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REVIEW

The antimicrobial peptides of the immune response of shrimp

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Abstract

The cultivation of penaeid shrimp is a worldwide economic activity which has the potential to contribute to increasing shrimp production. However, penaeid shrimps are susceptible to bacterial and viral diseases, and may thus cause significant losses to the aquaculture industry. In view of this, it is imperative to understand the immune response of shrimp against pathogens as this could help in devising efficient strategies to control, and eventually eradicate, shrimp diseases. At present, a considerable number of research studies on the identification and characterization of antimicrobial peptides/proteins (AMPs) in penaeid shrimps. Such research activities will contribute to finding solutions to shrimp diseases. AMPs are widespread in animals and plants, involved in their innate immunity, and considered as the front liners of host defense against pathogens. In penaeid shrimps, eight kinds of AMPs have been found. These are the penaeidins, whey acidic protein (WAP) domain containing proteins [crustins and single WAP domain containing peptides (SWD)], antilipopolysaccharide factors (ALFs), lysozymes, a C-type lectin, histones, anionic hemocyanins, and peritrophins. In this study, the structures, distributions, expression profiles, phylogenetic evolution, and functions of some AMPs are discussed, focusing on the WAP-domain containing peptides and ALF in penaeid shrimp.

Key words: antimicrobial peptides; innate defense effectors; innate immunity; penaeid shrimp

Introduction

The cultivation of penaeid shrimp is an important economic activity in the world. This industry, however, has been suffering serious problems brought by viral and bacterial diseases. One specific disease is the white spot syndrome virus (WSSV) infection which has caused a drastic decline in production and multi-national economic losses. Based on the report of the Fisheries and Aquaculture Department of Food and Agriculture Organization (2007), there is an exceeding 2.4 million tons per annum of shrimp global aquaculture production. However, up to 25 % of this production was estimated to have been lost due to diseases. Given that pathogenic diseases are one significant cause of production and economic loss in the shrimp industry, this phenomenon calls for an urgent understanding of the immune defenses of shrimp. At present, research studies are being conducted to examine the innate immunity of shrimp, and such activities have been continuously contributing to the development of shrimp aquaculture. In fact, it has been found that comparable to insects, the innate defense of shrimp is triggered by the pattern recognition receptors, such as the Gram-negative binding proteins (Vargas-Albores *et al.*, 1997; Yepiz-Plascencia *et al.*, 1998; Jimenez-Vega *et al.*, 2002; Roux *et al.*, 2002; Sritunyalucksana *et al.*, 2002; Romo-Figueroa *et al.*, 2004; Cheng *et al.*, 2005; Du *et al.*, 2007; Lin *et al.*, 2008) and the C-type lectins (Luo *et al.*, 2006; Liu *et al.*, 2007; Sun *et al.*, 2008). Generally, the recognition of non-self activates a proteolytic cascade of serine proteases that amplify the signal and trigger downstream effector responses. This becomes possible through the signal transduction pathways which lead to the elimination of the invader. Moreover, the serine proteinase and its inhibitors was found in shrimp (Okumura, 2007), and the Toll-like receptors and other signal pathway molecules also reported in several shrimp (Arts *et al.*, 2007; Yang *et al.*, 2007; Yang *et al.*, 2008).

Anti-microbial peptides (AMPs) are a diverse group of innate immune effector molecules in multi-

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cellular organisms. They are considered as effector molecules for immune cells that prevent or withstand microbial infection. Similar to those found in other animals, AMPs are also key factors in the innate immunity of shrimp (Bachère *et al.*, 2004).

Since AMPs play a significant role in the inherent immunity of shrimp, research studies have been actively focusing on the identification and characterization of AMPs in penaeid shrimp. In fact, eight kinds of AMPs have been found in penaeid shrimps. These are the penaeidins (Destoumieux *et al.*, 1997, 1999, 2000; Cuthbertson *et al.*, 2004; Muñoz *et al.*, 2004; Kang *et al.*, 2004, 2007), whey acidic protein (WAP) domain containing proteins [crustins and single WAP containing peptides (SWD)] (Gross *et al.*, 2001; Amparyup *et al.*, 2008a; Jia *et al.*, in press), antilipopolysaccharide factors (ALFs) (Gross *et al.*, 2001; Liu *et al.*, 2005; Liu 2006; Somboonwiwat *et al.*, 2005, 2008; Tharntada *et al.*, 2008), histones (Patat *et al.*, 2004), hemocyanin (Destoumieux-Garzon *et al.*, 2001; Zhang *et al.*, 2004), lysozymes (Hikima *et al.*, 2003 Sotelo-Mundo *et al.*, 2003; Bu *et al.*, 2008; de la Re Vega *et al.*, 2006; Burge *et al.*, 2007; Xing *et al.*, in press), a C-type lectin (Sun *et al.*, 2008), and peritrophins (Loongyai *et al*., 2007).

This study presents a discussion of the structures, distributions, phylogenetic evolution, expression profiles, and functions of some AMPs, particularly on the WAP-domain containing peptides and ALF from penaeid shrimp.

Whey acidic protein (WAP)-domain containing peptides (WDPs)

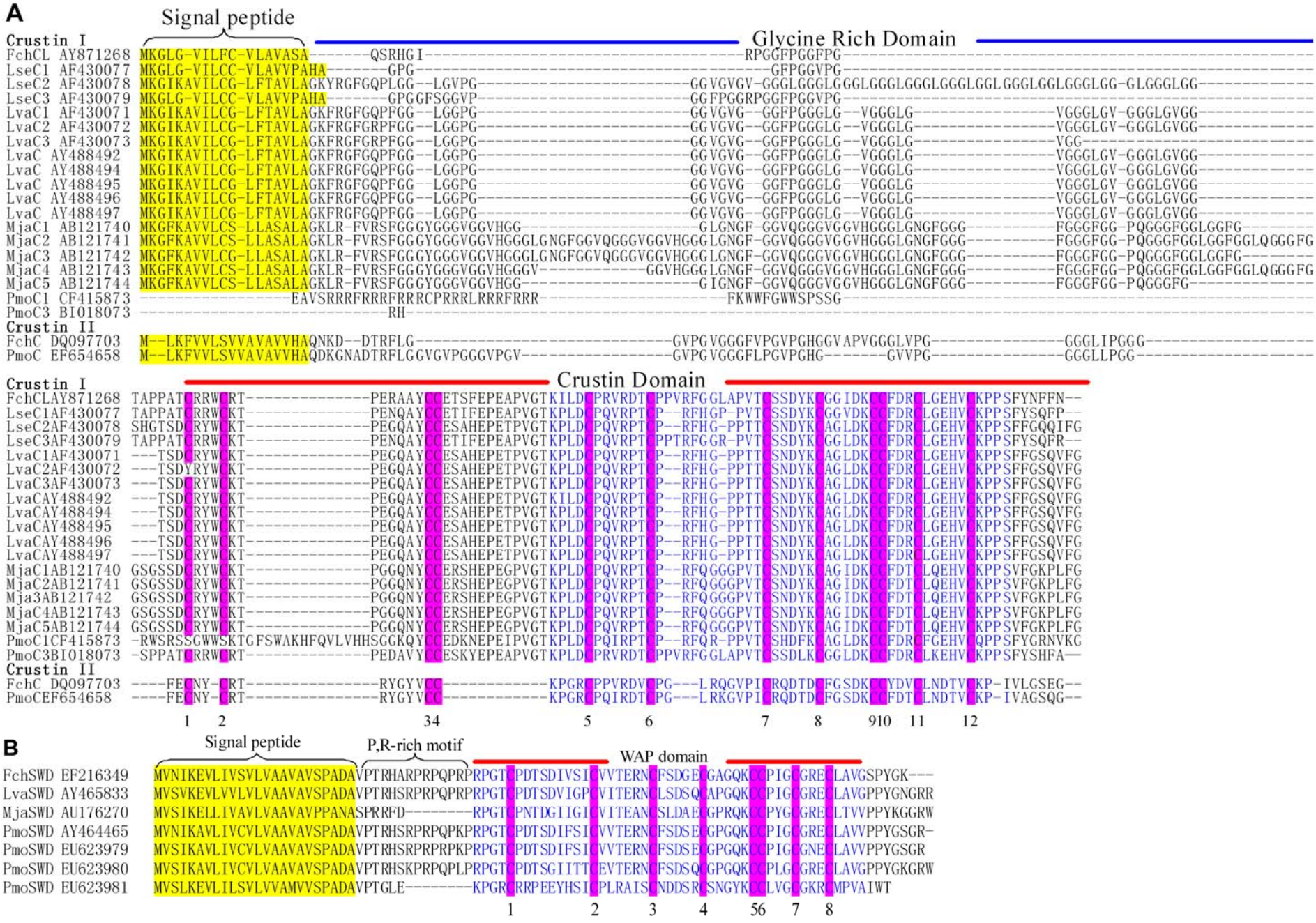
A major milk protein in most mammals, WAP, has eight cysteine residues arranged to form a tightly packed structure called a four-disulphide core (4-DSC) at the carboxyl terminus (Hennighausen and Sippel, 1982). These WAP domain-containing proteins are found to prevail among metazoans (Beg, 1995; Devinoy *et al.*, 1988; Ali *et al.*, 2002; Carro *et al.*, 2004; Furutani *et al.*, 2004). They are further found to have highly diverse biological functions, including proteinase inhibition (Ranganathan *et al.*, 1999; Schalkwijk *et al.*, 1999; Ota *et al.*, 2002), antimicrobial activity (Relf *et al.*, 1999; Hagiwara *et al.*, 2003), and association to ovulation (Garczynski *et al.*, 1997). In addition, WAP-domain proteins have antiviral functions, specifically against the human immunodeficiency virus (Alvarez *et al.*, 2008).

Crustins, which are anti-microbial peptides containing a WAP-domain, were first identified in the shore crab *Carcinus maenas*, characterized as cysteine-rich 11.5 kDa antimicrobial peptides which function against Gram-positive bacteria (Relf *et al.*, 1999). There have been more than 50 crustins or crustin-like peptides reported to have been found from a variety of decapods, including crabs, lobsters, shrimp, and crayfish (refer to the review of Smith *et al.*, 2008). In this study, they were termed as WAP-domain containing peptides (or WDPs) and were classified into two sub-families, namely, crustins and SWD (the justification for such is discussed below). These WDPs are apparently a large family of antimicrobial peptides ubiquitous among penaeid shrimp. In fact, the cDNAs of crustins and WDPs have been reported to be present in a variety of penaeid shrimp, including *Litopenaeus vannamei*, *Litopenaeus setiferus* (Gross *et al.*, 2001; Bartlett *et al.*, 2002; Vargas-Albores *et al.*, 2004), *Penaeus monodon* (Chen *et al.*, 2004; Supungul *et al.*, 2004, 2008; Amparyup *et al.*, 2008a, b), *Marsupenaeus japonicus* (Rattanachai *et al.*, 2004), *Fenneropenaeus chinensis* (Zhang *et al.*, 2007; Jia *et al.*, in press), *Farfantepenaeus paulensis*, *Farfantepenaeus subtilis*, *Farfantepenaeus brasiliensis*, and *Litopenaeus schmitti* (Rosa *et al.*, 2007). Accordingly, the crustins in shrimp are diverse in amino acid sequences. However, they are conserved with the C-terminus of 12 cysteine residues, thereby leading it to be termed as crustin-domain, in which a single WAP domain is contained. The SWDs have no crustin domain, and only contain a single WAP-domain (8 cysteine residues).

Classification of shrimp WDPs

Recently, the crustins (WPDs) in crustaceans were comprehensively reviewed by Smith *et al*. (2008). In the review they discussed three main types of crustins (Crustin type I, II and III) in crustaceans. Here, we focused on the crustins and SWDs in penaeid shrimp, including the new functions of the peptides. There have been several studies that have revealed the presence of crustins and SWDs in different penaeid shrimp species (refer to the above citations), in this study, most of the sequences WDPs in penaeid shrimp and in other crustaceans were collected, including some Expressed Sequence Tags (EST) from the GenBank database. A multiple alignment analysis for the amino acid sequences (Fig. 1) and the phylogenetic analysis of the proteins were performed. The neighbor-joining tree revealed that the WDPs in crustaceans could be divided into four different clusters (Fig. 2), namely, crustins I and II, carcinin and carcinin-like peptides, and the SWD. It is noteworthy to mention that in this study, our classification is considerably different from that of Smith *et al.* (2008). The crustin type I that they discussed in their study is similar to carcinin and carcinin-like peptide discussed in this study. Similarly, the crustin type II is equivalent to our crustins I and II, and the crustin type III is equivalent to our SWDs.

The WDPs in the penaeid shrimp are divided into three classes, namely, crustin I, crustin II, and SWDs. The first class is characterized by the following: (1) a relatively conserved signal peptide, (2) an N-terminal glycine-rich domain, and (3) a C-terminal cysteine-rich domain (12-cysteine crustin domain) with the following signature: $C1(X_3)C2(X_X)C3C4(X_{16})C5(X_6)C6(Xn)C7(X_5)C8(X_5)$ $C9(X_5)C9C10(X_3)C11(X_5)C12$. The second class of crustins have similar sequence domains as class I, but in terms of signal peptide, there are sequence differences between them. Another difference between them lies in the sequence of their crustin domain, as crustin II have the following signature: $C1(X_2)C2(X_7)C3C4(X_4)C5(X_6)C6(Xn)C7(X_5)C8(X_5)C9$ $C10(X_3)C11(X_5)C12$, specifically in the residue numbers between C4 and C5 (Fig.1A). Finally, the


A
Signal peptide
Glycine Rich Domain
Crustin Domain
Crustin I
Crustin II
FchCL AY871268
LseC1 AF430077
LseC2 AF430078
LseC3 AF430079
LvaC1 AF430071
LvaC2 AF430072
LvaC3 AF430073
LvaC AY488492
LvaC AY488494
LvaC AY488495
LvaC AY488496
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MjaC2 AB121741
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MjaC4 AB121743
MjaC5 AB121744
PmoC1 CF415873
PmoC3 BI018073
FchC DQ097703
PmoC EF654658
FchCLAY871268
LseC1AF430077
LseC2AF430078
LseC3AF430079
LvaC1AF430071
LvaC2AF430072
LvaC3AF430073
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MjaC1AB121740
MjaC2AB121741
Mja3AB121742
MjaC4AB121743
MjaC5AB121744
PmoC1CF415873
PmoC3BI018073
FchC DQ097703
PmoCEF654658
1 2 34 5 6 7 8 910 11 12
B
Signal peptide
P,R-rich motif
WAP domain
FchSWD EF216349
LvaSWD AY465833
MjaSWD AU176270
PmoSWD AY464465
PmoSWD EU623979
PmoSWD EU623980
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1 2 3 4 56 7 8

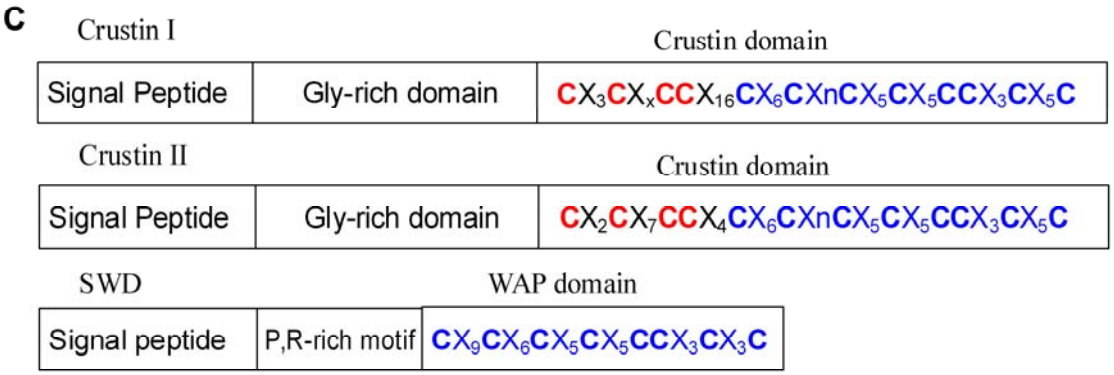

Fig. 1 Alignment of amino acid sequences of Crustins (A), and SWDs (B), and the domain signature of penaeid shrimp and other Crustacea (C). Fch, *Fenneropenaeus chinensis*; Lse, *Litopenaeus setiferus*; Lva, *Litopenaeus vannamei*; Mja, *Marsupenaeus japonicus*; Pmo, *Penaeus monodon*; The sequences of signal peptides are presented in yellow, the identical cysteines that characterize crustin or WAP domain are in purple, and the WAP domains are shown in blue.

class III WDPs are single WAP domain-containing peptides (SWD) which are characterized by the following: (1) a highly conserved signal peptide, (2) a proline- and arginine-rich motif between the signal peptide and the WAP domain, and (3) a WAP-domain (8 cysteine residues) in the C-terminus with the signature: C1(X_9)C2(X_6)C3(X_5)C4(X_5)C5C6(X_3)C(X_3)C (Fig.1B). In crustaceans, carcinin and carcinin-like peptides have a signal peptide and a crustin domain, without the N-terminal glycine-rich domain. Moreover, SWDs are significantly different from Crustins I and II, and Carcinins because they have no crustin-domain in their sequences. We therefore consider the idea that the four groups of WDPs in crustaceans should be divided into two sub-families, namely, the crustins (which present crustin-domain in their sequences) and the SWDs (which only have WAP-domain). As such, these two sub-families also have different functions in vitro (as discussed below).

Structure comparison of shrimp WDPs with other AMPs

To date, a wide variety of AMPs in metazoans have been identified. On the basis of sequence and structural features, these cationic AMPs can be grouped into three classes: (i) the linear peptides which form α-helices and do not contain cysteine residues; (ii) the cyclic peptides which contain cysteine residues; and (iii) the peptides with an over-representation in one or two residues, such as proline, glycine, arginine, and tryptophan (Bulet *et al.*, 2004).

Several antibacterial glycine-rich polypeptides have been isolated from various insect species. They are actually considered effective against Gram-negative bacteria and are inactive against Gram-positive bacteria, yeasts and mammalian cell lines (Mackintosh *et al*., 1998). Another example is that of short-chain proline-rich peptides, which are mostly active against Gram-negative bacteria, while the Gram-positive cells remain generally unaffected (Bulet *et al*., 1999). Furthermore, the cyclic peptides containing cysteine residues, like insect defensins, are active against a wide range of Gram-positive bacteria and only for a few Gram-negative bacteria, fungi and yeasts (Bulet *et al.*, 1999).

Crustins I and II are composed of an N-terminal glycine-rich domain, and a C-terminal region which contains 12 cysteine residues (crustin domain) organized in two doublets. These crustins are similar with the two classes of insect AMPs, that is glycine-rich peptides and cyclic peptides containing cysteine residues (Bulet *et al.*, 1999). On the other hand, the SWDs are composed of a short proline-arginine-rich region and a C-terminal region containing 8 cysteine residues (the WAP domain). They are also similar in terms of the two classes of insect AMPs, particularly the cyclic peptides containing cysteine residues and the proline- and arginine-rich peptides. Similar to the penaeidins found in shrimp, the crustins and the SWD are chimera molecules of glycine- or proline-rich AMPs and cysteine-rich AMPs.

Chimera-like features often reflect the multi-functional properties of a molecule, such that each domain performs different functions. For example, crustins I and II have glycine-rich and crustin domains, and therefore should have anti-Gram positive and negative bacterial activities. Supungul *et al.* (2008) reported that crustinPm1 (which belongs to crustin I) exhibited anti-microbial activity against only a Gram-positive bacteria, whereas the rCrus-like*Pm* (crustin II) showed remarkable anti-microbial activity against both Gram-positive and -negative bacteria (Amparyup *et al.*, 2008b). Likewise, the CruFc (crustin II) from *F. chinensis* exhibited high activity against Gram-positive bacteria but low activity was exhibited against Gram-negative bacteria and fungi (Zhang *et al.*, 2007). The SWDs have Pro- and Arg-rich and WAP domains. They exhibit the following activities: relatively high against Gram-positive and/or negative

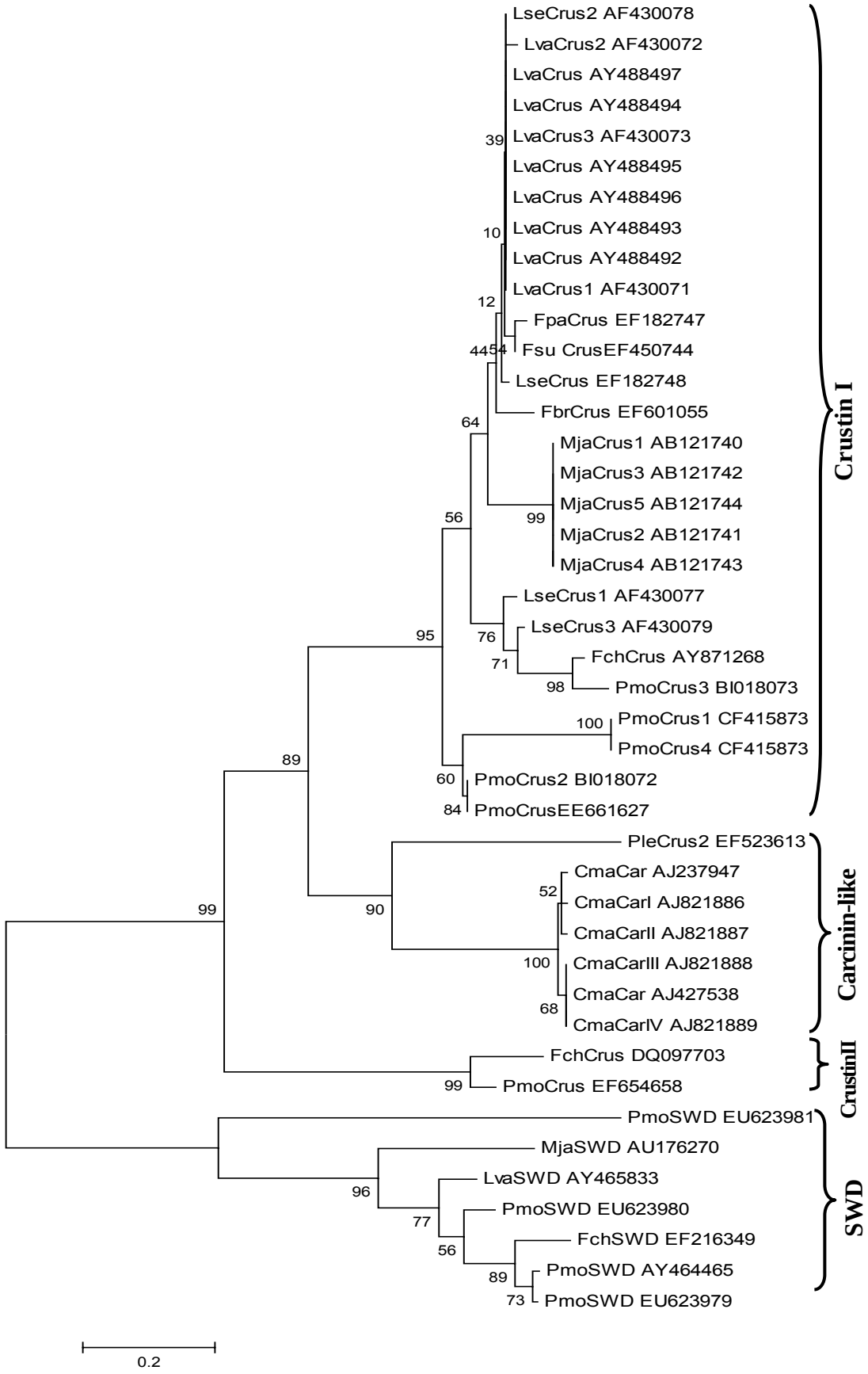

Fig. 2 Phylogenetic analysis of WAP containing proteins/ peptides in penaeid shrimps by MEGA 4. Five thousand bootstraps were performed for the neighbour-joining trees to verify the reliability of the results.
Cma, *Carcinus maenas*; Ham, *Homarus americanus*; Hga, *Homarus gammarus*; Par, *Panulirus argus*; Ple, P*acifastacus leniusculus*. Others are the same with Fig. 1.

bacteria, moderate against fungi, and strong antiproteinase activity, especially against the bacterial proteinases (Amparyup et al., 2008a; Jia *et al.*, in press). Therefore, they are bi-function peptides.

From above results, we can see that the primary structures of AMP are not corresponding to their functions. It need further study for their tertiary structures.

Expression profiles and functions of shrimp WDPs

The spatio-temporal expression of WDPs in shrimp was not well-understood. Most of them seem to be constitutively expressed in the hemocytes. Apparently, the expression patterns was only reported during the development of the larvae of shrimp, *P. monodon*. High level expression of a crustin are recorded at all stages of development from the nauplii stage IV to juvenile period(Jiravanichpaisal *et al.*, 2007).

Furthermore, the expression patterns of WDPs to bacterial challenge were reported in several shrimps, but the results showed no consistent patterns of change in expression subsequent to bacterial injection. Vargas-Albores *et al.* (2004) found two isoforms of crustin I in *L. vannamei*, which showed different expression patterns after bacterial inoculation. First, crustin-P seems to be constitutively expressed, and second, the crustin-I mRNA concentration drops after 6 h. Results also revealed that there was a decrease in the transcribed expression of crustin in *P. monodon* subjected to bacterial challenge (Supungul *et al.*, 2004). In hemocytes, the *M. japonicus* crustin-like peptide mRNA was identified, and the expression level of this peptide mRNA increased significantly 1, 3, and 7 days after peptidoglycan feeding (Rattanachai *et al.*, 2004). The Crus *Pm*1 (crustin I) in *P. monodon* was also expressed in hemocytes, but the expression profile was not analyzed (Supungul *et al.*, 2008). The mRNA transcript of a Crus-like *Pm2* (crustin II) in *P. monodon* was found to be abundantly expressed in hemocytes and was significantly up-regulated after *Vibrio harveyi* injection (Amparyup *et al.*, 2008b).

Jiménez-Vega *et al.* (2004) reported that the expression of the *SWD* gene in *L. vannamei* hemocytes increased after 3 to 6 h it was inoculated with *V. alginolyticus*, but slowly returned to non-stimulated levels within 12 to 24 h. The *Fc-SWD* from Chinese shrimp is constitutively expressed and increased in hemocytes 24 h after bacterial challenge (*Staphylococcus aureus* and *Vibrio anguillarum*). The results of the Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis revealed a weak expression in heart and gill and challenged stomachs in addition to hemocytes. Moreover, results showed that the signal from challenged tissues was stronger than from those unchallenged. Consequently, these results suggest that *Fc-SWD* is an inducible gene and is essential in responding to bacterial infection (Jia *et al.*, in press). The tissue distribution of *SWDs* in *P. monodon* was as well analyzed through RT-PCR. Results indicated the presence of all three *SWD* transcripts in hemocytes. The transcript expression of *SWDPm1* was down-regulated upon injection with *S. aureus* while no change was recorded in temrs of *SWDPm2* and *SWDPm3* expressions. Contrastingly, the results obtained from the WSSV injection showed that in a biphasic response, there was an up-regulation of the *SWDPm1* and *SWDPm2* transcripts at 6 h followed by a significant down-regulation by 24 h after infection (Amparyup *et al.*, 2008a).

The WDPs are a large family of antimicrobial effectors in shrimp immunity. In fact, more than 30 WDPs, including isoforms and ESTs, have been found in shrimp. Despite this, many of them are poorly characterized for their functions. One of the *P. monodon* crustins (crustin I), recombinant expressed in *E. coli*, exhibited an antimicrobial activity against only Gram-positive bacteria, specifically with strong inhibition against *S. aureus* and *Streptococcus iniae* (Supungul *et al.*, 2008). Zhang et al. (2007) similarly reported that the recombinant CrusFc (crustin II) had relatively high activities against Gram-positive bacteria and low activities against Gram-negative bacteria and fungi. Moreover, another recombinant crustin II (EF654658) from *P. monodon* has been recently reported to have a strong activity not only against Gram-positive bacteria, but also against Gram-negative bacteria, such as *Escherichia coli 363* and *Vibrio harveyi* (Amparyup *et al.*, 2008b).

In penaeid shrimp, there were less than 10 SWDs found, and two of them were studied for their functions in vitro. The functions of SWD molecule from Chinese shrimp were analyzed (Jia *et al.*, in press). The recombinant Fc-SWD has manifested antimicrobial activities against Gram-positive and Gram-negative bacteria and fungi, as well as a strong inhibitory activity against subtilisin A and protein K with an inhibition constant (Ki) of 2.14 nM and 2.27 nM, respectively; but a much lesser activity against trypsin was recorded. Amparyup *et al.* (2008a) also analyzed the biological functions of recombinant SWD*Pm*. Based on the results they obtained, recombinant SWD*Pm* exhibits activity against several Gram-positive, but not Gram-negative bacteria and is a competitive inhibitor of subtilisin A with an inhibition constant (Ki) of 1.98 nM. This phenomenon indicates the dual functions of SWDs, that is antimicrobial activity and anti-proteinase activity against pathogenic proteinase. Therefore, these SWDs might have an important role in the immunity of shrimp *in vivo*.

So far, it is generally accepted that both activities could not co-exist in the same (unique) domain. Why does SWD show both anti-microbial and anti-proteinase activities? In fact, similar situations were found in some peptides with a single domain, which is the Avian WAP (AWAP IV) originally found in chicks (Townes *et al.*, 2006). This has a broad-spectrum of antibacterial activities against both Gram-positive and Gram-negative bacteria. In addition to that, the Avian WAP lysate significantly inhibited the activities of the microbial serine proteinases subtilisin and proteinase K. Furthermore, Li *et al.* (2007) reported the occurrence of a small serine proteinase inhibitor with antimicrobial capability in a diskless-fingered odorous frog, *Odorrana grahami*. Based on a disulfide-bridged hendecapeptide loop of this serine

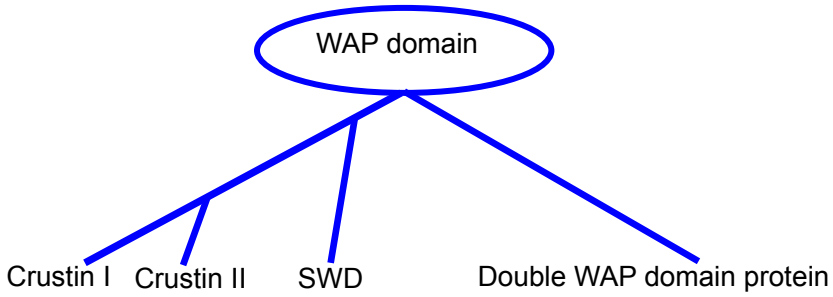

Fig. 3 The possible divergent evolution of WPDs in crustaceans.

proteinase inhibitor, a series of peptides have been synthesized. They found that seven synthetic peptides exhibited trypsin inhibitory activity, while the other five have both the trypsin inhibitory and antimicrobial activities. In terms of SWDs, the findings showed that they have high anti-proteinase activities to bacterial serine proteinases (subtilisin A and proteinase K) and antimicrobial activities. The recombinant WAP domain (Fc-WAPD) shows relatively low activities against the bacterial serine proteinases (Jia *et al.*, in press), and manifests quite a low activity against bacteria. Comparing their sequence, it was found that Fc-SWD contain higher positively charged amino acids than the Fc-WAPD. The net charge of Fc-SWD is +4, while that of Fc-WAPD is –2. In addition, it is generally known that most antimicrobial cationic peptides have the same unique features, that is, they are both polycationic (having a net positive charge of more than +2) and fold into amphipathic structures (having both a hydrophobic and a hydrophilic domain). As such, these characteristics enable them to interact with the negatively charged surface molecule of bacteria and to interact with and penetrate into the negatively charged cytoplasmic membranes of most bacteria (Hancock, 1997). This is therefore the reason for the high anti-microbial activity of Fc-SWD compared to that of Fc-WAPD's low activity against bacteria (Jia *et al.*, in press).

The possible divergent evolution of the WAP domain in crustaceans

The WAP domain was initially identified in the primary milk protein of rats and mice (Hennighausen and Sippel, 1982). WAP proteins have one or more WAP domains containing about 50 amino acids with eight highly conserved cysteine residues that form a four-disulphide core (4-DSC). Amino acids in the WAP domain, except for the conserved cysteine residues, are significantly diverse, and proteins with a WAP motif perform variety of functions. In fact, a large biological diversity exists between the proteins that contain one or two WAP domains, with many being identified as proteinase inhibitors or AMPs. The most studied WAP proteins, for example, are the elafin and antileukoproteinase, which are two serine-proteinase inhibitors with anti-microbial and inflammatory activity (Bouchard *et al.*, 2006). In crustaceans, several WAP domain-containing proteins were found. In addition to the aforementioned four WAP-containing proteins, double WAP domain containing proteins were also reported in shrimp [the *L. vannamei* secretory leukocyte proteinase inhibitor, EF467169; the *M. japonicus* double WAP domain-containing protein, EU095018; the *F. chinensis* double WAP domain-containing protein (our unpublished data)]. Many identified genes that code for WAP proteins in human are clustered on chromosome 20q12–13.1 (Bouchard *et al.*, 2006). The results of Southern analysis show that a large family of sequences related to the crustins is present in *L. vannamei* genome (Bartlett *et al.*, 2002). This may indicate some similarities in gene locations. Based on the domain structure and functions of the WAP-containing proteins in crustaceans, we therefore propose the possible divergent evolution of WAP domain in crustaceans (Fig. 3). In this proposal, the different groups may have different functions, including antimicrobial and anti-proteinase activities among others.

ALF factors

ALF, a basic peptide, was initially found as a potent anticoagulant from horseshoe crabs, *Limulus polyphemus* and *Tachypleus tridentatus*, which inhibited the endotoxin mediated activation of the coagulation cascade (Tanaka *et al.*, 1982). Thereafter, several studies demonstrated that the ALF from hemocytes of the horseshoe crab *L. polyphemus* have similar characteristics with that of the binding and neutralizing lipopolysaccharide (LPS). Additionally, ALF was indicated to have a strong antibacterial activity, particularly on the growth of Gram-negative bacteria (Morita *et al.*, 1985; Aketagawa *et al.*, 1986; Muta *et al.*, 1987). In shrimp, the cDNA clones homologous to the horseshoe crab ALFs were initially identified in hemocytes of *P. monodon* and *L. setiferus* by means of EST analysis (Gross *et al.*, 2001; Supungul *et al.*, 2004). In recent years, there have been a growing number of studies on shrimp ALF available to provide pertinent information. To note, several ALFs have been isolated and characterized from hemocytes in penaeid shrimp, *F. chinensis* (Liu

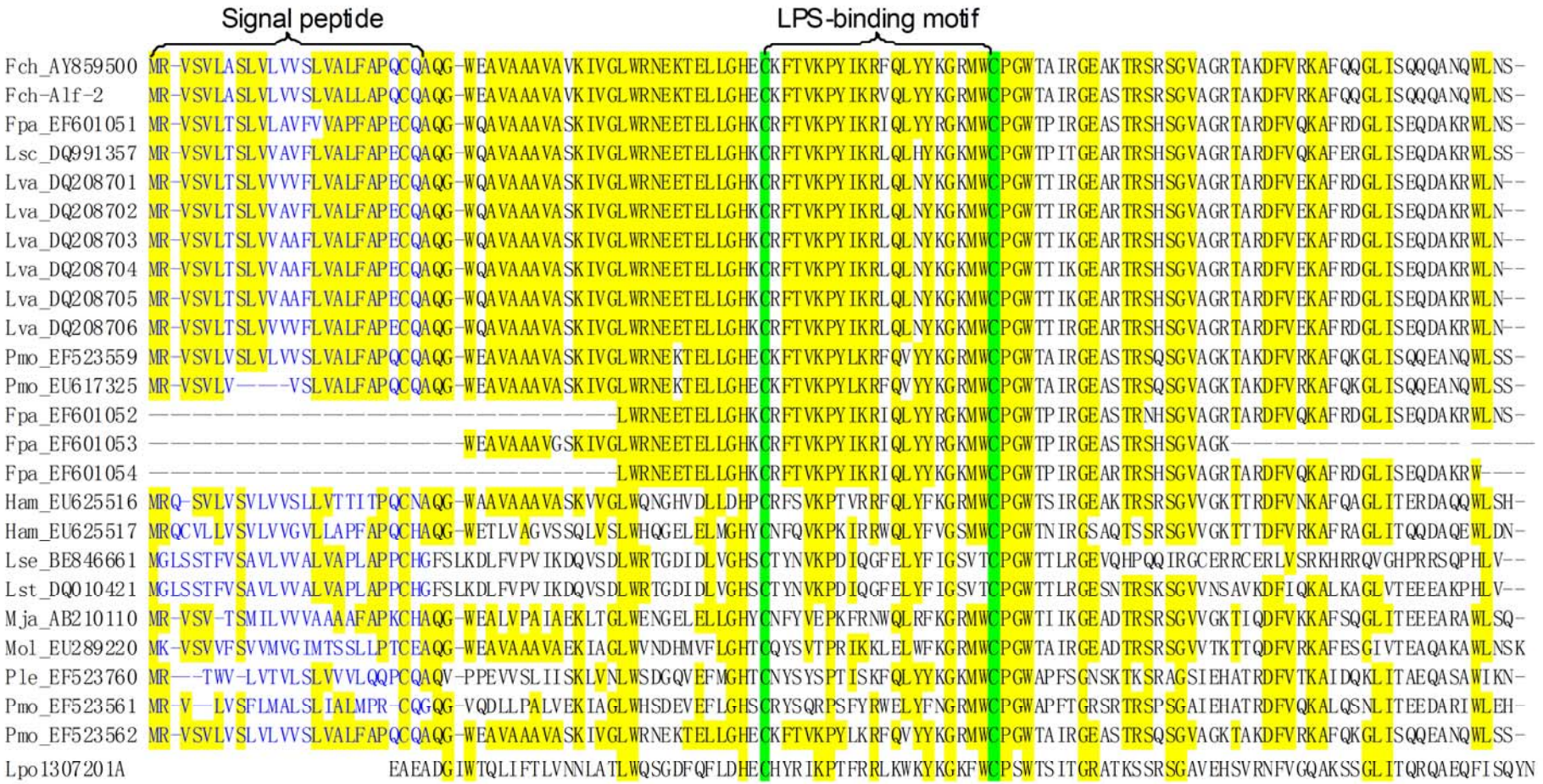

Fig. 4 Alignment of ALFs-based amino acid sequence. All sequences of ALFs are from GenBank. Fch, *Fenneropenaeus chinensis*; Fpa, *Farfantepenaeus paulensis*; Ham, *Homarus americanus*; Lse, *Litopenaeus setiferus*; Lsc, Lst; *Litopenaeus stylirostris*:*Litopenaeus schmitti*; Lva, *Litopenaeus vannamei*; Mja, *Marsupenaeus japonicus*; Mol, *Macrobrachium olfersii*; Pmo, *Penaeus monodon*; and Ple, P*acifastacus leniusculus*, Lpo, *Limulus polyphemus*.

et al., 2005; Zhou *et al.*, 2008) *M. japonicus* (Nagoshi *et al.*, 2006), *P. monodon* (Supungul *et al.,* 2004; Somboonwiwat *et al.*, 2005; Tharntada *et al.*, 2008), *L. vannamei* (de la Vega *et al.*, 2008), *L. setiferus* (Gross *et al.*, 2001), *F. paulensis* (EF601051, EF601054), *L. schmitti* (DQ991357), and *L. stylirostris* (DQ010421).

Classification of shrimp ALF

Twenty-nine crustacean ALF sequences collected from GenBank were aligned using the Alignment Editor of the MEGA 4 software (Tamura *et al.*, 2007). The results obtained by the multiple alignment (Fig. 4) revealed that all the molecules of ALFs contain two preserved cysteine residues which form a disulfide bridge. It was also found out that ALF contained a relatively conserved sequence of a positively-charged amino acid residue cluster within the disulfide loop. This structure of the β-hairpin loop in shrimp ALF suggests a conservation of the LPS binding activity.

With the use of neighbor joining method of MEGA 4, a phylogenetic tree was constructed (Tamura *et al.*, 2007). To attain and assess the reliability of the tree, bootstrapping using 5,000 replications was as well performed. The phylogenetic tree analysis categorized the various ALF proteins into three main groups. Accordingly, most ALFs from shrimp belong to cluster I, including the ALFs from *L. setiferus* (white gulf shrimp); then cluster II contains the *L. stylirostris* (blue shrimp) and *Eriocheir sinensis* (Chinese mitten crab). Finally, cluster III contains the ALFs from *L. vannamei* (Pacific white shrimp), *P. monodon* (black tiger shrimp) and other crustacean *Pacifastacus leniusculus* (signal crayfish), *L. polyphemus* (Atlantic horseshoe crab), and *Tachypleus tridentatus* (horseshoe crab) (Fig. 5). It can be observed that in *L. vanamei* and *P. monodon*, two kinds of ALFs.

Expression profiles and functions in shrimp innate immunity

The transcription of ALFs in some species was specified in tissues. In Chinese shrimp (*F. chinensis*), for example, the ALF (Cluster I) has high expression in hemocytes, gills, and intestine but exhibited low expression in ovary, hepatopancreas, and muscle (Liu *et al.*, 2005). Similar observations had been reported in *P. monodon*, in which ALF (Cluster I) was constitutively expressed in hemocytes, hearts, gills, intestines, and lymphoid organs, while there was no observed transcription in the hepatopancreas (Supungul *et al.*, 2004). Another example is that in the kuruma shrimp (*M. japonicus*), in which the ALF (Cluster I) was reported to be expressed at higher levels in hemocytes, lymphoid organs, hearts, intestines, and gills. The expression of ALFs, on the other hand, was found to be at lower levels in stomachs, hepatopancreas, and muscles (Nagoshi *et al.*, 2006). In *L. vannamei*, ALF1 (which belongs to Cluster III) was found to have high mRNA levels in the lymphoid organ and heart, intermediate levels in the gills, eyestalk, and hemocytes, and very low levels in the muscle and hepatopancreas (de la Vega *et al.*, 2008). The aforementioned reports are considerably in contrast with the patterns of many other AMPs in shrimp which are carried out primarily in hemocytes (Munoz *et al.*, 2002; Bachère *et al.*, 2004; Kang *et al.*, 2004). It is important to note that in shrimp, however, it is clear that ALF transcription, although it is tissue-specific, occurs in multiple organs and could thereby provide systemic protection against pathogens.

The transcription of the ALF is induced upon bacterial challenge in several shrimp (Supungul *et al.*, 2004; Liu *et al.*, 2005; Nagoshi *et al.*, 2006). In a study, ALF was induced upon WSSV infection or exposed to UV-inactivated WSSV (Liu *et al.*, 2006). On the other hand, in *L. vanname*, the infection with pathogenic bacterium, *Vibrio penaeicida* or fungus, *Fusarium oxysporum*, did not cause any significant change in the *Lv*ALF1 mRNA levels compared to saline-injected controls. An considerable increase was recorded in the *Lv*ALF1 mRNA expression in WSSV-infected shrimp at 54-h time point (de la Vega *et al.*, 2008). Similarly, in *L. vannamei*, ALF was significantly up-regulated in the infected viral hepatopancreas (Robalino *et al.*, 2007). This up-regulation of antimicrobial proteins as a response to viral infection has also been reported in *Drosophila* (Zambon *et al.*, 2005). Despite these multitude studies, the mechanism in anti-viral responses of ALF still needs to be clarified. The results reported by different authors indicate the inconsistent expression patterns of the ALFs after the shrimps have been injected with bacteria or viruses. As such, this further indicates the different functions *in vivo* of the ALFs.

To date, only a few ALFs and their characteristics have been analyzed. In *P. monodon*, ALF*Pm*3, a predominant antimicrobial peptide, was identified in both the unchallenged and *V. harveyi*-challenged shrimp (Supungul *et al.*, 2004). In their study, a strong activity against multiple Gram-positive and Gram-negative bacteria and filamentous fungi (Somboonwiwat *et al.*, 2005) has been recorded. Another study found out that a synthetic peptide, corresponding to the LPS-binding domain of Mj-ALF from *M. japonicus*, exhibited a LPS-neutralizing and hemolytic activities on LPS-sensitized human red blood cells (Nagoshi *et al.*, 2006). Moreover, ALF was found to have a potential in interfering with the replication of WSSV in crayfish *P. leniusculus* (Liu *et al.*, 2006).

The in vivo function of ALF in protecting shrimp from bacterial, fungal, and viral infections was also studied in *L vanamei* through the RNA interference (RNAi) method (de la Vega *et al.*, 2008). The injection of double-stranded RNA (dsRNA) corresponding to the *Lv*ALF1 message resulted in a significant reduction of the abundant *Lv*ALF1 mRNA levels. Following knockdown of the *Lv*ALF1, the shrimps were challenged with low pathogenic doses of pathogenic *V. penaeicida*, or *F. oxysporum*. The results showed a significant increase in the mortality among the *Lv*ALF1 low-level shrimps, specifically those with *V. penaeicida* and *F. oxysporum* infections, compared to the control shrimps. The result showed that this gene functions in protecting shrimp from both bacterial and fungal infections. In the viral challenge using WSSV, the ALF dsRNA injection caused no significant increase in mortality

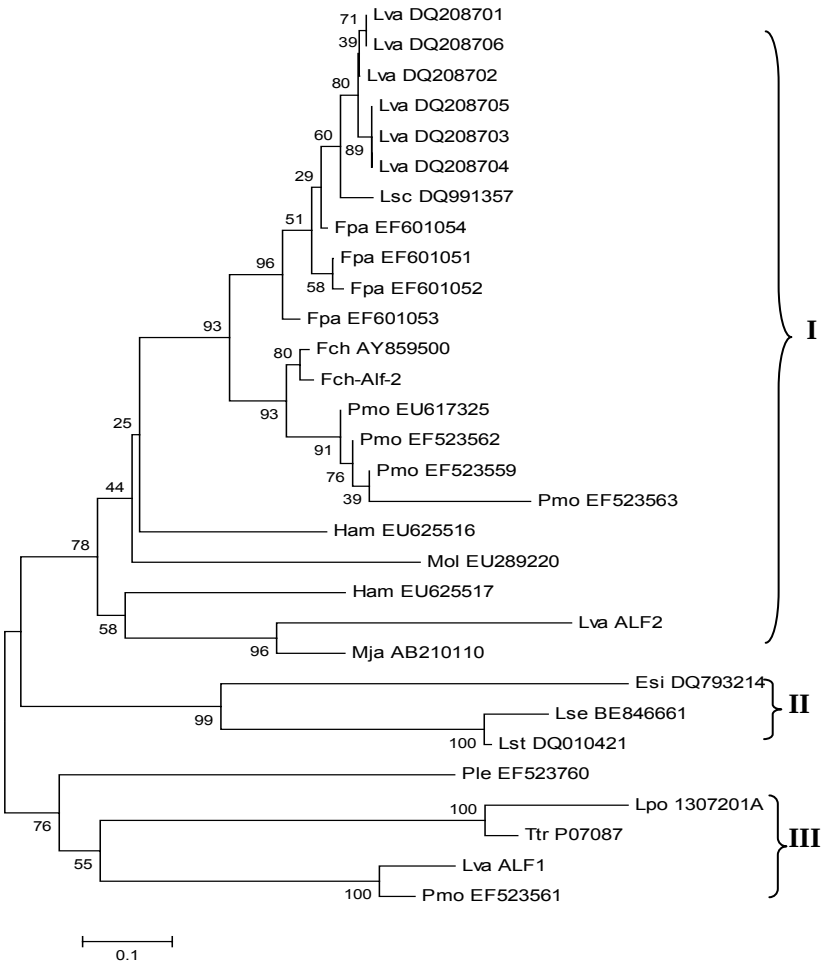

Fig. 5 Phylogenetic analysis of ALFs from shrimp using the sequence information from Fig. 4.

compared to the non-specific dsRNA controls in the *L. vannamei* (de la Vega *et al.*, 2008). Other experiments that focused on ALF RNAi in freshwater *P. leniusculus* indicated that ALF can protect against WSSV infection, as observed in the ALF low-level through RNAi, which specifically resulted in higher rates of viral propagation (Liu *et al.*, 2006). The differences in the two above-mentioned reports emphasize the diverse taxonomic groups of crustacea and their varying responses to infection and immunity mechanisms (de la Vega *et al.*, 2008).

Possible mechanisms of LPS binding and antimicrobial activity

ALFs contain two conserved cysteine residues which form a disulfide bridge. They also contain a relatively conserved sequence of a positively charged amino acid residue cluster within the disulfide loop, which is regarded as ALF's functional domain. Hoess *et al.* (1993) reported the high resolution structure of a recombinant *Limulus*-ALF (L-ALF). In their report, they stressed that it contains an N-terminal α-helix (that opens into a α-helix in its final turn), a simple four-stranded anti-parallel β-sheet, and two C-terminal α-helices. The three helices form a bundle that packs against the β-sheet and encloses a hydrophobic and highly-aromatic core. Their study performed a structural analysis of L-ALF through the X-ray crystallography, and the results demonstrated the β-hairpin loop with an alternating series of hydrophilic (mainly basic amino acids) in the central disulfide-bonded loop region thus obtaining an amphipathic protein molecule (Hoess *et al.*, 1993). This amphipathic loop structure is believed to be a LPS-binding motif which can bind a single fatty acid with the phosphoglucosamine portion of lipid A (the membrane anchor of LPS). This amphipathic loop of L-ALF were compared to the ALF sequences (Cys^{1st} to Cys^{2nd}) of shrimp, and the results revealed similarity between the alternating residues pattern (Fig. 4). As such, the structure of the β-hairpin loop in shrimp ALF suggests that there is a conservation of LPS binding activity.

Somboonwiwat *et al.* (2008) also studied the binding activity of rALF*Pm*3 and found that it could strongly bind to both Gram negative and Gram positive bacterial cells. Further analysis demonstrated that ALF*Pm*3 could bind to both the immobilized LPS and lipoteichoic acid (LTA), with a high dissociation constant (K_d) of 1.26×10^{-8} and 1.34×10^{-8} M, respectively. This suggested that they

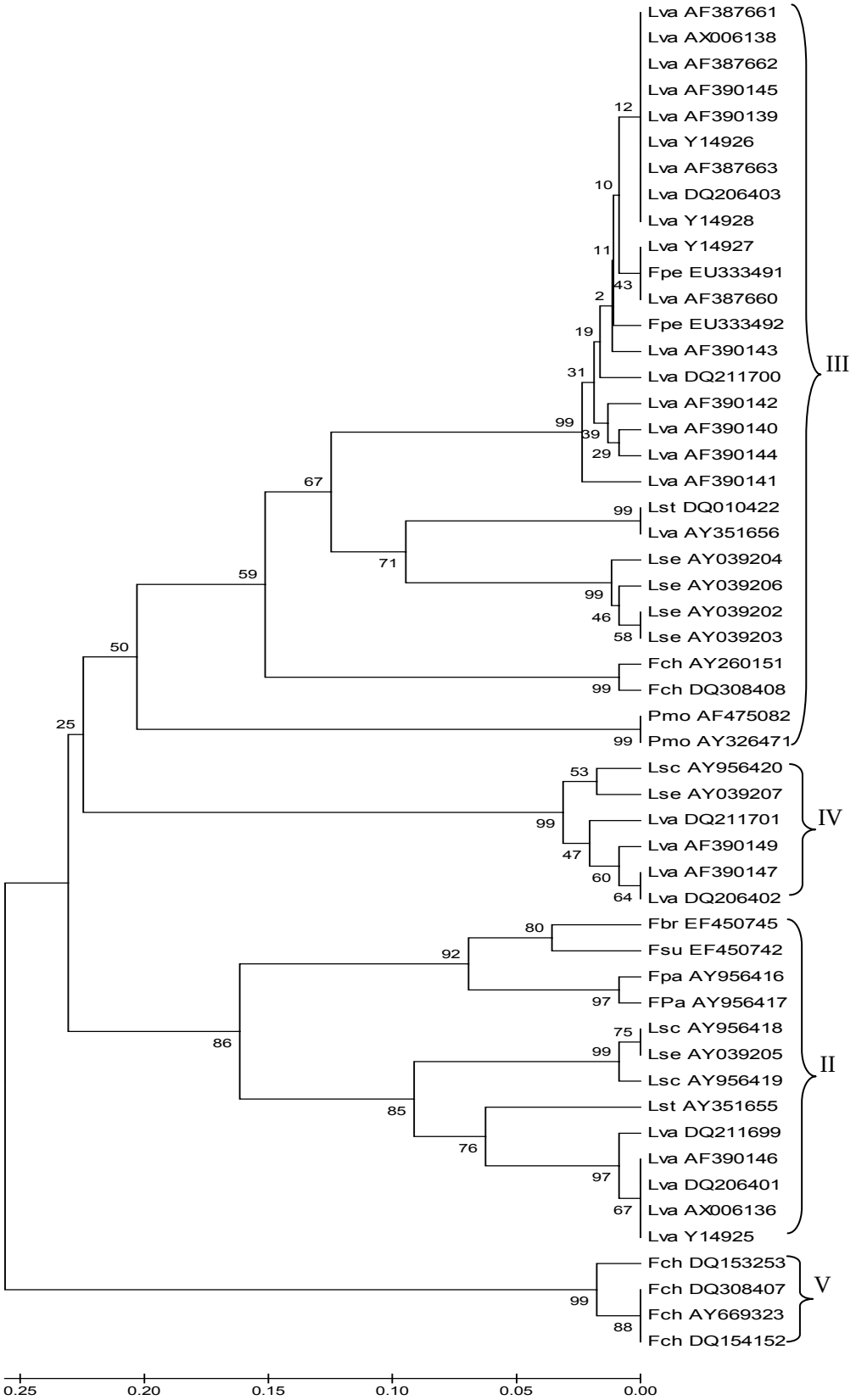

Fig. 6 Phylogenetic analysis of penaeidins from shrimp using the sequence information from GenBank. The UPGMA tree was obtained using MEGA with complete deletions of gaps. Bootstraps (5000) were performed for the UPGMA trees to verify repeatability and reliability of results. Fch, *Fenneropenaeus chinensis*; Fpa, *Farfantepenaeus paulensis*; Fpe, *Fenneropenaeus penicillatus*; Fsu, *Farfantepenaeus subtilis*; Fbr, *Farfantepenaeus brasiliensis*; Lva, *Litopenaeus vannamei*; Lst, *Litopenaeus stylirostris*; Lsc, *Litopenaeus schmitti*; Lse, *Litopenaeus setiferus*, and Pmo, *Penaeus monodon*

are at least one of the target molecules for the ALF*Pm*3 on Gram-negative and Gram-positive bacteria, respectively. Assuming that ALF binds to bacterial cells before they exterminate the cells, it is still unknown as to how such extermination is performed.

ALF has both the ability to inhibit the endotoxin or LPS mediated coagulation system and to exhibit strong anti-microbial activity against the Gram-negative and Gram-positive bacteria and fungi. Thus, ALF is also one of the pivotal effectors in shrimp immunity.

Penaeidins

Penaeidins, initially isolated from the Pacific white shrimp *L. vannamei* (Destoumieux *et al.*, 1997), are also a large family of AMPs that have been detected in several penaeid shrimp, including *L. setiferus*, *M. japonicus*, *P. monodon*, *F. chinensis* (Bachère *et al*., 2004; Kang *et al.*, 2004, 2007; Cuthbertson *et al.*, 2008), *L. stylirostris* (AAY33770), *L. schmitti* (AAX58698; AAX58697), *Fenneropenaeus penicillatus* (ABY56821), *F. paulensis* (AAX58696), *F. subtilis* (ABO93321), and *F. brasiliensis* (ABO93324). In fact, among the AMP family, the penaeidins are considered the most well-characterized in terms of the level of gene expression and biological activities. There are four classes of penaeidins (penaeidins 2, 3, 4, and 5) that have been characterized so far (Bachère *et al.*, 2004; Kang *et al.*, 2007) (Fig. 6). This classification and characterization of penaeidin isoforms have been summarized in the database, PenBase (Gueguen *et al.*, 2006) and were reviewed by several authors (Bachère et al., 2004; Cuthbertson *et al.*, 2008). The evolution pattern of these peptides was likewise analyzed (Padhi *et al.*, 2007). Table 1 presents the primary characteristics and functions of penaeidins.

Lysozymes

Lysozyme (muramidase, EC.3.2.1.17), an important antibacterial protein, catalyzes the hydrolysis of bacterial cell walls and acts as a non-specific innate immunity molecule against the invasion of bacterial pathogens (Jollés and Jollés, 1984). Initially found among eukaryotes and prokaryotes, the lysozymes are classified into six types. These are the chicken-type lysozyme (c-type), goose-type lysozyme (g-type), plant lysozyme, bacteria lysozyme, T4 phage lysozyme (phage-type), and invertebrate lysozyme (i-type) (Hikima *et al.*, 2003). Similarly, these lysozymes had been reported in several shrimps, such as *L. vannamei* (Sotelo-Mundo *et al.*, 2003; de-la-Re-Vega *et al.*, 2006; Burge *et al.*, 2007; Xing *et al.*, in press), *M. japonicus* (Hikima *et al.*, 2003), *P. monodon*, (Xing *et al.*, in press), *P. semisulcatus* (Xing *et al.*, in press) and *F. chinensis* (Bu *et al.*, 2008). Furthermore, most lysozymes identified in shrimps belong to a c-type lysozyme, like those from *L. vannamei* (AF425673), *M. japonicus* (BAC57467), *P. monodon* (B1784440), and *F. chinensis* (AAV83994). In addition, there were i-type lysozymes identified in shrimp, such as lysozymes from *L. vannamei* (BF023863, BF024192) and *L. setiferus* (BF024309) (Hikima *et al.*, 2003).

In some penaeid shrimp, the lysozymes are well-characterized, and they possess lytic activity against a range of Gram-positive and Gram-negative bacterial species, including pathogenic *Vibrio spp.* (Hikima et al., 2003; de-la-Re-Vega et al., 2006). Hikima *et al.* (2003) used the RT-PCR analysis and reported that the lysozyme from *M. japonicus* was strongly expressed in samples from hemocytes, moderately expressed in the epidermis, and weakly expressed in the gills, midgut, and muscle. Furthermore, the post-infection expression profile of a lysozyme EST was analyzed using the macro-array, Northern blot, and real-time PCR. The results revealed that the hemocyte lysozyme expression during the *V. penaeicida* challenge was significantly lower at 12 h after the infection, but had returned to control levels within 24-96 h after the challenge as observed in the surviving shrimps (de Lorgeril *et al.*, 2005). Similarly, the lysozyme mRNA from Chinese shrimp (*FcLyz*) was analyzed by semi-quantitative RT-PCR. The lysozyme was actually expressed in various tissues of the unchallenged shrimp and the expression of *FcLyz* was increased in the bacterial-challenged tissues of the hemocytes, heart, hepatopancreas, and gills as compared to the mock (saline)-challenged ones (Bu *et al.*, 2008).

C-type lectin

C-type lectins have diverse functions. Aside from their agglutinating activity and opsonic effects, some C-type lectins perform antimicrobial activities. A hepatopancreas specific C-type lectin, designated *Fc-hsL*, has been found from the hepatopancreas of the Chinese shrimp, *F. chinensis* (Sun *et al.*, 2008). This type of lectin was constitutively expressed in the hepatopancreas of normal shrimp, and its expression was up-regulated after the bacterial and viral challenge. In addition to Fc-hsL's calcium-dependent agglutinating and binding activity to some Gram-positive and Gram-negative bacteria, it also has high anti-microbial activity against some Gram-positive and Gram-negative bacteria and fungi. Therefore, Fc-hsL may act as a pattern recognition receptor and an effector molecule in the innate immunity of Chinese shrimp.

Other antimicrobial peptides/proteins

Hemocyanin, as the main protein component of hemolymph, is prevalent in several invertebrate animals. It typically represents up to 95 % of the total amount of protein (Sellos *et al.*, 1997). Meanwhile, the hexamer is the predominant form in the most primitive crustacean Decapoda, such as *Penaeus setiferus* or *P. monodon* (Sellos *et al.*, 1997). The hemocyanin, in relation to crustaceans, primarily functions as anthropods' oxygen carrier. It is also the multi-functional proteins involved in physiological processes, such as osmoregulation, protein storage or enzymatic activities. In some reports, the fragments generated from the C-terminus of hemocyanin of *L. vanamei* and *L. stylirostris* have exhibited a high anti-fungal activity (Destoumieux-Garzon *et al.*, 2001). Contrary to most

Table 1 Antimicrobial peptides/proteins in penaeid shrimp

AMPs	Expression tissues	Functions	References
1. Penaidins			
Penaeidin II Penaeidin III Penaeidin IV Penaeidin V	Primarily in hemocytes and in highly vascular tissues	Broad spectrum of anti-Gram-positive and anti-fungal activities and weak activity against Gram-negative strains	Destoumieux *et al.*, 1997, 1999, 2000; Bachère *et al*., 2004; Cuthbertson *et al*., 2004, 2008; Kang *et al.*, 2004, 2007
2. WDPs			
Crustin I	Hemocytes	Anti-Gram-positive activity	Supungul *et al.*, 2008
Crustin II	Hemocytes	Anti Gram-positive and anti-Gram-negative activities	Zhang *et al.*, 2007; Amparyuo *et al.*, 2008
SWD	Hemocytes	Anti-Gram-positive, anti-Gram-negative, anti-fungal and anti-proteinase activities	Amparyup *et al.*, 2008; Jia *et al.*, in press
3. ALFs			
ALF I	High expression in hemocytes, gills and intestine and low expression in ovary, hepatopancreas and muscle	Anti-Gram-positive, anti-Gram-negative and anti-fungal activities; LPS-neutralizing and hemolytic activities; interference with the replication of WSSV	Gross *et al*., 2001; Supungul *et al*., 2004; Liu *et al.*, 2005; Nagoshi *et al.*, 2006
ALF II	?	?	(DQ010421，BE846661)
ALF III	High level in lymphoid organ and heart, intermediate levels in the gills, eyestalk and hemocytes and very low levels in the muscle and hepatopancreas	Anti-Gram-positive, anti-Gram-negative and anti-fungal activities	de la Vega *et al.*, 2008; Somboonwiwat *et al.*, 2005; Zhou *et al.*, 2008
4. Lysozymes			
c-type lysozyme	Hemocytes, lymphoid organ, hepatopancreas, gill, heart, midgut, muscle, epidermis, and eyestalk	Anti Gram-positive and anti-Gram-negative activities	Sotelo-Mundo *et al.*, 2003; Hikima *et al*.,2003; de-la-Re-Vega *et al.*, 2006; Burge *et al.*, 2007; Xing *et al.*, in press; Bu *et al*., 2008
i-type lysozyme	?	?	(BF023863, BF024192) (BF024309)
5. C-type lectin			
HsL	Hepatopancreas	Anti-Gram-positive, anti-Gram-negative and anti-fungal activities	Sun *et al*., 2008
6. Hemocyanin-derived peptides			
C-terminal fragment 7.9 kDa, 8.3 kDa	Hepatopancreas	Anti-fungal activity	Destoumieux-Garzón *et al.*, 2001
Hemocyanin subunits	Hepatopancreas	Anti-viral activity	Zhang *et al.*, 2004
7. Histones			
H2A, H2B, and H4	Hemocytes	Anti-Gram-positive activity	Patat *et al.*, 2004
8. Peritrophin	ovary	Anti Gram-positive and anti-Gram-negative activities	Loongyai *et al*., 2007

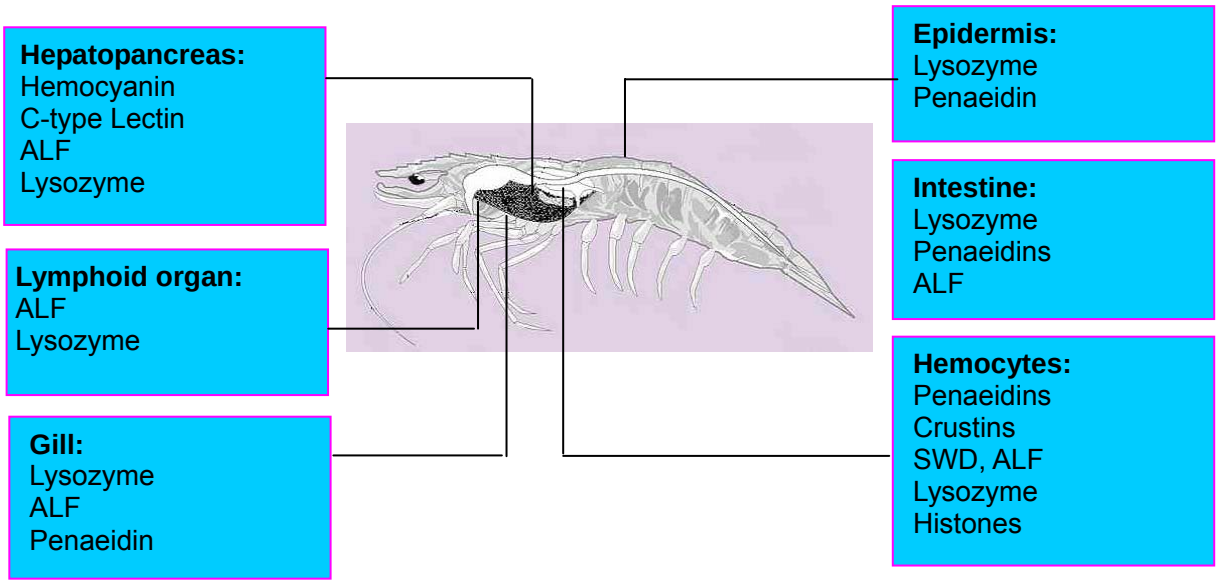

Fig. 7 Distribution of anti-microbial peptides/proteins in shrimp.

AMPs with highly-cationic charges, the antifungal substances present a negative net charge at physiological pH with a *p*I ranging from 5.65 to 6.54. Zhang *et al.* (2004) also reported that shrimp hemocyanin had antiviral property as it inhibited the virus replication.

Histone proteins are primarily involved in DNA packaging and regulation of DNA replication and transcription. A number of reports have shown that histone proteins or histone-derived peptides from various vertebrates possess antimicrobial activity (Hirsch, 1958; Robinette *et al.*, 1998; Fernandes *et al.*, 2002). Patat *et al.* (2004) as well reported that the hemocyte histone H2A, a mixture of histones H2B and H4 and an H1-derived fragment in *L. vannamei*, have anti-microbial activity against the tested bacterium. They believed that histone proteins or histone-derived peptides can be secreted to the cytoplasm from the nucleus and be localized in it along with other antimicrobial peptides.

Shrimp peritrophin, a major protein in jelly layer (JL) and cortical rods (CRs) (Du *et al.*, 2006; Loongyai *et al.*, 2007), was inducing expression in hemocytes, heart, stomach, intestine and gill, and was constitutively expressed in ovary (Du *et al.*, 2006). The recombinant peritropnin exhibited a chitinase activity and efficiently inhibited the growth of *Vibrio harveyi* and *S. aureus*, with minimum inhibitory concentrations of 2.4 and 15.7 μM, respectively (Loongyai *et al.*, 2007).

Conclusions

To summarize, shrimps have efficiently developed and used their innate immune system in defense against pathogenic microorganisms. To date, eight kinds of AMPs have been identified in penaeid shrimp, namely, penaeidins, WDPs (crustins and SWDs), ALFs, lysozymes, anionic hemocyanin, histones, and a C-type lectin. These kinds of AMPs have different distribution and expression profiles and functions in shrimp (Table 1 and Fig. 7). Furthermore, there are several subclasses or isoformes for each kind of AMPs in one species. An example is the three subgroups of WDPs (Crustin I, II, and SWD) in Chinese shrimp. This only suggests that they had different functions *in vivo* in the shrimp. Even though large groups of AMPs were found in penaeid shrimp, there is still a limited number of studies conducted about the AMPs' antibacterial properties and their other functions. In view of this, there is a need to have more studies that will delve on the functions and the anti-bacterial properties of AMPs, especially with respect to the diverse bioactivities of the natural proteins *in vivo*. As such, some AMPs in shrimp can be better candidates for clinical uses in the aquaculture.

Acknowledgements

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