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Selenoprotein M gene expression, peroxidases activity and hydrogen peroxide concentration are differentially regulated in gill and hepatopancreas of the white shrimp *Litopenaeus vannamei* during hypoxia and reoxygenation

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ABSTRACT

In many organisms, episodes of low O_2 concentration (hypoxia) and the subsequent rise of O_2 concentration (reoxygenation) result in the accumulation of reactive oxygen species and oxidative stress. Selenoprotein M (SelM), is a selenocysteine containing protein with redox activity involved in the antioxidant response. It was previously shown that in the white shrimp *Litopenaeus vannamei*, the silencing of SelM by RNAi decreased peroxidase activity in gill. In this work, we report the structure of the SelM gene (*LvSelM*) and its relative expression in hepatopancreas and gill after 24 h of hypoxia followed by 1 h of reoxygenation. The gene is composed by four exons interrupted by tree introns. In gills and hepatopancreas, SelM expression increased after 24 h of hypoxia followed by 1 h of reoxygenation, while peroxidases activity diminished in hepatopancreas but increased in gills. Hydrogen peroxide (H_2O_2) concentration was higher in hepatopancreas in response to hypoxia for 6 h and did not change after 24 of hypoxia followed by reoxygenation; conversely, no change was detected in gill. SelM appears to be a key enzyme in gill oxidative stress regulation, since the higher expression is associated with an increase in peroxidases activity while maintaining H_2O_2 concentration. In contrast, in hepatopancreas there is a higher expression after hypoxia and reoxygenation for 24 h, but peroxidases activity was lower and the change in H_2O_2 occurred after 6 h of hypoxia and this level was maintained during reoxygenation.

1. Introduction

Low environmental O_2 concentration (hypoxia) and subsequent reoxygenation to fully air-saturated conditions (normoxia) are physiological stressors for most multicellular obligatory aerobic organisms. Reoxygenation of hypoxic tissues restores the energy potential and results in generation of reactive oxygen species (ROS) (Li and Jackson, 2002) including hydrogen peroxide and superoxide anion. Modulation of ROS is vital in all known organisms. ROS are important regulators of metabolism and part of the antioxidant and humoral defense mechanism, but their accumulation can be harmful for many cellular components. In the shrimp *Litopenaeus vannamei*, superoxide anion ($O_2^{\bullet-}$) production increased while antioxidant capacity decreased in hepatopancreas during the first hours of reoxygenation (Zenteno-Savin et al., 2006) and this could lead to tissue damage, (Zenteno-Savin et al., 2006). Many crustaceans are known to be quite tolerant to hypoxia (McMahon, 2001), but the detailed mechanisms that sustain this response are still unknown.

Peroxidases catalyze the reduction of peroxide or hydroperoxides using a donor substrate (typically a thiol) that is oxidized, thereby regulating H_2O_2 levels. Many of them have heme as the prosthetic group, but other proteins contain selenocysteine, the 21st amino acid. Selenoprotein M (SelM) belongs to this group and plays an important role in maintaining the cellular redox balance (Ferguson et al., 2006) and its expression and regulation is actively studied (Stadtman, 2005, Lobanov et al., 2009).

Little is still known in aquatic animals about selenoproteins (Sels), but their importance is evident. In zebrafish, 21 selenoproteins mRNAs are differentially expressed during development, with specific tissue and ovarian stage profiles (Thisse et al., 2003). Glutathione peroxidase (GPx) and thioredoxin reductase are among the most studied Sels in crustaceans. Their expressions and activities are affected by pathogens, environmental and metabolic factors; their functions might be key factors to orchestrate the redox cellular balance. The physiological role of Selenoprotein M (SelM) is only beginning to be studied. The difference in expression during a disease and in different tissues indicates that SelM is involved in diverse regulatory responses. In *L. vannamei*, there are still questions about tissue-specific functions and whether it is involved in H_2O_2 regulation as a second messenger in the different

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tissues (Garcia-Triana and Yepiz-Plascencia, 2012). In the mitten crab *Eriocheir sinensis* reproduction, SelM plays a key role in the regulation of differential expression in the ovary, testis, and hepatopancreas, which indicates its importance in selenium metabolism (Lu et al., 2012). Also, in the shrimp *Penaeus monodon*, SelM is highly expressed in stage 2–4 ovaries and in hepatopancreas (Zhou et al., 2015). SelM also appears to be important in the regulation of ROS generated during infections. For instance, in the shrimp *L. vannamei*, SelM expression is transiently induced in gills after infection with the white spot syndrome virus (WSSV) in a time-dependent manner (Clavero-Salas et al., 2007), suggesting that it plays an important role in the defense against the virus. Also, in this same shrimp, the expression of the selenoprotein glutathione peroxidase (GPx) during recovery from tidally-driven hypoxia and hypercapnic hipoxia (4 h) decreases or is muted by more persistent exposure to these conditions (24 h), leaving shrimps potentially vulnerable to ROS damage during recovery (Kniffin et al., 2014). However, much remains to be investigated about SelM expression in normoxia conditions of the shrimp and in many other processes, such as hypoxia and reoxygenation, which might shed light about its function in specific conditions.

We reported that SelM is expressed differentially in juvenile shrimp *L. vannamei* gills and hepatopancreas, with 7-fold higher levels in hepatopancreas (Garcia-Triana et al., 2010a). It was also shown that SelM silencing by RNAi, resulted in low peroxidase activity in gills, but not in hepatopancreas. In contrast, no change in H_2O_2 concentration in gills and hepatopancreas occurred in the silenced shrimp (Garcia-Triana et al., 2010a).

Peroxidases play key roles in the degradation of ROS produced during oxidative stress. Peroxidase activity has been studied in shrimp to evaluate the role in the response to stress (Garcia-Triana et al., 2010a, 2010b). Changes in peroxidase activity were detected in some shrimp species in different tissues. During embryonic and larval developmental stages of the prawn *Macrobrachium malcolmsonii*, higher peroxidase activity was found in hepatopancreas than in gills (Arun and Subramanian, 1998). In the shrimp *Penaeus monodon* infected with WSSV, the peroxidase activity in gills and hepatopancreas decreased (Rameshthangam and Ramasamy, 2006). In contrast, no changes were found in response to osmotic alteration, since peroxidase activity in the hemolymph of the shrimp *Litopenaeus schmitti* is not affected by hyposaline environments of 18 and 8 ppt (Lamela et al., 2005). Differences are also due to the tissues studied. In the chinese shrimp *Fenneropenaeus chinensis*, reduced peroxidase activity was detected during hypoxia (Li et al., 2006), indicating the involvement of peroxidases in different processes and in response to stress. In *L. vannamei*, increased GPx activity and DNA damage was detected in gill, hepatopancreas and hemolymph compared with the control during the period of hypoxia (3.0 and 1.5 ppm), and then partially recovered during the period of reoxygenation (6.5 ppm for 24 h) (Li et al., 2015).

Comparison of H_2O_2 production in lobsters collected from field sites and then submitted to different levels of dissolved oxygen suggests an influence of dissolved oxygen in H_2O_2 production (Moss and Allam, 2006). There are still no reports relating the effect of hypoxia and reoxygenation to H_2O_2 production in shrimp tissues. To obtain more insights about the response of shrimp to hypoxic stress and the roles of antioxidant enzymes, in this work we obtained the gene sequence of SelM and evaluated the effect of hypoxia and reoxygenation on its expression in gills and hepatopancreas. Peroxidase activity and H_2O_2 concentration were also analyzed in the same tissues to investigate the association to SelM.

2. Materials and methods

2.1. Animals

White shrimp *L. vannamei* (average weight 15 ± 1.5 g) were donated by the shrimp farm Selecta at Kino Bay, Sonora. Shrimps were acclimated for 14 days at 28 °C, 37 ppt, pH 7, constant aeration (6 mg/L dissolved oxygen) and fed *ad libitum* twice daily with commercial feed (Camaronina 35®, Agribrands Purina, Mexico). One-third of the water volume was changed daily to prevent ammonia accumulation and maintain the pH; uneaten food particles and feces were removed daily. Randomly selected healthy intermolt shrimp were placed in separate aquaria.

2.2. Hypoxia and reoxygenation assay

L. vannamei adults (N = 40, n = 8) at intermolt stage were placed in 150 L glass fiber aquarium with seawater (37 ppt), temperature controlled (28 °C), pH (7) and constant O_2 concentration (6 mg/L) measured with an YSI 550 A oxymeter. Shrimp were randomly divided into five groups. The following treatments were included: Normoxia (Nor, 6 mg/L) during the complete experiment as control, hypoxia (Hyp, 1.5 mg/L) for 6 h (Hyp6), hypoxia for 6 h and reoxygenation for 1 h (Reo6, 6 mg/L), hypoxia for 24 h (Hyp24), hypoxia for 24 h and reoxygenation for 1 h (Reo24). Gills and hepatopancreas were dissected from shrimp at the end of the hypoxia and hypoxia-reoxygenation treatments, immediately frozen in liquid nitrogen and kept at −80 °C until used.

2.3. SelM gene cloning and sequences analyses

To obtain the complete SelM gene sequence, specific primers were designed based on the cDNA sequence from *L. vannamei* (Clavero-Salas et al., 2007), (Table 1) and used to amplify the complete SelM gene by PCR from genomic DNA (gDNA), prepared according to Bradfield and Wyatt (1983). High quality gDNA was isolated from 2 g of muscle using proteinase K digestion, phenol-choloroform extraction and ethanol precipitation. The gDNA fibers were resuspended in 10 mM Tris–HCl, pH 8, 1 mM EDTA and used as template for PCR. The gene sequence was obtained from two PCR amplicons, both derived by reamplifications from an amplicon of approximately 4000 bp. This amplicon was obtained with one primer that includes the initial MET codon and the other one includes the stop codon (SelMFw1 and SelMRva5). The first PCR reaction was done in a 25 μL reaction using the Advantage 2 PCR Kit (Clontech) containing 50 ng of gDNA, 0.5 μL of each primer (20 μM), 2.5 μL of 10X Advantage 2 PCR Buffer, 0.5 μL 50X dNTP mix, 0.5 μL 50X Advantage 2 Polymerase Mix and 20 μL PCR grade water. The PCR program had the following conditions: 5 cycles at 94 °C for 25 s, 68 °C for 5 min; 32 cycles of 94 °C for 25 s, 56 °C for 30 s and 68 °C for 5 min; and an overextension step of 68 °C for 7 min. For the 5′- and 3′-ends, two fragments were obtained by seminested PCR, using the following primers pairs SelMFw1 + SelMR3 and SelMF3 + SelMRva5, respectively, and using ten times diluted first amplicon. The PCR programs conditions were: 5 cycles at 94 °C for 1 min, 68 °C for 2.5 or 1.5 min; 32 cycles of 94 °C for 25 s, 56 °C for 30 s and 68 °C for 2.5 or 1.5 min; and an overextension step of 68 °C for 5 min. Both amplicons of approximately 2 and 1 Kbp were cloned in the pGEM T-Easy vector (Promega). The SelM full-length gene sequence was assembled by overlapping internal sequences obtained from the 5′- and 3′-ends. Purified recombinant plasmids were sequenced thoroughly in both strands and compared with the cDNA nucleotide sequence of SelM.

Table 1
Primers used for cloning the *LvSelM* gene and for RT-qPCR.

Primer name	Nucleotide sequence (5′–3′)
BSelMF	GAT TTG ACC AGG TTG TGG AG
BSelMR	AAG CTG CAT TTT GGA GTC TG
SelMFw1	ATG GCG AAA GGG AGT GTC CAG CTC CTC C
SelMRva5	TGC CCG AGC TTT CAT TTA
SelMF3	CTG CGG CGG ATG ACG TCT GAA CAG GCT C
SelMR3	GGG GAT GTC CTC GTG GAT GAA TTT CTT C