SHORT COMMUNICATION

Analysis of differently expressed proteins and transcripts in gills of *Penaeus vannamei* after yellow head virus infection

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In this proteomic analysis of gills from yellow head virus (YHV)-infected *Penaeus vannamei*, we identified 13 spots with up-regulated protein expression levels and five spots with down-regulated levels. LC-nanoESI-MS/MS indicated that the up-regulated proteins included enzymes in the glycolytic pathway, the tricarboxylic acid cycle and amino acid metabolism. The other up-regulated proteins were arginine kinase, imaginal disk growth factor (IDGF) and a Ras-like GTP binding protein. By contrast, expression levels were reduced for an SCP-calcium binding protein (SCP), actin-1, a valosin-containing protein, and Rab11. Time-course assays by real time RT-PCR revealed no significant increase in mRNA level of glycolytic enzymes and arginine kinase. However, a significant decrease in SCP mRNA was observed. The present results are consistent with previously published work and suggest that a decrease in SCP expression may play an important role in the shrimp response to viral infections in general.

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Introduction

Yellow head virus is a highly virulent shrimp pathogen that can cause mass mortalities in farmed penaeid shrimp. It was first reported in farmed *Penaeus monodon* near Bangkok in early 1990 and subsequently in more southerly farming areas on both sides of peninsular Thailand [1]. It was the first extremely virulent pathogen reported for the black tiger shrimp, causing total crop losses within 3–5 days. YHV has a single-stranded RNA (ssRNA) genome of positive sense and has recently been classified in the genus *Okavirus* in a new family *Roniviridae* in the order *Nidovirales* [2, 3]. Although YHV was originally described from *P. monodon*, laboratory [4] and field studies [5] have shown that it is also highly virulent for other species of cultivated shrimp including *Penaeus vannamei*.

To identify the proteins involved in the host–viral interaction, 2-DE was used to compare protein expression in a main target organ, gills [6], from normal shrimp and YHV-infected shrimp. To avoid the variability associated with cultivated shrimp derived from captured, wild broodstock, we used domesticated specific pathogen free (SPF) *P. vannamei* as the experimental animals. Proteins differentially expressed in the

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Abbreviations: EF-1α, elongation factor-1α; **hpi**, hours postinjection; **IDGF,** imaginal disk growth factor; **SCP,** SCP-calcium binding protein; **WSSV,** white spot syndrome virus; **YHV,** yellow head virus

YHV-infected gills were analyzed by LC-nanoESI-MS/MS to identify those that might play a significant role in YHV pathogenesis.

Juvenile *P. vannamei* (4–5 g) derived from imported, SPF broodstock were maintained in a controlled environment and fed a high protein commercial feed (Charoen Pokphand Public Company) for at least 1 day before use in the challenge experiments. Shrimps were injected intramuscularly at the 6th abdominal segment with 100 μL of YHV stock diluted 500-fold in PBS (100 μL/shrimp). Control shrimps were injected with 100 μL of PBS. Shrimps in the YHV-challenged group were collected at 6, 12, 24, 36, and 48 h postinjection (hpi) to check for YHV infection using a commercial PCR detection kit (Farming IntelliGene Tech. Taiwan). When cumulative mortality reached 40–60% in the YHV-challenged group, gills were removed from the surviving YHV-infected shrimps and from control shrimp and kept in liquid nitrogen. For comparison, frozen gills were also prepared from parallel white spot syndrome virus (WSSV) challenge tests that were carried out as previously described [7]. Each frozen gill sample was then subjected to 2-DE. This was followed by manual excision and in-gel digestion of the protein spot as previously described [7]. The digested peptides of protein spots were then analyzed by LC-nanoESI-MS/MS, and proteins were identified by MS/MS ion search using the search program MASCOT and NCBI and Swiss-Prot sequence databases using parameters described previously [7].

YHV was first detected in gills of the YHV-challenged shrimps at 12 hpi, and this YHV-challenged group approached 50% cumulative mortality by 48 hpi. At this time, 2-DE revealed 13 protein spots that appeared to be up-regulated and five proteins spots that appeared to be down-regulated relative to the YHV-negative PBS injected controls (Fig. 1). β-actin (spot 20) was used as the reference to normalize the intensity data of each protein spot. The intensity ratio of β-actin from YHV injected and PBS injected shrimps had a mean value of 1 ± 0.2.

Table 1 shows the 18 up- or down-regulated spots, plus the β-actin reference and a YHV nucleocapsid protein positive control. Protein spots successfully identified included glycolytic enzymes, enzymes involved in amino acid metabolism, and proteins involved in other cellular functions. Some of these protein spots were the same as those found in stomachs of *P. vannamei* after WSSV infection [7]. The up-regulated proteins (intensity ratio $\geq$1.5) (Table 1) included glycolytic enzymes such as triose-phosphate isomerase (spot 3), enolase (spots 4 and 8), isocitrate dehydrogenase (spots 5 and 7), and pyruvate kinase (spot 10). The dual spots observed for both enolase and isocitrate dehydrogenase may have resulted from protein modification.

Up-regulation of enzymes involved in the glycolytic pathway was similar to that reported for WSSV infection, and this response may be related to the additional energy required for viral replication in infected cells [7]. On the other hand, there was no change in the levels of other proteins that are involved in energy production, such as the beta subunit of mitochondrial ATP synthase (acc. no. ABF51410) (data not shown). This suggests that up-regulation of glycolytic enzymes during YHV infection may not be related to increased energy production, but may have some other

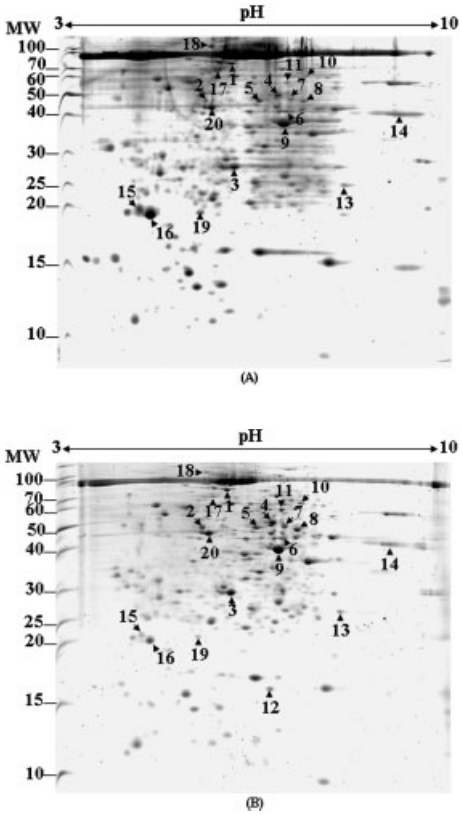

Figure 1. Example 2-DE profiles from gill extracts of *P. vannamei* stained with sypro ruby. (A) PBS control group and (B) YHV-injected group. Consistency is indicated by the same relative location of numbered spots and β-actin (spot 20) in both gels.

function instead. For example, α-enolase is also able to regulate the transcription of Sendai virus genome when associated with other cellular factors such as tubulin and phosphoglycerate kinase [8], while glycolytic enzymes such as hexokinase, lactate dehydrogenase (LDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and enolase have additional roles that include transcriptional regulation of apoptosis [9]. It is therefore possible that enolase may regulate YHV genome transcription or be involved in defense mechanisms to eliminate YHV from infected cells. Since there is apparently only one isoform of enolase in shrimp [10], these possibilities need to be further investigated.

Also up-regulated (Table 1) were glutamine synthetase (spot 6) and aspartate aminotransferase (spot 14), both of which are involved in amino acid metabolism. Up-regulation of these two proteins may be a biomarker for viral infection since they are also up-regulated in viral infections of humans and in cell cultures [11–14]. Arginine kinase (spot 9) was also up-regulated (Table 1). Since up-regulated arginine kinase has been observed in WSSV-infected spiny lobster [15] and *Penaeus stylirotris* [16], this up-regulation might constitute part of the general crustacean metabolic and physiological response to viral infection.

Table 1. Protein identification by LC-nanoESI-MS/MS and comparison of protein levels in 2-D gels during YHV and WSSV viral infections

Spot	Protein name (species)	Acc. no.	Exp. MW (kDa)/p*I*	Theor. MW (kDa)/p*I*	MS/mps	YHV/PBS Mean ± SD[b]	PBS	YHV infected	WSSV stomachs[c]	WSSV gills[c]
1	Hemocyanin (*Penaeus vannamei*)	CAA57880	80.7/5.76	74.9/5.27	247/6[a]	1.92 ± 0.58			+	=
2	Hemocyanin (*Penaeus vannamei*)	CAA57880	47.1/5.27	74.9/5.27	609/12	3.67 ± 0.4			ND	=
3	Triose-phosphate isomerase (*Archaeopotamobius sibiriensis)*	CAD29196	27.4/5.82	24.36/5.33	133/2[a]	1.9 ± 0.23			+	+
4	Enolase (*Penaeus monodon*)	ACC78141	49.7/6.64	47.24/6.18	777/33	2.25 ± 0.32			+	=
5	Isocitrate dehydrogenase (*Tribolium castaneum*)	XP_970446	47/6.29	48.95/8.72	316/9[a]	1.67 ± 1.07			+	=
6	Glutamine synthetase	Q04831	42.1/6.68	40.7/6.22	187/6	2.65 ± 0.21			ND	+
7	Isocitrate dehydrogenase (*Anopheles gambiae*)	XP_970446	47.9/6.89	48.95/8.72	278/4[a]	2.29 ± 0.46			=	+
8	Enolase (*Penaeus monodon*)	ACC78141	46.9/7.12	47.24/6.18	549/13[a]	2.64 ± 1.02			+	=
9	Arginine kinase (*Penaeus monodon*)	AAO15713	37.1/6.75	40.09/6.05	1003/39[a]	2.5 ± 0.62			=	=
10	Pyruvate kinase *(Aedes aegypti)*)	EAT35242	67.1/7.16	56.18/7.55	61/3	1 .5 ± 0.57			ND	+
11	Putative IDGF (*Diaprepes abbreviatus*)	AAV68692	60.4/6.83	49.4/6.69	51/1	2.26 ± 0.23			ND	+
12	YHV nucleocapsid protein	AAZ20367	16.2/6.57	16.3/9.98	426/11	-			ND	ND
13	Ras-like GTP binding protein (*Onchocerca volvulus*)	B48463	25.5/7.92	24.2/6.21	107/2	1.53 ± 0.05			ND	+
14	Aspartate amino-transferase (*Aedes aegypti*)	AAQ02892	40.8/9.22	47.23/9.14	127/6[a]	1.5 ± 0.01			=	-
15	SCP-calcium binding protein (*Penaeus sp.*)	P02636	19.6/4	21.97/4.58	146/4	0.46 ± 0.21			-	-
16	SCP-calcium binding protein (*Penaeus sp.*)	P02636	19/4.22	21.97/4.58	513/20	0.5 ± 0.17			-	-
17	Actin-1 (*Aedes aegypti*)	AAA62350	73.3/5.51	41.7/5.74	97/2	0.45 ± 0.23			ND	-
18	Valosin containing protein-1 (*Eisenia fetida*)	BAD91024	104.8/5.38	89.58/5.23	464/8[a]	0.47 ± 0.19			=	=
19	Rab11-2 (*Limulus polyphemus*)	AAP48704	19.2/5.17	24.4/5.7	58/1	0.47 ± 0.12			ND	=
20	β-actin (*Homarus gammarus*)	CAE46725	43.7/5.38	41.8/5.3	490/21[a]	1 ± 0.2			=	=

Ratios ≥1.5 indicate up-regulation while ratios ≤0.67 indicate down-regulation. "MS/Mps" = MOWSE score/match peptides.

a) The result of MOWSE score/match peptides retrieved from previous analysis of protein spots in 2-D gels of WSSV-infected stomachs [7].

b) Mean ± SD expression from 13 independent 2-D gels.

c) Results for expression in stomachs [7] and gills (present study) of WSSV-infected *P. vannamei*. A plus sign (+) indicates up-regulation, minus (–) indicates down-regulation, equals (=) indicates no change, and ND indicates not detected.

Up-regulated proteins involved in other cellular functions included hemocyanin (spots 1 and 2), putative imaginal disk growth factor (IDGF) (spot 11) and Ras-like GTP binding protein (spot 13). The up-regulation of hemocyanin may serve to provide more oxygen for energy production during viral replication. However, the molecular mass of hemocyanin in spot 2 (47.1 kDa) differed from the molecular mass of *P. vannamei* hemocyanin in GeneBank (74.9 kDa). Despite this, the result from LC-nanoESI-MS/MS strongly confirmed that it was hemocyanin of *P. vannamei*. Therefore, this spot may have resulted from cleavage of the intact hemocyanin polypeptide and it could be related to the massive damage and loss of hemocytes associated with YHV infection [17, 18]. Several studies have shown that hemocyanin may function not only as an oxygen transport protein but also as a defense molecule [19, 20]. Antibacterial and antifungal peptides isolated from shrimp are generated from the C-terminus of hemocyanin [20, 21], and both subunits of *P. monodon* hemocyanin were recently shown to have antiviral activity against fish viruses in fish cell cultures [22]. It has also been proposed [23] that *P. vannamei* hemocyanin is an IgA-like protein that reacts with antihuman IgA and aggregates eight species of shrimp pathogenic bacteria. Thus, it is possible that up-regulation of hemocyanin after YHV infection is part of the shrimp defense response to YHV. IDGF (spot 11) and Ras-like GTP binding protein (spot 13) may also be part of the shrimp response to any viral infection. These two proteins are involved in the cell division cycle and cell proliferation. Their up-regulation may facilitate viral replication as has been reported for NIH3T3 cells [24–26].

There was a significant down-regulation of SCP (alpha A and B chains) (spots 15 and 16) (intensity ratio $\leq$0.67) (Table 1). Additionally, time-course immunoblot analysis and immunofluorescent staining of this protein from gills of YHV-infected shrimp confirmed the decrease in SCP from 12 hpi through to 48 hpi (data not shown). SCPs normally bind to Ca^{2+} ions and function as a cytosolic Ca^{2+} buffer [27], and their down-regulation may impair Ca^{2+} binding. This might lead to the activation of phospholipase, protease, and endonuclease, which could damage or destroy the YHV-infected cells [28–30].

Other significantly down-regulated proteins involved in cellular functions included actin-1 (spot 17), valosin-containing protein (spot 18), and Rab11-2 (spot 19). Down-regulation of valosin containing protein-1 may be related to YHV pathogenesis, because its reduction causes up-regulation of multiple transcripts such as cell stress proteins and proteins involved in apoptosis in mammalian cells [31]. One of the major effects of YHV is induction of apoptosis [17].

A receptor that is capable of mediating the entry of YHV into shrimp cells has been characterized and is known to be expressed in gills [32]. Although the receptor's identity is presently unknown, it is therefore likely that YHV enters target cells *via* a receptor-mediated endocytotic pathway that is regulated by Rab family proteins [33]. Rab11 plays a role in the recycling of intracellular vesicles as they return to the cell surface [33], and its down-regulation in YHV-infected *P. vannamei* may prevent this recycling and thus enhance the efficiency of YHV entry into host cells.

YHV and WSSV protein spot positions in the 2-D gels were compared using PDQuest software (BioRad). Table 1, which also includes results from WSSV-infected stomach tissue [7], shows how expression levels varied according to viral challenge and tissue type. Thus, glutamine synthetase (spot 6), puruvate kinase (spot 10), putative IDGF (spot 11), and Ras-like GTP binding protein (spot 13) were up-regulated in the gills of both YHV-infected and WSSV-infected shrimps but were not detected in the stomachs of WSSV-infected shrimp [7]. By contrast, expression profiles for triose-phosphate isomerase (spot 3) and SCP (alpha A and B chains) (spots 15 and 16) in gills of YHV-infected shrimps were similar to those for both gills and stomachs [7] of WSSV-infected shrimps. Truncated hemocyanin (spot 2) was up-regulated in gills of YHV-infected shrimps but it was unchanged in the gills of WSSV-infected shrimps and it was not detected in WSSV-infected stomach tissues [7]. This cleaved fragment may therefore be up-regulated only as a response to the RNA virus YHV rather than the DNA virus WSSV. Whether this is a specific response to YHV or a generic response to an RNA virus remains to be determined. Curiously, the spot location for stomach hemocyanin (spot 1) was also up-regulated in YHV infected gills but not in WSSV-infected gills.

Isocitrate dehydrogenase (spot 7) was up-regulated in gills of both YHV-infected and WSSV-infected shrimps, but it was constant in stomachs of WSSV-infected shrimps [7]. Enolase (spots 4 and 8) was up-regulated in gills of YHV-infected shrimps and stomachs of WSSV-infected shrimps [7], but was not up-regulated in gills of WSSV-infected shrimps.

In addition to the 2-DE results, the mRNA expression levels for some of the up-regulated and down-regulated proteins were measured at 12 h intervals by real time RT-PCR in three independent YHV challenge experiments. Transcriptional levels were determined by a QuantiTect SYBR Green PCR kit (Qiagen) using primers that were previously designed for enolase and SCP [7] and the following additional primers: pyruvate kinase, GAT GAT GGA GGC TGG CAT GAA CAT CGC AC (forward), and CTG CTA TCT GTG GTC AAC TTG ATT GTG GCA C (reverse); triose-phosphate isomerase, GGT CAC GTG TTG TCT TGG CTT ATG AAC C (forward), and CTG AAC AAA GTC TGG CTT GAG AGC TGC (reverse); arginine kinase, CAA CGC TTG CCG CTA CTG (forward), and ATC TCC TTG ACA GCC TGG (reverse). The primers used for the detection of elongation factor-1α (EF-1α) and YHV were derived from previous studies [34, 35]. RT-PCR was carried out in a GeneAmp 7500 thermal cycler (PE Applied Biosystems) with a thermal profile of 95°C for 15 min followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and at 72°C for 45 s.

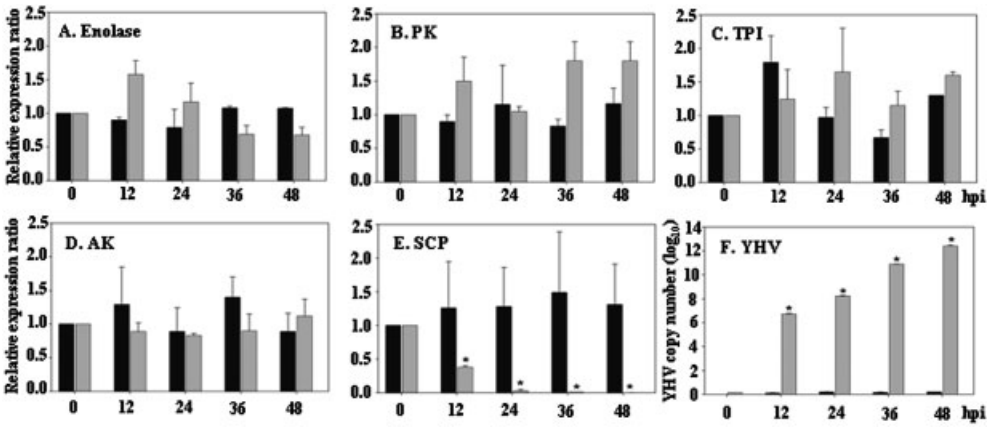

Figure 2. Quantitative real-time RT-PCR for five representative proteins from gills after YHV injection. Each bar (black = PBS control; gray = YHV injected) represents the average relative expression ratio (R) ± SD derived from three replicates at each time interval after YHV injection (normalized with EF-1α gene). Asterisks indicate a statistically significant difference from the value at time zero (p-value<0.02). (A) Enolase, (B) pyruvate kinase (PK), (C) triose-phosphate isomerase (TPI), (D) arginine kinase (AK), (E) SCP, (F) YHV copy number.

Data analysis was performed with the 7500 Sequence Detection System software (SDS version 1.3.1). For each time point, the quantity of target gene and a reference gene (EF-1α) was expressed as a Ct value (the threshold PCR cycle), and from this the expression level was calculated. Results were then presented as a normalized relative expression ratio (R) as previously described [36]. Statistical analysis of R was done using one-way analysis of variance (ANOVA), and *posthoc* comparison was carried out using the Bonferroni test. A p-value<0.02 was considered to indicate a significant difference.

The transcriptional levels (Fig. 2) of arginine kinase and some of the enzymes involved in the glycolytic pathway did not correlate with results from the 2-DE (Table 1). This discrepancy may have resulted from the effect of mRNA lability or from PTM mechanisms. Like other viruses, such as rabies virus [37], YHV may affect the translational level of host cell proteins but have no effect on the transcriptional levels. However, we note that the transcriptional level of SCP decreased dramatically by 12 hpi and remained low through to 48 hpi. YHV copy number increased steadily at successive sampling times.

In conclusion, the results presented here provide preliminary data on the interaction between YHV and shrimp. Although this study was not able to assign specific YHV-response status to any of the 13 up-regulated or five down-regulated genes identified after YHV challenge, it opens the way for more focused studies on their functions and possible interactions with shrimp or viral proteins.

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