Fish & Shellfish Immunology 48 (2016) 30–38

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Characterisation of a novel Type I crustin involved in antibacterial and antifungal responses in the red claw crayfish, *Cherax quadricarinatus*

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ARTICLE INFO

Article history:
Received 2 July 2015
Received in revised form
9 October 2015
Accepted 8 November 2015
Available online 14 November 2015

Keywords:
Crustin
Innate immunity
Pathogen-associated molecular patterns (PAMPs)
Cherax quadricarinatus

ABSTRACT

Antimicrobial peptides are important immune effectors involved in mediating innate immune responses against intruding pathogens. Here, we successfully isolated and characterized a novel Type I crustin from the red claw crayfish *Cherax quadricarinatus*. The full-length cDNA encoded by this gene, designated *CqCrs*, comprised 608 bp, containing a 5′-untranslated region (UTR) of 55 bp, a 3′-UTR of 229 bp with a poly (A) tail, and an open reading frame (ORF) of 324 bp encoding a polypeptide of 107 amino acids. The deduced amino acid sequence of *Cq*Crs exhibited a configuration typical of other crustacean Type I crustin orthologs, including one signal peptide region at the N-terminus between residues 1 and 16 and a long whey acidic protein (WAP) domain at the C-terminus between residues 60 and 107, along with a WAP-type "four-disulfide core" motif. Phylogenetic analysis showed that CqCrs was clustered first with other crustacean Type I crustins, then with other crustacean Type II crustins, and finally with other crustacean Type III crustins. Transcription of *CqCrs* was detected in all tissues, especially in immune tissues and was differentially induced in hemocytes post-stimulation with β-1, 3-glucan, lipopolysaccharides (LPS) and peptidoglycans (PG) at selected time-points. To clarify the biological activity of *Cq*Crs, the recombinant *Cq*Crs protein (r*Cq*Crs) was constructed and expressed in *Escherichia coli* BL21 (DE3). Purified r*Cq*Crs bound to diverse bacteria and inhibited the growth of different microbes to varying degrees. These findings suggest that *Cq*Crs is involved in a specific innate immune recognition and defense mechanisms against bacterial and fungal in *C. quadricarinatus*.

1. Introduction

The response of crustaceans to pathogens is believed to depend solely on innate, non-adaptive immune mechanisms [1], including phagocytosis, encapsulation, clotting, and a variety of soluble AMPs [2]. AMPs have been characterized primarily from insects but have also been isolated from crustaceans [2]. In the crustacean immune system, secreted AMPs constitute an important component of the humoral immune defense [3]. A group of WAP domain-containing AMPs, collectively termed the crustins [4] have the potential capability to normalize and/or destroy invading microbial pathogens and enhance immunity by functioning as immunomodulators [5,6] in the crustacean groups. Crustins may also be germline-encoded pattern recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharides (LPS) from Gram-negative bacteria, peptidoglycans (PG) from Gram-positive bacteria, double-stranded RNA (dsRNA) and β-1, 3-glucans from fungi [7]. Recently, increasing numbers of crustin orthologs have been found to play essential roles in non-specific host defense by preventing or limiting infections and selectively recognizing potential pathogens in crustaceans. Additionally, crustins are implicated as effective candidates for the development of novel antibiotics due to their antibacterial activity profile *in vivo* [8].

The antimicrobial activity of crustins against Gram-negative, Gram-positive, fungal, some viral and protozoan pathogens is well-established in crustacean organisms [8,9]. The first crustin was successfully isolated from the shore crab *Carcinus maenas* as a cysteine-rich antibacterial protein [10]. Subsequently, increasing numbers of crustin or crustin-like proteins have been characterized from diverse crustaceans, including crabs, shrimps and crayfish

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http://dx.doi.org/10.1016/j.fsi.2015.11.019
1050-4648/

[11]. All the reported crustin proteins can be divided into three types (Type I, Type II and Type III), designated by Smith et al. [12]. Type I crustin proteins contain a cysteine-rich region between the signal peptide and the WAP domain, which are mostly present in crabs, lobsters and crayfish [13,14]. Type II crustins contain a signal peptide region followed by a glycine-rich profile, a cysteine-rich profile and a WAP domain [15]. Type III crustins lack the cysteine-rich domain and glycine-rich domain, and instead possess a proline-arginine-rich region between the signal peptide and the WAP domain [12].

To date, many crustin proteins have been successfully characterized at the molecular and protein levels from various crustacean groups [13,14]; however, molecular characterization of crustins from *Cherax quadricarinatus* has not yet been reported. In the present study, the full-length cDNA of *CqCrs* was identified and characterized. The domain architecture of the *Cq*Crs protein was determined, and a phylogenetic comparison was made among crustaceans. The results showed that *CqCrs* can be induced differentially by diverse PAMPs, and the recombinant protein r*Cq*Crs binds mediates antimicrobial activity by binding directly to bacteria and fungus. These observations indicate crucial roles for the *Cq*Crs protein in the specific innate immune recognition and defense mechanisms of *C. quadricarinatus*.

2. Materials and methods

2.1. In vivo immune challenge and sample collection

Healthy red claw crayfish (n = 200; 90 ± 20 g wet weight) were collected from the Jin-Shan Aquafarm in Shanghai, China. After acclimation for one week at 23 ± 3 °C in filtered, aerated freshwater, crayfish were placed in an ice bath for 1–2 min until each was fully anesthetized. Hemolymph (approximately 2.0 ml per crayfish) was drawn from the hemocoel at the base of the fourth walking legs using a syringe and added to an equal volume of anticoagulant solution [16] (0.1 M glucose, 30 mM citrate, 26 mM citric acid, 0.14 M NaCl and 10 mM EDTA in 100 ml double-distilled water). The samples were then centrifuged for 30 min at 500 × *g* at 4 °C to isolate hemocytes. Other tissues (hepatopancreas, gill, muscle, stomach, intestine, testis, ovary, abdominal nerve, eye, brain and heart) were harvested, snap-frozen in liquid nitrogen, and stored at −80 °C prior to nucleic acid analysis. For cloning and subsequent in-depth analysis, each type of tissue from five crayfish were pooled, and ground with a pestle and mortar prior to extraction.

For stimulation by PAMPs, 140 crayfish were divided equally into four groups (sex ratio 1:1). The three experimental crayfish groups were injected into the base of the fourth walking legs with approximately 100 μl of LPS from *Escherichia coli* (Sigma–Aldrich, St. Louis, MO, USA), 100 μl of PG from *Staphylococcus aureus* (Sigma–Aldrich) and 100 μl of zymosan (β-1,3-glucan from *Saccharomyces cerevisiae*, Sigma–Aldrich) resuspended (500 μg/ml) in *C. quadricarinatus* saline (CQS, 0.2 M NaCl, 5.4 mM KCl, 10.0 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2.0 mM $NaHCO_3$; pH 7.4) [16–18]. The control group received 100 μl CQS (pH 7.4) in the same manner. Five crayfish were randomly selected at each time interval: 0 (as blank control), 3, 6, 12, 24, 36 and 48 h after administration of each type of PAMP. Hemocytes were harvested as described and stored at −80 °C after the addition of 1 ml Trizol® reagent (Invitrogen, Carlsbad, CA, USA) for subsequent RNA extraction.

2.2. Total RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from various tissues sampled as described in Section 2.1 using Trizol® reagent (RNA Extraction Kit, Invitrogen) according to the manufacturer's protocol. Following treatment with RNase-Free DNase I (Tiangen, China), the total RNA concentration and quality were estimated spectrophotometrically (absorbance at 260 nm) and by agarose gel electrophoresis, respectively.

Total RNA (5 μg) isolated from hemocytes was reverse-transcribed using the SMARTer™ RACE cDNA Amplification kit (Clontech, Mountain View, CA, USA) for cDNA cloning. For quantitative real-time RT-PCR (qRT-PCR) expression analysis, total RNA (4 μg) was reverse-transcribed using the PrimeScript™ Real-time PCR Kit (Takara, Shiga, Japan).

2.3. Cloning of full length CqCrs cDNA

The degenerate primers (**Crs-F and Crs-R**) (Table 1) were designed based on the conserved region of insect crustins to obtain partial sequence of *Cq*-Crs. The resulting PCR product was purified and cloned into a TOP10 vector (Tiangen) and sequenced. A partial cDNA sequence that obtained was extended using 5′- and 3′-RACE (SMARTer™ RACE cDNA Amplification kit, Clontech). The gene-specific primers (Table 1) were designed based on the obtained sequence. The 3′-RACE PCR reaction was carried out in a total volume of 50 μl containing 2.5 μl (800 ng/μl) of the first-strand cDNA reaction as the template, 5 μl 10 × Advantage 2 × PCR buffer, 1 μl 10 mM dNTPs, 5 μl (10 μM) gene-specific primers (Table 1), 1 μl Universal Primer A Mix (UPM; Clontech, USA), 34.5 μl sterile deionized water, and 1 U 50 × Advantage 2 polymerase mix (Clontech, USA). For the 5′-RACE, UPM was used as the forward primer in PCR reactions in conjunction with the reverse gene-specific primers (Table 1). PCR amplification conditions for both the 3′- and 5′-RACE reactions were as follows: 5 cycles at 94 °C for 30 s, 72 °C for 3 min; 5 cycles at 94 °C for 30 s, 70 °C for 30 s, and 72 °C for 3 min; 20 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. PCR amplicons were separated on the basis of size by

Table 1
PCR primer sequences used for *CqCrs* analysis.

Primer name	Sequences(5′→3′)
Degenerate primers	
CqCrs -DP-F	CCYGARYCCWAAGGGC
CqCrs -DP-R	AAGTGWGCWGGCTTGCAGGT
5′-RACE	
CqCrs-5′-1	TTCCTTGAGCGGAGGTGGG
CqCrs-5′-2	CTACCAGGGTGTTTCTGTTCT
CqCrs-5′-3	TTCCTTGAGCGGAGGTGGG
3′-RACE	
CqCrs-3′-1	TGTAGTCCACCTCACGTTCCA
CqCrs-3′-2	ACTGCTGTAGTCCACCTCACG
CqCrs-3′-3	TGCTGTAGTCCACCTCACG
UPM-Long	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UPM-Short	CTAATACGACTCACTATAGGGC
qRT-PCR	
CqCrs-RT-F	CTATGGTAGTGGTGGTTGTGG
CqCrs-RT-R	CCTCTGGTGCATTCCTTGA
β-actin-RT-F	CTGTGGTGGTGAAGGAGTAGCC
β-actin-RT-R	TACCATCCAGGCTGTGCTCTCC
GAPDH-RT-F	CGCATCGGTCGCCTTGTT
GAPDH-RT-R	CCATCCTCTTCCTTCACCTCCC
Recombinant expression	
pET32a-CqCrs-F	CCGGAATTCCCAGAAGGGCCCAACAAGGG
pET32a-CqCrs-R	CCGCTCGAGTTATCAGTATGCAGGCTTGCAGGTATG
Sequencing	
Mer-23	CGACTCACTATAGGGAGAGCGGC
Mer-24	AAGAACATCGATTTTCCATGGCAG
T7-Promotor	TAATACGACTCACTATAG
T7-Terminator	GCTAGTTATTGCTCAGCGG

Note: R = A/G,W = A/C/T, Y = C/T.