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Differential and additional expression of proteins in the subcellular organelles of *Penaeus monodon* (Fabricus) in response to white spot syndrome virus (WSSV) infection

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ABSTRACT

Currently the most common and devastating disease of shrimp is caused by the white spot syndrome virus (WSSV), which has spread throughout the world mainly by different species of crustaceans carrying the virus. *Penaeus monodon* were challenged with WSSV and the protein expression variation studied over a period of time. The gills, one of the main target organs of WSSV, showed upregulation of 24 proteins and expression of three novel proteins after infection. In the hepatopancreas, 20 upregulated proteins and six novel proteins were observed. The muscle showed upregulation of 11 proteins and one new protein. Subcellular organelles like nuclear and mitochondrial proteins of each tissue showed different profiles with either increased/decreased expression of few proteins or additional expression of novel proteins. The protein profiles resolved in the study provide a rich source of information on proteins in shrimp which may be involved in antiviral response.

Keywords: Host protein expression, *Penaeus monodon*, Protein expression profiling, SDS-PAGE,White spot syndrome virus, WSSV

Introduction

Viral diseases are a major recurring problem in shrimp culture worldwide. Serious viral outbreaks often cause catastrophic losses in shrimp harvest (Lightner *et al*., 1996). Under farming conditions the white spot syndrome virus (WSSV) infection can result in total crop losses within 3-10 days (Lightner *et al*., 1996). Viral infection cycles involve numerous interactions between the virus and the host. These interactions range from the initial binding between host membrane receptors and viral coat proteins, to the takeover of the host transcription machinery by replacing specificity-determining host factors by viral proteins. Most of these interactions are essential for viral propagation and can be potential targets for antiviral therapy (Huber *et al*., 1994). A better understanding of host response to WSSV will help to elucidate the pathogen's unique mechanisms of virulence and pathogenesis (Wang *et al*., 2007). To comprehend the pathogenesis of any viral disease, knowledge of the interactions between virus and host is essential. Virus-host interactions are involved in the immune responses against the invader, and may also result in changes in the expression levels of host genes that favour virus replication (Wang *et al*., 2007).

Till date, virus-host interactions of WSSV have been investigated at the transcription level only using expressed sequence tags (ESTs), RT-PCR, microarray chips, suppression subtractive hybridisation (SSH) and differential hybridisation (Gross *et al*., 2001; Astrofsky *et al*., 2002; Rojtinnakorn *et al*., 2002; Roux *et al*., 2002; Bangrak *et al*., 2002; Dhar *et al*., 2003; He *et al*., 2005; Pan *et al*., 2005). However, many of these studies focused on immune cells (lymphoid organ cells and haemocytes) and provided good insights into biodefense mechanisms, but these cells are not primary WSSV targets (Lo *et al*., 1997; Tsai *et al*., 1999; Wu and Muroga, 2004) and their cellular pathways are not necessarily representative of the host cells in which virus replication occurs (Wang *et al*., 2007). Consequently, little is known about the cellular events associated with WSSV infection in permissive cells. There is an urgent need to investigate the expression pattern of proteins in the virus infected hosts and to determine their functions. Identifying the protein profile consequent to WSSV infection is an important step towards improving our knowledge of the cellular pathways involved in WSSV infection (Wang *et al*., 2007).

Little is known about WSSV infection and morphogenesis *in vivo*. Research on virus replication and virion morphogenesis shows that DNA replication and *de novo* envelope formation take place in the nucleus (Durand *et al*., 1997; Wang *et al*., 2000). Comprehending the interaction between host and pathogen would be useful

in developing control strategies. Although an increasing number of immune function related genes of shrimp are being reported, the genes involved in the interaction between WSSV and shrimp still remain unclear (Wang *et al*., 2006). There may be a large number of immune related genes, but our knowledge in this area is extremely limited. Hence an alternate approach is to investigate the changes in the protein profile, which is of particular interest since the level of mRNA is not necessarily correlated with an abundance of protein. In the present study, the protein profile of main organs like gills, hepatopancreas and muscle of the WSSV infected *P. monodon* was investigated to unravel other factors in viral infection.

In addition to the total protein profile of the cells, the present study also investigated the changes specifically in the nuclear and mitochondrial proteins following WSSV infection. Due to the lack of suitable shrimp cell lines for culturing shrimp viruses, Huang *et al.* (2002) suggested that the tissues from various infection stages should reveal the early expressed proteins.The approach was to resolve the expressed proteins bythe use of one-dimensional electrophoresis of the tissue extracts of gill, hepatopancreas and muscle from WSS Vinfected as well as WSSV free tiger shrimp.Thus, the changes in the protein expression by way of upregulation or downregulation as well as the expression of novel proteins during WSSV infection were identified.

Material and methods

Experimental animals

Penaeus monodon (20 g body weight) which were PCR tested and found to be WSSV negative (Kimura *et al*., 1996) were reared in a continually aerated tank containing seawater at a constant salinity of 20 g l^{-1} and ambient temperature of 28 ± 1 ºC. Shrimp were acclimatised for one week. Animals in the intermoult stage were used for the study.

Real time quantification of viral sample and infection of animals

WSSV inocula were prepared by homogenising the infected shrimp muscle tissue in phosphate-buffered saline (PBS) (0.1 mol l^{-1}, pH 7.4). The homogenate was centrifuged at 5000 *g* for 10 min and the supernatant collected. This supernatant was used to inoculate an animal from which fresh inoculum was prepared to be injected into the experimental shrimp.

All the animals were injected with the viral inoculum (50 µl, 6× 10^7 copies) between the second and third tergal plates of the lateral side of the tail using a 1ml syringe (30G). The inoculum was quantified using real time PCR as described previously (Jeswin *et al*., 2013). The dose was previously optimised to ensure 100% mortality within 4 days post-infection (dpi). Control animals were injected with 50 µl PBS.

Tissue collection and sample preparation

Five infected shrimps were selected for each time point. The tissues *viz.,* hepatopancreas, muscle and gills were then excised from infected and control shrimps at 6, 12, 24, 36 and 48 h post-infection (hpi). To minimise deviations arising from individuals, the sample collected at each time point from five animals were pooled for both the control and WSSV injected samples. The collected tissues were flash frozen at -80°C for protein expression studies.

Mitochondrial and nuclear protein isolation

Mitochondrial and nuclear proteins were isolated from hepatopancreas, gills and muscle. For this 200 mg of tissue was minced and homogenised in 0.25 M sucrose and 0.0018 M calcium chloride under ice cold condition. The homogenate was layered on top of a solution consisting of 0.34 M sucrose and 0.18 mM Calcium chloride and centrifuged at 2000 *g* for 10 min at 4 °C. The fraction settled above the gradient was saved for mitochondrial protein isolation and pellet for nuclear protein isolation.

The nuclear pellet was dissolved in 10% SDS prepared in 0.025 M sucrose and 0. 18 mM calcium chloride and kept at room temperature for 15 min and then stored at -80 °C.

The mitochondrial fraction was centrifuged at 12,000 *g* for 20 min at 4 °C. The resulting pellet was dissolved in 10% SDS prepared in 0.025 M sucrose and 0.18 mM calcium chloride and kept at room temperature for 15 min and then stored at -80 °C.

Total protein isolation

Total proteins were isolated from hepatopancreas, gills and muscle of both infected and control animals. One hundred mg of each tissue was homogenised in TN buffer (20 mMTris –HCl, 400 mMNaCl, pH 7.4) in cold conditions and centrifuged at 10,000 *g* for 20 min at 4 °C. The supernatant containing total proteins was preserved at - 80 °C.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous denaturing electrophoresis was used to separate the proteins in the tissue samples according to their molecular weight. The protein was separated by 10% SDS-PAGE, following the method of Laemmli *et al*. (1970) and visualised using Coomassie Brilliant Blue R-250 staining. The molecular weight of the proteins was identified using Quantity One software (Bio-Rad).

Results

Pathogenesis of infection

In survival studies, animals injected with the WSSV inoculum showed typical signs of infection. At 24 hpi, the shrimps significantly reduced feed intake and exhibited a faded or reddish body colour. By 55 hpi, some of the infected shrimps were dead whereas the remaining ones appeared weak to moribund. All infected shrimps died before 96 hpi.

Analysing protein expression variations

The cells were fractionated into nuclear and mitochondrial fractions to reduce sample complexity for SDS PAGE and also to provide an excellent way to study the interaction of a nuclear replicating virus with the nuclear and mitochondrial proteins. The one dimensional SDS PAGE of the nuclear, mitochondrial and total protein extracts from the gills, muscle and hepatopancreas of the WSSV infected shrimps and WSSV free controls has revealed the expressional variations in the proteins upon WSSV infections. The time dependent expressional variation was resolved from the tissue samples analysed at 6, 12, 24, 36 and 48 hpi.

Host protein expressional variation in muscle

In response to the WSSV infection, while numerous proteins showed change in the expression level, a few novel proteins were also expressed. The total protein profile of muscle showed variation in the expression level of 141, 73 and 24 kDa. All of them were upregulated from 6 hpi and remained expressed upto 48 hpi (Fig. 1). Among the nuclear proteins of muscle, 31 and 24 kDa proteins were upregulated from 12 hpi onwards and remained so up to 48 hpi. Of the two, the 24 kDa protein was highly upregulated at 48 hpi. Another nuclear protein of 29 kDa was upregulated from 24 hpi onwards and it was highly expressed both at 24 and 36 hpi (Fig. 2). Upregulation of four mitochondrial proteins of the muscle were observed. They were 70, 45, 39 and 24 kDa. While the 70 kDa protein was upregulated from 6hpi onwards, the 45 kDa and 24kDa were upregulated from 12 hpi and all of them remained expressed upto 48 hpi. However, the 39 kDaprotein was upregulated from 36 hpi and intensely expressed at 48 hpi. A new mitochondrial protein of 34 kDa was expressed in the muscle at 12 hpiwhich was highly expressed at 48 hpi (Fig. 3).

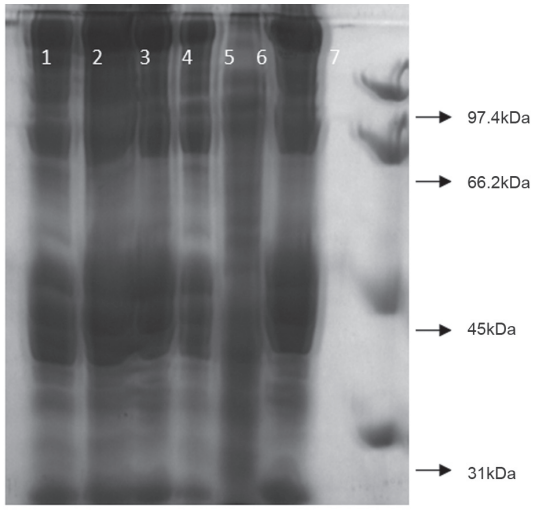

Fig. 1. Coomassie-Brilliant-Blue-stained SDS-PAGE gel (10%) of WSSV infected and uninfected total protein of muscle tissues of *P. monodon*. Uninfected muscle tissue (lane 1), WSSV infected muscle tissue (lane 2-6 as 6, 12, 24, 36 and 48 hpi) and Molecular weight marker (lane 7)

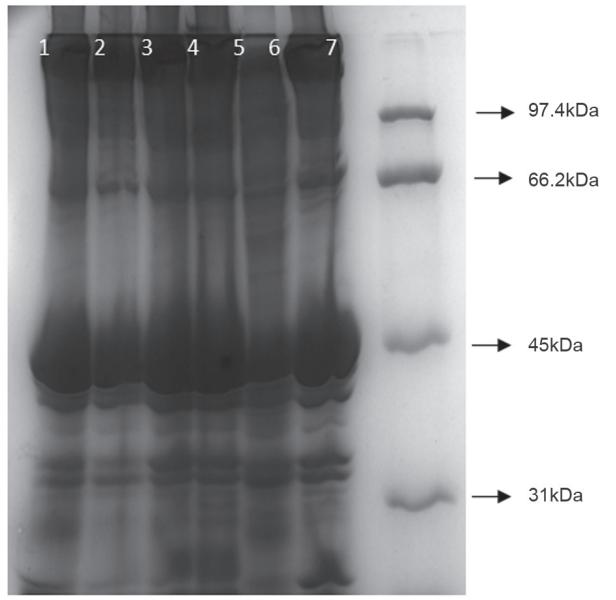

Fig. 2. Coomassie-Brilliant-Blue-stained SDS-PAGE gel (10%) of WSSV infected and uninfected nuclear protein of muscle tissues of *P. monodon*. Uninfected muscle tissue (lane 1), WSSV infected muscle tissue (lane 2-6 as 6, 12, 24, 36 and 48 hpi) and molecular weight marker (lane 7)

Host protein expressional variation in gills

The gill, which is one of the main target organs of WSSV, showed the highest expressional variations of proteins. The total protein profile of gills revealed upregulation of 77 and 64 kDa proteins from 12 hpi onwards and with highest level of expression at 48 hpi. Many low molecular weight fractions were found to be upregulated from 6 hpi onwards. They were 55, 48, 47, 45, 40, 37 and 35 kDa proteins, of which 45 kDaprotein was intensely expressed. Two new proteins were observed in gills after infection. Out of the two, the 24 kDa protein was expressed from 6 hpi onwards till 48 hpi, whereas the 144 kDa appeared at 48 hpi only (Fig. 4).

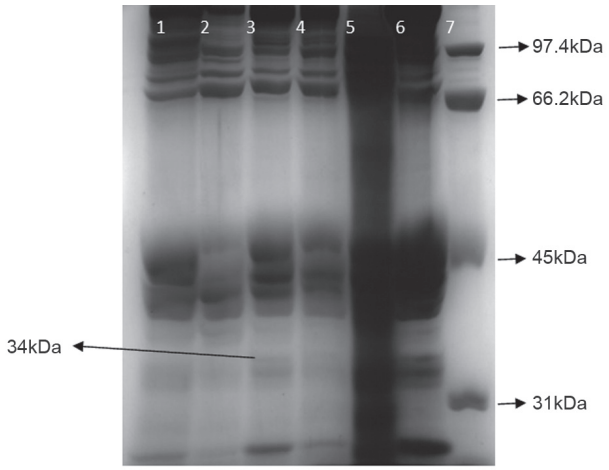

Fig. 3. Coomassie-Brilliant-Blue-stained SDS-PAGE gel (10%) of WSSV infected and uninfected mitochondrial protein of muscle tissues of *P. monodon*. Note the presence of new protein bands (mol. wt. 34 kDa) in WSSV infected muscle tissue (lane 2-6 as 6, 12, 24, 36 and 48 hpi) as compared with uninfected mitochondrial protein of muscle tissue (lane 1). Molecular weight marker (lane 7)

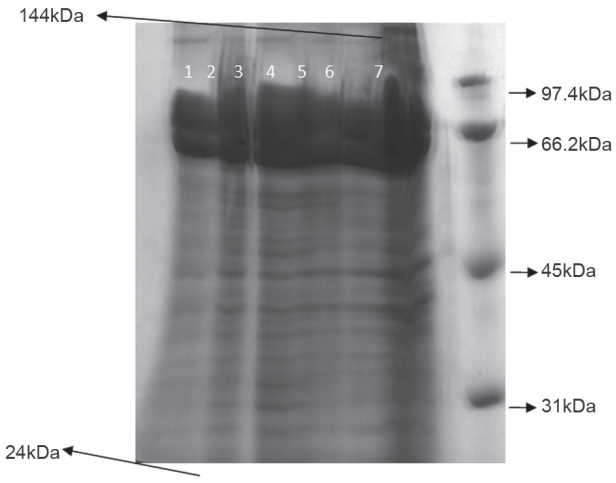

Fig. 4. Coomassie-Brilliant-Blue-stained SDS-PAGE gel (10%) of WSSV infected and uninfected total protein of gill tissues of *P. monodon*. Note the presence of new protein bands (mol. wt: 144, 24 kDa) in WSSV infected gill tissue (lane 2-6 as 6, 12, 24, 36 and 48 hpi) as compared with uninfected gill tissue (lane 1). Molecular weight marker (lane 7)

Many of the nuclear proteins of the gills also showed upregulation. The146 kDa protein started upregulation from 6 hpi and got highly expressed at 12 hpi, whereas the 37 kDa protein upregulated at 6 hpi, but later weakened to the original level. Two other proteins of 66 and 77 kDa size showed high upregulation from 6 hpi onwards and remained expressed upto 48 hpi. The 45 and 42 kDa proteins were upregulated from 12 hpi. The 64 kDa protein which was expressed only in the infected animal appeared at 6 hpi and was furtherupregulated upto 48 hpi.

Upregulated mitochondrial proteins of gills were 98, 82, 72, 64, 56 and 33 kDa. Of these, the 98, 82, 72 and 56 kDa proteins were upregulated from 6hpi onwards whereas the 64 and 33 kDa were upregulated, respectively, from 24 and 12 hpi onwards (Fig. 5).

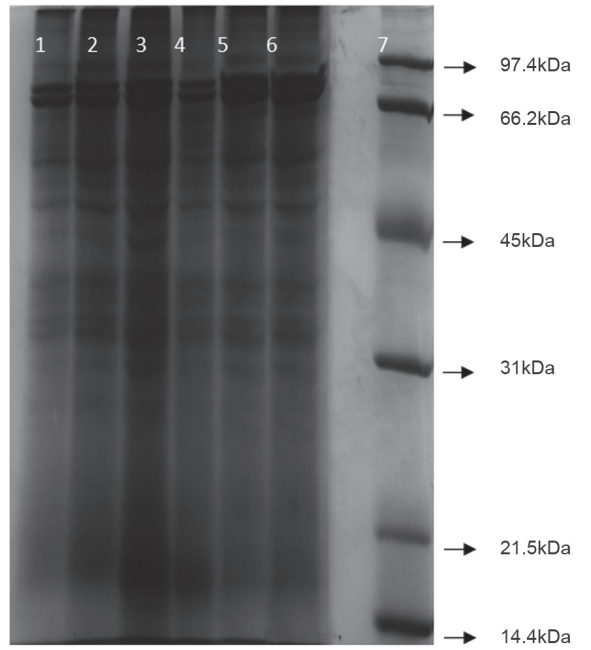

Fig. 5. Coomassie-Brilliant-Blue-stained SDS-PAGE gel (10%) of WSSV infected and uninfected mitochondrial protein of gill tissues of *P. monodon*. Uninfected gills tissue (lane 1), WSSV infected gill tissue (lane 2-6 as 6, 12, 24, 36 and 48 hpi) and Molecular weight marker (lane 7)

Host protein expressional variation in hepatopancreas

The protein expressional variation in hepatopancreas, was also monitored. The total protein profile of hepatopancreas showed high level of upregulation of 86, 61 and 49.5 kDa proteins at 48 hpi. A 31 kDa protein was upregulated from 6 hpi and was highly expressed at 48 hpi. The new proteins observed in the total protein profile was 77 kDa which was first detected at 48 hpi (Fig. 6). The nuclear proteins of hepatopancreas which showed differential expression were 45, 36, 26 and 23 kDa. The 45 kDa protein was highly expressed at 12 hpi. The 36 kDa protein was upregulated up to 12 hpi, and downregulated from 36 hpi onwards. The 26 and 23 kDa proteins were upregulated from 6 hpi onwards and expressed up to 48 hpi. The new nuclear proteins observed in the hepatopancreas of infected animals were 148, 114, 82, and 47 kDa. The 148 and 114 kDa proteins were observed at

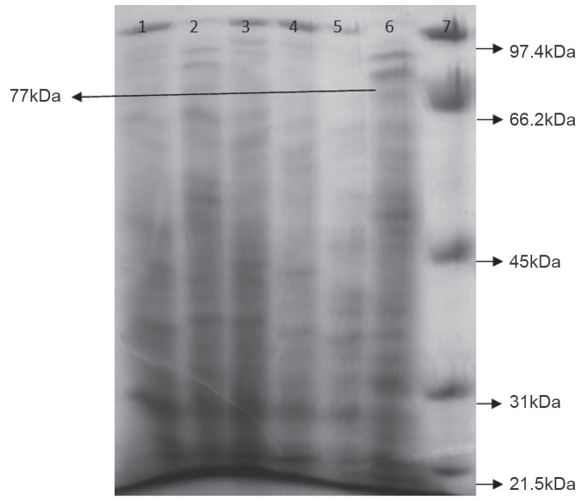

Fig. 6. Coomassie-Brilliant-Blue-stained SDS-PAGE gel (10%) of WSSV infected and uninfected total protein of hepatopancreatic tissues of *P. monodon*. Note the presence of new protein bands (mol. wt: 77 kDa) in WSSV infected hepatopancreatic tissue (lane 2-6 as 6, 12, 24, 36 and 48 hpi) as compared with uninfected hepatopancreatic tissue (lane 1). Molecular weight marker (lane 7)

24 hpi. While the 82 kDa was observed from 6 hpi and expressed upto 24 hpi, the 47 kDa was expressed at 12 and 24 hpi (Fig.7). Among the mitochondrial proteins of

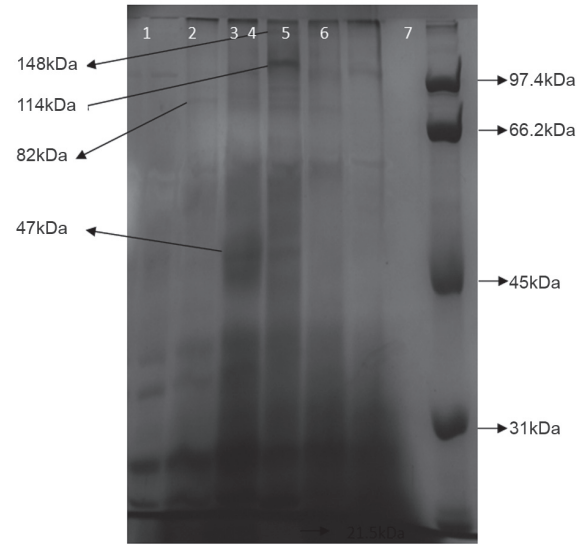

Fig. 7. Coomassie-Brilliant-Blue-stained SDS-PAGE gel (10%) of WSSV infected and uninfected nuclear protein of hepatopancreatic tissues of *P. monodon*. Note the presence of new protein bands (mol. wt: 148, 114, 82, 47 kDa) in WSSV infected hepatopancreatic tissue (lane 2-6 as 6, 12, 24, 36 and 48 hpi) as compared with uninfected hepatopancreatic tissue (lane 1). Molecular weight marker (lane 7)

hepatopancreas, the 106 kDa protein was upregulated till 12 hpi and thereafter, downregulated. The 54 kDa protein was upregulated at 6 hpi with the expression weakening in later hours. The 39 and 36 kDa proteins were up regulated at 6 hpi and slowly down regulated from 24 hpi onwards. The 28 kDa protein showed upregulation from 6hpi onwards and it remained upregulated up to 12 hpi and weakened to be observed barely above background. The 64 kDa protien was a new protein observed only in infected animals and was highly expressed at 48 hpi (Fig. 8).

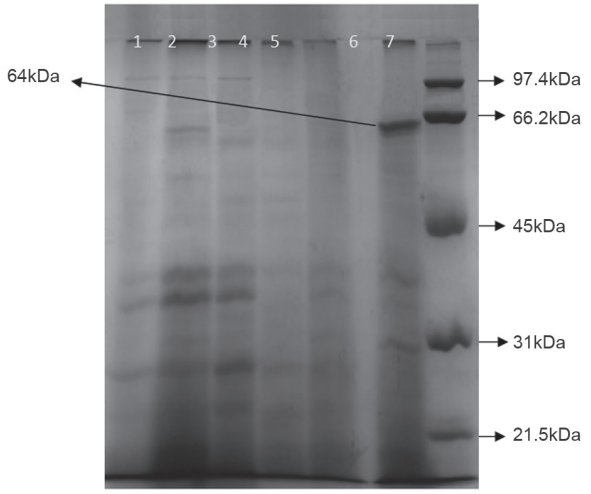

Fig. 8. Coomassie-Brilliant-Blue-stained SDS-PAGE gel (10%) of WSSV infected and uninfected mitochondrial protein of hepatopancreatic tissues of *P. monodon*. Note the presence of new protein bands (mol. wt: 64 kDa) in WSSV infected hepatopancreatic tissue (lane 2-6 as 6, 12, 24, 36 and 48 hpi) as compared with uninfected hepatopancreatic tissue (lane 1). Molecular weight marker (lane 7)

The present study shows that protein expression patterns in *P. monodon* are drastically altered by WSSV infection in experimentally infected hosts. The upregulated or novel proteins identified in the study are presented in Table 1.

Discussion

The aim of this study was to investigate the alterations in host protein expression in muscle, hepatopancreas and gills of WSSV challenged tiger shrimp. The SDS PAGE profiling was adopted to resolve the protein profile and analyse the molecular response during WSSV infection. Various subcellular fractions of cells were isolated to comprehend the protein expressional variations. A large number of upregulated/downregulated proteins or newly expressed proteins were observed in the subcellular fractions. These results provide valuable inputs for in-depthWSSV pathogenesis, emphasising on altered protein expression of host cells in response to WSSV challenge.

Table 1. The host protein expression variations observed in different tissues of *Penaeus monodon* after challenging with WSSV. The novel proteins are indicated in bold and those proteins that are expressed continuously in the following hours are shown by asterisk. Those that are upregulated only in a particular time period are represented by 'up'.

	Total protein			Nuclear protein			Mitochondrial protein		
Tissue	Muscle	Gill	Hepato	Muscle	Gill	Hepato	Muscle	Gill	Hepato
6 h	141*, 73*, 24*	55*, 48*, 47*, 45*, 40*, 35* 37*, **24***	31*		146*,77*, 66*, 37*, **64***	26*, 23*, 36^{up}, **82**	70*	98*, 82*, 72*, 56*	106 ^{up}, 39 ^{up}, 36 ^{up}, 28 ^{up}, 54 ^{up}
12 h		77*, 64*		31*, 24*	45*, 42*	45*, 36 ^{up}, **82, 47**	45*, 24*, **34***	33*	106 ^{up}, 39 ^{up}, 36 ^{up}, 28 ^{up}
24 h				29*		**148*, 114*, 82, 47**		64*	
36 h							39*		
48 h		**144**	86, 61, 49.5,.**77**						**64**

Protein profiling was repeatedly carried out at 6, 12, 24, 36 and 48 hpi to monitor the changes in the protein expression over a period of time. These time intervals are commonly used in WSSV studies and therefore allow comparison with results from previous studies. Ten novel proteins were observed on analysing different tissues. Low molecular weight proteins were found to be expressed in the initial hours (6 hpi). Out of these, 6 were observed in hepatopancreas, three in gills and one in muscle.

Considerable progress has been made to characterise the WSSV, but the comprehension of the defence mechanism in shrimp in response to viral infection is lacking. It is essential to consider the virus-host interaction while studying the change in virulence of WSSV rather than looking at the virus or shrimp alone (Stalinraj *et al*., 2009). Stalinraj *et al*. (2009) reported that some farmers in Tamil Nadu and Andhra Pradesh (South India) are able to obtain a reasonably good harvest despite the presence of WSSV infection. Severe white spots on the carapace positively identified the presence of the virus. Analysis of the protein profile of the two virus isolates (virulent isolate and non-virulent isolate) showed identical profiles with no discernible difference. On analysing haemolymph protein profile of host shrimp, it was found that the haemolymph of those shrimp that survived the WSSV infection had three proteins corresponding to 41, 33 and 24 kDa which were not observed in shrimp that succumbed to WSSV infection. These proteins may correspond to the gene products expressed by those shrimp that impart enhanced resistance or tolerance to WSSV.

Several transcriptional studies have reported that gill tissue is relevant for immune defense, like anti-lipopolysaccharide factor (ALF) transcripts found in gills of *Litopenaeus vannamei* (de la Vega *et al*., 2008) and penaeidins localised by immunohistochemistry (Destoumieux*et al*., 2000). The strong involvement of gills has been shown by numerous proteins that change expression profiles upon infection. Rameshthangam and Ramasamy (2005) reported two new proteins in the hepatopancreas at 20 and 62 kDa in WSSV infected moribund *P. monodon*. It is possible that proteins expressed in the initial hours may not express up to the stage of death. Pan *et al*. (2005) through suppression subtractive hybridisation, identified differentially expressed immune related genes in the hepatopancreas of the virus-resistant *Penaeus japonicus*. Immune recognition molecules are also reported in hepatopancreas of *L. vannamei* and *L. setiferus* (Gross *et al*., 2001). The reports of transcriptional studies of hepatopancreas have shown evidence of its major role in shrimp's defence system.

The nascent viral proteins might require host proteins for folding and modifications. On the other hand, defence mechanisms and physiological balance of host cells may have triggered the viral eradication. The proteins with increased levels during the WSSV infection are likely to play important roles in the defence mechanisms, while the proteins with lower levels might be involved in arrangements of cytoskeleton assembly, metabolism, and trafficking pathways (Bourchookarn *et al*., 2008). These findings can lead to a number of possible hypotheses, which deserve further investigations and may lead to novel insights into the virus-host interactions, particularly host defence mechanisms. In the present study, 10 new protein bands were detected only in infected animals. The molecular weight of these new proteins bands ranged from 24 to 148 kDa. In addition, a number of differentially expressed cellular proteins were also identified.

In conclusion, the present study revealed that there are variations in the *in vivo* translational expression profiles of the WSSV challenged and uninfected animals. The

muscle showed 11 upregulated proteins and one novel protein. In the gills, 24 upregulated proteins and 3 novel proteins were observed. The hepatopancreas showed 20 upregulated proteins and six novel proteins. Changes in the protein expression pattern may result not only from the responses of shrimp in its efforts to contain the virus infection but also from the attempt of the WSSV to subvert cellular functions for its multiplication. In addition, the new protein as well as the proteins with altered expression levels is a source of information for the identification of the proteins involved in shrimp antiviral response. This study provides a number of differentially expressed targets for further research and suggests that key events in pathogenesis may be established early in infection.

Acknowledgments

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