White Spot Syndrome Virus infection in *Penaeus monodon* is facilitated by housekeeping molecules

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White Spot Syndrome Virus (WSSV) is a major pathogen in shrimp aquaculture, and its rampant spread has resulted in great economic loss. Identification of host cellular proteins interacting with WSSV will help in unravelling the repertoire of host proteins involved in WSSV infection. In this study, we have employed one-dimensional and two-dimension virus overlay protein binding assay (VOPBA) followed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) to identify the host proteins of *Penaeus monodon* that could interact with WSSV. The VOPBA results suggest that WSSV interacted with housekeeping proteins such as heat shock protein 70, ATP synthase subunit β, phosphopyruvate hydratase, allergen Pen m 2, glyceraldehyde-3-phosphate dehydrogenase, sarcoplasmic calcium-binding protein, actin and 14-3-3-like protein. Our findings suggest that WSSV exploits an array of housekeeping proteins for its transmission and propagation in *P. monodon*.

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1. Introduction

Penaeus monodon is the commercially important marine shrimp species. In recent years production of *P. monodon* has been increasingly hampered by white spot syndrome disease caused by White Spot Syndrome Virus (WSSV) (Chou *et al.* 1995; Flegel 1997; Sahul Hameed *et al.* 1998; Escobedo-Bonilla *et al.* 2008). WSSV is one of the most devastating shrimp pathogen in cultured shrimp. WSSV infection can reach a cumulative mortality of up to 100% within 3–10 days (Lightner 1996). Clinical symptoms of WSSV include white spots on the inner side of the carapace, cuticle over abdomen, display signs of lethargy and reddish coloration of the hepatopancreas (Takahashi *et al.* 1994; Chou *et al.* 1995). Due to its serious impact on shrimp farming, there is an urgent need to understand virus–host interactions and unveil the underlying mechanisms involved in WSSV entry and pathogenesis in shrimp. Although considerable progress has been made in characterizing the virus, information on the cellular partners of WSSV in shrimp cells is limited.

The identification of virus interacting cellular factors is essential to understand how the virus exploits the cellular machinery and blocks the antiviral response to achieve efficient replication. This is especially important for a disease like WSSV with unprecedented mortality in cultured shrimps. So far, host cellular receptors of CHIKV have not been well documented. In early infection, subcuticular epithelial, stomach, gills and the connective tissue of the hepatopancreas are reported to be WSSV positive sites (Chang *et al.* 1996; Lightner 1996; Lo *et al.* 1996, 1997; Wang *et al.* 1999; Wu *et al.* 2007). To identify the cellular

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partners of WSSV in *P. monodon*, we have screened the WSSV interacting proteins from whole cell, membrane and cytosolic proteins from the subcuticular epithelial tissue of *P. monodon* using direct protein–protein interaction approach. Using a proteomics approach, based on virus overlay protein binding assay (VOPBA) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), we have identified proteins that interact with WSSV. The implication of these findings in understanding the biology of WSSV infection is discussed further.

2. Material and methods

2.1 *Shrimp collection*

Shrimps, *P. monodon* (20 to 25 g of body weight), were collected from the commercial shrimp culture farms near Thane, India. They were acclimatized in an aquarium at ambient temperature of 27°C to 29°C under controlled salinity of 20 to 25 ppt using water from the culture ponds. The animals were kept on artificial pellet feed. Shrimp were screened for the presence of WSSV by performing polymerase chain reaction (PCR) using WSSV detection kit (GeNei Merck, India) as per the manufacture's instructions.

2.2 *Preparation of viral inoculum*

WSSV-infected tissue (1 g) from *Penaeus monodon* was homogenized in 10 mL TNE buffer (20 mM Tris–HCl, 400 mM NaCl, 5 mM EDTA pH 7.5). After centrifugation at 5000 × g for 10 min, the supernatant was filtered (0.22 μm filter) and injected intramuscularly to shrimps using sterile syringe into healthy *P. monodon*. About 4–5 days later, dead *P. monodon* were collected and stored at −80°C until required for further experimentation. WSSV purification was carried as described by Xie *et al.* (2005). Briefly, 1 g of infected tissues were homogenized in 50 mL TNE buffer (50 mM Tris–HCl, 400 mM NaCl, 5 mM EDTA, pH 8.5) containing a protease inhibitor cocktail, and then centrifuged at 3500*g* for 5 min at 4°C. After filtering through nylon net (400 mesh), the supernatant was centrifuged at 30,000*g* for 30 min at 4°C. Then the upper loose pellet was rinsed out carefully, and the lower white pellet was suspended in 10 mL TM buffer (50 mM Tris–HCl, 10 mM MgCl2, pH 7.5). After centrifugation at 3500*g* for 5 min, the virus particles were sedimented by centrifugation at 30,000*g* for 30 min at 4°C, and then resuspended and kept in 1 mL TM buffer. In this experiment, tissues of uninfected *P. monodon* were used as control, but no white pellet could be obtained.

2.3 *Protein sample preparation*

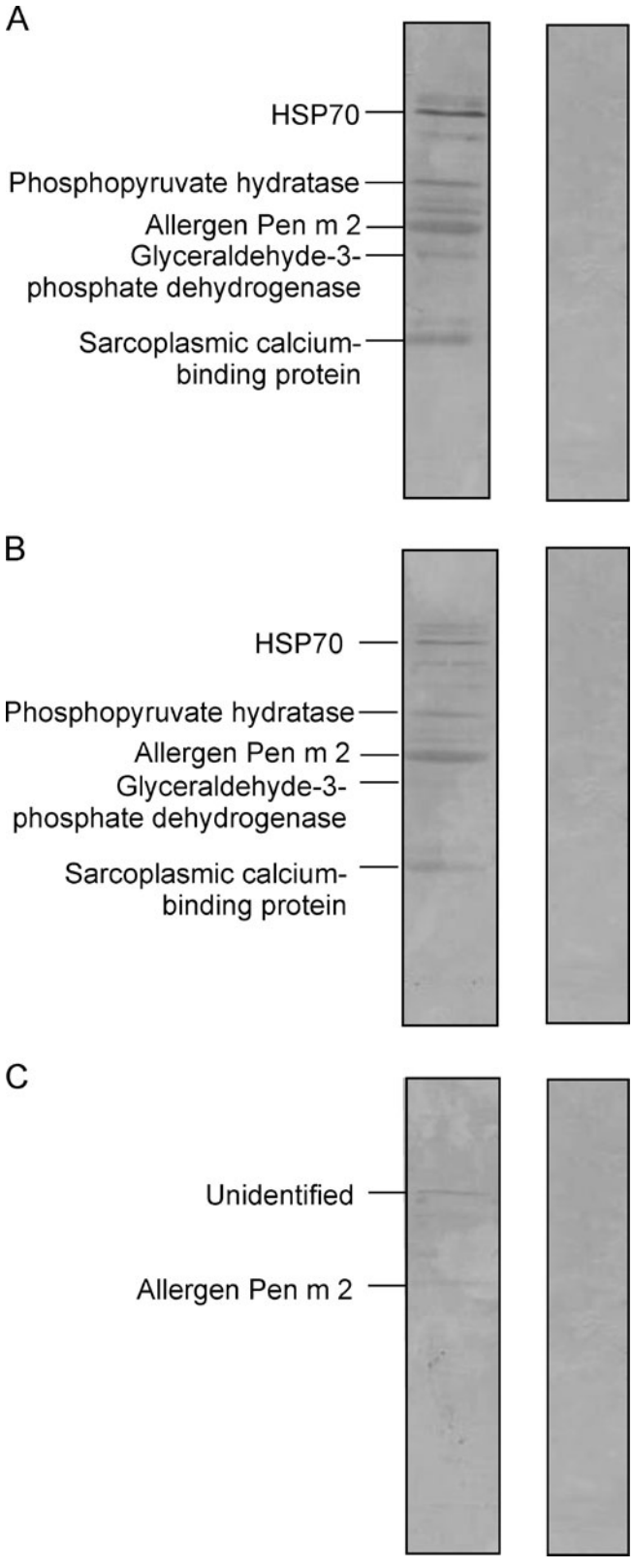
Membrane and cytosolic proteins were isolated from subcuticular epithelial tissue of *P. monodon* using Mem-PER® Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce, USA). Whole cell protein extract of subcuticular epithelial tissue of *P. monodon* was prepared using lysis buffer (20mM Tris-Cl pH 7.5, 20% glycerol, 0.5 mM EDTA, 0.5 mM PMSF, 100 mM NaCl, and 0.5 mM DTT). The isolated protein was quantified by using Bradford method.

2.4 *Two-dimensional gel electrophoresis (2-DE)*

All samples used for 2D gel electrophoresis were prepared using the Zoom 2D solubilizer Kit (Life technologies, USA) according to the manufacturer's instructions. 300 μg whole cell protein was solubilized in 200 μL of rehydration buffer (8 M urea, 2M thiourea 2% CHAPS, 10 mM DTT, 0.2% Bio-Lyte Ampholytes 3–10 pH range) at room temperature. ReadyStrip IPG, 11 cm long with pH range 4–7 (BioRad Laboratories, USA), was rehydrated overnight at room temperature using rehydration buffer. Subsequently, IEF (IEF PROTEIN CELL, BioRad, USA) was carried out at following voltage conditions: 300 V for 1 h, 500 V for 1 h, 1000 V for 2 h, 4000 V for 2 h, 8000 V for 10 h. The IEF strips were then incubated for 10 min in 1 mL equilibration buffer I (50 mM Tris-Cl, pH 8.8, 6 M urea, 2% SDS, 30% glycerol, 1% DTT) and 10 min in equilibration buffer II (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol, 2.5% w/v iodoacetamide). The strips were washed with distilled water and subjected for second dimension gel electrophoresis (12.5% SDS-polyacrylamide gel [SDS-PAGE]).

2.5 *Virus overlay protein binding assay*

Virus overlay protein binding assay (VOPBA) was performed to identify cell polypeptides involved in virus binding. Proteins were electrophoresed in a 1D and 2D (SDS-PAGE) on two parallel 12.5% gels. One gel was stained with Coomassie brilliant blue R-250 while proteins from the other gel were transferred to nitrocellulose membrane using transfer buffer (48 mM Tris, 39 mM glycine, and 20% (v/v) methanol) in a semidry blotting apparatus (BioRad Laboratories, USA). The membrane was blocked with 2% BSA (Sigma) in PBST (phosphate-buffered saline pH 7.4, 0.5% Tween-20) at 4°C and washed three times for 30 min with PBST. Membrane was then incubated with purified WSSV in PBS (10^5 PFU/mL) at 37°C for 60 min and washed three times for 30 min with PBST. It is then incubated with an Anti-White Spot syndrome virus VP28 antibody (1:400) (Abcam, UK) for 4 h. The membrane was



◀**Figure 1.** Virus overlay protein binding assay with WSSV: Whole cell lysate (lanes L1 and L2), cytosolic fraction (lanes C1 and C2) and membrane fractions (lanes M1 and M2) from *Penaeus monodon* tissue were subjected to 12.5% SDS–PAGE and transferred to nitrocellulose membrane (Trans-Blot, Bio-Rad Laboratories USA). Lanes L1, C1 and M1 were incubated with WSSV (10^5 PFU/mL) and Lanes L2, C2 and M2 with PBS pH 7.4 at 37°C. The putative WSSV binding proteins revealed after incubation with the anti-WSSV VP28 antibody and followed by goat anti rabbit IgG conjugated to peroxidise. Colour was developed with H_2O_2 and DAB. Identical results were obtained in all three independent experiments.

washed three times with PBST for 30 min each, followed by incubation for 2 hours at room temperature with goat anti rabbit IgG conjugated to peroxidase (1:1,000). Finally, membranes were washed three times for 30 min each with PBST. Colour reaction was developed with hydrogen peroxide (H_2O_2) and DABT (3, 3′–diaminodbenzidine tetrahydrochloride). Three independent experiments were carried out. Identical results were obtained in all three independent experiments. Identical results were obtained in all three independent experiments.

2.6 *Protein identification by MALDI-TOF MS*

Spots corresponding to the proteins of interest were excised from 1D and 2D SDS-PAGE gels and were subjected to alkylation followed by in-gel digestion with trypsin. The masses of resultant peptides were analysed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) on Voyager DE STR MALDI-TOF mass spectrometer (AB Sciex Instruments, USA). The m/z values generated by each protein sample was searched against NCBInr and Swissprot protein databases using MASCOT search engine and the proteins were identified based on homology.

3. Results and discussion

The virus overlay protein binding assay has been employed successfully to identify a number of putative virus interacting proteins (Upanan *et al.* 2008; Liang *et al.* 2010; Paingankar *et al.* 2010). Although VOPBA methodology has several limitations, the technique is still capable of selecting physiologically relevant binding molecules, possibly as a result of partial renaturation of proteins during the overlay process (Upanan *et al.* 2008; Paingankar *et al.* 2010). In current study we have used 1D as well as 2D VOPBA for identification of WSSV-interacting proteins of subcuticular epithelial tissue of *P. monodon*. In our study, 2D VOPBA not only helped in identification of the protein bands but also eliminated the problem of co-migrating bands in a single dimension. In 1D VOPBA, six proteins from whole cell extract and cytosolic extract of subcuticular epithelial tissue

Table 1. Molecular identification of WSSV-binding proteins from 1D VOPBA of *Penaeus monodon* subcuticular epithelial tissue

No.	Description	Source	Accession number	Molecular weight (kDa)		Mass value matched
				From VOPBA	From database	
1	Heat shock protein 70 [*Penaeus monodon*].	WC,CP	AAQ05768	70	71.482	13
2	ATP synthase subunit β [*Penaeus monodon*]	WC,CP,MP	AEB92164	64	65	7
3	Phosphopyruvate hydratase [*Penaeus monodon*]	WC, CP	AAC78141	45	47.265	8
4	Allergen Pen m 2 [*Penaeus monodon*]	WC,CP,MP	AAO15713	40	40.113	11
5	Glyceraldehyde-3-phosphate dehydrogenase (*Penaeus japonicus*) Glyceraldehyde-3-phosphate dehydrogenase OS=*Panulirus versicolor*	WC, CP	BAD95643	34	17.027 35.701	7 5
6	Sarcoplasmic calcium-binding protein [*Penaeus monodon*]	WC, CP	BAL72725	20	22.120	8

WC= whole cell lysate; MP= membrane proteins; CP= cytosolic protein.

were recognized as WSSV-VP28 interacting proteins (figure 1; table 1). Two proteins, Allergen Pen m 2 (AAO15713) and ATP synthase β subunit (AEB92164), were recognized as WSSV-VP28 binding proteins from membrane fraction of subcuticular epithelial tissue. In 2D VOPBA, 14 proteins of whole cell extract of subcuticular epithelial tissue were recognized as WSSV-VP28 interacting proteins (figure 2). Out of these 14 proteins we could identify eleven proteins Using MALDI TOF MS analysis (table 2). Five proteins, heat shock protein 70 (HSP70), ATP synthase β subunit, allergen Pen m 2, glyceraldehyde-3-phosphate dehydrogenase and sarcoplasmic calcium-binding protein were common 1D and 2D VOPBA. Identical results were obtained in all three independent 1D and 2D VOPBA experiments. We have not included functional studies in this present work because of unavailability of the relevant antibodies. Therefore this study is meant to be an exploratory study for identification of putative WSSV binding proteins from *P. monodon* tissues using 1D and 2D VOPBA as an interrogating tool.

A number of different proteins that were able to bind to WSSV were identified using VOPBA and MALDI TOF analysis. HSP70 has been shown as host interacting partner of various animal viruses (Xu *et al.* 2009; Paingankar *et al.* 2010; Zhu *et al.* 2012). It has been shown that VP28 of shrimp specifically interact with HSc70 and this interaction is ATP-dependent (Xu *et al.* 2009). The level of HSP 70 has been shown to increase in hemolymph of WSSV-infected crab *Scylla serrata* (Liu *et al.* 2011) whereas it has been also shown that HSP70 levels decreases after WSSV infection in subcuticular epithelium of *P. monodon* (Wu *et al.* 2007). Zhou *et al.* (2010) demonstrated that in addition to acting as a chaperon, HSP 70 may be involved in innate immunity against viral and bacterial infection. F-ATPases (F1F0-ATPases) present in mitochondria and plasma membranes and are the prime producers of ATP. In a recent study it has been demonstrated that F1-ATP synthase β subunit involved in WSSV infection in *Litopenaeus vannamei* (Liang *et al.* 2010; Zhan *et al.* 2013). The ATP synthase β subunit was significantly up-regulated when shrimps were infected with WSSV or Yellow Head Virus (YHV) (Wang *et al.* 2007; Bourchookarn *et al.* 2008). ATP binding and hydrolysis are essential for the chaperone activity of HSP70 proteins and this ATPase cycle is controlled by co-chaperons of J-domain family proteins (Laufen *et al.* 1999). The observations suggest that the HSP 70 plays important role in WSSV lifecycle and possible role of the ATP synthase β subunit in infection to provide the ATP in the chaperone process of HSP70. Additional studies are required to pin point the exact role of HSP70 in WSSV entry.

The earlier reports showed significant up-regulation of phosphopyruvate hydratase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Leu *et al.* 2007) and arginine kinase (Rattanarojpong *et al.* 2007; Somboonwiwat *et al.* 2010) in WSSV infection. It has been reported that cellular metabolic proteins have additional roles in immunity and transcriptional regulation of apoptosis (Kim and Dang 2005). Externalization of glycolytic enzymes is a common and early feature of cell death in different cell types triggered to die with different suicidal stimuli (Ucker *et al.* 2012). Apoptotic cells are recognized by phagocytes and trigger an active immunosuppressive response. It has been demonstrated that phosphopyruvate hydratase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sphingomyelin phosphodiesterase, serine proteinase-like protein and allergen Pen m2 are involved in induction of apoptosis (Egger *et al.* 2003; Kim and Dang 2005; Ucker *et al.* 2012). The allergen Pen m 2 protein, showed high

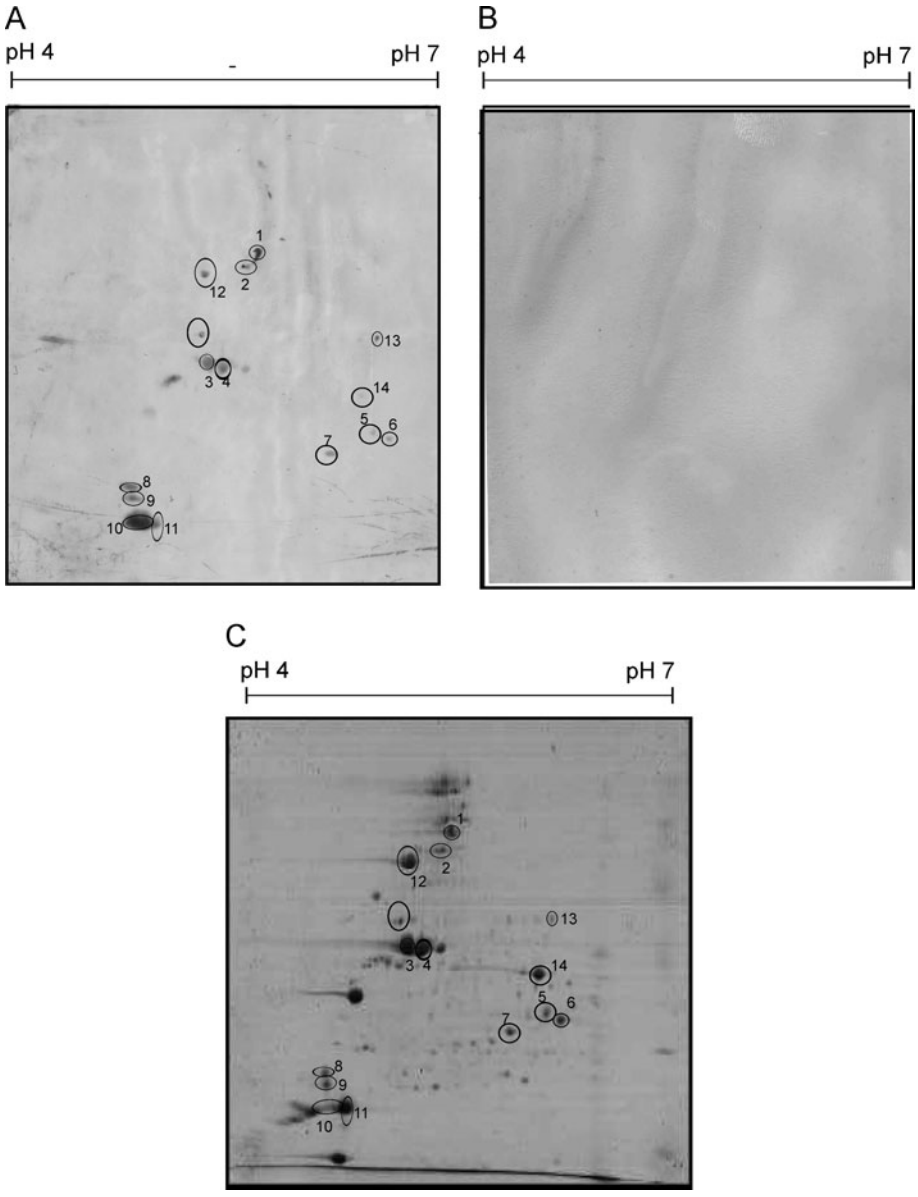


Figure 2. Two dimensional virus overlay protein binding assay with WSSV: Total whole cell protein extract (300 µg) of *Penaeus monodon* subcuticular tissue were subjected to 2-DE gel electrophoresis (ReadyStrip IPG, pH range 4–7, 11 cm, BioRad Laboratories, USA) and transferred to nitrocellulose membrane (Trans-Blot, Bio-Rad Laboratories USA). Membranes were incubated with (**A**) native WSSV (10^5 PFU/mL) or (**B**) PBS pH 7.4. The putative WSSV binding proteins revealed after incubation with the anti-WSSV VP28 antibody and followed by goat anti rabbit IgG conjugated to peroxidise. Colour was developed with H_2O_2 and DAB. (**C**) Two dimensional gel electrophoresis of total proteins of *P. monodon*; circled spots were reactive in the 2D VOPBA and identified using MALDI-TOF. Identical results were obtained in all three independent experiments.

sequence similarity to arginine kinase from crustaceans and had arginine kinase activity (Yu *et al.* 2003). Arginine kinase has been identified as a crustacean food allergen. It has been documented that arginine kinase and phosphoarginine are involved in generating ATP for driving phagocytosis in molluscan hemocytes (Coyne 2011). Signalling molecules such as sarcoplasmic calcium-binding protein 14-3-3 like protein, Allergen Pen m2 were found to interact with WSSV. The expression of the sarcoplasmic calcium-binding protein (SCP) α and β subunits (Leu *et al.* 2007), SCP-alpha chain (Wang *et al.* 2007) is strongly down-regulated after WSSV infection. These observations suggest that WSSV can

Table 2. Molecular identification of WSSV-binding proteins from 2D VOPBA of Penaeus monodon subcuticular epithelial tissue

No.	Description	Accession number	Molecular weight (kDa)		pI	Mass value matched
			From VOPBA	From database		
1	Heat shock protein 70 [*Penaeus monodon*].	AAQ05768	77	72	5.33	13
2	–	-	72	70	5.1	-
3	β-actin [*Penaeus monodon*]	AEW91436	43	41.972		7
4	Actin 2 [*Penaeus monodon*]	AAC78682	42	41.822	5.1	10
5	Glyceraldehyde-3-phosphate dehydrogenase (*Penaeus japonicus*) Glyceraldehyde-3-phosphate dehydrogenase OS=*Panulirus versicolor*	BAD95643	35	35.701	6.8	7 5
6	potential tRNA (adenine-N(1)-methyltransferase catalytic subunit TRMT61B-like [*Amphimedon queenslandica*]	XP_003385084	33	34	7	7
7	Similar to Sphingomyelin phosphodiesterase	XP_003385084	30	34.137	6.2	4
8	Serine proteinase like protein [*Penaeus monodon*]	ABD62888	21	25	4.5	8
9	–	-	20	25	4.5	-
10	14-3-3 like protein [*Penaeus monodon*]	AAY56092	19	22	4.5	7
11	Sarcoplasmic calcium-binding protein [*Penaeus monodon*]	BAL72725	18	22.106	4.76	5
12	ATP synthase subunit β [*Penaeus monodon*]	AEB92164	60	65	5	7
13	–	-	50	45	6.8	-
14	Allergen Pen m2 [*Penaeus monodon*]	AAO15713	45	40.087	6.8	10

potentially modulate the calcium ion concentration of infected cells by decreasing the expression of shrimp cell SCP and by simultaneously expressing the viral-encoded calcium-binding proteins. 14-3-3 family proteins are involved in important cellular processes such as signal transduction, cell-cycle control, apoptosis, stress response and malignant transformation. (Van Hemert *et al.* 2001; Mackintosh 2004). 14-3-3b protein is up-regulated after WSSV infection in subcuticular epithelium (Wu *et al.* 2007), stomach and lymphoid cells (Wang *et al.* 2007) of *P. monodon.* It has been also reported that14-3-3b protein is related to apoptosis in lymphoid cells but in stomach cells it is involved in other cellular processes that are often modulated during virus infection, such as cell-cycle control. These results suggest that WSSV induces apoptosis in infected cells by interacting with various apoptosis-inducing proteins.

Numerous viral proteins have been reported to interact directly interact with actin for several steps during their lifecycle like attachment, internalization and replication (Cudmore *et al.* 1997; Sodeik 2000). In innate immune response, polymerization of actin 2 is an important process in phagocytosis in multicellular organism including shrimp (Kaplan 1977). Previously it has been documented that β-actin of haemocyte in *Fraxinus chinensis* could specifically react with rVP26 using the IIFA, VOPBA and mass spectrometry (Liu *et al.* 2011). A similar result has also been reported in crayfish (Xie and Yang 2005). Actin might mediate the viral movement by interacting with VP26 after WSSV penetration and fusion into the host cytoplasm. Cytoskeletal protein actin 2 is seems to be up-regulated significantly upon *Vibrio harveyi* infection in hemocytes of *P. monodon* and it reflects that induction of phagocytosis in shrimp hemocytes in response to bacterial infection (Somboonwiwat *et al.* 2010). These observations suggest that a host cytoskeletal protein could be exploited by WSSV for vectorial transport in cytoplasm. It will be interesting to study the possible interplay of cytoskeletal protein in virus uptake and phagocytosis.

In conclusion, our analysis has resulted in identification of host proteins which interact with WSSV-VP28, a few of which could have receptor function. WSSV infection induces apoptosis in *P. monodon* by interacting with proteins like arginine kinase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphopyruvate hydratase. It would be interesting to further investigate and elucidate the role of each protein in WSSV infection. Understanding the host proteins involved in virus interaction not only provides information about the virus infection but may also lead to the development of strategies for interfering with virus–host interactions.

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