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Biotechnology of Marine Invertebrates —Recent Advances in Shrimp and Shellfish

Anchalee Tassanakajon,[1] Tipachai Vatanavicharn,[1] Premruethai Supungul,[1,2] Sureerat Tang,[2] Piti Amparyup,[1,2] Kunlaya Somboonwiwat,[1] Sirinit Tharntada,[1] Jun Takahashi[3] and Haruhiko Toyohara[3]*

[1]*Shrimp Molecular Biology and Genomics Laboratory*
Department of Biochemistry, Faculty of Science
Chulalongkorn University, Bangkok 10330, Thailand

[2]*National Center for Genetic Engineering and Biotechnology (BIOTEC)*
National Science and Technology Development Agency (NSTDA)
Pathumthani 12120, Thailand

[3]*Division of Applied Biosciences, Graduate School of Agriculture*
Kyoto University, Kyoto 608-8502, Japan

*E-mail: toyohara@kais.kyoto-u.ac.jp

Marine invertebrates possess a variety of interesting functions and some of them have been well studied from the standpoint of biotechnology. Shrimp and shellfish are useful animals among marine invertebrates. In this section, we describe the recent advances in biological defense system of shrimp and the biomineralization system of shellfish as examples of which molecular mechanisms have been well elucidated.

First, we focus on shrimp antimicrobial peptide (AMPs) which are considered to play a major role in the innate immunity against invading microbes. A total of 767 ESTs encoding putative AMPs were identified in the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th), which were comprised of homologues of the three AMP families; penaeidins, crustins and antilipopolysaccharide factors. Sequence analysis revealed that each AMP family had sequence diversity and constituted multiple isoforms or subgroups. The relevance of this sequence variability to the potential antimicrobial function was investigated. These shrimp AMPs with high selectivity to severe pathogens are potential candidates as an alternative to antibiotics in aquaculture.

As a second example, we present the recent advances in the molecular mechanism of shell formation. A variety of animals and plants have the ability to make hard biological structure called biominerals made of calcium, silicon and other minerals. Molluscan shell is a typical biomineral composed of $CaCO_3$, but a small amount of organic matrix proteins included in the shell direct the crystal growth that is specific for the shell species. Ca^{2+} adsorbed through specific transporters and HCO_3^{2-} synthesized from CO_2 are mixed under well regulated conditions, thereby forming shell.

KEYWORDS antilipopolysaccharides; antimicrobrial peptides; biomineral; calcification; crustins; penaeidins; shell

1. Shrimp Antimicrobial Peptides: Sequence Diversity and Functional Characteristics of Different Isoforms

1.1. Introduction

Organisms lacking an adaptive immunity rely on antimicrobial peptides (AMPs) as the first line of defense against invading pathogens (Hancock and Diamond 2000; Jenssen *et al.* 2006). AMPs are typically small cationic molecules (15 to 100 amino acids) which are considered to play an important role in the innate immune system. They are widely distributed in the whole living kingdom ranging from bacteria to plants, invertebrate and vertebrate species, including mammals. They display a broad spectrum of activity against bacteria, fungi, viruses, parasites and even tumor cells. Although they differ in amino acid sequences, most AMPs adopt an amphipathic secondary structure that is believed to be essential for their antimicrobial action.

Numerous AMPs are found in invertebrates, particularly in insects, where they play a major role in protection from invading pathogens (Bulet and Stocklin 2005). In crustaceans, AMPs were first described in the hemocytes of the shore crab *Carcinus maenas* (Schnapp *et al.* 1996) as a 6.5 kDa proline-rich cationic protein displaying activity against both gram-positive and gram-negative bacteria. Not until 1997 was the first shrimp AMP family, named penaeidin, discovered in the Pacific white shrimp, *Litopenaeus vannamei*, by means of reverse-phase chromatography (Destoumieux *et al.* 1997). Subsequently, penaeidin sequences have been found in several penaeid shrimps by genomic approaches (Cuthbertson *et al.* 2002; Kang *et al.* 2004; Supungul *et al.* 2004). The other two shrimp AMP families, the crustins and antilipopolysaccharide factors (ALFs), have been identified from haemocyte cDNA libraries of several shrimp species. The crustins, which are homologues of the shore crab 11.5 kDa antibacterial protein, were identified by expressed sequenced tag (EST) analysis from *L. vannamei* and *L. setiferus* (Bartlett *et al.* 2002; Vargas-Albores *et al.* 2004). Since then, crustins and crustin-like peptides have been identified from a variety of other species (Rattanachai *et al.* 2004; Hauton *et al.* 2006; Brockton *et al.* 2007; Jiravanichpaisal *et al.* 2007; Zhang *et al.* 2007; Supungul *et al.* 2008). Antilipopolysaccharide factors (ALFs), first reported in horseshoe crabs (Morita *et al.* 1985; Aketagawa *et al.* 1986; Muta *et al.* 1987), have also been identified in shrimps (Supungul *et al.* 2004; Liu *et al.* 2005; Somboonwiwat *et al.* 2005). Moreover, an anionic antimicrobial peptide, astacidin, which is derived from the limited proteolysis of hemocyanin, has been reported in *L. vannamei* (Destoumieux-Garzón *et al.* 2001). In this paper, we present a recent finding on the sequence diversity of cationic AMPs identified from the black tiger shrimp *Penaeus monodon* database (http://pmonodon.biotec.or.th), their antimicrobial properties and potential application for disease control in aquaculture.

1.2. AMPs identified from the *Penaeus monodon* EST Database

As of December, 2007, the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th) contained a total of 40,001 EST sequences. Approximately 30,000 additional ESTs were obtained after the first establishment of the database according to the report by Tassanakajon *et al.* (2006). Clustering and sequence assembly identified 10,536 unique sequences represented by 3,227 contigs and 7,309 singletons. Each unique sequence was subjected to similarity searches against the GenBank database using BLASTx and BLASTn with an e-value cut off of $<10^{-4}$. Of 10,536 unigenes, 5,648 (53.6%) showed a significant match whereas 4,888 (46.4%) did not match to any sequences in the database.

Among the known (annotated) genes, a total of 767 clones were identified as putative antimicrobial proteins. They were homologues of the three AMP families; penaeidins (284 clones), crustins (275 clones) and antilipopolysaccharide factors (208 clones). Sequence analysis revealed that each family of AMPs displays sequence diversity and constitutes multiple isoforms or subgroups (Table 1). The sequence and functional diversity of the different isoforms of *P. monodon* AMPs are described below.

1.3. Penaeidins

The shrimp AMPs in the penaeidin family contain a unique structure comprised of an N-terminal proline-rich domain and a C-terminal cysteine-rich domain with six conserved cysteine residues engaged in three disulfide bonds. Penaeidins 1, 2 and 3 (PEN1, -2 and -3) were first isolated from haemocytes of the white shrimp *L. vannamei* (Destoumieux *et al.* 1997). Subsequent phylogenetic analysis indicated that *Litvan*PEN1 is a variant of *Litvan*PEN2 (Cuthbertson *et al.* 2002). To date, penaeidin sequences have been identified from different penaeid shrimps by genomic approaches and can be classified into four different subgroups (PEN2, -3, -4 and -5) based on their primary sequence diversity. The conserved key residues that appear to be a signature for PEN-2, -3 and -4 have been established in PenBase, http://www.penbase.immunaqua. com (Gueguen *et al.* 2006) while the distinct sequence characteristics of PEN-5, recently identified in *Fenneropenaeus chinensis*, have also been described by Kang *et al.* (2007).

From the *Penaeus monodon* EST database, 284 ESTs representing penaeidins were identified and subjected to sequence analysis. Based on the sequence lengths and the conserved signature residues, they were classified into two different subgroups: *Penmon*PEN3 and -5 (Fig. 1). The major penaeidin sequences (260 clones) found were classified as PEN3 whereas PEN5 sequences (24 clones) were found much less frequently. *Penmon*PEN3 and -5 sequences encode predicted peptides of 74 and 79 amino acid residues, respectively, including a signal peptide of 19 amino acids. All *Penmon*PEN3 predicted peptide sequences possess the following signature: Gln^{1}, Gly^{5}, Arg^{13} Gly^{18}, Ser^{35}, His^{37}, Gln^{43} and Ala^{46}, similar to the conserved key residues of PEN3 described by Gueguen *et al.* (2006). However, *Penmon*PEN5 sequences do not contain all the reported conserved key residues (Gln^{1}, Ser^{5}, Arg^{13}, Ser^{18}, Arg^{37}, Gln^{43} and Ala^{46}) proposed by Kang *et al.* (2007), but show

Table 1. The abundance of putative antimicrobial peptides in the *Penaeus monodon* EST database (http://pmonodon.biotec.or.th).

Antimicrobial peptide	Frequency
Antilipopolysaccharide factors	**208**
ALF *Pm*1	1
ALF *Pm*2	2
ALF *Pm*3	184
ALF *Pm*4	1
ALF *Pm*5	2
ALF *Pm*6	16
ALF *Pm*7	2
ALF *Pm*8	1
Crustins	**275**
crustin *Pm*1	55
crustin *Pm*2*	2
crustin *Pm*3	1
crustin *Pm*4	158
crustin *Pm*5	1
crustin *Pm*6	2
crustin *Pm*7 (crus-like *Pm*)	56
Penaeidins	**284**
Penmon Pen3	260
Penmon Pen5	24

*not full length

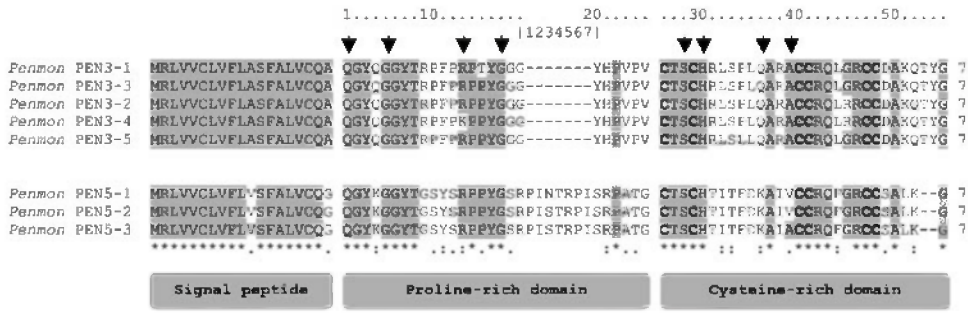

Fig. 1. Predicted amino acid sequence comparison of PEN3, -5 and their variants from *Penaeus monodon*. The full sequences of the penaeidins were aligned using ClustalW and gaps were introduced to optimize the alignment. Cysteines are in boldface, identical residues and conservative replacements are shaded. The asterisk (*) indicates amino acid identify and (.) and (:) indicate amino acid similarity. The arrows indicate conserved residues of penaeidin subgroups.

variations at Gly[5] and His[37] which are identical to the residues in PEN3, and at Ser[18] which is a specific residue in the subgroup 5 and was found in all *Penmon*PEN5 sequences. Variants of *Penmon*PEN3 (*Penmon*PEN3-1 to *Penmon*PEN3-5) and *Penmon*PEN5 (*Penmon*PEN5-1 to *Penmon*PEN5-3) show polymorphisms at four and two amino acid residues, respectively (Fig. 1). It should also be noted that the penaeidin-5 cDNA from *P. monodon* reported previously by Hu *et al.* (2006) is identical to *Penmon*PEN3-3 reported here. According to the conserved key residues for PEN3 proposed in PenBase by Gueguen *et al.* (2006) and those proposed for PEN5 by Kang *et al.* (2007), this cDNA sequence should be classified as a PEN3 isoform and not PEN5. Analysis of penaeidin sequences through PenBase, http://www. penbase. immunaqua.com, would allow a systematic nomenclature and classification of the penaeidin family and prevent confusion with the increasing number of independently derived penaeidin sequences from a variety of shrimp species.

In *L. vannamei* and *L. setiferus*, genes from each of the three penaeidin subgroups (PEN2, -3, and -4) have been found and shown to be expressed in a single individual (Cuthbertson *et al.* 2008), whilst in *P. monodon* only two subgroups (PEN3 and -5) were found with the expression level of the later being much less abundant. This probably indicates that *Penmon*PEN5 is not constitutively expressed but rather may be regulated in response to certain physiological stimuli. Further expression studies are needed to test this notion.

In general, penaeidins possess antibacterial activity predominantly directed against gram-positive bacteria and antifungal activity against filamentous fungi. PEN2 and -3 from *L. vannamei* both exhibit similar antimicrobial properties although PEN3 is more effective at low concentrations (Destoumieux *et al.* 1999). In *L. setiferus*, the antimicrobial properties of PEN3 and -4 were compared and it was found that *Litset*PEN3 has a broader range of microbial targets whilst *Litset*PEN4 is generally more effective against fungi (Cuthbertson *et al.* 2004). *Fenchi*PEN 5 displayed activities against gram-positive and gram-negative bacteria and fungi (Kang *et al.* 2007). Since antimicrobial properties of the four different subgroups of penaeidins have been well characterized by several research groups, we do not further characterize *Penmon*PEN3 and -5. The structure and function of penaeidins

have been subjected to extensive review elsewhere (Bachère *et al.* 2000; Cuthbertson *et al.* 2008).

1.4. Crustins

Crustins are homologues of 'carcinin', an 11.5 kDa antibacterial protein from the shore crab *Carcinus maenus*. The cDNA of crustins have been reported from a variety of crustaceans including *L. vannamei* (Bartlett *et al.* 2002; Vargas-Albores *et al.* 2004), *L. setiferus* (Bartlett *et al.* 2002), *P. monodon* (Supungul *et al.* 2004), *Marsupenaeus japonicus* (Rattanachai *et al.* 2004), *Fenneropenaeus chinensis* (Zhang *et al.* 2007), *Panulirus argus* (Stoss *et al.* 2004), *Homarus gammarus* (Hauton *et al.* 2006), *C. maenas* (Brockton *et al.* 2007) and *Pacifastacus leniusculus* (Jiravanichpaisal *et al.* 2007). Those crustins that have been described to date have diverse amino acid sequences but with a relatively conserved C-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 (Grütter *et al.* 1988). The WAP domain-containing proteins are widespread throughout vertebrates and invertebrates and have diverse biological functions including antibacterial activity and protease inhibitory activity (Sallenave 2000; Hagiwara *et al.* 2003).

From the *Penaeus monodon* EST database, 275 ESTs representing crustins were identified. Clustering analysis revealed the presence of five isoforms including the four isoforms reported previously (Crus*Pm*1–4) (Supungul *et al.* 2004). The amino acid sequence alignment of the full length sequences of all isoforms except crustin*Pm*2 suggested that isoform 3 is a subset of isoform 4. Each isoform was found at a different frequency (Table 1), and sequence variation was also observed within each isoform suggesting both differential expression and potential functional polymorphisms. The crustin isoforms are very different in length and primary sequence and they are the most diverged representatives of the AMP families in the species. Alignment of the crustin sequences revealed very diverse amino-terminal signal peptide and glycine-rich regions whilst the carboxyl-terminal region, where the WAP domain resided, is more conserved (Fig. 2). Crustin*Pm*4, one of the major isoforms, contained a very long glycine-rich repeat and this isoform is 92% identical at the amino acid sequence level to the *P. monodon* crustin reported by Chen *et al.* (2004). All isoforms contain 12 conserved cysteine residues in the C-terminus that participate in the formation of six disulfide bonds. The phylogenetic analysis of WAP domains from various invertebrate WAP domain-containing proteins showed a high diversity and could be divided into three distinct groups (Fig. 3). The first group composed of crustacean single WAP proteins. The second group contained crab carcinins, lobster and crayfish crustins. The last group contained all the shrimp crustins including all isoforms of *P. monodon* crustins. However, Crustin*Pm*7, later named the crus-like*Pm*, is quite distinct from the other isoforms and formed a subgroup with a crustin-like protein *Fc*1 from *F. chinensis* (Zhang *et al.* 2007). The difference of the WAP domains may indicate different biological activity of the proteins.

Expression analysis in various shrimp tissues revealed different patterns of expression of different crustin isoforms. For example, crustin*Pm*1 was expressed in all tissues examined including haemocytes, lymphoid organs, gill, intestine, eyestalk, hepatopancreas, epipodite and antennal gland whereas the crutin*Pm*5 transcript was observed only in epipodite and eyestalks (data not shown). The difference in tissue distribution of the crustin transcripts probably indicates diverse functions of crustins as immune effectors,

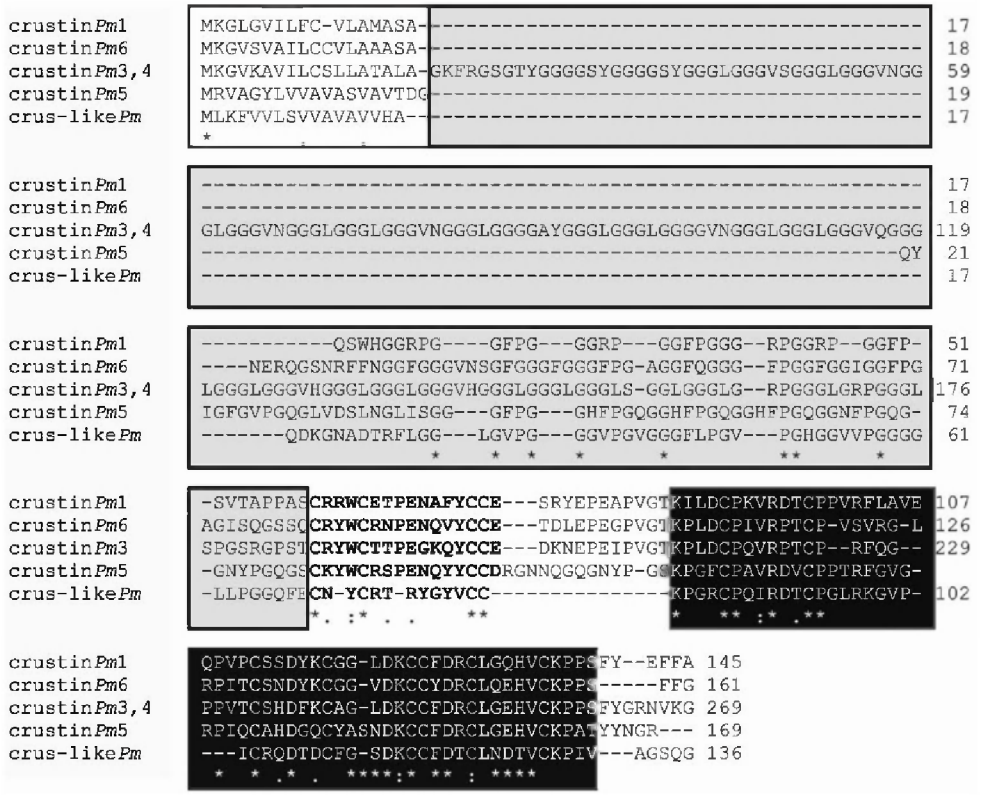

Fig. 2. Multiple alignment of deduced amino acid sequences of *Penaeus monodon* crustins. The asterisk (*) indicates amino acid identify and (.) and (:) indicate amino acid similarity. The signal peptides, glycine rich regions, and WAP domains are indicated by white, gray, and black boxes, respectively. The bolds letters correspond to the cysteine rich regions.

although multifunctional roles in development or differentiation cannot be excluded.

To further investigate the biological function of *P. monodon* crustins, three isoforms (crustin*Pm*1, -5 and crus-like*Pm*) were selected for the production of recombinant proteins in the *E. coli* system. The recombinant proteins expressed mainly in insoluble inclusion bodies were solubilized, purified and characterized for their antibacterial activity against several strains of gram-positive and gram-negative bacteria. Recombinant crus-like*Pm* showed the broadest spectrum of activity against various strains of bacteria including all five tested gram-positive bacteria (*Staphylococcus aureus*, *S. haemolyticus*, *Aerococcus viridans*, *Bacillus megaterium* and *Micrococcus luteus*) with MIC values ranging from 0.312 to 10 μM, and five of the six gram-negative bacteria (Table 2). However, potency against gram negative bacteria ranged from strong (*Escherichia coli* 363 and *Vibrio harveyi* with MIC values ranging from to 2.5 to 5 μM), intermediate (*Klebsiella pneumoniae*) to weak or no activity (*E. cloacae* and *S. thyphimurium*). In contrast, the other two isoforms were far less active (Table 2). Recombinant crustin*Pm*1 was active against gram-positive bacteria with the highest activity against *S. aureus* (MIC of 3.13 to 6.25 μM) and no detectable activity against

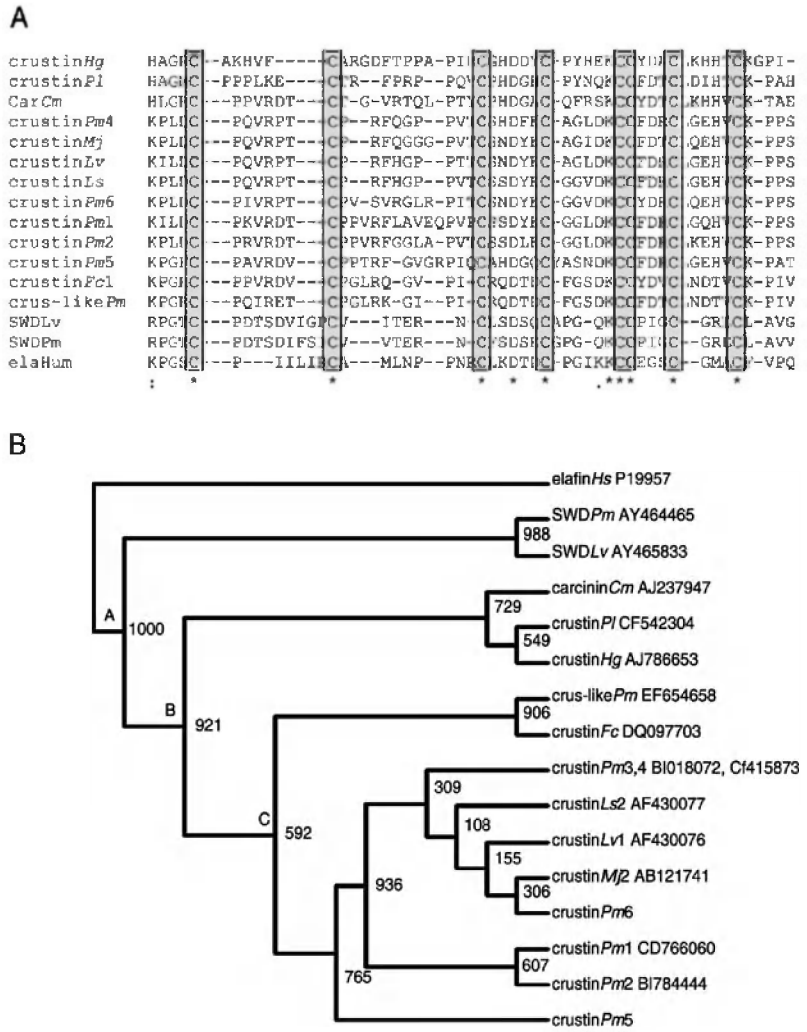

Fig. 3. Sequence alignment of the WAP domains in *Penaeus monodon* crustins and other crustacean WAP domain-containing proteins. SWD, single-whey acidic domain; *Hs*, *Homo sapiens*; *Pm*, *Penaeus monodon*; *Lv*, *Litopenaeus vannamei*; *Cm*, *Carcinus maenas*; *Pl*, *Pacifastacus leniusculus*; *Hg*, *Homarus gammarus*; *Fc*, *Fenneropenaeus chinensis*; *Ls*, *Litopenaeus setiferus*; *Mj*, *Marsupenaeus japonicus*. (**A**) ClustalW method. An asterisk indicates amino acid identity. Conserved Cys are shadowed and boxed. (**B**) Rooted phylogenetic tree constructed by Neighbor-Joining tree method (bootstrap = 1000). WAP domain of *Homo sapiens* elafin (elafin*Hs*) is defined as the out group.

five of the six gram-negative bacteria tested and a weak activity against *E. coli* 363 (MIC of 50 to 100 μM). Recombinant crustin*Pm*5 was only active against three of the six strains of gram-positive bacteria (*S. aureus*, *S. haemolyticus* and *M. lateus*) with MIC values ranging from 1.56 to 50 μM. Further studies on the mechanism of inhibition of crustin*Pm*1 (Supungul *et al.* 2008) and crustin-like *Pm* (Amparyup 2008) against bacteria has revealed crustins to have bactericidal effects. Besides the antimicrobial

Table 2. Antimicrobial activities of crus-like*Pm*, crustin*Pm*1 and crustin*Pm*5 peptides against gram-positive and gram-negative bacteria in a liquid growth inhibition assay

Microorganism	MIC value (mM)		
	crustin *Pm*1	crus-like *Pm*	crustin *Pm*5
Gram (+) bacteria			
Aerococcus viridans	50–100	0.312–0.625	>100
Staphylococus aureus	3.13–6.25	5–10	0.78–1.56
Staphylococus haemolyticus	50–100	2.5–5	12.5–25
Micrococcus luteus	25–50	2.5–5	25–50
Bacillus megaterium	6.25–12.50	1.25–2.5	>100
Gram (–) bacteria			
Enterobacter cloacae	>100	>100	>100
Klebsiella pneumoniae	>100	10–20	>100
Salmonella thyphimurium	>100	>100	>100
Escherichia coli 363	50–100	2.5–5	>100
Erwinia carotovora	>100	ND	>100
Vibrio harveyi 639	>100	2.5–5	>100

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 caused 100% growth inhibition. ND = not determined

activity, the WAP domain is also recognized as a signature motif for a serine protease inhibitor. However, the recombinant crustin*Pm*1, -5 and crus-like*Pm* do not posses a detectable protease inhibitory activity (data not shown).

Most crustins reported so far are active against only gram-positive bacteria, excepting the crus-like*Pm* which also kills some gram-negative bacteria. Although it is quite clear that crustins are antimicrobial proteins, some isoforms which showed low killing activity may be engaged in other biological functions, or else require different physiological conditions or partners to be active. Variations in the response of crustin expression to various pathogens have been observed in several shrimps (Supungul *et al.* 2004; Vargas-Albores *et al.* 2004; Amparyup *et al.* 2008) as well as in *H. gammarus* (Hauton *et al.* 2006) and *P. leniusculus* (Jiravanichpaisal *et al.* 2007). Moreover, we found that the expression of crustin*Pm*5, which is a novel crustin recently isolated from a gill-epipodite cDNA library of *P. monodon* was significantly up-regulated under heat stress when the animals were maintained at 35°C, compared to those at 30°C (unpublished data). Inconsistent patterns of correlation between expression of crustin isoforms in response to microorganisms and the response of some crustin isoforms to other physiological stress may indicate potential other functions for these proteins outside of immunity. Further investigation is required to elucidate the true functions of various crustins from crustacean species.

1.5. Antilipopolysaccharide factors

Antilipopolysaccharide factor (ALF) is a small basic protein originally isolated from hemocytes of the horseshoe crab, *L. polyphemus*, (LALF) and *Tachypleus tridentatus* (TALF) (Tanaka *et al.* 1982; Aketagawa *et al.* 1986). LALF binds to and

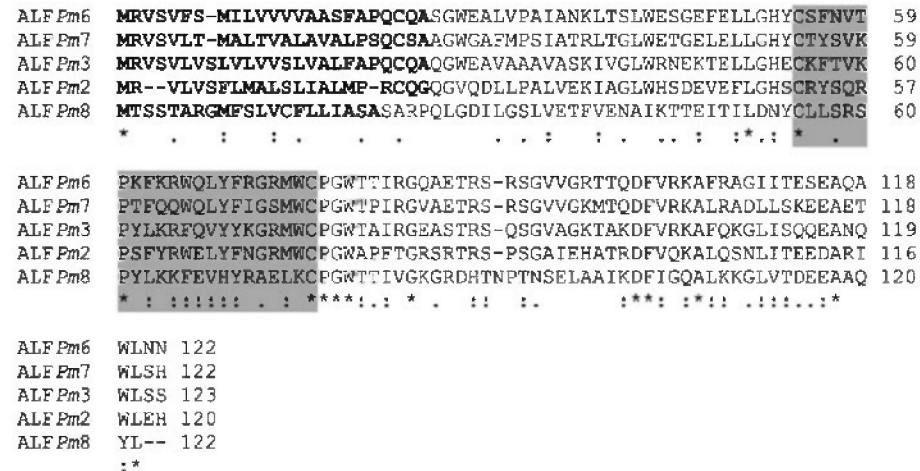

Fig. 4. Comparison of the deduced amino acid sequences of ALF cDNAs. The asterisk (*) indicates amino acid identity and (.) and (:) indicate amino acid similarity. The bold letters represent the putative signal sequences. The gray box indicates the putative LPS-binding site.

neutralizes LPS and exhibits an anti-bacterial effect on the growth of the gram-negative bacteria *Salmonella minnesota* but not on the gram-positive *S. aureus* (Morita *et al.* 1985). Since, the discovery of horseshoe crab ALFs, various lipopolysaccharide-binding proteins and derivatives have attracted great interest as candidate therapeutic agents for the management of septic shocks (Vallespi *et al.* 2000).

In penaeid shrimps, the cDNAs of ALF were identified and characterized in *Penaeus monodon* (Supungul *et al.* 2002, 2004), *L. setiferus* (Gross *et al.* 2001), *F. chinensis* (Liu *et al.* 2005) and *M. japonicus* (Nagoshi *et al.* 2006). Five different ALF sequences (ALF*Pm*1–ALF*Pm*5) previously reported in *P. monodon* could be divided into two groups: the ALF*Pm*1 and 2 as group A and the ALF*Pm*3, 4 and 5 as group B. Each group has a unique LPS binding site (CRYSQRPSFYRWELYFNGRMWC for group A and CKFTVKPYLKRFQVYYKGRMWC for group B). Recently, we showed that the two groups of *P. monodon* ALFs are encoded by different genomic loci (Tharntada *et al.* 2008) and that ALF*Pm*2 and 3 were the major or authentic ALFs in the haemocytes whereas the other isoforms may result from aberrant RNA splicing. In addition, sequence variation of the major isoform, ALF*Pm*3, has also been reported (Somboonwiwat *et al.* 2006).

From the *Penaeus monodon* database, 208 ESTs representing candidate ALF sequences were identified. Clustering analysis indicated the presence of five isoforms, and three of them (ALF*Pm*6, -7 and -8) are novel ALF*Pm* isoforms rather than aberrant RNA splices. Sequence alignment showed that all ALF sequences contained a signal peptide at the N-terminus and a predicted LPS binding site with clustered positive charges between two conserved cysteine residues (Fig. 4). Interestingly, each isoform contained quite a distinct putative LPS-binding site which suggested that they each might have a different ability to bind the microbial cell wall components or to different LPS isoforms.

ALF*Pm*2 and -3 isoforms were expressed as recombinant proteins in the yeast *Pichia pastoris* for further characterization of their

Table 3. Antimicrobial activities of ALF*Pm*3, *Livan*PEN-3 and crus-like*Pm* against gram-positive and gram-negative bacteria in a liquid growth inhibition assay

Microorganism	MIC [μM]		
	ALF*Pm*3	*Livan*PEN3 (Destoumieux *et al.* 1999)	crus-like*Pm*
Gram-positive bacteria			
Aerococcus viridans	1.56–3.12	0.3–0.6	0.312–0.625
Bacillus megaterium	0.19–0.39	2.5–5	1.25–2.5
Micrococcus luteus	1.56–3.12	1.25–2.5	2.5–5
Staphylococcus aureus	50–100	>40	5–10
Staphylococus haemolyticus	ND	ND	2.5–5
Gram-negative bacteria			
Enterobacter cloacae	3.12–6.25	>40	>100
Erwinia carotovora	1.56–3.12	>40	ND
Escherichia coli 363	0.095–0.19	5–10	2.5–5
Klebsiella pneumoniae	3.12–6.25	>40	10–20
Salmonella thyphimurium	6.25–12.5	>40	>100
Vibrio alginolyticus	0.39–0.78	>40	ND
Vibrio harveyi	0.78–1.56	>40	2.5–5
Vibrio penaeicida	25–50	>40	ND
Fungi			
Botrytis cinerea	3.12–6.25	5–10	ND
Fusarium oxysporum	1.56–3.12	5–10	ND

MIC are expressed as the interval a–b, where a = the highest concentration tested at which microorganisms are growing, and b = the lowest concentration that caused 100% growth inhibition. ND = not determined

antimicrobial properties. Overexpression of rALF*Pm*3 but not rALF*Pm*2 was attained. The purified rALF*Pm*3 and the crude protein of rALF*Pm*2 were then used for further characterization. The rALF*Pm*3 has a broad activity spectrum with anti-fungal properties against both tested filamentous fungi, and anti-bacterial activities against both gram-positive (4/4 tested species) and gram-negative (8/8 tested species) bacteria, with high potency against the natural shrimp pathogens, *V. harveyi* (MIC of 0.78 to 1.56 μM) and *V. alginolyticus* (MIC of 0.39 to 0.78 μM) (Table 3). The bactericidal activity against *E. coli* 363 and *B. megaterium* was also evidenced (Somboonwiwat *et al.* 2005). The synthetic peptide corresponding to a part of the LPS binding site of ALF*Pm*3 was shown to only exhibit activity against gram-positive bacteria suggesting the involvement of the full molecule for activity against gram-negative bacteria. The crude protein of rALF*Pm*2 was also active against the gram-positive bacteria, *B. megaterium*, and the gram-negative bacteria, *E. coli* 363 (data not shown). However, it would be interesting to compare the antimicrobial properties between the two isoforms once sufficient rALF*Pm*2 is available. ALF cDNAs have been identified in other shrimps but their antimicrobial properties remain to be characterized. Nevertheless, amongst the three families of shrimp AMPs, the ALF displayed the broadest spectrum of antimicrobial activities including against natural shrimp pathogens. The other two shrimp AMP

families, crus-like*Pm* and *Livan*PEN-3 revealed lower activities (*Livan*PEN-3) or a reduced target spectrum (crus-like*Pm*) under these conditions compared to ALF*Pm*3 (Table 3). Moreover, it has recently been shown by RNA interference (RNAi) in both whole animals and in cell cultures that the crayfish ALF can interfere with the replication of white spot syndrome virus (Liu *et al.* 2006). The exciting potential antiviral property of rALF*Pm*3 is now under investigation, but clearly signals the need for a more extensive screening of other host proteins including ALF proteins for anti-viral activity against the suite of shrimp viral pathogens.

1.6. The potential use of antimicrobial peptides for disease control in aquaculture

Aquatic animals including shrimps live in an environment enriched with pathogenic organisms and, therefore, antibiotics are commonly used in aquaculture to prevent or treat disease outbreaks. Most farmers use antibiotics prophylactically, some on a daily basis. In addition to the elevated costs, the continuous use of antibiotics has resulted in residues of varying concentrations of antibiotics in the water and mud, and subsequently, the development or selection for antibiotic resistance in bacteria in the environment. Moreover, there could be an adverse effect on species biodiversity which ultimately may affect the cultureed shrimps, humans and environment.

Antimicrobial peptides provide a good therapeutic alternative for the treatment of diseases in aquaculture. They are natural molecules with a broad spectrum of activity against a wide range of microorganisms, are easy to produce, and are less prone to induce resistance. Some antimicrobial peptides are already in clinical and commercial use (Reddy *et al.* 2004) but their use for disease control in aquaculture has yet to be demonstrated. However, it is quite promising that the shrimp AMPs reported here could be potential candidates as an alternative to antibiotics in shrimp farming. As shown here, rALF*Pm*3 possess broad spectrum of antimicrobial activity and effectively kills the shrimp pathogen, *V. harveyi*, at a MIC of 0.78 to 1.56 μM whereas the commonly used antibiotic in aquaculture, oxytetracyline kills the bacteria at a MIC of 72.1 to 90.3 μM (Vaseeharan *et al.* 2005). More recently, the shrimp AMPs *Litvan*Pen3 and ALF*Pm*3 were tested for their antiviral activity against herpes simplex virus type 1, human adenovirus respiratory strain, and rotavirus SA11 (Carriel-Gomes *et al.* 2007). Both shrimp AMPs exhibited significant activity against HSV-1 although they were active at near cytotoxic concentrations. The activity of shrimp AMPs against shrimp viruses has not yet been clearly demonstrated due to the lack of shrimp cell lines. Nevertheless, their potential use in shrimp farming to overcome severe disease outbreaks is quite promising. Besides their antimicrobial function, AMPs are also known to act as mediators of inflammation influencing diverse processes such as cell proliferation, wound healing, cytokine release and immune induction (Zaiou 2007). The application of AMPs as a food additive to enhance shrimp immunity is another strategy to combat microbial infections. Evidently, the genes encoding these AMPs represent good candidates for the genetic improvement of shrimps for resistance to severe pathogens.

2. Biomineralization of Marine Organisms

2.1. Biomineralization

A variety of animals including bacteria, fungus, plants and animals have the ability to synthesize mineral crystals called biominerals. Calcium phosphate in vertebrate bone and teeth, calcium carbonate in mollusk shell and some sea algae, silica in sponges and diatoms, calcium sulfate in jellyfish, small magnetic particles in chitons and magnetic bacteria are well-known examples of biominerals.

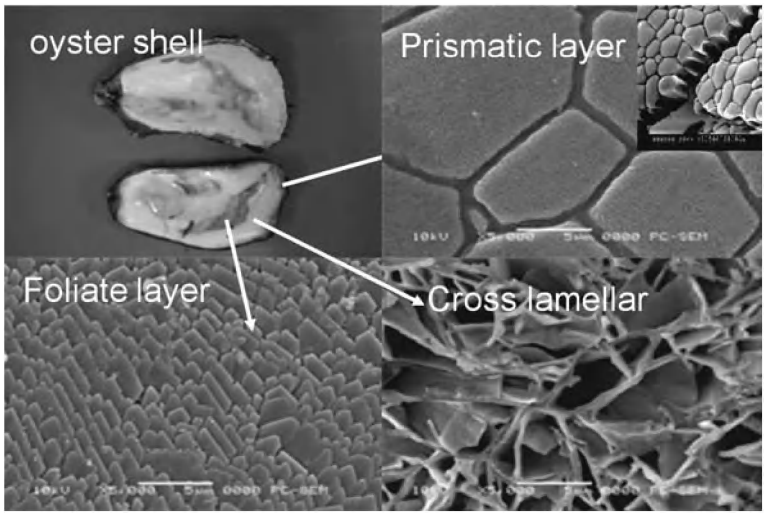

Fig. 5. Structure of oyster shell. Oyster shell is composed of prismatic layer, cross lamellar and foliate layer from outside to inside that are all made of $CaCO_3$. Small amount of organic matrix components possibly play important roles to produce the structure of crystal structures specific for each layer.

These biominerals function to maintain the framework of the body, besides regulation of the mineral balance in the body to function as a reservoir, recognition of the direction by detecting the earth magnet (biological magnets in birds), sense of hearing and body balance (statolith of fish and jellyfish), teeth of vertebrates and chitons.

A variety of biominerals have been utilized in human life: Coins, dishes and music apparatus, jewelries such as pearl, cameo and corals, industrial materials such as chalk, cement and marble, functional food additives such as chitin. Biominerals of fish statolith and fossil are biomarkers to evaluate the age of fish and era of the fossil.

In the 17th century, biominerals especially having economical value were studied by microscopic observation to judge the truth or fake. In the late 20th century, electron microscopy has been used for the observation, followed by the biochemical analysis of the components of the shell.

Synthesis of the shell proceeds under physiological condition, moderate temperature and pH, suggesting that there are so many merits in this system to be learned in the utilization for the implant of the bone and teeth in the medical field, production of light and tough materials in the industrial field.

2.2. Structure of shell

In this section, the author would like to explain the structure of the shell by taking oyster (*Crassostrea gigas*) as the example. Molluscan body consists of periostracum and ostracum. The structure of oyster shell is shown in Fig. 5. The periostracum that is not calcified and organic matrix covers the whole shell body. In the case of oyster, most of the periostracum is lost except for the edge of the shell.

The ostracum is calcified and separated into three parts, prismatic layer, cross lamellar and foliate layer from outside to inside. The prismatic layer is composed of column structure made of calcite. The cross lamellar, also called chalk, demonstrates the rough matrix structure formed with thin plate-like

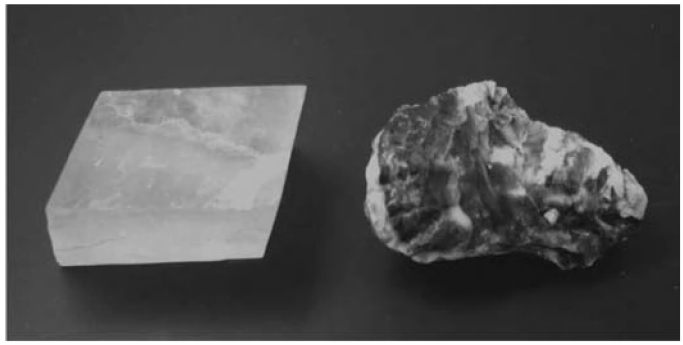

Fig. 6. Naturally obtained calcite (left) and oyster shell (right). Both have same crystal structure, but the apparent features are clearly distinctive.

crystal. Foliate layer is composed with distinct plate-like crystals accumulating each other.

Naturally, $CaCO_3$ forms calcite or aragonite crystals or otherwise amorphous structure. In the case of shell, most $CaCO_3$ crystal is calcite (Fig. 6), but the inner side of pearl oyster is aragonite, which produces rarely a pearl. Pearl is composed of thousands of $CaCO_3$ layer having a 0.4 μm thickness. When light shines into pearl layers, beautiful pink interfering color occurs by the mechanism of multiple layer interference. Pigments among $CaCO_3$ layers sometimes causes specific colors such as gold and black. The color of the bottom layer derived from organic materials sometimes causes a beautiful blue color.

After hatching, oyster larvae float 2–3 weeks and settle on the bottom of the sea with their left side shell down. Since most oysters live on the mud of estuaries under natural condition, they suffer from the change in mud condition according to the tidal change. They have selected two living strategy. One is that they settle on the other adult oyster of the prior generation to maintain the gill opened to the seawater. Another strategy is to lower the shell weight not to be buried in the mud. Chalk structure of the shell is helpful for them to lower the body weight. Due to their living strategy, the morphology of oyster is quite different from other bivalves.

2.3. Function of organic substances for biomineralization

Although $CaCO_3$ is a main constituent of the shell, morphology of the shell is highly species-specific (Fig. 7). In addition, the structure of the shell is different even in the same species as mentioned above. A possible assumption so far proposed is an implication of organic matrix compounds contained in the shell. Although the amount of the organic matrix component is very small (0.1–10%), these components possibly lead the shell structure specific for the shell species and portion specific of the shell even in the one species. The organic matrix components are roughly classified into soluble and insoluble fractions into water or EDTA solution. Generally, water or EDTA soluble fraction contains aspartate rich proteins and insoluble fraction contains glycine, serine and alanine rich proteins (Crenshaw 1996, Sudo 1997).

Calcification in the shell proceeds according to the following reaction.

$$Ca^{2+} + 2HCO_3^{2-} \rightarrow H_2O + CO_2 + CaCO_3$$

Ca^{2+} is adsorbed in the body through specific transporters expressed in the membrane of the epithelial cells of mantle, gill and

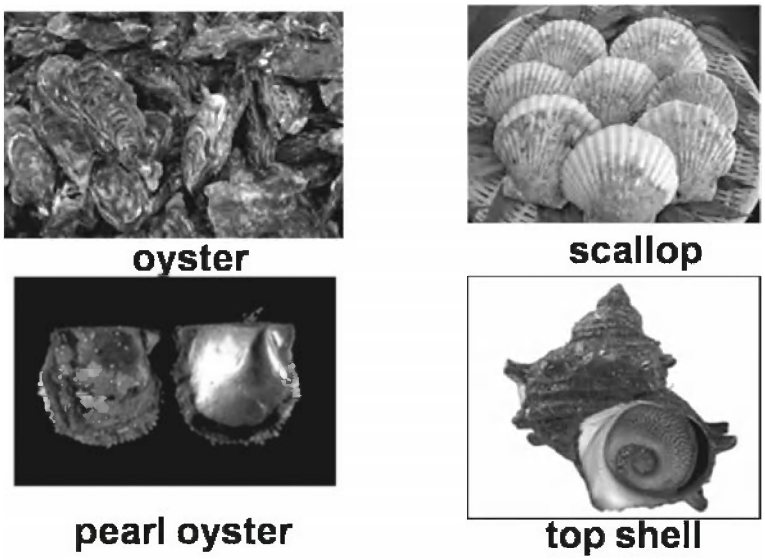

Fig. 7. Species specific structure of mollusk shells. All of shells are composed of $CaCO_3$ crystals, but the apparent features are clearly different.

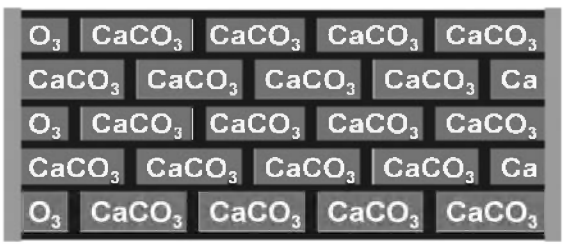

Fig. 8. Putative functions of organic matrix proteins. These proteins assume to play the function to combine the $CaCO_3$ crystals and lead the form of these units by suppressing the growth of the crystal formation.

intestine. HCO_3^{2-} is partly adsorbed from the surrounding water, but most of them are synthesized from CO_2 produced through TCA cycle.

Ca^{2+} and HCO_3^{2-} are mixed and concentrated in the specific space around the specific area surrounded by the shell and the epithelia of the mantle and crystallized there. The more the core materials exist, crystallization starts under the lower concentration of both compounds. Some kind of organic materials are possibly involved in the initiation of crystallization. After crystallization begins, sheets composed of other organic materials surround the crystals and lead the form of crystals to the expected form.

Some matrix protein fractionated in the soluble fraction exhibits an enzyme activity, Nacrein isolated from pearl oyster (*Pinctada fucata*) in 1996 showed carbonic anhydrolase activity that catalyzed the formation of CO_3^{2-} from HCO_3^{2-} (Miyamoto 1996).

Figure 8 demonstrates the putative function of organic matrix proteins. Direct

evidences to show the function of organic matrix proteins are not available at present, but these proteins assume to play the function to combine the $CaCO_3$ crystals and lead the form of these units by suppressing the development of the crystal formation.

2.4. Common proteins involved in biomineralization among animals

Among studies so far reported, evolutional relationship of shell matrix proteins are few. Dermatopontin gene has been detected in mammals, arthropods and sponge, and Nacrein and N66 found in pearl oyster has been also found among various animals (Sarashina 2006).

Perlucin, perlustrin and perlwapin reported from abalone demonstrates homology with known proteins. Perlucin is a kind of C-type lectin having carbohydrate binding domain and shares a variety of function involved in biomineral formation of mammal, bird, fish and sea urchin (Mann *et al.* 2000). Perlustrin shows a homology with N-terminal domain of insulin-like growth factor binding protein (Weiss *et al.* 2001). Perlwapin exhibits a homology with mammalian whey acidic protein (Treccani 2006).

As described above, there have been only limited reports suggesting the evolutional relationship of shell matrix proteins. Thus, most proteins involved in the synthesis of biominerals have evolved independently. Acidic amino acids such as aspartate and glutamate implicating in the chelating Ca^{2+} may play an important function in the biomineralization and the proteins having these amino acids apparently showed homology among different animals.

2.5. Transportation of Ca^{2+} for biomineralization

Metal ions are essential for a variety of biological systems in animals. Metal ions are transported into cells by membrane associated proteins including divalent metal transporters (DMTs), which have been identified from various species (Andrews *et al.* 1999). DMT1 (also called DCT1 or Nramp2) was reported as a homologous protein of Nramp which was implicated in natural resistance to infection by intracellular parasites in mouse (Vidal *et al.* 1993; Gruenheid *et al.* 1995; Govoni and Gros 1998). DMT1 was identified in rat as DCT1 through expression cloning to search for an mRNA that promoted Fe^{2+} uptake activity (Gunshin *et al.* 1997). Mutations in the DMT1 gene caused a defect in intestinal iron absorption and red cell iron utilization in rat and mouse, suggesting that DMT1 plays an important role in iron absorption by intestinal cells (Fleming *et al.* 1997, 1998; Su *et al.* 1998). The presence of an iron regulatory element (IRE) in mammalian DMTs suggests that DMT mRNA is implicated in post-transcriptional regulation by intracellular Fe^{2+} concentration (Lee *et al.* 1998; Andrews 1999). Accordingly, iron deficiency in rat induced a remarkable accumulation in the mRNA level of DMT1 due to the function of IRE (Gunshin *et al.* 2001).

We cloned a DMT gene from scallop *Mizuhopecten yessoensis* as a transporter possibly involved in the extraordinary accumulation of cadmium (Toyohara *et al.* 2005). Scallop DMT (scDMT) mRNA was strongly expressed in the gill and intestine. As a result of the functional analysis using electrophysiological method using *Xenopus laevis* oocytes, scDMT transported Fe^{2+} and Cd^{2+}, the same as mammalian DMT. Interestingly, scDMT differs from mammalian DMT in possessing an ability to transport Ca^{2+}.

It must be stressed that scDMT most effectively transports Ca^{2+} at 10 mM (Takagi *et al.* 2007). Considering that the Ca^{2+} concentration of sea water is approximately 10 mM, this result indicates that scallop possibly absorbs Ca^{2+} directly from sea water by scDMT. For further characterization of

scDMT, it is necessary to examine the Ca^{2+} transport activity in a wide range of Ca^{2+} concentration.

Ca^{2+} transport activity of DMT, specifically detected for the mollusks, suggests the implication of mollusks DMT in the shell formation process. Recently, it was reported that the crystal of $CaCO_3$ is formed in the hemocytes, not in the mantle, and released $CaCO_3$ crystals are used for the shell synthesis (Mount *et al.* 2004). Together with the fact that the higher level of mRNA expression of scDMT was detected in the gill and intestine, Ca^{2+} in sea water is transported by scDMT localized in the epithelium of these tissues and presumably transferred into the hemocytes to make $CaCO_3$ crystals.

2.6. Conclusions

In this paper, we described the recent advance in shrimp antimicrobial peptide and shell formation as examples of the studies of marine invertebrates. Marine invertebrates include a variety of animals and some of them are very important for human life. Shrimp and shell are the most useful animals among marine invertebrates, but there remain so many animals to be studied. We hope the studies of marine invertebrates will serve the development of biotechnology possibly promising the prosperous human life in the future.

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References

Aketagawa J, Miyata T, Ohtsubo S, Nakamura T, Morita T, Hayashida H, Miyata T, Iwanaga S, Takao T, Shimonishi Y. Primary structure of limulus anticoagulant anti-lipopolysaccharide factor. *J. Biol. Chem.* 1986; **261**: 7357–7365.

Amparyup P, Kondo H, Hirono I, Aoki T, Tassanakajon A. Molecular cloning, genomic organization and recombinant expression of a crustin-like antimicrobial peptide from black tiger shrimp *Penaeus monodon*. *Mol. Immunol.* 2008; **45**: 1085–1093.

Andrews NC. The iron transporter DMT1. *Int. J. Biochem. Cell Biol.* 1999; **31**: 991–994.

Andrews NC, Fleming MD, Gunshin H. Iron transport across biologic membranes. *Nutrition Review* 1999; **57**: 114–123.

Bachère E, Destoumieux D, Bulet P. Penaeidins, antimicrobial peptides of shrimp: a comparison with other effectors of innate immunity. *Aquaculture* 2000; **191**: 71–88.

Bartlett TC, Cuthbertson BJ, Shepard EF, Chapman RW, Gross PS, Warr GW. Crustins, homologues of an 11.5-kDa antibacterial peptide, from two species of penaeid shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus*. *Mar. Biotechnol. (NY)* 2002; **4**: 278–293.

Brockton V, Hammond JA, Smith VJ. Gene characterisation, isoforms and recombinant expression of carcinin, an antibacterial protein from the shore crab, *Carcinus maenas*. *Mol. Immunol.* 2007; **44**: 943–949.

Bulet P, Stocklin R. Insect antimicrobial peptides: structures, properties and gene regulation. *Protein Pept. Lett.* 2005; **12**: 3–11.

Carriel-Gomes MC, Kratz JM, Barracco MA, Bachere E, Barardi CR, Simoes CM. *In vitro* antiviral activity of antimicrobial peptides against herpes simplex virus 1, adenovirus, and rotavirus. *Mem. Inst. Oswaldo Cruz* 2007; **102**: 469–472.

Chen JY, Pan CY, Kuo CM. cDNA sequence encoding an 11.5-kDa antimicrobial peptide of the shrimp *Penaeus monodon*. *Fish Shellfish Immun.* 2004; **16**: 659–664.

Crenshaw MA. The Soluble matrix from *Mercenaria mercenaria* shell. *Biomineralization* 1996; **6**: 6–11.

Cuthbertson BJ, Shepard EF, Chapman RW, Gross PS. Diversity of the penaeidin antimicrobial peptides in two shrimp species. *Immunogenetics* 2002; **54**: 442–445.

Cuthbertson BJ, Bullesbach EE, Fievet J, Bachere E, Gross PS. A new class (penaeidin class 4) of antimicrobial peptides from the Atlantic white shrimp (*Litopenaeus setiferus*) exhibits target specificity and an independent proline-rich-domain function. *Biochem. J.* 2004; **381**: 79–86.

Cuthbertson BJ, Deterding LJ, Williams JG, Tomer KB, Etienne K, Blackshear PJ, Bullesbach EE, Gross PS. Diversity in penaeidin antimicrobial peptide form and function. *Dev. Comp. Immunol.* 2008; **32**: 167–181.

Destoumieux D, Bulet P, Loew D, Van Dorsselaer A, Rodriguez J, Bachère E. Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). *J. Biol. Chem.* 1997; **272**: 28398–28406.

Destoumieux D, Bulet P, Strub JM, Van Dorsselaer A, Bachere E. Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. *Eur. J. Biochem.* 1999; **266**: 335–346.

Destoumieux-Garzón D, Saulnier D, Garnier J, Jouffrey C, Bulet P, Bachere E. Crustacean immunity: antifungal peptides are generated from the C-terminus of shrimp hemocyanin in response to microbial challenge. *J. Biol. Chem.* 2001; **276**: 47070–47077.

Fleming MD, Trenor CC, Su MA, Foernzler D, Beier DR, Dietrich WF, Andrews NC. Microcytic anaemia mice have a mutation in Nramp2, a candidate iron transporter gene. *Nature Genetics* 1997; **16**: 383–386.

Fleming MD, Romano MA, Su MA, Garrick LM, Garrick MD, Andrews NC. *Nramp2* is mutated in the anemic Belgrade (*b*) rat: Evidence of a role for Nramp2 in endosomal iron transport. *Proc. Natl. Acad. Sci. USA* 1998; **95**: 1148–1153.

Govoni G, Gros P. Macrophage NRAMP1 and its role in resistance to microbial infections. *Inflamm. Res.* 1998; **47**: 277–284.

Gross PS, Bartlett TC, Browdy CL, Chapman RW, Warr GW. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. *Dev. Comp. Immunol.* 2001; **25**: 565–577.

Gruenheid S, Cellier M, Vidal S, Gros P. Identification and characterization of a second mouse *Nramp* gene. *Genomics* 1995; **25**: 514–525.

Grütter MG, Fendrich G, Huber R, Bode W. The 2.5 A X-ray crystal structure of the acid-stable proteinase inhibitor from human mucous secretions analysed in its complex with bovine alpha-chymotrypsin. *EMBO J.* 1988; **7**: 345–351.

Gueguen Y, Garnier J, Robert L, Lefranc MP, Mougenot I, de Lorgeril J, Janech M, Gross PS, Warr GW, Cuthbertson B, Barracco MA, Bulet P, Aumelas A, Yang Y, Bo D, Xiang J, Tassanakajon A, Piquemal D, Bachere E. PenBase, the shrimp antimicrobial peptide penaeidin database: Sequence-based classification and recommended nomenclature. *Dev. Comp. Immunol.* 2006; **30**: 283–288.

Gunshin H, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, Nussberger S, Gollan JL, Hediger MA. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 1997; **388**: 482–488.

Gunshin H, Allerson CR, Polycarpou-Schwarz M, Rofts A, Rogers JT, Kishi F, Hentze MW, Rouault TA, Andrews NC, Hediger MA. Iron-dependent regulation of the divalent metal ion transporter. *FEBS Lett.* 2001; **509**: 309–316.

Hagiwara K, Kikuchi T, Endo Y, Huqun, Usui K, Takahashi M, Shibata N, Kusakabe T, Xin H, Hoshi S, Miki M, Inooka N, Tokue Y, Nukiwa T. Mouse SWAM1 and SWAM2 are antibacterial proteins composed of a single whey acidic protein motif. *J. Immunol.* 2003; **170**: 1973–1979.

Hancock RE, Diamond G. The role of cationic antimicrobial peptides in innate host defences. *Trends Microbiol.* 2000; **8**: 402–410.

Hauton C, Brockton V, Smith VJ. Cloning of a crustin-like, single whey-acidic-domain, antibacterial peptide from the haemocytes of the European lobster, *Homarus gammarus*, and its response to infection with bacteria. *Mol. Immunol.* 2006; **43**: 1490–1496.

Hu SY, Huang JH, Huang WT, Yeh YH, Chen MHC, Gong HY, Chiou TT, Yang TH, Chen TT, Lu JK, Wu JL. Structure and function of antimicrobial peptide penaeidin-5 from the black tiger shrimp *Penaeus monodon*. *Aquaculture* 2006; **260**: 61–68.

Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. *Clin. Microbiol. Rev.* 2006; **19**: 491–511.

Jiravanichpaisal P, Lee SY, Kim YA, Andren T, Soderhall I. Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: characterization and expression pattern. *Dev. Comp. Immunol.* 2007; **31**: 441–455.

Kang CJ, Wang JX, Zhao XF, Yang XM, Shao HL, Xiang JH. Molecular cloning and expression analysis of Ch-penaeidin, an antimicrobial peptide from Chinese shrimp, *Fenneropenaeus chinensis*. *Fish Shellfish Immunol.* 2004; **16**: 513–525.

Kang CJ, Xue JF, Liu N, Zhao XF, Wang JX. Characterization and expression of a new subfamily member of penaeidin antimicrobial peptides (penaeidin 5) from *Fenneropenaeus chinensis*. *Mol. Immunol.* 2007; **44**: 1535–1543.

Lee PL, Gelbart T, West C, Halloran C, Beutler E. The human *Nramp2* gene: Characterization of the gene structure, alternative splicing, promoter region and polymorphisms. *Blood Cells Mol. Disease* 1998; **24**: 199–215.

Liu F, Liu Y, Li F, Dong B, Xiang J. Molecular cloning and expression profile of putative antilipopolysaccharide factor in Chinese shrimp (*Fenneropenaeus chinensis*). *Mar. Biotechnol. (NY)* 2005; **7**: 600–608.

Liu H, Jiravanichpaisal P, Soderhall I, Cerenius L, Soderhall K. Antilipopolysaccharide factor interferes with white spot syndrome virus replication *in vitro* and *in vivo* in the crayfish *Pacifastacus leniusculus*. *J. Virol.* 2006; **80**: 10365–10371.

Mann K, Weiss IM, Andre S, Gabius HJ, Fritz M. The amino-acid sequence of the abalone (*Haliotis laevigata*) nacre protein perlucin. Detection of a functional C-type lectin domain with galactose/mannose specificity. *Eur. J. Biochem.* 2000; **267**: 5257–5264.

Miyamoto H. A carbonic anhydrase from the nacreous layer in oyster pearls. *Proc. Natl. Acad. Sci.* 1996; **18**: 9657–9660.

Morita T, Ohtsubo S, Nakamura T, Tanaka S, Iwanaga S, Ohashi K, Niwa M. Isolation and biological activities of limulus anticoagulant (anti-LPS factor) which interacts with lipopolysaccharide (LPS). *J. Biochem. (Tokyo)* 1985; **97**: 1611–1620.

Mount AS, Wheeler AP, Paradkar RP, Snider D. Hemocyte-mediated shell mineralization in the eastern oyster. *Science* 2004; **304**: 297–300.

Muta T, Miyata T, Tokunaga F, Nakamura T, Iwanaga S. Primary structure of anti-lipopolysaccharide factor from American horseshoe crab, *Limulus polyphemus*. *J. Biochem. (Tokyo)* 1987; **101**: 1321–1330.

Nagoshi H, Inagawa H, Morii K, Harada H, Kohchi C, Nishizawa T, Taniguchi Y, Uenobe M, Honda T, Kondoh M, Takahashi Y, Soma G. Cloning and characterization of a LPS-regulatory gene having an LPS binding domain in kuruma prawn *Marsupenaeus japonicus*. *Mol. Immunol.* 2006; **43**: 2061–2069.

Rattanachai A, Hirono I, Ohira T, Takahashi Y, Aoki T. Cloning of kuruma prawn *Marsupenaeus japonicus* crustin-like peptide cDNA and analysis of its expression. *Fisheries Sci.* 2004; **70**: 765–771.

Reddy KV, Yedery RD, Aranha C. Antimicrobial peptides: premises and promises. *Int. J. Antimicrob. Agents* 2004; **24**: 536–547.

Sallenave JM. The role of secretory leukocyte proteinase inhibitor and elafin (elastase-specific inhibitor/skin-derived antileukoprotease) as alarm antiproteinases in inflammatory lung disease. *Respir. Res.* 2000; **1**: 87–92.

Sarashina I. Molecular Evolution and Functionally Important Structures of Molluscan Dermatopontin: implications for the origins of Molluscan Shell Matrix Proteins. *J. Mol. Evol.* 2006; **62**: 307–318.

Schnapp D, Kemp GD, Smith VJ. Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. *Eur. J. Biochem.* 1996; **240**: 532–539.

Somboonwiwat K, Marcos M, Tassanakajon A, Klinbunga S, Aumelas A, Romestand B, Gueguen Y, Boze H, Moulin G, Bachère E. Recombinant expression and anti-microbial activity of anti-lipopolysaccharide factor (ALF) from the black tiger shrimp *Penaeus monodon*. *Dev. Comp. Immunol.* 2005; **29**: 841–851.

Somboonwiwat K, Supungul P, Rimphanitchayakit V, Aoki T, Hirono I, Tassanakajon A. Differentially expressed genes in hemocytes of *Vibrio harveyi*-challenged shrimp *Penaeus monodon*. *J. Biochem. Mol. Biol.* 2006; **39**: 26–36.

Stoss TD, Nickell MD, Hardin D, Derby CD, McClintock TS. Inducible transcript expressed by reactive epithelial cells at sites of olfactory sensory neuron proliferation. *J. Neurobiol.* 2004; **58**: 355–368.

Su MA, Trenor CC, Fleming JC, Fleming MD, Andrews NC. The G185R mutation disrupts function of the iron transporter Nramp2. *Blood* 1998; **92**: 2157–2163.

Sudo S. Structures of mollusk shell framework proteins. *Nature* 1997; **387**: 563–564.

Supungul P, Klinbunga S, Pichyangkura R, Jitrapakdee S, Hirono I, Aoki T, Tassanakajon A. Identification of immune-related genes in hemocytes of black tiger shrimp (*Penaeus monodon*). *Mar. Biotechnol.* 2002; **4**: 487–494.

Supungul P, Klinbunga S, Pichyangkura R, Hirono I, Aoki T, Tassanakajon A. Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach. *Dis. Aquat. Organ.* 2004; **61**: 123–135.

Supungul P, Tang S, Maneeruttanarungroj C, Rimphanitchayakit V, Hirono I, Aoki T, Tassanakajon A. Cloning, expression and antimicrobial activity of crustin*Pm*1, a major isoform of crustin, from the black tiger shrimp *Penaeus monodon*. *Dev. Comp. Immunol.* 2008; **32**: 61–70.

Takagi M, Takiya J, Nakao J, Kaneko S, Toyohara H. Ca^{2+} transport activity of scallop divalent metal transporter at the concentration of seawater. *Fish. Sci.* 2007; **73**: 741–743.

Tanaka S, Nakamura T, Morita T, Iwanaga S. Limulus anti-LPS factor: an anticoagulant which inhibits the endotoxin mediated activation of limulus coagulation system. *Biochem. Biophys. Res. Commun.* 1982; **105**: 717–723.

Tassanakajon A, Klinbunga S, Paunglarp N, Rimphanitchayakit V, Udomkit A, Jitrapakdee S, Sritunyalucksana K, Phongdara A, Pongsomboon S, Supungul P, Tang S, Kuphanumart K, Pichyangkura R, Lursinsap C. *Penaeus monodon* gene discovery project: the generation of an EST collection and establishment of a database. *Gene* 2006; **384**: 104–112.

Tharntada S, Somboonwiwat K, Rimphanichayakit V, Tassanakajon A. Anti-lipopolysaccharide factors from the black tiger shrimp, *Penaeus monodon*, are encoded by two genomic loci. *Fish Shellfish Immunol.* 2008; **24**: 46–54.

Toyohara H, Yamamoto S, Hosoi M, Takagi M, Hayashi I, Nakao K, Kaneko S. Scallop DMT functions as a Ca^{2+} transporter. *FEBS Lett.* 2005; **579**: 2727–2730.

Treccani H. Perlwapin, an abalone nacre protein with three four-disulfide core (whey acidic protein) domains, inhibits the growth of calcium carbonate crystals. *Biophysical J.* 2006; **91**: 2601–2608.

Vallespi MG, Glaria LA, Reyes O, Garay HE, Ferrero J, Arana MJ. A Limulus antilipopolysaccharide factor-derived peptide exhibits a new immunological activity with potential applicability in infectious diseases. *Clin. Diagn. Lab. Immunol.* 2000; **7**: 669–675.

Vargas-Albores F, Yepiz-Plascencia G, Jiménez-Vega F, Ávila-Villa A. Structural and functional differences of *Litopenaeus vannamei* crustins. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 2004; **138**: 415–422.

Vaseeharan B, Ramasamy P, Murugan T, Chen JC. *In vitro* susceptibility of antibiotics against *Vibrio* spp. and *Aeromonas* spp. isolated from *Penaeus monodon* hatcheries and ponds. *Int. J. Antimicrob. Agents* 2005; **26**: 285–291.

Vidal SM, Malo D, Vogan K, Skamene E, Gros P. Natural resistance to infection with intracellular parasites: Isolation of a candidate for Bcg. *Cell* 1993; **73**: 469–485.

Weiss IM, Gohring W, Fritz M, Mann K. Perlustrin, a *Haliotis laevigata* (abalone) nacre protein, is homologous to the insulin-like growth factor binding protein N-terminal module of vertebrates. *Biochem. Biophys. Res. Commun.* 2001; **285**: 244–249.

Zaiou, M. Multifunctional antimicrobial peptides: therapeutic targets in several human diseases. *J. Mol. Med.* 2007; **85**: 317–329.

Zhang J, Li F, Wang Z, Xiang J. Cloning and recombinant expression of a crustin-like gene from Chinese shrimp, *Fenneropenaeus chinensis*. *J. Biotechnol.* 2007; **127**: 605–614.