Hsp70 expression in shrimp *Litopenaeus vannamei* in response to IHHNV and WSSV infection

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Abstract The effects of environmental changes, on non-target organisms may impact and bring consequences at the molecular level in marine organisms. There is a lack of data supporting the hypothesis according to which environmental stress modulates the immune system, leading to an increased susceptibility to infectious agents in shrimps. The present study was focused on changes occurring in cellular defense proteins in *Litopenaeus vannamei* infected by virus infection hypodermal and hematopoietic necrosis (IHHNV) and white spot syndrome (WSSV). Western blot analysis was used to evaluate expression of protein Hsp70, in gill tissue. Up-regulation levels were supported by immune detection analysis, suggesting that IHHNV and WSSV-shrimp infection promotes changes in the expression of these proteins.

Keywords Hsp70 · IHHNV · WSSV · *Litopenaeus vannamei*

Introduction

Marine shrimp in the family *Penaeidae* have gained status as a very important international seafood trade product of particular economic importance in shrimp farming countries [9]. During the past 20 years, viral diseases have been among the main causes of economic losses in shrimp farming, particularly in Asia and South America [2]. Factors contributing to the spread of diseases pre-selection of stocks of animals for growth rather

than disease resistance, intensive farming practices that favor the transmission of pathogens and immunosuppressive effect by high densities and stress transport [20]. Invertebrates rely on innate immunity to respond to the entry of foreign microorganisms [7]. Consequently, to investigate the molecules and mechanisms relevant to immune response in order to develop SPR (specific pathogen resistant) shrimp is still necessary work and should be a priority [3]. The Heat Shock Proteins (HSP) comprises a family of proteins with a high degree of homology. Its expression and synthesis is a part of the cellular response to physical and/or chemical stress. They act as molecular chaperones, maintaining homeostasis and acting against the proteotoxic effects [6, 22]. Hsp70 play important roles in resisting environmental stresses and stimulating innate immune system [6]. Hsp was found to be both constitutive and inducible, and was highly expressed in haemocytes and almost all tissues examined, including muscle, stomach, heart, hepatopancreas and gill tissue [4]. These proteins have also been associated with inhibition of viral replication. Increased expression of HSPs, particularly Hsp70, is considered a good biomarker changes in metabolic activity. Recently, it was demonstrated that the immune system responds vigorously to infection by pathogens with increased expression of Hsp70 [26]. This work aim evaluate the expression of Hsp70 in white shrimp *Litopenaeus vannamei* in response to infection by virus infection hypodermal and hematopoietic necrosis (IHHNV) and white spot syndrome (WSSV).

Materials and methods

Animals

Juvenile intermolt shrimp (8.5 ± 2 cm in body length, weight ± 1.0 g, n : 10 shrimps/group) were obtained from a

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Fig. 1 Electrophoresis in 2 % agarose gel stained with ethidium bromide. Animals 1–5 and 7 negative, 6, 8, 9 and 10 positive for IHNV, C– (negative control), C+ (positive control), MK (molecular marker 100 bp)

local shrimp farm in Northern of Santa Catarina State (Brazil), maintained in a 6,000 L fiberglass tank, with water temperature at 23 ± 1 °C, pH 8.5, Dissolved oxygen 6.0 mg/L and salinity of 20 g L^{-1} . Shrimp were held for a week to acclimate to laboratory conditions prior to experimental use. During this period, shrimp were fed once daily with commercial shrimp feed. Feeding was stopped 24 h before treatment.

DNA extraction and virus detection analysis

IHNV

The prevalence of IHNV virus was monitored by PCR, the conditions were initially standardized. Primers were designed from specific sequences of the viral genome, GenBank using the software OLIGO 3.0 as follow: initial denaturation step at 94 °C 3 min and then 30 cycles (94 °C 30 s, 55 °C 45 s, 72 °C 45 s), 20 °C 10 min and keep 4 °C. The set of primers used in the reaction were designed based in the sequence amplified by the PCR reaction: IHNV-F (5'-TCG-GAA-AAC-TGA-ACA-CTG-GCC-T-3') and IHNV-R (5'-CGG-CGT-GTT-CTT-CGT-CTT-CAT-T-3') [13]. Each amplification reaction was performed using 1 µL of DNA (up to 200 ng/µL), extracted from gills of shrimp, Taq DNA Polymerase (5U) (Gibco BRL), reaction buffer Taq DNA Polymerase 1X (10 × PCR Buffer : 200 mM Tris-HCl pH 8.4, 500 mM KCl), 2.5 mM MgCl₂, 0.2 mM of each dNTP, primers IHNV-F and IHNV-R 1 µM each primer. The final reaction volume was 25 µL. The fragment size to be amplified using the primers described above corresponding to 512 bp (Fig. 1). The reactions were performed in Mastercycler personal thermocycler (Eppendorf®).

WSSV

DNA extraction was performed in shrimp pleopods. Primers were designed from specific sequences of the viral genome, GenBank using the software OLIGO 3.0 as follow: initial denaturation step at 95 °C for 5 min and then

Fig. 2 Electrophoresis in 2 % agarose gel stained with ethidium bromide. 1–10 animals positive for WSSV, C– (negative control), C+ (positive control), MK (molecular marker 100 bp)

35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 10 s. The set of primers used in the reaction were designed based in the sequence amplified by the nested PCR reaction WS146 F1 (5'-ACT-ACT-AAC-TTC-AGC-CTA-TCT-AG-3') and WS146 R1 (5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-3') internal prime pairs WS146 F2 (5'-GTA-ACT-GCC-CCT-TCC-ATC-TCC-A-3') and WS146 R2 (5'-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3') [11, 15]. The final reaction volume was 25 µL. The size of the fragment to be amplified using the primers described corresponds to 941 bp (Fig. 2). The reactions were performed in Mastercycler personal thermocycler (Eppendorf®). After screening by PCR the groups were divided into three categories according to the virus infection: positive to IHNV or WSSV and Negative (control).

Immunodetection of proteins (Western blotting)

Gills homogenates at protein concentration range from $50 \mu\text{g } \mu\text{L}^{-1}$, were subjected to polyacrylamide gel electrophoresis (PAGE) under denaturing conditions (0.1 % SDS), according to established method [10]. Following electrophoresis, proteins were transferred to nitrocellulose membrane (Hybond, ECL). The membranes were incubated for 2 h with anti-Hsp70 (1:3000), followed by incubation with AP-conjugated, anti-mouse or anti-rabbit (1:2000) secondary antibodies (GE Healthcare or Calbiochemistry). The immune complex was detected by chemiluminescence with Luminol reagent and densitometry of immune reactive bands was performed with Gel Quant software (AMPL) [5]. All measurements were made in duplicate. The results were subjected to analysis of variance (ANOVA) followed by Tukey test, performed by GraphPad software.

Results

Significant differences were observed ($p < 0.05$) in the expression of Hsp70 between animals negative and positive

for both the IHHNV as for WSSV. Protein expression was investigated by the detection of specific immune complexes in gills of shrimp infected with IHHNV and WSSV. In gill homogenates immune reactive bands were detected by cross-reactivity with specific antibodies, as seen in Fig. 3, corresponding to the presence of Hsp70 (70 kDa). The virus IHHNV and WSSV was able to promote significant changes in Hsp70 expression on infected shrimps from farms (Fig. 4). Immunoreactive bands of Hsp70 also revealed significant induction, when compared to the levels in the control group.

Discussion

The results suggest that Hsp70 may act as a biomarker of alertness of the immune system in the presence of an intruder. The levels of this protein could be used to assess the health of aquatic organisms on culture conditions. However, its potential use for monitoring early triggering a defense response must be better evaluated. A number of studies have addressed the sensitivity of Hsp70 synthesis in response to environmental assault and showed that Hsp70 was induced by different mechanisms and suggested the use HSPs are a suite of highly conserved proteins well known for their quick responses in cell to environmental stresses [1, 8, 14, 16]. According to the results, was observed that the shrimp positive to IHHNV or WSSV

infection with changes in Hsp70 fold had a higher rate of survival during the period of cultivation in comparison with others. In the same way Hsp70 was predominantly induced in hepatopancreas and gills of *Fenneropenaeus chinensis* when the shrimp were exposed to heat shock and *Vibrio anguillarum*-challenged stresses [25]. Hsp70 genes in marine invertebrates have also been experimentally shown to decrease the mortality rate of certain organisms. The expression of Hsp70 was increased as a part of an immune response against *Vibrio harveyi* in *P. monodon* [19]. Hsp70 is one of the binding partners of VP28 during virus infection and its expression was enhanced by WSSV infection at the early stage of the process [24]. Crayfish *Procambarus clarkii* infected with WSSV during hyperthermia showed a rapid induction of Hsp70 4 h after infection, suggesting that Hsp70 plays a positive regulatory role in resistance to WSSV [23]. This work show that IHHNV and WSSV induce expression of Hsp70 and this can act as a regulatory immune system factor to combat the viral infection. It suggests that the induction of Hsp70 by shrimps infected with virus improves its responsiveness and decrease the mortality rate. Heat Shock Proteins (HSPs) comprise a group of highly conserved proteins that have general protective functions against pathogens in all living organisms [17]. In aquatic animals, Hsp70 has been shown to play an important role in relation to the host response to environmental pollutants, food toxins, and bacterial or viral infections [18]. Elucidating associations between Hsps, the immune system and pathogen resistance in shrimp represents an intriguing challenge [12]. Shrimps may express or suppress proteins in response to the viral infection. In this context the protein expression helps to elucidate the host response mechanisms against microorganism invasion and pathogenicity [21]. Comprehension these mechanisms can help in finding solutions to the problem of viral infection in cultured shrimp, thus

Fig. 3 Immunodetection of HSP70 in gills of *L. vannamei*. Animals 1-4 negative, 5-6 positive for IHHNV, and 7-8 positive for WSSV

Fig. 4 Columns from GraphPad software showing expression in arbitrary units (AU) the value of Hsp70 in animals IHHNV and WSSV positive and negative

improving the survival rate and avoiding production breaks.

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