Journal of Aquaculture Research & Development

Chen et al., J Aquac Res Development 2017, 8:11
DOI: 10.4172/2155-9546.1000517

Research Article Open Access

Up-regulated Expressions of Immune Parameters, ppa, propo, sod, and hsp70 in White Shrimp *Litopenaeus vannamei* Reared at Unfavorable Low Salinities

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Abstract

The expressions of prophenoloxidase activating enzyme (ppA), prophenoloxidase (proPO) I, proPO II, and antioxidant enzymes like cytosolic manganese dismutase (cytMnSOD), mitochondrial manganese dismutase (mtMnSOD), and extracellular copper and zinc dismutase (ecCuZnSOD) as well as heat shock protein 70 (HSP70) were examined in white shrimp *Litopenaeus vannamei* reared for 24 weeks at salinities of 2.5%, 5%, 15%, 25%, and 35%. The expression levels of ppA, proPO I, proPO II, cytMnSOD, mtMnSOD, ecCuZnSOD, and HSP70 of shrimp reared at 2.5% and 5% were significantly higher than in shrimp reared at 15%, 25%, and 35%. We concluded that white shrimp kept under long-term culture at 2.5% and 5% were under chronic stress had up-regulated expressions of ppA, proPO, MnSOD, CuZnSOD, and HSP70 that may provide candidate biomarkers in low salinity shrimp farming.

Keywords: *Litopenaeus vannamei*; Salinity; Prophenoloxidase activating enzyme (ppA); Prophenoloxidase (proPO); Superoxide dismutase (SOD); Heat shock protein 70 (HSP70); Gene expression

Introduction

The white shrimp *Litopenaeus vannamei* is an important species in world aquaculture, having top commercial value among the fish, crustaceans, and mollusks under global aquaculture production [1]. White shrimp inhabit a wild range of salinities, from 1%~2% to 40% [2]. This species exhibits hyper-osmotic regulation at low salinity levels and exhibits hypo-osmotic regulation at high salinity levels, with an iso-osmotic point of 718 mOsm/kg (equivalent to 25%) [3]. White shrimp reared at 15%~20%, or even to 25%, grow much better [4-6]. However, shrimp farming is liable to encounter various environmental stressors such as water temperature, salinity, pH, hypoxia, and pollutants that lead to increased susceptibility to foreign pathogen infection [7-10]. Like other invertebrate, shrimp do not have acquired (adaptive) immune system, instead relying on cellular and humoral innate (natural) immune responses to defend against invading microbes or foreign particles. Three types of hemocytes, comprised of hyaline cells (HCs), semi-granular cells (SGCs), and granular cells (GCs), which play important roles in the activation of immune response, are recognized based on cell size and degree of granularity [11,12]. The immune response is initiated through the recognition and binding of bacterial wall components like β-glucan, lipopolysaccharide, and peptidoglycan known as pathogen-associated molecular patterns (PAMPS) by pattern recognition proteins (PRPs) like lipopolysaccharide and β-glucan binding protein (LGBP) on the surface of hemocytes [13]. Both SGCs and GCs are induced by foreign particles to degranulate granules and release prophenoloxidase activating enzyme (ppA), prophenoloxidase (proPO), peroxinectin (PX), and other enzymes [14]. In the presence of minute amounts of cell-wall molecules, ppA transforms inactive proPO into the active form phenoloxidase (PO), which catalyzes oxygenation and the oxidation of phenols to o-quinones and leads to melanin formation [15,16]. ppA and proPO are important enzymes in the proPO system, and two sub-distinct groups of proPO I and proPO II have been identified in white shrimp *L. vannamei* [17, 18]. HCs and SGCs are involved in phagocytosis, an important and earlier cellular reaction. During post-phagocytosis, a series of reactions initiated by NADPH oxidase produce superoxide anions ($O_2^{\cdot-}$) and other reactive oxygen species (ROS) as a consequence of cellular oxygen metabolism. The superoxide anion is catalyzed by superoxide dismutase (SOD) to produce hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and single oxygen ($^1O_2$), which subsequently leads to the production of very reactive hypochlorous acid (HOCl) and nitric oxide (NO) [19,20]. SOD catalyzes the conversion of superoxide anion to H_2O_2 and oxygen that pass freely through a membrane [21]. Three types of SOD, known as cytosolic manganese SOD (cytMnSOD), mitochondrial manganese SOD (mtMnSOD), and extracellular copper and zinc SOD (ecCuZnSOD) have been identified in white shrimp, and they provide an important role in the antioxidant system [18-22]. White shrimp *L. vannamei* under long-term culture at 15%, 25%, and 35% salinities have significantly higher levels in their immune parameters, including phenoloxidase (PO) activity, respiratory burst (RB), superoxide dismutase (SOD) activity, and lysozyme activity, but have significantly lower expression levels of LGBP, peroxinectin (PX), integrin β, and α2-macroglobulin (α2-M) than shrimp reared at 2.5% and 5% [6]. Shrimp reared at such low salinity levels are considered to be in an unfavorable condition that brings about chronic stress. Heat shock protein 70s (HSP70s) are chaperone proteins known for their response to environmental stresses [23,24]. However, nothing was known about the expression levels of ppA, proPO, SOD, and HSP70 in shrimp following chronic salinity stress. Therefore, the aim of the present study was to examine the expressions of ppA, proPO I, proPO II, cytMnSOD, mtMnSOD, ecCuZnSOD, and HSP70 in white shrimp *L. vannamei* reared at salinity levels of 2.5%, 5%, 15%, 25%, and 35%.

Materials and Methods

Experimental design

About 20000 white shrimp postlarvae (PL_{5-6}) obtained from a hatchery farm in Kaohsiung, Taiwan were shipped to the laboratory and reared in fiberglass tanks filled with filtered natural 35% salinity

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Received November 02, 2017; **Accepted** December 08, 2017; **Published** December 15, 2018

Citation: Chen JC, Lin YC, Chen YY, Huang CL (2017) Up-regulated Expressions of Immune Parameters, ppa, propo, sod, and hsp70 in White Shrimp *Litopenaeus vannamei* reared at Unfavorable Low salinities. J Aquac Res Development 9: 517. doi: 10.4172/2155-9546.1000517

seawater at room temperature. They were initially fed live *Artemia* nauplii and later given an artificial diet until they grew a weight of about 0.48 g. They were then separated into five tanks with final acclimated salinity levels of 2.5%, 5%, 15%, 25%, and 35%, and reared for 24 weeks following previously described procedures [6]. Shrimp in the intermoult stage were sampled for the study [25].

Total RNA isolation and quantitative real-time (q)PCR analysis of gene expression

Eight shrimp each from the 24-week rearing salinity levels were sampled and used for the study. Five hundred microliters of hemolymph were individually withdrawn, placed in a tube containing 500 µl of an anticoagulant solution (30 mM trisodium citrate, 340 mM sodium chloride, and 10 mM EDTA at pH 7.55, with the osmolality adjusted to 718 mOsm/kg with 115 mM glucose), and centrifuged at 800 ×*g* and 4°C for 20 min. The hemocyte pellet was washed with an anticoagulant solution and centrifuged again. Trizol reagent (Invitrogen, Carlsbad, CA, USA) at 1 ml was added to the hemocyte pellet to isolate total RNA. First-strand complementary (c) DNA was generated in a 25-µl reaction volume containing 3 µg DNase I-treated total RNA, 400 pM oligo dT_{18}, 0.4 mM dNTP, 20 U of an RNase inhibitor (Invitrogen), 100 U ReverTra Ace RTase (Toyobo, Tokyo, Japan), and 1x reverse-transcription (RT) buffer. The reaction was conducted at 42°C for 1 h. After first-strand cDNA synthesis, a PCR of the housekeeping gene, elongation factor (EF)1α, was performed to check the RT reaction. Transcripts of target genes (ppA, proPO I, proPO II, cytMnSOD, mtMnSOD, ecCuZnSOD, and HSP70), and the internal control (EF1α) were measured by a qPCR as described previously [26]. Primer sets for each gene were designed based on published *L. vannamei* genes using Beacon Designer Software vers. 6.0 (Table 1). The recombinant plasmids containing ppA, proPO I, proPO II, cytMnSOD, mtMnSOD, ecCuZnSOD, and HSP70 qPCR fragments were all quantified to 1 µg/µl. A series of concentrations of recombinant plasmids of 10^{-5}~10^{-11} µg/µl was diluted with DEPC-treated water to construct the ppA, proPO I, proPO II, cytMnSOD, mtMnSOD, ecCuZnSOD, and HSP70 qPCR standard curves. Relationships between the threshold concentration (Ct) and copy number calculated based on the molecular weight of the target genes were established. Target gene expressions were quantified based on their relationships with the Ct and copy number.

Statistical Analysis

All data were subjected to a one-way analysis of variance (ANOVA). If significant differences were indicated at the 0.05 level, then Tukey's multiple-comparison test was conducted to examine for significant differences among treatments using SAS computer software (SAS Institute, Cary, NC, USA). Statistical significance of differences required that p be <0.05.

Results

The expressions of ppA, proPO I, and proPO II are shown in Figure 1. The expression levels of ppA, proPO I, and proPO II of shrimp reared at 2.5% and 5% were significantly higher than in shrimp reared at 15%, 25%, and 35%. No significant differences in the expressions of ppA, proPO I, or proPO II were observed among shrimp reared at 15%, 25%, and 35%. No significant differences in the expressions of ppA and proPO I was observed between the shrimp reared at 2.5% and 5%. Similar trends were observed in the expressions of cytMnSOD, mtMnSOD, ecCuZnSOD, and HSP70 (Figure 2). The expression levels of cytMnSOD, mtMnSOD, ecCuZnSOD, and HSP70 in shrimp reared at 2.5% and 5% were significantly higher than in shrimp reared

Gene	Primer name	Sequence 5' to 3'	Amplicon	*Reference/ GenBank*
ppA	Liva ppA qPCR F	CTA GAG ACG TCG GTG TCA TCA CC	151 bp	*AY368151*
	Liva ppA qPCR R	AAC TTG CCG TCC GAA GTG CG		
proPO I	Liva proPO I qPCR F	ACG TCA CTT CCG GCA AGC GA	156 bp	*AY723296*
	Liva proPO I qPCR R	CCT CCT TGT GAG CGT TGT CAG G		
proPO II	Liva proPO II qPCR F	ACC ACT GGC ACT GGC ACC TCG TCT A	161 bp	*EU373096*
	Liva proPO II qPCR R	TCG CCA GTT CTC GAG CTT CTG CAC		
cytMn-SOD	Liva cytMnSOD qPCR F	TGA CGA GAG CTT TGG ATC ATT CC	155 bp	*DQ029053*
	Liva cytMnSOD qPCR R	TGA TTT GCA AGG GAT CCT GGT T		
mtMnSOD	Liva mtMnSOD qPCR F	CAG ACT TGC CCT ACG ATT AC	216 bp	*KP099968*
	Liva mtMnSOD qPCR R	AGA TGG TGT GAT TGA TGT GAC		
ecCuZn-SOD	Liva CuZnSOD qPCR F	CGC GGG AGA CAC AGC TGA TTT C	164 bp	*HM371157*
	Liva CuZnSOD qPCR R	GAA ATC CAG GGT GCC GGA GA		
HSP70	Liva Hsp70 qPCR F	CCT CCT ACG TCG CCT TCA CAG ACA	233 bp	*AY645906*
	Liva Hsp70 qPCR R	GGG GTA GAA GGT CTT CTT GTC TCC C		
EF1α	Liva EF1α F	ATG GTT GTC AAC TTT GCC CC	500 bp	*GU136229*
	Liva EF1α R	*TTG ACC TCC TTG ATC ACA CC*		

Table 1: Primers used for the quantitative real-time PCR study of elongation factor 1-alpha (EF 1α) and eleven immune-related genes of white shrimp *Litopenaeus vannamei*.

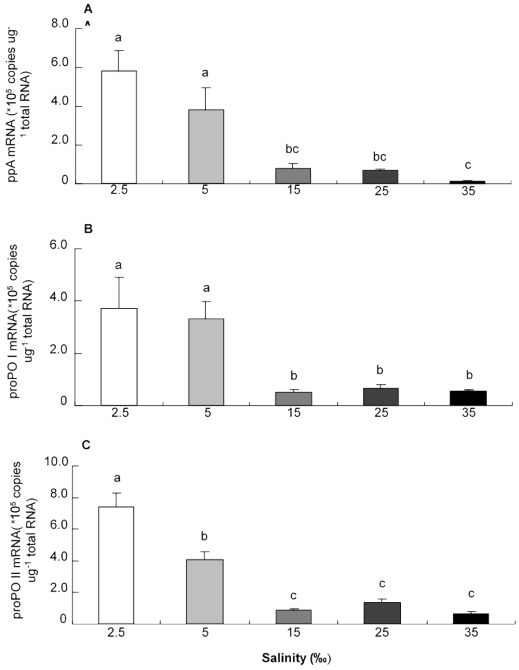

Figure 1: Real-time RT-PCR analysis of prophenoloxidase activating enzyme (ppA) (A), prophenoloxidase I (proPO I) (B), and proPO II (C) in the hemocytes of white shrimp *Litopenaeus vannamei* reared for 24 weeks at salinity levels of 2.5%, 5%, 15%, 25%, and 35%. Each bar represents mean values with the statistical error (SE) from eight determinations. Bars with different letters significantly differ (p<0.05) among salinity levels.

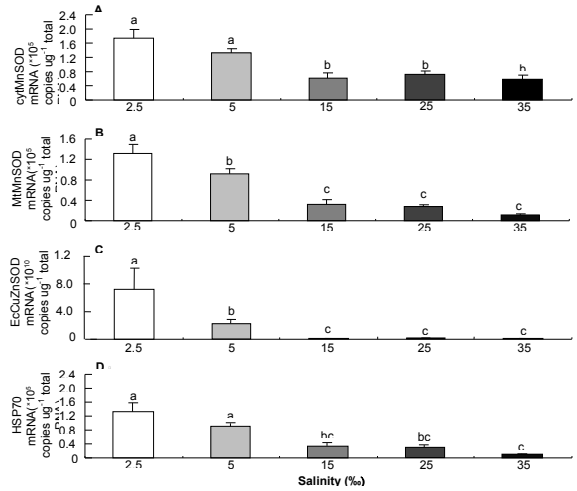

Figure 2: Real-time RT-PCR analysis of cytosolic manganese dismutase (cytMnSOD) (A), mitochondrial manganese dismutase (mtMnSOD) (B), extracellular copper and zinc dismutase (ecCuZnSOD) (C), and heat shock protein 70 (HSP70) (D) in the hemocytes of white shrimp *Litopenaeus vannamei* reared for 24 weeks at salinity levels of 2.5%, 5%, 15%, 25%, and 35%.

at 15%, 25%, and 35%. No significant differences in the expressions of cytMnSOD, mtMnSOD, ecCuZnSOD, and HSP70 were observed among shrimp reared at 15%, 25%, and 35%. No significant differences in cytMnSOD expression were observed between the shrimp reared at 2.5% and 5%. No significant differences in HSP70 expression were observed between shrimp reared at 2.5% and 5%.

Discussion

Immune modulation and immune dysfunction in teleosts and invertebrates caused by environmental stressors like temperature, salinity, pH, hypoxia, and pollutants have recently received attention and study, and have review in several respects [27,28]. Environmental stress can be divided into acute stress and chronic stress. Shrimp reared under favorable salinity, temperature, and pH environmental conditions will be acutely stressed upon encountering sudden changes in those parameters, whereas shrimp are chronically stressed when reared under unfavorable environmental conditions. Sudden change in environmental condition affects the resistance of shrimp against pathogens. For instance, white shrimp *L. vannamei* and Kuruma shrimp *Marsupenaeus japonicus* reared at 25% and then subjected to low salinity stress (5% and 15%) have decreased resistance against *V. alginolyticus* and *Photobacterium damselae* [9,29]. Fleshy shrimp *Fenneropenaeus chinensis* reared at 22% subjected to low salinity stress (14%) and Kuruma shrimp reared at 33% subjected to low salinity stress (9%, 17%, 25%) have decreased resistance to WSSV [30,31]. White shrimp *L. vannamei* reared at 28°C subjected to high temperature (32°C) have decreased resistance against *V. alginolyticus* [32]. White shrimp *L. vannamei* reared at pH 8.2 subjected to low pH (pH 6.5) and high pH (pH 10.1) have decreased resistance against *V. alginolyticus* [10]. Therefore, shrimp reared at a given salinity, temperature or pH level and then subjected to a sudden salinity, temperature or pH change exhibit decreased resistance against pathogens. Sudden change in environmental condition also affects the immune parameters in shrimp. For instance, white shrimp *L. vannamei* and kuruma shrimp *M. japonicus* reared at 25% subjected to low salinity stress (5% and 15%) have decreased total hemocyte count (THC), PO activity, RB, SOD activity [9,29]. Kuruma shrimp reared at 33% subjected to low salinity stress (9%, 17%, 25%) or a combination of WSSV infection and low salinity stress have decreased THC and PO activity [30]. Fleshy shrimp *F. chinensis* reared at 22% subjected to low salinity stress (14%) have decreased THC and PO activity after 24~72 h [31]. White shrimp *L. vannamei* reared at 28°C subjected to high temperature (32°C) have decreased THC, PO activity, RB, and SOD activity [32]. White shrimp *L. vannamei* reared at pH 8.2 subjected to low pH (pH 6.5) and high pH (pH 10.1) have decreased THC, PO activity, and SOD activity [10]. Therefore, shrimp reared at a certain salinity, temperature or pH level and then subjected to a sudden salinity, temperature or pH stress exhibit decreased immune parameters. Shrimp are susceptible to *Vibrio* and WSSV under sudden salinity, temperature, or pH stressing due to declines in immune parameters. Shrimp under long-term cultures at unfavorable environmental conditions decrease the immune parameters and increase susceptibility against pathogens. For instance, white shrimp under long-term low salinity culture at 2.5% and 5.0% decreased immune parameters and increased susceptibility to *V. alginolyticus* and WSSV infections [6]. White shrimp under long-term culture at low pH (pH 6.8) decreased immune parameters and increased susceptibility to *V. alginolyticus* infection [33]. Therefore, shrimp under long-term culture at 2.5% and 5%, under long-term culture at pH 6.5, or under long-term culture at unfavorable environmental condition are considered chronically stressed. Sudden change in environmental condition affects the expressions of immune-related genes. For instance, the expression levels of proPO and PX in the white shrimp *L. vannamei* and tiger shrimp *P. monodon* are down-regulated in response to high temperature stress (from 26°C to 34°C) [34,35]. The expression level of HSP60 in the white shrimp is up-regulated in response to high temperature stress (raised from 24°C to 37°C) [36]. The expression level of HSP70 was up-regulated in tiger shrimp that subjected to desiccation [37]. The expression levels of HSP60, HSP70, and HSP90 in the white shrimp are up-regulated in response to high temperature stress (raised from 27°C to 36°C) [24]. The expression level of HSP60 in the white shrimp is up-regulated in response to low salinity stress (lowered from 33% to 10%) [36]. The expression level of C-type lectin in white shrimp is down-regulated in response to low salinity stress (lowered from 20% to 10%) [38]. The expression levels of proPO and PX in the blue shrimp *Litopenaeus stylirostris* are down-regulated in response to ammonia exposure [8]. Therefore, the expression levels of proPO, PX, and C-type lectin are down-regulated, whereas the expression levels of HSP60, HSP70, and HSP90 are up-regulated in shrimp subjected to sudden change in environmental condition or sudden environmental stress.

Environmental chronic stress also affects the expressions of immune-related genes. For instance, the expression levels of cytMnSOD, ecCuZnSOD, glutathione peroxidase (GPx), lysozyme, and penaeidin 3 a are up-regulated with low levels of PO activity, RB and SOD activity in the white shrimp *L. vannamei* following long-term culture at low pH (pH 6.8) [33]. The expression levels of lipopolysaccharide and β-glucan binding protein (LGBP), PX, integrin β (IB), and α2-macrobulin (α2-M) are up-regulated, with low levels of PO activity, RB and SOD activity in the white shrimp following long-term culture at 2.5% and 5% [6]. In the present study, the expression levels of ppA, proPO I, and proPO II of white shrimp reared at 2.5% were 8.8-, 7.3-, and 5.5-fold higher than in shrimp reared at 15%, 25%, and 35%, respectively, and the expression levels of cytMnSOD, mtMnSOD, ecCuZnSOD, and HSP70 of shrimp reared at 2.5% were 2.4-, 4.8- and 64-, and 2.6-fold higher than in shrimp reared at 15%, 25%, and 35%, respectively. Therefore, shrimp reared under unfavorable environmental conditions like salinity and pH had up-regulated expressions of immune-related genes like LGBP, ppA, proPO, SOD, PX, IB, and HSP70, and had lower levels of PO activity, RB, and SOD activity indicating the modulation of immunity homeostasis [6,33].

Conclusion

In conclusion, white shrimp under long-term culture at low salinity levels (2.5% and 5%), an unfavorable environmental condition, are considered to be chronically stressed, exhibited up-regulated expression levels of immune-related genes including LGBP, PX, IB, ppA, proPO I, proPO II, α2-M, cytMnSOD, mtMnSOD, ecCuZnSOD, and HSP70. The up-regulated expression levels of these genes may provide candidate biomarkers in low salinity shrimp farming.

Acknowledgements

This research was supported by a grant from the National Science Council (NSC98-2815-C-019-016-B), and partial support from the Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung, Taiwan. We also thank Mr. C. C. Li for his assistance in drawing the graphs.

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