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# A thymosin repeated protein1 reduces white spot syndrome virus replication in red claw crayfish *Cherax quadricarinatus*

Dong-li Li, Xue-jiao Chang, Xiao-lu Xie, Shu-cheng Zheng, Qiu-xia Zhang, Shu-ao Jia, Ke-jian Wang, Hai-peng Liu*

*State Key Laboratory of Marine Environmental Science, Xiamen University, Fujian Collaborative Innovation Center for Exploitation and Utilization of Marine Biological Resources, State-Province Joint Engineering Laboratory of Marine Bioproducts and Technology, Xiamen 361102, Fujian, PR China*

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ABSTRACT

The β-thymosins are a group of structurally related, highly conserved intracellular small peptides in vertebrates with various biological functions, including cytoskeletal remodeling, neuronal development, cell migration, cell survival, tissue repair and inhibition of inflammation. In contrast to vertebrates, the function of β-thymosin is not fully understood in crustaceans. Previously, we found that a thymosin-repeated protein1 (*CqTRP1*) gene was up-regulated after white spot syndrome virus (WSSV) challenge in hematopoietic tissue (Hpt) cells from the red claw crayfish *Cherax quadricarinatus*. To further identify the effect of CqTRP1 on WSSV infection, a full length cDNA sequence of β-thymosin homologue was cloned and analyzed from red claw crayfish followed by functional study. The *CqTRP1* cDNA contains an open reading frame of 387 nucleotides encoding a protein of 129 amino acids with a putative molecular mass of 14.3 kDa. The amino acid sequence showed high identity with other β-thymosins and contained three characteristic thymosin β actin-binding motifs, suggesting that *CqTRP1* was a member of the β-thymosin family. Tissue distribution analysis revealed a ubiquitous presence of *CqTRP1* in all the examined tissues with the highest expression in hemocytes, Hpt and gonad at the transcriptional level. Interestingly, the gene silencing of endogenous *CqTRP1* by RNAi enhanced the WSSV replication in Hpt cells. Meanwhile, the WSSV replication was significantly reduced in the Hpt cell cultures if overloaded with a recombinant CqTRP1. Taken together, these data clearly indicated that CqTRP1 was likely to be associated with the anti-WSSV response in a crustacean *C. quadricarinatus*, which provides new strategy against white spot disease in crustacean aquaculture.



## 1. Introduction

The white spot syndrome virus (WSSV) is one of the most serious pathogens which affect the cultivation of global economic shrimp species. In addition to the impact of the virus on the economy, the natural marine ecology is also threatened by this virus as WSSV is able to infect a large number of crustaceans including crabs and freshwater crayfish (Lo et al., 1996; Wang et al., 1998). The WSSV virions can be found in most tissues and can circulate in the hemolymph of the infected crustaceans (Witteveldt et al., 2004). It is well known that crustaceans are lacking of adaptive immune response and dependent solely on the innate immune response to recognize and destroy the invading exogenous pathogens (Lee and Söderhäll, 2002). The host defense mechanisms and particularly host anti-viral defense of crustacean are still poorly understood (Gross et al., 2001). Hence, it is important to have a better understanding of relationship between WSSV infection and the host innate immune factors which will benefit the novel control strategies against white spot disease.

Thymosin, a family of polypeptide hormones isolated from thymus tissue, has been found from calf thymus first (Huff et al., 2001). Furthermore, thymosins were named and classified according to its capacity to stimulate lymphocytopoiesis and their isoelectric points: α-thymosins with pI below 5.0, β-thymosins with pI range from 5.0 to 7.0, and γ-thymosins with pI above 7.0 (Huff et al., 2001). Thymosin-β4 (Tβ4) is the most abundant member and representing about 70–80% of the total β-thymosin content, with a 43 amino acid peptide and about 5 kDa molecular mass (Goldstein et al., 2005; Huff et al., 2001; Low and Goldstein,

* Corresponding author.
*E-mail address:* Haipengliu@xmu.edu.cn (H.-p. Liu).

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1982; Low et al., 1981). Importantly, Tβ4 is highly conserved from mammals to echinoderms and plays important role in development and immune response (Kang et al., 2011; Sanders et al., 1992; Stoeva et al., 1997; Zhang et al., 2008). To date, the β-thymosin-like genes have been reported in invertebrates like *Drosophila melanogaster* (Koshikawa et al., 2010), *Caenorhabditis elegans* (Van et al., 2004) and *Helicoverpa armigera* (Saelee et al., 2013) and *Hodotermopsis sjostedti* (Koshikawa et al., 2010). Furthermore, in aquaculture animals, the studies on β-thymosins are also limited to only a few species, such as Chinese mitten crab *Eriocheir sinensis* (Gai et al., 2009), red swamp crayfish *Procambarus clarkia* (Shi et al., 2015) and disk abalone *Haliotis discus discus* (Kasthuri et al., 2013). Most reports about β-thymosin in invertebrates are related to its ability to promote growth and resist pathogens invasion, even to regulate the proliferation and differentiation of hematopoietic stem cells. However, the knowledge about the roles of invertebrate β-thymosin is still largely unknown, especially in terms of anti-viral immunity.

Previously, we found that a partial sequence of thymosin-repeated protein1 (*CqTRP1*) was responsive to WSSV infection in red claw crayfish Hpt cells (Liu et al. 2011). To reveal the role of *CqTRP1* in WSSV infection, in the present study, we obtained the full-length cDNA sequence of *CqTRP1* and determined its gene expression profile in various tissues. Then the effect on WSSV replication in *CqTRP1* silenced hematopoietic tissue (Hpt) cells or r*CqTRP1* overloaded Hpt cells by protein transfection were further examined. Our results found that *CqTRP1* showed strong reduction on WSSV replication, which provided new information on *CqTRP1* function in antiviral immunity in a crustacean and further useful strategy for white spot disease control.

## 2. Materials and methods

### 2.1. Experimental animals and preparation of Hpt cells

The healthy freshwater red claw crayfish, *C. quadricarinatus*, were purchased from Source Sentai Agricultural Science and Technology Co., Ltd of Zhangzhou, Fujian Province, China, and kept in tanks in aerated tap water at 26 °C. The Hpt cells of crayfish were prepared and cultured according to Söderhäll et al. (2005). The hematopoietic tissue was dissected from the dorsal side of the stomach, and washed with CPBS (phosphate buffer saline of crayfish: 10 mM $Na_2HPO_4$; 10 mM $KH_2PO_4$; 150 mM NaCl; 10 mM $CaCl_2$ and 10 mM $MnCl_2$; pH 6.8) and then incubated in 500 μL of 0.1% collagenase (type I) and 0.1% collagenase (type IV) (Sigma) in CPBS at room temperature for 45 min. The Hpt was centrifuged at 800 × g for 3 min at room temperature and to remove the collagenase solution. The samples were washed twice with 1 mL CPBS in the same method described above and the cells were isolated by gently pipetting and to remove the undigested tissues, and then the isolated Hpt cells were resuspended in L-15 medium (Söderhäll et al., 2005). Hpt cells were seeded in a 24-well plates at a density of $5 \times 10^5$ cells/500 μL and supplemented with plasma (a crude astakine preparation from red claw crayfish) (Söderhäll et al., 2005) after about 30 min attachment at 20 °C.

### 2.2. Virus preparation

The WSSV was kindly provided by Prof. Xun Xu (Third Institute of Oceanography, SOA, Xiamen, Fujian, China). The virus was prepared as described in the article of Xie et al. and quantified via absolute quantification by PCR (Xie et al., 2005).

### 2.3. RNA extraction and cDNA synthesis

Different tissues of crayfish were collected and total total RNA was extracted from all the tissues with TRIzol reagent (Roche, USA) according to the manufacturer's instructions. RNase-Free DNase I (Ambion, USA) was used to eliminate genome DNA contamination in the extracted RNA. The RNA samples were analyzed in 1.0% agarose electrophoresis and quantitated at 260 nm with NanoDrop 2000 spectrophotometer (Thermo Scientific, USA), and all OD260/OD280 were between 1.8 and 2.0. Total RNA (1 μg) was used for first strand cDNA synthesis using the PrimeScript™ RT Reagent Kit (TaKaRa, Japan) according to the manufacturer's instructions. The 3′ and 5′ ends RACE cDNA templates were synthesized using SMARTer™ cDNA Kit (Clontech, USA) following the protocol of the manufacturer.

### 2.4. Gene cloning of the full-length cDNA of CqTRP1

A partial *CqTRP1* cDNA sequence of *C. quadricarinatus* was obtained from a transcriptome library of Hpt cells post WSSV infection in our lab (Liu et al., 2011). BLAST analysis showed that this *Cherax β-thymosin* showed high level of identity (or similarity) with *β-thymosins* of other shrimps. Based on the transcriptome library sequence data of *CqTRP1*, its 3′ and 5′ ends were obtained using SMARTer™ cDNA Amplification Kit (Clontech, USA). For 3′ RACE, the PCR reaction was conducted using the primer F1 and the anchor primer UPM (Table 1). The PCR reaction conditions were 98 °C for 2 min, 30 cycles of 98 °C for 30 s, 65 °C for 30 s and 72 °C for 40 s, and 72 °C for 10 min. For 5′ RACE, as well as those described above.

All amplified PCR fragments were subjected to electrophoresis on 1.0% agarose gel to determine length differences, and the target spot was purified by PCR Gel Extraction Kit (Sengong Biotech, Co., Ltd., Shanghai, China). The final purified products were cloned into PMD18-T vector (TaKaRa, Japan), following the instructions provided by the manufacturer. The vectors were transformed into *E. coli* DH5α cells. Recombinant bacteria were confirmed by PCR and further sequenced at Xiamen Borui Biotech Company, China.

### 2.5. Sequence analysis and domain search analysis

The cDNA sequence of *CqTRP1* had been published previously (Genbank accession no: AEL23126.1). The nucleotide and deduced amino acid sequences of *CqTRP1* cDNA were analyzed and compared using the BLASTn and BLASTp search programs (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). A search for conserved domains and motifs was performed using the conserved domain database of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the prediction map from SMART (http://smart.emblheidelberg.de). The signal peptide was identified using SignalP 4.1 Server program (http://www.cbs.dtu.dk/services/SignalP/). Glycosylation site were elucidated using the Web-based-tools (http://www.cbs.dtu.dk/services/NetNGlyc/). The 3D structure of *CqTRP1* protein was predicted by SWISS-MODEL server (http://swissmodel.expasy.org/).

### 2.6. Tissue distribution profile of CqTRP1 mRNA

Hemocytes, Hpt, gonad, nerve, stomach, heart, gill, hepatopancreas, muscle, eyestalk, intestine and epithelium were dissected from three random individuals free of WSSV for total RNA extraction, respectively. Total RNA was extracted as described above. The mRNA expressions of *CqTRP1* in different tissues were determined by quantitative real-time RT-PCR (RT-qPCR). The PCR solution was performed in a total volume of 20 μL, containing 10 μL of 2 × SYBR Green Mix (Roche, USA), 1 μL each of primers (10 mM), 1.0 μL of