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EXPRESSION OF DYSTROPHIN GENE IN THE WHITE SPOT SYNDROME VIRUS INFECTED *MACROBRACHIUM ROSENBERGII*

Truong The Quang

Institute of Applied Science and Technology
Van Lang University, Vietnam
email: quangtruongthe@gmail.com

Abstract

We observed messenger ribonucleic acid (mRNA) expression of dystrophin gene and change of intracellular calcium ion concentration $[Ca^{2+}]$ in the white spot syndrome virus (WSSV) infected prawn *(Macrobrachium rosenbergii)* at 0, 4, 8, 12, 26, 38 and 48 hours post infection. We demonstrated the relationship between the dystrophin gene and the $[Ca^{2+}]$ in the WSSV infected prawn. Experimental results showed that the expression of the dystrophin gene increased sharply from 26 hours to 48 hours post WSSV infection. The $[Ca^{2+}]$ in muscle tissue of the infected prawn samples increased from 1.52 fold to 1.80 fold compared to the uninfected control sample.

1 Background

The giant freshwater prawn (*Macrobrachium rosenbergii*) is one of the most popular freshwater prawn species cultured today, and is considered to be a strong disease resistance when compared to other freshwater prawn species.

The white spot syndrome virus (WSSV) is one of the dangerous pathogens in farmed crustaceans (Figure 1). The disease occurs around the world, is one of the causes of high prawn mortality and seriously affects the industrial prawn farming industry.

Key words: Expression of dystrophin gene, Giant freshwater prawn, Intracellular calcium ion concentration, White spot syndrome virus.

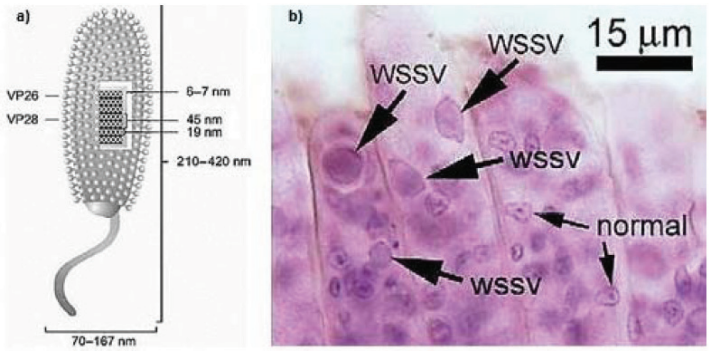

Figure 1. a) Morphological schematic of WSSV [1]; b) Hematoxylin and eosin section of WSSV infected gill tissue of prawn [5]

Dystrophin of *Macrobrachium rosenbergii* is a large and complex gene responsible for maintaining the structural integrity of muscle fibers [10]. The dystrophin gene is involved in the dystroglycan and sarcoglycan proteins, which work together in muscle contraction in the cellular framework. Mutations in the dystrophin gene can lead to muscle structural instability and subsequently muscular dystrophy, muscular disorder [16]. In addition, the intracellular calcium ion concentration $[Ca^{2+}]$ is involved in the regulation of muscle contraction and other muscle-related activities such as protein metabolism, growth. Studies have continued to show that changes of the $[Ca^{2+}]$ are often associated with muscular diseases [3, 7]. An increase in the total amount of calcium in dystrophin-affected muscles has been observed in cases of muscular degeneration, membrane damage and disorders of the $[Ca^{2+}]$ balance [9, 15].

Up to now, there have been many studies on the resistance to WSSV for the giant freshwater prawn. However, these studies were conducted under different conditions and at different stages on test subjects. Therefore, we conducted a WSSV infection experiment in the giant freshwater prawn with standard laboratory conditions, aimed at assessing the sensitivity of the prawn (*Macrobrachium rosenbergii*) to WSSV by how to investigate the mRNA expression of the dystrophin gene and the $[Ca^{2+}]$ in muscle tissue of the WSSV infected prawn.

1. Materials and methods

1.1 Experimental animals

The healthy giant freshwater prawn samples with size from 5 to 8 g were taken from the An Phat prawn breeding company, Can Tho city, Vietnam and taken to the laboratory of molecular biology, Phu Sa biochemical company, Can Tho

city, Vietnam (Figure 2). DNA from muscle tissue of prawn was extracted using PHUSA-HPKit according to the procedure of Phu Sa biochemical company. DNA extraction was performed on each prawn at each study time, with three replications for each prawn. The concentration and purity of the extracted DNA samples were checked by nucleotide quantification using the two wavelength measurement method of 260 nm and 280 nm and measured by a T60U spectrophotometer (PG Instruments, United Kingdom) [12, 13].

Figure 2. The giant freshwater prawn (*Macrobrachium rosenbergii*) in experiment

Total DNA of prawn was screened by polymerase chain reaction (PCR) using specific primers for the WSSV, including:

WSSV-f 5'-AGGTGTGGAACAACACATCAAG-3'
and WSSV-r 5'-TGCCAACTTCATCCTCATCA-3'.

We checked the healthy prawn without WSSV infection by PCR. After that, the prawn were acquainted for 7 days in 100 liters tanks at 27 ^{0}C. A total of 10 prawns were placed in each tank throughout the study. The prawn were fed once daily with the Grower C302 commercial prawn feed, Vietnam.

1.2. Estimate of WSSV copy numbers

A standard WSSV sample with a known copy number (6.50 10^6 copies/μl) was taken from Phusa BioChem and diluted 10 times continuously from 6.50 10^{10} copies/μl to 6.50 10^6 copies/μl. The quantitative real-time polymerase chain reaction (qPCR) was performed by the 7500 Real-Time PCR system (Applied Biosystems, USA). The standard curve was constructed using the cycle threshold values (Ct) obtained from the qPCR of the continuously diluted virus standard samples. This standard curve was then used to estimate the virus copy number at each stage of infection after immunological testing in accordance with the Mendoza-Cano methodology [8].

1.3. Immunization test for the giant freshwater prawn by WSSV

The WSSV used in this study was extracted from muscular tissue of the infected prawn samples. The infected tissues were homogenized in TN buffer of 0.1 g/ml (20 mM Tris-HCl, 400 mM NaCl, pH = 7.4) and centrifuged at 2000 rpm for 10 minutes. The isolated virus was diluted with NaCl (1 %, w/v) in a ratio of 1:5 (v/v) and filtered using a 0.45 μm syringe filter. The filtrate was stored at 80 ^{0}C before injection into the prawn samples. The copy number of the WSSV infected filtrate was determined by the qPCR as described in section 1.2 and compared with the standard curve. The WSSV copy number of the viral filtrate was diluted to 2.50×10^6 copies/ml and injected into the fourth abdomen of each prawn using the sterile syringes. Five prawn samples were selected and separated for each tank to control group. Each control sample was injected by 5 ml of phosphate salt buffer solution. Three single prawns infected by WSSV were randomly collected at the following intervals: 0 hours, 4 hours, 8 hours, 12 hours, 26 hours, 38 hours and 48 hours post infection. The samples were anesthetized and dissected in sterile conditions before being snap-frozen in liquid nitrogen and extracted the total RNA.

1.4. Total RNA isolation and cDNA convertion

Total RNA was isolated from 50 mg of the prawn muscle tissue. The prawn muscle tissue samples were preserved frozen in liquid nitrogen. Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, USA), then DNA was eliminated by RNAse solution (5 Prime GmbH, Hamburg, Germany). Get 4 μl of total RNA was reverse transcribed using GoScript reverse transcriptase (Promega, WI, USA) and Oligo $(dT)_{15}$ Primer (Promega, WI, USA). The concentration and purity of the cDNA were checked before being used to assess expression of dystrophin gene, using the T60U spectrophotometer (PG Instruments, United Kingdom) at 260 nm and 280 nm.

1.5. Quantification of dystrophin expression

The expression of the *Macrobrachium rosenbergii* dystophin gene (dystrophin) at different post infection time (hours) was determined by a qPCR using SYBR Green supermix on a 7500 Real-Time PCR System (Applied Biosystems, USA). The qPCR was carried out using the converted cDNA as a template and with primers: Dys-f 5'-TAGCTGTTTTGCATCGTGTTG-3' 3'and Dys-r 5'-TGGGGTGAGTGATCTTGTGA-3'.

The amplifications reactions were performed in 15 μl containing cDNA template (50 ng/μl), 1 *times* SYBRgreen Mastermix (Applied Biosystems, USA), Dys-f (0.30 μM) and Dys-r (0.30 μM). The thermal profile consisted of an initial step at 50 ^{0}C for 2 minutes, followed by 95 ^{0}C for 10 minutes and 45 cycles of 95

^{0}C for 15 seconds and 60 ^{0}C for 1 minutes. Cycle threshold (Ct) values were estimated by the inbuilt ABI 7500 SDS software. The specificity of the qPCR amplification was verified through a melt curve analysis. An internal control gene, elongation factor 1-alpha (ELF-1) [2] was quantified using the reaction mixture with two different primers: ELF-f 5'-CGCCGAACTGCTGACCAAGA-3'(0.30 μM) and ELF-r 5'-CCGGCTTCCAGTTCCTTACC-3' (0.30 μM). The relative expression of the dystrophin gene compared to the internal control gene was estimated in accordance with the comparative CT method [6]. The qPCR was carried out 3 times for each interval to achieve the permitted level of reliability.

1.6. Estimate of intracellular calcium ion concentration

The Calcium Assay Kit (Metallogenics, CHB, JAP) was used to quantify the intracellular calcium ion concentration in the muscle tissues of the prawn samples. The prawn muscle tissue (50 mg) was submerged in a 3 % trichloroacetic acid (TCA) solution. Then, it was aseptically crushed and sterile vortexed before incubation at 4 ^{0}C for 30 minutes. The mixture was centrifuged 10,000 rpm for 15 minutes. The absorbance of the supernatant was calculated by a UV-ViS molecular absorption spectrophotometer (T60U, PG Instruments) at 570 nm and 700 nm. The calcium ion concentration was measured using a standard calibration curve for calcium solution (10 mg/dl). The calcium ion concentration was calculated using the equation (1) [4].

$$\left[Ca^{2+}\right] = \frac{OD_{\text{sample}} - OD_{\text{blank}}}{OD_{\text{std}} - OD_{\text{blank}}} \cdot 10 \tag{1}$$

where $[Ca^{2+}]$ is the intracellular calcium ion concentration (mg/dl), OD_{sample} is the absorbance of test sample, OD_{blank} is the absorbance of blank sample, OD_{std} is absorbance of standard sample.

1.7. Statistical analysis

All of the quantifications were conducted in three replicates and analyzed by analysis of variance (ANOVA) tests at a significance level of $P < 0.05$, using Microsoft Excel 2010 software [11].

2. Results and discussion

2.1. WSSV infection of *Macrobrachium rosenbergii*

Symptoms of infection were not observable in the early stages from 0 to 12 hours after the injection of WSSV into the abdomen of the healthy prawn. After being infected for 12 hours, the prawn began to comatose and showed an appetite. Other clinical symptoms due to WSSV infection such as discoloration of muscle and loose cuticles were observed at 26 hours post infection. The symptoms of

infection continue to develop until 48 hours later. The prawn mortality was not seen, although at 48 hours post infection, visual observation showed that prawn was debilitated, swimming slowly.

Analysis with a confidence level of $P < 0.05$, the WSSV copy numbers in muscle tissues of the WSSV infected prawn by qPCR showed that there was no significant change in dystrophin gene response level in the prawn at 26 hours post infection. The WSSV copy numbers at 4, 8, 12 and 26 hours post infection were $5.15 \times 10^3, 6.47 \times 10^4, 8.65 \times 10^4$ and 5.72×10^5 copies/μl, respectively. However, the presence of WSSV increased drastically to 4.79×10^7 copies/μl at 38 hours post infection, and continued to increase exponentially to 1.97×10^9 copies/μl at 48 hours post infection. This showed that the expression of the dystrophin gene increased sharply from 26 hours to 48 hours post infection (Table 1) [14].

Table 1. WSSV copy number corresponds to time post WSSV infection

Time post WSSV infection (hours)	4	8	12	26	38	48
WSSV copy number (copies/µl)	$^{f}5.15_{\times}10^{3}$	$^{e}6.47_{\times}10^{4}$	$^{d}8.65_{\times}10^{4}$	$^{c}5.72_{\times}10^{5}$	$^{b}4.79_{\times}10^{7}$	$^{a}1.97_{\times}10^{9}$

2.2. Expression of dystrophin gene in the WSSV infected prawn

The qPCR was conducted on both dystrophin and WSSV at 0, 4, 8, 12, 26, 38 and 48 hours post infection, with a significant difference of $P < 0.05$. The mRNA expression levels of dystrophin were analyzed and standardized relative to the expression of ELF1 reference gene. The dystrophin expression and the WSSV copy number both began to increase sharply at 26 hours post infection (Figure 3).

The expression level of dystrophin gene in muscle tissues of the infected prawn sample group increased slowly 0.21, 0.56, 0.61 and 0.88 fold at 4, 8, 12 and 26 hours post infection respectively, compared to the levels in the uninfected control group. The expression level of dystrophin gene then gradually increased from 26 hours post infection onwards, rising to 1.50 fold and 2.03 fold higher than in the control sample group at 38 hours and 48 hours post infection, respectively (Figure 3).

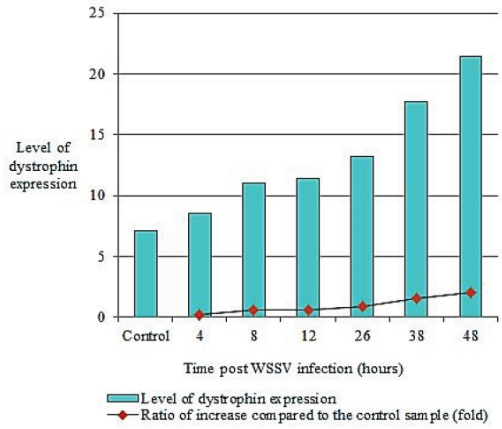

Figure 3. Expression level of dystrophin gene corresponds to time post WSSV infection

2.3. Intracellular calcium ion concentration in the prawn muscle tissue

The quantitative results of the intracellular calcium ion concentration $[Ca^{2+}]$ in the muscle tissue of WSSV infected *M. rosenbergii* showed a significant increase at 4 hours post WSSV infection, from 4.31 mg/dl in the control samples to 6.70 mg/dl. A further increase in the $[Ca^{2+}]$ was observed until a peak of 7.75 mg/dL at 8 hours post infection. The $[Ca^{2+}]$ then fell to 6.53 mg/dl at 12 hours post infection, before rising again slightly and then fluctuating, with levels of 7.35 mg/dl, 7.06 mg/dl and 7.14 mg/dl at 26 hours, 38 hours and 48 hours post infection, respectively (Table 2) [14].

Table 2. The $[Ca^{2+}]$ in the muscle tissue of WSSV infected *M. rosenbergii*

Time post WSSV infection (hours)	Control	4	8	12	26	38	48
Intracellular calcium concentration $[Ca^{2+}]$ (mg/dl)	4.31^{g}	6.70^{e}	7.75^{a}	6.53^{f}	7.35^{b}	7.06^{d}	7.14^{c}

The results were collected at 4, 8, 12, 26, 38 and 48 hours post WSSV infected prawn. The Ca^{2+} concentrations were obtained from three replicates at each time point, calibrated with a 10 mg/dl calcium solution. The intracellular calcium ion concentration in the infected prawn samples increased from 1.52 to 1.80 fold compared to the control sample with significant differences ($P < 0.05$) (Figure 4).

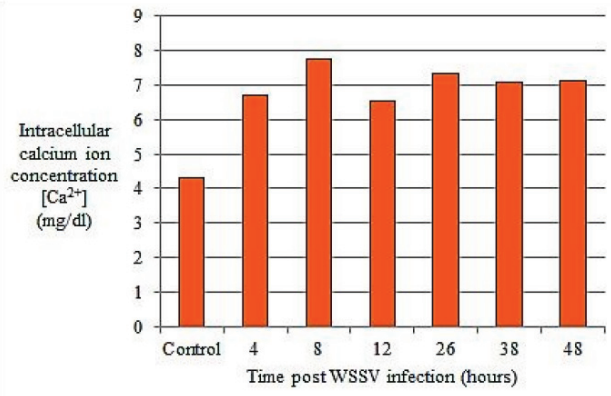

Figure 4. The [Ca^{2+}] corresponds to time post WSSV infection of *M. rosenbergii*

3. Conclusions

The study results demonstrated the relationship between the dystrophin gene and the intracellular calcium ion concentration [Ca^{2+}] in the muscle tissue of WSSV infected *Macrobrachium rosenbergii.* Specifically, we observed mRNA expression of dystrophin gene and the [Ca^{2+}] changes in the muscle tissues of WSSV infected *M. rosenbergii* at 0, 4, 8, 12, 26, 38 and 48 hours post infection.

Study of the WSSV copy number in the muscle tissues of WSSV infected M. rosenbergii by qPCR showed that there was no significant change of dystrophin gene response level in the prawn at 26 hours post infection. The WSSV copy numbers at 4, 8, 12 and 26 hours post infection were $5.15\times10^3, 6.47\times10^4, 8.65\times 10^4$ and 5.72×10^5 copies/μl. However, the presence of WSSV increased significantly to 4.79×10^7 copies/μl at 38 hours post infection and continued to increase to 2.10×10^9 copies/μl at 48 hours post infection. This showed that the expression of the dystrophin gene increased sharply at 38 hours and 48 hours post infection.

The intracellular calcium ion concentrations [Ca^{2+}] in the muscle tissue of WSSV infected *M. rosenbergii* showed a significant increase at 4 hours post infection, from 4.31 mg/dl in the control sample to 6, 70 mg dl. The [Ca^{2+}] peaked at 7.75 mg/dl at 8 hours post infection, then decreased to 6.53 mg/dl at 12 hours post infection, before increasing again and increasing gradually, with the [Ca^{2+}] of 7.35 mg/dl, 7.06 mg/dl, and 7.14 mg/dl at 26 hours, 38 hours and 48 hours post infection, respectively. In summary, The [Ca^{2+}] in the muscle tissue of WSSV infected prawn samples increased from 1.52 fold to 1.80 fold compared to the control sample.

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