Published in final edited form as:

Dev Comp Immunol. 2008 ; 32(3): 167–181.

Diversity in penaeidin antimicrobial peptide form and function

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Abstract

Penaeidins are a diverse family of two-domain antimicrobial peptides expressed in shrimp. Variation in penaeidin sequence results in functional diversity, which was discovered using synthetic reproductions of native penaeidins. An isoform of penaeidin class 3 from *L. setiferus* (Litset Pen3–4) was synthesized using native ligation and compared directly with the synthetic penaeidin class 4 known to be expressed in the same organism. New antimicrobial activity data is included in this review that emphasizes differences in effectiveness that are apparent from a direct comparison of two classes. A novel approach to intact penaeidin analysis is presented in the form of Fourier Transform Ion-Cyclotron Resonance Mass Spectrometry, which has implications for the identification of individual penaeidin isoforms without chemical modification or enzymatic cleavage. The new information included in this review helps gather the perspective on relevance of penaeidin diversity to antimicrobial function, the use of synthetic peptides as tools to evaluate specific immune functions and the application of high mass resolution, top-down sequencing methods to the intact analysis of individual penaeidin isoforms.

Keywords

Antimicrobial peptide; proline-rich; cysteine-rich; yeast; *Cryptococcus*; *Candida*; mass spectrometry; MALDI-TOF MS; FTICR MS; ECD

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Overview

Antimicrobial peptides (anti-MPs) are a diverse group of innate immune effector molecules that are utilized by multicellular organisms to prevent or combat infection by microbes. Anti-MPs have been the subject of extensive review and several categories of anti-MPs have been described based on structural characteristics with certain themes being repeated throughout the living world [1-4]. Two prominent themes of anti-MP form that are relevant to this particular review include linear, proline-rich peptides, and cysteine-rich peptides that fold into a tertiary structure stabilized by disulfide bonds. These two separate themes provide a lead-in to the peptide family that is the primary focus of this review, the penaeidin family of anti-MPs.

Many examples of short anti-MPs found in the literature contain a considerable percentage of Pro residues associated with other specific amino acids, particularly Arg, in repetitive motifs [5-7]. Such peptides have been well characterized in insects and mammals. Apidaecin, abaecin, drosocin, formaecin, pyrrhocorin and lebecin are all members of the extended apidaecin family of proline-rich peptides that was originally discovered in honeybees and now found to be widespread in insects [3,5,8-10]. Apidaecin family members primarily function as anti-bacterial agents, with no notable anti-fungal activity described. The mechanism of action of certain apidaecin family members appears to be more complex than bacterial cell lysis through membrane disruption as experimental evidence supports a targeted mechanism of action through direct interaction with bacterial DnaK (a 70kDa heat shock protein) [9,11-13].

Mammalian cathelicidins constitute a broad category of anti-MPs that all share a similar *N*-terminal prepropeptide segment; however, the mature, processed *C*-terminal segment varies considerably in length and amino acid composition [6,14,15]. The batenecins are a subset of the cathelicidin family that include linear, proline-rich antimicrobial peptides (e.g. Bac5 and Bac7) formed of repetitive motifs of proline, cationic (Arg and His) and hydrophobic (Leu, Ile, Phe and Tyr) amino acids [16,17]. In contrast to apidaecin-like peptides from insects, Bac5 exhibited some level of antifungal activity apparently affiliated with the *N*-terminal segment of this anti-MP [18]. Moreover, the structure induced by the position of proline residues appears to strongly influence the range of activity of Bac5 [18]. The membrane penetrating properties of batenecins are not only interesting because of the obvious effect on targeted microbes, but also because they confer the ability to penetrate other types of biological membranes. For instance, the *N*-terminal portion of Bac7 will penetrate mammalian cells and has potential application in drug delivery devices [19].

Disulfide bond stabilized tertiary structure is a theme, where considerable variation in the disulfide stabilized tertiary fold is observed, that is repeated many times over among cysteine-rich anti-MPs [2-4,7,20,21]. Insect defensins are some of the most notable examples and they retain a specific cysteine-stabilized α -helix and β -sheet configuration (CS $\alpha\beta$) [4,7,8,20]. Defensin-like molecules are widespread in nature, and are not limited to animals. Multiple examples have been described in plants, such as hevien [22,23]. In general, defensins target a wide range of microbes with notable activity against Gram(+) bacteria [4,20,24,25]. Other defensin-like Cys-rich anti-MP, such as drosomycin, appear to be primarily antifungal agents, thereby exhibiting selective antimicrobial activity [4,26]. Human α - and β -defensins constitute a family of anti-MPs that exhibit broad antimicrobial activity and are produced by various cell types and found in many tissues [27,28]. In addition to their antimicrobial activity, human defensins are known to act as cell signaling molecules [29,30]. Recently, a new type of primate defensin has been characterized, θ -defensin, that is cyclic and has three disulfide bonds [31]. θ -defensins are active against a wide range of microbes including human pathogens [32]. Even though a considerable body of work has been amassed in the field of defensin research the mechanism of action is still under investigation. Currently two models have been put forth for anti-MP mechanism of action [2,5,33] which are the barrel-like (barrel-stave) pore forming

mechanism and the carpeting mechanism. Each of these would result in the compromising of the microbial membrane, and much evidence corroborating both of these mechanisms has been reviewed previously [2,5,33].

Penaeidins are antimicrobial peptides from shrimp that are unique in that they are composed of two very different domains [34–36], an *N*-terminal proline-rich domain (PRD) linked to the *C*-terminal cysteine-rich domain (CRD) [37,38]. While the PRD forms an extended structure, the folded structure of CRD contains an α -helix stabilized by disulfide bonds [37,38]. Penaeidins were the subjects of two review articles [39,40] soon after their discovery and initial characterization, and they have since been subjects of extensive, in-depth research projects. Fig. 1 illustrates the relatedness of a representative penaeidin (Litset Pen4–1) to proline-rich anti-MP including Bac5 from cow (Fig. 1A) and a 6.5kDa peptide from crab (Fig. 1B), and examples of defensin-like proteins including β -defensin1 (Def1) from human (Fig. 1A) and heliomicin (Heli) from budworm (Fig. 1C). While penaeidins themselves are very unique and do not appear to have a clear orthologue in arthropods, their two different domains are surprisingly similar to separate types of anti-MPs that are prevalent throughout the living world. The similarity of the crab 6.5kDa protein sequence to penaeidin PRDs (Fig. 1, [40]) implies that there may be other Crustacea that express penaeidin like proteins, but the current definition of penaeidins includes the presence of the CRD as well [36]. Genome studies have yet to reveal clear evidence of how penaeidins evolved relative to other arthropod anti-MP except for the fact that the two domains are encoded by separate exons in some genes [41,42]. A plausible hypothesis is that the two exon structure of genes encoding two different penaeidin classes may reflect a situation where separate genes became juxtaposed during the evolution of Crustacea forming the separate exons of a potential penaeidin ancestral gene.

Penaeidin discovery and evaluation of function

Penaeidins were first discovered in the Pacific whiteleg shrimp (*Litopenaeus vannamei*) by extraction of proteins from a pool of hemocytes combined through collection from many individuals [34]. Total proteins were separated by reversed phase chromatography and each of the many fractions collected from this separation were used in antimicrobial assays. These native penaeidins from the shrimp hemolymph were examined for their range of antimicrobial activity and the individual peptides were sequenced [34]. Three unique sequences were obtained from the analysis of more than one active fraction and these were named penaeidin classes 1, 2 and 3. Only two of these classes were detectable in a cDNA library made from shrimp hemocytes from the same species, but three different isoforms of class 3 were detected in the cDNA library [34]. Class 1 has since been defined as a class 2 variant [35,36]. This approach followed similar experiments that successfully sought antimicrobials and other natural products from arthropods [10,43,44]. In fact, using this type of screen, fragments of the oxygen transport protein hemocyanin, which are increased in hemolymph after stimulation, were found to have anti-MP properties [45].

In the initial description of penaeidins and in the follow-up examination of recombinant forms of penaeidin [46], it was apparent that penaeidins are active primarily against Gram(+) bacteria, with some effect on fungi at higher concentrations. Making recombinant penaeidins proved challenging for two reasons including: the observed antimicrobial activity against bacteria, and the presence of conserved cysteines, indicative of disulfide bonds. A *Saccharomyces cerevisiae* based expression system proved useful for the production of penaeidin class 3 with respect to the antimicrobial activity spectrum of this class, but non-native glycosylation [46] was a limitation that required troubleshooting [37] and the final product was not *C*-terminally amidated like native penaeidins. Recently, a penaeidin from *Fenneropenaeus chinensis* was successfully produced using a methylotrophic yeast expression system [47]. In this case the product included additional residues at the *N*-terminus which may have affected overall

function. These studies show that penaeidins can be produced in appreciable quantities using recombinant yeast systems, but that non-natural modifications may occur. As the range of activity of penaeidin classes 2 and 3 from *L. vannamei* were examined in detail using recombinant peptides, it was revealed that the functional differences between these two classes were not very appreciable outside of the fact that class 3 appeared to be effective at a lower concentration than class 2 [46].

Expression of penaeidin genes occurs primarily in hemocytes and in highly vascular tissues [39,42,48-51]. The first significant discovery of penaeidin peptide expression and distribution revealed that penaeidins are stored in hemocytes until their release upon challenge [39,50]. This work provided significant findings indicating that penaeidins are responsive to pathogens and may provide surface protection to prevent or combat infection. Dramatic changes in penaeidin expression in circulating hemocytes in response to microbial challenge were observed [39,50], and upon further investigation, data to support the assertion that penaeidin-expressing hemocytes migrate to sites of infection was obtained [50]. Furthermore, evidence was gathered that indicates hemocytes that contain penaeidins in granules release their contents intracellularly and may lyse in the process of delivering penaeidins to the site of infection [48]. Moreover, the penaeidins may be involved in coating microbes for immune cell (phagocyte) recognition in addition to their antimicrobial activities [48]. Much of this work has been corroborated independently in different species [51] with more evidence being put forth of novel penaeidin activities such as agglutination of bacteria.

Penaeidin diversity

Diversity in penaeidins exists much like diversity in other anti-MP families (e.g. defensins) [36] in the form of multiple isoforms that constitute distinct classes of peptides. A feature of the penaeidin family is the intra-class diversity, particularly for class 3 where multiple unique isoforms have been documented in several studies [34-36,51,52]. Class 3 isoform diversity is not only observed within a species, but also within an individual organism [35]. Substantial differences in length and amino acid composition are observed when comparing the PRD of class 3 isoforms from different species, a characteristic that is reminiscent of the differences between batenecins, yet more pronounced within this particular penaeidin class. In fact, *L. setiferus* class 3 exhibits only 65% identity to *L. vannamei* class 3 with most of this identity observed in the CRD (78% identity) and the first 14 residues of the PRD (78.5% identity). It is possible that each domain evolves somewhat independently, with the PRD consistently being more evolutionarily flexible across isoforms of class 3 from different species, and the CRD being more conserved. The same is true when comparing the different classes to each other; the CRD is the more conserved domain.

A new class of penaeidin (class 4) was discovered through analysis of cDNA libraries in *L. vannamei* and its relative *L. setiferus* [35,53], and shown to be expressed as a mature peptide in hemocytes [53]. Class 4 stood out for two main reasons: (1) it is highly conserved across species [35,36] and (2) it is composed of the shortest penaeidin isoforms (47 amino acids in length). These characteristics make class 4 a natural root for phylogenetic trees constructed with a full spectrum of penaeidin sequences through various means [35,42,54]. Additionally, class 4 deduced amino acid sequences differ considerably in the PRD with those from classes 2 and 3 [35,36]. So far three classes (classes 2, 3 and 4) have been characterized with a penaeidin 5 being introduced recently [54] as a sub-family member. A recent study presented a thorough phylogenetic analysis of all identified penaeidin sequences and underscored how the ongoing observations of penaeidin diversity contribute to challenges with classification and nomenclature [55].

Synthesis of penaeidins

The question of diversity in penaeidin function arose with the discovery of the new class 4 and the unique class 3 isoforms from *L. setiferus*. Isolation of sufficient amounts of a penaeidin isoform from the native source (i.e. hemocytes), free of contaminating isoforms, did not seem like a reasonable way to approach the characterization of new penaeidins based on results of the initial characterization of penaeidin [34]. The very possibility of variation in function between classes and the fact that non-native modifications were observed previously through recombinant expression were two reasons to avoid recombinant expression systems for production of new penaeidins. Penaeidins are post-transcriptionally modified by C-terminal amidation, and modifications such as this can be easily introduced chemically. Also, undesired modifications, such as non-native glycosylation, could be avoided through a chemical synthesis approach.

Chemical synthesis proved to be an efficient way to produce penaeidins despite the fact that the full-length peptides are too long for routine solid phase peptide synthesis (SPPS). The use of native ligation, a method that links two peptide segments together, permitted the efficient synthesis of a penaeidin class 4 isoform (Litset Pen4-1) in a way that reflected native penaeidin characteristics. Penaeidins are excellent examples for such an approach since both the PRD and the CRD can be synthesized efficiently within the limits of automated peptide synthesis and CRD provides the cysteine that is necessary to “ligate” the two domains [56,57]. Furthermore, the characteristics of the full-length class 4 ligation product permitted efficient separation from the individual domain reactants and minor synthesis impurities by reversed phase HPLC [53]. The total chemical synthesis of class 4 is illustrated in Fig. 2.

Another challenge to the synthesis of penaeidins was the folding and disulfide formation. This was accomplished through a chemically mediated approach described by Tam and co-workers [58] utilizing the oxidant dimethyl sulfoxide [53]. Disulfide formation and folding at a range of pH conditions was examined (from pH 5.5 to 7.5 in pH 0.5 intervals), in the presence or absence of sodium chloride, to assess the variation in the final folded product under different conditions [38,53]. Consistently, a single disulfide isomer, observed as a symmetrical peak with the greatest area on a chromatogram was observed at neutral pH in the absence of NaCl. The prominent disulfide isomer was purified by reversed phase HPLC and no impurities were detectable by analytical HPLC, amino acid analysis or by mass spectrometry [38,53]. The disulfide pattern for the final Litset Pen4-1 product was determined by mass spectrometry using a partial reduction, cyanilation and cleavage approach [38]. Identified fragments from disulfide analysis confirmed the arrangement as predicted from the NMR structure of the recombinant *L. vannamei* class 3 [37]. Soon after, the solution structure for class 4 was solved, and comparisons were made to class 3 [38].

The Litset Pen4-1 isoform and a class 3 isoform, Litset Pen3-4, were both identified in the same individual as deduced amino acid sequences (previously named *Ls3k* and *Ls4d* respectively [35]) from on cDNA clones. Subsequently, predicted masses for both the Litset Pen3-4 and Litset Pen4-1 matched singly charged ions that were detected by MALDI-TOF MS of total *L. setiferus* hemocyte extracts [38,52,53]. Thus a direct comparison of penaeidin class 3 and 4 isoforms known to be expressed in the same organism could be achieved if the Litset Pen3-4 peptide was synthesized now that a class 4 had been successfully produced.

When the approach used for Litset Pen4-1 was attempted to generate Litset Pen3-4, the synthesis of the CRD failed because clusters of β -branched amino acids sterically hindered the peptide bond formation. However, a three-segment approach proved successful for making the full-length Litset Pen3-4 molecule. This synthesis was detailed recently [52] and is illustrated in Fig. 2 (right panel) alongside the general approach to the synthesis of class 4 (left panel).

As with Litset Pen4–1, the disulfide pattern for the final Litset Pen3–4 product was determined by mass spectrometry [38,52] revealing that the disulfide pattern is conserved across all three penaeidins examined thus far. Both synthetic penaeidins were completely soluble in water, and no impurities were detectable by analytical HPLC, amino acid analysis or by mass spectrometry.

The success of the synthesis of Litset Pen4–1 and Litset Pen3–4 illustrates the advantage of using native ligation for peptides of this size, particularly when two distinct domains are present. Another advantage of the use of native ligation for penaeidins was that additional amounts of the PRD segment of each isoform remained after the full-length isoform synthesis, and these could be used for direct comparisons in functional assays. A group studying the Tiger Shrimp, *Penaeus monodon* [59], described a synthetic penaeidin from this species, and synthetic reproductions of several well-characterized anti-MPs to be used as standards. A qualitative assessment of the synthetic products was not presented, and the general lack of effectiveness of the synthetic anti-MP standards [59] suggest that the synthesis products used in this study were not accurate reproductions. The results of this study underscore the value of synthesis in combination with native ligation for producing penaeidins that are reflective of native peptides. On the other hand, the PRD appears to synthesize quite efficiently as an individual segment [53] and studies that focus on one domain alone might not require the use of native ligation.

Functional comparisons of individual penaeidin isoforms

A direct comparison of the antimicrobial spectrum of the synthetic Litset Pen4–1 from *L. setiferus* to recombinant class 3 from *L. vannamei* (Litvan Pen3–1) confirmed the hypothesis that considerable diversity in penaeidin function exists [53]. In addition to confirming the legitimacy of class 4 as an anti-MP, antimicrobial activity for the PRD of class 4 alone was discovered [53]. Evidence presented implied that this domain may play a role in target specificity of this class [53] and that the CRD is not necessary for antimicrobial function. Class 4 was generally more effective against fungi than class 3 [38,53]. Penaeidin class 3 has a broader range of microbial targets based on what is known thus far, and is more effective against certain bacterial species than class 4 [38,46,53]. Microbial target species specific levels of activity were observed between Litvan Pen3–1 and Litset Pen4–1, particularly for Gram(+) bacteria and fungi. Now that the newly synthesized class 4 had been characterized it could be used as a standard for comparison to the Litset Pen3–4 that was identified in the same individual. This comparison had the potential to provide insight into penaeidin functional diversity within an individual, and possibly reveal fine differences between isoforms that are part of the same immune system.

Functional comparisons between Litset Pen4–1 and Litset Pen3–4 were focused on bacterial genera that showed potential for being differentially targeted by penaeidins based on the previous study [53], including *Micrococcus* and *Bacillus* species (Table 1). PRDs of class 3 (PRD Pen3–4) and class 4 (PRD Pen4–1) were tested in parallel, and proved to be useful tools in the evaluation of overall penaeidin function. The direct comparison of Litset Pen3–4, Litset Pen4–1 and each PRD is new data presented in this review article (Table 1). Standard growth inhibition assays were carried out using the method that has been described previously [13, 38,46,52,53] and the results are presented as minimum inhibitory concentration (MIC) ranges, where lower numbers indicate greater effectiveness.

Differences in effectiveness were observed when comparing the class 3 and 4 isoforms directly for several species of Gram(+) bacteria. *Micrococcus luteus*, *Bacillus megaterium* and *B. subtilis* each showed a greater than two fold difference in susceptibility to the different penaeidin isoforms (Table 1), with the Litset Pen3–4 molecule being most effective in each

case. Identical levels of effectiveness were observed for the class 3 and 4 isoforms against a *Micrococcus* species that was isolated from coral off the coast of Florida against *B. cirroflagellosus* another marine Gram(+) species [60]. Overall, class 4 retains an appreciable level of anti-bacterial activity, but appears to be more selective with respect to certain microbial targets. As with other penaeidins, little or no activity was observed for Litset Pen4–1 against Gram(-) bacteria. The antimicrobial activity of PRD Pen4–1 was reproduced in this study, and its range of targets overlapped considerably with the range observed for its full-length congener, similar to previous studies [38,53]. These results further emphasize the possibility that the PRD may play a role in influencing specificity in the case of class 4.

From a direct comparison of class 3 and 4 from the same individual it appears that class 3 may have greater scope of antibacterial function relative to class 4. Based on this assertion each class may have specialized relevance to the self-defense capabilities of an individual shrimp. We have not yet tested Litset Pen3–4 and Litset Pen4–1 in combined assays to test a potential synergistic effect, but previously no synergistic effect was observed when combining classes 2 and 3 [46]. It is possible that class 3 is evolving as a flexible tool that is used by a single shrimp species to counter new pathogenic threats, particularly bacteria, while class 4 is conserved. Class 4 may serve as an effective agent against microbes that may be encountered more in more general environments, by many shrimp species.

Activity of penaeidins against human pathogens

Antifungal activity in penaeidins has been a primary point of interest that was brought to the surface more recently through the characterization of class 4 and the activity of its PRD [38, 52,53]. Based on this general characteristic of penaeidins an examination of the activity of penaeidins against fungi that have direct relevance to human health was undertaken [52]. Both synthetic full-length penaeidins exhibited antifungal activity against multiple species of *Candida*, including those that exhibit multiple antibiotic resistances, and four sterofoms of *Cryptococcus*, which also exhibit antibiotic resistance [52]. Overall the relative activity against these fungal species was in the range of that observed for standard bacteria and fungi that have been used repeatedly in penaeidin antimicrobial assays. In each case, where an activity was apparent and a MIC range was established, the upper MIC value corresponded to an assay well that was subsequently plated on rich medium after 48hours. No growth was observed 72hours after plating, indicating that the effect observed was fungicidal in nature.

The results obtained for PRD Pen4–1 indicated that this peptide may be an interesting candidate for development of a therapeutic for several reasons. First, this proline-rich segment exhibited greater antifungal effectiveness than full-length penaeidins, in some cases, with up to five-fold greater activity against select species when overall length was considered in the context of the MIC value [52]. Secondly, preliminary cytotoxicity assays revealed that neither PRD tested seemed to affect the growth of human monocytes in culture, similar to vehicle (water) controls [52]. Moreover, rabbits injected with the full-length Litset Pen4–1 for antiserum production did not produce levels of anti-PRD Pen4–1 antibodies that could be detected by western blot using enzyme conjugated anti-rabbit IgG, whereas antibodies against the full-length molecule were detected (data not presented). Next, the relative abundance of proline residues may lend resistance to common proteases that are present in human tissues.

Some of the more notorious resistant fungal infections occur in surface tissues, such as *Candida spp.* infection in the oropharynx of HIV positive patients [61,62]. In such cases many of the challenges encountered with the use of peptide antibiotics that come from the presence of proteases within tissues or underneath the skin may be avoided. PRD Pen4–1 synthesized very efficiently because it is short (22 amino acids) and linear in nature, suggesting that high yields of this peptide could be produced for development of peptide based therapeutics. Efforts to

define the core functional unit of PRD Pen4–1 may point to a shorter section of this molecule that could provide even greater yields when produced by SPPS and a shorter peptide segment may face even less challenges with respect to cytotoxicity, immunogenicity and delivery.

Intact analyses of Litset Pen3–4 by FTICR MS

To study the role of specific penaeidin peptides in the shrimp immune response it is important that the expressed proteins be identified conclusively. Initial approaches for determining the amino acid sequence of purified penaeidin peptides have traditionally been achieved by the MS/MS analysis of the peptide fragments generated from the enzymatic cleavage of full-length molecules [34,63]. More recently, the MS/MS analyses of intact, full-length penaeidin peptides (also referred to as a top-down approach) without chemical modification and/or enzymatic cleavage has been accomplished; thereby resulting in amino acid sequence information from the intact peptide [53]. MALDI-TOF/TOF MS (a tandem mass spectrometry technique employing collision induced dissociation and time-of-flight) analyses were performed on native penaeidin class 4 peptides obtained from immunoprecipitation [53]. The results of these experiments included the observation of many singly charged ions over a wide mass range of 2000–5500 m/z, and of these ions, several abundant species were observed that corresponded in mass to the predicted masses of Litset Pen4–1 fragments [53].

MALDI-TOF analyses of a pooled *L. setiferus* hemocyte extract is shown in Fig. 3. Typically, when analyzing shrimp hemocyte extract, penaeidins are represented as some of the most abundant ions detected by MALDI-TOF in the 5,000 – 6,500 m/z range. The noticeable differences between these two spectra in the m/z range corresponding to possible penaeidin isoforms illustrate the potential variability in proteins observed in a cellular extract across individuals. Analysis of m/z values in this range does not provide information about protein identity and the variability of penaeidins compounds the problem of determining isoform identity. Up to this point, the examination of penaeidin expression has focused almost exclusively on immunodetection in tissue sections [48-51]. Clearly, the identification of specific isoforms from the same class that differ slightly in amino acid sequence is not a reasonable goal using either a general m/z survey by MALDI-TOF or by immunodetection. The question of class specificity is not even addressed through immunodetection unless purified class representatives are included in parallel western blots that validate the specificity of the antibodies or antisera being used.

Identifying penaeidins using antibodies is a challenging prospect given the diversity of expressed penaeidin peptides in individual animals [35]. The non-specific nature of immunodetection of penaeidin using anti-penaeidin rabbit serum was illustrated when an immunoprecipitate was thoroughly analyzed [53] (Fig. 4). Anti-Litset Pen4–1 antibodies were enriched from rabbit serum, linked to a stationary resin and used to affinity purify the native penaeidin class 4 from a pool of shrimp hemocytes [53]. The immunoprecipitate was subjected to HPLC followed by MALDI-TOF MS analysis of selected fractions and MALDI-TOF MS/MS (TOF/TOF) analyses to obtain sequence information. The resulting HPLC chromatogram implied that a complex mixture was isolated, which was confirmed by MALDI-TOF analyses of individual HPLC fractions (Fig. 4). Despite the fact that Litset Pen4–1 (m/z, 5300.84) was the most abundant HPLC component of the immunoprecipitate [53], two other HPLC fractions were examined by MALDI-TOF MS and they contained ions within the mass range that penaeidins are typically observed (Fig. 4). These ions may correspond to other isoforms of penaeidin class 4, isoforms of penaeidin class 2, or other hemocyte proteins that are not penaeidin family members. Regardless, these results emphasize the potential lack of specificity of an anti-penaeidin serum that was generated against the synthetic Litset Pen4–1 [53], and further identification strategies for precise evaluation of the exact protein(s) immunoprecipitated are necessary. As reported [53], this serum was generated using only the

purified Litset Pen4–1 final product. It is plausible that sera generated against conjugated peptides may show even less specificity, resulting in the immunoprecipitation of an even broader range of proteins.

The ability to conclusively identify ions of this size without further manipulation and/or enzymatic cleavage would be advantageous to researchers who are interested in examining subtle changes in penaeidin expression patterns between individuals and/or possibly even between different tissues within an individual. Immunodetection does have many advantages if proper controls are included, but identification at the level of a specific isoform or post-translationally modified variant is beyond the resolution of this approach. While the TOF/TOF experiments were useful in the identification of native Litset Pen4–1, advances in peptide fragmentation have been reported that hold promise for the intact analysis of penaeidins [64–67]. One of these technologies is electron capture dissociation (ECD) in conjunction with Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) [66,68,69].

In an effort to further explore MS/MS approaches for fragmentation of intact penaeidins, FTICR MS in combination with ECD was used to generate fragments of the full-length Litset Pen3–4 without prior reduction of the disulfide bonds (Fig. 5). Using this approach, primarily singly charged *c*-ions [70] from the PRD of Litset Pen3–4 were observed. Other fragments corresponded in mass to *z*-ions [70] and internal fragmentations of the Litset Pen3–4 peptide were generated, but at lower abundance. The extensive *c*-ion series observed permitted the assignment of the first 23 residues of the synthetic Litset Pen3–4. The *N*-terminal PRD region of all the penaeidins described thus far is unique; hence, the class and isoform identity can be determined by the sequence of this domain alone. The high mass accuracy, resolution, and information-rich ECD spectra that can be obtained by FTICR MS has implications for further studies of the diversity of expressed penaeidin peptides in circulating hemocytes and in tissues.

FTICR MS in conjunction with ECD is an approach to the intact analysis of larger peptides that, as shown here, has the potential to provide high mass resolution, information-rich sequence data for individual peptides. This approach permits the identification of specific penaeidin isoforms present in a mixture and elimination of other ions that may have a similar mass, but may not be legitimate proteins of interest. In this case a few microliters of high nanomolar concentration was sufficient for the analysis, which is far less than what is required for antimicrobial assays (typically performed in the micromolar range). A distinct advantage of the FTICR MS approach is that a high level of uniform purity, comparable to that for antimicrobial assays, is not necessary since specific ions can be selected for fragmentation.

Structural comparisons of classes 3 and 4

NMR structures for isoforms of penaeidin classes 3 and 4 permit the correlation of observed functional differences to structural characteristics. While the Litvan Pen3–1 and Litset Pen4–1 structures represent penaeidins from different species they show a common theme, an extended structure for the PRD and a globular structure for the CRD. Direct comparisons of the two NMR structures sheds light on the effect of sequence divergence between classes on structural characteristics, and, at the same time, emphasizes how amino acid sequence conservation in the CRD forms the basis of a conserved tertiary structure that is constrained by disulfide bonds. For discussion purposes, a homology model of Litset Pen3–4 was produced by fitting the sequence onto one of the Litvan Pen3–1 known solution structure isomers using DeepView [71–73]. Figure images for the homology model and for isomers of the two known penaeidin structures were rendered using POV-Ray v3.6 (available at www.povray.org). This basic model was generated without energy minimization or other manipulations in order to provide a conceptual alternative to basic sequence alignments. Comparisons of a modeled

Litset Pen3–4 structure to the NMR structures of Litvan Pen3–1 and Litset Pen4–1 are shown in Figures 6 and 7.

Penaeidin proline-rich domains are quite variable with 47.6% identity in comparable sequence between Litvan Pen3–1 and Litset Pen4–1, and these differences are reflected in function [38,53]. Differences in length are apparent for penaeidin PRDs in addition to variability in comparable stretches of amino acids, which is illustrated in Fig. 6A. PRD Pen3–4 shows some interesting characteristics when compared to PRD Pen3–1, its *L. vannamei* homologous domain, including a reduction in proline concentration from 9 Pro out of 31 amino acids (29.0%) for PRD Pen3–1, to 4 Pro out of 24 amino acids (16.6%) for PRD Pen3–4 (Fig. 6A). The difference is emphasized when looking at the 3D models (Fig. 6B) where PRD Pen3–1 reveals a Pro-Pro-Pro region that forms a polyproline II helix. It is possible that the local restriction of the PRD Pen3–1 structure by the abundant proline residues prevents flexibility that could be required for antimicrobial activity when the peptide interacts with the microbial membrane surface. PRD Pen3–1 is the only penaeidin PRD tested thus far that lacks activity at the concentration ranges commonly used in penaeidin antimicrobial assays.

In addition to the arrangement of key amino acids, such as Pro and Arg, the characteristics endowed by amino acid content are likely to affect function. Isoelectric point (pI) may be a factor that influences the activity of the PRD alone. PRD Pen4–1 has a pI of 13, while the PRDs of Pen3–1 and Pen3–4 have pIs of 11.8 and 11.4 respectively, as calculated using DS Gene v1.5 (Accelrys Inc., Burlington, MA). It is possible that the ionic character of PRD Pen4–1 endows a higher affinity for microbial membranes compared to class 3 PRDs. Furthermore, PRD Pen4–1 appears to be more hydrophobic near the C-terminus (Fig. 6) which becomes clearer when the hydrophobicity is specifically analyzed and plotted by various approaches (data not presented). The relevance of the PRD function to the shrimp immune system is not yet known beyond the possibility that it may influence specificity and may augment activity in the full-length molecules.

Once the full-length peptides start to become degraded the PRD itself may become free to act independently since the unique amino acid composition of the PRD may confer resistance to proteases. Resistance to proteases is observed for other proline-rich anti-MP [5,14]. Penaeidins have not been examined to this level of detail *in vivo*, but advances in the use of mass spectrometry to conclusively determine the amino acid sequence of penaeidin peptides, such as the use of ECD in combination with FTICR MS presented in this manuscript, may serve as a platform from which to work from to examine the presence of free penaeidin PRDs in hemolymph or in tissue fluid extract where penaeidins have been secreted.

The cysteine-rich domain (CRD) of penaeidins contrasts with the PRD in many ways including general conservation of length and specific amino acids (Fig. 7A). The CRD consistently shows a structured configuration [37,38] that relies on conserved Cys residues that participate in three disulfide bonds and the presence of a helix. Aside from the general secondary and tertiary structural similarities, the specific amino acids that form the exposed surface of the CRD differ considerably between Litvan Pen3–1 and Litset Pen4–1, as implied by the 61.5% amino acid conservation [38]. Based on the NMR structures, exposed residues on the helix exhibit a certain level of conservation across isoforms of class 3 (Fig. 7B). This conservation extends, to some degree, across classes (Fig. 7B). Two Arg residues (blue) are conserved in their general location across all three penaeidin helices presented (Fig. 7B). The first Arg in each case (R37, R45, R35 – left to right) is highly conserved across all penaeidins described [36]. Additionally, there is a cluster of polar amino acids (yellow) at the N-terminal beginning (bottom) of the helix in each case, with a preference for Ser or Thr. Litset Pen3–4 and Litset Pen4–1 have Thr-Thr-Thr or Ser-Ser-Thr at this general location respectively. Nearly all penaeidin sequences described have at least two Ser or Thr residues, or combination thereof, at this respective

position [36]. Contrastingly, considerable variation is observed for aromatic residue content along this helix with Litset Pen4–1 having Phe39 and Tyr41, Litvan Pen3–1 having only Phe41 and Litset Pen3–4 lacking any aromatic residues in this area. At this point all that can be ascertained about the content of the helix with respect to function is that the exposed combination of basic and polar residues must be important for the function of a full-length penaeidin. Variation at interspersed amino acids, particularly aromatic residues, may be responsible for functional variation between isoforms and classes. In the future, site directed changes in such residues might provide insight into the effect of single residues on the activity and specificity of penaeidins. The helix itself may provide a stable framework for antimicrobial mode of action in full-length penaeidins, and the disulfides most likely contribute substantially to this stable framework.

Conclusion

Penaeidin proteins are probably highly regulated in their expression, potentially at multiple levels that include transcription, translation, modification and delivery. There is a great potential for class-specific utility of penaeidins within the immune system of a shrimp, and even specific isoforms may be used differentially, a possibility that is illustrated by the preferential expression of the Litvan Pen3–1 isoform in circulating hemocytes. It is possible that each class and/or specific isoforms of a class are used as a specific defense mechanism against the pathogenesis of microbes. Much has yet to be learned about the immune response of the shrimp in regulating penaeidin expression. Ultimately, the location of expression of particular penaeidin classes or isoforms may be of great importance for combating or preventing infection or the spread of infection. The diversity that is observed in penaeidin function between classes and between isoforms of a single class from different species shows the potential for *in vivo* diversity of immune function and immune (antibiotic) specificity in penaeidins. Expressing specific penaeidin isoforms in different tissues, depending on the type of immunogen detected by the shrimp and the way in which it was detected, may prove to be a most efficient way of responding to immune insult and pathogenic aggressiveness of microbes.

Increasing sensitivity and refinement of mass spectrometry approaches for intact analysis of penaeidins provides the background for evaluation of the expression of particular isoforms in an individual animal. Together with the genomic sequence data, a penaeidin isoform can be positively identified. The accessibility of synthetic penaeidins with characteristics that accurately reflect native peptides permits detailed biological investigations into microbial target specificity and specialization of innate immune functions.

Applying synthesis approaches to the study of penaeidins has facilitated the discovery of diversity in antimicrobial activity. Penaeidins have the potential to be used by the immune system to target specific types of microbes that cause infection, infestation and the spread of disease. Furthermore, the variability in this peptide family provides a reservoir of diversity generated in nature that may hold promise for the treatment of human disease. The characteristics of penaeidins that make them relevant to the treatment of human disease might not have been discovered if the hypothesis of functional variation in penaeidins had not been investigated and the synthesis of penaeidin 4 had not been undertaken. Moreover, the relative gain of function of the PRD in class 4 and its promise as a peptide therapeutic peptide might not have been discovered without the synthesis approach that was developed for full-length penaeidins.

Acknowledgements

We would especially like to thank Dr. Gregory Warr for initiating the collaboration between the Montpellier and Charleston based groups that investigate penaeidins. Dr. Evelyne Bachère and her collaborators contributed

substantially to the foundation of work that is reviewed herein. Drs. André Aumelas and Yinshan Yang solved the NMR structures that are reviewed here. Dr. Shawn Polson kindly provided *Micrococcus* and *Bacillus* strains isolated from coral off the coast of Florida. Special thanks to Dr. Joseph Krahn for helpful discussions and to Drs. Yanhong Liao, Suraj Dhungana and David Armstrong for providing NIEHS internal reviews of this manuscript. This work was supported by the National Science Foundation, the South Carolina Department of Natural Resources, and by the intramural program of the NIH, National Institute of Environmental Health Sciences.

Abbreviations

anti-MP, antimicrobial peptide; PRD, proline-rich domain; CRD, cysteine-rich domain; MIC, minimum inhibitory concentration; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; TOF/TOF, MALDI-TOF based tandem mass spectrometry; FTICR MS, Fourier Transform Ion-Cyclotron Resonance Mass Spectrometry; ECD, electron capture dissociation, standard amino acid abbreviations (e.g. proline = Pro or P)..

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Figure 1.

Penaeidin class 4 (Litset Pen4-1) abbreviated as Pen4 aligned with selected proline-rich and cysteine-rich antimicrobial peptides. (A) Here Bactenecin 5 (Bac5) from *Bos taurus* (#B36589) is aligned with the proline-rich domain of Pen4 (PRD Pen4-1), and human β -defensin 1 (Def1) (#CAA90650) is aligned with the cysteine-rich domain of Pen4 (CRD Pen4-1). Considerable identity is observed between Bac5 and PRD Pen4-1 with 53% identity in a specific region (boxed), containing shared Pro, Arg, Ile and Phe residues (bold text). (B) Alignment of a segment of a 6.5kDa proline-rich peptide (6,5k) (#P82964) isolated from a crab [74], hypothesized to be a penaeidin family member [40], compared to PRD Pen4-1; several residues are shared (bold text). (C) Heliomicin (Heli) (#P81544), a defensin-like antimicrobial peptide from budworm [75,76], is aligned with CRD Pen4-1 emphasizing, in addition to similarities in amino acid sequence, the relative location of the known helix structure for both peptides [38,75]. In this case the placement of the helix overlaps considerably in the aligned region. The italicized numbers above the alignments indicate the amino acid position within the mature peptide sequence. Accession numbers for each protein sequences are listed here in parentheses and highlighted by the pound (#) symbol.

Figure 2.

Synthesis of *L. setiferus* penaeidins. Class 4 (left) was the product of one native ligation step combining two peptide segments [53]. The synthesis of class 3 (right) required two steps of native ligation; one to make the complete CRD followed by a second step to produce the full-length molecule [52]. Both full-length peptides were folded in aqueous buffer at neutral pH using dimethylsulfoxide to promote disulfide formation. The purified, folded products were thoroughly analyzed for purity, uniformity of disulfide bonding and synthesis accuracy [38, 52, 53]. Included is a MALDI-TOF spectrum for each finished product emphasizing synthesis accuracy and purity (shown with singly and multiply charged ions labeled). The final folded products are rendered here as ribbon images with Pro (green) and Arg (red) side chains shown in each PRD and disulfide bonds (yellow) arranged according to experimentally determined patterns [38, 52]. The ribbon structure shown for Litset Pen4-1 was determined experimentally [38], and the image included for Litset Pen3-4 is a homology model described in this manuscript that is included for discussion purposes. More detailed renderings are shown in Fig. 6 and 7. The amino acid position is noted at the top of each peptide segment by italic numbers.

Figure 3.

MALDI-TOF spectra from hemocyte extracts of two *L. setiferus* individuals. Hemocytes were lysed in acidified water (0.1% trifluoroacetic acid) and the total lysate was subjected to MALDI-TOF MS analysis [53]. These two spectra illustrate the different masses that can be observed when looking at ions within the full-length penaeidin mass range from different *L. setiferus* individuals. Insight into the regulation of penaeidin expression might be gained from such extracts under controlled conditions if the identity of the proteins that represent the peaks in this mass range could be conclusively identified. The new FTICR MS/MS data presented in Fig. 5 for Litset Pen3–4 has implications for penaeidin identification in mixed samples.

Figure 4.

MALDI-TOF MS analysis of a pool of hemocyte extracts from many *L. setiferus* animals [53] is shown in the top panel. The middle and bottom panels show spectra obtained from two fractions that were obtained by immunoprecipitation followed by two reversed phase separation steps [53]. Another fraction that contained Litset Pen4-1 was subject to TOF/TOF analysis and was detailed previously [53], so it is not presented here. The two fractions that produced the spectra in the middle and bottom panels of this figure contained prominent ions that were co-immunoprecipitated with rabbit anti-Litset Pen4-1 serum. These results emphasize the potential for cross-reactivity by an antiserum from an individual rabbit generated against synthetic Litset Pen4-1, and might reflect a common antigenic determinant on

penaeidins. The conclusive identification of peptides that potentially represent penaeidins within this mass range would have provided information about the specificity of this antiserum for penaeidins.

Figure 5.

FTICR MS/MS data for Litset Pen3–4 were acquired on an IonSpec quadrupole Fourier Transform ICR mass spectrometer with a 9.4 Tesla actively shielded magnet, equipped with a Waters Z-spray electrospray interface. For these analyses, ions were accumulated for 500 milliseconds in the hexapole region and then pulsed into the ICR cell and then fragmented by electron capture dissociation (ECD). Electrons were generated with a cathode filament operated at 7 volts. Dissociation was performed by using a 250 ms pulse of electrons from an initial voltage of +5 V to a voltage of –1.00 V. For the MS/MS analyses, the ICR transient was measured at 1 MHz with 512k data points (~0.5 sec transient) and 20 scans were accumulated. Each transient was processed with a Blackman windowing function, zero-filled once, and Fourier transformed to the frequency domain. The resultant c and z ions (asterisks) are labeled and noted on the Litset Pen3–4 sequence. M+5H, M+6H and M+7H ions represent multiply charged versions of the full-length Litset Pen3–4 peptide. This approach provided sufficient information about the isoform specific PRD of Litset Pen3–4 to result in conclusive identification. Such an approach could be used to distinguish closely related penaeidin isoforms from one another in mixed samples, and from un-related peptides of a similar mass.

Figure 7.

(A) The CRD sequences for Litset Pen3–4, Litvan Pen3–1 and Litset Pen4–1 are aligned. Amino acid types presented in Fig. 7A and 7B are colored according to the following classification: basic (blue), acidic (red), proline (green), polar (yellow), non-polar (grey). Numbers above the alignment indicate relative position across consensus of this domain only, and do not reflect the exact residue number, which are used in Fig. 7B and 7C. (B) Exposed surface residues of the conserved alpha-helix are highlighted. The orientation is *N*-terminal to *C*-terminal, bottom to top for the helices, indicated by the directional arrow (top). In addition to the conserved Cys residue pair that is part of each helix (not shown, found on opposing side of helix) each helix contains a cluster of polar residues at the *N*-terminus and two Arg residues, including a canonical Arg residue that is found mid-way into the helix and is conserved across all penaeidin sequences [36]. (C) A ribbon image is shown to illustrate the conserved penaeidin CRD fold including the disulfide arrangement, which has been determined experimentally for all three proteins either by NMR or chemical means [37, 38, 52]. The rendering of Litset Pen3–4 is based on a homology model generated for conceptualization and discussion purposes. Numbering in Fig. 7B and 7C is indicative of exact residue position in each mature peptide.

Table 1

Antimicrobial properties of *L. setiferus* penaeidins from classes 3 and 4 were examined directly in parallel with their corresponding proline-rich domains. Target specific activity was observed for the new Litset Pen 3–4, Litset Pen 4–1 and PRD Pen 4–1 as observed against Gram(+) bacterial species.

Micro-organisms	MIC (μM) ^a			
	Litset Pen 4–1	PRD- Pen 4–1	Litset Pen 3–4	PRD- Pen 3–4
Gram (+) bacteria				
<i>Micrococcus luteus</i>	1.9–2.25 ^c	6.25–12.5 ^c	0.4–0.8	>50
<i>Micrococcus coral 649</i>	0.4–0.8 ^c	3.12–6.25 ^c	0.4–0.8	>50
<i>Bacillus cereus</i>	>50	>50	>50	>50
<i>Bacillus megaterium</i>	>50 ^b	>50	12.5–25 ^b	>50
<i>Bacillus subtilis</i>	12.5–25 ^b	NA	1.6–3.12 ^b	>50
<i>Bacillus epiphytus</i>	0.4–0.8 ^c	3.12–6.25 ^c	0.2–0.4	>50
<i>Bacillus cirroflagellus</i>	0.4–0.8	NA	0.4–0.8	>50
<i>Bacillus coral 644</i>	0.8–1.6 ^c	6.25–12.5 ^c	0.4–0.8	>50
<i>Staphylococcus aureus</i>	>50	>50	NA	>50
<i>Staphylococcus haemolyticus</i>	>50	>50	NA	>50
Gram (-) bacteria				
<i>Escherichia coli</i>	25–50	>50	25–50	>50
<i>Vibrio alginolyticus</i>	>50	>50	NA	>50
<i>Vibrio penaeicida</i>	>50	>50	NA	>50
<i>Vibrio vulnificus</i>	>50	>50	NA	>50
<i>Salmonella thyphimurium</i>	>50	>50	NA	>50
<i>Klebsiella pneumoniae</i>	>50	>50	NA	>50
<i>Enterobacter cloacae</i>	>50	>50	NA	>50
<i>Erwinia carotovora</i>	>50	>50	NA	>50

NA — this peptide was not available when assays were done with this species

^a MIC values are observed as a range of concentration, X — Y, where X is the highest peptide concentration with normal growth and Y is the lowest peptide concentration with complete growth inhibition.

^b Target specificity is observed for Gram(+) species

^c Instances where Litset Pen 4–1 and PRD Pen 4–1 act on the same species