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* (crustin*Pm*s) 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 (crustin*Pm*s1-

Table 1
Types, number of sequences, diversity and antimicrobial activities of the *Penaeus monodon* AMPs from the EST database (http://pmonodon.biotec.or.th)

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

*ND is no data available.

6 and crustin-like*Pm*) whereas Type III crustin or single WAP domain containing protein (SWD) comprises three isoforms of SWD (SWD*Pm*s 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*Pm*s 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*Pm*s was clearly observed (Tassanakajon *et al*., 2008). Recent data showed that crustin*Pm*s 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*Pm*5 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

the crustin*Pm*5 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 SWD*Pm* is composed of 3 subgroups (SWD*Pm*1, SWD*Pm*2 and SWD*Pm*3) (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 SWD*Pm*2 (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 (ALF*Pms*) constitute at least five different isoforms (Supungul *et al.,* 2002) which are grouped into groups A

(ALF*Pm*1 and 2) and B (ALF*Pm*3-5), derived from different genomic loci, according to nucleotide sequence analysis (Tharntada *et al*., 2008). Group A ALF*Pm* 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, ALF*Pm*s 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). ALF*Pm* 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 ALF*Pm* 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 ALF*Pm* 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. mondon* 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-like*Pm* gene is highly expressed in *P. monodon* hemocytes and weakly expressed in gills and intestines. On the other hand, crustin*Pm*5 gene is somehow expressed only in epipodites and eyestalks but not in hemocytes. In addition, crustin*Pm*5 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.

SWD*Pm*s are all mainly expressed in hemoctyes. SWD*Pm*1 and SWD*Pm*3 mRNA expressions are hemocyte specific whilst SWD*Pm*2 transcripts are abundant in hemoctyes but lower amounts are also found in gills and hepatopancreas. There is no SWD*Pm* transcripts observed in the lymphoid organ, intestine and heart.

So far, all ALF*Pm* 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-like*Pm* was significantly

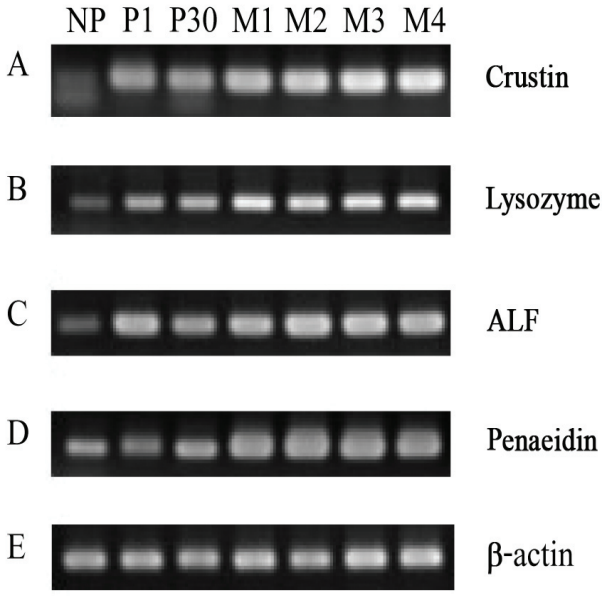

Figure 1 Semi-quantitative RT-PCR analysis of expression of representative *Penaeus monodon* AMP genes: The whole shimp at the different developmental stages: nauplius (NP), post-larvae 1(P1), post-larvae 30 (P30), 1-4 months old shrimp (M1-M4) were collected and total RNA was extracted. The first strand 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.

up-regulated at 24 h post *V. harveyi* challenge (Amparyup *et al.*, 2008b). SWD*Pm*1 mRNA level was decreased sharply at 6 h after *Staphylococcus aureus* injection where no change in expression level of SWD*Pm*2 and SWD*Pm*3 was evident (Amparyup *et al.*, 2008a). *V. harveyi* infection also causes rapid up-regulation of ALF*Pm*3 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 ALF*Pm*2, ALF*Pm*3, Crustin*Pm*1, Crustin-like*Pm*, SWD*Pm*1-3, 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 ALF*Pm*3, ALF*Pm*6, SWD*Pm*1, SWD*Pm*2 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 ALF*Pm*2, ALF*Pm*3, crustin*Pm*1, crustin-like*Pm*, crustin*Pm*5, SWD*Pm*2, c-type lysozyme and i-type lysozyme, wwere 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 ALF*Pm*3 protein (rALF*Pm*3) that showed wide range and strong activity against Gram-positive and Gram-negative bacteria and fungi. The recombinant proteins of crustin*Pm*1, crustin*Pm*5 and SWD*Pm*2 exhibit only anti-Gram-positive bacteria whilst the recombinant crus-like*Pm* 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 ALF*Pm*3 and crustin*Pm*1 were a result of bactericidal effect. In addition, SWD*Pm*2 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 rALF*Pm*3 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 rALF*Pm*3 at the lowest concentration of 6.25 μM resulting in 100% shrimp survival after systemic challenge. The rALF*Pm*3 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 rALF*Pm3* against the most severe pathogen in shrimp, the white spot syndrome virus (WSSV), was recently elucidated. WSSV propagation was significantly reduced after rALF*Pm3* was added together with WSSV into the crayfish hematopoietic cell cultures. The rALF*Pm3* also exhibited WSSV neutralization activity resulted in inhibition of WSSV replication in *P. monodon* (Tharntada *et al.,* 2009). In addition, the rALF*Pm3* 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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