Proteomic analysis of differentially expressed protein in hemocytes of wild giant freshwater prawn *Macrobrachium rosenbergii* infected with infectious hypodermal and hematopoietic necrosis virus (IHHNV)

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A R T I C L E   I N F O

A B S T R A C T

Epizootic diseases cause huge mortality and economical loses at post larvae stages in freshwater prawn aquaculture industry. These prawns seem less susceptible to viral diseases except for infectious hypodermal and hematopoietic necrosis virus (IHHNV). During viral infection in prawns, hemocytes are the primary organ that shows immunological response within the early stages of infection. We applied proteomic approaches to understand differential expression of the proteins in hemocytes during the viral disease outbreak. To aid the goal, we collected *Macrobrachium rosenbergii* broodstocks from the local grow out hatchery which reported the first incidence of IHHNV viral outbreak during larvae stage. Primarily, application of the OIE primer targeting 389 bp fragments of IHHNV virus was used in identification of the infected and non-infected samples of the prawn breeding line. Analysis of two-dimensional gel electrophoresis showed specific down-regulation of Arginine kinase and Sarcoplasmic calcium-binding protein and up/down-regulation of Prophenoloxidase1 and hemocyanin isoforms. These proteins were validated using semi quantitative RT-PCR and gene transcripts at mRNA level. These identified proteins can be used as biomarkers, providing a powerful approach to better understanding of

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the immunity pathway of viral disease with applications in analytic and observational epidemiology diagnosis. Proteomic profiling allows deep insight into the pathogenesis of IHHNV molecular regulation and mechanism of hemocyte in freshwater prawns.

Introduction

Viral diseases have not been a serious threat to the freshwater prawn aquaculture until recently when a viral disease called the white tail disease began afflicting adult *Macrobrachium rosenbergii*. The disease is caused by the *M. rosenbergii* nodavirus (MrNV) and extra small virus (XSV) (Bonami and Sri widada, 2011). Another virus, IHHNV has also been reported to cause mass mortalities of *M. rosenbergii* at the juvenile stage (Hsieha et al., 2006). In contrast, viral diseases caused the largest loss in the aquaculture of penaeids. In recent years, worldwide trafficking and industrialization of known farmed species, caused infections to spread easily and rapidly, leading to economic losses mostly during acute epizootics (Sudhakaran et al., 2007) In Malaysia, there were reported cases of mortality at hatchery during nursery phase. In this study, *M. rosenbergii* was infected with infectious hypodermal and hematopoietic necrosis virus (IHHNV) which was listed by OIE (Office of International Epizootics of World Organization for Animal Health) (OIE, 2010) was used to show its international significance in aquaculture. It is a shrimp virus that has recently been classified as *Penaeus stylirostris brevidensovirus* (PstDNV) from the family Parvoviridae (Tattersall et al., 2005). IHHNV was first described from high mortality disease outbreaks in cultured *Penaeus (Litopenaeus) stylirostris* in the United States (Lightner et al., 1983). The pathogenesis of IHHNV varies in hosts it infects. In *M. rosenbergii*, IHHNV can cause mortalities mostly in juvenile's stage. However, the effect of IHHNV infection in adult *M. rosenbergii* is unknown. However, the *M. rosenbergii* and other shrimps that share a similar immune system which is innate immune response have same response to disease. In the case of bacterial infection, both the defense systems, cellular and humoral immune responses are activated (Imler and Hoffmann, 2000) by host surface conserved protein recognition. This determines the pathogens such as ß-1,3-glucans from fungi peptidoglycan (PGN), lipoteichoic acid (LTA) and lipopolysaccharide (LPS) from bacteria (Medzhitov and Jnr, 2002) to be eliminated. However, these proteins are absent when the prawn has a viral infection. Until now the molecular mechanisms of IHHNV pathogenesis is virtually unknown. Immune-related proteins and antimicrobial peptides are synthesized in hemocytes and transferred into the plasma. Both proteomic and mRNA-based approaches were combined to study the innate immunity complexity (Levy et al., 2004). For the investigation of the real mediator of innate immunity for fluids such as hemolymph, at the protein level, proteomic analysis is required and genomic approaches are not suitable. *Drosophila* hemolymph protein 2D map was constructed as a reference database for researchers to investigate the protein level changes occurring at different physiological conditions or developmental stages after infection (Vierstraete et al., 2003). The present study was aimed to gain more insights to hemocyte's protein function in the innate immunity of *M. rosenbergii* upon IHHNV infection. Meanwhile, the potential role of identified proteins in 2D-gels was discussed after IHHNV infection. These identified proteins can be used as biomarkers, providing a powerful approach to have better understanding of the immunity pathway of viral disease with applications in analytical and observational epidemiology diagnosis. The present research is the first attempt to document the potential biomarkers in *M. rosenbergii* which were not reported yet.

Material and methods

Sample collections

The collection of *M. rosenbergii* broodstocks was from the same local grow out farms reporting the first incidence of viral outbreak during juvenile stage at the hatchery phase in September 2009 (Hazreen Nita et al., 2012). A total of 50 wild adult broodstocks were sampled randomly from the brooders used in hatchery systems. The study was carried out in the National Prawn Center of Fisheries in Malaysia. The research is part of a breeding program initiated by the Department of Fisheries. The work on breeding and genetics is a joint

collaboration of both parties. No specific permission was required to carry out the activity as the study did not involve any endangered or protected species. The animals were brought in for a breeding program and it was compulsory to screen them for disease under the biosecurity for food safety. During the routine screening, infectious prawns were killed and discarded immediately. The sampling method only targeted prawns that followed through the biosecurity protocols. The selected individuals were transported to Monash Medical School, Kuala Lumpur, Malaysia. The serum and pleopods of each individual were collected for virus screening and proteomic study.

Serum and DNA isolation

Hemolymph was collected through puncture using 1.5 ml syringe and centrifuged at 12,000 rpm at 4 °C for 15 min. The supernatant serum (100–150 μl) was transferred in to a 1.5 ml micro tube and kept in −80 °C for proteomic use. A frozen DNA sample (−80 °C) extracted from *M. rosenbergii* and infected with IHHNV was used as a local reference sample. Total DNA was extracted from pleopods of the *M. rosenbergii* samples using the C-TAB method (Sambrook and Russell, 2001). DNA was quantified by UV spectrophotometry (A_{260} nm) and kept in −80 °C for virus detection.

Polymerase chain reaction (PCR) of IHHNV infection and analysis

Fragments of IHHNV genomic sequences from *M. rosenbergii* samples were amplified by using the newly designed IHHNV 389 F/R specific primers which were previously reported to discriminate infectious IHHNV from non-infectious (Hazreen Nita et al., 2012). PCR was conducted in a 30 μL reaction volume containing 3 μL PCR buffer (10×), 1 μL MgCl2 (50 mM), 0.6 μL dNTP (10 mM), 1 μL of each forward and reverse primer (10 mM), 22.2 μL water, 0.2 μL (1U) Taq DNA polymerase, and 1 μL DNA template. Primer IHHNV389F/R, being developed specifically to detect infectious-type IHHNV but not IHHNV related sequence integrated into the *Penaeus monodon* genome (Hsieha et al., 2006; Tang et al., 2007). The PCR amplification protocol consisted of initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 2 min, with a final extension at 72 °C for 7 min.

The PCR amplification protocol consisting of initial denaturation at 94 °C for 5 min, was followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 50 s, with a final extension at 72 °C for 2 min, and the expected amplicon size was 389 bp. The gel photo shows detection of IHHNV in the infected hemocytes using specific primer and Crude IHHNV virus as positive reference line 1, (P). Deionized water was used as negative reference line2, (N). Non-infected samples (N-IN) showed no PCR product line 10 to 14. The 389 bp PCR product of swimming leg DNA was observed in infected samples line 4, (IN) (Fig. 1).
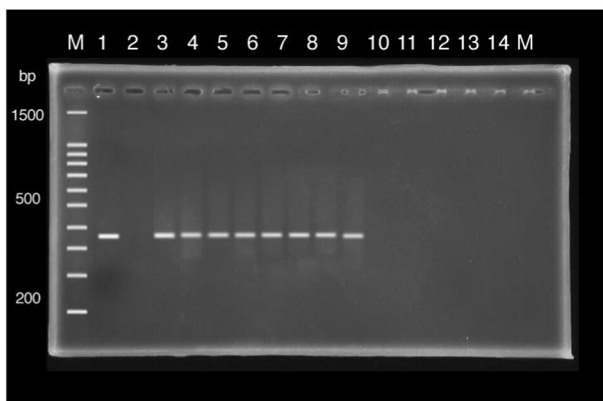

Fig. 1. Detection of IHHNV in the infected Hemocyte. Detection was done by suing specific primer and Crude IHNNV virus as positive reference line 1. Deionized water was used as negative reference line 2. 11 to 14 non-infected samples (N-IN) did not show any PCR product in their line. The 389 bp PCR product of swimming leg DNA was observed in infected samples line 3 to 10, (IN).

Protein quantification

The protein concentration in serum was estimated using Bradford Assay for protein quantification (Bradford, 1976). Bradford reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid. The mixture was then topped to 1 L with deionized water and stirred overnight. The final mixture was filtered and stored in room temperature away from light.

Two dimensional gel electrophoresis (2-DE)

Rehydration of the dry strips was done by rehydration solution at 250 μl per strip in the presence of 8 M urea, 2% v/v CHAPS, trace of bromophenol blue, 50 mM DTT, 2.5 μl IPG buffer and dry-strip cover liquid. The first dimension of 2-DE was performed in IPGphor IEF system. A total of 250 μg of proteins from each prawn was loaded onto each IPG strip (nonlinear pH 4–7; 13-cm long). Four IPG strips, presenting IHHNV infected prawns and four IPG strips, presenting non-infected prawns. The focused IPG strip was incubated for 15 min in an equilibration buffer containing 87% w/v glycerol, 60.06 Mw Urea, 2% w/v SDS, 50 mM Tris–HCl (pH 8.8), 100 mM DTT, and trace of bromophenol blue. Before running second dimension the strip was then further equilibrated for 15 min in a similar buffer, which replaced 100 mM DTT with 250 mM of iodoacetamide. The IPG strip was placed onto the top of 12.5% SDS-PAGE gel and sealed with hot agarose (1% w/v). After the run, the gel was fixed in a fixative solution containing 500 ml ethanol and 120 ml acetic acid, 500 μl formaldehyde 35%, 380 ml dH2O and stained with silver nitrate, containing 2 g silver nitrate, 759 μl formaldehyde 35%2.5, developed reaction with developing solution containing 60 g sodium carbonate, 4 mg sodium thiosulfate, 500 μl formaldehyde 35%, and 12% acetic acid as terminating solution.

Protein identification

Gel images were captured using a GS-800TM calibrated densitometer at a resolution of 360 pixels and was analyzed with the PDQuest software according to the protocols provided by the manufacture (BioRad). After scanning, spots of interest were excised from the gels and were digested using trypsin (Bradford, 1976). The gel pieces were washed with 100 mL of 25 mM NH_4HCO_3, dehydrated twice with 100 mL of acetonitrile, and dried with a Speed Vac evaporator before reduction (10 mM DTT in 25 mM NH_4HCO_3) and alkylation (55 mM iodoacetamide in 25 mM NH_4HCO_3). For the tryptic digestion, the gel pieces were resuspended in 3 gel volumes of trypsin (12.5 ng/L) freshly diluted in 25 mM NH_4HCO_3 and incubated overnight at 35 °C. The digested peptides were then extracted from the gel in a buffer containing 25% H_2O, 70% acetonitrile, and 5% HCOOH. The LC–MS/MS was preferred using CapLC system. The samples were concentrated on a precolumn, and the peptides were separated on a 15 cm 75 mm i.d. column packed with 3 mm 100 Å C18 PepMap (LC-Packings). The MS and MS/MS analyses were performed with two hybrid quadruple time-of-flights (Q-TOF) mass spectrometer equipped with a Z-spray ion source. LC/MS/MS raw data were processed automatically with the Protein Lynx Process module to create peak list.

Data analysis was performed with Mascot against The National Center for Biotechnology Information (NCBI) database. The peptide tolerance was set to 130 ppm for MS and set to 0.3 Da for MS/MS. Double and triple charged peptides were used for the research in database. One missed cleavage by trypsin is accepted, and prophenoloxidase and hemocyanin were set as variable modifications. The closest reference organism was the *Locusta migratoria* and *Gammarus roeseli*, respectively. The Mascot score cut-off value for a positive protein hit was set to 60 and peptide with a score below 40 were manually interpreted to validate or discard the identification.

Total RNA extraction and first strand cDNA synthesis

Total RNA was isolated from the tissues of each animal using TRI Reagent following manufacturer's protocol (Guangzhou Dongsheng Biotech, China). Total RNA was treated with RNase free DNA set (5 Prime GmbH, Hamburg, and Germany) to remove the contaminating DNA. The total RNA concentration was measured spectrophotometrically (NanoVue Plus Spectrophotometer, GE Healthcare UK Ltd, England). First-stranded cDNA was synthesized from total RNA by rev-transcriptase (Promega, USA) following the manufacturer's protocol.

Prophenoloxidase1 mRNA expression by qRT-PCR

The relative expression of *M. rosenbergii* prophenoloxidase in the Hemocyte was measured by quantitative real time polymerase chain reaction (qRTPCR). qRT-PCR was carried out using a ABI 7500 Real time Detection System (Applied Biosystems) in 20 µl reaction volume containing 4 µl of cDNA from each tissue, 10 µl of Fast SYBR_ Green Master Mix, 0.5 µl of each primer (20 pmol/ll) (MrProAE-III F1: AACAACTGGGA GTCACCCTTG AGT; MrProAE-III R2: TGACG GCATC TCTGGACAACTTCA) (Arockiaraj et al., 2012) and 5 µl dH2O. The qRT-PCR cycle profile was 1 cycle of 95 °C for 10 s, followed by 35 cycles of 95 °C for 5 s, 58 °C for 10 s and 72 °C for 20 s and finally 1 cycle of 95 °C for 15 s, 60 °C for 30s and 95 °C. The same qRT-PCR cycle profile was used for the internal control gene, elongation factor primers were designed based on EST of *M. rosenbegrii* (Table 1). After the PCR program, data were analyzed with ABI 7500 SDS software (Applied Biosystems). To maintain consistency, the baseline was automatically set by the software. All data were given in terms of relative mRNA expressed as means ± standard deviation. To compare the relative MrProI mRNA expression, statistical analysis was performed using one way ANOVA and mean comparisons were performed by Tukey's Multiple Range Test using SPSS 11.5 at 5% significant level (Fig. 3).

Results

PCR detection of IHHNV isolates results

A single 389 bp amplicon in this assay indicates the presence of IHHNV infectious and non-amplified one indicates non-infected samples. Infected samples *M. rosenbergii* from Malaysia gave positive PCR results (an amplicon of 389 bp) with primers IHHNV389F/R (Hazreen Nita et al., 2012).

2D electrophoresis analyses

Approximately 300 spots were reproducibly detected from thirty six independent gels. The data of each gel represented three repeats of three individual shrimps, four replicates for each infected and non-infected gels represented 12 shrimps for each group. A pH of 4–7 was found to be suitable for the separation of hemocytic proteins. Twenty proteins were identified by PDQuest protein analysis software as significantly up and down regulated (Fig. 2 A, B).

These twenty proteins were analyzed by MALDITOF-TOF mass spectrometer using a 5800 Proteomics Analyzer [AB Sciex]. Spectra were analyzed to identify protein of interest using Mascot sequence matching software [Matrix Science] with Ludwig NR database and taxonomy set to other Metazoa. Most of these proteins were successfully identified (except only spot 20 which remain unidentified) (Table 2). Then, these proteins were divided into 2 groups: Group I significantly up-regulated proteins (>0.5 fold compared to the control), and Group II significantly down-regulated proteins which showed significantly decreased expression level (<0.5 fold compared to the control).

Except for prophenoloxidase, all other identified proteins were not identified in *M. rosenbergii* previously, and the great majority of them shows homologies with proteins described in crustaceans and insects. Most of the spot value based on gel estimated molecular weight matched well with the corresponding theoretical value of homologous proteins isolated in other arthropods. However, several of them showed some variations of the expected values of molecular weight that can be explained by amino acid sequence modifications,

Table 1
Primer used in PCR and RT-PCR analysis.

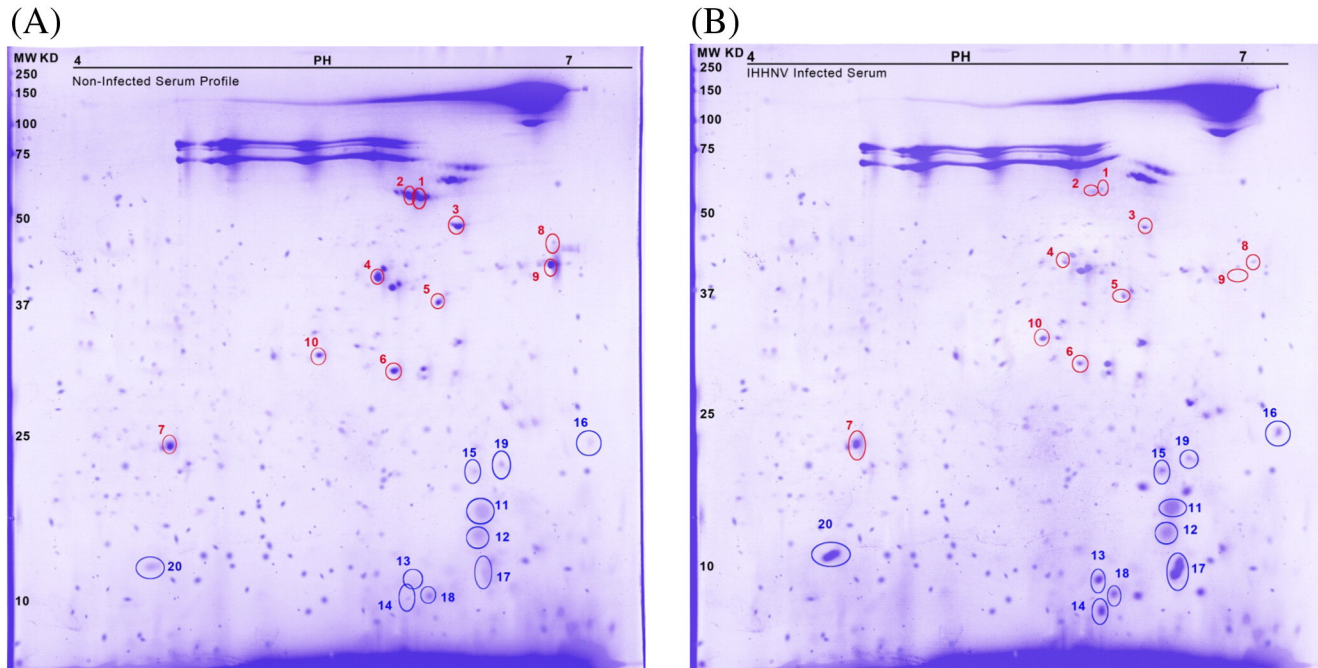| Name	Target	Sequence (5′–3′ direction)
IHHNV detection	309-F	TCCAACACTTAGTCAAAACCAA
	309-R	TGTCTGCTACGATGATTATCCA
Elongation factor-1	EF-1-F	CATCTCATCTACAAATGCG
	EF-1-R	ATGAAATCACGATGGCCTG
Prophenolxidase-1	PPo-1-F	TTCGATGCCTCTAACCGGGCGA
	PPo-1-R	GCGGGATGCGGTTACCGGATTCA

Fig. 2. 2-DE profiles from infected and non-infected hemocyte. Hemocyte profile of *M. rosenbergii* protein separation on two-dimensional electrophoresis gel over the pH range 4–7. Stained with coomasie brilliant blue. (A) Indicate non-infected group and (B) indicate IHHNV infected group. Consistency is indicated by the same relative location of numbered spots in both gels. Blue circles show up-regulated proteins and red circle indicates down-regulated proteins, based on PDQuest software analysis.
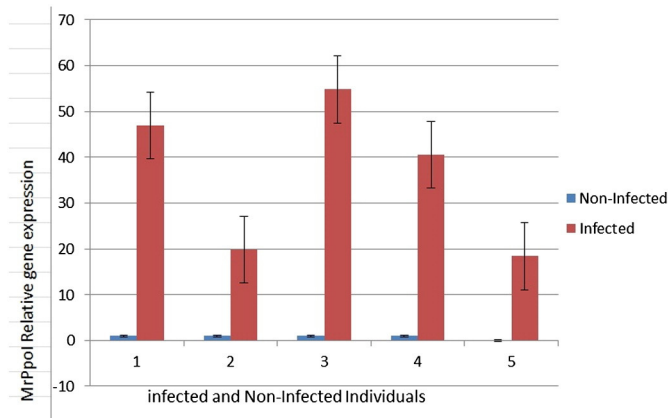
Fig. 3. Gene expression Prophenoloxidase1 mRNA by qRT-PCR. Data are expressed as a ratio of MrPpol mRNA expression in hemocyte. The different colors are statistically significant at $P < 0.05$ level by one-way ANOVA. The individual IHHNV infected of MrPpol mRNA expression in hemocyte are compared with five non-infected individuals.

including arginine kinase1 and enolase, in some less conserved regions compared with other species as it shown in Table 2.

The major problem of proteomic research is the lack of complete genome and proteome data in crustacean. These results confirm the possibility to identify proteins from animal's proteomes of which the genome are still poorly characterized. Actually, proteomics studies have been hindered by a relative lack of molecular proteomics information and tools suitable for high throughput assessment of protein expression (Rabilloud and Lelong, 2011).

Results of proteomic expression level compression by transcriptome data

Sample preparation & Solexa RNA-seq

Poly-A-containing mRNAs, extracted previously for both IHHNV-infected and non-infected shrimps, were purified using beads with oligo (dT) and cleaved into smaller pieces of RNA. The RNA fragments were reversely transcribed into cDNA using Invitrogen reverse transcriptase. This was followed by second strand cDNA synthesis using Invitrogen RNaseH and DNA polymeraseI by New England BioLabs. The cDNA libraries were constructed with accordance to the Illumina GA platform sequencing protocols. Transcriptome data was obtained from the IHHNV infected and non-infected hepatopancreas of *M. rosenbergii* and shows the expression of some of this protein at gene level. Based on transcriptome data it could be confirmed that arginine kinase and sarcoplasmic calcium-binding protein were both down regulated in infection level and that Prophenoloxidase1 and hemocyanin are both up/down regulated at infection level. This is another confirmation of the existence of these protein isomers with structural differences before and after infection (Table 2). This data also confirms that protein down/up regulation would happen in hemocyte.

Discussion

Proteomic analysis allows the present researchers to study the levels and patterns of protein expression in organs, cells or subcellular compartments. Such studies provide a better understanding of the cell's response to various external factors, such as viral and bacterial pathogens, under several different circumstances (Zhang et al., 2010).

To date the understanding of immune responses at a molecular level of invertebrates, especially shrimps, toward off viral infection is extremely limited. In this study, a gel-based proteomics approach was used to investigate the molecular responses of *M. rossenbergii* during IHHNV infection. Our results showed that an average 20 out of 300 protein spots in each prawn hemocyte gel underwent considerable alterations in their expression levels upon IHHNV infection as reported similarly in previous study by (Triwit et al., 2007).

Table 2

Comparative level of expression for protein spots from IHHNV-infected versus normal prawn determined using PDQuest image analysis software Biorad.

Spot	Protein name	Species	ACC. no.	Exp. Mw(KDa)/PI	Theor. Mw(KDa)/PI	MS/mps	Control	IHHNV Infected	IHHNV/Contr Mean ± SD
1	Carbonic anhydrase 2	*Bos taurus*	P00921	29.096/5–6	29.78/6.41	54			0.51 ± 0.00
2	Predicted protein (fragment)	*Nematostella vectensis*	A7RVD2	13.9716/5–6	16.97/10.4	44			0.75 ± 0.2
3	Enolase	*Callinectes sapidus*	Q6QWP6	39.852/6–7	85.39/5.89	42.6			0.34±/.00
4	Hemocyanin subunit	*Gammarus roeseli*	Q571R4	76.304/5–6	74/5.27	45			0.19 ± .00
5	Hemocyanin subunit L	*Penaeus japonicus*	B0L611	77.184/6–7	74/5.27	52			0.33 ± .03
6	Hemocyanin subunit 1	*Gammarus roeseli*	Q571R4	76.304/5–6	74/5.27	42			0.31 ± 0.01
7	Sarcoplasmic calcium-binding protein	*Procambarus clarkii*	Q2XT28	21.761/4–5	21.97/4.58	44			0.37 ± 0.01
8	Arginine kinase 1	*Neocaridina denticulata*	Q6QWP6	39.520/7	40/6.5	59.6			0.50 ± 0.00
9	Arginine kinase 1	*Neocaridina denticulata*	Q6QWP6	39.520/7	40/6.5	59.6			0.61 ± 0.00
10	Pro-phenoloxidase 1	*Locusta migratoria*	C0LV9	80.766/5–6	39.514/6.17	33.5			0.48 ± 0.00

11	Pro-phenoloxidase 1	*Locusta migratoria*	C0LV9	80.766/6–7	39.514/6.17	31.5			1.76 ± 0.02
12	Pro-phenoloxidase 1	*Locusta migratoria*	C0LV93	80.766/6–7	39.514/6.17	34			2.11 ± 0.02
13	Pro-phenoloxidase 1	*Locusta migratoria*	C0LV93	80.766/6–7	39.514/6.17	33.5			2.17 ± 0.02
14	Pro-phenoloxidase 1	*Locusta migratoria*	C0LV93	80.766/6–7	39.514/6.17	32.5			1.26 ± 0.00
15	Pro-phenoloxidase 1	*Locusta migratoria*	C0LV93	80.766/6–7	39.514/6.17	36			1.64 ± 0.02
16	Putative uncharacterized protein	*Ixodes scapularis*	B7Q192	38.314/6–7	26.92/9.38	16			1.91 ± 0.03
17	Putative uncharacterized protein	*Trichoplax adhaerens*	B3S8W0	36.166/6–7	26.92/6.5	20			1.89 ± 0.03
18	Pro-phenoloxidase 1	*Locusta migratoria*	B9VR33	80.766/6—7	39.514/6.17	24.5			2.99 ± 0.09
19	Hemocyanin	*Fenneropenaeus chinensis*	B9VR33	77.538/6—7	74/5.27	20.5			1.97 ± .02
20	NS	—	—	10—20/4—5	—	–			1.65 ± 0.02

Two-dimensional gel electrophoresis showed that the complex proteome of shrimp hemocyte expression was significantly up-regulated or down-regulated according to PDQuest image analysis software (Biorad) analysis in 2 cohorts with the serum IHHNV infected and non-infected shrimp from the collected wild population (Table 2) Twenty differentially expressed protein spots (ten up-regulated and ten down-regulated) were annotated via mass spectrometry. One of the samples from the 20 selected samples was not identified from the annotated databases. However, the other 9 up-regulated and 10 down-regulated protein spots were successfully annotated. In view of the fact that the complete genome sequence of the prawn species is still unavailable (Somboonwiwat et al., 2010), the origins of all these unidentified protein spots are uncertain, though it is likely that they are mostly prawn proteins.

Twelve of the total protein spots were annotated as isoforms of three proteins, hemocyanin, arginine kinase and prophenoloxidase2, which differ in either isoelectric points (pI) or mass (Table 2). Assuming that the different protein spots were correctly annotated (highly likely), these differences may be the result of portion post-translational modifications of the same protein population (Somboonwiwat et al., 2010).

These fluctuating proteins comprising most of the up-regulated proteins (i.e., hemocyanin, prophenoloxidase) had significant functional roles in the defensive mechanisms of the shrimp. The down-regulated proteins on the other hand had significant physiological effects in self-defense. These proteins play important roles in energy production and catabolism (arginine kinase1, Carbonic anhydrase2, Sarcoplasmic calcium-binding protein). After database analysis, the 20 detected proteins were categorized into 8 groups according to their cellular function. The divided groups are immune system related protein (33%), cell function and physiology (14%), energy production and catabolism (14%), antioxidants (5%), ATP-buffering and environmental stress (5%), calcium homeostasis (5%), cell structure (5%) and oxygen transportation (19%).

The innate immune response is a conserved trait shared by invertebrates and vertebrates, defending the organism against pathogens and parasites through detection and signaling pathways and the initiation of defense mechanisms (Hoffmann, 2003; Leulier et al., 2003) In crustaceans, circulating hemocytes, which play a significant role in the innate immune response, include the release of non-self-recognition proteins, clotting proteins, antimicrobial peptides, and prophenoloxidase. Activated phenoloxidase (tyrosinase) catalyzes the hydroxylation of monophenols to diphenols such as dopamine and dopa. In a second step (catecholoxidase reaction), the enzyme oxidizes the diphenol to an ortho-quinone, a highly reactive molecule that involve in encapsulation and melanization of foreign organisms. Phenoloxidase is also important in sclerotization (hardening) of the new exoskeleton after wound-repair and molting (NB, 1999; Soderhall and Cerenius, 1998).

The oxygen-transport protein hemocyanin functions as a phenoloxidase under certain conditions (Decker et al., 2001; Jaenicke and Decker, 2004). Hemocyanin belongs to the same family of copper proteins as phenoloxidase. Therefore, hemocyanin, hemocytes and phenoloxidase are related players in the crustacean immune response. The basic mechanisms for activation of arthropod hemocyanins and phenoloxidases and non-arthropod tyrosinases have recently been discussed (Matoba et al., 2006; Terwilliger, 2007).

In this study, two different types of hemocyanin subunits were observed in non-infected samples which included hemocyanin subunit 1 (Mass: 76,304) and L (Mass: 77,184). Another type of hemocyanin (Mass: 77,538) was observed in infected samples at the same pH with pro-phenoloxidase. This difference is likely to be the result of a response to the virus. These different subunits may be the activated form of hemocyanin which is the main substance in the prophenoloxidase activating system. It may also show the relative contributions of hemocyte phenoloxidase and hemocyanin in the physiological ratio.

The proPO activating system is an important immune response that produces melanin and a reactive oxygen species in order to kill, trap and eliminate invading microorganisms. For invertebrates, this system is integral in the immune response toward viral and bacterial infections. Two different proPOs, proPO-1 and proPO-2, which individually play crucial roles in the proPO activating system, have been identified in several shrimp species including *P. monodon*, *Fenneropenaeus chinensis* and *Litopenaeus vannamei* (Kunlaya Somboonwiwat et al., 2010). These proPOs have now been identified and their functional studies have been conducted in *M. rosenbergii* (Arockiaraja et al., 2011b).

Seven spots (Spots Number 10, 11, 12,13,14,15 and 18) were presumed to be the same protein (proPO-2) judged by their partial amino acid sequences and molecular masses, however, these proteins may have different PI and posttranslational modifications. Expression of proPO-2 (Spots Number 11, 12, 13 and 14) was up-regulated in infected hemocytes, while the other proPO-2 isoform (Spot Number 10) was

down-regulated, suggesting a possible PI altering posttranslational modification of the proPO-2 of spots 10, 11, 12, 13 and 9 (Somboonwiwat et al., 2010).

Since the up-regulated expression of proPO-2 was observed in non-infected samples, it can be hypothe-sized that the inactive form of proPO-2 is produced by the shrimp to restore the level of proPO-2 in the hemocyte in order for the shrimp to promptly fight against pathogenic microorganisms.

The up-regulation of hemocyanin may provide oxygen for an increase in energy production during viral replication. Massive damage of hemocyanin might be associated with IHHNV infections identical to infections of YHV (Triwit et al., 2007). The ion uptake mechanisms in shrimps have not been systematically studied, though in other groups of decapods crustaceans (e.g., crabs, lobsters and crayfish) specific ion transport proteins and transport-related enzymes which play a role in the active uptake of the major ions Na^+ and Cl^- have been identified (Mantel and L.L., 1983). One of the central proteins in this mechanism is believed to be the transport-related enzyme carbonic anhydrase (CA). CA is known to be present and active as catalytic enzyme in high levels in the gill catabolism. The activity of CA is sensitive to changes in environmental salinity. CA is essential to most biological process involving in any general mechanisms of cation–anion transport. Because of its general function and its universal presence in gill tissue of aquatic species, CA is believed to be critical to the osmoregulatory function of the gills of all aquatic invertebrates (Henry, 1984, 1988)

Shrimps are able to withstand a wide range of salinity fluctuations by maintaining their hemolymph osmotic and ionic concentrations within narrow levels. The enzyme carbonic anhydrase appears to be important in both hyper and hypo osmotic regulation because it is induced in response to exposure to both low and high salinities (Roy et al., 2007).

Enolase is a well-known enzyme involved in cell metabolism and associates with a multifunctional role in disease. The enolases family is composed of 3 isozyme subunits alpha, beta and gamma which can form homodimers or heterodimers. Alpha-enolase performs many functions in both eukaryotes and prokaryotes, and plays an important role in various pathophysiological processes. Antibodies (Abs) against alpha-enolase have been detected in individuals affected by a large variety of infectious and autoimmune diseases. Still, the reasons why the spectrum of associated diseases is so wide and the exact pathogenic role of this auto Abs remain unclear. Alpha-enolase may have a direct or indirect role in the immune response of shrimps to viral infection (Terrier et al., 2007). Further studies are still needed to improve our knowledge on the pathogenic role of alpha-enolase in viral diseases.

Arginine kinase is the most widely distributed phosphagen kinase, being found in all invertebrates (Suzuki et al., 1997). The phosphagen kinases have been studied primarily in muscle tissues, in which their function is to buffer the ATP supply during periods of high energy demand by regenerating depleted ATP supplies (Walliman et al., 1992).

In some tissues, phosphagen kinases may also function as energy shuttles between the mitochondria and the cytosol. This process is facilitated by the presence of separate isoforms in different parts of the cell (Bessman and Carpenter, 1985).

The insignificant contribution of mitochondrial arginine kinase to the total activity in the posterior gills suggests that arginine kinase serves mainly as an ATP buffer rather than as part of a high energy phosphate shuttle system. The immunolocalization of arginine kinase in the mitochondria of heart muscle cells in the blue crab Callinectes sapidus is of particular interest to the present study (Pineda and R, 1998), because it supports an earlier indication of a mitochondrial arginine kinase in the hepatopancreas of the same species (Chen and Lehnisger, 1973). In the present study, arginine kinase was a significantly down regulated protein in M. rosenbergii serum after IHHNV infection and it may show the impact of viruses on the cell energy system. The functional studies of these genes were validated by (Arockiaraja et al., 2011a). The most significant up-regulation was found to be for arginine kinase Spot Number 8 and 9. These two protein spots had identical peptide fragment sequences. However, arginine kinase Spot Number 8 which was constantly expressed subsequently was of a slightly higher MW than arginine kinase Spot Number 9. These two arginine kinases differ from each other in PI. Therefore, it is likely that these may represent separate alleles or isoforms under a separate regulation and not simple interchanges of posttranslational modifications.

Sarcoplasmic Calcium-binding Protein (SCP) is believed to function as the invertebrate equivalent of vertebrate parvalbumin, which is used to "buffer" cytosolic Ca^2. Its expression works as a function of molting stage in non-epithelial and epithelial tissues (Gao et al., 2006). SCPs from other crustaceans, including shrimp and lobster, similarly exist as dimers of two different polypeptide chains (Takagi and K, 1984a, 1984b), SCPs

exhibit a high degree of polymorphism and evolutionary drift between phyla (Cox, 1990) having evolved to satisfy cell-specific needs.

The transcriptional level of arginine kinase1 and some of the proteins involved in the glycolytic pathway and immune response are correlated with the results from the 2-DE (Table 2). Although changes in the expression level of arginine kinase in an IHHNV challenge suggest that arginine kinase expression correlates closely with the shrimp's immune response (Arockiaraja et al., 2011a). The gene expression of ppo1 in comparative analysis is also another proof to support protein expression in IHHNV infected samples that shows ppo's important role in shrimp's immune response.

In conclusion, the pathogenesis of IHHNV varies in hosts it infects. This study is the first report showing nine up-regulated and ten down-regulated proteins identified after the IHHNV infection in surviving brood stocks. It also shows some key proteins such as propehnoloxidase and hemocyanin which play an important role in the survival of the prawns. Thus, this paves way for functional studies to elucidate the role of these proteins.

The data presented might help in understanding the interactions between the virus and host that caused by IHHNV infection in the normal metabolic hemostasis of shrimp. Future studies are necessary to determine whether the differences observed during IHHNV infection are because of virus replication or protein–protein interaction in host cells. Finally, the data might provide a deep insight into the pathogenesis of IHHNV molecular regulation and mechanism of hemocyte in shrimp. The deeper understanding of host response will now allow new strategies for control of virus in shrimp.

Acknowledgments

References

Arockiaraj, J., Easwvaran, S., Vanaraja, P., Singh, A., Othman, R.Y., S, B., 2012. Prophenoloxidase activating enzyme-III from giant freshwater prawn Macrobrachium rosenbergii: characterization, expression and specific enzyme activity. Mol. Biol. Rep. 39, 10. http://dx.doi.org/10.1007/s11033-011-0872-5.

Arockiaraja, Jesu, Vanarajaa, Puganeshwaran, Easwvarana, Sarasvathi, Singhb, Arun, Alinejaid, Tahereh, Othmana, Rofina Yasmin, Subha, B., 2011a. Gene profiling and characterization of arginine kinase-1 (MrAK-1) from freshwater giant prawn (Macrobrachium rosenbergii). Fish Shellfish Immunol. 31.

Arockiaraja, Jesu, Easwvarana, Sarasvathi, Vanarajaa, Puganeshwaran, Singhb, Arun, Othmana, Rofina Yasmin, Bhassua, S., 2011b. Prophenoloxidase activating enzyme-III from giant freshwater prawn Macrobrachium rosenbergii: Characterization, expression and specific enzyme activity. Mol. Biol. Report. 39, 1377–1386.

Bessman, S.P., Carpenter, C.L., 1985. The creatine-creatine phosphate energy shuttle. Annu. Rev. Biochem. 54, 831–862.

Bonami, J.R., Sri widada, J., 2011. Viral diseases of the giant fresh water prawn Macrobrachium rosenbergii: a review. J. Invertebr. Pathol. 106, 131–142.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chen, Chung-Ho, Lehnisger, A., 1973. Respiration and phosphorylation by mitochondria from the hepatopancreas of the blue crab (Callinectes sapidus). Arch. Biochem. Hiophysics 154, 449–459.

Cox, J.A., 1990. Calcium vector protein and sarcoplasmic calcium binding proteins from invertebrate muscle. pp. 83–107.

Decker, H., Ryan, M., Jaenicke, E., Terwilliger, N., 2001. SDS induced phenoloxidase activity of hemocyanins from Limulus polyphemus, Eurypelma californicum and Cancer magister. J. Biol. Chem. 276, 17796–17799.

Gao, Yongping, MGillen, Christopher, Wheatly, M.G., 2006. Molecular characterization of the sarcoplasmic calcium-binding protein (SCP) from crayfish Procambarus clarkii. Comp. Biochem. Physiol. 144 (B), 478–487.

Hazreen Nita, M.K., Kua, B.C., Bhassu, S., Rofina, Y.O., 2012. Detection and genetic profiling of Infectious hypodermal and hematopoietic necrosis virus (IHHNV) infections in wild berried freshwater prawn, Macrobrachium rosenbergii collected for hatchery production. Mol. Biol. Rep. 39 (4), 3785–3790.

Henry, R.P., 1984. The role of carbonic anhydrase in blood ion and acid-base regulation. Am. Zool. J. 24, 241–253.

Henry, R.P., 1988. Subcellular distribution of carbonic anhydrase activity in the gills of the blue crab, Callinectes sapidus. J. Exp. Zool. 245, 1–8.

Hoffmann, J.A., 2003. The immune response of Drosophila 426. Nature America Inc., pp. 33–38.

Hsieha, Chia Y., Chuanga, Po C., Chenb, Li C., Tuc, Chien, Chiend, Maw S., Huange, Kwo C., Tsaia, S.S., 2006. Infectious hypodermal and haematopoietic necrosis virus (IHHNV) infections in giant freshwater prawn, Macrobrachium rosenbergii. Aquaculture 258, 73–79.

Imler, J.L., Hoffmann, J., 2000. Signaling mechanisms in the antimicrobial host defence of Drosophila. Curr. Opin. Microbiol. 3, 16–22 (doi: 1061016/ S 13695274(99) 0004 5-4).

Jaenicke, Decker, 2004. Functional changes in the family of type 3 copper proteins during evolution. Chem. Biol. Chem. 5, 163–176.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J., Caroff, M., Lee, W., B, L., 2003. The Drosophila immune system detects bacteria through specific peptidoglycan recognition. Nat. Immunol. 4, 478–484.

Levy, F., Bulet, P., L, E.S., 2004. Peptidomic and proteomic analysis of the systemic immune response of Drosophila. Mol. Cell. Proteomics 3, 156–166. http://dx.doi.org/10.1074/MCP.M300114-MCP200.

Lightner, D.V., Redman, R.M., Bell, T.A., 1983. Infectious hypodermal and hematopoietic necrosis: a newly recognized virus disease of penaeid shrimp. J. Invertebr. Pathol. 42, 62–80.

Mantel, L.H., LL., F., 1983. Osmotic and ionic regulation. In: Mantel, L.H. (Ed.), The Biology of Crustacea. Internal Anatomy and Physiological Regulation 5, pp. 54–162.

Matoba, Y., Kumagai, T., Yamamoto, A., Yoshitsu, H., M, S., 2006. Crystallographic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. J. Biol. Chem. 281, 8981–8990.

Medzhitov, R., Jnr, J., 2002. Decoding the patterns of self and nonself by the innate immune system. Science 296, 298–300. http://dx.doi.org/10.1126/science.1068883.

NB, T., 1999. Hemolymph proteins and molting in crustaceans and insects. Am. Zool. 39, 589–599.

OIE, 2010. Aquatic Animal Health Code 13. World Organization for Animal Health.

Pineda Jr., A., R, E.W., 1998. Immunogold transmission electron microscopic localization of arginine kinase in arthropod mitochondria. J. Exp. Zool. 281, 73–79.

Rabilloud, Thierry, Lelong, C., 2011. Two-dimensional gel electrophoresis in proteomics. A tutorial. J. Proteome 74 (10), 1829–1841.

Roy, Luke A., Davis, Allen, Saoud, Patrick, Henry, R.P., 2007. Branchial carbonic anhydrase activity and ninhydrin positive substances in the Pacific white shrimp, *Litopenaeus vannamei*, acclimated to low and high salinities. Comp. Biochem. Physiol. 147 (Part A), 404–411.

Sambrook, Russell, 2001. Molecular Cloning: A Laboratory Manual 3.

Soderhall, Kenneth, Cerenius, L., 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. Curr. Opin. Immunol. 10, 23–28.

Somboonwiwat, Kunlaya, Chaikeeratisak, Vorrapon, Wang, Hao-Ching, Fang Lo, Chu, Tassanakajon, A., 2010. Proteomic analysis of differentially expressed proteins in *Penaeus monodon* hemocytes after Vibrio harveyi infection. Proteome Sci. 8 (39). http://dx.doi.org/10.1186/1477-5956-8-39.

Sudhakaran, R., Parameswaran, V., A.S., S. H., 2007. In vitro replication of *Macrobrachium rosenbergii* nodavirus and extra small virus in C6/36 mosquito cell line. J. Virol. Methods 146, 112–118.

Suzuki, Tomohiko, Kawasaki, Yoshitada, Furukohri, Takahiro, Ellington, R., 1997. Evolution of phosphagen kinase. VI. Isolation, characterization and cDNA-derived amino acid sequence of lombricine kinase from the earthworm Eisenia foetida, and identification of a possible candidate for the guanidine substrate recognition site. Biochim. Biophys. Acta 1348, 152–159.

Takagi, T., K, K., 1984a. Amino acid sequence of the beta chain of sarcoplasmic calcium binding protein (SCP) obtained from shrimp tail muscle. J. Biochem. 96, 59–67.

Takagi, T., K, K., 1984b. Amino acid sequence of alpha chain of sarcoplasmic calcium binding protein obtained from shrimp tail muscle. Biochem. Biophys. Res. Commun. 95, 1603–1615.

Tang, K.F.J., Navarro, S.A., D.V., L., 2007. PCR assay for discriminating between infectious hypodermal and hematopoietic necrosis virus (IHHNV) and virusrelated sequences in the genome of *Penaeus monodon*. Dis. Aquat. Org. 74, 165–170.

Tattersall, P., Bergoin, M., Booom, M.E., Brown, K.E., Linden, R.M., Muzyczka, N., P, T., 2005. Virus taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier, Amsterdam, pp. 353–369.

Terrier, Benjamin, Degand, Nicolas, Guilpain, Philippe, Servettaz, Amélie, GuillevinLoïc, Luc, M., 2007. Alpha-enolase: A target of antibodies in infectious and autoimmune diseases. Autoimmunity 6, 176–182.

Terwilliger, Nora B., 2007. Hemocyanins and the immune response: defense against the dark arts. Integr. Comp. Biol. 47 (4), 662–665.

Triwit, Rattanarojpong, Wang, Hao-Ching, Lo, Chu-Fang, Flegel, T.W., 2007. Analysis of differently expressed proteins and transcripts in gills of *Penaeus vannamei* after yellow head virus infection. Proteomics 7, 3809–3814.

Vierstraete, E., Cerstiaens, A., Baggerman, G., Van den Bergh, G., De Loof, A., L, S., 2003. Proteomics in Drosophila melanogaster: first 2D database of larval hemolymph proteins. Biochem. Biophys. Res. Commun. 304 (4), 831–838. http://dx.doi.org/10.1016/S0006-291x(03)00683-1.

Walliman, Theo, Markus, W.Y.S.S., Brdiczkar, Dieter, Nicolay, Klaas, Eppenberger, H.M., 1992. Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem. J. 281, 21–40.

Zhang, Jinkang, Li, Fuhua, Jiang, Hao, Yu, Yang, Liu, Chengzhang, Li, Shihao, Xiang, Jianhai, 2010. Proteomic analysis of differentially expressed proteins in lymphoid organ of *Fenneropenaeus chinensis* response to Vibrio anguillarum stimulation. Fish Shellfish Immunol. 29, 186–194.