Journal of Marine Science and Technology

Volume 22 | Issue 1 Article 1

SHRIMP IMMUNE SYSTEM -SPECIAL FOCUS ON PENAEIDIN

Yen-Ling Song
Institute of Zoology, National Taiwan University, Taipei, Taiwan, R.O.C. Department of Life Science, National Taiwan University, Taipei, Taiwan,R.O.C, song@ntu.edu.tw

Ching-Yu Li
Institute of Zoology, National Taiwan University, Taipei, Taiwan, R.O.C

SHRIMP IMMUNE SYSTEM -SPECIAL FOCUS ON PENAEIDIN

Acknowledgements

The authors greatly appreciate Dr. Vinu S Siva for thorough editing of this manuscript

Journal of Marine Science and Technology, Vol. 22, No. 1, pp. 1-8 (2014)
DOI: 10.6119/JMST-013-0813-1

SHRIMP IMMUNE SYSTEM – SPECIAL FOCUS ON PENAEIDIN

Yen-Ling Song[1, 2] and Ching-Yu Li[1]

Key words: clotting system, proPO-activating system, astakine, penaeidin.

ABSTRACT

In this review paper, we summarize the immune system of penaeid shrimp. This review includes the clotting system comprising clot protein and clotting enzyme (transglutaminase II), as well as the prophenoloxidase (proPO) activation system including proPO-activating enzyme (PPAE), proPO and clip domain serine protease homolog (c-SPH). In addition, several shrimp cytokines or cytokine-like molecules found in tiger shrimp *Penaeus monodon* participating in the defense responses are also discussed. C-SPH could enhance hemocyte adhesion. Shrimp astakine promoted hemocyte proliferation in the hematopoietic tissues and could be down-regulated at the post-transcriptional level by the binding of intracellular molecules such as transglutaminase I and crustin Pm4 to its 3'-untranslatd region. Penaeidin is found to be a dual function molecule as an antimicrobial peptide and an autocrine-acting cytokine. Yet, other antimicrobial substances such as quinone and melanine which are generated during the proPO activation pathway and the reactive oxygen and nitrogen intermediates (ROI and RNI) which are generated during the phagocytic process can also kill and clear the invading microorganisms directly. In addition, Dscam (Down syndrome cell adhesion molecule), a member of the immunoglobulin superfamily (IgSF) which plays an essential role in the alternative adaptive immune system of invertebrates, has also been characterized in shrimp.

Penaeid shrimp is one of the most important commercially available aquaculture resources. Although, the culture techniques and field management skills have significantly improved in the recent years, infectious diseases are still a threat to the industry. The study of shrimp immune system could be important in designing the strategies against pathogen infection. For shrimp, the innate immune responses play a major role in combatting invading pathogens and prevent them against diseases. The immune responses involve different factors such as physical barrier, bacteria clearance, encapsulation, clotting reactions, prophenoloxidase system, reactive oxygen intermediates, and antimicrobial activity. The innate immune system uses a variety of pattern recognition receptors (PRR) on the hemocyte membrane to detect the pathogen-associated molecule pattern (PAMP) and transduce signals during pathogen invasion. Hemocytes are then activated and the defense molecules are degranulated against the pathogens.

I. THE CLOTTING SYSTEM

Coagulation (clot formation) forms a physical barrier to prevent the loss of body fluid and the dissemination of microbes into the haemocoel after an injury or infection. The rapidity and efficiency of coagulation are essential for the survival of invertebrates that rely solely on innate immunity. Transglutaminase (TG) catalyses intermolecular or intramolecular 3-(g-glutamyl) lysine bond formation, resulting in protein polymerization, and plays a role in blood coagulation and post-translational protein remodeling. In shrimp, coagulation is initiated by the activation and lysis of hyaline cells, which release hemocyte components to react with plasma factors [45]. One of the hemocyte components is believed to be a transglutaminase. The plasma factor which has been purified is known as clottable protein (CP) in *P. monodon* [66]. *P. monodon* CP resembles the vitellogenin family clotting protein in crayfish [28]. Also, a *P. monodon* transglutaminase (designated STGI) was cloned from the hemocytes, but no clotting activity was detected. STGI is ubiquitously expressed with a low mRNA level in circulating hemocytes, but a high level in mitotic cells of hematopoietic tissue was shown in an *in situ* hybridization assay [33]. RT-PCR experiment also showed that STGI transcript decreased when hemocytes matured and were released into the hemolymph. Taken together, these results suggest that transcription and translation of STGI in the hemocytes possibly take place in early developmental stages but are down-regulated in later developmental stages [67].

Chen *et al*. [15] cloned a TG from shrimp hemocyte cDNA, which has been designated as *P. monodon* transglutaminase II (STGII). RT-PCR results showed a significant level of

Paper submitted 01/31/13; revised 07/31/13; accepted 08/13/13. Author for correspondence: Yen-Ling Song (e-mail: song@ntu.edu.tw).
[1] Institute of Zoology, National Taiwan University, Taipei, Taiwan, R.O.C.
[2] Department of Life Science, National Taiwan University, Taipei, Taiwan, R.O.C.

*Pm*TGII expression in hemocytes but not in hepatopancreas, in contrast to STGI which is ubiquitously expressed. The genetic distance between STGII and STGI is much farther than the distance between STGII and the *Mj*TG of the kuruma shrimp (*Marsupenaeus japonicus*). Evidence based on tissue distribution and genetic distance suggests that there exist at least two types of shrimp TGs exist that are encoded at different chromosomal locations. This is not consistent with TG of insects such as fruit flies and mosquitoes whose genomes were sequenced and showed that only one locus encodes for TG with a functional domain organization. This implies that the TG of the fruit flies and mosquitoes plays multiple physiological functions. Besides, the recombinant STGII (rSTGII) can incorporate a TG-specific substrate, dansylcadaverine, into CP in a calcium dependent manner [15]. Other hemocyte- or plasma-derived TG substrate is not required for CP polymerization, but may be necessary for the stable clot formation. The rSTGII catalysed clottable proteins that were reflected as a long chain under transmission electron microscopy (TEM) observation. Conclusively, STGII is characterized as a hemocyte TG and is involved in coagulation. In a recent study, STGI associates with crustin Pm4 in serving as RNA binding proteins and in down-regulating astakine-mediated hematopoiesis at the post-transcriptional level [12].

The coagulation system seems to be a major target for virus attacks. In shrimps infected with Taura syndrome virus, the transglutaminase activity declined concomitantly [53]. A detailed understanding of shrimp transglutaminase and clotting regulation is therefore not only a scientific researchable area but will also contribute significantly to the disease control in the invertebrate aquaculture industry.

II. PROPHENOLOXIDASE (PROPO) ACTIVATION SYSTEM

The prophenoloxidase (proPO) activation system is one of the major invertebrate immune systems. The process is controlled by the key enzyme, phenoloxidase (PO), which catalyses the early steps in the pathway of melanin synthesis [56]. PO catalyzes hydroxylation of monophenols into o-diphenols, and further oxidation of o-diphenols into o-quinones, which can nonspecifically crosslink with neighboring molecules to form the insoluble melanin. The production of o-quinones that are intermediates for cuticle sclerotization, and the sclerotized cuticle turned to be a barrier for infection. The highly toxic quinone substances and melanization around pathogens help in killing the pathogens directly. The process is initiated by the recognition of lipopolysaccharides or peptidoglycans from bacteria and **β**-1,3-1,6-glucans from fungi. Generally, the recognition of pattern recognition receptors (PPRs) activates extracellular serine proteinase (SP) cascades that amplify the signal and trigger downstream molecules, which leads to the killing of pathogens. In invertebrates, extracellular SP cascades involving the clip domain serine protease family play important roles in the signaling cascades in both embryonic development and defense responses, such as hemolymph coagulation in horseshoe crab, antimicrobial peptide synthesis and activation of proPO in insects and crustaceans.

The activation of proPO is mediated by a serine proteinase cascade. The serine proteinases which catalyze the proteolysis of proPO to active PO have been named as proPO-activating proteinases (PAPs) or proPO-activating enzymes (PPAEs). Four controlling mechanisms are known for PPAE-regulation such as gene induction upon microbial infection, activation by another proteinase, requirement for masquerade-like serine proteinae homologues (SPHs) as cofactors and inhibition by serine proteinase inhibitors [60].

PPAEs, SPHs and proteinases that function on activating the PO system can be referred as prophenoloxidase activating factors (PPAFs) [10]. The PPAFs contain a highly conserved C-terminal serine proteinase domain and one or more N-terminal clip domains. Clip domains, 30~65 residues long, include six strictly conserved cysteine residues that form three-disulfide bonds [35]. This structural unit is widely found in arthropod serine proteinases and SPHs [48], and is believed to possess important regulatory functions through their interaction with other proteins. The serine proteinase domains of PPAEs and SPHs are similar in sequence to those of serine proteinases, but the typical active-site serine residue of PPAE is changed to glycine in SPHs, and thus, SPHs lack proteolytic activity.

In crustaceans, a prophenoloxidase-activating enzyme (ppA) of the freshwater crayfish *Pacifastacus leniusculus* was purified [3] and cloned [59] from blood cells. The purified ppA is capable of cleaving proPO into active PO without additional protein cofactor [3, 4]. The N-terminal half of ppA contains a cationic glycine-rich domain, a cationic proline-rich domain and a clip domain. The C-terminal half of ppA is composed of a typical serine proteinase domain [59]. Additionally, a masquerade-like protein has been cloned from crayfish hemocytes, but this protein binds bacteria and appears to function as pattern recognition and cell adhesion protein rather than a PPAF [34, 38]. In addition, a masquerade-like SPH (cMasII) has been isolated and cloned from granular haemocytes from a crayfish [55]. A cDNA encoding a PPAF has been cloned from the hypodermal tissue of the blue crab *Callinectes sapidus* [10].

1. Shrimp proPO-Activating Enzyme (PPAE)

Charoensapsri *et al*. [13] reported that gene silencing of a prophenoloxidase activating enzyme (later designated *Pm*PPAE1) in the tiger shrimp *P. monodon* increases its susceptibility to *Vibrio harveyi* infection. The same team found a new class of crustacean PPAE (designated *Pm*PPAE2) from the hemocytes of the shrimp which possibly mediates the activation of *Pm*proPO1 [14]. *Pm*PPAE2 showed structural features of the clip domain serine proteinase (c-SP) family whereas its transcripts were mainly expressed in hemocytes. Double-stranded RNA-mediated suppression of *Pm*PPAE2 transcript levels resulted in a significant decrease of the total

hemolymph PO activity (41%) but an increase in shrimp susceptibility to *V. harveyi* infection. Genomic organization analysis revealed that *Pm*PPAE1 and *Pm*PPAE2 are encoded by different genomic loci. The *Pm*PPAE1 gene consists of ten exons and nine introns, whereas *Pm*PPAE2 gene has eight exons interrupted by seven introns. *Pm*PPAE1 and *Pm*proPO2 transcripts were expressed in all larval stages (nauplius, protozoea, mysis and post-larvae), whereas *Pm*PPAE2 and *Pm*proPO1 transcripts were mainly presented in the late larval developmental stages (mysis and post-larvae).

2. Shrimp Clip Domain Serine Protease Homolog (c-SPH)

The clip domain serine proteinases (clip-SPs) play critical roles in the signaling processes during embryonic development and in the innate immunity of invertebrates. Rattanachai *et al*. [47] reported a cDNA encoding a pseudo-clip SPH of kuruma shrimp *Marsupenaeus japonicus* and characterized the peptidoglycan inducible mRNA expression. Lin *et al*. [42] reported a clip domain serine protease homologue (*Pmo* c-SPH) from *P. monodon*. Besides one clip domain, *Pmo* c-SPH possesses one catalytically inactive serine protease-like domain, because glycine instead of serine replacement is present in the catalytic domain of the sequence of SP. *Pmo* c-SPH is expressed mostly in tissues with the highest hemocyte abundance. Although the molecular mechanism(s) by which crustacean serine proteinase homologues regulate the proPO system was not clarified, the sequences of *Pmo* c-SPH close to the amino acid residues forming the substrate specificity pocket in catalytically active serine protease are well conserved. This implies that the substrate specificity pocket in the *Pmo* c-SPH plays a role such as protein-protein interaction. Expression of c-SPH transcript in hemocytes being up-regulated at the 12-day post yeast β-glucan immersion of shrimp suggests that it is involved in the innate immune responses against pathogen infection. However, the direct antimicrobial and opsonization activities of *Pmo* c-SPH are not detected. The recombinant *Pmo* c-SPH protein significantly enhancing hemocyte adhesion suggests that it serves as a cell adhesion molecule (CAM) and possibly plays a role during shrimp embryonic development.

Amparyup *et al*. [1] also cloned and characterized a masquerade-like serine proteinase homologue (*Pm*MasSPH) cDNA from the hemocyte cDNA library of *P. monodon*. *Pm*MasSPH shared most of the structural characteristics of insect PPAEs but showed a non-catalytic activity similar to *Pm* c-SPH as reported by Lin *et al*. [42]. However, the transcript of *Pm*MasSPH molecule in hemocytes was found to increase within 24 h after *V. harveyi* infection, and this reveals that *Pm*MasSPH, in comparison to *Pmo* c-SPH, can be an early-response molecule against pathogen infection.

Amparyup *et al*. [2] identified the other clip domain serine proteinase (*Pm*ClipSP1) homolog from shrimp hemocytes. This molecule shares a predicted structural similarity to the clip-SPs of other arthropod species and possesses a clip domain at the N-terminus and an enzymatically active serine proteinase domain at the C-terminus. Surprisingly, *Pm*ClipSP1 does not participate in the prophenoloxidase activation but plays a role in antibacterial defense in shrimp.

The clip domain-serine protease categorized as a PPAE must fulfill the requirement of being catalytically active with proPO as its specific substrate. According to the genome sequences of *Drosophila melanogaster*, *D. pseudoobscura*, *Anopheles gambiae* and *Apis mellifera*, more than one c-SPH isoform were identified in these species. There are two c-SPHs (mas-like and low molecular mass mas-like molecules) found in the granules of crayfish hemocyte, and three c-SPHs are in *Manduca sexta*. In the genome of the fruit fly, 13 c-SPH sequences have been identified. Questions arise as to why there are so many catalytically inactive enzymes, what roles they play and how they proceed. Further study is required to address these questions.

III. ASTAKINE, A SHRIMP HEMATOPOIETIC CYTOKINE

Significant decrease in the number of hemocytes is always found in crustaceans after infections. Thus, generation of new hemocytes (hematopoiesis) in hematopoietic tissue is essential to maintain homeostasis which is vital to the animals. Cytokines secreted from various types of cells have been demonstrated to regulate hematopoiesis and immune responses in vertebrates. A cytokine-like factor, called astakine containing a prokineticin (PK) domain, was purified and cloned from fresh water crayfish, *Pacifastacus leniusculus*. Addition of purified crayfish astakine protein could prolong the hematopoietic cell culture and cell proliferation. Injection of recombinant protein to crayfish and subsequent gene silencing of astakine *in vivo* altered the total hemocyte count in hemolymph and suggested that crayfish astakine is involved in hematopoiesis [49]. Recently, the hematopoietic process of the crayfish was found under circadian control and is tightly regulated by astakines [63]. Shrimp astakine (PmAst) was cloned and the role of the recombinant protein which promoted hematopoiesis was elucidated *in vivo*. The protein belongs to PK superfamily and is mainly expressed in hemocytes. When shrimp health is challenged, PmAst is released from the hemocytes and circulates through the hemolymph to the hematopoietic tissue where it promotes hemocyte proliferation. Subsequently, newly generated hemocytes are released into the peripheral hemolymph for maintaining homeostasis [32]. Interestingly, PmAst-mediated hematopoiesis was down-regulated at the post-transcriptional level with the binding of STGI and crustin Pm4 to its 3'UTR [12].

IV. PENAEIDIN PLAYS DUAL FUNCTION AS ANTIMICROBIAL PEPTIDE (AMP) AND CYTOKINE

Since the discovery of the first AMP cecropin in 1981 [7], many AMPs have been identified and characterized from a

wide variety of vertebrate and invertebrate species. Approximately 103 antiviral peptides, 549 antifungal peptides, 105 anticancer peptides and 1380 antibacterial peptides have been recorded in the antimicrobial peptide database [61]. AMPs possess common properties such as amphipathic amino acid structure, low molecular weight and positive charge. In the mammalian study, AMPs play critical roles in both innate and adaptive immunity [54].

1. History of Penaeidin

Penaeidins are members of antimicrobial peptides originally isolated from *Litopenaeus vannamei* hemolymph. They are present in stored form in the granulocytes and secreted after microbial stimulation [23, 25]. Penaeidins appear to be ubiquitous in penaeid shrimps where they are major actors in the immune response [6]. The three penaeidins named as penaeidin-1, -2, and -3 with molecular weight between 5.5 and 6.6 kDa, and pI value range of 9.34~9.84 were found functionally active against Gram-positive bacteria and filamentous fungi. Penaeidin molecules consisted of an N-terminal proline-rich domain (PRD) and a C-terminal cysteine-rich domain (CRD). Thirty nine different penaeidins have been found in 8 species of shrimps so far. Comparing these penaeidins, penaeidin-4 from *P. setiferus* showed the strongest bactericidal activity, whereas penaeidin-3 was the most abundant. In some cases, penaeidins from *P. monodon* and *P. chinensis* are referred to independently as penaeidin-3 or penaedin-5 [16, 36, 64]. Suppression of *P. monodon* pen-5 transcript led to an increased susceptibility of shrimps to WSSV infection, suggesting a possible immunodulation role of penaeidin in shrimp's antiviral immunity [64]. *P. monodon* penaeidin is more similar to *L. vannamei* penaeidin-3 (52% amino acid sequence similarity) than to *P. setiferus* penaeidin-4 (34% amino acid sequence similarity) [20, 22, 29]. Still, the nomenclature is an issue in the categorization of pen-3 and pen-5.

2. Structure of Penaeidin

According to the classification of AMP, most structures are composed of a single character of α-helix, linear, looped or intra-molecular disulfide bond [31]. Penaeidin is a unique AMP because it is composed of both an N-terminal linear PRD and a C-terminal CRD with three pairs of disulfide bonds [25, 43]. The amphipathic structure of penaeidin is located in the CRD and has been proposed as the pathogen binding domain. Li *et al*. [40] demonstrated that only the intact *P. monodon* penaeidin molecule and neither PRD nor CRD possesses the bactericidal activity. Nevertheless, the PRD alone of penaeidin-4 possesses bactericidal activity in high concentration [22, 65].

3. Two Domains of Penaeidin are Functionally Different

In an individual domain basis, no bactericidal activity was observed in each domain of penaeidin except penaeidin-4, of which PRD was reported to be bactericidal [22]. The PRD was found as a cytokine which promotes granulocyte migration through an autocrine action. Penaeidin knock-down in both hemocytes and shrimp decreases the expression of cell adhesion-associated molecules such as integrin –β and collagen, respectively, but increases the expression of collagenase [40]. These results demonstrate that penaeidin/PRD directs granulocyte migration towards the inflammatory foci which assists in the inflammatory responses [39]. However, no similar cytokine feature of CRD was found in hemocytes. Nevertheless, some studies indicate that AMPs possess the various cytokine functions in various tissues [8, 51]. From an HPLC study, CRD was shown to bind with chitin [25, 43].

What is the possible function of CRD? A positive charge could be the key factor in the bactericidal activity of AMPs [68]. The 55 amino acids of mature *P. monodon* penaeidin contain 8 positively charged amino acids and no negatively charged amino acids. Among the 8 positively charged amino acids, 6 are located in the CRD and 2 in the PRD. Furthermore, in a study on penaeidin structure, the membrane-binding amphipathic character is observed in CRD [6]. Although the bactericidal mechanism of penaeidin is still not fully understood, an interaction between penaeidin and pathogen membrane may depend on CRD, while destruction of the pathogen membrane may depend on PRD. Neither of the two domains alone has the capacity to destroy pathogens. We propose that the CRD could be the regulatory domain which directs the PRD onto the pathogen surface to destroy it.

Tight connections between integrin-β, collagen and collagenase regulate cell adhesion, and interaction between collagen and collagenase controls the structure and stability of extra cellular matrix (ECM). A decrease in collagen and an increase in collagenase destroy the integrity of ECM, whereas more collagen and less collagenase strengthen the rigidity of ECMs. Integrins are essential for cells anchoring on ECMs and transduce adherence signals within the cells. Since collagen possesses a repeated RGD (Arg-Gly-Asp) motif which is recognized by integrins, thus the addition of RGDS (Arg-Gly-Asp-Ser, an integrin competitor) competitively decreases the number of adhesive hemocytes in the culture system. This is similar with that induced by penaeidin knock-down hemocytes. Combining these results indicates that penaeidin-regulated hemocytes are dependent on the interaction between CAM and ECM [40].

4. Penaeidin: Tissue Expression and Regulation

Penaeidin was first identified in the hemocytes, then in the hematopoietic tissue, gill and hepatopancreas. Based on the immunohistochemistry and Transmission Electron Microscope (TEM) studies, penaeidin distribution in tissues apart from hemocytes might be due to hemocyte infiltration [23, 29]. During infection by *Aerococcus viridians, Fusarium oxysporum* or *V. alginolyticus*, total peripheral hemocytes and penaeidin positive granulocytes concomitantly decrease at 24 hours post injection. Afterward, the hemocyte numbers and penaeidin expression recovered gradually [23, 43]. Both pe-

naeidin molecules and penaeidin positive granulocytes are concentrated in the inflammatory foci but decreased in the peripheral. These results imply that the missing penaeidin positive granulocytes in the peripheral after challenge may migrate toward the inflammatory foci. This phenomenon is proposed as penaeidin-dependent positive feedback through the regulation of integrin expression [39].

Not like inducible AMPs such as defensin, drosomycin and cathelicidin, penaeidin is thought to be a non-inducible constitutively expressed AMP [30, 44]. However, penaeidin expression fluctuates during developmental stages and molting cycles. Neither Toll receptor nor Imd pathway is involved in the signal transduction of penaeidin activation. Penaeidins were reportedly detected as early as 1-4 day embryo. Its expression gradually increased to the highest at nauplius I and postlarval stages, decreased to the lowest after molting and then gradually recovered in the premolting stage [16]. Thus, penaeidin regulation of molting cycle may be another postulate function [25, 43].

5. Penaeidin in Clinical Application

Several aqua-cultural and human pathogens had been tested for the penaeidin bactericidal activity. These microbes include Gram-positive and Gram-negative bacteria, filamentous fungi and yeast. No significant anti-viral activity was reported [17, 20, 23, 24, 29, 36, 41]. Biosynthetic recombinant penaeidins produced from yeast and insect cell expression systems show bactericidal activities successfully. Chemically synthesized penaeidins show similar bactericidal activities [20, 22, 40]. There is slight difference in the bactericidal activity between the native and recombinant penaeidins. Anti-yeast activity of penaeidins is not observed from yeast expression system. Whereas, the chemically synthesized penaeidin can act against the human antibiotic-resistant fungi including *Cryptococcus* and *Candida* [21].

6. Penaeidin in the Wound Healing

Many AMPs appear in the wound or injection site [57]. The mammalian AMPs such as HBD and LL-37 chemoattract white blood cells and promote epithelial cell migration during the wound healing process [46, 51, 54]. Hemocytes play critical roles in invertebrate immune and wound-repair systems [5]. **A** study on hemocytes in fruit fly wound-repair procedures indicates hemocyte concentration, spreading and obstruction of the wound to alleviate bleeding. The wound-repair processes in shrimps also include concentration of hemocytes into the wound [27]. We suggest that penaeidin is involved in shrimp wound-healing process. Penaeidin released in the wound area as an AMP can clean up invasive pathogens and enhances hemocyte adhesion and spreading for wound obstruction as a cytokine.

Penaeidin reveals a cytokine activity in the wound tissue which is corroborated in the previous study [37]. In contrast to other AMPs, penaeidin level was significantly decreased in hemolymph post-wound. However, the average penaeidin expression in each granulocyte did not change but the number of penaeidin-positive peripheral granulocytes decreased after injury. These indicate that the decrease in peripheral penaeidin could be due to integrin-dependent migration of penaeidin-positive granulocytes chemotactically towards the wound tissue via the circulatory system [39].

Many AMPs are thought to facilitate the wound healing process or participate in it [2, 11, 46]. Penaeidin was shown to promote granulocyte migration towards wound tissue within 2 h post-injury in the previous studies. Neutrophil is the type of leukocytes firstly appearing in the acute inflammatory response in vertebrates [50, 58]. Neutrophils release cytokines and AMPs by degranulation and engulf pathogens by phagocytosis or restrict the pathogens by neutrophil extracellular traps (NETs) [9]. These characteristics imply that penaeidin-positive granulocytes are functionally similar to vertebrate neutrophils and are also the subtype of hemocytes that appear first in the wound tissue and release AMPs. To assess whether penaeidin promotes or obstructs the wound healing process in shrimps, it will take more than one-week or until the next molting to observe complete sealing of the open wound. Li and Song [39] observed that shrimp mortality within 32 h was higher when penaeidin-specific siRNA was injected (23%; 15/65) in comparison to those injected with unrelated siRNA (0.7%; 4/65). It is possible that penaeidin has other unknown yet important functions in homeostasis or immunomodulation [39].

V. OTHER ANTIMICROBIAL SUBSTANCES

Besides penaeidins and the highly toxic quinone and melanin substances produced in the PO activation pathway, the other reactive oxygen and nitrogen intermediates (ROI and RNI) generated during the phagocytic process [52] could also kill and clear the invading microorganisms directly.

VI. ALTERNATIVE ADAPTIVE IMMUNITY

Down syndrome cell adhesion molecule (Dscam) seems likely to play a key role in the "alternative adaptive immunity" as reported in invertebrates. In some invertebrate animals, such as mosquito and *Drosophila*, the innate immune responses are supplemented by a novel immunity that exhibits specificity and memory. The mechanism is not well understood, but a pathogen-specific receptor capable of high diversity is presumably required. Different means evolved from different invertebrates to achieve this goal. In arthropods, the diversity is seen in Dscam. The studies of Watson *et al*. [62] have identified a homologue of Dscam in fruit flies (*Drosophila*) that is expressed in key phagocytic cell types in this insect's immune system. These workers have estimated that 18,000 isoforms of Dscam could be formed by sequence variation in the three immunoglobulin-like domains of this molecule, and these variations probably allowed Dscam to show differential binding to foreign agents in an analogous way as

vertebrate antibody binds different antigens. In a related invertebrate (the mosquito) further experimentation has revealed the production of different Dscam-like molecules with varying pathogen interaction specificities after challenge with different pathogens [26] which is analogous to the clonal selection mechanism of vertebrate immunity. Dscam consists of a cytoplasmic tail that is involved in signal transduction and a hypervariable extracellular region that might use a pathogen recognition mechanism similar to that used by the vertebrate antibodies. Dscam was isolated from shrimps such as *L. vannamei* and *P. monodon* and occurred in both membrane-bound and tail-less forms [18, 19].

ACKNOWLEDGMENTS

The authors greatly appreciate Dr. Vinu S Siva for thorough editing of this manuscript.

REFERENCES

1. Amparyup, P., Jitvaropas, R., Pulsook, N., and Tassanakajon, A., "Molecular cloning, characterization and expression of a masquerade-like serine proteinase homologue from black tiger shrimp *Penaeus monodon*," *Fish & Shellfish Immunology*, Vol. 22, No. 5, pp. 535-546 (2007).
2. Amparyup, P., Wiriyaukaradecha, K., Charoensapsri, W., and Tassanakajon, A., "A clip domain serine proteinase plays a role in antibacterial defense but is not required for prophenoloxidase activation in shrimp," *Developmental & Comparative Immunology*, Vol. 34, No. 2, pp. 168-176 (2010).
3. Aspán, A. and Söderhäll, K., "Purification of prophenoloxidase from crayfish blood cells, and its activation by an endogenous serine proteinase," *Insect biochemistry*, Vol. 21, No. 4, pp. 363-373 (1991).
4. AsPAN, A., Huang, T. S., Cerenius, L., and Söderhäll, K., "cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation," *Proceedings of the National Academy of Sciences*, Vol. 92, No. 4, pp. 939-943 (1995).
5. Babcock, D. T., Brock, A. R., Fish, G. S., Wang, Y., Perrin, L., Krasnow, M. A., and Galko, M. J., "Circulating blood cells function as a surveillance system for damaged tissue in *Drosophila* larvae," *Proceedings of the National Academy of Sciences*, Vol. 105, No. 29, pp. 10017-10022 (2008).
6. Bachere, E., Gueguen, Y., Gonzalez, M., De Lorgeril, J., Garnier, J., and Romestand, B., "Insights into the anti-microbial defense of marine invertebrates: the penaeid shrimps and the oyster *Crassostrea gigas*," *Immunological reviews*, Vol. 198, No. 1, pp. 149-168 (2004).
7. Boman, H. and Steiner, H., "Humoral immunity in *Cecropia pupae*," *Current topics in Microbiology and Immunology*, Vol. 94, pp. 75-91 (1981).
8. Braff, M. H., Mi'i, A. H., Di Nardo, A., Lopez-Garcia, B., Howell, M. D., Wong, C., Lin, K., Streib, J. E., Dorschner, R., and Leung, D. Y. M., "Structure-function relationships among human cathelicidin peptides: dissociation of antimicrobial properties from host immunostimulatory activities," *The Journal of Immunology*, Vol. 174, No. 7, pp. 4271-4278 (2005).
9. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., and Zychlinsky, A., "Neutrophil extracellular traps kill bacteria," *Science*, Vol. 303, No. 5663, pp. 1532-1535 (2004).
10. Buda, E. S. and Shafer, T. H., "Expression of a serine proteinase homolog prophenoloxidase-activating factor from the blue crab, *Callinectes sapidus*," *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, Vol. 140, No. 4, pp. 521-531 (2005).
11. Chamorro, C. I., Weber, G., Grönberg, A., Pivarcsi, A., and Ståhle, M., "The human antimicrobial peptide LL-37 suppresses apoptosis in keratinocytes," *Journal of Investigative Dermatology*, Vol. 129, No. 4, pp. 937-944 (2008).
12. Chang, Y.-T., Lin, C.-Y., Tsai, C.-Y., Siva, V. S., Chu, C.-Y., Tsai, H.-J., and Song, Y.-L., "The new face of the old molecules: crustin Pm4 and transglutaminase type I serving as RNPs down-regulate astakine-mediated hematopoiesis," *PLoS One*, Vol. 8, No. 8, e72793 (2013).
13. Charoensapsri, W., Amparyup, P., Hirono, I., Aoki, T., and Tassanakajon, A., "Gene silencing of a prophenoloxidase activating enzyme in the shrimp, *Penaeus monodon*, increases susceptibility to *Vibrio harveyi* infection," *Developmental & Comparative Immunology*, Vol. 33, No. 7, pp. 811-820 (2009).
14. Charoensapsri, W., Amparyup, P., Hirono, I., Aoki, T., and Tassanakajon, A., "*Pm* PPAE2, a new class of crustacean prophenoloxidase (proPO)-activating enzyme and its role in PO activation," *Developmental & Comparative Immunology*, Vol. 35, No. 1, pp. 115-124 (2011).
15. Chen, M. Y., Hu, K. Y., Huang, C. C., and Song, Y. L., "More than one type of transglutaminase in invertebrates? A second type of transglutaminase is involved in shrimp coagulation," *Developmental & Comparative Immunology*, Vol. 29, No. 12, pp. 1003-1016 (2005).
16. Chiou, T. T., Lu, J. K., Wu, J. L., Chen, T. T., Ko, C. F., and Chen, J. C., "Expression and characterisation of tiger shrimp *Penaeus monodon* penaeidin (mo-penaeidin) in various tissues, during early embryonic development and moulting stages," *Developmental & Comparative Immunology*, Vol. 31, No. 2, pp. 132-142 (2007).
17. Chiou, T. T., Wu, J. L., Chen, T. T., and Lu, J. K., "Molecular cloning and characterization of cDNA of penaeidin-like antimicrobial peptide from tiger shrimp (*Penaeus monodon*)," *Marine Biotechnology*, Vol. 7, No. 2, pp. 119-127 (2005).
18. Chou, P. H., Chang, H. S., Chen, I., Lee, C. W., Hung, H. Y., and Han-Ching Wang, K., "*Penaeus monodon* Dscam (PmDscam) has a highly diverse cytoplasmic tail and is the first membrane-bound shrimp Dscam to be reported," *Fish & Shellfish Immunology*, Vol. 30, No. 4, pp. 1109-1123 (2011).
19. Chou, P. H., Chang, H. S., Chen, I., Lin, H. Y., Chen, Y. M., Yang, H. L., and Wang, K., "The putative invertebrate adaptive immune protein *Litopenaeus vannamei* Dscam (LvDscam) is the first reported Dscam to lack a transmembrane domain and cytoplasmic tail," *Developmental & Comparative Immunology*, Vol. 33, No. 12, pp. 1258-1267 (2009).
20. Cuthbertson, B. J., Büllesbach, E. E., Fievet, J., Bachère, E., and Gross, P. S., "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," *Biochemical Journal*, Vol. 381, No. Pt 1, pp. 79-86 (2004).
21. Cuthbertson, B. J., Büllesbach, E. E., and Gross, P. S., "Discovery of synthetic penaeidin activity against antibiotic-resistant fungi," *Chemical Biology & Drug Design*, Vol. 68, No. 2, pp. 120-127 (2006).
22. Cuthbertson, B. J., Yang, Y., Bachère, E., Büllesbach, E. E., Gross, P. S., and Aumelas, A., "Solution structure of synthetic penaeidin-4 with structural and functional comparisons with penaeidin-3," *Journal of Biological Chemistry*, Vol. 280, No. 16, pp. 16009-16018 (2005).
23. Destoumieux, D., Bulet, P., Loew, D., Van Dorsselaer, A., Rodriguez, J., and Bachère, E., "Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda)," *Journal of Biological Chemistry*, Vol. 272, No. 45, pp. 28398-28406 (1997).
24. Destoumieux, D., Bulet, P., Strub, J. M., van Dorsselaer, A., and Bachère, E., "Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp," *European Journal of Biochemistry*, Vol. 266, No. 2, pp. 335-346 (2001).
25. Destoumieux, D., Muñoz, M., Cosseau, C., Rodriguez, J., Bulet, P., Comps, M., and Bachère, E., "Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge," *Journal of Cell Science*, Vol. 113, No. 3, pp. 461-469 (2000).
26. Dong, Y., Taylor, H. E., and Dimopoulos, G., "AgDscam, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system," *PLoS Biology*, Vol. 4, No. 7, e229 (2006).

27. Fontaine, C. and Lightner, D., "Observations on the process of wound repair in penaeid shrimp," *Journal of Invertebrate Pathology*, Vol. 22, No. 1, pp. 23-33 (1973).
28. Hall, M., Wang, R., Van Antwerpen, R., Sottrup-Jensen, L., and Söderhäll, K., "The crayfish plasma clotting protein: a vitellogenin-related protein responsible for clot formation in crustacean blood," *Proceedings of the National Academy of Sciences*, Vol. 96, No. 5, pp. 1965-1970 (1999).
29. Ho, S. H., Chao, Y. C., Tsao, H. W., Sakai, M., Chou, H. N., and Song, Y. L., "Molecular cloning and recombinant expression of tiger shrimp *Penaeus monodon* penaeidin," *Fish Pathology*, Vol. 39, No. 1, pp. 15-24 (2004).
30. Ho, S. H. and Song, Y. L., "Cloning of penaeidin gene promoter in tiger shrimp (*Penaeus monodon*)," *Fish & Shellfish Immunology*, Vol. 27, No. 1, pp. 73-77 (2009).
31. Hof, W., Veerman, E. C. I., Helmerhorst, E. J., and Amerongen, A. V., "Antimicrobial peptides: properties and applicability," *Biological Chemistry*, Vol. 382, No. 4, pp. 597-619 (2001).
32. Hsiao, C. Y. and Song, Y. L., "A long form of shrimp astakine transcript: Molecular cloning, characterization and functional elucidation in promoting hematopoiesis," *Fish & Shellfish Immunology*, Vol. 28, No. 1, pp. 77-86 (2010).
33. Huang, C. C., Sritunyalucksana, K., Söderhäll, K., and Song, Y. L., "Molecular cloning and characterization of tiger shrimp (*Penaeus monodon*) transglutaminase," *Developmental & Comparative Immunology*, Vol. 28, No. 4, pp. 279-294 (2004).
34. Huang, T., Wang, H., Lee, S. Y., Johansson, M. W., Söderhäll, K., and Cerenius, L., "A cell adhesion protein from the crayfish *Pacifastacus leniusculus*, a serine proteinase homologue similar to *Drosophila* masquerade," *Journal of Biological Chemistry*, Vol. 275, No. 14, pp. 9996-10001 (2000).
35. Jiang, H. and Kanost, M. R., "The clip-domain family of serine proteinases in arthropods," *Insect Biochemistry and Molecular Biology*, Vol. 30, No. 2, pp. 95-105 (2000).
36. Kang, C. J., Xue, J. F., Liu, N., Zhao, X. F., and Wang, J. X., "Characterization and expression of a new subfamily member of penaeidin antimicrobial peptides (penaeidin 5) from *Fenneropenaeus chinensis*," *Molecular Immunology*, Vol. 44, No. 7, pp. 1535-1543 (2007).
37. Lai, Y. and Gallo, R. L., "AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense," *Trends in Immunology*, Vol. 30, No. 3, pp. 131-141 (2009).
38. Lee, S. Y. and Söderhäll, K., "Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*," *The Journal of Immunology*, Vol. 166, No. 12, pp. 7319-7326 (2001).
39. Li, C. Y. and Song, Y. L., "Proline-rich domain of penaeidin molecule exhibits autocrine feature by attracting penaeidin-positive granulocytes toward the wound-induced inflammatory site," *Fish & Shellfish Immunology*, Vol. 29, No. 6, pp. 1044-1052 (2010).
40. Li, C. Y., Yan, H. Y., and Song, Y. L., "Tiger shrimp (*Penaeus monodon*) penaeidin possesses cytokine features to promote integrin-mediated granulocyte and semi-granulocyte adhesion," *Fish & Shellfish Immunology*, Vol. 28, No. 1, pp. 1-9 (2010).
41. Li, L., Wang, J. X., Zhao, X. F., Kang, C. J., Liu, N., Xiang, J. H., Li, F. H., Sueda, S., and Kondo, H., "High level expression, purification, and characterization of the shrimp antimicrobial peptide, Ch-penaeidin, in *Pichia pastoris*," *Protein Expression and Purification*, Vol. 39, No. 2, pp. 144-151 (2005).
42. Lin, C. Y., Hu, K. Y., Ho, S. H., and Song, Y. L., "Cloning and characterization of a shrimp clip domain serine protease homolog (c-SPH) as a cell adhesion molecule," *Developmental & Comparative Immunology*, Vol. 30, No. 12, pp. 1132-1144 (2006).
43. Munoz, M., Vandenbulcke, F., Saulnier, D., and Bachère, E., "Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp," *European Journal of Biochemistry*, Vol. 269, No. 11, pp. 2678-2689 (2002).
44. O'Leary, N. A. and Gross, P. S., "Genomic structure and transcriptional regulation of the penaeidin gene family from *Litopenaeus vannamei*," *Gene*, Vol. 371, No. 1, pp. 75-83 (2006).
45. Omori, S. A., Martin, G. G., and Hose, J. E., "Morphology of hemocyte lysis and clotting in the ridgeback prawn, *Sicyonia ingentis*," *Cell and Tissue Research*, Vol. 255, No. 1, pp. 117-123 (1989).
46. Otte, J. M., Werner, I., Brand, S., Chromik, A. M., Schmitz, F., Kleine, M., and Schmidt, W. E., "Human beta defensin 2 promotes intestinal wound healing *in vitro*," *Journal of Cellular Biochemistry*, Vol. 104, No. 6, pp. 2286-2297 (2008).
47. Rattanachai, A., Hirono, I., Ohira, T., Takahashi, Y., and Aoki, T., "Peptidoglycan inducible expression of a serine proteinase homologue from kuruma shrimp (*Marsupenaeus japonicus*)," *Fish & Shellfish Immunology*, Vol. 18, No. 1, pp. 39-48 (2005).
48. Ross, J., Jiang, H., Kanost, M. R., and Wang, Y., "Serine proteases and their homologs in the *Drosophila melanogaster* genome: an initial analysis of sequence conservation and phylogenetic relationships," *Gene*, Vol. 304, pp. 117-131 (2003).
49. Söderhäll, I., Kim, Y. A., Jiravanichpaisal, P., Lee, S. Y., and Söderhäll, K., "An ancient role for a prokineticin domain in invertebrate hematopoiesis," *The Journal of Immunology*, Vol. 174, No. 10, pp. 6153-6160 (2005).
50. Serhan, C. N. and Savill, J., "Resolution of inflammation: the beginning programs the end," *Nature Immunology*, Vol. 6, No. 12, pp. 1191-1197 (2005).
51. Shaykhiev, R., Beißwenger, C., Kändler, K., Senske, J., Püchner, A., Damm, T., Behr, J., and Bals, R., "Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure," *American Journal of Physiology-Lung Cellular and Molecular Physiology*, Vol. 289, No. 5, pp. L842-L848 (2005).
52. Song, Y. L. and Hsieh, Y. T., "Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances: Analysis of reactive oxygen species," *Developmental & Comparative Immunology*, Vol. 18, No. 3, pp. 201-209 (1994).
53. Song, Y. L., Yu, C. I., Lien, T. W., Huang, C. C., and Lin, M. N., "Haemolymph parameters of Pacific white shrimp (*Litopenaeus vannamei*) infected with Taura syndrome virus," *Fish & Shellfish Immunology*, Vol. 14, No. 4, pp. 317-331 (2003).
54. Soruri, A., Grigat, J., Forssmann, U., Riggert, J., and Zwirner, J., "β-Defensins chemoattract macrophages and mast cells but not lymphocytes and dendritic cells: CCR6 is not involved," *European Journal of Immunology*, Vol. 37, No. 9, pp. 2474-2486 (2007).
55. Sricharoen, S., Kim, J. J., Tunkijjanukij, S., and Söderhäll, I., "Exocytosis and proteomic analysis of the vesicle content of granular hemocytes from a crayfish," *Developmental & Comparative Immunology*, Vol. 29, No. 12, pp. 1017-1031 (2005).
56. Sritunyalucksana, K., "The proPO and clotting system in crustaceans," *Aquaculture*, Vol. 191, pp. 53-69 (2000).
57. Tanji, T., Hu, X., Weber, A. N. R., and Ip, Y. T., "Toll and IMD pathways synergistically activate an innate immune response in *Drosophila melanogaster*," *Molecular and Cellular Biology*, Vol. 27, No. 12, pp. 4578-4588 (2007).
58. Toumi, H., F'guyer, S., and Best, T. M., "The role of neutrophils in injury and repair following muscle stretch," *Journal of Anatomy*, Vol. 208, No. 4, pp. 459-470 (2006).
59. Wang, R., Lee, S. Y., Cerenius, L., and Söderhäll, K., "Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*," *European Journal of Biochemistry*, Vol. 268, No. 4, pp. 895-902 (2002).
60. Wang, Y. and Jiang, H., "Purification and characterization of *Manduca sexta* serpin-6: a serine proteinase inhibitor that selectively inhibits prophenoloxidase-activating proteinase-3," *Insect Biochemistry and Molecular Biology*, Vol. 34, No. 4, pp. 387-395 (2004).
61. Wang, Z. and Wang, G., "APD: the antimicrobial peptide database," *Nucleic Acids Research*, Vol. 32, No. suppl 1, pp. D590-D592 (2004).
62. Watson, F. L., Püttmann-Holgado, R., Thomas, F., Lamar, D. L., Hughes, M., Kondo, M., Rebel, V. I., and Schmucker, D., "Extensive diversity of

Ig-superfamily proteins in the immune system of insects," *Science*, Vol. 309, No. 5742, pp. 1874-1878 (2005).
63. Watthanasurorot, A., Saelee, N., Phongdara, A., Roytrakul, S., Jiravanichpaisal, P., Soderhall, K., and Soderhall, I., "Astakine 2--the dark knight linking melatonin to circadian regulation in crustaceans," *PLoS Genet*, Vol. 9, No. 3, e1003361 (2013).
64. Woramongkolchai, N., Supungul, P., and Tassanakajon, A., "The possible role of penaeidin5 from the black tiger shrimp, *Penaeus monodon*, in protection against viral infection," *Developmental & Comparative Immunology*, Vol. 35, No. 5, pp. 530-536 (2011).
65. Yang, Y., Poncet, J., Garnier, J., Zatylny, C., Bachère, E., and Aumelas, A., "Solution structure of the recombinant penaeidin-3, a shrimp antimicrobial peptide," *Journal of Biological Chemistry*, Vol. 278, No. 38, pp. 36859-36867 (2003).
66. Yeh, M. S., Chen, Y. L., and Tsai, I. H., "The hemolymph clottable proteins of tiger shrimp, *Penaeus monodon*, and related species," *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, Vol. 121, No. 2, pp. 169-176 (1998).
67. Yeh, M. S., Kao, L. R., Huang, C. J., and Tsai, I. H., "Biochemical characterization and cloning of transglutaminases responsible for hemolymph clotting in *Penaeus monodon* and *Marsupenaeus japonicus*," *Biochimica et Biophysica Acta (BBA)-Proteins & Proteomics*, Vol. 1764, No. 7, pp. 1167-1178 (2006).
68. Zasloff, M., "Antimicrobial peptides of multicellular organisms," *Nature*, Vol. 415, No. 6870, pp. 389-395 (2002).