

Temporal analysis of molecular changes in shrimp (*Penaeus vannamei*) tissues with respect to white spot disease

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Revised: 13 April 2015 / Accepted: 27 April 2015 / Published online: 7 May 2015
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Abstract In spite of lot of attention and significant research efforts, White spot disease (WSD) is the major cause for shrimp mortality in aquaculture industry. This is due to the limited understanding in White Spot Syndrome Virus (WSSV) pathogenesis. To understand shrimp molecular responses towards WSSV infection, proteome and protease profiles of various shrimp tissues (gill, muscle, gut and hepatopancreas) were studied at different time-intervals post-infection (pi) using SDS-PAGE analysis and In-gel gelatin zymography, respectively. Expression of new proteins along with up-regulation, down-regulation and varied expression of many host proteins were observed. These variations were observed as early as 6 h pi and the maximum variations were observed at the time-intervals 6 h pi and 12 h pi representing the early stage of infection. Protease profile analysis had revealed that most of the host proteases were down-regulated during WSSV infection. Among the tested shrimp tissues, gill, gut and hepatopancreas are the most affected due to WSSV infection, while muscle is the least affected one with minimum proteolytic activity, whereas hepatopancreas is highly enriched with active proteases. These results suggest that during WSSV infection, both protein and protease profiles of shrimp tissues gets drastically altered and down-regulation of the host proteases is the major step in WSSV-pathogenesis. These observations are significant for intervening with the

early stages and delaying the morbidity and mortality, so that shrimps could be harvested at profitable incubation time and reduce the impact on shrimp aquaculture industry.

Keywords WSSV · Shrimp · Protein · Protease · SDS-PAGE · Zymography · Pathogenesis

Introduction

Shrimp is the most widely consumed seafood across the globe as it is highly nutritious, owing to its high protein content (23.98 g/100 g), zero glycemic index and low calories (USDA SR 27 2014). Shrimp farming is centuries old practice which was usually done in small scale. The industrialization of shrimp farming became a reality only after the research efforts of Motosaku Fujinaga in year 1933. At present shrimp aquaculture is more than 20 billion dollar industry, though it makes up only 4 % of global aquaculture production by weight, but contributes almost 15 % by value (FAO 2014). Asia is the major producer of shrimp in the world, accounting for 80 % of total global shrimp production, in which China, Thailand, Vietnam, Indonesia and India are the major contributors (GOAL 2013). But this industry is being threatened by various diseases, especially the White Spot Disease (WSD) (Flegel 2012). It was first reported in 1992 in Taiwan, and is caused by White Spot Syndrome Virus (WSSV) (Chou et al. 1995). Near 100 % mortality in shrimp farms within 3–10 days post-infection, results in great economic losses to shrimp aquaculture industry (Jiravanichpaisal et al. 2001). The outbreak of 1992–93 in Asia resulted in loss of US dollars 6 billion, whereas the 1999 outbreak in America cost more than a billion US dollar loss. (Lightner et al. 2012). According to the recent survey conducted in India, WSSV attributes the loss of more than 21,000 metric tonne shrimp production, worth

Electronic supplementary material The online version of this article (doi:10.1007/s13197-015-1866-4) contains supplementary material, which is available to authorized users.

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US dollars 77 million annually to the nation (Kalaimani et al. 2013).

Currently shrimp research is getting more and more focused in understanding the molecular mechanisms underlying shrimp-WSSV encounter. Shrimp being an invertebrate lacks adaptive immunity and depends on innate immunity for its defense against pathogens (Hoffmann et al. 1999). It includes both humoral responses comprising prophenoloxidase (proPO) system, the clotting cascade, anti-oxidant defense enzymes and a wide variety of antimicrobial peptides (Hoffmann et al. 1996) and cellular responses comprising apoptosis, encapsulation, phagocytosis and nodule formation (Lackie 1988).

Most of the above mentioned innate immune cascades are dependent on proteases for their activation. Melanization, an important innate immune response gets activated by proPO cascade. In this cascade a serine protease converts prophenoloxidase (inactive form) to phenoloxidase (active form) by limited proteolysis. Phenoloxidase (PO) oxidize phenols to quinones, which polymerize non-enzymatically to melanin (Nappi and Vass 1993). Low levels of PO during WSSV infection (Roux et al. 2002) indirectly indicates the down regulation of serine proteases involved in activation of PO. Apoptosis or programmed cell death, resulting in destruction of damaged and diseased cells is a major player in invertebrate immune system and is dependent on aspartate specific cysteine proteases also known as caspases for its activation (Wang et al. 2008). Caspases, involved in apoptosis activation are significantly down-regulated during the WSSV infection (Leu et al. 2008). Blood coagulation or clotting is an important part of hemostasis. It is highly conserved in both invertebrates and vertebrates. Though the processes of blood coagulation differ in invertebrates and vertebrates but they both are dependent on proteolytic cascade for their activation (Iwanaga 2002). The significance of proteases in shrimp innate immunity indicates that, proteases have to be one of the most interactive effector molecules in Shrimp-WSSV interaction.

Studies with respect to the changes during the early stages of WSSV infection helps in understanding the pathogenesis of the disease. During WSSV infection anti-oxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase were progressively down-regulated and lipid peroxidation was up-regulated from zero hour to moribund stage in various shrimp tissues (Mohankumar and Ramasamy 2006). Alterations were noticed in protein profiles of infected shrimp (*P.monodon*) tissues at moribund stage when compared to uninfected shrimp tissues analyzed in SDS-PAGE (Rameshthangam and Ramasamy 2005). Differentially expressed proteins analyzed using 2-D electrophoresis showed up-regulation of 24 proteins and down-regulation of 19 proteins in shrimp hemocytes at 24 h post WSSV infection (Li et al. 2014). Protein profiling of 48 h post WSSV infected

P.vannamei gut revealed up-regulation of 75 proteins when compared to uninfected controls (Wang et al. 2007).

Most of the above mentioned studies were limited to one or few shrimp tissues and their analysis at one or few post-infection time-points. This study is an attempt to analyze the effect of progressive WSSV infection on various shrimp tissue protein and protease profiles at different time intervals post-infection. This study enriches the understanding of WSSV pathogenesis as it demonstrates the significant changes in both proteases and protein profiles of infected shrimp tissues when compared to uninfected shrimp tissue samples. Further analysis of these findings could possibly result in formulating new significant strategies against WSSV and hence helping in securing the shrimp aquaculture industry from WSD.

Materials and methods

Collection of experimental animals and screening

Fifty healthy, specific pathogen free shrimps (*P.vannamei*) weighing 20–25 g each, were collected from the local farm in Chennai and were maintained in well-aerated 500ltr Fibre Reinforced Plastic (FRP) tanks at room temperature (24–29 °C) with salinity of 20–25 ppt. Shrimps were fed with commercial feed (CP aquaculture) ad-libitum. For screening the presence WSSV infection, pleopods of five randomly picked shrimps were subjected to genomic DNA isolation by QIAamp DNA kit (Qiagen) as per the manufacturer's protocol. 20 ng of the isolated shrimp genomic DNA was used as template in PCR analysis using VP28 primers VP28-F 5'-GCGCGCGGATCCAAT CATGGATCTTTCTTTCAC-3', VP28-R 5'-GCGCGCGAATTCTTACTCGGTCTCAGTCC-3' in the PCR program of 95 °C for 5 min, 34 cycles of 95 °C for 30s, 55 °C for 30s, 72 °C for 40s and final extension of 72 °C for 5 min. The amplicons were then analyzed by 1 % agarose gel electrophoresis run at 100 volts for 20 min. After confirming the absence of WSSV infection, the shrimps were acclimatized in the same conditions for 7 days prior to WSSV challenge.

WSSV challenge experiment

After acclimatization, 10 shrimps were injected with 100 µl of 1X PBS (1.8 mM KH₂PO₄, 10 mM Na₂HPO₄, 2.7 mM KCl, 137 mM NaCl, pH-7.4) and were maintained separately as uninfected controls. Remaining shrimps were injected intramuscularly in the third ventral abdominal segment with 100 µl of 10⁻⁴ dilution WSSV inoculum, prepared from infected hemolymph as described elsewhere (Sahul Hameed et al. 2006). In the dose standardization experiment, it was found that 100 µl of 10⁻⁴ dilution WSSV infected hemolymph results in 100 % mortality within 3–6 days post injection (Results

not shown). Five shrimps were randomly sacrificed from WSSV infected group at different time intervals (0, 6, 12, 18, 24, 48, 72 and 96 h) and for the experimental control, five shrimps from uninfected group were sacrificed at 96 h time-point. The samples (Gills, Muscle (site of injection), Gut and Hepatopancreas) were dissected from sacrificed shrimps and collected in 1.5 ml microfuge tubes, followed by immediate immersion in liquid nitrogen for instant freezing and then preserved at -80°C freezer until further experimentation. The pleopods of the moribund shrimp were subjected to genomic DNA isolation followed by PCR analysis using VP28 primers as mentioned above to confirm the WSSV infection in the virus injected group.

SDS-PAGE analysis

Preserved tissue samples (50 mg) collected at various time-points post-injection from both infected and uninfected shrimps were taken in triplicates representing 3 individual shrimp in fresh 1.5 ml microfuge tubes and homogenized using micro pestle in 200 μl of TN buffer (20 mM Tris HCl, 400 mM NaCl pH-7.4). The homogenate was centrifuged at 8,000 g for 10 min at 4°C . The supernatant was collected in fresh 1.5 ml microfuge tube and the total protein content was estimated using Quick StartTM Bardford Protein Assay Kit (Bio-Rad) as per the manufacturer's protocol. Equal amount of estimated protein of each tissue sample was prepared in 3:1 dilution with 4X laemmli loading buffer (62.5 mM Tris-HCl, pH 6.8, 10 % glycerol, 1 % SDS, 10 % β -mercaptoethanol, 0.005 % bromophenol blue) and denatured by heating at 100°C in dry bath incubator (Labnet International Inc.) for 10 min. The prepared samples were loaded in 12 % SDS-PAGE and run at constant voltage of 100 V till the dye-front reaches the bottom of the gel. The gel was then stained with Coomassie Brilliant Blue (CBB) G-250 staining solution (0.25 % CBB dye in 4.5:4.5:1 ratio of methanol, water and acetic acid) for 20 min, and destained with destaining solution (4:4:2 ratio of methanol, water and acetic acid) till clear protein bands could be visualized.

In-gel gelatin zymogram

Equal amount of estimated protein of each tissue sample was prepared in laemmli loading buffer devoid of β -mercaptoethanol (reducing agent) and without heat denaturation. The prepared samples were loaded in 12 % SDS-PAGE impregnated with 0.1 % gelatin. The gel was run at constant voltage of 100 V till the dye-front reaches the bottom of the gel. For gill and muscle tissue samples, the gels were incubated in renaturation buffer (2.5 % TritonX-100) at room temperature in rocking condition for 30 min followed by the incubation in developing buffer (100 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl_2 pH 7.4) at 37°C for 40 min. While for the gut

and hepatopancreas tissue samples, the gels were incubated in renaturation buffer for 15 min and in developing buffer for 10 min. Post incubation, the gels were stained in CBB-G250 staining solution and destained with destaining solution as mentioned above.

Results and discussion

Understanding early stages of host biochemistry is crucial for not only understanding pathogenesis but also for rationalizing intervention processes. In this regard, shrimp transcriptomics has been extensively studied (Leu et al. 2007) to understand the WSSV pathogenesis but only a few studies report on the proteomics (Chai et al. 2010; Wu et al. 2007) and hardly any on proteases. It is well known that proteases form a critical proteomic entity both in defense and in response to infection; for animals with primitive immune system like shrimp, which are heavily dependent on innate immune mechanisms, this entity is all the more critical (Wang and Wang 2013). Even in these limited investigations, tissue-wise and temporal variations during post-infection are not reported. Hence, this study, for the first time, looks into the temporal variations (0 to 96 h starting from 6 h post-infection) of their proteome and proteases (selective proteome) in four major WSSV-targeted shrimp tissues for clues to identify early molecular events in this viral pathogenesis. The shrimps acquired for this study were free from WSSV infection, while the shrimps challenged with WSSV during the experiment showed positive signs of white spot disease, which was later confirmed by VP28 PCR analysis as shown in Fig. 1.

The changes in the WSSV-infected shrimp tissue protein/protease profile were categorized into four types: New (expressed as a result of WSSV infection), Up-regulated (showing higher expression level compared to uninfected control), Down-regulated (showing lower expression level compared to uninfected control) and Varied (up-regulation followed by down-regulation in due course of infection or vice-versa). This categorization of variations in protein and protease profiles of shrimp gill, muscle, gut and hepatopancreas is summarized in composite figures: Figs. 2, 3, 4 and 5, respectively.

Protein and protease analysis in gills

Analysis of protein profile variations of shrimp gills as a consequence of WSSV infection showed that there is drastic change in these profiles as shown in composite Fig. 2 (for original source, please refer to the [Online Resource 1](#)). The protein profile changes were visible right after 6 h of injection of the virus. However, this profile further changed in subsequent stages. Out of which the prominent changes were; distinct expression of ~ 21 kDa (new protein) at 18 h post-

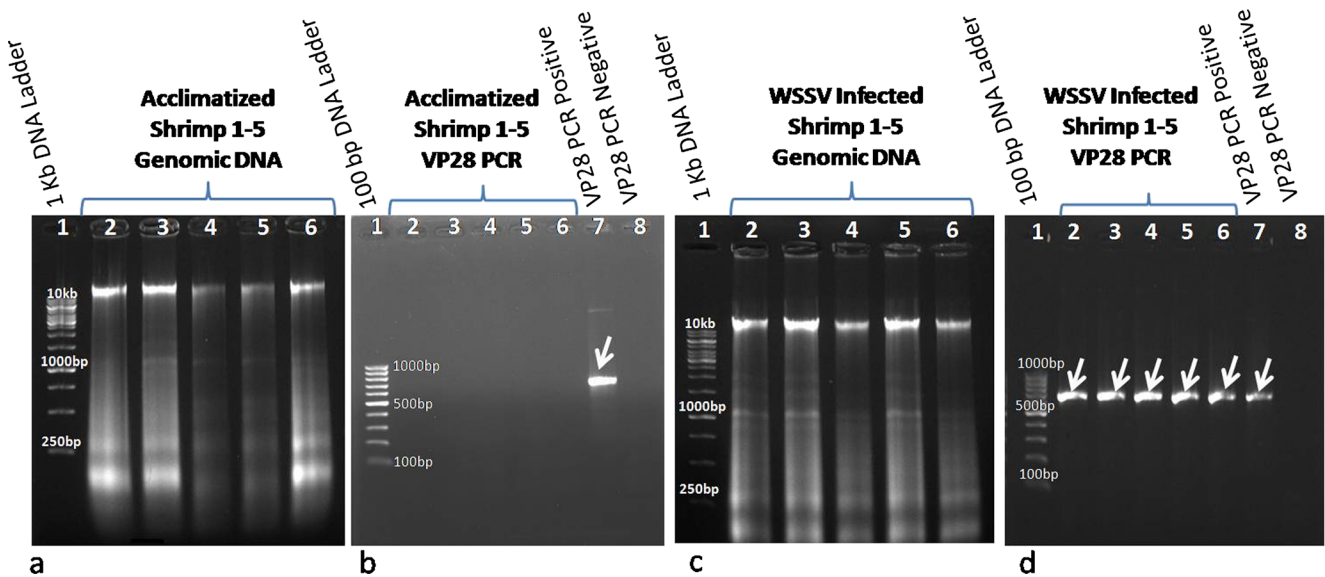


Fig. 1 WSSV screening of uninfected and WSSV challenged shrimps using VP28 PCR analysis **a** Unchallenged, acclimatized shrimp genomic DNA analysis on 0.8 % agarose gel. Lane 1–1 kb DNA ladder (Thermo Scientific). Lane 2 to 6–2 μ l of isolated genomic DNA from the five randomly selected, uninfected shrimps was loaded per well. **b** PCR analysis of uninfected shrimp genomic DNA on 1 % agarose gel. Lane 1–100 bp DNA ladder (Thermo Scientific). Lane 2 to 6–10 μ l PCR product of VP28 from uninfected shrimps was loaded in each well. Lane 7 and 8 - PCR positive and negative controls of VP28 gene,

respectively. **c** Genomic DNA analysis of WSSV infected moribund shrimp on 0.8 % agarose gel. Lane 1–1 kb DNA ladder (Thermo Scientific). Lane 2 to 6–2 μ l of the isolated genomic DNA of WSSV infected shrimps loaded per well. **d** PCR analysis of WSSV-infected shrimp genomic DNA on 1 % agarose gel. Lane 1–100 bp DNA ladder (Thermo Scientific). Lane 2 to 6–10 μ l PCR product of VP28 from WSSV infected shrimps was loaded in each well. Lane 7 and 8 - PCR positive and negative controls of VP28 gene, respectively

injection which got down-regulated to undetectable levels in subsequent stages. Another protein at ~39 kDa was variedly expressed throughout the infection. The progressive up-regulation of a prominent ~85 kDa protein is noteworthy.

The proteolytic profile of gills showed 12 proteases ranging from ~87 to ~21 kDa as shown in composite Fig. 2 (for original source, please refer to the [Online Resource 2](#)). Dramatic changes were observed as a result of WSSV infection, in that there was sudden drop in the number of proteases at 6 h with only a few proteases being moderately expressed. Two of the prominent proteases at ~25 and ~23 kDa were down-regulated at 6 h post-injection and were further down-regulated at late stage of infection. A protease of about 78 kDa remained at somewhat higher level than at the time of injection throughout the infection and at the time of death. These profound changes in this important organ appear to be an important early event in the pathogenesis of WSSV.

Gills are reported to be immunologically significant organ for marine animals like fish as evident from recent descriptions of immunohistological features and it is also known to be probably the first organ to sense and respond to the viral infection (Austbø et al. 2014). Though previous studies had reported the presence of immune genes in shrimp gill (Tassanakajon et al. 2013), it is now more evident through our study that, shrimp gill is the first to respond to WSSV

infection among the organs we studied. In gill, the proteome and protease changes observed as early as 6 h post-injection, whereas in other organs, the significant changes could be observed after 12 and 24 h post injection.

Protein and protease analysis in muscle

In this study shrimps were infected by injecting the virus in the abdominal muscle and therefore the muscle tissue at the site of infection was chosen for analysis of protein and protease profile variation under the presumption that it should show dramatic changes. However, contrary to the expectation, as can be seen from the composite Fig. 3 (for original source, please refer to the [Online Resource 3](#)), there are apparently not many differences, unlike in the case of gills. A few changes were observed at 6 h when the proteins ~170 kDa and below ~17 kDa remained variedly expressed. Another protein ~80 kDa got progressively up-regulated from 6 h post-injection to 96 h post-injection.

There was almost little proteolytic activity observed in the muscle and there was no significant alteration to this status throughout the infection period as shown in composite Fig. 3 (for the original source, please refer to the [Online Resource 4](#)). Lack of proteases in muscle tissue was reported earlier (Sriket et al. 2011). Obviously, WSSV infection did not induce proteases in muscle. Interestingly, contrary to the expectations, both from physiological consequences and pathogenesis,

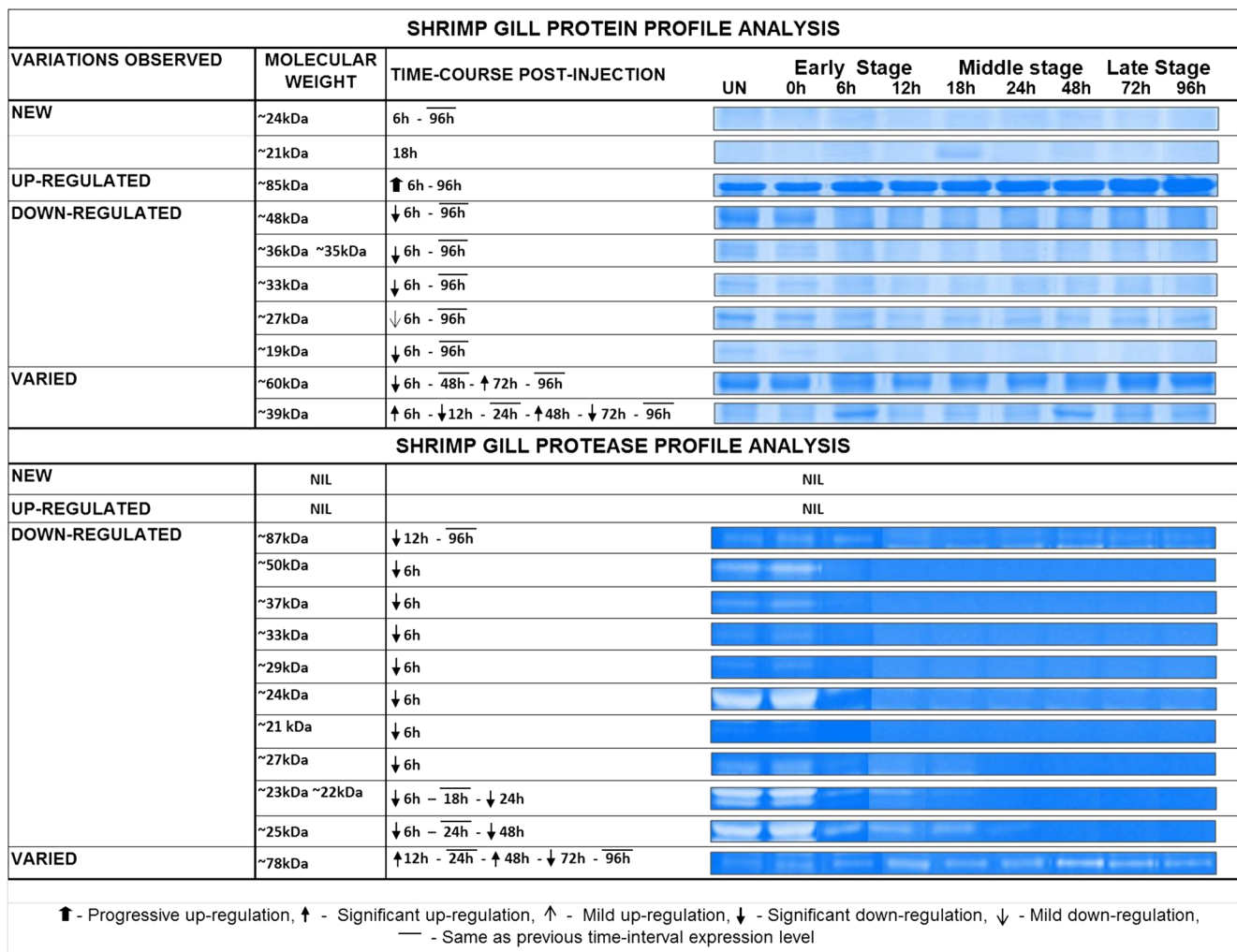


Fig. 2 Temporal variation analysis in shrimp gill protein and protease profile during WSSV infection

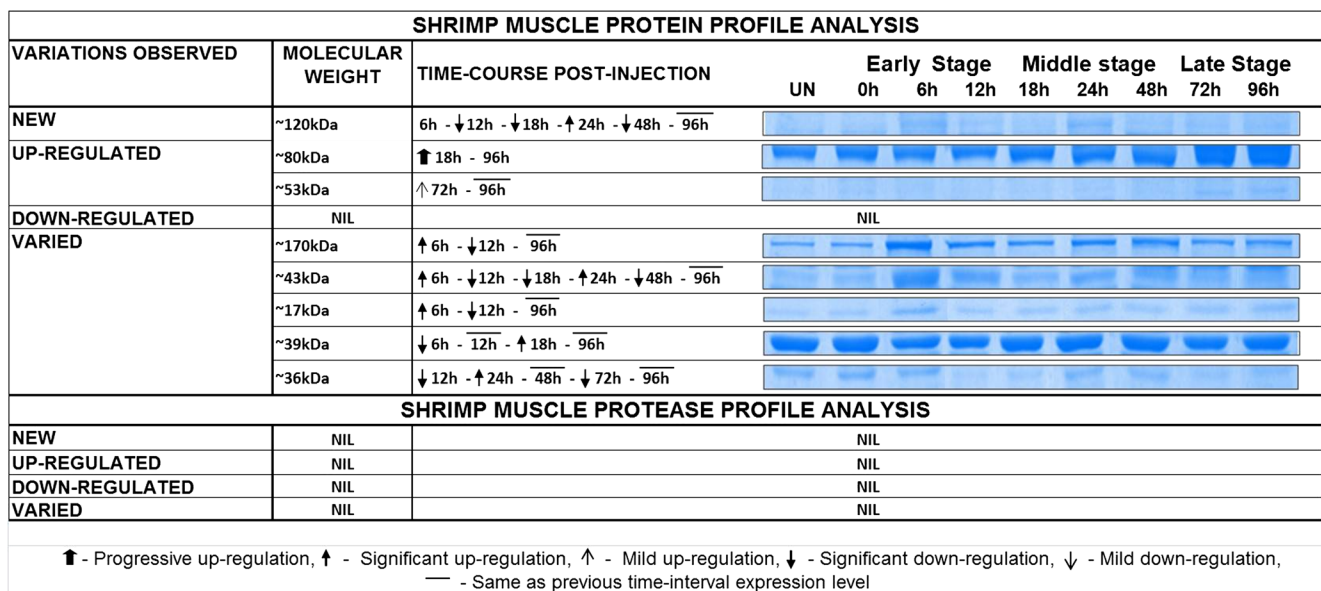


Fig. 3 Temporal variation analysis in shrimp muscle protein and protease profile during WSSV infection

muscle appeared to be undergoing very few proteomic changes with negligible protease activity, indicating that muscle might not be the major target tissue of the virus.

Protein and protease analysis in gut

Gut has remained an organ of interest in viral pathogenesis, for in field conditions oral route is the major mode of infection and subversion of mucosal immunity seems to play critical role in the pathogenesis of the virus. Gut is also known to be the site of digestive enzymes and prior to death, lack of appetite and weakness may be related to drastic biochemical and physiological changes in this organ. The variations of protein were shown in composite Fig. 4 (for original source, please refer to the Online Resource 5). The changes in the protein profiles, especially above 35 kDa, were observed as up and down-regulation of proteins. Significantly, expression of a ~54 kDa protein preceded the death phase.

As expected, a number of proteases could be identified in the gut as shown in composite Fig. 4 (for original source, please refer to the Online Resource 6). But, most strikingly, almost all, including the prominent ones at about 20 kDa, were down-regulated to very low levels, especially coinciding with the time (12 h and afterwards) of lack of appetite.

Protein and protease analysis of hepatopancreas

Hepatopancreas is another significant organ in terms of the viral pathogenesis. The proteome of this organ undergoes drastic changes during the entire time course of this study as shown in composite Fig. 5 (for original source, please refer to the Online Resource 7). The changes were marked by progressive stages of morbidity and mortality. Though the changes were subtle at 6 h point, the changes from the 12 h point were significant. During the later stages, especially at death stages, hardly two proteins were visible in the gel, thus showing drastic reduction in protein expression.

One of the surprising results of this study was the presence of a number of highly active proteases in this organ as shown in composite Fig. 5 (for original source, please refer to the Online Resource 8). The amount of shrimp hepatopancreatic sample used for protease profile analysis had to be cut down to nanogram level in order to obtain a good zymogram. Even as low as 10 ng of the total protein tissue sample was sufficient to show up a number of proteases especially some of them at molecular sizes less than 25 kDa. Like the proteins, the proteases were also down-regulated gradually from 12 h onwards. Prominent low molecular size proteases variedly expressed during the infection period. Hepatopancreas has been shown to be an important immune organ of shrimp in mounting both humoral and cellular immune responses (Pan et al. 2005).

SHRIMP GUT PROTEIN PROFILE ANALYSIS									
VARIATIONS OBSERVED	MOLECULAR WEIGHT	TIME-COURSE POST-INJECTION	Early Stage		Middle stage		Late Stage		
			UN	0h	6h	12h	18h	24h	48h
NEW	NIL		NIL						
UP-REGULATED	~64kDa ~62kDa	↑ 12h - 96h							
DOWN-REGULATED	~53kDa	↓ 48h - 96h							
	~40kDa	↓ 48h - 96h							
	~43kDa	↓ 48h - 96h							
VARIED	~180kDa	↓ 12h - 24h - ↑ 48h - 96h							
	~100kDa	↓ 12h - ↑ 18h - 24h - ↓ 48h - 72h - ↑ 96h							
	~85kDa	↑ 12h - ↓ 18h - 24h - ↓ 48h - 72h - ↑ 96h							
	~55kDa	↑ 12h - ↓ 18h - 24h - ↑ 48h - 72h - ↓ 96h							
SHRIMP GUT PROTEASE PROFILE ANALYSIS									
NEW	NIL		NIL						
UP-REGULATED	NIL		NIL						
DOWN-REGULATED	~125kDa ~105kDa ~83kDa	↓ 6h							
	~54kDa ~27kDa ~23kDa	↓ 12h - ↓ 18h							
	~24kDa	↓ 12h - ↓ 18h - ↓ 24h							
VARIED	~25kDa	↓ 6h - ↓ 12h - ↓ 18h - ↓ 24h - ↑ 48h - 96h							

↑ - Progressive up-regulation, ↑ - Significant up-regulation, ↑ - Mild up-regulation, ↓ - Significant down-regulation, ↓ - Mild down-regulation, — - Same as previous time-interval expression level

Fig. 4 Temporal variation analysis in shrimp gut protein and protease profile during WSSV infection

SHRIMP HEPATOPANCREAS PROTEIN PROFILE ANALYSIS									
VARIATIONS OBSERVED	MOLECULAR WEIGHT	TIME-COURSE POST-INJECTION	Early Stage			Middle stage		Late Stage	
			UN	0h	6h	12h	18h	24h	48h
NEW	~116kDa	18h - 24h							
UP-REGULATED	NIL		NIL						
DOWN-REGULATED	~110kDa ~100kDa ~96kDa ~85kDa ~30kDa	↓ 48h							
	~33kDa ~28kDa ~27kDa	↓ 12h - 24h - ↓ 48h							
	~25kDa	↓ 12h - 24h - ↓ 48h - 96h							
	~19kDa ~18kDa	↓ 12h - 24h - ↓ 48h							
	~63kDa ~60kDa ~57kDa	↓ 12h - 24h - ↓ 48h							
VARIED	~40kDa	↓ 12h - ↑ 18h - 24h - ↓ 48h							
	~32kDa	↑ 6h - ↓ 12h - ↑ 18h - 24h - ↓ 48h							
SHRIMP HEPATOPANCREAS PROTEASE PROFILE ANALYSIS									
NEW	NIL		NIL						
UP-REGULATED	NIL		NIL						
DOWN-REGULATED	~32kDa	↓ 6h - 12h - ↓ 18h							
	~25kDa	↓ 6h - ↓ 12h							
VARIED	~50kDa	↓ 6h - ↓ 12h - 48h - ↓ 72h - ↑ 96h							
	~24kDa ~23kDa ~22kDa	↑ 12h - 24h - ↓ 48h - 96h							
	~21kDa ~20kDa	↑ 72h - ↓ 96h							

↑ - Progressive up-regulation, ↑ - Significant up-regulation, ↑ - Mild up-regulation, ↓ - Significant down-regulation, ↓ - Mild down-regulation, — - Same as previous time-interval expression level

Fig. 5 Temporal variation analysis in shrimp hepatopancreas protein and protease profile during WSSV infection

Therefore, the correlation between its potent proteolytic activity and innate immunity is interesting.

The notable observation is the down regulation of some of the tissue proteases from early stages within the first 24 h post-injection. Eleven proteases from gill and gut, and three proteases from hepatopancreas were down-regulated during the early stage of WSSV infection. Up-regulation of proteolytic inhibitors has been reported (Tonganunt et al. 2005). The temporal down-regulation of gut proteases correlated with loss of appetite in shrimp during the intermediary stage of WSSV infection. Though the significance of these down-regulations in terms of molecular mechanisms of host-pathogen interactions and molecular pathogenesis is not clear, the study has opened up leads to look into in understanding such processes. Identification of the proteins and proteases using mass spec analysis and partial sequencing will greatly enhance our knowledge to identify disease markers and intervention strategies thus protecting the shrimp aquaculture industry from WSSV impact.

It is further noteworthy that the shrimp hepatopancreas possessing such highly active proteases is often discarded as shrimp waste but it could be used as cost-effective alternative for industries that are dependent on protease applications especially in food processing

industry. In these industries, the proteases have wide applications like selective tissue degradation, meat tenderization, fermentation and curing of fish, production of hydrolyzed products, extraction of pigments, recovery of lipids from fish processing waste, coagulation of protein and waste management (Rai et al. 2014; Shahidi and Kamil 2001; Swapna et al. 2010). Furthermore shrimp proteases could also be used for the production of bio-active peptides from food, which is a major area of interest in functional food research (Sanjukta et al. 2015).

Conclusions

In this study we have shown that gill is one of the first organs to respond against WSSV. Shrimp protein and protease profiles get drastically altered and most of the shrimp tissue proteases were down-regulated during progressive WSSV infection which can be a potential strategy of WSSV pathogenesis. Among the shrimp tissues, hepatopancreas is found to be enriched with highly active proteases, which could be used as effective alternative in several protease dependent industries especially in the food industry.

Acknowledgments We thank (the late) Dr V. Murugan, Centre for Biotechnology, Anna University for his initial contributions and we dedicate the work to his memory. The financial support of the DBT for InNoVacc project (Grant – BT/AAQ/Indo-Norway/183196/2007) is gratefully acknowledged. The investigators of InNoVacc project are acknowledged for their valuable support. We thank Mr. S. Kumar, Research Scholar, Centre for Biotechnology for his help in procuring the shrimps and in the experimentation. PAK thanks DBT for the Senior Research Fellowship.

Conflict of interest The authors declare that they have no competing interests.

Authors contribution PAK and KS participated in design, interpretation of data and revision of manuscript. PAK performed the shrimp study and tissue analysis, and drafted the manuscript.

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