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A novel crustin from *Marsupenaeus japonicus* promotes hemocyte phagocytosis

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ABSTRACT

Crustins are cationic cysteine-rich antimicrobial peptides (AMPs) that contain multiple domains (glycine-rich, cysteine-rich, or proline-rich) at the N-terminus and whey acidic protein (WAP) domains at the C-terminus. Crustins have multiple functions, including protease inhibition and antimicrobial activity. Other functions of crustins need to be clarified. In this study, a novel crustin with a cysteine-rich region, and a single WAP domain, belonging to type I crustins, was identified in *Marsupenaeus japonicus* and designated as *Mj*Cru I-1. *Mj*Cru I-1 was expressed in various tissues. The expression of *MjCru I-1* was upregulated in the hemocytes of shrimp challenged with bacteria. *MjCru I-1* could bind to bacteria by binding to the cell wall molecules of the bacteria, such as lipopolysaccharide (LPS), peptidoglycan (PGN), and lipoteichoic acid (LTA). The synthesized WAP domain of *Mj*Cru I-1 but not synthesized Cys-rich domain has antibacterial and agglutinative activities. Scanning electron microscope assay showed that the bacterial cells treated with s*Mj*Cru I-1 appeared to be disrupted and cracked compared with those of the control samples. The knockdown of *MjCru I-1* could reduce bacterial clearance and injection of *Mj*Cru I-1 could significantly increase the survival rate of shrimp infected with *Vibrio anguillarum* and *Staphylococcus aureus* compared with those of the control samples. Further study discovered that *Mj*Cru I-1 could increase the hemocyte phagocytosis against *V. anguillarum* and *S. aureus*. These results suggest that *Mj*Cru I-1 has dual functions, bactericidal and phagocytosis promoting activities, in the antibacterial immunity of shrimp.

1. Introduction

Invertebrates including shrimp have no real adaptive immunity and mainly depend on innate immunity against a variety of pathogens. Antimicrobial peptides (AMPs) are important effectors in innate immunity, which is the front line of host defense against infection of microbes including bacteria, fungi, and viruses (Brown and Hancock, 2006; Hancock et al., 2006; Silva et al., 2013). Several types of AMPs, such as penaeidins, anti-lipopolysaccharide factors, crustins, and lysozymes, have been discovered in shrimp (Tassanakajon et al., 2011). Crustins are cationic cysteine-rich AMPs that contain a glycine-rich, cysteine-rich, or proline-rich region at the N-terminus and whey acidic protein (WAP) domains at the C-terminus (Hauton et al., 2006; Smith et al., 2008; Supungul et al., 2004). The WAP domain contains eight cysteines with a conserved arrangement forming four disulfide bridges (Smith et al., 2008). The first crustin was identified in hemocytes of shore crab, *Carcinus maenas* (Relf et al., 1999).

Recent studies revealed that crustins were present in various crustaceans, including crabs, lobsters, crayfish, and shrimps (Donpudsa et al., 2010; Mu et al., 2011; Pisuttharachai et al., 2009; Sun et al., 2010). Crustins are currently divided into three types, namely, crustin types I, II, and III (Smith et al., 2008). Most of studies reveal that crustins have protease inhibitory and antimicrobial activities (Arockiaraj et al., 2013; Krusong et al., 2012; Smith et al., 2008). However, the studies of some other aspects of functions need to be undertaken (Smith et al., 2008).

In this study, a novel type I crustin in kuruma shrimp, *Marsupenaeus japonicus*, was discovered. This type of crustin, termed *Mj*Cru I-1, contains a signal peptide, a cysteine-rich region, and a WAP domain. The *MjCru I-1* expression was upregulated in the hemocytes of shrimp challenged with *V. anguillarum* or *S. aureus*. *Mj*Cru I-1 exhibited antibacterial activity. Further study indicated that *Mj*Cru I-1 was associated with phagocytosis of hemocytes.

2. Materials and methods

2.1. Challenge of shrimp and collection of tissues

M. japonicus samples (6–8 g each) were obtained from a fishery market in Jinan, Shandong Province, China, and cultured in

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laboratory aquarium tanks with aerated seawater at 22 °C. Bacteria were collected at the logarithmic phase and then washed with PBS and suspended in PBS to obtain a final cell number of 1×10^7 CFU/mL for injection. Afterward, each normal shrimp was injected in the abdomen with 1×10^5 CFU of *V. anguillarum* or *S. aureus*, obtained from Shandong University Organism Culture Collection (SDMCC). The control groups were injected with phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4; pH 7.4). Total RNA was extracted from the tissues of hemocytes, heart, hepatopancreas, gills, stomach, and intestine with the use of Trizol reagent (Cwbio, Beijing, China) 12 h post injection. For hemocyte collection, the hemolymph was extracted from three shrimp by using a syringe preloaded with 1 mL of anti-coagulant buffer (0.45 M of NaCl, 10 mM of KCl, 10 mM of EDTA, and 10 mM of HEPES, pH 7.45) and immediately centrifuged at 800 *g* for 10 min (4 °C). The other tissues (heart, hepatopancreas, gills, stomach, and intestine) were also collected from at least three shrimp for further analysis.

2.2. cDNA cloning and sequence analysis

The full-length cDNA sequence of the crustin was obtained from intestine transcriptome sequence. Similarity analysis of crustins was conducted using BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The corresponding cDNA was translated, and the deduced proteins were predicted using ExPASy (http://www.expasy.org/). Signal sequencing and domain prediction were performed using SMART (http://smart.embl-heidelberg.de/). MEGA 6 was used for phylogenetic analysis.

2.3. Tissue distribution and expression profiles of Mj*Cru I-1* analyzed by quantitative real-time PCR

The tissue distribution of *MjCru I-1* in the hemocytes, heart, hepatopancreas, gills, stomach, and intestine was analyzed using quantitative real-time PCR (qRT-PCR) with the primers RT-F and RT-R of *MjCru I-1* (Table 1). *β-actin* was used as the control with the primers Actin F and Actin R (Table 1). The qRT-PCR was programmed at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, and 60 °C for 60 s. The plate was read at 78 °C for each cycle. The final product was analyzed through melting curve analysis from 65 °C to 95 °C.

The expression profiles of *MjCru I-1* were detected in the hemocytes of shrimp challenged with *V. anguillarum* or *S. aureus*. Mock challenge of the shrimp with PBS was used as the control. The experiments were repeated at least three times using individual templates. The relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The unpaired sample *t*-test was used for statistic analysis, and the significant difference was accepted at $p < 0.05$.

Table 1
Sequences of primers used in this study.

Primers	Sequence(5′–3′)
*Mj*Cru -RT-F	TGCTCAGAACTCCCTCCACC
*Mj*Cru -RT-R	TTGAATCAGCCCATCGTCG
*Mj*Cru -ExF	TACTCAGAATTCACCATGATGATCCGCCTG
*Mj*Cru -ExR	TACTCACTCGAGTCAGTGTGGGAACTGAGC
Actin F	CAGCCTTCCTTCCTGGGTATGG
Actin R	GAGGGAGCGAGGGCAGTGATT
*Mj*Cru -Fi	GCGTAATACGACTCACTATAGGTGCCGATCCTCGCTCACTCG
*Mj*Cru -Ri	GCGTAATACGACTCACTATAGGTCAGTGTGGGAACTGAG
GFP-Fi	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGGAAC
GFP-Ri	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC

2.4. Recombinant expression, purification, and anti-serum preparation of Mj*Cru I-1*

The *Mj*Cru I-1 with a His-tag was recombinantly expressed in *Escherichia coli*. *MjCru I-1* was amplified from the hemocytes using the primers ExF and ExR (Table 1). The PCR procedure was as follows: 1 cycle at 95 °C for 3 min; 35 cycles at 94 °C for 30 s, at 55 °C for 45 s, and at 72 °C for 45 s; and 1 cycle at 72 °C for 10 min. The PCR products were inserted into the pET30a(+) vector (Novagen). The pET30a(+)-*MjCru I-1* expression vector was transformed into competent *E. coli* BL21 (DE3). Isopropyl thiogalactoside was added to a final concentration of 0.5 mM to induce protein expression at 37 °C for 4 h. The *Mj*Cru I-1 recombinant protein with a His-tag (expressed as inclusion body) was purified according to the previously described method (Du et al., 2006). The anti-serum preparation of *Mj*Cru I-1 using New Zealand rabbits was performed as previously described (Du et al., 2007).

2.5. Western blot analysis of Mj*Cru I-1*

The cytoplasmic proteins of the stomach were obtained by following the instruction of Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce, Thermo) and via Western blot analysis. After 12.5% SDS–polyacrylamide gel electrophoresis (Laemmli, 1970), the tissue proteins were transferred onto the nitrocellulose membrane. The membrane was blocked for 1 h with 3% nonfat milk in Tris-buffered saline (TBS) (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) and incubated with 1/100 diluted *Mj*Cru I-1 anti-serum in TBS with 3% nonfat milk for 2 h. The alkaline phosphatase-conjugated goat anti-rabbit IgG (1/10,000 diluted in TBS) was added. After incubation with the membrane for 2 h, unbound IgG was washed out. The membrane was dipped into the reaction system (10 mL of TBS, 45 μL of NBT, and 35 μL of BCIP) in the dark for 5 min to visualize the target protein.

2.6. Immunocytochemical assay

The hemolymph obtained from shrimp was fixed with 1 mL of mixture containing an anti-coagulant (pH 7.4) and 4% paraformaldehyde and then centrifuged with 600 *g* for 10 min at 4 °C. Collected hemocytes were resuspended with PBS and then incubated in 0.2% Triton X-100 at 37 °C for 5 min. The hemocytes were washed with PBS, blocked with 3% bovine serum albumin (BSA) at 37 °C for 30 min, and then incubated with anti-*Mj*Cru I-1 (1:400 in 3% BSA) at 4 °C overnight. After incubation, the hemocytes were washed with PBS and subsequently incubated with the second antibody of goat anti-rabbit-IgG H&L (Alexa Fluor 488) (1:1000, diluted in 3% BSA) in the dark at 37 °C for 1 h. The hemocytes were washed three times, stained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI, AnaSpec, San Jose, USA; 1 μg/mL in PBS) for 10 min at room temperature, and washed again six times. The fluorescence of the hemocytes was observed under the Olympus BX51 fluorescence microscope.

2.7. Binding of recombinant Mj*Cru I-1 to microorganisms and bacterial cell wall components*

Gram-negative bacteria (*E. coli, V. anguillarum*) and gram-positive bacteria (*S. aureus*, *Bacillus subtilis*) were used to detect r*Mj*Cru I-1 binding activity to microorganisms. The bacteria were cultured in 3 mL of Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) and then gathered by centrifugation at 6000 *g* for 8 min. After washing two times with TBS, the bacteria were resuspended in TBS to the OD_{600} of 1.0. The bacteria (500 μL) in TBS were incubated with purified r*Mj*Cru I-1(100 μg) for 20 min at 25 °C with rotation, collected by centrifugation, and washed four