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RESEARCH REPORT

Betaine aldehyde dehydrogenase is regulated during WSSV infection in white shrimp

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Abstract

The white spot syndrome virus (WSSV) is the most lethal virus that infect shrimps, generating economic losses for aquaculture industry. Osmotic stress compromises the immune response in WSSV-infected shrimp. Betaine aldehyde dehydrogenase (BADH E.C. 1.2.1.8) is an enzyme that participates in the regulation of osmotic stress, and that is up-regulated in shrimp exposed to changes in salinity concentration. However, different gene expression studies in shrimp during WSSV infection focused in changes on the mechanisms of energy production and cellular defense genes, without considering genes sensitive to osmotic variation. In this work, we evaluate the mRNA levels and activity of the BADH enzyme, as well as glycine betaine (GB) accumulation, during WSSV infection in white shrimp. Shrimps were inoculated with WSSV and samples from the hepatopancreas were collected at 24 and 48 h post-infection. Our results demonstrate that WSSV infection increases the expression of BADH 1.5-fold at 48h and the enzyme activity increase 4.28- and 4.59-fold at 24 and 48 h, respectively. Also, higher accumulation of GB was observed during WSSV infection. These results suggest that BADH could participate in the cellular response during WSSV infection in white shrimp.

Key Words: betaine aldehyde dehydrogenase; white spot syndrome virus; glycine betaine; mRNA expression; shrimp

Introduction

Twenty-six year ago, the white spot syndrome virus (WSSV) emerged as one of the most severe shrimp pathogens, with high rates of mortality and prevalence in Penaeid shrimp (Oidtmann and Stentiford, 2011). Liu *et al*. (2009) summarized some of the genes and proteins that are up-regulated and respond or interact with the WSSV virus in crustaceans. Most of the molecules described are related to inflammatory and adaptative response affecting survival rates, but little is known about the mechanism and interaction of impactful pathways during the disease. In addition, different aspects of the infection in invertebrates, and how the virus access the host cell is yet to be elucidated, and all the analyses available to this day are based on the response described in vertebrates.

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Several studies have shown that variations in salt concentration compromise the shrimp response to the WSSV infection (Liu *et al*., 2006, 2009; Joseph and Philip, 2007; Vaseehara, *et al*., 2013; Ramos-Carreño *et al.*, 2014; Amrillah *et al*., 2015; Raj *et al*., 2015; Xhue *et al*., 2015; Thuong *et al.*, 2016; Qianqian *et al*., 2017). However, to date, there are no studies that demonstrate the relation of osmotic regulation and WSSV, or other viral, infections. Therefore, the importance of the enzyme BADH from white shrimp *Litopenaeus vannamei* (LvBADH), which belongs to the ALDH9 family in the invertebrate clade, arises, as it is involved in the synthesis and accumulation of the organic osmolyte glycine betaine (GB, N,N,N-trimethylglycine) (Delgado-Gaytán *et al*., 2017). GB is a water-soluble organic molecule with the ability to bind a significant amount of water molecules to keep hydration and therefore, stabilize proteins under stress conditions. Also, GB can bind inorganic ions acting as bioprotectant to proteins (Fedotova and Kruchinin, 2017). Most of the studies of GB has been done in mammals, where it has been reported that GB has a role in cell metabolism, in addition to

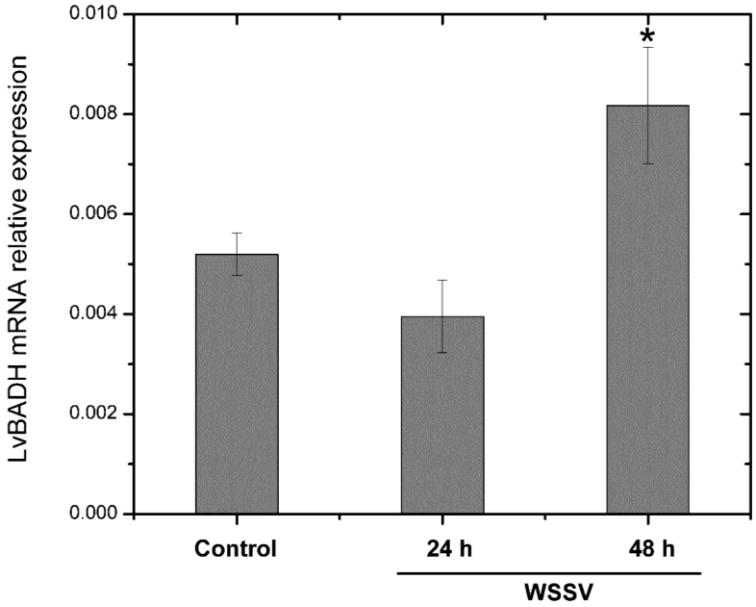

Fig. 1 Relative expression of LvBADH during WSSV-infection. The mRNA levels were normalized with L8 as a constitutive gene. Asterisks denote statistically significant ($P < 0.05$) difference from the control group.

its functions as osmolyte or osmoprotector (Figueroa-Soto and Valenzuela-Soto, 2018). On the other hand, in white shrimp it has only been shown that NAD-dependent aldehyde dehydrogenase is up-regulated by the WSSV infection at high temperature (32 °C), and that ALDH interacts with the 70 kDa heat shock proteins (Hsp70), which inhibits viral replication of the WSSV (Lin *et al*., 2011).

Recently, we demonstrated that LvBADH is expressed in a tissue-specific manner in white shrimp , and the activity of the LvBADH enzyme is modulated by changes in the salinity, suggesting that LvBADH is involved in mechanisms of stress adaptation (Delgado-Gaytán *et al*., 2015; Delgado-Gaytán *et al*., 2017). Fan *et al*. (2016) described an increase in the osmoregulatory system under WSSV infection in crustacea *Cherax quadricarinatus*. Considering the role of LvBADH as a detoxifying and stress-response enzyme, in this work we evaluate the mRNA levels and activity of LvBADH enzyme, as well as GB accumulation during WSSV infection in white shrimp.

Materials and methods

Animal handling

A total of 30 juvenile shrimp *Litopenaeus vannamei* were collected from a farm located in the State of Sonora, México. The shrimps were acclimated in individual systems for 4 weeks in 200 L aquaria, temperature 28-30 °C, 35 ppt of salinity, and were fed with commercial pellet (35% protein). The virus inoculum for experimental infection was obtained from white shrimp muscle of moribund shrimp infected with the WSSV. The tissue was homogenized in buffer TRIS-NaCl (20 mM Tris pH 7.4, containing 400 mM NaCl). The supernatant was obtained by centrifugation of the homogenate at 9,402 *x g* during 9 minutes at 4 °C and then filtered with 0.8 μm pore filters. The inoculum was diluted with saline buffer (1.4 x 10^5 WSSV copies total) and injected intramuscularly to shrimps for cumulative mortality reached 100% at 48 to 72 h posterior to WSSV infection. The hepatopancreas (n =7) were collected from the three groups as follows: A) Saline solution (SS) group at 24 h after starting the experiment, B) WSSV group at 24 h post-infection, and C) WSSV group at 48 h post-infection.

mRNA quantification

Total RNA was isolated from individual hepatopancreas using the TRIzol reagent (Invitrogen) following the manufacturer instructions. Total RNA concentration and integrity was evaluated by measuring the absorbance at 260/280 nm and by 1% agarose gel, while contamination of genomic DNA was eliminated by digestion with RQ1 RNase-Free DNase (Promega). Total DNA-free RNA (1 μg) was used to synthesize one cDNA, for each tissue sample collected, using oligo-dT and the GoScript™ Reverse Transcriptase kit (Promega). For LvBADH quantification we used the LvBADHF3 and LvBADHRv3 primers previously reported (Delgado-Gaytán *et al*., 2015). The quantitative PCR reactions were performed separately for LvBADH and normalized by the expression of the ribosomal protein L8 (Miranda-Cruz *et al.,* 2018). Three PCR reactions for each sample were run (Step One Real-Time PCR Systems, Applied Biosystems) in a final volume of 15 μL containing 7.5 μL of SsoAdvanced Universal SYBR Green Supermix (BioRad), 6 μL of H_2O, 0.25 μL of each primer (20 μM), and 1 μL of cDNA

(equivalent to 125 ng of DNA-free RNA). After an initial denaturing step at 94 °C for 10 min, amplifications were performed for 40 cycles at 94 °C for 15 s and 63 °C for 1 min with a single fluorescence measurement, and a final melting curve program increasing 0.3 °C each 20 s from 60 to 95 °C. Positive and negative controls were included for each gene. Standard curves for LvBADH and L8 were run together with the cDNA samples to determine the efficiency of amplification and the mRNA concentration, which was calculated based on the concentrations of the standard curve dilutions ($5E^{-4}$ to $5E^{-8}$ ng μL^{-1} of PCR fragments) by the StepOne™ Software v2.3 (Applied Biosystems). The expression level (ng/µL) of LvBADH was normalized to L8 and expressed as relative values (LvBADH/L8).

Activity assay

Activity of BADH was evaluated following the plate-based spectrophotometric quantification of aldehyde dehydrogenases, reported by Sreerama and Sládek (2005) and adapted to the specific BADH assay conditions reported by Guzman-Partida and Valenzuela-Soto (1998). A 96-well flat-bottom microtiter plate was set up with 180 µL of the reaction solution (HEPES 100 mM pH 8.0 with 1 mM NAD, 0.5 mM Betaine Aldehyde (BA) and 14 mM β-mercaptoethanol) and 20 µL of the cytoplasmic extract from each animal tissue (samples were diluted at 1:2 ratio). The protein extracts from hepatopancreas of shrimp was obtained following the directions of the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific). The enzyme activity was evaluated by triplicate following the reduction of NAD^+ at 340 nm for a total of 10 minutes. The rate of reaction (ΔA/min) was multiplied by a factor of 1639 to obtain the enzyme activity as nmol of NADH/min/mL. The protein content of the extracts was determined using the Quick Start Bradford Protein Assay (Bio-Rad) to express the activity as nmol/min/mg of protein.

Glycine betaine quantification

GB concentration in the cytoplasmic extract was evaluated following the assay proposed by Grieve and Grattan (1983). The assay was adapted for the analysis using a microplate reader, considering the minimum time for the effective aqueous extraction and optimal acidity for periodide precipitation. The extracts were diluted 1:1 with 2N H_2SO_4 prior addition of potassium triiodide and the glycine betaine crystals were obtained as previously described. A standard curve of GB (50-200 ug/mL) was prepared in 1-2-dichloroethane for analysis at 365 nm.

Statistics

The normality of the data from each experiment was evaluated using the Kolmogorov-Smirnov test, and one-way ANOVAs were used to determine statistical differences for each group using the Tukey and Fisher LSD means comparison; the non-infected shrimps were used as control ($P<0.05$). All data was analyzed using OriginPro 9.0 software OriginLab, Northampton, MA, USA.

Results and discussion

Analysis of LvBADH *mRNA expression and enzyme activity*

The accumulation of the osmolyte GB is primordial for osmoregulation and protection of proteins to avoid denaturalization (Timasheff, 1993; Yancey, 2005). In this sense, the GB synthesis is regulated in a two-step irreversible pathway. The first step involves the oxidation of choline to betaine aldehyde by the action of choline dehydrogenase (CDH EC 1.1.99.1) and the second step the oxidation of betaine aldehyde to glycine betaine by the enzyme betaine aldehyde dehydrogenase (BADH EC 1.2.1.8) (Perrino and Pierce, 2000; Muñoz-Clares and Valenzuela-Soto, 2008). Although investigations of GB in crustaceans are limited, there is evidence of the use of organic osmolytes to virus capsid formation and reduce the viral infectivity (Tafur *et al*., 2013). Therefore, in this work we evaluate the mRNA and activity of BADH enzyme, as a crucial participant in the GB synthesis, as well as the accumulation of GB during WSSV in white shrimp

Our results demonstrate that LvBADH is expressed in the hepatopancreas of non-infected shrimps, and that infection with the WSSV increases the expression of mRNA levels. The LvBADH expression increased 1.5-fold at 48 h post-infection with the WSSV, compared to the control group, while non-statistical significance was found at 24 h post-infection (Figure 1). In contrast, the activity of LvBADH increased 4.28- and 4.59-fold at 24 and 48h post-infection with the WSSV, respectively, compared to the control group (Figure 2).

In agreement with our results, a NAD-dependent enzyme (ALDH) was previously described as a crucial protein to suppress the WSSV replication at high temperatures (32 °C). Moreover, the knock-out of the ALDH enzyme promoted a severe WSSV infection in the K.O. shrimps. The authors suggest that the ALDH enzyme detoxifies the aldehyde compounds, which inhibits DNA-replication and protein synthesis, protecting the cell during WSSV infection (Lin *et al*., 2011), as these inhibitions prevent the virus from replicating. Although the authors did not analyze the sequence of the ALDH reported (GenBank Accession No. CK571503), it had 86% identity with a predicted ALDH4A1, which is involved in the proline degradation pathway; and it is well known that proline has osmoprotective and osmoregulatory properties under stress conditions.

It is known that expression and activity of BADH are increased in response to stress conditions in several organisms from bacteria, mammals, and plants with a broad range of catalytic properties and roles against different stress conditions (McCue and Hanson, 1992; Legaria *et al*., 1998; Velasco Garcia *et al*., 2006; Singh *et al*., 2013; Delgado-Gaytán *et al*., 2015; Rosas-Rodríguez *et al*., 2017). To the best of our knowledge, our study represents the first report of BADH modulation under a viral infection in invertebrates.

It has been described that the activity of antioxidant enzymes and membrane-bound ATPases are reduced during WSSV infection, which

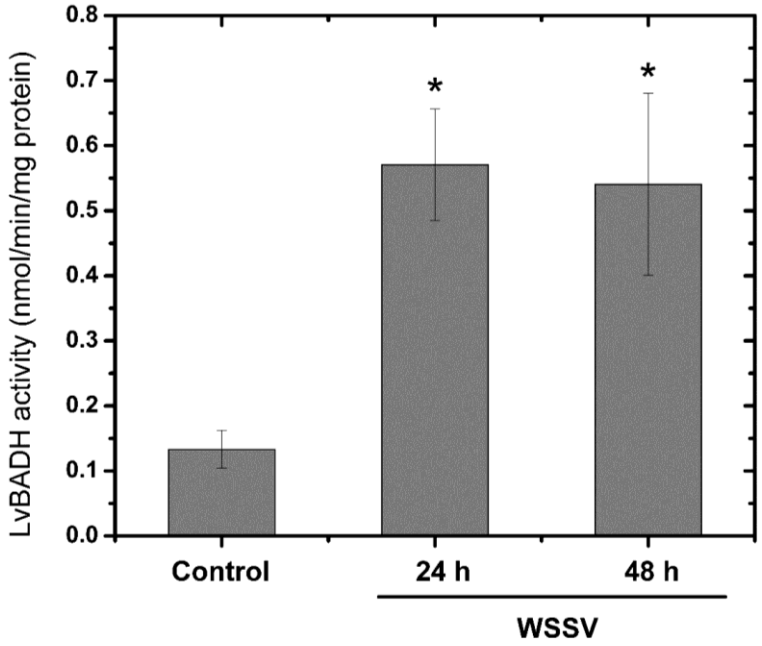

Fig. 2 LvBADH activity during WSSV-infection. Asterisks denote the statistically significant (*P* < *0.05*) difference from the control group.

exposes shrimp cells into a stress environment and alterations in ion concentration (Rameshthangam and Ramasamy, 2006). This is important for BADH activity, previous studies from animals indicate that stress conditions and monovalent cations, mainly Na^+ and K^+ ions, can enhance BADH activity (Valenzuela-Soto *et al*., 2003). In our results, the activity of LvBADH increases at 24 and 48 h post-infection while mRNA expression increases only at 48 h. These results suggest an effect from post-translational regulation of LvBADH during WSSV infection.

Analysis of glycine betaine accumulation

The accumulation of GB was evaluated in the hepatopancreas of healthy and WSSV-infected shrimps. The standard curve of GB (50-200 ug/mL) for the quantification of the osmolyte in all the standards presented a good correlation with an R-square value of 0.9904. The GB is accumulated in the hepatopancreas of non-infected shrimps with a concentration of 37.05 ± 1.73 μg/mL. The WSSV-infection increased the GB accumulation in the hepatopancreas 1.49- and 1.23-fold at 24 and 48h, respectively, compared to the control group (Figure 3).

To date, the accumulation of GB is related to the osmotic pressure and changes in salinity in crustacea, but little is known about GB response to biotic stress (Moran and Pierce, 1984; Jahn *et al*., 2006; Romano and Zeng, 2012; Freire *et al*., 2013). Gonçalves-Soares *et al*., 2012 described that hypoosmotic stress induces changes in the expression of genes involved in cellular defense and energy production, but the infection with WSSV does not change the transcription profile of the mentioned genes. In this work, we demonstrate that GB responds to the WSSV infection, probably as a defense mechanism of the shrimp against the virus.

Our results support studies where GB levels increase during WSSV infection to balance osmotic pressure, altered by the virus, and balance the phosphorylcholine –related to choline metabolism and membrane biosynthesis–, which may benefit the synthesis of virus envelope and virus assembly (Fan *et al*., 2016). Pointing out that GB accumulation in *L. vannamei* during WSSV infection could be regulated by LvBADH expression and activity.

However, the increase on GB accumulation in hepatopancreas has not a direct correlation with the increase in the enzyme activity. This behavior was previously observed in shrimp, with a lack of correlation between mRNA expression, activity and GB accumulation during salinity stress (Delgado-Gaytan *et al*., 2015). We must consider that GB synthesis could take place in hepatopancreas to further migrate to other tissues and facilitate them with osmotic substrates/ions and further osmoprotection. Also, it has been demonstrated that GB could migrate using membrane transporters (Burg and Ferraris, 2008), which strengthens the hypothesis beforementioned of GB migration after its synthesis in hepatopancreas.

The mechanisms of response from shrimp to the WSSV infection are not clear, while some evidence indicates that apoptosis is induced as a defense strategy during WSSV infection, increasing the mortality in shrimp, some anti-apoptosis proteins are described in shrimp cells infected with WSSV, suggesting that virus decreases the apoptotic signals

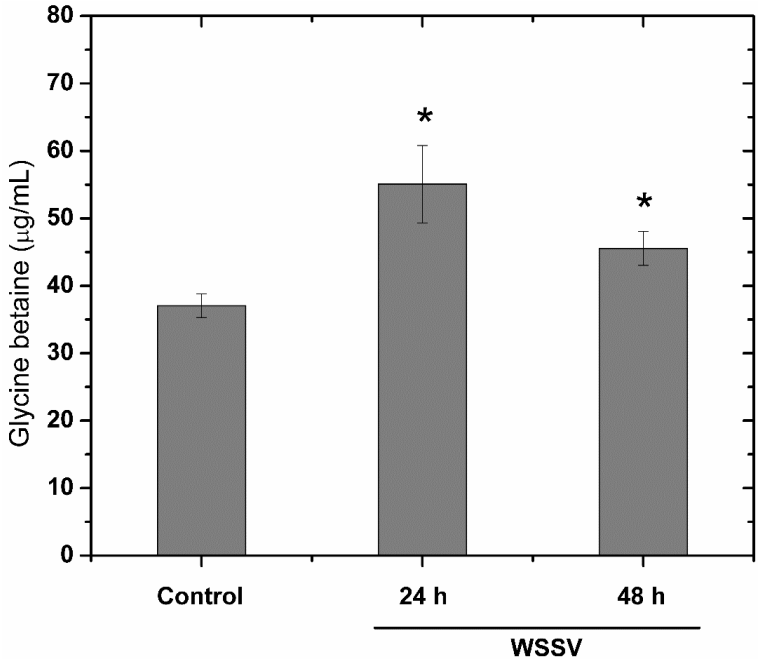

Fig. 3 Glycine betaine accumulation during WSSV-infection. Asterisks denote the statistically significant ($P < 0.05$) difference from the control group.

(Wang *et al*., 2004). DNA damage, caspases family activation, and regulatory factors activate the apoptosis mechanism, which functions as innate host defense in the shrimp. However, some of the cells with apoptotic characteristics did not contain any WSSV virons, suggesting that the apoptotic response might not be discriminated, targeting cells with or without the infection alike, maybe to prevent further spreading among neighboring cells to the ones infected, but further studies beyond the scope of this study are required to validate those suggestions (Li *et al*., 2019). The results obtained in this study are important considering that regulation of cell volume and homeostasis by GB plays a crucial role in the apoptosis process (Panayiotidis *et al*., 2006; Zhao *et al*., 2018).

In summary, we demonstrate that LvBADH expression and activity increase during WSSV infection in shrimp in a time-dependent manner, along with GB concentration. Our results suggest that BADH is involved in the response against WSSV-infection in shrimp. Due to the ability of BADH to metabolize distinct amino aldehydes, further studies are required to elucidate if LvBADH response is related to the defense system of the shrimp against viral infections or used by the WSSV for further viral replication.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest.

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