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Expression and silencing of Selenoprotein M (SelM) from the white shrimp *Litopenaeus vannamei*: Effect on peroxidase activity and hydrogen peroxide concentration in gills and hepatopancreas

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ABSTRACT

Selenoprotein M (SelM), is a selenocysteine containing protein with redox activity involved in the antioxidant response. In the white shrimp *Litopenaeus vannamei*, SelM expression in gills is induced transiently during viral infection by the White Spot Syndrome Virus (WSSV). We report that SelM expression was detected in healthy shrimp *L. vannamei* in gills, muscle, hepatopancreas and pleopods, with more abundance in the hepatopancreas and gills. SelM transcripts were silenced by intramuscular injection with double-stranded RNAs (dsRNAs). In gills and hepatopancreas, all shrimp injected with long dsRNAs had lower SelM transcripts levels compared with controls. Peroxidase activity and hydrogen peroxide concentration were measured to detect effects on antioxidants. Peroxidase activity decreased upon silencing of SelM in gills, but no significant effect was detected in hepatopancreas. In contrast, total cell hydrogen peroxide concentration did not change in gills and hepatopancreas of silenced shrimp. Non-heme peroxidases are new players in the oxidative stress system that need to be addressed in detail, as well as selenium as a critical micronutrient for the antioxidant and innate immune systems in crustaceans.

1. Introduction

Modulation of reactive oxygen species (ROS) is critical in all known organisms. ROS are part of the defense mechanisms and important regulators of metabolism, but their accumulation can be detrimental for many cellular components. Peroxidases are antioxidant enzymes that catalyze the reduction of peroxide or hydroperoxides using a donor substrate that is oxidized, regulating H_2O_2 levels. Many of them have heme as the prosthetic group but some others more recently discovered contain selenocysteine, the 21st amino acid. Selenoprotein M (SelM) is a selenocysteine containing protein with redox activity (Ferguson et al., 2006) and its expression and regulation is actively studied (Stadtman, 2005). Selenoproteins are differentially expressed. During development of zebrafish embryos, twenty-one selenoproteins mRNAs were analyzed and all of them exhibited expression patterns restricted to specific tissues and developmental stage (Thisse et al., 2003). The introduction of human SelM in a transgenic rat, resulted in higher peroxidase activity than in the non-transgenic animals, furthermore, the enzyme activity varied in the different tissues examined (Hwang et al., 2008).

Very little information is available about crustacean SelM physiology. The first marine invertebrate SelM was reported in the demosponge *Suberites domuncula*, (Muller et al., 2005) followed by its identification in the shrimp *Litopenaeus vannamei* (Clavero-Salas et al., 2007). *L. vannamei* SelM expression is transiently induced in gills after infection with the white spot syndrome virus (WSSV) on a time dependent manner (Clavero-Salas et al., 2007), suggesting that it plays an important role in the defense against the virus. However, much remains to be investigated about SelM in normal conditions of the shrimp and in many other processes.

In non-model organisms as shrimp, silencing by RNA interference (RNAi) is an alternative to understand gene function, opening an opportunity and a strategy when knowledge and methodology for classical genetics are lacking. Until now, there is no information about selenoprotein M expression and silencing in crustaceans.

Peroxidases play key roles in the degradation of ROS produced during the oxidative stress. Peroxidase activity has been studied in shrimps and prawns to evaluate their roles in metabolism and in the response to stress. Important changes in peroxidase activity have been detected 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, peroxidase activity in gills and hepatopancreas decreased (Rameshthangam and Ramasamy, 2006). In contrast, no

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changes were found in response to osmotic alteration, since peroxidase activity in the haemolymph of the shrimp *Litopenaeus schmitti* is not affected by hyposaline environments as 18 and 8 psu (Lamela et al., 2005). Selenoproteins are widely distributed among eukaryotes. In mouse, mutations that render the cell unable to incorporate selenium into proteins result in embryonic lethality (Stillwell and Berry, 2005). Phylogenetic analysis shows that aquatic organisms apparently retained and sometimes expanded their selenoproteomes, whereas the selenoproteomes of some insects and other terrestrial organisms were reduced or completely lost (Chapple et al., 2009).

We detected the expression of SelM in different shrimp tissues and evaluated the effect of silencing by RNAi in the transcripts of gills and hepatopancreas. As indicators of an effect in antioxidants, peroxidase activity and H_2O_2 concentration after silencing with SelM dsRNA were determined in gills and hepatopancreas.

2. Materials and methods

2.1. Animals

White shrimp *L. vannamei* (average weight 15 g) were donated by the University of Sonora, DICTUS, Kino Bay Unit. Shrimp were acclimated for 14 days at 28 °C, 37 psu (Practical Salinity Units), under 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, and uneaten food particles and feces were removed daily. Before injecting the shrimp, randomly selected healthy intermolt shrimp were placed in separate aquaria.

2.2. Total RNA preparation, first strand cDNA synthesis and SelM mRNA detection

Gills, muscle, hepatopancreas and pleopods were dissected from shrimp, immediately frozen in liquid nitrogen and kept at −80 °C until used. Total RNA was extracted using Trizol® (Invitrogen) according to instructions of the manufacturer. Reverse transcription (RT) was performed using Quantitect Reverse transcription (Qiagen)®. For this, 1 μg of total RNA was reverse transcribed using oligo dT (12–18). For a 20 μL final volume PCR reaction, the following were added: 18 μL of Platinum® PCR Supermix (Invitrogen), 0.5 μL of each primer (20 μM) and 1 μL of cDNA (equivalent to 50 ng of total RNA). Detection of SelM transcripts was done using the primers BSelMF (5′-GATTTGAC-CAGGTTGTGGAG-3′) and BSelMR (5′-AAGCTGCATTTTGGAGTCTG-3′) designed based on our previously reported sequence (positions 255–648 of the sequence, GenBank accession no. DQ907947). These primers amplify a 393 bp fragment and were used under the following conditions: 95 °C, 1 min, 95 °C, 30 s, 60 °C, 1 min, 68 °C, 1 min (31 cycles); 72 °C, 10 min and kept at 4 °C until used. A ribosomal protein L8 producing a 428 bp fragment, (positions 72–500 of the sequence, DQ316258) was amplified side by side for comparisons using the forward and reverse primers (5′-GAAGAGGATCGGTGTTCAAGT-3′) and (5′-CTCCTGAAGGAAGCTTTACAC-3′), respectively and using the conditions mentioned before. Positive and negative controls were included. The PCR products were analyzed by agarose gel electrophoresis.

2.3. Synthesis of dsRNA

Two DNA templates were used to produce separately each ssRNA by *in vitro* transcription for each strand of SelM. Also, ssRNA was synthesized from a clone for a mango ethylene receptor (ER) that was used as an unrelated negative dsRNA control. The DNA templates were first cloned in the pGEM T Easy Vector (Promega®) in both orientations. A 454 pb fragment of the coding sequence of SelM (positions 194–648 of the sequence, DQ907947) was obtained with the primers SelMFwa1 (TTCCACAATGCCAAGTTCAA) and BSelMR (5′-AAGCTGCATTTTGGAGTCTG-3′), while an 850 pb of the ER clone was used (clone graciously donated by Dr. Islas-Osuna, unpublished data). The clones obtained in pGEM T Easy contain a T7 promoter adjacent to the inserted DNA fragments. The T7 primer was combined with specific primers to obtain from each clone a PCR product containing the T7 promoter. Single-stranded RNA (ssRNA) was transcribed *in vitro* from the PCR products templates using T7 phage RNA polymerases RiboMAX (Promega®). Then the DNA template was degraded with DNase I using 1 U/μg of template DNA. The *in vitro* synthesized RNAs were purified according to the manufacturer's protocol. Equal amounts of the two cRNA strands were mixed and annealed by incubation at 80 °C for 10 min and slowly cooled to room temperature for 50 min. The formation of dsRNA was detected by the changes in migration of the dsRNA vs the ssRNA by agarose gel electrophoresis.

2.4. Knockdown assay for Sel M

L. vannamei adults at intermolt stage were placed in a 50 L glass aquarium with seawater (37 psu), temperature controlled (28 °C) and under constant aeration. Shrimp were randomly divided into seven groups. The following treatments were included: non-injected (NI), injected with shrimp saline solution (ISS, 400 mM NaCl, 20 mM Tris, pH, 7.5), injected with 20 μg of dsRNA mango ethylene receptor (ER) as controls, and with varying quantities of SelM dsRNA including 5, 10, 20 and 40 μg; all the dsRNA were resuspended in shrimp saline solution. The shrimps were injected intramuscularly through the dorsal area of the second abdominal segment. The samples were collected 24 h after injection of the dsRNA, frozen on liquid nitrogen and stored at −80 °C. SelM and ribosomal protein L8 transcripts were detected by real time quantitative RT-PCR (qRT-PCR) in gills and hepatopancreas. Two separate cDNA reactions and two PCR reactions for each individual shrimp and tissue were done ($n=8$) for qRT-PCR on an iQ5 Real-Time PCR Detection System (Bio-Rad) in 20 μL final volume containing 10 μL of iQ SYBR Green Supermix (Bio-Rad), 8 μL of H_2O, 0.5 μL of each primer (20 μM) and 1 μL of cDNA (equivalent to 50 ng of total RNA). A fragment of 393 bp for SelM was obtained using the primers BSelMF and BSelMR under the following conditions: 95 °C, 5 min, 95 °C, 30 s, 65 °C, 35 s, 75 °C, 55 s (40 cycles). A single fluorescence measurement and a final melting curve program increasing 0.3 °C each 20 s from 60 °C to 95 °C were run to discard unspecific amplifications. The L8 cDNA (positions 333–500 of the sequence, GenBank accession no. DQ316258) was amplified side by side for comparisons using the L8F2 (5′-TAGGCAATGTCATCCCCATT-3′) and L8R2 (5′-TCCTGAAGGGAGCTTTACACG-3′) primers, producing a fragment of 167 bp and under the same conditions. Positive and negative controls were included. Standard curves of SelM and L8 were run to determine the efficiency of amplification using dilutions from 5×10^{-3} to 5×10^{-8} ng/μL of PCR fragments. For each measurement, expression levels (ng/μL) were normalized to L8 and expressed as relatives values (SelM/L8).

2.5. Peroxidase activity

This activity was measured using guaiacol as the substrate (Pérez-Tello et al., 2009) with the following modifications. Hepatopancreas and gills (60 mg, $n=8$) were homogenized in 120 μl of 0.1 M Tris–HCl, 5 mM β-mercaptoethanol, pH 8, centrifuged at 12,000 g/20 min/4 °C and the aqueous extract separated. In a 96-well microplate, 25 μL of the extract from gills or hepatopancreas was mixed with 160 μL of 0.01 M sodium acetate, 0.5% guaiacol, pH 5.3, and then 25 μL of 0.1% H_2O_2 were added. This reaction was immediately mixed and the absorbance was recorded at 490 nm after 60 s in a microplate reader. Activity was expressed as the difference in absorbance after 60 s of reaction per mg of protein. Protein concentration was determined