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The crustin-like peptide plays opposite role in shrimp immune response to *Vibrio alginolyticus* and white spot syndrome virus (WSSV) infection

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ABSTRACT

Crustin is an antimicrobial peptide (AMP) that plays a key role in innate immunity of crustaceans. In this study, we cloned the entire 660 bp crustin-like sequence with a 507 bp open reading frame encoding a 168 amino acid from *Marsupenaeus japonicus*. The crustin-like gene was primarily expressed in gills and over-expressed in shrimp hemocytes after challenge with WSSV or *Vibrio alginolyticus*. After knockdown crustin-like gene using specific double-stranded RNA (CRU-dsRNA), IMD, Rab7, L-lectin, mitogen-activated protein kinase, p53, prophenoloxidase and Rho were down-regulated and nitric oxide synthase, myosin and tumor necrosis factor-α were up-regulated in hemocytes at 24 h post dsRNA treatment. After WSSV challenge, The mortality, WSSV copy number and expressions of WSSV immediate early genes (IE1, IE2, DNA polymerase, VP28) were both decreased but the apoptosis rate was increased in CRU-dsRNA-treated shrimps, indicating that WSSV may take advantage of crustin-like to benefit its replication. After silenced the crustin-like, the results of phagocytosis showed that the phagocytic rate of shrimp hemocytes on WSSV decreased significantly. In contrast, the absence of crustin-like in shrimps increased the mortality following *V. alginolyticus* challenge, indicating that crustin-like may play a positive role in the antibacterial process. The phagocytosis experiment showed there was a higher phagocytosis rate of hemocytes after CRU-dsRNA treatment. The result indicated that *V. alginolyticus* may be able to use crustin-like to avoid phagocytosis of shrimp hemocytes. These results further added to our understanding of the function of crustin-like peptide and also provided its potential role in innate immunity in shrimp.

1. Introduction

All crustaceans, including *Marsupenaeus japonicus*, are considered to lack a highly specific adaptive immune system, opposing to foreign pathogens primarily via innate immunity [1]. As two of the most serious diseases in shrimps, vibriosis and white spot syndrome virus (WSSV) have caused irreversible damage to the shrimp culture industry worldwide [2]. However, there are currently no efficient measures to control these diseases.

Numerous antimicrobial peptides (AMPs) have been identified in crustaceans, insects, mammals and plants. As the major component of the innate immune defense system in marine invertebrates [3], AMPs represent suitable candidates for the development of novel antibiotics because of their antimicrobial activities against Gram-negative and -positive bacteria, fungi and some viral and protozoan pathogens [4]. AMPs may also serve as templates for the development of therapeutic agents [5]. The first crustin, carcinin, was identified in hemocytes of the shore crab *Carcinus maenas* [6]. Crustins are cationic, cysteine-rich AMPs with a molecular weight of 7–14 kDa, with an isoelectric point of 7.0–8.7, and containing one whey-acidic protein (WAP) domain at the carboxy terminus [7]. Many crustins have been characterized in various crustaceans, including crab, lobster, crayfish and shrimp, and previous studies have reported that crustins are widely distributed AMPs among shrimp [7]. Crustins have various biological activities, including antimicrobial, protease inhibitory and immunoregulatory activities during recovery from wounding or physiological stress [7,8].

However, although the antibacterial activity of shrimp crustin is well known, its role in the process of virus infection has been less

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well-studied. In this study, we identified a novel crustin-like AMP in *M. japonicus* and investigated its role in WSSV replication.

2. Methods and materials

2.1. Shrimps and tissue preparation

The healthy adult *M. japonicas* (approximately 15 g and 10–12 cm each)were obtained from a seafood market of Hangzhou. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang A & F University (Hangzhou, China). The muscles, hepatopancreas, gills, sex glands and hemolymph were collected from health or challenged shrimps. The samples were used immediately for RNA extraction, in order to prevent total RNA degradation. WSSV (GenBank accession no. AF332093.1) was purified and used in challenge experiments, as described previously [9]. *Vibrio alginolyticus* was cultured and used to challenge the shrimps according to the previous report [10].

2.2. Rapid amplification of cDNA ends (RACE)

Total RNA was extracted from muscle of the *M. japonicus* using PureLinkTM RNA Mini Kit (Ambion, USA), following the protocol of the manufacturer. The concentration and quality of total RNA were determined by the Nanodrop Trace Spectrophotometer and 1% agarose gel electrophoresis detection, respectively. The RACE technique was utilized to clone the full-length cDNA sequence of a gene, based on the known middle fragment using 5'/3′ RACE Kit, 2nd Generation (Roche, Germany), following the protocol of the manufacturer. The synthesized cDNA were kept at −20 °C, used for the 3′/5′ -RACE PCR with 3′ gene-specific primer (3GSP, 3NGSP) or 5′ GSP (5SP1, 5SP2, 5SP3), designed on the basis of middle sequence (the primer's sequences are shown in Table 1). The PCR products were purified using MiniBEST DNA Fragment Purification Kit Ver.3.0 (Takara, Japan), following the manufacturer's instruction. The PCR products were purified using AxyPrep DNA Gel Extraction Kit (Axygen, USA), following the manufacturer's instruction. Amplified cDNA fragments were transferred into the pMD19-T vector (Takara, Japan). Recombinant bacteria were identified by blue/white screening and confirmed by PCR and sent to sequencing company (Sangon, China). Nucleotide sequences of the cloned cDNA were sequenced by double pass. All primers used in this experiment were designed using Primer Premier 5.0.

2.3. Nucleotide sequence and bioinformatics analyses

The nucleotide sequence similarities were examined by BLAST software (http://www.ncbi.nlm.nih.gov/BLAST/). The 5′and 3′sequences from RACEs were assembled with the partial cDNA sequences corresponding to each fragmental sequence by DNAMAN 5.0. The protein prediction was performed using the open reading frame (ORF) Finder tool. Multiple sequence alignment was created by using the ClustalX 1.81. And the phylogenetic trees based on the amino acid sequences were performed by the neighbor-joining method using Molecular Evolutionary Genetics Analysis, MEGA7.1.

2.4. The quantitative real-time PCR

Relative crustin-like mRNA expression levels in various adult tissues were measured by qRT-PCR using a SYBRII® Premix Ex Taq (Tli Rnase Plus) (TaKaRa, Japan). Total RNA was isolated from various tissues of healthy adult prawns and from hemocytes of shrimps challenged by intramuscular injection of 0.1 ml of viral or bacterial suspension including WSSV (10^5/mL) or *V. alginolyticus* (10^5 colony-forming units [CFU]/mL), respectively, for different

Table 1
Universal and specific primers used in this study. Crustin-like was marked as CRU.

Primer Name	Nucleotide sequence (5′→3′)	Purpose
3′ race GSP	CGCACAGCACGCAAAGAAG	CRU first primer for 3′RACE
3′ race NGSP	TGGAGTTCCTGGAGCCGTAG	CRU second primer for 3′RACE
5′race sp1	CTGGGAATCCACCTCCGACT	CRU first primer for 5′RACE
5′race sp2	TCCACCTACGGCTCCAGGAACTC	CRU second primer for 5′RACE
5′race sp3	CACCGCCACGACGGACAATAC	CRU third primer for 5′RACE
CRU-F	TCATCGCACAGCACGCAAAG	for CRU expression
CRU-R	ACTTACGGCTCCAGGAACTCC	for CRU expression
CRU dsRNA F	CCCAAGCTT ACTTGTAGTATTGTCCGTCGTGGCG	for CRU RNAi
CRU dsRNA R	CGGGGATCC CGTTTAGACAGCGGTCGAAGCAG	for CRU RNAi
hemocyanin-F	AACCCTGAACAAAGAGTTGCCTAT	for hemocyanin expression
hemocyanin-R	AACGGACGGTAAGTTGATGATGT	for hemocyanin expression
IMD-F	ATTCATCCGTCTACCTCCCTACA	for IMD expression
IMD-R	GAGCTGAGTCTGTCTTAATGTTATCC	for IMD expression
L- lectin-F	ATGTTATGCCATCTGCCTCGTATTT	for L- lectin expression
L- lectin-R	CTTTCGCTGCTGCTCTTTCTGTT	for L- lectin expression
MAPK-F	CGCATCACTGTTGAGGAGG	for MAPK expression
MAPK-R	GCAGGTCATCAAGTTCCATCT	for MAPK expression
NOS-F	CCAGGATCTTCTTGTTGGTGTTG	for NOS expression
NOS-R	CCCTCATCTGTAGCATAAAGTTCTC	for NOS expression
p53-F	TTCCTGCCTGGCTGACTCTA	for p53 expression
p53-R	CACCCAATCTTCCAACATCACAT	for p53 expression
proPO-F	TTCTACCGCTGGCATAAGTTTGT	for proPO expression
proPO-R	TATCTGCCTCGTCGTTCCTCAC	for proPO expression
STAT-F	TGGCAGGATGGATAGAAGACAAG	for STAT expression
STAT-R	TGAATAAGCTGGGATACGAGGGA	for STAT expression
TNF-F	ACAGACGGTCCAGAGTCCCAAAG	for TNF expression
TNF-R	GCGACGAAGTGAGCCACAGTAA	for TNF expression
EGFP-dsRNA F	CCCAAGCTTCACCATCTTCTTCAAGGACGAC	
EGFP-dsRNAR	CCGGAATTCCCAGCCAGGACCATGTGAT	