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Original Article

Crustin protein Amk1 from black tiger shrimp (*Penaeus monodon*) inhibits *Vibrio harveyi* and *Staphylococcus aureus*

Moltira Tonganunt[1]*, Korkiat Wongmanee[2], Sonchai Sartthai[1], Wilaiwan Chotigeat[1] and Amornrat Phongdara[1]

[1] *Center for Genomics and Bioinformatics Research, Faculty of Science*

[2] *Department of Biochemistry, Faculty of Science*
Prince of Songkla University, Hat Yai, Songkhla, 90110 Thailand.

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Abstract

A crustin gene (*Amk1*) was identified from a haemocyte library of the black tiger shrimp, *Penaeus monodon*. The full-length cDNA consists of 411 bp encoding a deduced precursor of 136 amino acids with a signal peptide of 17 amino acids. Amk1 contains a hydrophobic and a Gly-rich region at the N-terminus and a 12 conserved cysteine domain (6-DSC) at the C-terminus. Transcripts of *Amk1* are mainly detected in haemocytes and gills by RT-PCR analysis. A recombinant Amk1 was overexpressed and purified from *Escherichia coli*. This has a molecular mass of 43.66 kDa with a predicted *pI* of 8.23. Antibacterial assays demonstrated that recombinant Amk1 exhibited antibacterial activity against Gram-positive and Gram-negative bacteria with strong inhibition against *Staphylococcus aureus* and *Vibrio harveyi*.

Keywords: crustin, antibacterial peptide, *Penaeus monodon*, glycine rich protein, WAP

1. Introduction

Antimicrobial peptides (AMPs) are host defense compounds that have recently drawn attention, due to their properties and diversity. These compounds are generally small cationic proteins that have direct antimicrobial activities and act as part of the immune systems found in all organisms ranging from bacteria to plants and animals. In crustaceans, AMPs are considered to be a main component of innate immunity (Smith and Chisholm 2001). Several antimicrobial peptide families have been discovered, the first one being the anti-lipopolysaccharide factor (ALF) isolated from the horseshoe crab *Limulus polyphenus* (Tanaka *et al.*, 1982). The penaedin family are a group of antimicrobial peptides isolated from shrimp *Litopenaeus vannamei* (Destoumieux *et al.*, 1997; Li *et al.*, 2005), *P. monodon* (Supungul *et al.*, 2002), *L. setiferus* (Gross *et al.*, 2001) and *F. chinensis* (Liu *et al.*, 2005). Crustin, a hydrophobic molecule containing a cysteine-rich region and a whey acidic domain (WAP), is another member of AMPs that is distributed in a wide range of decapods (Gross *et al.*, 2001; Bartlett *et al.*, 2002; Stoss *et al.*, 2004; Vargas-Albores *et al.*, 2004). In shrimp, crustins have been isolated from *L. vannamei* and *L. setiferus* (Thomas *et al.*, 2002), *Marsupenaeus japonicus* (Ratanachai *et al.*, 2004), *Fenneropenaeus chinensis* (Zhang *et al.*, 2007), *Homarus gammarus* (Hauton *et al.*, 2006), *Pacifastaeus leniusculus* (Jiravanichpaisal *et al.*, 2007) and *P. monodon* (Supungul *et al.*, 2004)

In *Penaeus monodon*, Crustin*Pm1* and a Crus-like *Pm* (Amparyupa *et al.*, 2008) have been cloned and characterized. Both peptides contain a Gly-rich region at the amino-terminus and a single whey, acidic protein (WAP) domain at the carboxyl-terminus. A recombinant crustinPm1 exhibited antimicrobial activity against only Gram-positive bacteria, and strongly on *Staphylococcus aureus* and *Streptococcus*

*Corresponding author.
Email address: tonganunt@yahoo.com

iniae, while a recombinant Crus-like *Pm* showed strong antibacterial activity against both Gram-positive and Gram-negative bacteria including *Vibrio harveyi*, a well known shrimp pathogen.

Two isoforms of crustin-like genes, *CruFc* and *CshFc*, were cloned from haemocytes of *Fenneropenaeus chinensis. CruFc* consists of 134 amino acids with a putative signal peptide of 17 amino acids. The open reading frame (ORF) sequence contains a whey-acidic protein (WAP) domain at its C-terminal. Transcripts of *CruFc* were mainly detected in haemocytes and gills. The other gene, *CshFc* contains an amino acid sequence lacking the WAP-type 'four-disulfide core' domain. A fusion protein containing CruFc did inhibit the growth of Gram-positive bacteria *in vitro*, whereas the recombinant CshFc did not under the same conditions. This evidence indicated that the four-disulfide core domain of crustin could play an important role in its antibacterial activities.

In this paper, we present data on a new isoform of crustin peptide from *P. monodon*. The recombinant protein shows antibacterial activity against Gram-positive and Gram-negative bacteria and demonstrates high potency against *Vibrio harvyei*. The amino acid sequences of this protein that might determine its activity against bacterial strains will be discussed.

2. Materials and Methods

2.1 Animals, vector and bacterial strains

Shrimp, *Penaeus monodon*, were obtained from a farm in Satun province, Thailand. The animals were checked for pathogens and maintained in an aquarium as described in a previous work (Bangrak *et al.*, 2002). Bacterial strains for use in the antibacterial assays (*Bacillus megaterium*, *Enterococcus faecalis, Micrococcus luteus*, *Staphylococcus aureus*, *Enterobacter cloacae*, *Escherichia coli, Salmonella* Typhimurium and *Vibrio harveyi*) were kindly provided by Department of Microbiology, Faculty of Science, Prince of Songkla University, and were maintained in our laboratory. *Escherichia coli* strain BL21 and expression vector pGEX-4T-1 (Amersham Biosciences) were used in the production of the recombinant protein.

2.2 Isolation and construction of the crustin expression plasmid

The full length sequence of the crustin-like cDNA (GenBank accession no. EU 103630) was obtained from a clone in the EST library of haemocytes infected with WSSV (Tassanakajon *et al.*, 2006). The clone is named *Amk1*. The ORF corresponding to a mature antibacterial peptide was cloned into an *E. coli* expression vector, pGEX- 4T-1. The recombinant plasmid was then transformed into an *E. coli* strain BL21 for the production of protein.

2.3 Analysis of *Amk1* expression in various tissues

Expression of *Amk1* in different tissues was measured by RT-PCR. Total RNA from haemocytes, hepatopancreas, lymphoids, gills, intestines and hearts were extracted by homogenization with TRIzol reagent (Invitrogen) following the manufacturer's protocol. The specific primers (*Amk1* Forward: 5' GAATTCAAAATGAAGGGTCTCGGAG 3' and *Amk1* Reverse: 5' CTCGA GGGCAAAAAATTCATAGAAG 3') were designed. EF- 1 was used as the internal control : (EF Forward: 5' GTCTTCCCCTTCAGGACGTC 3' and EF Reverse: 5'GTCGATCTTGGTCAGCAGTTC 3'). The amplification products were visualized after electrophoresis on 1.5% agarose gels.

2.4 Purification of the recombinant protein Amk1 from *E. coli*

E. coli BL21 harboring *Amk1-GST* was cultured at 37°C. Expression of the antibacterial peptide was induced by adding IPTG to a final concentration of 0.1mM. Cells were harvested by centrifugation at 5,000 rpm for 5 minutes and resuspended in phosphate-buffered saline, pH 7.4. After analysis of the protein fractions, Amk1 was found in inclusion bodies and the protein was purified by the washing method (Violand, 2001). Purified proteins were analyzed in 12% SDS-PAGE. Western blot analysis was used to confirm the identity of the expressed recombinant Amk1.

2.5 Antibacterial Assay

The spectrum of antibacterial activity of recombinant Amk1 and the minimal inhibition concentrations (MICs) was determined by a liquid growth inhibition assay (Bulet *et al.*, 1993). The antibacterial activities were tested against the Gram-positive bacteria (*Bacillus megaterium*, *Enterococcus faecalis, Micrococcus luteus* and *Staphylococcus aureus*) and Gram-negative bacteria (*Enterobacter cloacae*, *Escherichia coli, Salmonella* Typhimurium and *Vibrio harveyi*). The stock solution of recombinant protein and purified GST as the negative control were serially diluted with two-fold amounts of PBS (pH7.4). Aliquots 80 ml from each dilution were transferred to a 96-well polypropylene microliter plate, and each well was inoculated with 100 µl of a suspension of mid-log phase bacteria (10^8 CFU/ml) grown with LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl (w/v), pH 7.5), and growth was measured by its absorbance at 630 nm using a microplate reader (Li *et al.*, 2005).

3. Results

3.1 Cloning of the full-length *Amk1*

The complete nucleotide sequence of *Amk1* contains a 411 bp open reading frame (ORF) (Figure 1). The Amk1

```
TCGGATNTGCNTNCGCGTGGATCCAAAGAATTCGGCACGAGGGCGACCATGAAGGGTCTC 60
                                                M  K  G  L

GGAGTCATTCTGTTCTGCGTCCTGGCGATGGCATCAGCCCAGAGTTGGCACGGAGGTCGA 120
 G  V  I  L  F  C  V  L  A  M  A  S  A  Q  S  W  H  G  G  R

CCCGGAGGCTTCCCTGGTGGAGGTAGACCCGGAGGTAGACCCGGAGGCTTCCCAAGCGTC 180
 P  G  G  F  P  G  G  G  R  P  G  G  R  P  G  G  F  P  S  V

ACAGCCCCACCCGCCTCCTGTAGGCGCTGGTGCGAAACTCCAGAGAATGCTTTTTACTGC 240
 T  A  P  P  A  S  C  R  R  W  C  E  T  P  E  N  A  F  Y  C

TGCGAGTCAAGGTATGAACCCGAGGCACCCGTGGGCACCAAGATACTTGACTGCCCAAAA 300
 C  E  S  R  Y  E  P  E  A  P  V  G  T  K  I  L  D  C  P  K

GTCCGTGACACCTGCCCACCGGTACGTTTTCTTGCAGTAGAGCAGCCAGTACCTTGCTCC 360
 V  R  D  T  C  P  P  V  R  F  L  A  V  E  Q  P  V  P  C  S

AGTGACTACAAGTGCGGCGGCCTTGACAAGTGCTGCTTCGACAGGTGTTTGGGACAACAC 420

 S  D  Y  K  C  G  G  L  D  K  C  C  F  D  R  C  L  G  Q  H

GTGTGCAAGCCACCTTCCTTCTATGAATTTTTTGCCTGAAAAGGAAATTTGGAAGTAATT 480
 V  C  K  P  P  S  F  Y  E  F  F  A  *

ACCGATCCATGTAGAATCTGTGACTAATAAAGTGTTTTTCAGAGCATGATTTCAAAAAAA 540

AAAAANAAAAAAAAAAAAAAA                                        561
```

Figure 1. Nucleotide and deduced amino acid sequences of *Amk1*. The bold type indicates the start codon (ATG), "*" indicates the stop codon (TGA) and the polyadenylation signal (AATAAA). The underlined amino acid sequence identifies a signal peptide sequence, the bold and underlined amino acid are residues that differ from crustinPm1.

```
BAD15064: DCRYWCKTPGGQNYCCERSHEPEGPVGTKPLDCPQVRPTCP--RFQGGG-PVTCSNDYKCAGIDKCCFDTCLQEHVCKPPVF----
AAL36892: DCRYWCKTPEGQAYCCESAHEPETPVGTKPLDCPQVRPTCP--RFGH-P-PTTCSNDYKCAGLDKCCFDRCLGEHVCK--------
AAL36896: TCRRWCKTPENQAYCCETIFEPEAPVGTKPLDCPQVRPTCP--RFGH-P-PVTCSSDYKCGGVDKCCFDRCLGEHVCKPPSFYSQF
AAX63906: TCRRWCRTPERAAYCCETSFEPEAPVGTKILDCPRVRDTCPPVRFGGLA-PVTCSSDYKCGGIDKCCFDRCLGEHVCKPPSF----
CD766060: SCRRWCETPENAFYCCESRYEPEAPVGTKILDCPKVRDTCPPVRFLAVEQPVPCSSDYKCGGLDKCCFDRCLGQHVCKPPSF----
AMK1    : SCRRWCETPENAFYCCESRYEPEAPVGTKILDCPKVRDTCPPVRFLAVEQPVPCSSDYKCGGLDKCCFDRCLGQHVCKPPSF----
           1  2       34                 5    6             7    8     910  11   12
```

Figure 2. Multiple alignment of the deduced amino acid sequences of the 12 conserved cysteine domain (6-DSC) of Amk1 from *Penaeus monodon* and those of *Litopenaeus setiferus* (accession no. AAL 36892, 36896), *Marsupenaeus japonicus* (accession no. BAD15064), *Fenneropenaeus chinensis* (accession no. AAX6390) and *P.monodon* (accession no. CD766060) and (-) represents a gap/deletion. The 12 conserved cysteines (6-DSC) are indicated by number.

ORF (GenBank accession no. EU103630) encodes 136 amino acids with a predicted molecular weight (MW) of 14.669 kDa and theoretical isoelectric point (p*I*) of 8.23. The analysis with the SignalP software revealed that the N-terminal segment included a high proportion of hydrophobic amino acid residues and a signal peptide with a predicted cleavage site between Ala 17 and Glu 18. The deduced amino acid sequence of Amk1 contained a number of glycine-rich repeats between positions 22 and 42, consisting of five repeats of the tetrapeptide amino acid sequence GG(R/P)P. The C-terminal segment included a Cys-rich region, with 12 Cys residues that might participate in the formation of 6-disulfide bonds as found in crustinPm1 (Rattanachai *et al.*, 2004, Figure 2). As predicted by the ScanProsite program, a whey-acidic protein (WAP) domain signature of 52 amino acids was present starting from K (78) to S (130) at the C-terminal.

The amino acid sequence of our Amk1 is very similar to crustinPm1 from *Penaeus monodon* (GenBank accession no. CD766060) except for a 9 amino acids gap and we suggest that this is another isoform of the crustin protein.

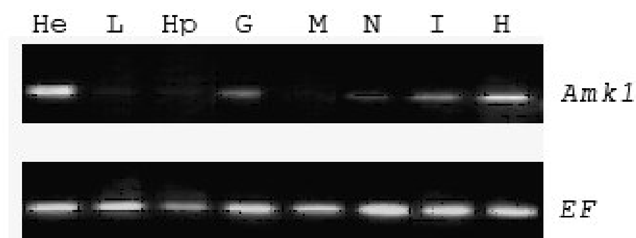

Figure 3. *Amk1* detection by Semi-quantitative RT-PCR using total RNA from various organs and the expression of EF-1 gene as an internal control; analysis by electrophoresis in a 1.5% agarose gel He: haemocyte; L: lymphoid organs; Hp: hepatopancreas; G: gill; M: muscle; N: nervous system; I: intestine and H: heart respectively.

The distinction between these two isoforms is that Amk1 contains a single region of G F P G G G R P G while CD 766060 crustinPm1 contains two such regions (Figure 1).

3.2 Distribution of *Amk1* mRNA in shrimp tissues

Based on the RT-PCR analysis, *Amk1* is expressed in all tissues tested. It is highly expressed in the haemocytes and heart and moderately expressed in the gill, intestine and nerve (Figure 3).

3.3 Expression, purification and immunobloting analysis of recombinant *Amk1*

The *Amk1* cDNA was expressed in the *E. coli* BL21 expression system. The recombinant protein with an approximate size of 43.66 kDa was isolated from a BL21 cell lysate after induction with 0.1 mM IPTG. However, most of the recombinant protein appeared in the insoluble inclusion bodies. We used the washing method (Violand, 2001) to purify the protein. The yields of purified Amk1 were 0.05 mg/ml culture media. The identity of recombinant Amk1 protein was confirmed by Western immunoblotting using anti-GST antibody. A major band at about 43.66 kDa was identified.

3.4 Antibacterial activity

The spectrum of antibacterial activity of recombinant Amk1 was investigated against several Gram-positive and Gram-negative bacteria. The MIC of inhibited bacteria was determined and summarized in Table 1. The recombinant Amk1 was active with highest activity against *Staphylococcus aureus* (MIC = 0.0066-0.026 mM) and *Vibrio harveyi* (MIC = 0.0066-0.053 mM). No activity was observed against the other bacteria tested.

4. Discussion

Shrimp and invertebrates lack an adaptive immune system. They rely on their innate immunity to effectively fight against invading pathogens using humoral and cellular reactions such as synthesis of antimicrobial proteins (AMPs), nodule formation, and melanization (Ratcliffe, 1985; Brey and Hulmark, 1998). In shrimp, AMPs such as the penaedin, crustinPm1, anti-lipopolysaccharide factor, proline/glycine-rich antibacterial peptides, and antifungal proteins are found in the haemolymph (blood) to combat invading microorganisms. Penaedin is active against Gram-positive and Gram-negative bacteria as well as fungi, whereas crustinPm1 kills only Gram-positive bacteria, most strongly *Staphylococcus aureus* and *Streptococcus iniae* (Supungal *et al.*, 2008). Another protein named crus-like Pm was recently isolated from *P. monodon*, the recombinant crus-like Pm compound show strong antibacterial activity against both Gram-positive and Gram-negative, particularly against *V. harveyi*.

Table 1. Antibacterial activity of crustin against Gram-positive bacteria measured by a liquid growth inhibition assay, MIC are expressed as the interval a-b, where a is the highest concentration tested at which microorganisms are growing and b the lowest concentration that causes 50% growth inhibition.

Microorganism	MIC (mM)	
	a	b
Gram-positive bacteria		
Bacillus megaterium	na	na
Enterococcus faecalis	0.0356	0.230
Micrococcus luteus	0.0111	0.0117
Staphylococcus aureus	0.0066	0.026
Gram-negative bacteria		
Enterobacter cloacae	na	na
Escherichia coli	na	na
Salmonella Typhimurium	na	na
Vibrio harveyi	0.0066	0.053

na = no activity

In this study, we have reported the cloning and characterization of another crustin-like gene from *P.monodon* named *Amk1*. The peptide is similar to the CrustinPm1 of the *P.monodon*. Our full-length *Amk1* consists of an ORF of 411 bp coding for a putative protein of 136 amino acid residues. Like other crustins, the *Amk1* protein also contains a signal peptide, a glycine-rich repeat region, 12 cysteine residues and a WAP- domain. The difference of the amino acid sequence of Amk1 and CrustinPm1 is that Amk1 has the deletion of 9 amino acid residues G F P G G G R P G.

The vast majority of AMPs appear to kill microorganisms by permeabilization of the cell membrane, the disruption of membrane integrity kills the target microbes. Their activity and selectivity are governed by the physicochemical parameters of the peptide chains as well as the properties of the membrane system itself. Recent studies also indicate that some of the peptides may enter the target cell through disrupted membrane and have other killing mechanisms

associated with intracellular interactive targets. They bind to specific molecules and interfere with their metabolic function. These mechanisms may act independently or synergistically with membrane permeabilization. For example, a derivative of buforin I from the Asian toad, *Bufo garagriozans*, might inhibit the cellular functions of the pathogen by binding to its DNA or RNA. Cecropin PR 39 inhibits both DNA and protein synthesis and induces selective transcription of the stress related genes micF and osmY in *E. coli* at sublethal concentrations (review by Toke, 2005).

The molecular structure of Amk1 is rich in glycine residues near the N-terminal region. The C-terminal can be categorized as a cysteine-bridged peptide. A two-step mechanism of membrane permeabilization (binding followed by insertion) has been proposed for several cysteine-bridged AMPs, including protegrin-1176 and the cyclic b-defensin, RTD-1. The results for Amk1 and CrustinPm1 show differences in their antimicrobial activity against *V. harveyi* yet the two peptides are only different at the Glycine rich domain, and it is likely that the 6 cysteine bridge of Amk1 initiates entry into the host cell. The glycine rich domain may play an important role in determining the specificity to microorganisms by enhancing membrane permeabilization and/or target binding inside the cell and leading to antimicrobial activity against *V. harveyi*. Kustanovich *et al.* (2002) demonstrated that the amphipathicity of peptides is important for the function of the potency of the peptides and the selectivity appears to be effected by the hydrophobic properties of the peptide chain. These observations raised the possibility of achieving a higher antibacterial selectivity by modifying the hydrophobicity of the peptide chain. This possibility is confirmed by our work showing that Amk1 has less hydrophobicity than crustinPm1 because of the deletion of a 9 amino acid region, G F P G G G R P G.

Both Amk1 and Crus-like Pm1 peptides have activities against *V. harveyi*, therefore it is interesting for future work to identify the molecular signature at the N-terminus that might specify the activity against *V. harveyi*. This might clarify the reason for the diversity of AMPs and their mode of action that differs from that of conventional antibiotics. The exploitation of the properties of these peptides will be of benefit not only to shrimp farmers but also to future medicinal applications.

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