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# Arginine kinase of *Litopenaeus vannamei* involved in white spot syndrome virus infection

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ABSTRACT

Virus–host interaction is important for virus infection. White spot syndrome virus VP14 contains transmembrane and signal peptides domain, which is considered to be important for virus infection. Until now, the function of this protein remains undefined. In this study, we explored the interaction of VP14 with host cell. A new shrimp protein (arginine kinase of *Litopenaeus vannamei*, LvAK) is selected and its localization in shrimp cells is also confirmed. Cellular localization of LvAK protein in shrimp hemocytes showed that LvAK was primarily located at the periphery of hemocytes and was scarcely detectable in the nucleus. Tissue distribution indicated that arginine kinase gene was spread commonly in the tissues and was highly present in shrimp muscle tissue. The expression of LvAK mRNA in muscle was significantly up-regulated after WSSV stimulation. Indirect immunofluorescence assay showed that LvAK interacted with VP14 in WSSV-infected shrimp. Injection of LvAK protein enhanced the mortality of shrimp infected with white spot syndrome virus (WSSV). These results showed that LvAK is involved in WSSV infection. Future research on this topic will help to reveal the molecular mechanism of WSSV infection.


## 1. Introduction

White spot syndrome virus (WSSV) is a major shrimp pathogen that causes shrimp death with a mortality rate of 90%–100% (Chou et al., 1995; Wongteerasupaya et al., 1995; Zhan et al., 1998). WSSV is an enveloped ellipsoid virus, which belongs to the genus Whispovirus of the Nimaviridae family (Vlak et al., 2005). So far, the genome from three different WSSV isolates has been sequenced (Chen et al., 2002; van Hulten et al., 2001; Yang et al., 2001). Sequence analysis showed that WSSV contains approximately 500 putative open reading frames (ORFs), most of which have no homology with any known genes or proteins in public databases. Till now, more than 50 structural and non-structural proteins were identified (Li et al., 2007; Tsai et al., 2006; Xie et al., 2006; Zhang et al., 2004).

Viral structural proteins play key functions in viral assembly, infection, cell adhesion and recognition. Virus–host interaction is important for virus infection. Understanding the interaction between host and pathogen will be helpful in controlling the infectious diseases in shrimp. A number of WSSV binding proteins have been reported to bind to viral particles or viral protein components (Sritunyalucksana et al., 2012). However, there is still only limited data on how WSSV interacts with its hosts, and the pathogenesis of the disease is not completely understood.

The VP14 is one of the newly identified envelope proteins in WSSV genome encoded by ORF wsv349 (Li et al., 2007). The open reading frame (wsv349) contains 273 bp, presumably encodes a protein of 91 aa, with an apparent molecular weight of about 14 kDa and is therefore referred to as VP14 in this study. VP14 contains transmembrane and signal peptides domain which is considered to be important for virus infection (Xie et al., 2006). Until now, the function of this protein remains undefined. In this study, we explored the interaction of VP14 with host cell. A new shrimp protein (arginine kinase of *Litopenaeus vannamei*, LvAK) is selected and its localization in shrimp cells is also confirmed. Neutralization assay indicated that LvAK increased WSSV infection in the early stage. Far-western blot and ELISA result confirmed the multi-interaction of LvAK with envelope protein of WSSV. These results collectively showed that LvAK is involved in WSSV infection. Future research on this topic will help to reveal the molecular mechanism of WSSV infection.

## 2. Materials and methods

### 2.1. Expression of VP14

The sequence of vp14 was amplified using the specific forward primer (5′-CGCTCGAGATGGATTCAAATGATTC-3′) and reverse primer (5′-CTAAGCTTCCCTGGTGGAATTGTTCT-3′). The amplified DNA and plasmid vector pBAD/gIIIA were digested with *Xho*I and *Hin*dIII. After

*Abbreviations:* WSSV, White spot syndrome virus; LvAK, Arginine kinase of *Litopenaeus vannamei*; ORFs, Open reading frames; aa, Amino acid; PBS, Phosphate-buffered saline; FITC, Fluorescein isothiocyanate; GMPs, Gill membrane proteins; TBS, Tris-buffered saline; DIG, Digoxigenin; AP, Alkaline phosphatase; NBT-BCIP, Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate; DAPI, 4′-6′-Diamidino-2-phenylindole dihydrochloride; SD, Standard deviation.

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purification, the vp14 gene was ligated into the vector. The recombinant plasmid was confirmed by double enzyme digestion (*Xho*I and *Hind*III) and DNA sequencing and transformed into *Escherichia coli* Top10 competent cells. The expression of VP14 was assayed by SDS–PAGE and confirmed by western blot with mouse anti-(His)×6-antibodies (Clontech). Purification of the VP14 was performed using $Co^{2+}$ beads according to the recommended protocol.

### 2.2. Recombinant expression of AK in E. coli

Total RNAs were extracted from *L. vannamei* using Trizol regent (Life Technologies, USA). cDNAs were prepared from total RNAs by reverse transcription kit (TaKara, Japan) according to the instructions. The LvAK was cloned by RT-PCR using forward primer (5′-TACTCGAGAT GGCTGACGCTGCTGT-3′) and reverse primer (5′-GCTCTAGAGGCATCTC CTTCTCAATCT-3′) and ligated with His-tag vector pBAD/gIIIA. Recombinant LvAK was confirmed by double enzyme digestion and sequence analysis. The correct rLvAK was expressed in *E. coli* Top10. The rLvAK was confirmed by western blot analyses with mouse anti-(His)×6-antibodies and purified by affinity chromotograph as described above.

### 2.3. Antibody preparation

To raise the specific antibody against VP14 and LvAK, VP14 and LvAK were used as antigen to immunize mice and rabbit, respectively. The titers of the antisera were determined by ELISA assay, and pre-immune serum was used as control. The titers of mouse anti-VP14 antibody and rabbit anti-LvAK antibody were approximately 1:20000, respectively.

### 2.4. VP14 binding with shrimp hemocytes

The hemolymph was withdrawn from the shrimp heart with a needle on a 2-ml syringe containing 1 ml anticoagulant (3.8% sodium citrate). It was then centrifuged at 600 ×*g* for 10 min to separate cells and overlayed with 12-well plates. After the monolayer had formed, the cells were washed by phosphate-buffered saline (PBS) and incubated with purified VP14 protein. After incubation and washing, mouse anti-VP14 antibody was added. Following incubation and washing, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Beyotime, China) was added, and the nucleus was stained with 4′-6′-diamidino-2-phenylindole dihydrochloride (DAPI). The fluorescent signals were examined using Nikon microscopy. Pre-immune serum from mouse was used as a negative control.

### 2.5. Identification of VP14-binding proteins

The shrimp gill membrane proteins (GMPs) (Liu et al., 2009) were separated by SDS–PAGE, and then the proteins were electroblotted onto PVDF membrane. The membrane was blocked with Tris-buffered saline (TBS) (20 mM Tris–HCl [pH 7.5], 0.15 M NaCl) containing 5% BSA at 4 °C overnight. After being rinsed with TBS containing 0.5% Tween 20, the membrane was incubated with DIG-labeled rVP14 (10 μg) for 1 h at room temperature on a rocking platform. The membrane was washed three times with TBS containing 0.5% Tween 20. Then anti-DIG antibody conjugated with alkaline phosphatase (AP) was added. After rinsing with TBS, the interaction proteins were detected by exposing the membrane to nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) substrates. DIG-labeled BSA was used as a negative control. The positive protein spot was excised from gels and identified by mass spectrometry analysis.

### 2.6. Far-western overlay and ELISA assay

To identify LvAK interacted with VP14, a far-western overlay assay was performed. VP14 and VP51 (recombinant WSSV-VP51 constructed previously as control) were subjected to SDS–PAGE and transferred to PVDF membranes, blocked with 5% nonfat milk in TBST for 1 h at room temperature. After washing with TBST, the membranes were incubated with DIG-labeled LvAK (10 μg in TBST) for 1 h at room temperature. Then anti-DIG antibody conjugated with AP was added. The interaction proteins were detected by exposing the membrane to NBT-BCIP substrates. For ELISA assay, purified rVP14 was coated the 96-well microtiter plates (1 μg/well) overnight at 4 °C with the binding buffer. The plates were washed three times and blocked with blocking buffer containing 2% BSA in PBS-T for 2 h at 37 °C. After washing, the plates were incubated with various amounts of rLvAK (0, 0.1, 1.0 and 2.0 μg/well) for 1 h. Then rabbit anti-LvAK serum was added followed by incubation with peroxidase-conjugated secondary antibody (goat anti-rabbit immunoglobulin G antibody, 1:200 dilution in PBS-T; Beyotime, China) for 2 h. After the plates were rinsed with PBS-T, the bound peroxidase activity was determined by a reaction with 3,5′,5,5′-tetramethylbenzidine (TMB), and the optical density(OD) value was measured by spectrophotometry, reading at 450 nm. All ELISAs were performed in triplicate, with the data given in mean values. Wells coated with bovine serum albumin (BSA) were used as negative controls. $P/N = (OD - OD_{blank}) / (OD_{BSA} - OD_{blank})$. A value of *P/N* greater than 2 indicated significant difference.

### 2.7. Localization of LvAK in shrimp hemocytes

Healthy shrimps were swabbed with 75% ethanol, and hemolymph was withdrawn from the shrimp heart with a needle on a 2-ml syringe containing 1 ml anticoagulant (3.8% sodium citrate). It was then centrifuged at 600 ×*g* for 10 min to separate cells that were then resuspended in 2xL-15 culture medium. After the monolayer had formed, the cells were washed by phosphate-buffered saline (PBS) and incubated with purified polyclonal antiserum against LvAK (diluted with 3% BSA, 1:10000) for 2 h at 37 °C. Cells were washed three times with PBS and then incubated with the secondary antibody, FITC-conjugated goat anti-rabbit IgGs (Beyotime, China), for 1 h at 37 °C. Background staining was removed by washing with PBS three times. After staining the nuclear DNA with DAPI for 10 min, the cells were examined under a confocal laser scanning microscope. Pre-immune serum from rabbit was as negative control.

### 2.8. Tissue distribution of LvAK

Real-time RT-PCR was performed to investigate the tissue distribution of LvAK mRNA in *L. vannamei*. Briefly, total RNAs were extracted from different tissues (hemocytes, gills, muscle, hepatopancreas, lymphoid and intestine) using TRIzol Reagent (Invitrogen). After treatment with DNase I, first-strand cDNA was synthesized using kit (TaKara, Japan). Such cDNA was then subjected to PCR amplification using SYBR Green I real-time PCR Master Mix (TaKara) with LvAK specific primer (forward primer, 5′-CAAGGACTTCGGTGATGTGA-3′; reverse primer, 5′-AGGCTAGAAAGGGTAGAGGAAA-3′). β-Actin (forward primer, 5′-GCCCATCTACGAGGGATA-3′; reverse primer, 5′-GGTGGTCGTGAA GGTGTAG -3′) was used as an internal control.

The real-time RT-PCR was carried in a Rotor Gene 3000. The amplifications were carried out in total volumes of 20 μl containing 10 μl 2× SYBR Premix Ex Taq (Takara, China) according to the manufacturer's instructions. PCR amplification was performed in triplicate wells, with the following program: 95 °C for 30 s, followed by 35 cycles of 95 °C for 5 s, 55 °C for 20 s, and 72 °C for 15 s. Melting curve analysis of amplification products was performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected. Data were analyzed with Rotor Gene software.

To maintain consistency, the baseline was set automatically by the software. Quantitative data were expressed as the fold-change in expression, relative to the β-actin gene, by the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001) and given as means ± SD (standard deviation).