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Molecular cloning, genomic organization and recombinant expression of a crustin-like antimicrobial peptide from black tiger shrimp *Penaeus monodon*

Piti Amparyup[a,b], Hidehiro Kondo[c], Ikuo Hirono[c], Takashi Aoki[c], Anchalee Tassanakajon[b,*]

[a] *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Paholyothin Rd., Klong1, Klong Luang, Pathumthani 12120, Thailand*
[b] *Shrimp Molecular Biology and Genomics Laboratory, Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand*
[c] *Laboratory of Genome Science, Tokyo University of Marine Science and Technology, Konan 4-5-7, Minato-ku 108-8477, Japan*

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Abstract

A novel crustin-like antimicrobial peptide (Crus-like*Pm*) was identified from haemocytes of *Penaeus monodon*. The deduced amino acid sequence of a Crus-like*Pm* consists of 124 amino acid residues of the mature peptide and a signal peptide of 17 amino acid residues. The mature peptide contains a glycine-rich domain at the N-terminus and 12 conserved cysteine residues containing a single WAP domain at the C-terminus. Phylogenetic tree and sequence comparison clearly confirmed a distinct between a Crus-like*Pm* and other shrimp crustins. Genomic organization and upstream region of a Crus-like*Pm* gene was investigated. The gene consisted of two exons and one intron. The 5′-flanking regions of a Crus-like*Pm* gene contain multiple putative transcription factor binding sites. mRNA transcript of a Crus-like*Pm* was found to be abundantly expressed in haemocyte and highly up-regulated after *Vibrio harveyi* injection. The mature Crus-like*Pm* was cloned into the pET28b with an N-terminal hexa-histidine tag fused in-frame, and expressed in *E. coli*. The purified recombinant Crus-like*Pm* showed strong antimicrobial activity against both Gram-positive and Gram-negative bacteria including *V. harveyi*, a major pathogenic bacteria in shrimp aquaculture.

Keywords: Shrimp; *Penaeus monodon*; Crustin; Whey acidic protein; Antimicrobial peptide; Genomic organization

1. Introduction

Antimicrobial peptides (AMPs) are generally small cationic molecules widely distributed in the whole living kingdom, and they are thought to be essential for organisms lacking adaptive immunity. During the past several years, a variety of AMP families were characterized from shrimps. Penaeidins are 5.5–6.6 kDa peptides with an N-terminal proline-rich domain and a C-terminal domain containing six cysteine residues and have antimicrobial activity against Gram-positive bacteria and fungi (Destoumieux et al., 1997). Antilipopolysaccharide factors (ALFs), originally identified from the amebocyte of the horseshoe crab *Limulus polyphemus* (Tanaka et al., 1982), are small basic protein, which binds and neutralizes lipopolysacharide (LPS) and have strong antimicrobial effects on Gram-negative bacteria (Somboonwiwat et al., 2005; Imjongjirak et al., 2007).

Crustins are homologues of carcinin that was first isolated from the shore crab *Carcinus maenas* and characterized as a cysteine-rich 11.5 kDa AMP with antimicrobial activity against Gram-positive bacteria (Relf et al., 1999; Brockton et al., 2007). The cDNA of crustins have been reported from a variety of crustaceans including *Litopenaeus vannamei* (Bartlett et al., 2002; Vargas-Albores et al., 2004), *L. setiferus* (Bartlett et al., 2002), *Penaeus monodon* (Supungul et al., 2007), *Marsupenaeus japonicus* (Rattanachai et al., 2004), *Fenneropenaeus chinensis* (Zhang et al., 2007a), *Panulirus argus* (Stoss et al., 2003), *Homarus gammarus* (Hauton et al., 2006), *C. maenas* (Brockton et al., 2007) and *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2007). Crustins that have been described to date are classified by diverse amino acid sequences with conservation in the C-

* Corresponding author. Tel.: +66 2 218 5439; fax: +66 2 218 5414.
E-mail address: anchalee.k@chula.ac.th (A. Tassanakajon).

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terminus of 12 cysteine residues including a single whey acidic protein (WAP) domain. The WAP domain generally consists of 50 amino acid residues with eight cysteine residues at defined positions. They form four intracellular disulfide bonds creating a tightly packed structure. The WAP domain-containing proteins are widespread throughout the Metazoan and have diverse biological functions such as the secretory leukocyte protease inhibitor (SLPI) and elafin which have both antibacterial activity and antiprotease activity (Sallenave, 2002) and mouse single WAP motif protein1 (SWAM1) and SWAM2, as antibacterial proteins (Hagiwara et al., 2003).

Previously, we reported different crustin-like cDNA sequences of haemocytes-EST library of *P. monodon*. Among these crustin-like sequences, a partial cDNA (accession no. BI784446) showing the lowest sequence identity to other crustins was also reported. Further study on the characterization of this crustin-like antimicrobial peptide (hereafter named Crus-like*Pm*) may lead to a better understanding of immune system in shrimp. In the present study, we described the molecular characterization of a full-length cDNA and genomic organization of a new Crus-like*Pm* gene from *P. monodon*. The expression profiles in various tissues and in response to infection with pathogenic bacteria were examined. In addition, the recombinant expression and the antimicrobial activity of a Crus-like*Pm* were also investigated.

2. Material and methods

2.1. RNA isolation and genomic DNA extraction

Haemolymph of shrimp was collected from the ventral sinus at 0–72 h after *Vibrio harveyi*-(10^6 CFU) and saline-injection in an anticoagulant solution of 10% (w/v) of trisodium citrate dihydrate. Haemocytes were isolated by centrifugation at 8000 rpm for 10 min at 4 °C. Total RNA from haemocytes was extracted using TRI REAGENT®. Genomic DNA was extracted from pleopod of individual shrimp by phenol-chloroform extraction method.

2.2. Rapid amplification of cDNA end (RACE)-PCR

The 5′-RACE reaction was performed with SMART RACE cDNA Amplification kit (Clontech, USA). The first strand 5′-RACE-ready cDNA samples from *P. monodon* were prepared according to the manufacturer's protocol and used as templates for RACE-PCR. Gene specific primers (HC72-R1 and HC72-R2; Table 1) were designed from EST sequence (accession number BI784446). The amplification reaction was performed as described in Amparyup et al. (2007). The RACE-PCR product was ligated to pGEM®-T Easy vector (Promega, USA). The recombinant plasmid DNA was extracted and sequenced (Macrogen Inc., Korea).

2.3. Isolation of genomic structure and promoter region of a Crus-likePm gene

The genomic structure of a Crus-like*Pm* gene was obtained by amplification of genomic DNA using the primers 5UTRC72-F and HC72-R1 (Table 1). PCR was performed with the BD Advantage™ Genomic PCR Kit (Clontech, USA). The expected DNA fragments of PCR product were cloned and sequenced. The upstream sequence of a Crus-like*Pm* gene was isolated by Universal Genomewalker Kit (Clontech, USA) following the manufacturer's instructions. First round PCR was performed using AP1 and the gene specific primer, Crus72-GWR1 (Table 1) using genomic DNA libraries as templates. Nested PCR was carried out with AP2 and the gene specific primer, Crus72-GWR2 using primary PCR products as templates. The PCR product was cloned and sequenced. The amplification of single fragment of a Crus-like*Pm* gene was carried out by PCR using primer C72g-F and HC72-R1 (Table 1). The PCR product was cloned and sequenced in both directions.

Table 1
Sequences of oligonucleotide primers used for amplification of a Crus-*likePm* gene

Primer	Sequence (5′–3′)
HC72-R1	AATTGATGAGTCGAACATGCAGGCCTAT
HC72-R2	CAGGCCTATCCCTGAGAACCTGCCA
HC72-F	CCCTGGAGGTCAATTCGAGTGC
5UTR72-F	CGTTCATCGCACAGCCGAGAGAGGA
Crus72-GWR1	TCCACCTAGGAAGCGAGTATCGGCATTG
Crus72-GWR2	CAGCCACAGCGACAACGGATAATACTAC
Crus72-F	CGGCAGGTGTCCACAGATTCG
Crus72-R	GCAGACGGTGTCGTTCAAGCA
C72g-F	TCCAGTCTAAAACGCGACAGGTGATCTC
EF1α-F	GGTGCTGGACAAGATGAAGGA
EF1α-R	CGTTCCGGTGATCATGTTCTTGATG
NcoI-C72-F	CATGCCATGGGCCATCATCATCATCATCATA TGGATACTCGCTTCCTAGGTGGAGTTGGA
NotI-C72-R	ATAAGAATGCGGCCGCCTATCCCTGAGAACC TGCCACGATG

2.4. Real-time RT-PCR analysis

A real-time RT-PCR analysis was performed on iCycler-iQ™ system (Bio-Rad Laboratories, USA) by SYBR Green I dye detection. The amplification and thermal profile for SYBR Green real-time RT-PCR was performed using HC72-F and HC72-R1 primers as described in Amparyup et al. (2007). The relative quantification analyzes the amount of target transcript relative to an internal standard, elongation factor 1-alpha gene (EF-1α) in the same sample of *V. harveyi*-injected shrimp haemocytes. The Ct values of *V. harveyi* injected sample at each time point were normalized with saline-injected samples. A mathematical model was used to determine the relative expression ratio (Pfaffl, 2001).

2.5. Tissue specific expression of a Crus-likePm

Expression of a Crus-like*Pm* in different tissues was measured by RT-PCR. Total RNA from the tissues of haemocytes, hepatopancreas, lymphoids, gills, intestines and hearts was extracted from *P. monodon*. The specific primers (Crus72-F and Crus72-R) were designed (Table 1). The EF1α was used