Fish & Shellfish Immunology 43 (2015) 387–395

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Four crustins involved in antibacterial responses in *Marsupenaeus japonicus*

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ARTICLE INFO

Article history:
Received 25 August 2014
Received in revised form
1 January 2015
Accepted 2 January 2015
Available online 9 January 2015

Keywords:
Marsupenaeus japonicus
Antibacterial peptide
Crustin
Innate immunity

ABSTRACT

Crustins are a family of cationic, cysteine-rich antimicrobial peptides with a whey acidic protein (WAP) domain in the C-terminal. They have diverse functions in antimicrobial immune responses. Four groups of crustins (crustins I, II, III, and IV) have been identified in crustaceans, but type I crustins have not been reported in penaeid shrimp until now. In this study, we identified four crustins in kuruma shrimp *Marsupenaeus japonicus*, and named them *Mj*Crus I-2, 3, 4 and 5. These four crustins belong to type I crustins, which contain a signal peptide, cysteine-rich region at the N-terminus, and WAP domain at the C-terminus. Tissue distribution demonstrated that *Mj*Crus I-2, 3 and 5 had high expression levels in hemocytes, gills and stomach. whereas *Mj*Crus I-4 was distributed in all tissues detected. *Mj*Crus I-2 to 5 showed different expression patterns in different tissues after Gram-positive bacterial (*Staphylococcus aureus*), Gram-negative bacterial (*Vibrio anguillarum*), and white spot syndrome virus (WSSV) challenge. The expression of *Mj*Crus I-2 to 5 was upregulated by bacterial or WSSV challenge. The three crustins were recombinantly expressed in *Escherichia coli*, and the purified proteins showed few antimicrobial activities. Three *Mj*Crus Is could bind to different bacteria. *Mj*Crus I-2 and 3 showed different inhibitory abilities to secreted bacterial proteases. *Mj*Crus I-4 could not inhibit bacterial proteases. After knockdown of *Mj*Crus I-3, the bacterial scavenging ability to *V. anguillarum* was impaired. These results suggested that type I crustins played an important role in the innate immunity of shrimp.

1. Introduction

Antimicrobial peptides (AMPs) are the most important players of humoral immunity in innate immunity. AMPs are almost ubiquitous in the entire biological world. As of this writing, 20 antimicrobial peptides in *Drosophila* have been found; they can be divided into seven groups, namely, diptericin, attacin, drosocin, cecropin, defensin, drosomycin, and metchnikowin [1]. In crustaceans, many types of AMPs have been discovered, including penaeidins [2–4], crustins [5–7], antilipopolysaccharide factors [8–10], lysozymes [11,12], and astacidin [13,14]. Moreover, several reviews about the classification and functions of AMPs in shrimp have been published [15,16].

The crustin family has similar structural characteristics: a signal peptide and multi-domain region (glycine-rich, proline-rich, or cysteine-rich) at the N-terminus, and a whey acidic protein (WAP) domain at the C-terminus. Three types of crustins were divided by Smith et al [6]. Type I crustins have variable sequences between the signal peptide and WAP domain; this region contains a cysteine-rich region, and such crustins mainly exist in crabs, lobsters, and crayfish [17–19]. Type II crustins possess not only a cysteine-rich region but a glycine-rich region between the signal peptide and WAP domain. The glycine-rich region contains 40 to 80 amino acid residues, and the number of glycine residues varies in different species; type II crustins mainly exist in shrimp [7]. Type III crustins, also called single WAP domain-containing peptides (SWDs), contain a short proline and/or arginine-rich region between the signal peptide and WAP domain. They have been discovered in many shrimp, such as black tiger shrimp [20], kuruma shrimp [21], and Chinese white shrimp [22].

Crustins have various biological activities, including antimicrobial activity, protease inhibitory activity, and immune regulatory activity in recovery from wounding, trauma, or physiological stress [6]. For example, *Fc*SWD from Chinese white shrimp exhibits dual functions of antimicrobial and anti-protease activities [22]. Most

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http://dx.doi.org/10.1016/j.fsi.2015.01.001
1050-4648/

crustins in crustaceans can kill Gram-positive bacteria [6,7,23]. As of this writing, two types of crustins, type II and type III, have been discovered in penaeid shrimp. Type I crustins were reported only in crabs, lobsters, and crayfish.

We identified four crustins in kuruma shrimp *Marsupenaeus japonicus*, and designated them as *Mj*Crus I-2 to 5. These four crustins were type I crustins, which contain a signal peptide, cysteine-rich domain with two disulfide bridges at the N-terminus, and single WAP domain at the C-terminus. The distribution, expression patterns, and functions of the crustins were analyzed in this study.

2. Materials and methods

2.1. Immune challenge and tissue collection

Marsupenaeus japonicus (10 g–20 g/shrimp) were purchased from a seafood market in Jinan City, Shandong Province, China. They were cultured in an aquarium with seawater in the laboratory. After acclimation for 48 h, shrimp were divided into five groups. In bacterial challenge groups, each shrimp was injected with 2×10^7 CFU of *Vibrio anguillarum* or *Staphylococcus aureus* into the abdomen. For viral challenge, each shrimp was infected with 3.2×10^7 copies of WSSV [24]. In the control group, the same volume of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) was injected into each shrimp. The shrimp without any treatment were used as the blank control group. Tissues, including the heart, hepatopancreas, gill, stomach, and intestine, were collected from the shrimp of five groups at 2, 6, 12, 24, 36, and 48 h post-injection for total RNA and protein extraction. Hemocytes were collected following a previously reported method [25].

2.2. Sequence analysis

The full-length sequences of four crustins were obtained by hemocyte and gill transcriptome analysis. Sequence homology was analyzed using the online program BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). cDNA sequences were translated with the online software ExPASy-translation tool (http://www.au.expasy.org/). SMART (http://www.smart.embl-heidelberg.de/) was used for analysis of signal peptides and functional domains. ClustalW and GENDOC were used for sequence alignment. MEGA 6 was used to construct the phylogenetic tree.

2.3. Quantitative real-time PCR

The tissue distributions of four crustins were analyzed by quantitative RT-PCR with the following primer pairs: *Mj*Crus I-2 RT-F/RT-R, *Mj*Crus I-3 RT-F/RT-R, *Mj*Crus I-4 RT-F/RT-R, and *Mj*Crus I-5 RT-F/RT-R (Table 1). qRT-PCR was performed as follows: 10 min at 95 °C; 40 cycles of 95 °C for 15 s, 60 °C for 50 s, and read at 72 °C for 2 s, followed by melting curve analysis from 65 °C to 95 °C. The gene *EF1α* was used as a control with the primers *EF1α*F and *EF1α*R (Table 1).

The time course expression patterns of four *Mj*Crus after *V. anguillarum*, *S. aureus*, and WSSV challenge were analyzed by qRT-PCR with the same primer pairs. EF1α was used as the internal control. All samples were repeated in triplicate for PCR analysis. The qRT-PCR data were calculated by $2^{-\Delta\Delta CT}$. The figures were constructed using GraphPad Prism software. Significant differences between groups after the same challenge were calculated using one-way ANOVA followed by Tukey's multiple comparison test.

2.4. Expression and purification of recombinant peptides of three crustins

Three pairs of primers, namely, *Mj*Crus I-2 EX-F/EX-R, *Mj*Crus I-3 EX-F/EX-R, and *Mj*Crus I-4 EX-F/EX-R (Table 1), were used to amplify the sequences encoding the mature peptide of the three crustins. The amplified three fragments and pET30a(+) or pGEX-4T-1 were then digested with *Bam* HI and *Xho*I. T_4 DNA ligase was used to link the fragments and plasmid for 16 h at 16 °C. The recombinant plasmids were transformed into competent *Escherichia coli* BL21-DE3 or BL21 cells to determine the expression of recombinant peptides.

The recombinant proteins of *Mj*Crus I-2 and 4 were successfully expressed from *E. coli* BL21-DE3 with pET30a/*Mj*Crus I-2 and 3 induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). *Mj*Crus I-4 were expressed from *E. coli* with pGEX-4T-1/*Mj*Crus I-4 after 0.5 mM IPTG induction. Recombinant *Mj*Crus I-2 and 3 were purified by high-affinity Ni-IDA resin following the manufacturer's instructions. *Mj*Crus I-4 was purified using high-affinity GST resin following the manufacturer's instructions. The concentrations of recombinant proteins were detected by the Bradford method [26].

2.5. Protease inhibitory activity of MjCrus against secreted bacterial proteases

Gram-positive bacteria *Bacillus megaterium*, *S. aureus*, and *Bacillus subtilisin*, and Gram-negative bacteria *E. coli*, *V. anguillarum*,

Table 1
Sequences of primers used in this study.

Primer	Sequence (5′–3′)	Direction
Crus 2 RT-F	GCGTTTTCGTCTTCGTCCTG	Forward
Crus 2 RT-R	AGTCCTTTCCGCCGTCACA	Reverse
Crus 3 RT-F	CTCCACCACTCTCGCACTAACA	Forward
Crus 3 RT-R	TGATGGTCTCAGATTGGGGC	Reverse
Crus 4 RT-F	TACTGTTGGCAGCCGTGTCT	Forward
Crus 4 RT-R	GGTTGAATCTGGGTTTGAGGA	Reverse
Crus 5 RT-F	ATCGGCAAACCCGCAGTCTCTCT	Forward
Crus 5 RT-R	CCGCTCTTCGTCGCAGCAGTAATAGT	Reverse
Crus 2 EX-F	TCATACGGATCCTGCCGCTATTATTGCATCAA	Forward
Crus 2 EX-R	TCATACCTCGAGCTATTTTCCCTGGTTCGCCT	Reverse
Crus 3 EX-F	TACTCAGGATCCCTGACGACGAGTGTCCTTCC	Forward
Crus 3 EX-R	TACTCACTCGAGTTAATTATATTCAGCTGGCT	Reverse
Crus 4 EX-F	TACTCAGGATCCCAACGCTGGTGTAATCACGA	Forward
Crus 4 EX-R	TACTCAGAATTCCTACACACGCCGACAGATCC	Reverse
Crus 3 RNAi-F	TAATACGACTCACTATAGGGCATCGTCTCAGCATCCTCATC	Forward
*Crus*3 RNAi-R	TAATACGACTCACTATAGGGACCAGGCAATAAATACCAACATA	Reverse
GFP RNAi-F	TAATACGACTCACTATAGGGGGGTGGTCCCAATTCTCGTGGAAC	Forward
GFP RNAi-R	TAATACGACTCACTATAGGGCTTGTACAGCTCGTCCATGC	Reverse
EF1αF	GGATTGCCACACCGCTCACA	Forward
EF1αR	CACAGCCACCGTTTGCTTCAT	Reverse