) as a fusion protein. According to SDS-PAGE, VP31 with the 6-His tag was successfully expressed in the induced *E. coli* with the expected molecular mass of approximately 35 kDa in comparison with induced empty plasmid bacteria or non-induced bacteria (Fig.1, lanes 1–3). After purification by Co^{2+} affinity chromatography, the purified rVP31 was confirmed as a single band of approximately 35 kDa by SDS-PAGE (Fig.1, lanes 4–5). The purified rVP31 band was extracted in SDS-12% polyacrylamide gel for mass spectrometry analysis. The results of NCBI Mascot search for MALDI-TOF/TOF MS data showed that rVP31 resembles WSSV envelope protein VP31 (GenBankID: gi|17158442, wsv340, shrimp white spot syndrome virus) with 28 matching peptides. The protein sequence coverage was about 41.8%.

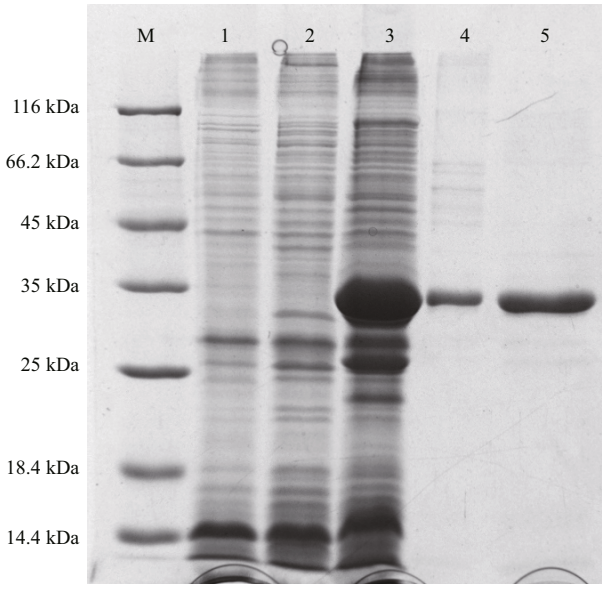

Fig.1 Expression and purification of rVP31

Lane M: protein marker (MBI); lane 1: induced pET30a (+); lane 2: non-induced pET30a (+)-VP31; lane 3: 1 mmol/L IPTG induced pET30a (+)-VP31; lanes 4 and 5: purified rVP31.

3.2 rVP31 binding with shrimp hemocytes

To study the binding activity of rVP31 with shrimp hemocytes, the interaction of FITC labeled rVP31 and hemocytes was observed under a laser confocal microscope. Some of the hemocytes showed significant green fluorescent signals under the laser confocal microscope after incubation with FITC-rVP31 (Fig.2a), while no green fluorescent signal was observed on any of the adhered cells in FITC-BSA group (Fig.2b). This indicated that the rVP31 could bind with shrimp hemocytes.

3.3 Identification of VP31 binding proteins by affinity chromatography

To identify VP31 binding proteins, shrimp gill cellular proteins were tested in affinity chromatography.

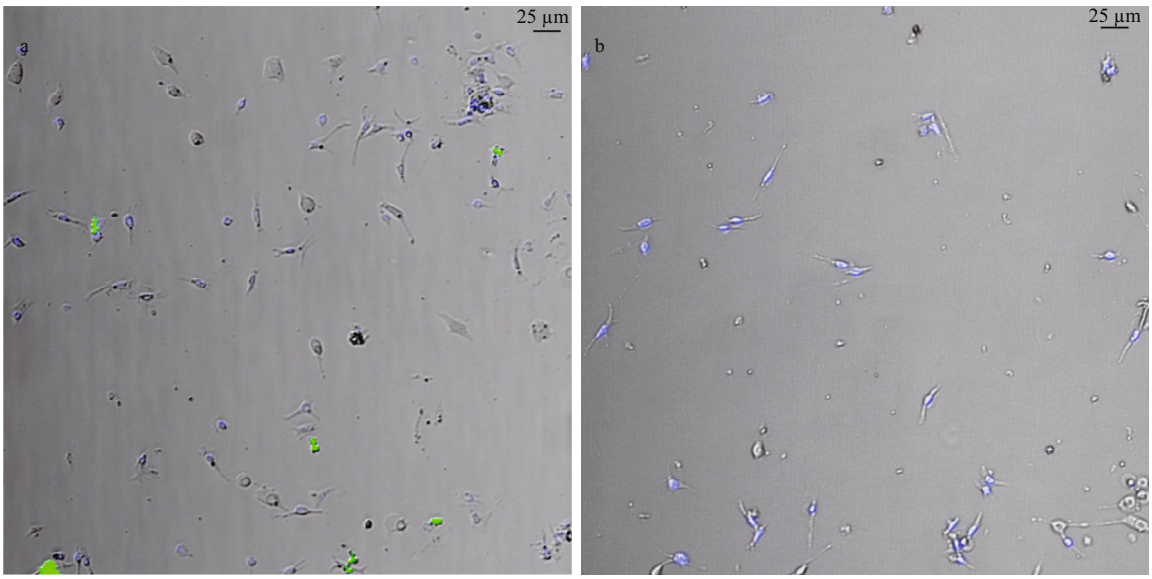

Fig.2 In vitro binding assay between rVP31 and primary cultured shrimp hemocytes

a: rVP31 binding group; b: BSA control group.

Table 1 Peptide mass fingerprint (PMF) search results of three VP31 binding proteins (1, 2, 3) using Mascot Search with NCBInr databases

Rank	Database	Accession name	Mass (Da)	Queries matched	Protein description	Score	Sequence coverage (%)
1	NCBInr	gi|854403	74 934	13	Hemocyanin	179	18.4
2	NCBInr	gi|10304437	41 841	12	Beta-actin	305	33.8
3	NCBInr	gi|56182374	40 104	27	Arginine kinase	359	66.9

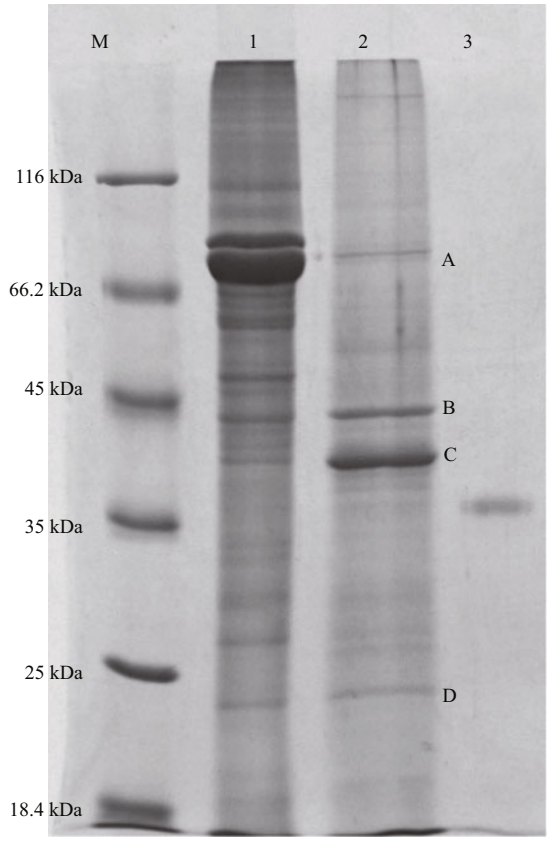

Fig.3 SDS-PAGE analysis of rVP31 binding proteins from shrimp gill cellular proteins

Lane M: protein marker; lane 1: shrimp gill cellular proteins; lane 2: eluted proteins from rVP31; lane 3: the purified rVP31; A: hemocyanin; B: β-actin; C: arginine kinase; D: unknown protein.

The refolded rVP31 was concentrated and coupled to Sepharose 4B, mixed with shrimp gill cellular protein preparation and packed into an affinity column. SDS-PAGE analysis showed that pulled down fractions contained different amounts of more than 10 proteins, among which four bands were obviously noticed (Fig.3). In comparison with the bands shown in the lane containing the shrimp gill cellular proteins, band C was significantly enriched in the eluted fraction of the affinity chromatography. The four main protein bands (A, B, C, and D) were cut off from the gel and analyzed by mass spectrometry. Peptide mass fingerprint (PMF) search results showed that protein band A is hemocyanin (Table 1 Rank 1, Fig.4), protein band B is beta-actin (Table 1 Rank 2, Fig.5), protein band C is arginine kinase (Table 1 Rank 3, Fig.6) and protein band D is an unknown protein. Arginine kinase was about 65% of the total weight of the four eluted proteins by digital image analysis for band quantitation.

3.4 rAK expression, purification, and mass spectrometric analysis

The *F. chinensis* AK gene, encoding a protein of 356 amino acids with a theoretical molecular mass of 40 kDa was inserted into the pET30a (+) vector and expressed in *E. coli* BL21 (DE3) as a fusion protein by 0.1 mmol/L IPTG induction. According to SDS-PAGE, rAK with the 6-His tag was expressed successfully in the induced *E. coli*, with the expected molecular mass of about 46 kDa in comparison with non-induced bacteria (Fig.7a, lanes 1 and 2). After purification by Co^{2+} affinity chromatography, the purified rAK was confirmed as a single band of approximately 46 kDa by SDS-PAGE (Fig.7b, lanes 2 and 3). The purified AK band was extracted from a 12% SDS-PAGE for mass spectrometry analysis. The results of NCBI Mascot search for MALDI-TOF/TOF MS data showed that rAK resembles *F. chinensis* arginine kinase (GenBankID: gi| gi|56182374, arginine kinase [*F. chinensis*]) with 28 matching peptides. The protein sequence coverage was about 39%.

3.5 rAK binding activities to rVP31 and WSSV

A binding assay on a 96-well plate was used to examine the binding activities of rAK to rVP31 and WSSV. The results indicated that both rVP31 and WSSV could bind rAK (P/N>2.1). The rVP31 binding activity with rAK was comparable to that with the same amount of WSSV. The binding assay results also showed that there was no significant difference between the interaction of rAK with 2 μg of rVP31 or with 4 μg of rVP31 (P>0.05) (Fig.8). In general, the binding assay results confirmed our findings from the pull down assay.

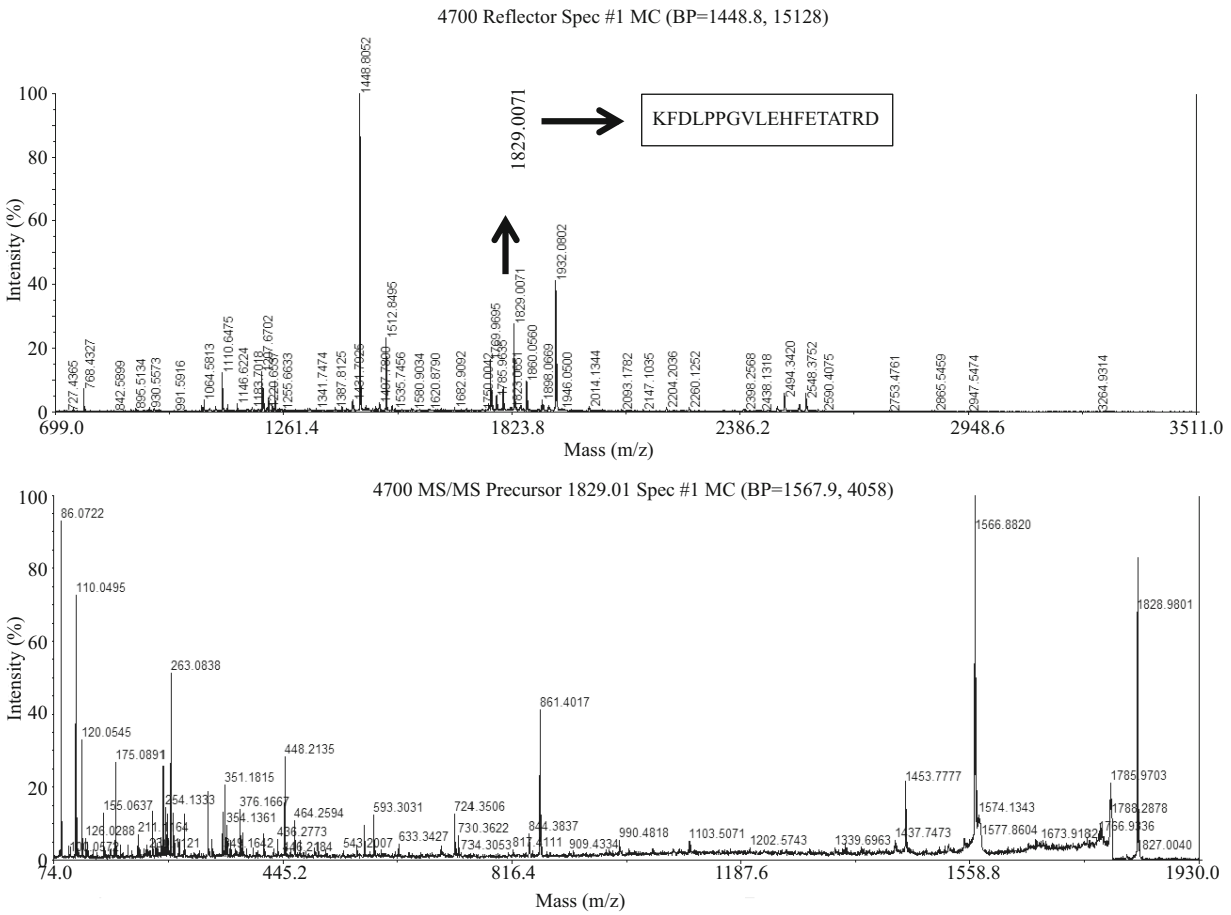

Fig.4 PMF analysis of gel spot (VP31 binding protein band A) in MALDI TOF/TOF and MS/MS analysis on the molecular ion peak of 1 829.0071 in MALDI TOF/TOF

4 DISCUSSION AND CONCLUSION

WSSV is a devastating pathogen causing a huge loss to shrimp aquaculture worldwild. Functional analyses of its genes and encoded proteins have provided a nearly panoramic understanding for its infection mechanism in the recent decade since its whole genome sequencing completed. Studying the interaction between WSSV and its host cells is one way to reveal the function of WSSV proteins during infection. It is widely reported that the RGD motif mediates attachment to host cells and virus infectivity (Mandl et al., 1989; Verdaguer et al., 1995). VP31 has been identified as an envelope protein involved in WSSV infection (Li et al., 2005). It contains an RGD motif, which is required for cell adhesion (Li et al., 2011). These previous research indicated that VP31 might play an important role in the process of WSSV infection. Studies on VP31 might lead to a better understanding of how the virus enters into host cells and starts its life cycle. In this study, we identified four proteins from host shrimp cells that might act as putative receptors of VP31: hemocyanin, β-actin, arginine kinase and an unknown protein. Furthermore, AK was the dominant binding protein within these candidate cellular receptors of VP31.

Recently, researchers have studied the role of AK in the process of recognition and immune response. AK is highly expressed in shrimp, especially in muscle (France et al., 1997; Yao et al., 2009). cDNA or amino acid sequences of crustacean AK and their expressions in various conditions have been reported, including those from *F. chinensis* (Yao et al., 2005), *Homarus gammarus* (Dumas and Camonis, 1993), *Marsupenaeus japonicas* (Furukohri et al., 1994), *P. monodon* (Yu et al., 2003), *Litopenaeus vannamei* (Yao et al., 2009), *Carcinus maenas* (Kotlyar et al., 2000), *Callinectes sapidus* (Kotlyar et al., 2000; Kinsey and Lee, 2003), and *Portunus trituberculatus* (Song et al., 2012). Studies have indicated that AK shows different transcription levels in different tissues after WSSV infection (Wang et al., 2008; Yao et al., 2009; Li et al., 2014; Valentim-Neto et al., 2014). Up or down regulation of AK might depend on the

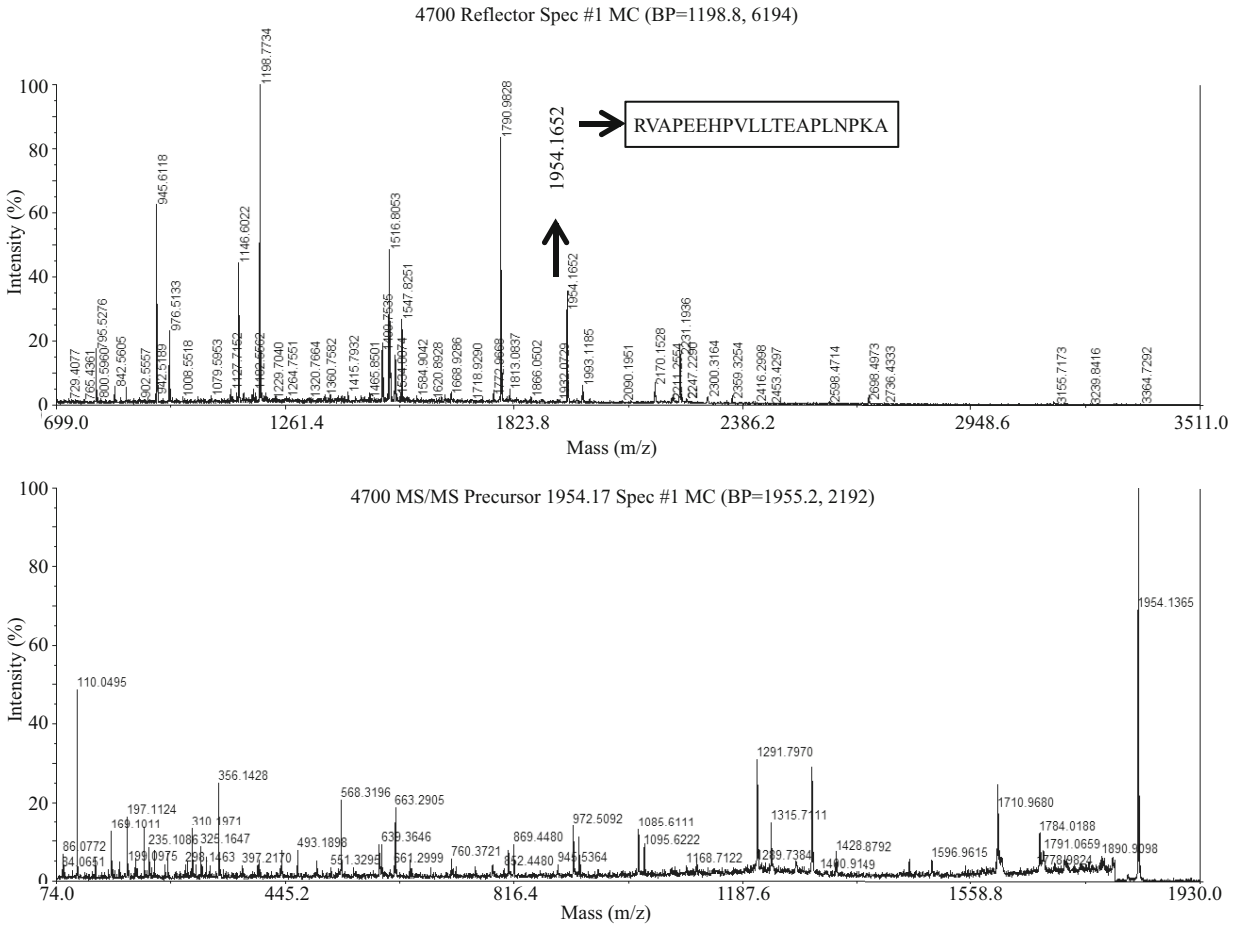

Fig.5 PMF analysis of gel spot (VP31 binding protein band B) in MALDI TOF/TOF and MS/MS analysis on the molecular ion peak of 1 954.165 2 in MALDI TOF/TOF

progress of virus infection. It has been reported that AK could interact with VP14, VP19, VP28, and four other WSSV proteins (Ma et al., 2014; Liu et al., 2014) including VP31. AK was also proven to bind to homologous repeat regions of WSSV genomic DNA (Li et al., 2003). This could explain why the same amount of rVP31 and WSSV had comparable binding activity with rAK as shown in Fig.8, because the WSSV binding activity with rAK involves not only VP31, but also from AK binding proteins and DNA. Previously, we identified the subcellular localization of *L. vannamei* AK (*Lv*AK) as primarily in the cytoplasm of hemocytes (Ma et al., 2014), which is similar to the subcellular localization of AK in *Callinectes sapidus* and *Limulus polyphemus* (Pineda and Ellington, 1998). AK is a member of the phosphatase kinase family. It plays a key role in vertebrates' energy metabolism by catalyzing the reversible phosphorylation of arginine by MgATP to form phosphoarginine and MgADP (Strong and Ellington, 1995). VP31 might be phosphorylated during its interaction with rAK (Liu et al., 2014). Based on the results in this study that AK interacted with VP31, we hypothesized that AK might be involved in the host defense against WSSV infection during the viral replication process.

Hemocyanins are extracellular giant copper containing proteins present in mollusks and arthropods hemolymph (Terwilliger, 1998). Hemocyanins have phenoloxidase and antimicrobial activities (Decker and Jaenicke, 2004), and are considered as important immune molecules in mollusks and arthropods. Hemocyanins also has been proved to have non-specific antiviral properties (Zhang et al., 2004). We performed a binding assay that demonstrated that hemocyanin could bind WSSV virions and VP37, and the binding activity with WSSV was stronger than with VP37 (Liu et al., 2008). This suggested that there are other WSSV envelope proteins that interact with hemocyanin. The present findings indicated that VP31 might be one of these proteins interacting with hemocyanin.

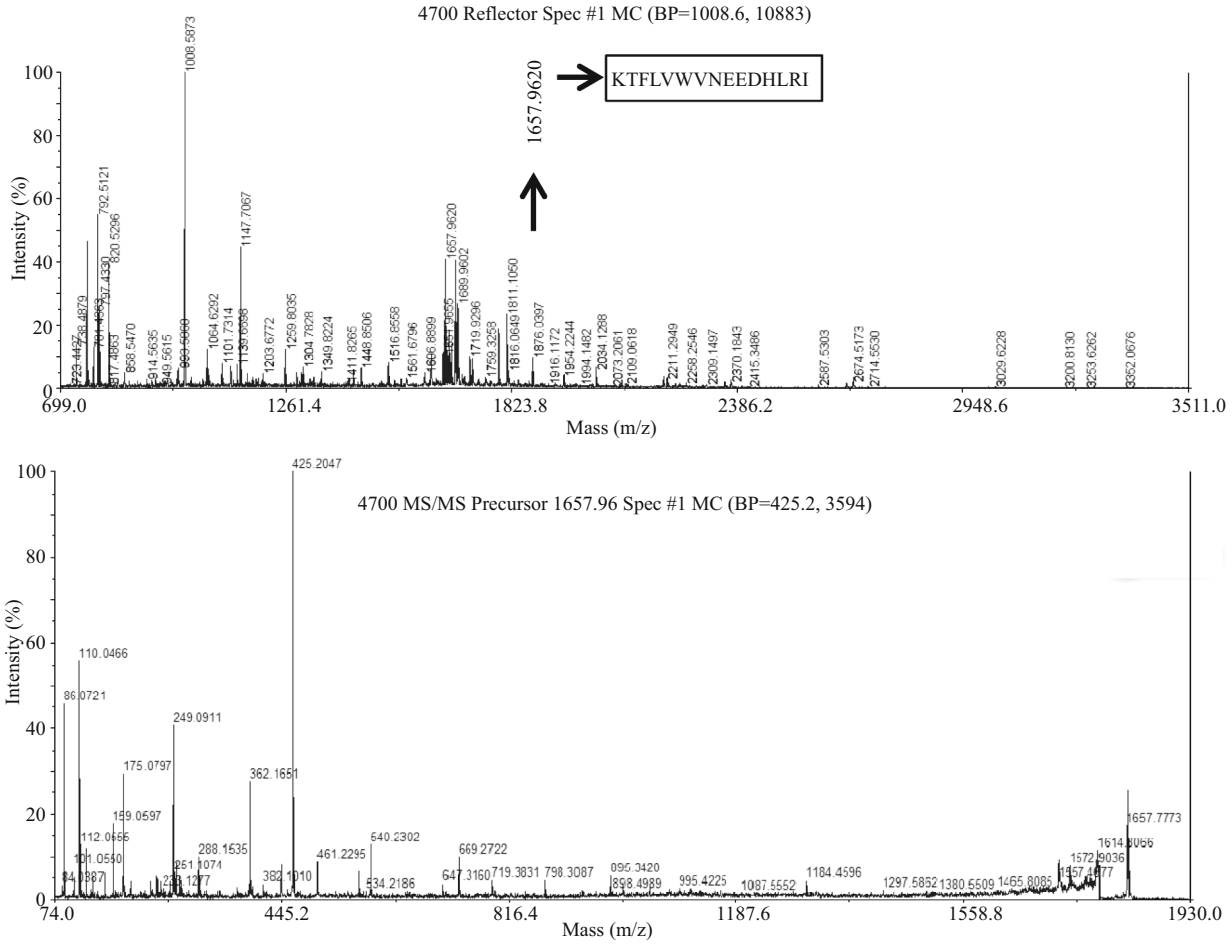

Fig.6 PMF analysis of gel spot (VP31 binding protein band C) in MALDI TOF/TOF and MS/MS analysis on the molecular ion peak of 1 657.962 0 in MALDI TOF/TOF

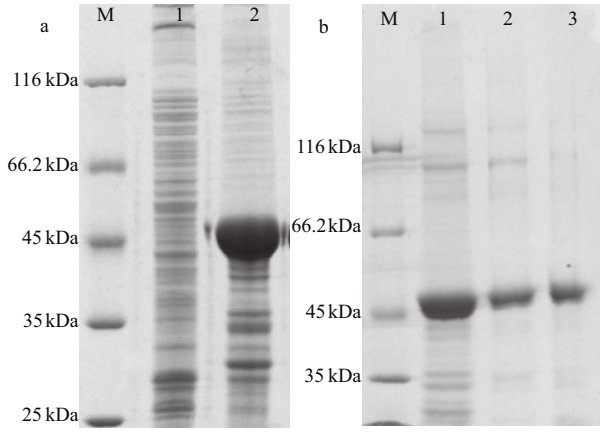

Fig.7 SDS-PAGE analysis of rAK expression and purification

a: lane M: protein marker; lane 1: non-induced pET30a (+)-AK; lane 2: 0.1 mmol/L IPTG induced pET30a (+)-AK; b: lane M: protein marker; lane 1: 0.1 mmol/L IPTG induced pET30a (+)-AK; lanes 2 and 3: purified rAK.

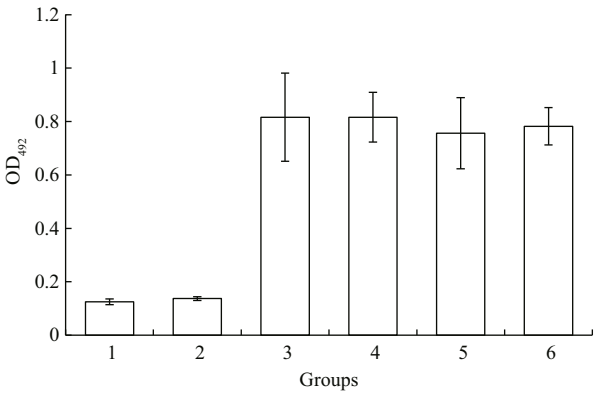

Fig.8 ELISA results of the interaction of rAK with rVP31 and WSSV

Group 1: coated with coating buffer; group 2: coated with 2% skim milk; group 3: coated with 2 μg rVP31; group 4: coated with 4 μg rVP31; group 5: coated with 2 μg WSSV; group 6: coated with 4 μg WSSV. Data are shown as the mean±SEM of three individual value.

The pull down assay also showed that VP31 could bind to shrimp β-actin. Studies have indicated that cytoskeleton components, especially actin filaments, play an important role in virus transportation in host cells. VP26 of WSSV was found to interact with actin by affinity-chromatography and confirmed by co-immunoprecipitation (Xie and Yang, 2005). To enter

host cells, viruses usually exploit the cytoskeleton and cellular motor proteins (Smith and Helenius, 2004). For example, there is evidences that actin cables may be involved in the transport of baculoviral nucleocapsids into the host nucleus (Lanier and Volkman, 1998). Similar evidence was found in HIV-1 (Liu et al., 1999). Here, we speculated that the interaction between VP31 and actin may occur in early time of WSSV infection and VP31 might help the virus nucleocapsid to move towards the cell nucleus taking advantage of the cytoskeleton.

Cell surface binding assays are used to visualize the binding activity between a virus and its host cells in vitro (Liang et al., 2003; Ma et al., 2014). FITC-labeled VP28 and VP37 showed strong and pervasive binding activity on the adhesive hemocytes (Xu et al., 2009; Liu et al., 2009). The identified binding proteins of WSSV-VP28 and WSSV-VP37, such as *Pm*Rab7 and F_1ATP synthase beta subunit, were proven to be present on the shrimp cell surface (Sritunyalucksana et al., 2006; Liang et al., 2010). In this study, the binding of FITC-labeled VP31 on the hemocyte culture showed a different pattern, only binding to a small portion of cells in the shrimp hemolymph. This suggested that the binding protein of VP31 might not be distributed on the surface of most hemocytes. Two binding proteins of VP31, AK and β-actin, with the most significant binding activities in the affinity chromatography assay are intracellular proteins (Smith and Helenius, 2004; Ma et al., 2014), while another known binding protein, hemocyanin, is a secretory protein that is distributed inside hemolymph of shrimp (Terwilliger, 1998). It remains unknown whether the other VP31 binding proteins are cellular membrane proteins distributed on a small portion of the cells from shrimp hemolymph. Hemocytes function in non-self-recognition and phagocytosis in crustacean immune system (Cerenius et al., 2010). It was reported that WSSV could enter circulating hemocytes of shrimp, but no WSSV genome replication or structural protein expression was observed in the hemocytes; conversely, hematopoietic cells support WSSV replication (Wu et al., 2015). There is a possibility that only cells newly released from hematopoietic tissue represent the small portion of cells in the shrimp circulation system that support WSSV infection, on which the unknown VP31 binding protein may be a specific membrane receptor. The unknown protein will be an interesting subject for further study. The findings on the new membrane protein will extend our knowledge of the interaction between WSSV and host cells.

References

Cerenius L, Jiravanichpaisal P, Liu H P, Soderhall I. 2010. Crustacean immunity. *In*: Söderhäll K ed. Invertebrate Immunity: Advances in Experimental Medicine and Biology. Springer, US. **708**: 239-259.

Chai C Y, Yoon J, Lee Y S, Kim Y B, Choi T J. 2013. Analysis of the complete nucleotide sequence of a white spot syndrome virus isolated from Pacific white shrimp. *J. Microbiol*., **51**(5): 695-699.

Chen L L, Leu J H, Huang C J, Chou C M, Chen S M, Wang C H, Lo C F, Kou G H. 2002. Identification of a nucleocapsid protein (VP35) gene of shrimp white spot syndrome virus and characterization of the motif important for targeting VP35 to the nuclei of transfected insect cells. *Virology*, **293**(1): 44-53.

Decker H, Jaenicke E. 2004. Recent findings on phenoloxidase activity and antimicrobial activity of hemocyanins. *Dev. Comp. Immunol*., **28**(7-8): 673-687.

Dumas C, Camonis J. 1993. Cloning and sequence analysis of the cDNA for arginine kinase of lobster muscle. *J. Biol. Chem*., **268**(29): 21 599-21 605.

France R M, Sellers D S, Grossman S H. 1997. Purification, characterization, and hydrodynamic properties of arginine kinase from Gulf shrimp (*Penaeus aztecus*). *Arch. Biochem. Biophys*., **345**(1): 73-78.

Furukohri T, Okamoto S, Suzuki T. 1994. Evolution of phosphagen kinase (III). Amino acid sequence of arginine kinase from the shrimp *Penaeus japonicus*. *Zoolog. Sci*., **11**(2): 229-234.

Huang J, Song X L, Yu J, Yang C H. 1995. Baculoviral hypodermal and hematopoietic necrosis-study on the pathogen and pathology of the explosive epidemic disease of shrimp. *Marine Fisheries Research*, **16**(1): 1-10 (in Chinese with English abstract)

Kinsey S T, Lee B C. 2003. The effects of rapid salinity change on *in vivo* arginine kinase flux in the juvenile blue crab, *Callinectes sapidus*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol*., **135**(3): 521-531.

Kotlyar S, Weihrauch D, Paulsen R S, Towle D W. 2000. Expression of arginine kinase enzymatic activity and mRNA in gills of the euryhaline crabs *Carcinus maenas* and *Callinectes sapidus*. *J. Exp. Biol*., **203**(Pt 16): 2 395-2 404.

Lanier L M, Volkman L E. 1998. Actin binding and nucleation by *Autographa california* M nucleopolyhedrovirus. *Virology*, **243**(1): 167-177.

Li L, Lin Z Y, Xu L X, Yang F. 2011. The RGD motif in VP31 of white spot syndrome virus is involved in cell adhesion. *Arch. Virol*., **156**(8): 1 317-1 321.

Li L, Xie X X, Yang F. 2005. Identification and characterization of a prawn white spot syndrome virus gene that encodes an envelope protein VP31. *Virology*, **340**: 125-132.

Li Q, Yang F, Zhang J H, Chen Y J. 2003. Proteomic analysis of proteins that binds specifically to the homologous repeat regions of white spot syndrome virus. *Biol. Pharm. Bull*., **26**(11): 1 517-1 522.

Li W, Tang X Q, Xing J, Sheng X Z, Zhan W B. 2014. Proteomic analysis of differentially expressed proteins in *Fenneropenaeus chinensis* hemocytes upon white spot syndrome virus infection. *PLoS One*, **9**(2): e89962.

Liang Y, Cheng J J, Yang B, Huang J. 2010. The role of F_1 ATP synthase beta subunit in WSSV infection in the shrimp, *Litopenaeus vannamei*. *Virol*. *J*., **7**(1): 144.

Liang Y, Huang J, Song X L, Zhang P J, Xu H S. 2005. Four viral proteins of white spot syndrome virus (WSSV) that attach to shrimp cell membranes. *Dis*. *Aquat*. *Organ*., **66**: 81-85.

Liang Y, Song X L, Huang J, Zhang P J. 2003. DIG labeled WSSV and observation of binding phenomenon between WSSV and it's host cell. *Oceanologia et Limnologia Sinica*, (S): 1-9. (in Chinese with English abstract)

Lightner D V. 1996. A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp. World Aquaculture Society, Tucson, Arizona, U.S.A.

Liu B D, Dai R K, Tian C J, Dawson L, Gorelick R, Yu X F. 1999. Interaction of the human immunodeficiency virus type 1 nucleocapsid with actin. *J. Virol*., **73**(4): 2 901-2 908.

Liu C, Zhao P, Liang Y, Gao Q, Liu S P, Huang J, 2014. Preliminary study on the conjunction between arginine kinase of *Fenneropenaeus chinensis* and the structure proteins of white spot syndrome virus (WSSV). *Marine Sciences*, **38**(3): 104-110 (in Chinese with English abstract)

Liu Q H, Zhang X L, Ma C Y, Liang Y, Huang J. 2009. VP37 of white spot syndrome virus interact with shrimp cells. *Lett*. *Appl*. *Microbiol*., **48**(1): 44-50.

Ma F F, Liu Q H, Guan G K, Li C, Huang J. 2014. Arginine kinase of *Litopenaeus vannamei* involved in white spot syndrome virus infection. *Gene*, **539**(1): 99-106.

Mandl C W, Guirakhoo F, Holzmann H, Heinz F X, Kunz C. 1989. Antigenic structure of the flavivirus envelope protein E at the molecular level, using tick-borne encephalitis virus as a model. *J. Virol*., **63**(2): 564-571.

OIE. 2012. Manual of Diagnostic Tests for Aquatic Animals. Chapter 2.2.6 White Spot Disease. World Organisation of Aquatic Animal Health.

Pierschbacher M D, Ruoslahti E. 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature*, **309**(5963): 30-33.

Pineda Jr A, Ellington W R. 1998. Immunogold transmission electron microscopic localization of arginine kinase in Arthropod mitochondria. *J. Exp*. *Zool*., **281**(2): 73-79.

Sánchez-Paz A. 2010. White spot syndrome virus: an overview on an emergent concern. *Vet*. *Res*., **41**(6): 43.

Smith A E, Helenius A. 2004. How viruses enter animal cells. *Science*, **304**(5668): 237-242.

Song C W, Cui Z X, Liu Y, Li Q Q, Wang S Y. 2012. Cloning and expression of arginine kinase from a swimming crab, *Portunus trituberculatus*. *Mol*. *Biol*. *Rep*., **39**(4): 4 879-4 888.

Sritunyalucksana K, Wannapapho W, Lo C F, Flegel T W. 2006. PmRab7 is a VP28-binding protein involved in white spot syndrome virus infection in shrimp. *J. Virol*., **80**(21): 10 734-10 742.

Strong S J, Ellington W R. 1995. Isolation and sequence analysis of the gene for arginine kinase from the chelicerate arthropod, *Limulus polyphemus*: insights into catalytically important residues. *Biochim*. *Biophys*. *Acta*, **1246**(2): 197-200.

Terwilliger N B. 1998. Functional adaptations of oxygen-transport proteins. *J. Exp*. *Biol*., **201**(Pt 8): 1 085-1 098.

Tsai J M, Wang H C, Leu J H, Hsiao H H, Wang A H J, Kou G H, Lo C F. 2004. Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. *J. Virol*., **78**(20): 11 360-11 370.

Valentim-Neto P A, Fraga A P M, Marques M R F. 2014. Differential expression of proteins in the gills of *Litopenaeus vannamei* infected with white spot syndrome virus. *Aquacult*. *Int*., **22**(5): 1 605-1 620.

van Hulten M C W, Witteveldt J, Peters S, Kloosterboer N, Tarchini R, Fiers M, Sandbrink H, Lankhorst R K, Vlak J M. 2001. The white spot syndrome virus DNA genome sequence. *Virology*, **286**(1): 7-22.

Verdaguer N, Mateu M G, Andreu D, Giralt E, Domingo E, Fita I. 1995. Structure of the major antigenic loop of foot-and-mouth disease virus complexed with a neutralizing antibody: direct involvement of the Arg-Gly-Asp motif in the interaction. *EMBO J*., **14**(8): 1 690-1 696.

Wang B, Li F H, Luan W, Xie Y S, Zhang C S, Luo Z, Gui L, Yan H, Xiang J X. 2008. Comparison of gene expression profiles of *Fenneropenaeus chinensis* challenged with WSSV and *Vibrio*. *Mar*. *Biotechnol*., **10**(6): 664-675.

Wu J J, Li F, Huang J J, Xu L M, Yang F. 2015. Crayfish hematopoietic tissue cells but not hemocytes are permissive for white spot syndrome virus replication. *Fish Shellfish Immunol*., **43**(1): 67-74.

Xie X X, Yang F. 2005. Interaction of white spot syndrome virus VP26 protein with actin. *Virology*, **336**(1): 93-99.

Xu H, Yan F, Deng X B, Wang J C, Zou T T, Ma X, Zhang X, Qi Y P. 2009. The interaction of white spot syndrome virus envelope protein VP28 with shrimp Hsc70 is specific and ATP-dependent. *Fish Shellfish Immunol*., **26**(3): 414-421.

Yang F, He J, Lin X H, Li Q, Pan D, Zhang X B, Xu X. 2001. Complete genome sequence of the shrimp white spot bacilliform virus. *J. Virol*., **75**(23): 11 811-11 820.

Yao C L, Ji P F, Kong P, Wang Z Y, Xiang J H. 2009. Arginine kinase from *Litopenaeus vannamei*: cloning, expression and catalytic properties. *Fish Shellfish Immunol*., **26**(3): 553-558.

Yao C L, Wu C G, Xiang J H, Dong B. 2005. Molecular cloning and response to laminarin stimulation of arginine kinase in haemolymph in Chinese shrimp, *Fenneropenaeus chinensis*. *Fish Shellfish Immunol*., **19**(4): 317-329.

Yu C J, Lin Y F, Chiang B L, Chow L P. 2003. Proteomics and immunological analysis of a novel shrimp allergen, Pen m 2. *J Immunol*., **170**(1): 445-453.

Zhang X B, Huang C H, Qin Q W. 2004. Antiviral properties of hemocyanin isolated from shrimp *Penaeus monodon*. *Antiviral Res*., **61**(2): 93-99.