Anti-*Vibrio* Response of Carcinin*Pm* 1 from *Penaeus monodon* and Its Heterologous Expression

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Abstract. Crustins are crucial antimicrobial peptides in shrimp and play very important roles in innate immunity. In this research, a Type I crustin from *Penaeus monodon* (Carcinin*Pm*1) contained 108 residues was studied. The first 16 residues are signal peptide. It contained ten cysteines but did not form an intact whey acidic protein (WAP) domain. Carcinin*Pm*1 was observed to widely distribute in all tissues, while highly expressed in intestine. The expression level of Carcinin*Pm*1 in hepatopancreas was up-regulated 12-20 times during 4-12h post challenged by *Vibrio parahaemolyticus*. And the transcription in heart, stomach and gills was also significantly enhanced at 4h post challenge. The mature peptide was expressed successfully in *Eschericha coli* by fusing to a SUMO protein, with protein production around 8 mg/mL. After cleavage with SUMO protease, carcinin*Pm*1 was obtained indicating its potential applications.

1 Introduction

Antimicrobial peptides (AMPs) are small and active peptides widely distributed in various organisms, as an important part of innate immunity of organisms and exhibited broad-spectrum of antimicrobial activities against variety of bacteria, virus and cancer cells [1].

Penaeus monodon, also called black tiger shrimp, is the second most widely cultured shrimp species in the world. However, the outbreak of diseases seriously reduced the production and economic benefit, especially infections caused by *Vibrios*[2] or viruses [3]. As invertebrates do not have acquired immune system, they depend on innate immunity to defend invading microbes[4]. Innate immunity includes humoral and cellular immunity. Antimicrobial peptides (AMP) are important humoral immunity molecules, as they can kill invading microbes directly and/or regulate other immune response[5,6]. Several types of AMPs were identified in shrimps, including crustins, penaeidins, anti-lipopolysaccharide factors (ALFs), stylicins, haemocyanin-derived peptides, lysozymes and histones and derived fragments [6–8].

Crustin is one of the largest families of AMPs in invertebrate. There are in total four types of crustins in crustaceans [9]. Type I crustins are constituted by a signal peptide, a cysteine-rich domain with two disulfide bridges and a single WAP (whey acidic protein) domain at the C-terminus. Compared to Type I crustins, the type II crustins contain a long glycine-rich region to the N-terminus of the cysteine-rich domain. Type III crustins, also called single WAP domain (SWD) containing proteins, only contain a short proline and arginine-rich region in front of the WAP domain [10,11]. The type IV crustins (DWD crustins) have two WAP domains [11].

So far, four types of crustins have been found in *P. monodon*. Only two Type I crustins have been mentioned, while at least ten kinds of Type II crustins were discovered [12]. CrustinPm1 and crustinPm5 exhibit antimicrobial activity only against Gram-positive bacteria, while crustinPm7 is active against both Gram-positive and Gram-negative bacteria [13–15]. Type III crustin from *P. monodon* exhibits anti-Gram-positive, but not anti-Gram-negative bacteria activity and it is a competitive inhibitor of subtilisin A [16]. However, the DWD crustins do not show apparent activity [17].

Type I crustins are present mainly in crabs[18,19] but also found in crayfish[20–22] and shrimps [23–25]. Some shrimps contain more than one isoforms of Type I crustins. The first Type I crustin was isolated in 1999, from the granular haemocytes of the shore crab, *Carcinus maenas* and named carcinin *Cm*1. It was heat stable and active only against Gram-positive bacteria [26]. There are in total five type I crustins identified in *Marsupenaeus japonicas* [24-25].

The type I crustin Carcinin*Pm*1, was first reported by Suchao Donpudsa, *et.al.*[12]. However, it was not fully characterized. In this research, further studies were performed to reveal its sequence similarity with other type I crustin, the tissue distribution and expression trend during *Vibrio* infection. All the results suggest that it could play a very important role in the innate immunity system of *P. monodon*.

2 Materials and Methods

2.1 Bioinformatics analysis of Carcinin*Pm*1

The nucleotide sequence of Carcinin*Pm*1 was obtained from transcriptome sequencing data of the hepatopancreas of *Penaeus monodon*. The Open Reading Frame and amino acid sequence of Carcinin*Pm*1 were deduced by ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).The signal peptide was predicted with signalP 4.0 server. DNAman version 6 was used for generating gene structure information. Homologous sequences of Carcinin*Pm*1 were obtained by Basic Local Alignment Search Tool (BLASTP)and multisequences alignment was performed with clustalw (https://www.genome.jp/tools-bin/clustalw). The online software ESPript 3.0 was used to generate the alignment result [27]. The physicochemical properties were predicted with the online software Protparam (http://web.expasy.org/protparam/).

2.2 Immune challenging of shrimps and tissues collection

Individual shrimps (about 16 cm in length) were collected from a prawn breeding base (Dapeng, Shenzhen), and cultured in 50 L glass boxes, each containing 10 L of filtered seawater at 25±1℃ for one day before experiments.

Vibrio parahaemolyticus (SIV) was cultured with Luria-Bertani broth containing 3% NaCl shaken at 30℃. The overnight cultured bacteria were inoculated to a fresh media and cultured until OD540 reached 0.3 (about 5×10^7 CFU/ml). Cells were harvested, washed twice with sterilized PBS and then resuspended with PBS to a final concentration of 1×10^7 CFU/ml.

The shrimps were divided into two groups, Group P and Group V. Each group contained at least 20 individuals. For shrimps in group V, 100μl suspended bacteria were injected into the ventral blood sinusand shrimps in group P were injected with 100μl PBS as a control.Hepatopancreas of Group P and Group V shrimps were collected at 0, 2, 4, 8, 12 and 24 h post injection. Heart, gills, stomach, intestine and hemocytes were collected from unchallenged and 4h post-challenged shrimps.

To collect hemocytes, at least 600μl hemolymph was drawn out from pericardial sinus of shrimps with a 1 ml syringe preloaded 100μl anticoagulant (0.1M Sodium Citrate, 0.25M sucrose, 0.01M Tris-HCl, pH 7.6) and centrifuged at 830g, 4°C for 10 min. The collected cells were washed with 1ml anticoagulant and then suspended in 600μl lysis buffer to proceed with the subsequent total RNA extraction.

2.3Total RNAs extraction and cDNAs synthesis

Total RNAs were extracted from all the tissues prepared above using RNeasy Mini Kit (Qiagen, USA), according to the manufacturer's instruction. cDNAs were synthesized with PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan).

2.4Confirmation of the Carcinin*Pm*1 gene

Reverse-transcription PCR(RT-PCR) was performed with primers Carcinin*Pm*1 conF and Carcinin*Pm*1 conR (Table 1) to amplify the ORF of Carcinin*Pm*1. The PCR product was connected to a T-vector (pMD™18-T Vector Cloning Kit, Takara) and sequenced.

Table 1.Primers used in the present study.

Name	**Primer sequence** (5'-3')
Carcinin*Pm*1 conF	AACGAGTTCATCGTCAAGCAAATTC
Carcinin*Pm*1 conR	GCGCATCCGATTCCAAGTTG
Carcinin*Pm*1 F	GGTGCCGTCTTCTCCCAAAC
Carcinin*Pm*1 R	GGATGTCCAGCTCCCTCTGC
EF-1α F	GGACAGCACCGAGCCCAAG
EF-1α R	TGCTTCTTCCACCAGCCCATA

2.5Tissue distribution of Carcinin*Pm*1

Semi-quantitative real-time PCR was used to test the relative expression level of Carcinin*Pm*1 in different tissues of *P. monodon*. One pair of primers (Carcinin*Pm*1 F and Carcinin*Pm*1 R) was used and EF-1αwas used as a control. The cDNAs of six different tissues from unchallenged shrimps were used as templates. The PCR protocol is as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 15 s, and 72 °C for 10 min. The PCR product was checked by electrophoresis on a 2% agarose gel.

2.6Response of Carcinin*Pm*1 to SIV challenge

Quantitative real-time PCR was performed to check the expression trend of Carcinin*Pm*1 in hepatopancreas and other tissues after *Vibrio* challenge. The qRT-PCR was performed on ViiA7 Real-time PCR system (ABI), with SYBR Premix Ex Taq II (TaKaRa, Japan).The same primers and control gene as in 2.4 were used. The cDNAs from PBS or *V. parahaemolyticus* challenged shrimps were used as templates. The qRT-PCR was performed with 95 °C, 30s; 40 cycles of 95 °C, 5s and 60 °C, 34 s; and a melt from 60°C to 95 °C. The experiment was repeated three times with individual templates. $2^{-\Delta\Delta CT}$ method was used for expression profile analysis.

2.7Gene synthesis and vector construction

The mature peptide of Carcinin*Pm*1 was fused to the C-terminus of a SUMO protein with His-tag and

overexpressed in *Escherichia coli*BL21 (DE3). The nucleotide sequence of His-SUMO-Carcinin*Pm*1 with NdeI and SacI cut sites in both ends was codon optimized for *E. coli* and chemically synthesized (General Biosystems, Inc., Hefei, China). The synthesized DNA was then linked to pColdIV vector by NdeI and SacI cut sites. The reconstituted plasmid was transformed to *E. coli* BL21 Rosetta (DE3), named BL21-carpm1.

2.8 Protein expression and purification

Adding 20 mL of overnight cultivated BL21-carpm1 intothe fresh 2 L LB broth medium containing ampicillin (50 μg/mL) and then cultivating them at 37 °C with shaking at 200 rpm until the absorbance reached 0.5 at 600 nm. IPTG was added to the culture at a final concentration of 1 mM. The induction was performed at 16°C for 12 hours. Cells were harvested by centrifugation and the cell pelletswere washed and resuspended in PBS. The bacterial suspensions were then disrupted by ultrasonication. The supernatant (soluble fraction) was collected and analyzed by SDS-PAGE.

The fusion protein was purified by Ni-NTA Sepharose Fast Flow (Ruidahenghui, Beijing, China), and the his-tagged protein was eluted with buffer contained 50 mM PBS, 300 mM NaCl and 200 mM imidazole. Elutes were further analyzed by SDS-PAGE.

The eluted protein was further dialyzed against 50mM Tris-HCl, 200mM NaCl to remove the imidazole and then quantified by Bradford reagent (Sangon, China).100μg protein was mixed with 1U SUMO protease (General Biosystems, Inc., Hefei, China), and incubated at 4°C overnight to remove the SUMO tag.

3 Results

3.1 Sequence information of Carcinin*Pm*1

The nucleotide sequence of Carcinin*Pm*1 in this research was obtained from transcriptome data of hepatopancreas of *P. monodon*and was confirmed by RT-PCR. The deduced amino acid sequence is the same as the Carcinin*Pm*1 identified from EST database of *P. monodon*by Suchao Donpudsa, *et.al.*, and it was classified as Type I crustin [12].The *carcininPm1* gene is 327bp in length, encoding a 108-residues peptide and the first 16 residues constituted a signal peptide. The 92 residues mature peptide contained 10 cysteines in total (**Figure 1**). The first four cysteines were in the cysteine rich region and the rest six cysteines constituted an incomplete WAP domain. The molecular weight is 10.6kDa and the theoretical p*I* is 5.47.

3.2Alignment of CarcininPm1 with other Type I crustins

Carcinin*Pm*1 showed 70 % identity with crustinI-5 from *Penaeus japonicas* (ANA91277.1), and 33%-40% sequence alignment indicated that the ten cysteines and their flanking residues are conserved. Notably, dentity with the other four crustins in **Figure 2**. The Carcinin*Pm*1 and CrustinI-5 contained less cysteine residues in the mature peptide region compared to the other four crustins.

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1     AACGAGTTCATCGTCAAGCAAATTCTCCTCAAACAAAATGTTGCGTCTGTGCGTAATGTT
1                                           M  L  R  L  C  V  M  L

61    GGCTGTGGTCGGTGCCGTCTTCTCCCAAACGTACCCCGATCCGCTGGCGAATATTAACTG
21    A  V  V  G  A  V  F  S  Q  T  Y  P  D  P  L  A  N  I  N  C

121   CGAGAACTGGTGTAACCTTAGCAGTACTACCTATTACTGCTGCGACGAACATCGTGAAAT
41    E  N  W  C  N  L  S  S  T  T  Y  Y  C  C  D  E  H  R  E  I

181   TGATGGAGGGCGCTCCGGCAAGTGCCCAGCCACCCCGATTTCGCAGAGGGAGCTGGACAT
61    D  G  G  R  S  G  K  C  P  A  T  P  I  S  Q  R  E  L  D  I

241   CCTCAGGGATCTCGGGGATCACAACGCTCTCAACTGCAAGCATGACAGAGAGTGCGAGGT
81    L  R  D  L  G  D  H  N  A  L  N  C  K  H  D  R  E  C  E  V

301   CGGGGAAAAGTGTTGCTACGCTAAGGAAAGTCAGCACTACAGGATCTGCCGCTTTTCGTT
101   G  E  K  C  C  Y  A  K  E  S  Q  H  Y  R  I  C  R  F  S  F

361   CTAGATGTCCTTGGACATGTAGTTCGCTCGAGGCCTCTTGAATCCCGTCAACTTGGAATC
121   *

421   GGATGCGC
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Fig. 1. Nucleotide and amino acid sequence of Carcinin*Pm*1. The initiation and termination codons were bolded; the signal peptide was underlined; the cysteines were in boxes.

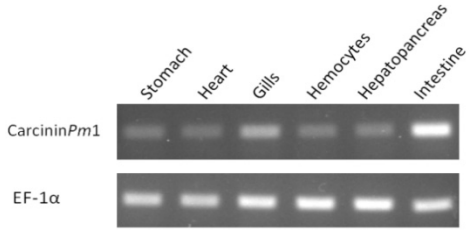

Fig. 3. Tissue distribution of Carcinin*Pm*1 in *P. monodon* tested by semi-quantified RT-PCR. EF-1α was used as a control.

3.3Tissue distribution of Carcinin*Pm*1 in *P. monodon*

The tissue distribution results (**Figure 3**) showed that Carcinin*Pm*1 could be detected in all tissues of *P. monodon* tested. It was highly expressed in intestine and the expression level in gills was relatively higher compared to that in the other four tissues.

3.4Response of Carcinin*Pm*1 to *Vibrio* challenge

The expression of Carcinin*Pm*1 in hepatopancreas of *P.monodon* was up-regulated significantly when the shrimp was challenged by SIV(**Figure 4A**). About 12-20 times upregulation was detected at 4 and 12h post challenge. And then the expression returned to the normal level post 24 hours. This is in accordance with the transcriptome sequencing result, which showed that the expression level of Carcinin*Pm*1 increased 16 times at 3h post challenge. The expression level of Carcinin*Pm*1 in other tissues also increased significantly at 4h post infection (**Figure 4B**). The most significant upregulation was in heart, stomach and gills (12-15 fold). The transcription was up-regulated about 8 times in hemocytes and about 3 times in intestine.

3.5Vector construction, recombination expression of CarcininPm1

The mature peptide of Carcinin*Pm*1 contained 92 residues was linked with SUMO protein with His- tag (**Figure 5**). The His-SUMO-Carcinin*Pm*1 was linked to pCold IV vector. The molecular weight of Carcinin*Pm*1 was 10.64 kDa as shown on SDS-PAGE (**Figure 6C**).

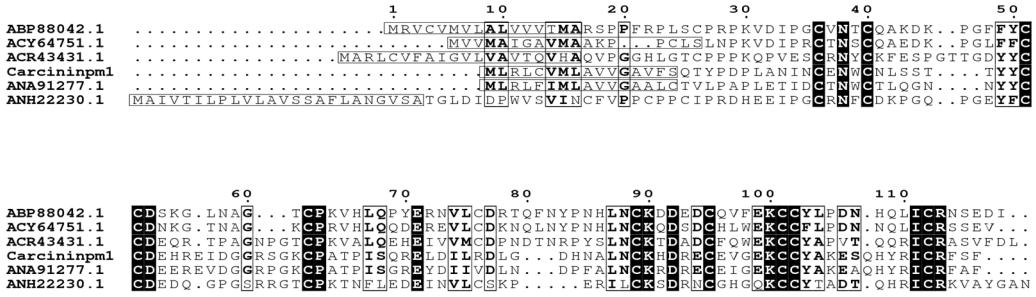

Fig. 2. Alignment of Carcinin*Pm*1 with other crustins. Identical residues were indicated with reverse color; similar residues were in bold and boxed. The signal peptides were in boxes.The GenBank accession number: ANA91277.1, crustinI-5 (*Penaeus japonicas*); ACR43431.1, crustin type I (*Macrobrachium rosenbergii*); ANH22230.1, crustin (*Macrobrachium rosenbergii*); ABP88042.1, Pl-crustin 1 (*Pacifastacus leniusculus*); ACY64751.1, crustin 1 (*Procambarus clarkia*)

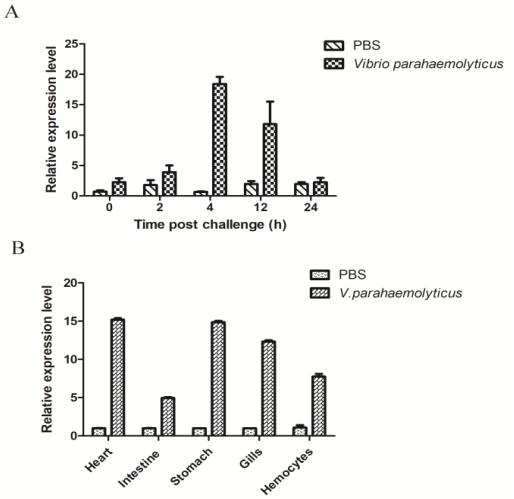

Fig. 4. Expression patterns ofCarcinin*Pm*1 in *P.monodon*post *V. parahaemolyticus* challenge. A,Relative expression levelofCarcinin*Pm*1 in hepatopancreas at different time point post challenge. B. Relative expression level of Carcinin*Pm*1 in different tissues at 4h post infection. Shrimps were injected with *V. parahaemolyticus* or PBS as control. qPCR was used to test the relative expression level.

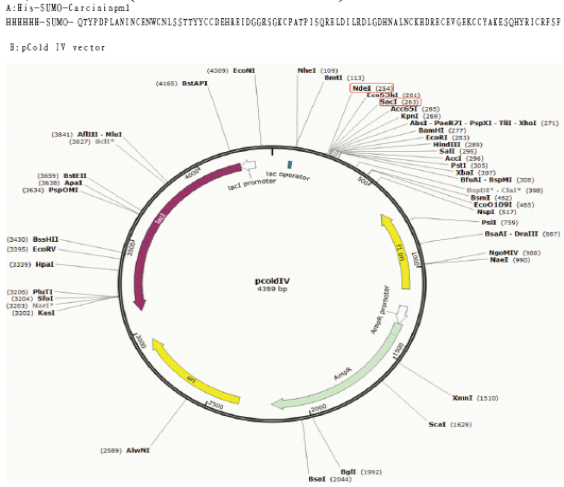

Fig.5. Construction of recombination plasmid. A, Amino acid sequence of His-SUMO-Carcinin*Pm*1. B, Schematic representation of pCold IV vector.

3.6 Purification of CarcininPm1

The His-SUMO-Carcinin*Pm*1 fusion protein was highly expressed in *E.coli* induced with IPTG at low temperature and half protein was in the supernatant (**Figure 6A**). The fusion protein was purified with a Ni-NTA column and eluted by 500mM imidozol as a pure protein (**Figure 6B**). The yield of purified SUMO-Carcinin*Pm*1 was around 8 mg/mL, measured by bradford reagent.And then the SUMO tag was successfully removed by cutting with a SUMO protease, shown that a ~10.64 kDa band was detected on SDS-PAGE (**Figure 6C**).

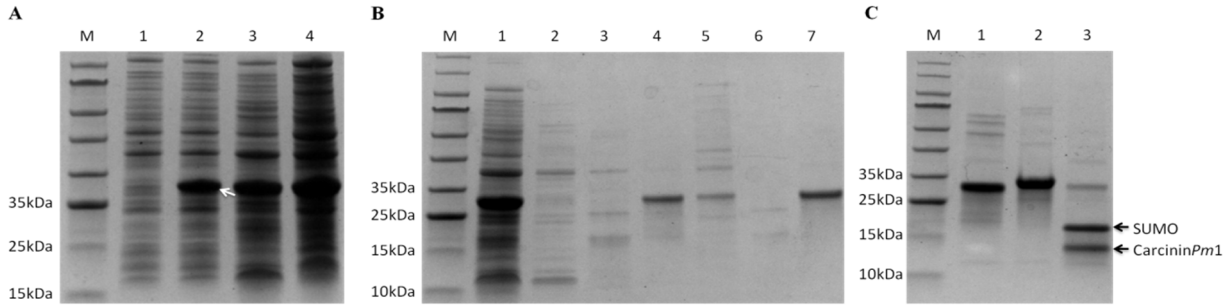

Fig. 6. Heterologous expression and purification of Carcinin*Pm*1 analyzed by SDS-PAGE. M, Protein marker. A, Over-expression of Carcinin*Pm*1 in *E. coli* (BL21). 1, total protein of *E. coli* without induction; 2, total protein of *E. coli* induced with 1mM IPTG at 16°C for 12 hours, the fusion protein is indicated by an arrow; 3, supernatant after cell disruption; 4, sediment after cell disruption. B, Purification of Carcinin*Pm*1 by Ni-NTA column. 1, sample, which is the supernatant after cell disruption; 2, flowthrough; 3 and 5, wash off protein with 60mM imidazole; 4 and 7, eluted proteins with 200mM and 500mM imidazole. C, SUMO tag cleavage. 1, purified protein before cleavage; 2, SUMO protease; 3, protein after cleavage, with SUMO and Carcinin*Pm*1 indicated.

4 Discussion

Type I crustins normally contain a cysteine-rich domain and a WAP domain. The cysteine-rich domain forms two disulfide bridges and the WAP domain contains four disulfide bridges[20]. However, the WAP domain of CarcininPm1was not intact, as it lacked two cysteins, the second and seventh cysteines in the WAP domain. And these two cysteines normally form a disulfide bridge. This indicates that there were in total three disulfide bridges in the WAP domain of Carcinin*Pm*1. The MjCru I-4 and 5 are also ten-cysteine crustins [24]. And there are some Type Icrustins contain sevencysteines in the WAP domain [21]. However, most of Type I crustins have 12 cysteines with an intact WAP domain [23].

Although there are many types of crustins discovered in *P. monodon*, the trancriptome sequencing data showed that most of them were not up-regulated during *Vibrio* challenge, but the expression level of Carcinin*Pm*1 increased about 15 times post infection, which means that it might play an important role in the innate immunity of *P. monodon*. The most significant up-regulation was in hepatopancreas, heart, stomach and gills. As the normal expression level in gills was high, Carcinin*Pm*1 was abundantly transcribed in gills post challenge. The up-regulationcould be observed at 2h post challenge, which means that Carcinin*Pm*1 responded to *Vibrio* challenge in a very short time. And the up-regulation lasted at least 12 hours in hepatopancreas. Besides *Vibrio*, Type I crustinscould also be up-regulated by other pathogens. A type-I crustin from red swamp crayfish *Procambarus clarkii* was significantly induced by *Staphylococcusaureus,Vibrio anguillarum* and *Aeromonas hydrophila* stimulations, with the relative expression level only increased about 5 times. It could also be induced by WSSV in hemocyte from 48 h post-infection[20]. MjCru I-1 was also observed to up-regulate about 15 and 10 times at 12h post challenged by *S. aureus*and *V. anguillarum*, respectively [25].

In this research, we used a novel method to express Carcinin*Pm*1, and the expression and purification were successful, which indicates that this method could be applied for overexpression of other crustins.

In summary, Carcinin*Pm*1 is a very important molecule in *P. monodon* during fighting against *Vibrios*. However, it is still unknown about how it functioned*in vivo* and the *in vitro* activities need to be further clarified.

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