Mar Biotechnol (2014) 16:111–123
DOI 10.1007/s10126-013-9538-8

ORIGINAL ARTICLE

Comparison of Protein Expression Profiles of the Hepatopancreas in *Fenneropenaeus chinensis* Challenged with Heat-inactivated *Vibrio anguillarum* and White Spot Syndrome Virus

Hao Jiang · Fuhua Li · Jiquan Zhang · Jinkang Zhang · Bingxin Huang · Yang Yu · Jianhai Xiang

Received: 23 March 2013 / Accepted: 15 July 2013 / Published online: 22 September 2013

Abstract *Fenneropenaeus chinensis* (Chinese shrimp) culture industry, like other Penaeidae culture, has been seriously affected by the shrimp diseases caused by bacteria and virus. To better understand the mechanism of immune response of shrimp to different pathogens, proteome research approach was utilized in this study. Firstly, the soluble hepatopancreas protein samples in adult Chinese shrimp among control, heat-inactivated *Vibrio*-challenged and white spot syndrome virus-infected groups were separated by 2-DE (pH range, 4–7; sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and pH range, 3–10; tricine-SDS-PAGE). Then the differentially expressed protein spots (≥1.5-fold or ≤0.67-fold averagely of controls) were analyzed by LC-ESI-MS/MS. Using Mascot online database searching algorithm and SEQUEST searching program, 48 and 49 differentially expressed protein spots were successfully identified in response to *Vibrio* and white spot syndrome virus infection, respectively. Based on these results, we discussed the mechanism of immune response of the shrimp and shed light on the differences between immune response of shrimp toward *Vibrio* and white spot syndrome virus. This study also set a basis for further analyses of some key genes in immune response of Chinese shrimp.

Keyword *Vibrio* · WSSV · Shrimp · Proteomics · 2-DE

H. Jiang · F. Li · J. Zhang · J. Zhang · B. Huang · Y. Yu · J. Xiang (✉)
Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, China
e-mail: jhxiang@qdio.ac.cn

H. Jiang · J. Zhang · Y. Yu
University of Chinese Academy of Sciences, Beijing 100049, China

Abbreviations

*Fc*DB	*Fenneropenaeus chinensis* peptides database
PDI	Protein disulfide isomerase
ROS	Reactive oxygen species
WSSV	White spot syndrome virus

Introduction

The Chinese shrimp, *Fenneropenaeus chinensis* (*F. chinensis*), is one of the most commercially important cultured shrimp in China. However, the worldwide outbreaks of viral and bacterial diseases have caused the menaced penaeid shrimp aquaculture a big economic loss. Among all known pathogens of shrimp, the *Vibrio* has been implicated as the main bacterial pathogen (Baticados et al. 1990 ; Bachère 2000), and white spot syndrome virus (WSSV) is the most popular viral pathogen (Lightner 1996; Yang et al. 2001).

It's well-known that shrimp adopt innate immune system to prevent them from pathogen invasion since lacking adaptive immune systems (Bachère et al. 2004). In the recent years, more studies about crustacean immunology have been reported (Li and Xiang 2013a). Many major effectors of the crustacean immune system, including the pattern-recognition proteins (Cheng et al. 2005; Liu et al. 2007), prophenoloxidase system (Liu et al. 2006), antimicrobial peptides (Destoumieux et al. 2000; Liu et al. 2005), and signaling pathways regulating innate immune responses (Flegel and Sritunyalucksana 2011; Li and Xiang 2013b; Wen et al. 2013), were studied using biochemical and molecular biological approaches. In recent years, the bioinformatic data of shrimp are accumulating at dramatic rate (Zhang et al. 2010b; Leu et al. 2011; Andriantahina et al. 2013), which enable researchers to perform some high-throughput analysis to rapidly screen a plenty

of important genes or proteins involved in shrimp immune response, without knowing the detailed molecular mechanisms of immune responses. In transcriptional level, expressed sequence tags analyzing, suppression subtractive hybridization, differential display PCR, and cDNA microarray have been applied to isolate differentially expressed genes of shrimp in response to immune stimulation (Somboonwiwat et al. 2006; Wang et al. 2006; Wang et al. 2008; Robalino et al. 2009; Aoki et al. 2011; Li et al. 2013); the information of mRNA expression level reflected the expression potential of the corresponding proteins. To comprehensively study the gene expression pattern, it is necessary to collect information of protein expression profiles, as proteins take center stage in directing the work of living cell. As a high-throughput characterization method in translational level, proteomics was utilized to identify differentially expressed proteins of shrimp in response to pathogen challenge (Chongsatja et al. 2007; Wang et al. 2007; Rattanarojpong et al. 2007; Chai et al. 2010; Zhang et al. 2010a; Chaikeeratisak et al. 2012).

To develop control strategies to prevent different infectious diseases of shrimp, it is necessary to study the response of shrimp toward different types of pathogen, especially the similar and differential molecular reactions of the shrimp immune system toward different pathogens. The differentially expressed gene in response to WSSV or *Vibrio* infection within the same batch of shrimp have been screened and compared by cDNA microarray (Wang et al. 2008). However, there is no report on comparing the protein expression patterns of shrimp stimulated by different pathogens. In this study, differentially expressed proteins in the hepatopancreas, a main immune organ in shrimp, were investigated through proteomic approach when shrimp were challenged by WSSV and *Vibrio*, aiming to compare protein expression patterns in shrimp response to different pathogens.

Materials and Methods

Shrimp and Pathogens Challenge

Healthy Chinese shrimp with an average length of 9.4–10.6 cm were obtained from a local shrimp farm near Qingdao. They were cultured in air pumped circulating seawater at 21 °C with 32‰ salinity at least 10 days prior to the experiment. Artificial diet was given two times per day. Shrimp were randomly selected and divided into *Vibrio*-challenged, WSSV-challenged, and control groups.

In the *Vibrio* challenge experiment, shrimp were injected with 20 μL heat-inactivated *Vibrio anguillarum* suspended in normal saline (10^7 cells/mL). In the WSSV challenge experiment, shrimp were injected with 20 μL tissue homogenate isolated from WSSV-infected shrimp. To prepare the tissue homogenate, 10-g tissues of WSSV-infected shrimp were homogenized in 10 mL PBS-His on ice (Huang et al. 1999). The homogenized tissue was centrifuged at 3,800×*g* at 4 °C for 15 min. The supernatant was transferred to a fresh tube, sucrose was added to 30 % (*w*/*v*) and then the mixture was centrifuged at 38,000×*g* at 4 °C for 50 min. The pellets were resuspended in 25 mL of normal saline for injection. The control shrimp were injected with 20 μL normal saline. Twelve shrimp from each group were taken out randomly after 24 h. The hepatopancreas were dissected out for further proteomic analysis.

Extraction of Hepatopancreas Proteins

The total protein of shrimp hepatopancreas from *Vibrio*-challenged, WSSV-challenged, and control groups were extracted as previously described (Jiang et al. 2009). Protein concentration was measured using the Quick Start Bradford Protein Assay Kit 1 (Bio-Rad, Hercules, USA).

2-DE

A pool of hepatopancreas samples isolated from four shrimps (within the same group) was used for resolving the hepatopancreas proteins in a two-dimensional electrophoresis (2-DE) gel. Three independent pools per group (totally twelve shrimps per group) were used for 2-DE analysis. A Bio-Rad Protean IEF Cell System (Bio-Rad, Hercules, USA) was used for the isoelectric focusing (IEF) dimension. Approximately 300 and 600 μg of total protein was loaded onto each 18 cm immobilized pH gradient (IPG) strip pH 4–7 and pH 3–10NL (GE Healthcare, Sweden) separately. The IEF conditions and the following equilibration were as described in the previous study (Jiang et al. 2009). The second dimension electrophoresis was carried out at 14 °C on a Bio-Rad Protean II xi Cell System (Bio-Rad, Hercules, USA). Fourteen percent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and 0.165 T, 3 %C tricine-SDS-PAGE were used for the pH 4–7 and pH 3–10NL IPG strips separately. Then the gel was stained with CBB G-250 colloid staining method.

Image Analysis

ImageMaster 2D Platinum 6.0 (GeneBio, Geneva, Switzerland) was used for analyzing 2-DE images including matching spots, quantifying spots, and identifying differences of spot intensity. The spot intensity was quantified by total spot volume normalization and comparison was according to the spot volume percentage. Spots with significant and reproducible changes were considered to be differentially expressed proteins.

In-gel Protein Digestion

The differentially expressed protein spots were manually excised from the gels using 200 μL tips and grinded into small pieces. The following gel destain, dehydration, digestion, and peptides collection procedures were operated as previous described (Jiang et al. 2009).

LC-ESI-MS/MS

Twenty microliters of peptide samples were loaded onto a C-18 column, 100×0.18 mm, 5 μm (Thermo Electron Corporation, Waltham, USA) and separated using a gradient of 5 % A and 95 % B for 15 min, 65 % A and 35 % B for 45 min, and 95 % A and 5 % B for 25 min, where A was acetonitrile with 0.1 % formic acid and B was water with 0.1 % formic acid. The C-18 column was connected directly to the LCQ DECA XP^{plus} ion trap mass spectrometer (ThermoQuest, San Jose, USA). Full-scan spectra were recorded in positive mode over the mass range of 300–1,500 Da. The MS/MS data were automatically acquired on the three most intense precursor ions in each full-scan spectrum.

Database Searching

The MS/MS data were firstly searched against the NCBInr and expressed sequence tag (EST) databases using the Mascot online (http://www.matrixscience.com). The searching parameters were set as previously described. Identifications with individual ions scores indicating identity or extensive homology ($p<0.05$) were defined positive.

Then the MS/MS spectra were searched again using SEQUEST in the BioWorks 3.1 software package against *F. chinensis* peptides database (*Fc* DB). The construction of *Fc* DB and searching parameters were as previously described (Jiang et al. 2009). Those with Xcorr score equal to or above 1.5, 2.2, or 3.5 for singly, doubly, or triply charged precursor ions, respectively, and Delta Cn above 0.1 was defined as positive identifications.

Results

Comparison of Protein Expression Profiles of Hepatopancreas Between *Vibrio*-Challenged Shrimp, WSSV-Challenged Shrimp, and Normal Shrimp

As shrimp hepatopancreas protein mainly ranged from 15,000 to 90,000 Da, pH 4–7 (Jiang et al. 2009), we first run pH 4–7 strips and 14 % SDS-PAGE gels to decrease the disturbance of high abundance proteins in acidic area and enhance the resolution of 2-DE gels (Fig. 1a–c). Approximately 680–710 spots on each gel were distinguished by ImageMaster 2D. We also hope to get a complete view of the shrimp hepatopancreas protein ranging from pI acidic to basic, molecular weight low to high, especially low molecular weight proteins that might contain antibacterial peptides. So we run a parallel gel of pH 3–10NL strip and 0.165 T, 3 %C tricine-SDS-PAGE (Fig. 1d–f). Approximately 760–800 spots on each gel were distinguished.

Using ImageMaster 2D software to analyze the total 18 2-DE gels, 53 protein spots showed significantly differential expression in the hepatopancreas of *Vibrio*-challenged shrimp compared with control shrimp (Fig. 1), including 8 upregulated (expression levels were ≥1.5-fold of controls) spots and 45 downregulated (expression levels were ≤0.67-fold of controls) spots; 52 protein spots showed significantly differential expression in the hepatopancreas of WSSV-challenged shrimp compared with control shrimp (Fig. 1), including 11 upregulated (expression levels were ≥1.5-fold of controls) spots and 41 downregulated (expression levels were ≤0.67-fold of controls) spots.

Protein Identification

The differential expression proteins were analyzed by LC-ESI-MS/MS. By combining results of searching MS/MS spectra data against NCBInr and EST database by Mascot and against *Fc* DB by SEQUEST, more abundant and accurate peptide identifications were achieved (Porubleva et al. 2001; Kim et al. 2004). In hepatopancreas of Chinese shrimp challenged with heat-inactivated *Vibrio anguillarum*, 48 of the total 53 differentially expressed protein spots including 6 upregulated (representing 5 proteins) and 42 downregulated (representing 31 proteins) were successfully identified, including 5 protein spots related to energy production, 5 protein spots related to immune, 224 protein spots involved in the metabolism, 4 antioxidant protein spots, 7 chaperone spots, 1 translation related protein spot, and 2 ungrouped protein spots; in hepatopancreas of Chinese shrimp challenged with WSSV, 49 differentially expressed protein spots including 11 upregulated (representing 9 proteins) and 38 downregulated (representing 27 proteins) were successfully identified, including 7 protein spots related to energy production, 9 protein spots related to immune, 21 protein spots involved in the metabolism, 5 antioxidant protein spots, and 7 chaperone spots. Thirty-one of these identified altered expressed protein spots were found in both *Vibrio*-challenged and WSSV-challenged groups, and most of them had the similar alteration trend response to *Vibrio* and WSSV challenge. Seventeen or 18 of these identified altered expressed protein spots were found only in *Vibrio*-challenged group or WSSV-challenged group (shown in Table 1). Some different spots were identified as the same proteins, and this might be the results of different protein isoforms or posttranslational modifications.

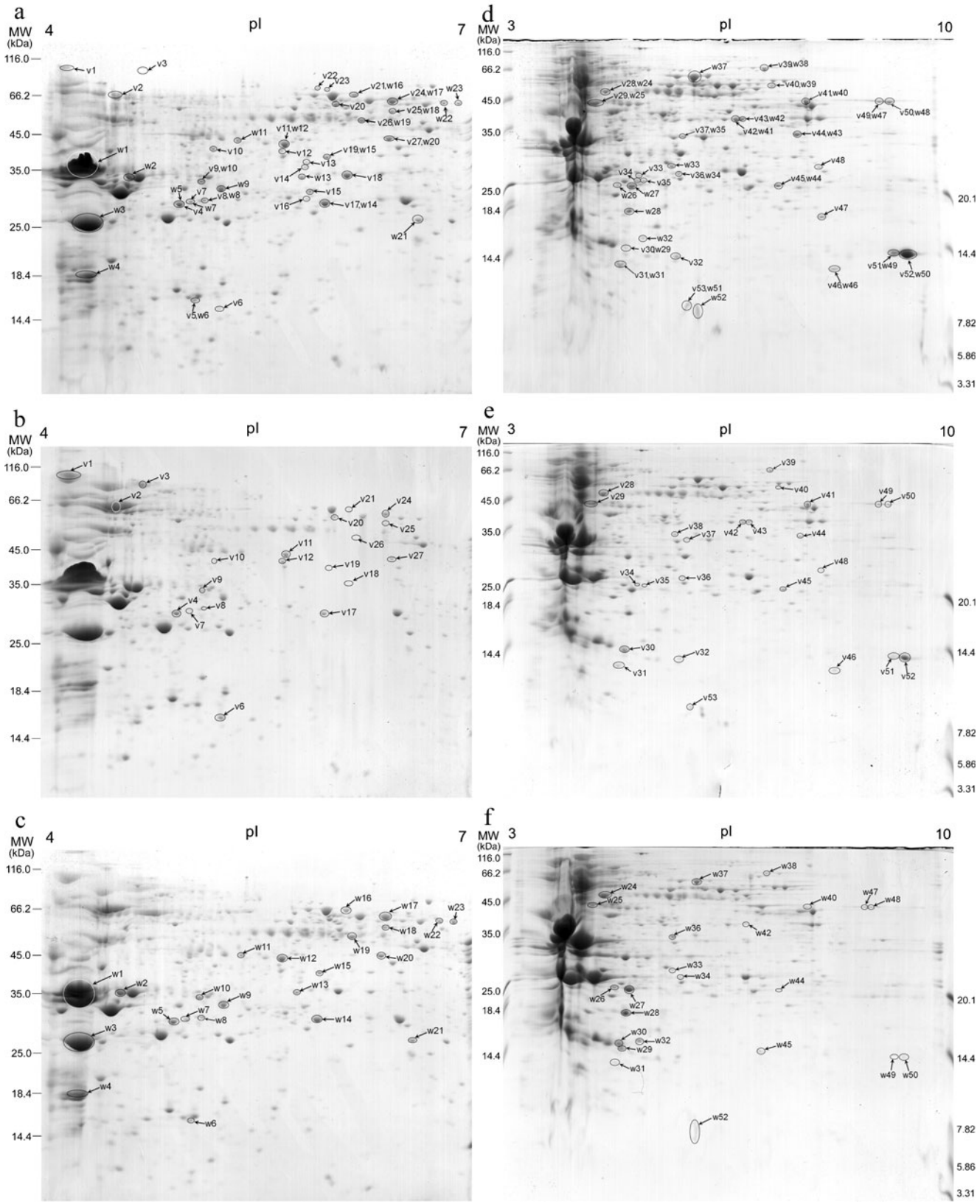

Fig. 1 2-DE maps of Chinese shrimp hepatopancreas. **a** IPG 4–7, SDS-PAGE; and control, **b** IPG 4–7, SDS-PAGE, and heat-inactivated *Vibrio anguillarum*-challenged group; **c** IPG 4–7, SDS-PAGE, and WSSV-challenged group; **d** IPG 3–10NL, tricine-SDS-PAGE, and control; **e** IPG 3–10NL, tricine-SDS-PAGE, and heat-inactivated *Vibrio anguillarum*-challenged group; and **f** IPG 3–10NL, tricine-SDS-PAGE, and WSSV-challenged group

Table 1 Differentially expressed proteins identified by LC-ESI-MS/MS in hepatopancreas of Chinese shrimp challenged by *Vibrio* and WSSV

Spot number		Protein [species]	Accession number	Predicted	Observed	Mascot scores/ match peptides		SEQUEST scores/match peptides	Infection/control Mean ± SD	
Vibrio	WSSV			MW (kDa)/pI	MW (kDa)/pI	NCBInr	EST	*Fc*DB	*Vibrio*	WSSV
Proteins related to energy production										
v9	w10	Preamylase 1 [*Litopenaeus vannamei*]	CAA54524	57/5.21	34/4.90	65/1	NA	NA	0.28±0.11	2.05±0.88
v17	w14	Triosephosphate isomerase [*Archaeopotamobius sibiriensis*]	CAD29196.1	24/5.33	29/5.50	363/6	708/11	130.34/6	0.28±0.10	0.59±0.35
v19	w15	Arginine kinase [*Fenneropenaeus chinensis*]	AAV83993	40/5.92	39/5.50	80/2	68/2	NA	0.55±0.41	0.67±0.25
v25	w18	Phosphopyruvate hydratase [*Penaeus monodon*]	AAC78141	47/6.18	53/6.00	330/7	183/2	120.17/5	0.26±0.06	2.84±0.60
v27	w20	Arginine kinase [*F. chinensis*]	AAV83993	40/5.92	41/5.90	478/9	416/7	556.20/14	0.00±0.00	0.63±0.12
	w9	Preamylase 1 [*L. vannamei*]	CAA54524	57/5.21	32/4.90	61/1	NA	NA		0.66±0.15
	w45	Triosephosphate isomerase [*F. chinensis*]	ABB81879	27/5.55	15/6.30	159/4	159/4	210.1/4		∞
Proteins with immune function										
v46	w46	Macrophage migration inhibitory factor [*Anisakis simplex*]	ABM30179	13/6.71	9/7.50	NA	105/2	NA	0.65±0.21	0.00±0.00
v1		Neprilysin 2 [*Apis mellifera*]	XP_393860	89/6.21	95/4.10	NA	129/2	NA	2.92±1.05	
v2		Chitinase [*L. vannamei*]	AAT80625	50/4.71	60/4.40	321/5	138/2	170.14/3	1.92±0.52	
v14		Predicted: similar to TBC1 domain family, member 19 [*Ciona intestinalis*]	XP_002119373	61/6.61	35/5.40	NA	102/2	NA	0.00±0.00	
v35		Cathepsin l [*L. vannamei*]	CAA68066	36/5.18	25/5.10	NA	128/2	NA	0.38±0.06	
	w26	Zinc proteinase Mpc1 [*L. vannamei*]	ABD65301	28/5.30	24/4.80	83/2	83/2	NA		2.35±0.29
	w27	Zinc proteinase Mpc1 [*L. vannamei*]	ABD65301	28/5.30	25/4.90	91/2	132/2	NA		1.81±0.35
	w30	Chitinase [*L. vannamei*]	AAT80625	50/4.71	16/4.80	85/1	85/1	NA		∞
	w32	Chitinase [*L. vannamei*]	AAT80625	50/4.71	16/5.00	138/3	138/3	30.1/1		1.76±0.33
	w1	Trypsin [*L. vannamei*]	CAA75311	28/4.31	32/4.20	85/2	99/2	NA		0.51±0.21
	w2	Carboxypeptidase A2 (pancreatic) (predicted), isoform CRA_a [*Rattus norvegicus*]	EDM15254	37/5.54	35/4.50	75/1	184/3	NA		0.62±0.15
	w3	Chymotrypsin BI [*L. vannamei*]	CAA71672	29/5.56	27/4.30	65/1	85/1	260.2/1		0.60±0.22
	w4	Zinc proteinase Mpc1 [*L. vannamei*]	ABD65301	28/5.30	18/4.30	74/1	74/1	NA		0.53±0.11
Proteins involved in the metabolism										
v38	w36	Adenosine kinase [*Aedes aegypti*]	XP_001652432	38/4.72	33/5.40	NA	113/3	260.2/2	∞	∞
v11	w12	Adenosine kinase [*A. aegypti*]	XP_001652432	38/4.72	41/5.20	NA	192/4	400.21/5	0.61±0.1	0.62±0.26
v5	w6	Blo t aldehyde dehydrogenase allergen [*Blomia tropicalis*]	AAQ24547	45/5.64	63/6.00	NA	123/2	78/1	0.00±0.00	0.64±0.30
v21	w16	Pyrroline-5-carboxylate dehydrogenase [*A. aegypti*]	XP_001650875	63/8.90	66/5.70	NA	198/4	NA	0.59±0.29	0.66±0.25
v26	w19	S-adenosyl-L-homocysteine hydrolase [*Anopheles gambiae*]	AAC29475	48/5.62	48/5.80	130/3	170/4	NA	0.62±0.40	0.59±0.31
v37	w35	Predicted: similar to esterase D/formylglutathione hydrolase [*A. mellifera*]	XP_395656	32/5.54	31/5.50	75/1	416/7	350.2/4	0.22±0.11	0.00±0.00
v39	w38	Transketolase [*A. aegypti*]	XP_001649141	68/6.54	66/6.50	132/4	246/4	NA	0.40±0.18	0.45±0.25

Table 1 (continued)

Spot number		Protein [species]	Accession number	Predicted	Observed	Mascot scores/ match peptides		SEQUEST scores/match peptides	Infection/control Mean ± SD	
Vibrio	WSSV			MW (kDa)/pI	MW (kDa)/pI	NCBInr	EST	*Fc*DB	*Vibrio*	WSSV
v30	w29	Transketolase [*C. quinquefasciatus*]	XP_001849453	68/6.56	15/4.80	NA	93/1	NA	4.67±1.88	3.59±0.91
v41	w40	Cystathionine beta-lyase [*Culex quinquefasciatus*]	XP_001844736	44/6.23	43/6.70	NA	133/3	NA	0.40±0.02	0.26±0.02
v42	w41	Fructose-1 6-biphosphatase [*Phytophthora infestans*]	AAN31471	36/5.34	38/6.00	74/1	354/5	90.1/2	0.33±0.05	0.00±0.00
v43	w42	Fructose-1 6-biphosphatase [*P. infestans*]	AAN31471	36/5.34	38/6.10	72/1	176/3	40.1/2	0.41±0.08	0.25±0.11
v49	w47	Aspartate aminotransferase [*A. aegypti*]	XP_001655159	47/9.14	43/8.60	NA	135/2	NA	0.63±0.06	0.65±0.15
v50	w48	Aspartate aminotransferase [*A. aegypti*]	XP_001655159	47/9.14	43/8.80	63/2	201/4	NA	0.54±0.08	0.66±0.13
v53	w51	Homocysteine S-methyltransferase, putative [*Ixodes scapularis*]	EEC00912	43/7.91	8/5.70	NA	114/2	NA	0.45±0.20	0.00±0.00
v12		Adenosine kinase, putative [*I. scapularis*]	EEC15268	38/5.46	40/5.20	NA	164/3	300.23/3	1.98±0.45	
v13		Fructose-1,6-bisphosphatase [*Bombyx mori*]	NP_001040381	36/8.40	37/5.40	NA	169/1	NA	0.00±0.00	
v15		Hydroxyacylglutathione hydrolase [*Danio rerio*]	NP_956337	29/6.60	31/5.40	NA	79/1	NA	0.00±0.00	
v16		Transketolase [*C. quinquefasciatus*]	XP_001849453	68/6.56	30/5.40	61/2	108/3	NA	0.00±0.00	
v18		Predicted: similar to esterase D/formylglutathione hydrolase [*A. mellifera*]	XP_395656	32/5.54	33/5.70	58/1	394/9	250.17/11	0.23±0.21	
v20		Predicted: similar to aldehyde dehydrogenase 7 family, member A1 [*Tribolium castaneum*]	XP_969882	56/6.34	60/5.60	NA	173/4	NA	0.50±0.09	
v22		10-formyltetrahydrofolate dehydrogenase [*Homo sapiens*]	AAC35000	99/5.68	80/5.40	64/1	NA	NA	0.00±0.00	
v23		Formyltetrahydrofolate dehydrogenase [*Xenopus (Silurana) tropicalis*]	NP_001011027	100/5.69	80/5.50	114/3	248/5	NA	0.00±0.00	
v33		Predicted: similar to 3-hydroxyacyl-coa dehydrogenase [*T. castaneum*]	XP_973042	34/8.90	27/5.00	134/3	377/6	150.1/5	0.00±0.00	
v34		Predicted: similar to glutamate carboxypeptidase [*Nasonia vitripennis*]	XP_001604790	58/5.79	25/5.00	NA	107/3	NA	0.36±0.11	
	w28	Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase [*Pongo abelii*]	NP_001128995	47/6.94	17/4.90	NA	NA	32.1/2		1.74±0.31
	w11	Beta-galactosidase precursor, putative [*I. scapularis*]	EEC10856	71/5.49	41/5.00	NA	118/2	NA		0.46±0.23
	w13	Adenosine kinase [*A. aegypti*]	XP_001652432	38/4.72	33/5.30	NA	76/1	NA		0.59±0.35
	w23	Formyltetrahydrofolate dehydrogenase [*X. tropicalis*]	NP_001011027	100/5.69	59/6.60	201/5	321/5	NA		0.56±0.38
	w33	Aldehyde dehydrogenase 16 family, member A1 [*X. tropicalis*]	AAI21296	89/5.94	30/5.40	NA	138/2	NA		0.38±0.03
	w37	Predicted: similar to pyrroline-5-carboxylate dehydrogenase [*T. castaneum*]	XP_969408	63/8.67	60/5.70	60/2	274/6	NA		0.62±0.19
	w52	Homocysteine S-methyltransferase, putative [*I. scapularis*]	EEC00912	43/7.91	8/5.80	NA	132/2	NA		0.52±0.22
Antioxidant proteins										
v44	w43	Similar to carbonyl reductase [NADPH] 1 [*A. mellifera*]	XP_623474	31/8.64	32/6.70	385/7	NA	410.2/5	0.17±0.01	0.00±0.00
v45	w44	Mitochondrial manganese superoxide dismutase [*F. chinensis*]	ABB05539	24/7.09	26/6.40	265/5	234/4	100.18/3	0.52±0.28	0.67±0.44
v51	w49	Fatty acid-binding protein 10 [*L. vannamei*]	ABD65306	8.2/4.82	10/9.00	NA	61/3	40.08/2	0.18±0.02	0.12±0.02

Table 1 (continued)

Spot number		Protein [species]	Accession number	Predicted	Observed	Mascot scores/ match peptides		SEQUEST scores/match peptides	Infection/control Mean ± SD	
Vibrio	WSSV			MW (kDa)/pI	MW (kDa)/pI	NCBInr	EST	*Fc*DB	*Vibrio*	WSSV
v52	w50	Fatty acid-binding protein 10 [*L. vannamei*]	ABD65306	8.2/4.82	10/9.20	48/2	97/6	80.08/4	0.15±0.02	0.25±0.01
	w21	Mitochondrial manganese superoxide dismutase [*F. chinensis*]	ABB05539	24/7.09	26/6.30	150/3	150/3	350.17/3		0.58±0.05
Chaperone										
v28	w24	Predicted: similar to protein disulfide isomerase [*N. vitripennis*]	XP_001605359	56/4.71	48/4.80	66/2	NA	150.29/6	1.62±0.25	2.25±0.69
v7	w7	Similar to protein disulfide isomerase precursor (PDI) [*T. castaneum*]	XP_975184	91/5.20	29/4.90	NA	121/3	98.08/2	0.08±0.02	0.04±0.03
v8	w8	Protein disulfide isomerase [*L. vannamei*]	ACN89260	55/4.64	29/4.90	NA	103/2	20.1/2	0.06±0.04	0.06±0.03
v29	w25	Protein disulfide isomerase [*L. vannamei*]	ACN89260	55/4.64	43/4.70	NA	116/2	NA	0.52±0.12	0.67±0.22
v31	w31	Heat shock protein 70 [*Chelon labrosus*]	ABF55695	21/5.22	13/4.80	131/2	119/3	692.1/4	0.55±0.08	0.40±0.07
v36	w34	Predicted: similar to proteasome beta subunit [*N. vitripennis*]	XP_001605493	26/5.76	29/5.70	NA	82/2	NA	0.32±0.10	0.27±0.06
v4	w5	Similar to Protein disulfide isomerase precursor (PDI) [*T. castaneum*]	XP_975184	91/5.20	29/4.80	NA	204/4	60.10/4	0.62±0.25	0.40±0.22
Translation										
v48		Similar to ribosomal protein L5 [*Xenopus laevis*]	NP_001079437	34/9.70	30/7.20	NA	187/3	36.3/3	0.36±0.06	
Ungrouped proteins										
v32		Heat-responsive protein 12 [*Mus musculus*]	EDL08846	17.6/8.55	14/5.60	NA	267/5	NA	0.60±0.16	
v47		Oncoprotein nm23 [*L. vannamei*]	ABI93176	17/6.74	17/7.20	216/5	216/5	920.3/7	0.00±0.00	

Discussion

The aim of this study was to investigate the protein expression changes in the hepatopancreas of Chinese shrimp challenged with two different pathogens and get a better understanding of shrimp response to immune stimulation. Here we successfully identified 48 and 49 spots in *Vibrio* and WSSV-challenged group, respectively, and most of them have important biological functions and should play important roles in immune response of Chinese shrimp.

Proteins Related to Energy Production

In this study, we observed the expression level of some proteins related to energy production changed after pathogen challenge, and some of them had different alteration trend between *Vibrio* and WSSV-challenged group. Alpha-amylase, the most important glucosidase in shrimp, catalyzes the hydrolysis of internal alpha-1,4-glucan links in polysaccharides, yielding a mixture of maltose and glucose, the fermentable substrate (Wormhoudt and Sellos 1996). In this study, we observed one spot (v9) identified as preamylase 1, the precursor of alpha-amylase, showed downregulation after *Vibrio* challenge, which would lead to a decrease in ATP production due to decreased fermentable substrate. In WSSV-challenged group, the same spot (w10) showed upregulation, and another spot (w9) also identified as preamylase 1 showed downregulation. Meanwhile, we observed that two key glycolytic enzymes, phosphopyruvate hydratase and triosephosphate isomerase showed downregulation after *Vibrio* challenge (v25 and v17). In WSSV-challenged group, only w14 (same spot as v17) was downregulated, while w18 (same spot as v25) and w45 were upregulated. This may reflect that after *Vibrio* challenge, the energy production was depressed, while after WSSV challenge, the energy production was maintained in shrimp. We also observed that two spots identified as arginine kinase were downregulated in both *Vibrio*- and WSSV-challenged groups. Arginine kinase catalyzes the reversible transfer of high-energy phosphate from arginine phosphate to ADP for regenerating ATP when acute lack of energy occurs in invertebrate (Yao et al. 2005). The downregulation of arginine kinase might be a result of the decreased concentration of arginine phosphate and ATP due to acute energy consumption. This observation indicated that shrimp confront severe energy shortage after *Vibrio* or WSSV challenge.

Based on the results above, we concluded after *Vibrio* challenge, the energy production in shrimp reduced as a result of glycolysis depression, and after WSSV challenge, energy production was at least maintained as before. However, both *Vibrio*- and WSSV-challenged shrimp confronted energy shortage, so as the energy reservoir, arginine phosphate were consumed. It seems probable that this different energy metabolism strategy between *Vibrio*- and WSSV-challenged shrimp is because WSSV replication requires more energy in WSSV-challenged group.

Proteins Related to Immune

In our study, we also observed the expression of several immune-related proteins varied after pathogen challenge. Chitinase, an enzyme involved in digestion of chitin-containing food in crustacean hepatopancreas, may play an important role in innate immunity. In *Crassostrea gigas*, chitinase mRNA was upregulated in hemocytes after bacterial challenge (Badariottia et al. 2007). In the stomach of WSSV-infected *L. vannamei*, chitinase also showed upregulation (Wang et al. 2007). In this study, one spot in *Vibrio*-challenged group and two spots in WSSV-challenged group identified as chitinase also showed upregulation. Macrophage migration inhibitory factor, initially identified as a T cell cytokine, plays an important role in the innate immune response. It was reported that macrophage migration inhibitory factor-deficient macrophages were hyporesponsive to lipopolysaccharide and gram-negative bacteria (Nishihira 2000). In this study, one macrophage migration inhibitory factor spot was downregulated in both *Vibrio*- and WSSV-challenged shrimp which might reflect that the phagocytosis in shrimp was depressed after pathogen challenge.

The expressions of four proteases, including chymotrypsin BI, trypsin, carboxypeptidase, and zinc proteinase Mpc1 varied in WSSV-challenged shrimp but did not vary in *Vibrio*-challenged shrimp. Chymotrypsin BI and trypsin, the major proteinase component in shrimp hepatopancreas, not only are involved in digestion but also participate in many aspects of invertebrate immunity (Gorman and Paskewitz 2001). In insects, chymotrypsin-like serine protease has been found related to immune defense reactions against bacteria, yeast, and baculovirus (Finnerty et al. 1999; Guedes et al. 2005), and in *F.chinensis*, chymotrypsin-like serine protease showed upregulation after pathogen infection (Shi et al. 2008). In crustaceans, trypsin can significantly trigger the activation of prophenoloxidase, whose active form, phenoloxidase, catalyzes the early steps in the pathway to melanin formation in innate immunity and is also responsible for pigmentation (Söderhäll and Cerenius 1998; Zufelato et al. 2000; Lai et al. 2005; Thomas-Guyon et al. 2009). In most cells of a mammalian, carboxypeptidase participates in the formation of protease–proteoglycans complex, an important component in innate immune system of a mammalian (Stevens and Adachi 2007). In WSSV-challenged group, these three proteins were all downregulated, and this might reflect that the immune system in shrimp was defected after WSSV challenge. A similar result was also observed in the stomach of *L. vannamei* challenged with WSSV (Wang et al. 2007). Zinc

proteinase Mpc1 belongs to metalloendoprotease and participates in many biological processes, including the rebuilding of connective tissue and signal peptide removal (Dumermuth and Sterchi 1991). It was reported that in *L. vannamei* and *Marsupenaeus japonicus*, the transcriptional level of zinc proteinase Mpc1 in WSSV-resistant individuals was higher than the susceptible ones (Pan et al. 2005; Zhao et al. 2007). In this study, we observed three altered expressed spots identified as zinc proteinase Mpc1 in WSSV-infected shrimp. Two of them showed upregulation (w26, w27), and one of them showed downregulation (w4), reflecting that zinc proteinases with different posttranslational modifications had different functions in anti-WSSV defense response. Neprilysin is also a zinc metalloendopeptidase, involved in the metabolism of many regulatory peptides in the mammalian nervous, cardiovascular, inflammatory, and immune systems (Turner et al. 2001). For invertebrate, the putative zinc-dependent protease homologous to mammalian neprilysin was proved to be crucial to the immune response in *Manduca sexta* (Willott and Tran 2002). In this study, neprilysin was upregulated after *Vibrio* stimulation, reflecting that the zinc proteinases may be also important in anti-*Vibrio* defense response.

Cathepsin L is a kind of cysteine proteases of papain family, stored in lysosomes in the form of proenzyme. It participates in a number of biological processes, such as prohormone activation, antigen presentation, and the development of tissues or organs. In shrimp, the main function of cathepsin L is digestion (Hu and Leung 2007). It was reported that the expression of cathepsin L was downregulated in WSSV-infected *Penaeus monodon* in transcriptional level (Pongsomboon et al. 2008). In this study, we found that cathepsin L was downregulated in *Vibrio*-challenged Chinese shrimp but did not change in WSSV-challenged group. We also found that a signal transduction protein related to immune was downregulated in the hepatopancreas of *Vibrio*-challenged Chinese shrimp. This protein, containing a TBC1 domain, is a Rab-like GTPase. Rab GTPases play important roles in regulating the trafficking of membrane including phagosome formation, phagosome movement, and membrane fusion (Rak et al. 2000). It was reported recently that in *Marsupeneaus japonica*, phagocytic percentage was significantly decreased when the Rab gene was silenced by RNAi, and the overexpression of Rab gene led to the increase of phagocytic percentage, suggesting that Rab was essential in the regulation of hemocytic phagocytosis of shrimp (Zong et al. 2008). The downregulation of Rab GTPase in this study might reflect phagocytosis of Chinese shrimp decreased after *Vibrio* challenge, leading to weakened immunity of Chinese shrimp.

The expression pattern of immune-related proteins after pathogen challenge supports very important information to explore the immune response mechanism in Chinese shrimp. From the abovementioned, we could see that there were few in common of the differentially expressed proteins related to immune function between shrimps challenged with *Vibrio* and WSSV. This was a persuasive evidence that the shrimp adapted very different strategies toward different pathogen infection, which was observed in the study of screening differentially expressed genes at the mRNA level in Chinese shrimp challenged with WSSV and *Vibrio* (Wang et al. 2008).

Proteins Involved in the Metabolism

According to the expression profiles of differently expressed proteins related to energy production in shrimp after *Vibrio* and WSSV challenge that, we can see after *Vibrio* or WSSV challenge, shrimp suffer the energy shortage. In order to maintain ATP demand and supply balance, shrimp were inclined to suppress energy-consuming processes such as reducing metabolic rate. In our present study, a number of enzymes participated in the metabolism of nucleoside, lipid, and amino acid, and one-carbon unit showed differential expression after pathogen challenge, and most of them were downregulated.

Adenosine kinase, which catalyzes the phosphorylation of adenosine to AMP, is a key enzyme in purine salvage pathway (Decking et al. 1997). Transketolase, catalyzing transfer of a two-carbon fragment from a ketose to an aldose in pentose phosphate pathways, has a controlling role in the supply of ribose units for nucleoside biosynthesis (Turner 2000). Phosphoribosylaminoimidazole carboxylase is an enzyme involved in nucleotide synthesis. It catalyzes the conversion of 5′-phosphoribosyl-5-aminoimidazole into 5-phosphoribosyl-4-carboxy-5-aminoimidazole (Meyer et al. 1999). These three enzymes which participated in the metabolism of nucleoside were all found differentially expressed in Chinese shrimp challenged with *Vibrio* and WSSV. Three spots were identified as adenosine kinase in *Vibrio*-challenged group with two upregulated (v38, v12) and one downregulated (v11). There were also three spots identified as adenosine kinase in WSSV-challenged group. The spot w36 (same spot as v38) showed upregulation, while w12 (same spot as v11) and w13 showed downregulation. Two spots (v16, v39) identified as transketolase showed downregulation in *Vibrio*-challenged group, while v30 were upregulated. In WSSV-challenged group, w38 (same spot as v39) also showed downregulation, but w29 (same spot as v30) showed upregulation. Phosphoribosylaminoimidazole carboxylase was found upregulated only in WSSV-challenged group. To sum up, the enzymes related to nucleoside metabolism tended to be downregulated in *Vibrio*-challenged group, reflecting a suppression of nucleoside biosynthesis took place to save energy; while the upregulation of these enzymes in WSSV-challenged group might be a result of the virus duplication.

Cystathionine beta-lyase catalyzes the alpha, beta-elimination of cystathionine to produce homocysteine, pyruvate, and NH_3, and is a key enzyme in methionine biosynthesis (Ravanel et al. 1996). Pyrroline-5-carboxylate dehydrogenase oxidizes pyrroline-5-carboxylate which is a physiological intermediate in the interconversions of proline, ornithine,

glutamate, and arginine, to glutamate (Mohamed et al. 2008). These two enzymes involved in amino acid metabolism showed obvious downregulation in both *Vibrio*- and WSSV-challenged group which might reflect depressions of some amino acid-related metabolism pathways. Pyruvate, an intermediate of several metabolic pathways, can convert to alanine and take part in amino acid metabolism (Cody et al. 2000). Hydroxyacylglutathione hydrolase participates in pyruvate metabolism, with the common substrate methylglyoxal, the product is D-lactate (Vander 1993). Aspartate aminotransferase, also known as glutamic oxaloacetic transaminase, catalyzes the reversible reaction of aspartate and 2-oxoglutarate yielding glutamate and oxaloacetate (Kuramitsu et al. 1985). These four enzymes showed downregulation in *Vibrio*-challenged group, and three of them except hydroxyacylglutathione hydrolase were downregulated in WSSV-challenged group. This reflected that some amino acid-related metabolism pathways were depressed to save energy after pathogen infection.

One-carbon metabolism can supply organisms to the one-carbon units, which has important biological significant in synthesizing proteins, nucleic acids, pantothenate, and many methylated molecules (Bailey and Gregory 1999). Here, we identified five enzymes related to one-carbon metabolism. Homocysteine *S*-methyltransferase catalyzes the *S*-adenosylmethionine, a universal methyl donor of numerous methylation reactions and L-homocysteine to produce *S*-adenosyl-L-homocysteine and L-methionine, resulting to the one-carbon unit transferring. Formyltetrahydrofolate dehydrogenase participates in folate metabolism, and its downregulation would decrease the concentration of *S*-adenosylmethionine (Lu 2000; Anguera et al. 2006). *S*-Adenosyl-L-homocysteine hydrolase catalyzes the reversible hydration of *S*-adenosyl-L-homocysteine, a potent inhibitor of all methylation reactions, to adenosine and homocysteine. In eukaryotic cells, *S*-Adenosyl-L-homocysteine hydrolase is believed to activate methyl cycle by inhibiting *S*-adenosyl-L-homocysteine (Shu et al. 2006). Glutamate carboxypeptidase is a folate hydrolase, playing an important role in folate metabolism (Rawlings and Barrett 1997). Formylglutathione hydrolase is a thioesterases involved in detoxification of formaldehyde which is synthesized from choline oxidation (Hanson et al. 2000). In our study, except glutamate carboxypeptidase that was observed downregulated in *Vibrio*-challenged group, other four enzymes related to one-carbon metabolism showed downregulation in both *Vibrio*- and WSSV-challenged groups. We could conclude that some one-carbon units involved in metabolisms were suppressed in the hepatopancreas of Chinese shrimp after pathogen infection, which lead to a suppression of other metabolic pathway.

Two proteins involved in carbohydrate metabolism and one-lipid metabolism-related enzyme also showed downregulation in shrimp after pathogen infection. Fructose-1,6-biphosphatase converts fructose-1,6-bisphosphate to fructose 6-phosphate in gluconeogenesis, which is an energy-consuming process, and is suppressed when energy deficits happen. This enzyme was downregulated in both *Vibrio*- and WSSV-challenged groups. Beta-galactosidase, catalyzes the hydrolysis of beta-galactosides into glucose and galactose, showed downregulation in WSSV-challenged group. 3-hydroxyacyl-CoA dehydrogenase is an oxidoreductase that participates in the fatty acid metabolism, and this enzyme was downregulated in *Vibrio*-challenged group.

Aldehyde dehydrogenase catalyzes the conversion of aldehydes to its corresponding acids by means of an $NAD(P)^+$-dependent irreversible reaction. It was reported that aldehyde dehydrogenase was expressed at basal level under hypoxic conditions in fungus (Shimizu et al. 2009). In this study, we also observed two kinds of aldehyde dehydrogenase that showed downregulation in *Vibrio*- or WSSV-challenged groups (v20 and w33), which might be a result of the reducing environment in hepatopancreas of shrimp after pathogen infection.

Antioxidant Proteins

Phagocytosis is an important immune defense reaction in shrimp hemocytes (Lee and Söderhäll 2002). During this course, the host enhances the production of reactive oxygen species (ROS), known as respiratory burst, to kill foreign invaders efficiently (Bogdan et al. 2000). However, the excessive ROS may cause serious damage to cellular macromolecules, such as nucleic acids, lipids, and proteins (Halliwell and Gutteridge 1999). To maintain the proper functions of cells, excessive ROS should be eliminated, which is performed by antioxidants. In this study, we observed three antioxidant proteins, mitochondrial manganese superoxide dismutase, fatty acid-binding protein 10, and carbonyl reductase (NADPH) 1, that were downregulated in both *Vibrio*- and WSSV-challenged groups. Mitochondrial manganese superoxide dismutase catalyzes the dismutation of two molecules of superoxide anion into water and hydrogen peroxide (Zhang et al. 2007). Fatty acid-binding protein 10 can bind long-chain fatty acid oxidative products with high affinity and capacity to scavenge them (Rajaraman et al. 2007). Carbonyl reductase 1 detoxificates the lipid peroxidation produced under oxidative conditions (Forrest and Gonzalez 2000). The depressed expression of these three antioxidant proteins in this study might reflect that in the hepatopancreas of Chinese shrimp, it was not an oxidative environment as a result of respiratory burst after pathogen stimulation. It was reported that in the hemocytes of *L. vannamei* injected with *Vibrio alginolyticus*, the respiratory burst and superoxide dismutase activity varied at different time points. In the first 24 h after infection, respiratory burst and superoxide dismutase activity decreased as a result of the weakened shrimp's immunity, while a slow recovery of respiratory burst and superoxide dismutase activity happened during 72–96 h postinjection (Li et al. 2008). This might explain

why in some reports antioxidant protein expression was provoked by pathogen stimulation (Chai et al. 2010).

Chaperones and Proteins Related to Translation

We found three differentially expressed chaperone proteins in hepatopancreas of shrimp after pathogen infection. Protein disulfide isomerase (PDI) catalyzes the native disulfide bonds formation and rearrangement and participates in protein folding, assembly, and posttranslational modification (Freedman et al. 1994; Tu and Weissman 2004). In the present study, we identified five spots as PDI in *Vibrio*-challenged group, including one upregulated and four downregulated spots. The corresponding spots in WSSV-challenged group showed the same alteration trend as that in *Vibrio*-challenged group. The complex PDI expression pattern might reflect that the changes in posttranslational modifications of PDI played important roles in immune response, which should be further studied. Heat shock protein 70, a kind of ubiquitously expressed molecular chaperone, assists the folding of newly synthesized peptides in the cell and helps to protect cells from stress (Mayer and Bukau 2005). Proteasomes are very large barrel-like protein complexes, and beta subunits are components of the core structure. The main function of the proteasome is to degrade unneeded or damaged proteins into peptides by proteolysis, a chemical reaction that breaks peptide bond. These peptides are further degraded into amino acid molecules for synthesizing new proteins (Peters et al. 1994). Ribosomal protein L5 is a component of 50S subunit in ribosome, a molecular machine for translation in cells. In this study, heat shock protein 70 and proteasome beta subunit showed downregulation in both *Vibrio*- and WSSV-challenged Chinese shrimp, and ribosomal protein L5 was downregulated in *Vibrio*-challenged group. Taking together, the PDI showed downregulated; we conclude that protein synthesis was greatly suppressed in response to pathogens stimulation as a result of energy shortage. This result is also in agreement with the heat shock protein 70 expression pattern in epithelium of *Penaeus monodon* in response to WSSV infection (Wu et al. 2007).

Ungrouped Proteins

Oncoprotein nm23 is a nucleoside diphosphate kinase, which plays roles in maintaining a pool of nucleoside triphosphate required for biosynthesis. It was reported that overexpression of nucleoside diphosphate kinase could protect cell from excessive production of ROS under oxidative stress (Benhar et al. 2002). We also found that in shrimp under hypoxic condition, oncoprotein nm23 was downregulated at protein level (Jiang et al. 2009). In the present study, nm23 was observed downregulated after *Vibrio* infection, and this might relate to the suppression of ROS in response of *Vibrio* stimulation deduced in this study. Heat-responsive protein 12 had significant similarity to DnaK/heat shock protein 70, expressed highly in mouse liver and kidney and was upregulated after heat shock (Samuel et al. 1997). In this study, it was downregulated after *Vibrio* stimulation, but the significance of this phenomenon is unknown.

From the protein expression patterns of Chinese shrimp in this study, we found that the mechanisms of Chinese shrimp in response to *Vibrio* and WSSV challenge had something in common and also had their own characteristics. Many proteins participated in the interaction between host and pathogens; their different expression patterns between shrimps challenged with *Vibrio* and WSSV indicated that host adapted different strategies to resist different pathogens. This study set basis for dissecting the immune mechanisms in shrimp and provided important information to develop anti-disease strategy, aiming at different pathogens infection in shrimp culture. Some results in this study was not absolutely in agreement with the existing studies; this might be because of the differences in shrimp species, organs, pathogens, and the immune stage of the shrimp (Wu et al. 2007; Qiao et al. 2011). Further studies on shrimp immune response in other organs and at other time points should be performed. We found some spots identified as one protein that showed opposite alteration trends after pathogen challenge; this might be because they were same proteins with different post translational modifications that played different roles in immune response. The function of these proteins should be focused in the future studies. We used the inactivated but not live *Vibrio* to challenge shrimp in this study, so the host's responses were caused by pattern-recognition molecules, without the influences of live pathogen to host. Future efforts would also be focused on the protein expression profiles of shrimp in response to other kinds of pattern-recognition molecules and live pathogens.

Acknowledgments This work was financially supported by Major State Basic Research Development Program of China (973 program) (2012CB114403) and National Natural Science Foundation of China (41076101).

References

Andriantahina F, Liu X, Feng T, Xiang J (2013) Current status of genetics and genomics of reared penaeid shrimp: information relevant to access and benefit sharing. Mar Biotechnol (NY) 15(4):399–412. doi:10.1007/s10126-013-9500-9

Anguera MC, Field MS, Perry C, Ghandour H, Chiang E-P, Selhub J, Shane B, Stover PJ (2006) Regulation of folate-mediated one-carbon metabolism by 10-formyltetrahydrofolate dehydrogenase. J Biol Chem 281:18335–18342

Aoki T, Wang H-C, Unajak S, Santos MD, Kondo H, Hirono I (2011) Microarray analyses of shrimp immune responses. Mar Biotechnol (NY) 13:629–638

Bachère E (2000) Shrimp immunity and disease control. Aquaculture 191:3–11

Bachère E, Gueguen Y, Gonzalez M, Lorgeril JD, Garnier J, Romestand B (2004) Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*. Immunol Rev 198:149–168

Badariottia F, Thuaub R, Lelonga C, Dubosa M-P, Favrela P (2007) Characterization of an atypical family 18 chitinase from the oyster *Crassostrea gigas*: evidence for a role in early development and immunity. Dev Comp Immunol 31:559–570

Bailey LB, Jesse F, Gregory I (1999) Folate metabolism and requirements. J Nutr 129:779–782

Baticados MCL, Lavilla-Pitogo CR, Cruz-Lacierda ER, Pena LDDL, Sunaz NA (1990) Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *V.splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. Dis Aquat Org 9: 133–139

Benhar M, Engelberg D, Levitzki A (2002) ROS, stress-activated kinases and stress signaling in cancer. EMBO Rep 3:420–425

Bogdan C, Röllinghoff M, Diefenbach A (2000) Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol 12:64–76

Chai Y-M, Yu S-S, Zhao X-F, Zhu Q, Wang J-X (2010) Comparative proteomic profiles of the hepatopancreas in *Fenneropenaeus chinensis* response to white spot syndrome virus. Fish Shellfish Immunol 29:480–486

Chaikeeratisak V, Somboonwiwat K, Wang H-C, Lo CF, Tassanakajon A (2012) Proteomic analysis of differentially expressed proteins in the lymphoid organ of *Vibrio harveyi*-infected *Penaeus monodon*. Mol Biol Rep 39:6367–6377

Cheng W, Liu C-H, Tsai C-H, Chen J-C (2005) Molecular cloning and characterisation of a pattern recognition molecule, lipopolysaccharide- and beta-1,3-glucan binding protein (LGBP) from the white shrimp *Litopenaeus vannamei*. Fish Shellfish Immunol 18:297–310

Chongsatja P-O, Bourchookarn A, Lo CF, Thongboonkerd V, Krittanai C (2007) Proteomic analysis of differentially expressed proteins in *Penaeus vannamei* hemocytes upon Taura syndrome virus infection. Proteomics 7:3592–3601

Cody GD, Boctor NZ, Filley TR, Hazen RM, Scott JH, Sharma A, Yoder HS Jr (2000) Primordial carbonylated iron-sulfur compounds and the synthesis of pyruvate. Science 289:1337–1340

Decking UKM, Schlieper G, Kroll K, Schrader J (1997) Hypoxia-induced inhibition of adenosine kinase potentiates cardiac adenosine release. Circ Res 81:154–164

Destoumieux D, Munoz M, Bulet P, Bachère E (2000) Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda). Cell Mol Life Sci 57:1260–1271

Dumermuth E, Sterchi EE (1991) The astacin family of metalloendopeptidases. J Biol Chem 266:21381–21385

Finnerty CM, Karplus PA, Granados RR (1999) The insect immune protein scolexin is a novel serine proteinase homolog. Protein Sci 8:242–248

Flegel TW, Sritunyalucksana K (2011) Shrimp molecular responses to viral pathogens. Mar Biotechnol (NY) 13:587–607

Forrest GL, Gonzalez B (2000) Carbonyl reductase. Chem Biol Interact 129:21–40

Freedman RB, Hirst TR, Tuite MF (1994) Protein disulphide isomerase: building bridges in protein folding. Trends Biochem Sci 19:331–336

Gorman MJ, Paskewitz SM (2001) Serine proteases as mediators of mosquito immune responses. Insect Biochem Mol Biol 31:257–262

Guedes SDM, Vitorino R, Domingues R, Tomer K, Correia AJF, Amado F, Domingues P (2005) Proteomics of immune-challenged *Drosophila melanogaster* larvae hemolymph. Biochem Biophys Res Commun 328:106–115

Halliwell B, Gutteridge JMC (1999) Free radical biology and medicine. Oxford University Press, Oxford

Hanson AD, Gage DA, Shachar-Hill Y (2000) Plant one-carbon metabolism and its engineering. Trends Plant Sci 5:206–213

Hu K-J, Leung P-C (2007) Food digestion by cathepsin L and digestion-related rapid cell differentiation in shrimp hepatopancreas. Comp Biochem Physiol B Biochem Mol Biol 146:69–80

Huang J, Song X-L, Yu J, Zhang L-J (1999) The components of an inorganic physiological buffer for *Penaeus chinensis*. Methods Cell Sci 21:225–230

Jiang H, Li F, Xie Y, Huang B, Zhang J, Zhang J, Zhang C, Li S, Xiang J (2009) Comparative proteomic profiles of the hepatopancreas in *Fenneropenaeus chinensis* response to hypoxic stress. Proteomics 9:3353–3367

Kim H-J, Lee D-Y, Lee D-H, Park Y-C, Kweon D-H, Ryu Y-W, Seo J-H (2004) Strategic proteome analysis of Candida magnoliae with an unsequenced genome. Proteomics 4:3588–3599

Kuramitsu S, Okuno S, Ogawa T, Ogawa H, Kagamiyama H (1985) Aspartate aminotransferase of Escherichia coli: nucleotide sequence of the *aspC* gene. J Biochem 97:1259–1262

Lai C-Y, Cheng W, Kuo C-M (2005) Molecular cloning and characterisation of prophenoloxidase from haemocytes of the white shrimp, *Litopenaeus vannamei*. Fish Shellfish Immunol 18:417–430

Lee SY, Söderhäll K (2002) Early events in crustacean innate immunity. Fish Shellfish Immunol 12:421–437

Leu J-H, Chen S-H, Wang Y-B, Chen Y-C, Su S-Y, Lin C-Y, Ho J-M, Lo C-F (2011) A review of the major penaeid shrimp EST studies and the construction of a shrimp transcriptome database based on the ESTs from four penaeid shrimp. Mar Biotechnol (NY) 13:608–621

Li F, Xiang J (2013a) Recent advances in researches on the innate immunity of shrimp in China. Dev Comp Immunol 39:11–26

Li F, Xiang J (2013b) Signaling pathways regulating innate immune responses in shrimp. Fish Shellfish Immunol 34:973–980

Li CC, Yeh ST, Chen JC (2008) The immune response of white shrimp *Litopenaeus vannamei* following *Vibrio alginolyticus* injection. Fish Shellfish Immunol 25:853–860

Li S, Zhang X, Sun Z, Li F, Xiang J (2013) Transcriptome analysis on Chinese shrimp *Fenneropenaeus chinensis* during WSSV acute infection. PLoS ONE 8:e58627

Lightner DV (1996) Epizootiology, distribution and the impact on international trade of two penaeid shrimp viruses in the Americas. Rev Sci Tech 15:579–601

Liu F, Liu Y, Li F, Dong B, Xiang J (2005) Molecular cloning and expression profile of putative antilipopolysaccharide factor in Chinese shrimp(*Fenneropenaeus chinensis*). Mar Biotechnol (NY) 7:600–608

Liu C-H, Tseng D-Y, Lai C-Y, Cheng W, Kuo C-M (2006) Molecular cloning and characterisation of prophenoloxidase cDNA from haemocytes of the giant freshwater prawn, *Macrobrachium rosenbergii*, and its transcription in relation with the moult stage. Fish Shellfish Immunol 21:60–69

Liu Y-C, Li F-H, Dong B, Wang B, Luan W, Zhang X-J, Zhang L-S, Xiang J-H (2007) Molecular cloning, characterization and expression analysis of a putative C-type lectin (Fclectin) gene in Chinese shrimp *Fenneropenaeus chinensis*. Mol Immunol 44:598–607

Lu SC (2000) S-adenosylmethionine. Int J Biochem Cell Biol 32:391–395

Mayer MP, Bukau B (2005) Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 62:670–684

Meyer E, Kappock TJ, Osuji C, Stubbe J (1999) Evidence for the direct transfer of the carboxylate of N^5-carboxyaminoimidazole ribonucleotide (N^5-CAIR) to generate 4-carboxy-5-aminoimidazole ribonucleotide catalyzed by *Escherichia coli* PurE, an N^5-CAIR mutase. Biochemistry 38:3012–3018

Mohamed SA, Mohamed TM, Fahmy AS, El-Badry MO, Abdel-Gany SS (2008) Fasciola gigantica: enzymes of the ornithine-proline-glutamate pathway-characterization of Δ^1-pyrroline-5-carboxylate dehydrogenase. Exp Parasitol 118:47–53

Nishihira J (2000) Macrophage migration inhibitory factor (MIF): its essential role in the immune system and cell growth. J Interferon Cytokine Res 20:751–762

Pan D, He N, Yang Z, Liu H, Xu X (2005) Differential gene expression profile in hepatopancreas of WSSV-resistant shrimp (*Penaeus japonicus*) by suppression subtractive hybridization. Dev Comp Immunol 29:103–112

Peters JM, Franke WW, Kleinschmidt JA (1994) Distinct 19S and 20S subcomplexes of the 26S proteasome and their distribution in the nucleus and the cytoplasm. J Biol Chem 269:7709–7718

Pongsomboon S, Wongpanya R, Tang S, Chalorsrikul A, Tassanakajon A (2008) Abundantly expressed transcripts in the lymphoid organ of the black tiger shrimp, Penaeus monodon, and their implication in immune function. Fish Shellfish Immunol 25:485–493

Porubleva L, Velden KV, Kothari S, Oliver DJ, Chitnis PR (2001) The proteome of maize leaves: use of gene sequences and expressed sequence tag data for identification of proteins with peptide mass fingerprints. Electrophoresis 22:1724–1738

Qiao J, Du Z, Zhang Y, Du H, Guo L, Zhong M, Cao J, Wang X (2011) Proteomic identification of the related immune-enhancing proteins in shrimp *Litopenaeus vannamei* stimulated with vitamin C and Chinese herbs. Fish Shellfish Immunol 31:736–745

Rajaraman G, Wang GQ, Yan J, Jiang P, Gong Y, Burczynski FJ (2007) Role of cytosolic liver fatty acid binding protein in hepatocellular oxidative stress: effect of dexamethasone and clofibrate treatment. Mol Cell Biochem 295:27–34

Rak A, Fedorov R, Alexandrov K, Albert S, Goody RS, Gallwitz D, Scheidig AJ (2000) Crystal structure of the GAP domain of Gyp1p: first insights into interaction with Ypt/Rab proteins. EMBO J 19: 5105–5113

Rattanarojpong T, Wang H-C, Lo C-F, Flegel TW (2007) Analysis of differently expressed proteins and transcripts in gills of *Penaeus vannamei* after yellow head virus infection. Proteomics 7:3809–3814

Ravanel S, Job D, Douce R (1996) Purification and properties of cystathionine β-lyase from *Arabidopsis thaliana* overexpressed in *Escherichia coli*. Biochem J 320:383–392

Rawlings ND, Barrett AJ (1997) Structure of membrane glutamate carboxypeptidase. Biochim Biophys Acta Protein Struct Mol Enzymol 1339:247–252

Robalino J, Carnegie RB, O'leary N, Ouvry-Patat SA, Vega EDL, Prior S, Gross PS, Browdy CL, Chapman RW, Schey KL, Warr G (2009) Contributions of functional genomics and proteomics to the study of immune responses in the Pacific white leg shrimp *Litopenaeus vannamei*. Vet Immunol Immunopathol 128:110–118

Samuel SJ, Tzung SP, Cohen SA (1997) Hrp12, a novel heat-responsive, tissue-specific, phosphorylated protein isolated from mouse liver. Hepatology 25:1213–1222

Shi X-Z, Zhao X-F, Wang J-X (2008) Molecular cloning and expression analysis of chymotrypsin-like serine protease from the Chinese shrimp, *Fenneropenaeus chinensis*. Fish Shellfish Immunol 25:589–597

Shimizu M, Fujii T, Masuo S, Fujita K, Takaya N (2009) Proteomic analysis of *Aspergillus nidulans* cultured under hypoxic conditions. Proteomics 9:7–19

Shu S, Mahadeo DC, Liu X, Liu W, Parent CA, Korn ED (2006) S-adenosylhomocysteine hydrolase is localized at the front of chemotaxing cells, suggesting a role for transmethylation during migration. Proc Natl Acad Sci U S A 103:19788–19793

Söderhäll K, Cerenius L (1998) Role of the prophenoloxidase-activating system in invertebrate immunity. Curr Opin Immunol 10:23–28

Somboonwiwat K, Supungul P, Rimphanitchayakit V, Aoki T, Hirono I, Tassanakajon A (2006) Differentially expressed genes in hemocytes of Vibrio harveyi-challenged shrimp *Penaeus monodon*. J Biochem Mol Biol 39:26–36

Stevens RL, Adachi R (2007) Protease-proteoglycan complexes of mouse and human mast cells and importance of their β-tryptase-heparin complexes in inflammation and innate immunity. Immunol Rev 217:155–167

Thomas-Guyon H, Gagnaire B, Bado-Nilles A, Karine Bouillyb SL, Renault T (2009) Detection of phenoloxidase activity in early stages of the Pacific oyster *Crassostrea gigas* (Thunberg). Dev Comp Immunol 33:653–659

Tu BP, Weissman JS (2004) Oxidative protein folding in eukaryotes: mechanisms and consequences. J Cell Biol 164:341–346

Turner NJ (2000) Applications of transketolases in organic synthesis. Curr Opin Biotechnol 11:527–531

Turner AJ, Isaac RE, Coates D (2001) The neprilysin (NEP) family of zinc metalloendopeptidases: genomics and function. BioEssays 23:261–269

Vander JD (1993) Glyoxalase II: molecular characteristics, kinetics and mechanism. Biochem Soc Trans 21:522–527

Wang B, Li F, Dong B, Zhang X, Zhang C, Xiang J (2006) Discovery of the genes in response to white spot syndrome virus (WSSV) infection in *Fenneropenaeus chinensis* through cDNA microarray. Mar Biotechnol (NY) 8:491–500

Wang H-C, Wang H-C, Leu J-H, Kou G-H, Wang AH-J, Loc C-F (2007) Protein expression profiling of the shrimp cellular response to white spot syndrome virus infection. Dev Comp Immunol 31:672–686

Wang B, Li F, Luan W, Xie Y, Zhang C, Luo Z, Gui L, Yan H, Xiang J (2008) Comparison of gene expression profiles of *Fenneropenaeus chinensis* challenged with WSSV and *Vibrio*. Mar Biotechnol (NY) 10:664–675

Wen R, Li F, Sun Z, Xiang J (2013) Shrimp MyD88 responsive to bacteria and white spot syndrome virus. Fish Shellfish Immunol 34:574–581

Willott E, Tran HQ (2002) Zinc and *Manduca sexta* hemocyte functions. J Insect Sci 2:6–14

Wormhoudt AV, Sellos D (1996) Cloning and sequencing analysis of three amylase cDNAs in the Shrimp *Penaeus vannamei* (Crustacea Decapoda): evolutionary aspects. J Mol Evol 42:543–551

Wu J, Lin Q, Lim TK, Liu T, Hew C-L (2007) White spot syndrome virus proteins and differentially expressed host proteins identified in shrimp epithelium by shotgun proteomics and cleavable isotope-coded affinity tag. J Virol 81:11681–11689

Yang F, He J, Lin X, Li Q, Pan D, Zhang X, Xu X (2001) Complete genome sequence of the shrimp white spot bacilliform virus. J Virol 75:11811–11820

Yao C-L, Wua C-G, Xiang J-H, Dong B (2005) Molecular cloning and response to laminarin stimulation of arginine kinase in haemolymph in Chinese shrimp, *Fenneropenaeus chinensis*. Fish Shellfish Immunol 19:317–329

Zhang Q, Li F, Wang B, Zhang J, Liu Y, Zhou Q, Xiang J (2007) The mitochondrial manganese superoxide dismutase gene in Chinese shrimp *Fenneropenaeus chinensis*: Cloning, distribution and expression. Dev Comp Immunol 31:429–440

Zhang J, Li F, Jiang H, Yu Y, Liu C, Li S, Wang B, Xiang J (2010a) Proteomic analysis of differentially expressed proteins in lymphoid organ of *Fenneropenaeus chinensis* response to *Vibrio anguillarum* stimulation. Fish Shellfish Immunol 29:186–194

Zhang X, Zhang Y, Scheuring C, Zhang H-B, Huan P, Wang B, Liu C, Li F, Liu B, Xiang J (2010b) Construction and characterization of a bacterial artificial chromosome (BAC) library of Pacific white shrimp, *Litopenaeus vannamei*. Mar Biotechnol (NY) 12:141–149

Zhao Z-Y, Yin Z-X, Weng S-P, Guan H-J, Li S-D, Xing K, Chan S-M, He J-G (2007) Profiling of differentially expressed genes in hepatopancreas of white spot syndrome virus-resistant shrimp (*Litopenaeus vannamei*) by suppression subtractive hybridisation. Fish Shellfish Immunol 22:520–534

Zong R, Wu W, Xu J, Zhang X (2008) Regulation of phagocytosis against bacterium by Rab GTPase in shrimp *Marsupenaeus japonicus*. Fish Shellfish Immunol 25:258–263

Zufelato MS, Bitondi MMG, Simões ZLP, Hartfelder K (2000) The juvenile hormone analog pyriproxyfen affects ecdysteroid-dependent cuticle melanization and shifts the pupal ecdysteroid peak in the honey bee (*Apis mellifera*). Arthropod Struct Devel 29:111–119