Antimicrobial peptides from the black tiger shrimp *Penaeus monodon* – a review

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**ABSTRACT**

Black tiger shrimp *Penaeus monodon* is one of the major shrimp species being cultured in Asian countries. Disease outbreaks have resulted in a decline of *P. monodon* cultivation and severe losses in the shrimp production. To combat harmful microorganisms, shrimp produce diverse classes of antimicrobial peptides (AMPs) as part of their immune defense. In *P. monodon*, several cDNA sequences of AMPs were identified by means of expressed sequence tag (EST) approach. These DNA sequences encode different putative AMPs, such as crustins, penaeidins, anti-lipopolysaccharide factors (ALFs) and lysozymes. In this review, we present the recent information on sequence diversity, expression and antimicrobial properties of *P. monodon* AMPs. The information indicates the importance of these AMPs as the effective host defense molecules against the invasion of pathogenic microorganisms.

**Keywords:** *Penaeus monodon*, shrimp, antimicrobial peptides, innate immunity, expressed sequence tag

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INTRODUCTION

Antimicrobial peptides (AMPs), ubiquitously found in all living organisms, are important immune effectors with an ability to neutralize and/or kill invading microorganisms (Brown and Hancock, 2006). AMPs share common features of small size (about 15 to 100 amino acids) with amphipathic properties but they differ in the primary sequences, secondary structures and spectra of activities. Lacking the adaptive immunity, shrimp rely primarily on immune molecules including AMPs to combat the varieties of microorganisms. The first AMP family discovered in shrimp was penaeidin of the Pacific white shrimp, *Litopenaeus vannamei* (Destoumieux et al., 1997) which displayed antimicrobial activity against Gram-positive bacteria and fungi. Thereafter, other shrimp AMP families: crustins, anti-lipopolysaccharide factors (ALFs) and lysozymes (Amparyup et al., 2008a; Amparyup et al., 2008b; Bartlett et al., 2002; Hikima et al., 2003; Sotelo-Mundo et al., 2003; Supungul et al., 2004), had been reported in various shrimp species. These shrimp AMPs have been characterized for their expression and antimicrobial activities. In the black tiger shrimp *Penaeus monodon*, Supungul et al. (2002; 2004) reported the discovery of cationic AMPs from the hemocyte cDNA libraries. The most recent data was reported by Tassanakajon et al. (2006) describing the identification of putative antimicrobial sequences from the *Penaeus monodon* EST database http://pmonodon.biotec.or.th. In this paper, we summarized a recent information on *P. monodon* AMPs concerning the sequence variations, gene expression in various tissues and in response to pathogen infections and antimicrobial properties.

SEQUENCE DIVERSITY

AMPs found in *P. monodon* were discovered mainly by means of expressed sequence tag (EST) analysis. A total of 889 ESTs encoding putative AMPs were identified in the *Penaeus monodon* EST database of 40,001 EST sequences. These AMP sequences represent four major AMPs: penaeidins, crustins including single WAP domain (SWD) proteins, anti-lipopolysaccharide factors (ALFs) and lysozymes. Of these AMPs, crustins were the most abundant followed by penaeidins, ALFs and lysozymes, respectively (Table 1).

Crustins are cationic cysteine-rich antibacterial peptides consisting of a single whey acidic protein (WAP) domain (Smith et al., 2008). The WAP domain located at the C-terminus of the molecule contains 50 amino acid residues with eight cysteine residues at defined positions (Ranganathan et al., 1999). The first crustin molecule, an 11.5 kDa antimicrobial protein namely ‘carcinin’, was isolated from hemocytes of the crab *Carcinus maenas* (Smith and Chisholm, 2001). The putative crustin genes of *P. monodon* (crustinPms) have been identified from the hemocyte and the gill-epipodite cDNA libraries (Supungul et al., 2004; Tassanakajon et al., 2008; Vatanavicharn et al., 2009). In comparison with other *P. monodon* AMPs, the crustin family exhibits the highest sequence variation both in length and their primary sequences. According to the classification of crustins proposed by Smith et al. (2008), *P. monodon* crustins belong to Type II and III crustins. Type II crustin constitutes the majority of *P. monodon* crustin sequences consisting of seven isoforms (crustinPms1-
6 and crustin-like Pm) whereas Type III crustin or single WAP domain containing protein (SWD) comprises three isoforms of SWD (SWDPms 1-3).

Type II crustins contains a signal peptide followed by a long glycine-rich domain and a cysteine-rich domain (four cysteine residues) at the N-terminal region, and a WAP domain (eight cysteine residues) at the C-terminal region. Based on the alignment of amino acid sequences, all Type II crustin Pms possess 12 cysteine residues, eight of which participate in a four disulfide core (4DSC) or WAP domain. High sequence diversity both in length and primary sequence of different isoforms of crustin Pms was clearly observed (Tassanakajon et al., 2008). Recent data showed that crustin Pms were encoded by different genes and had quite different promoter and regulatory sequences. Genome organization study of crustin-like Pm gene (716 bp) reveals that it consists of two exons (38 and 487 bp) and one intron (191 bp) (Amparyup et al., 2008b). The 5’UTR sequence contains a putative TATA box and several potential cis-acting elements, e.g. putative binding sites for GATA binding factor, STAT5, NF-kappaB, AP-1 and C/EBP-b. Crustin Pm5 gene (1,394 bp) contains four exons of 31, 75, 242 and 194 bp, respectively, interrupted by three introns of 606, 148 and 116 bp. Instead of the putative GATA and NF-kappaB sites, interestingly, the upstream sequence of

### Table 1

Types, number of sequences, diversity and antimicrobial activities of the *Penaeus monodon* AMPs from the EST database (http://pmonodon.biotec.or.th)

<table>
<thead>
<tr>
<th>Types</th>
<th>No. of sequences</th>
<th>No. of isoforms</th>
<th>Major isoform</th>
<th>Antimicrobial activities</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crustins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type II</td>
<td>275</td>
<td>7</td>
<td>Crustin*Pm 1</td>
<td>Gram +</td>
<td>Supungul et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Crustin*Pm 5</td>
<td>Gram +</td>
<td>Vatanavicharn et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Crustin-like Pm</td>
<td>Gram +</td>
<td>Amparyup et al., 2008a</td>
</tr>
<tr>
<td>Type III (SWD)</td>
<td>50</td>
<td>3</td>
<td>SWDPm2</td>
<td>Gram +, Gram-, anti-</td>
<td>Amparyup et al., 2008b</td>
</tr>
<tr>
<td>ALFs</td>
<td>208</td>
<td>6</td>
<td>ALFPm3</td>
<td>Gram +, Gram-, fungi,</td>
<td>Somboonwiwat et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ALFPm2</td>
<td>Anti-virus</td>
<td>Thartada et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gram +, Gram-</td>
<td>Thartada et al., 2008</td>
</tr>
<tr>
<td>Penaeidins</td>
<td>284</td>
<td>2</td>
<td>PEN3</td>
<td>ND*</td>
<td>Tassanakajon et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>PEN 5</td>
<td>ND*</td>
<td>Tassanakajon et al., 2008</td>
</tr>
<tr>
<td>Lysozymes</td>
<td>72</td>
<td>2</td>
<td>C-type</td>
<td>Gram+, Gram-</td>
<td>Supungul et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>I-type</td>
<td>Gram+, Gram-</td>
<td></td>
</tr>
</tbody>
</table>

*ND is no data available.*
The crustinPm5 gene contains a complete heat shock regulatory element (HSE) suggesting that it is the heat-inducible gene (Vatanavicharn et al., 2009).

The less well-characterized P. monodon Type III crustin or SWDPm is composed of 3 subgroups (SWDPm1, SWDPm2 and SWDPm3) (Amparyup et al., 2008a). The Type III crustin does not contain the glycine-rich and the cysteine-rich regions but instead has a proline-arginine rich region between the signal sequence and the WAP domain. Analysis of the exon-intron organization of Type III crustin genes from P. monodon SWD (Chen et al., 2006) and SWDPm2 (Amparyup et al., 2008a) reveals that they both contain three exons and two introns. The differences between these two SWD proteins are the size of the first intron and the microsatellite sequences in the second intron. At the 5’ upstream sequences, putative transcription factor binding sites, such as cap, hsf, GATA-1, SRY, Tst-1, HNF-3b, CF2-II, Oct-1, AP-1 and USF, are found but not the putative TATA box (Chen et al., 2006).

As mentioned before, the penaeidin family is the first AMP identified and characterized in penaeid shrimp (Destoumieux et al., 1997). Penaeidins are cationic peptides with a molecular mass of 5.5 to 6.6 kDa. They have two domains: one is an N-terminal proline-rich domain and the other is a C-terminal cysteine-rich domain containing six cysteine residues. A collection of penaeidin sequences has been reported in PenBase www.penbase.immunaqua.com established in 2006 (Gueguen et al., 2006). Penaeidins have variation in the primary sequences and can be grouped into four classes (PEN2, PEN3, PEN4 and PEN5). Amino acid sequence characteristics and conserved key residues proposed as penaeidin signature of PEN2, PEN3 and PEN4 were previously described in PenBase whilst those of PEN5 were proposed by Kang et al. (2007). However, recent alignment of known penaeidin sequences revealed that there were variations in a few key amino acid residues in the proposed penaeidin signatures (Tassanakajon et al., 2011).

In P. monodon, only PEN3 and PEN5 were identified and PEN3 is the most abundant class (Tassanakajon et al., 2006). The members of PEN3 show the differences in the number and sequences of amino acids across the shrimp species, but within the species the members of PEN3 from P. monodon have the same amino acid residue in length (74 aa). Generally, the variants arise from the amino acid variations in the proline-rich domain. The P. monodon PEN5 exhibits 72% sequence identity to those from Fenneropenaeus chinensis (Woramongkolchai et al., 2011). Unlike the intronless PEN3 gene from L. vannamei (O’Leary and Gross, 2006), genomic structure analysis of PEN3 from P. monodon by Chiou et al. (2007) indicated that it contains an intron of 680 bp. Also, the PEN5 from P. monodon contains an intron of 620 bp (Woramongkolchai et al., 2011).

ALFs are the antimicrobial peptides firstly isolated from the hemocytes of the horseshoe crabs, Tachypleus tridentatus and Limulus polyphemus (Tanaka et al., 1982; Muta et al., 1987). ALF genes have, then, been identified from several species of crustacean including shrimp (Supungul et al., 2002; Liu et al., 2006; Nagoshi et al., 2006; Rosa et al., 2008; Beale et al., 2008; Imjongjirak et al., 2007). The P. monodon ALFs (ALFPms) constitute at least five different isoforms (Supungul et al., 2002) which are grouped into groups A
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(ALFPm1 and 2) and B (ALFPm3-5), derived from different genomic loci, according to nucleotide sequence analysis (Tharntada et al., 2008). Group A ALFPm contains three exons interrupted by two introns, but group B is composed of four exons interrupted by three introns. The different isoforms within the group derived from the alternative RNA splicing phenomenon. However, ALFPms share common features of high hydrophobicity at the N-terminal region and the conserved disulfide loop containing positively charge cluster, described as the putative LPS-binding domain (Hoess et al., 1993). ALFPm genes contain an open reading frame encoding a protein of 98 - 123 amino acid residues.

The cis-regulatory elements involving in immune response and/or regulating the expression of antimicrobial peptides, such as NF-kappaB, GATA, AP-1, GAAA and Oct-1 motifs are found in the genomic sequences at the 5' upstream region of both ALFPm groups A and B. However, the AP-1 and NF-kappaB binding sites are only predicted in the ALF group A genes. It should be noted that the presence of AP-1 (Karin et al., 1997; Douglas et al., 2003) together with the GAAA motifs (Au et al., 1993) infers that the ALFPm group A genes expressed in response to viral infection.

Lysozyme is an important antibacterial protein that hydrolyzes bacterial cell wall leading to cell lysis. Lysozyme, normally found in both eukaryotes and prokaryotes, can be divided into six types: plant lysozyme, chicken-type lysozyme (c-type), goose-type lysozyme (g-type), invertebrate lysozyme (i-type), bacteria lysozyme and T4 phage lysozyme (phage-type). In P. monodon, a total of 72 EST sequences encoding lysozymes have been identified. Of these, only two types of lysozyme, c-type and i-type have been identified (Table 1) (Tassanakajon et al., 2008; Supungul et al., 2010). The information on sequence diversity and the genomic structures of P. monodon lysozymes are being analyzed.

GENE EXPRESSION PATTERNS

Gene expression of P. monodon AMPs in various shrimp tissues, during the developmental stages and in response to pathogen infection, has been extensively investigated and is discussed herein.

Generally, AMP genes are highly expressed in shrimp hemocytes but lower expression is observed in other tissues such as intestine, heart, gill, and lymphoid organ (Tassanakajon et al., unpublished data). However, some isoforms of P. monodon AMPs are specifically expressed in particular tissues which imply the specific functions or responses of those isoforms.

The crustin-likePm gene is highly expressed in P. monodon hemocytes and weakly expressed in gills and intestines. On the other hand, crustinPm5 gene is somehow expressed only in epipodites and eyestalks but not in hemocytes. In addition, crustinPm5 gene expression is found to be up-regulated in response to heat and hyperosmotic salinity stresses (Vatanavicharn et al., 2009). These data perhaps indicate the function of crustins other than antimicrobial activity as effectors or mediators in physiological stress responses.
SWDPms are all mainly expressed in hemocytes. SWDPm1 and SWDPm3 mRNA expressions are hemocyte specific whilst SWDPm2 transcripts are abundant in hemocytes but lower amounts are also found in gills and hepatopancreas. There is no SWDPm transcripts observed in the lymphoid organ, intestine and heart.

So far, all ALFPm isoforms are shown to be specifically expressed in hemocytes with variation in the expression level (Somboonwiwat et al., 2006; Somboonwiwat et al., 2008; Tharntada et al., 2008). Hemocyte is also the main site of penaeidin gene expression as reported in L. vannamei (Destoumieux et al., 2000). Expression analysis of F. chinensis PEN5 also reveals that it is constitutively expressed in hemocytes, heart, gills, intestine and ovary (Kang et al., 2007). In our study, P. monodon PEN3 is expressed in all tissues tested: hemocyte, lymphoid organ, gill, hepatopancreas, heart and intestine, with the highest level in hemocyte (Tassanakajon et al., unpublished data).

Shrimp is exposed to a variety of microorganisms in the environment throughout their developmental stages. During P. monodon development, it is found that all AMPs are expressed at the very early developmental stages starting from the nauplii stage to adult (Tassanakajon et al., unpublished data) (Fig. 1). The results suggest that the presence of AMPs is vital to the shrimp throughout their development.

Under pathogenic infections, P. monodon AMP genes have different expression patterns. Vagas-Albores et al. (2004) reported that the crustin isoform I mRNA of L. vannamei was down-regulated at 12 to 24 h post-Vibrio alginolyticus injection but the isoform P transcript was constantly expressed. On the contrary, the crustin-likePm was significantly

![Figure 1 Semi-quantitative RT-PCR analysis of expression of representative Penaeus monodon AMP genes: The whole shrimp at the different developmental stages: nauplius (NP), post-larvae 1(P1), post-larvae 30 (P30), 1-4 months old shrimp (M1-M4) were collected and total RNA was extracted. The first strand cDNA was then synthesized and used for semi-quantitative RT-PCR analysis of Crustin gene (A), Lysozyme gene (B), ALF gene (C) and PEN3 gene (D). These genes were amplified using the optimum concentration of specific primer pairs for 40 cycles. The β-actin gene (E) was used as an internal control.](image-url)
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up-regulated at 24 h post *V. harveyi* challenge (Amparyup et al., 2008b). SWDPm1 mRNA level was decreased sharply at 6 h after *Staphylococcus aureus* injection where no change in expression level of SWDPm2 and SWDPm3 was evident (Amparyup et al., 2008a). *V. harveyi* infection also causes rapid up-regulation of ALFPm3 gene expression in hemocytes (Somboonwiwat et al., 2006; Somboonwiwat et al., 2008).

Upon white spot syndrome (WSSV) infection, the expression of AMPs from *P. monodon* such as ALFPm2, ALFPm3, CrustinPm1, Crustin-likePm, SWDPm1-3, PEN3 and PEN5, was investigated by a semi-quantitative RT-PCR (Amparyup et al., 2008a; Tassanakajon et al., unpublished data). The data revealed that the expression of ALFPm3, ALFPm6, SWDPm1, SWDPm2 and PEN5 considerably was increased at different time points, whereas those of others were not dramatically changed. The results suggested the important role of *P. monodon* AMPs in antiviral immune responses.

**ANTIMICROBIAL ACTIVITY**

In order to study the biological activity of AMPs from *P. monodon*, the recombinant proteins of the major isoforms of each AMP family including ALFPm2, ALFPm3, crustinPm1, crustin-likePm, crustinPm5, SWDPm2, c-type lysozyme and i-type lysozyme, were produced and tested for their activities (Somboonwiwat et al., 2005; Supungul et al., 2008; Amparyup et al., 2008a, Amparyup et al., 2008b; Vatanavicharn et al., 2009). Antimicrobial assays demonstrated that almost all recombinant AMPs from *P. monodon* showed narrow spectrum of activity against specific target bacteria except for the recombinant ALFPm3 protein (rALFPm3) that showed wide range and strong activity against Gram-positive and Gram-negative bacteria and fungi. The recombinant proteins of crustinPm1, crustinPm5 and SWDPm2 exhibit only anti-Gram-positive bacteria whilst the recombinant crus-likePm has strong antimicrobial activity against both Gram-positive and Gram-negative bacteria. The preliminary data on the activity of *P. monodon* lysozyme indicated that both c-type and i-type lysozymes were active against Gram-negative bacterium *V. harveyi*. Moreover, the recombinant c-type lysozyme also inhibited the growth of a Gram-positive bacterium *Micrococcus luteus*. Inhibition study had revealed that the antimicrobial activities of ALFPm3 and crustinPm1 were a result of bactericidal effect. In addition, SWDPm2 also has antiproteinase activity against subtilisin A. It should be also emphasized herein that not all but several AMPs are active against *V. harveyi*, a major pathogenic bacteria in shrimp aquaculture.

In *vivo* neutralization activity and protective effects of rALFPm3 were recently reported in *P. monodon* challenged with *V. harveyi* (Ponprateep et al., 2009). It was found that *V. harveyi* at a lethal dose was completely neutralized by pre-incubation of live bacteria with rALFPm3 at the lowest concentration of 6.25 µM resulting in 100% shrimp survival after systemic challenge. The rALFPm3 injection into the shrimp followed by *V. harveyi* challenge clearly showed the reduction of the cumulative mortality of shrimp suggesting its potential prophylactic effect.
Besides the antibacterial activity, the antiviral property of rALFPm3 against the most severe pathogen in shrimp, the white spot syndrome virus (WSSV), was recently elucidated. WSSV propagation was significantly reduced after rALFPm3 was added together with WSSV into the crayfish hematopoietic cell cultures. The rALFPm3 also exhibited WSSV neutralization activity resulted in inhibition of WSSV replication in *P. monodon* (Tharntada *et al.*, 2009). In addition, the rALFPm3 was shown to be significantly active against non-shrimp pathogenic DNA viruses: herpes simplex virus types 1 (HSV-1) and human adenovirus respiratory strain (AdV-5), in mammalian cell lines (Carriel-Gomes *et al.*, 2007).

**CONCLUSIONS**

From the current information, it has been found that diverse classes of AMPs are produced in *P. monodon*. The target specificity and strength of antimicrobial activity are diverged among the AMPs. Variable in the expression pattern in response to infection has been observed among AMPs indicating perhaps the different mechanisms of antimicrobial action. These AMPs play crucial roles in shrimp innate immunity enabling the shrimp to survive in nature that normally contains the varieties of pathogenic microorganisms.

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