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# Crustins, Homologues of an 11.5-kDa Antibacterial Peptide, from Two Species of Penaeid Shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus*

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**Abstract:** The response of crustaceans to pathogens is believed to depend solely on innate, nonadaptive immune mechanisms, including phagocytosis, encapsulation, clotting, and a variety of soluble antimicrobial activities. Arthropod antimicrobial peptides, while characterized primarily from insects, also have been isolated from crustaceans. Expressed sequence tag analysis of hemocyte complementary DNA libraries from 2 species of shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus,* revealed transcripts with strong sequence similarity to an 11.5-kDa antibacterial peptide (crustin *Cm*1) found in *Carcinus maenas.* Crustins were also observed to contain motifs common to proteinase inhibitors. Analysis of these cDNA libraries yielded at least 3 different isoforms of this peptide in *L. vannamei* (crustin *Lv*1–*Lv*3) and 3 in *L. setiferus* (crustin *Ls*1–*Ls*3). Further analysis of a second *L. vannamei* cDNA library revealed the presence of 3 more possible isoforms (crustin *Lv*4–*Lv*6), which differed from those seen in the first *L. vannamei* cDNA library. Genomic Southern blot analysis revealed a complex family of crustin-related sequences. However, full-length crustin appears to be encoded by a much more restricted subset of sequences within this family.

**Key words:** antibacterial peptide, crustin, penaeid shrimp, innate immunity, proteinase inhibitor.

## INTRODUCTION

The Pacific white shrimp (*Litopenaeus vannamei*) and the Atlantic white shrimp (*Litopenaeus setiferus*) are 2 species of commercially important penaeid shrimp. *Litopenaeus vannamei* is widely aquacultured, especially in the Pacific Rim, while *L. setiferus* supports a trawl fishery in the U.S. mid-Atlantic coastal waters. The aquaculture of shrimp has been adversely affected by infectious disease, with losses totaling hundreds of millions of dollars (Lightner, 1996, 1999). The impact of disease and the mitigation of its effects on commercially important wild stocks of crustaceans are highly significant aspects of resource management (EPA, 1999). Crustacean host defense mechanisms are poorly understood at the molecular level. This is particularly so in the case of the response to viral infections. The

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management of both aquacultured and wild crustacean stocks will require an understanding of the nature of their immune responses to pathogens.

Like other invertebrates, crustaceans possess only innate, nonadaptive cellular and humoral immune responses. A major effector in the crustacean cellular defense system is the hemocyte, participating in phagocytosis, encapsulation, agglutination, and the formation of reactive oxygen intermediates (see Chisholm and Smith, 1995). The 3 hemocyte cell types (hyaline, semigranular, and granular cells) are associated with different defense reactions. Hyaline cells have been shown to be involved in coagulation (Omori et al., 1989), while semigranular cells are involved in encapsulation (Persson et al., 1987; Kobayashi et al., 1990), and both semigranular and granular cells participate in phagocytosis (Gargioni and Barracco, 1998), cytotoxicity (Söderhäll et al., 1985), and arthropod antimicrobial peptide (AMP) production (Destoumieux et al., 1999). Crustaceans have a number of humoral response elements, including the prophenoloxidase (pPO) cascade and related components (e.g., β-1,3-glucan binding protein [β-1,3-GBP] and lipopolysaccharide binding protein [LPSBP], lectins, and antimicrobial peptides (for review, see Söderhäll and Cerenius, 1992).

A widespread mechanism of host defense, AMPs are present in bacteria, protozoa, invertebrates, vertebrates, and plants, and demonstrate a broad spectrum of activity (for review, see Bulet et al., 1999; Epand and Vogel, 1999). Of the 400 or so AMPs reported, over 50% have been identified in invertebrates. Since the first characterization of cecropin from the cecropia moth *Hyalophora cecropia* (Steiner et al., 1981), more than 170 AMPs have been identified in insects alone (Bulet et al., 1999). As the incidence of microbial resistance to existing antibiotics increases, AMPs are a promising resource for therapeutic and pharmaceutical applications. Clinical trials are already in progress for 2 AMPs as topical agents, one based on magainins from the African clawed frog *Xenopus laevis,* and the other on protegrins from pig leukocytes (see Ganz and Lehrer, 1999). Both magainins and cecropins have been clinically tested against multidrug-resistant strains of *Acinetobacter baumannii* isolated from immunocompromised patients (Giacometti et al., 2000), and cecropins have been shown to reduce lethality of septic shock in rats (Giacometti et al., 2001). AMPs may also be useful in the treatment of some infectious viruses, since tachyplesins (from the horseshoe crab) have been reported to inhibit influenza A virus, vesicular stomatitis virus, and human immunodeficiency virus (Murakami et al., 1991). AMPs with antifungal activity may also have applications in agriculture as many phytopathogenic fungal strains such as *Nectria haematococca, Alternaria brassicola, Neurospora crassa,* and *Botrytis cinerea* were shown to be sensitive to penaeidins (Destoumieux et al., 1999). These effectors of innate immunity are generally small, cationic molecules, and it is this cationic nature that allows for their interaction with the anionic phospholipids of bacterial membranes (Epand and Vogel, 1999). Common primary and secondary structure features have led to the classification of AMPs into 4 major families: (1) linear peptides, forming α-helices and lacking in cysteine residues; (2) cysteine-rich peptides with intramolecular disulfide bridges, forming hairpin-like β-sheets or α-helical β-sheet mixed structures; (3) proline-rich peptides; and (4) glycine-rich peptides (for review, see Hetru et al., 1998). Although AMPs of marine invertebrates have only recently attracted much attention, we predict that marine organisms will prove to synthesize a large number (and variety) of these molecules, rivaling that seen in insects. The following brief review of AMPs includes such information as is known for marine organisms, but relies, of necessity, very heavily on the large body of knowledge from insects.

## Linear α-Helical Antimicrobial Peptides

Perhaps the best studied AMPs are those that form amphipathic α-helical structures. The cecropin family best exemplifies this group of AMPs. Since their original description, cecropins have been found in a number of insect species either by peptide isolation or by complementary-DNA sequencing. They are, however, apparently restricted to the orders Lepidoptera and Diptera (for review, see Boman, 1994). Studies of the 37-residue cecropin A show the molecule folds into 2 helical regions (Steiner, 1982; Holak et al., 1988). The amino-terminal region forms a nearly perfect α-helix and is strongly amphipathic. In contrast, the carboxy-terminal region is much more hydrophobic. Interestingly, the carboxyl terminus of this class of AMPs is often amidated (see Boman, 1995). The insect cecropins A and B demonstrate activity against different strains of *Escherichia coli* and other gram-negative bacteria as well as activity against gram-positive bacteria such as *Bacillus megaterium* (Steiner et al., 1981). The cytotoxic effect of cecropins has been attributed to their ability to compromise the integrity of the bacterial membrane (Gazit et al., 1994). They appear to interact with lipid membranes to form channels of different sizes (Christensen et al., 1988). Alternatively, it has been proposed that

cecropins act as quaternary detergents, rather than forming selective channels (Vaara and Vaara, 1994). In addition, cecropins have been shown to inhibit protein import and respiration (Hugosson et al., 1994).

Moricin, an AMP isolated from the silkworm *Bombyx mori* (Hara and Yamakawa, 1995a) is a 42 amino acid novel peptide that does not exhibit similarities with any other known protein or peptide. It is highly basic and possesses a predicted amino-terminal amphipathic α-helix, analogous to the structure of cecropins. The proposed α-helix structure is based on the spacing of positively charged amino acids in the amino-terminal region, a characteristic of other AMPs containing the amphipathic α-helix (Cociancich et al., 1994a; Kreil, 1994). Unlike cecropins, moricin tends to show higher activity against gram-positive bacteria and slight antifungal activity. It appears that the basic carboxy-terminal region first interacts with the negatively charged surface of the bacterial membrane, and then the amino-terminal amphipathic α-helix region changes the permeability of the membrane (Hara and Yamakawa, 1995a).

## Cysteine-rich Antimicrobial Peptides

Cysteine-rich peptides are characterized by the formation of hairpin-like β-sheets or α-helical–β-sheet mixed structures, stabilized by intramolecular disulfide bonding (for review, see Dimarcq et al., 1998). The defensin group is the best characterized of the cysteine-rich AMPs and is widely distributed throughout divergent taxa, including plants, arthropods, mollusks, and vertebrates. Insect defensins are cationic peptides, generally composed of 34 to 46 residues, and possess a consensus motif of 6 cysteines, which form 3 intramolecular disulfide bonds. The cysteine interactions result in a stabilized αβ motif (CSαβ), which stabilizes an α-helix on one strand of the β-sheet through 2 disulfide bridges (Cornet et al., 1995). The defensin-like CSαβ signature has also been characterized in AMPs from 2 scorpion species, *Leiurus quiquestriatus* (Cociancich et al., 1993b) and *Androctonus australis* (Ehret-Sabatier et al., 1996), and recently in the blue mussel *Mytilus edulis* (Charlet et al., 1996) and the Mediterranean mussel *Mytilus galloprovincialis* (Hubert et al., 1996). Mainly active against gram-positive bacteria, defensins are also occasionally active against gram-negative bacteria or viruses. They display a rapid bactericidal effect involving pore formation (Cociancich et al., 1993a). The big defensin, a 79-residue, 8-kDa antimicrobial peptide, found in the hemocyte granules of *Tachypleus tridentatus* (Saito et al., 1995), possesses 2 distinct functional domains. The amino-terminal hydrophobic portion of the peptide exhibits activity against gram-positive bacteria, whereas the cationic, cysteine-rich carboxy-terminal region is effective against gram-negative bacteria (Saito et al., 1995).

Drosomycin, another cysteine-rich AMP with the CSαβ motif, was the first inducible antifungal peptide to be characterized in insects and has only been found in *Drosophila* (Fehlbaum et al., 1994). Unlike insect defensins, this 44-residue peptide contains 8 cysteine residues engaged in the formation of 4 intramolecular disulfide bonds (Michaut et al., 1996). Drosomycin bears little sequence similarity with insect defensins; however, it does show striking homology (34% identity in amino acid sequence) with a potent cysteine-rich antifungal peptide from the wild radish *Raphanus sativus* (Terras et al., 1992). Drosomycin, which is secreted by the fat body, inhibits spore germination and hyphal growth of filamentous fungi, but is ineffective against yeast and bacteria (Fehlbaum et al., 1996). The actual mode of action of drosomycin on filamentous fungi is unknown.

Tachyplesins (also considered members of the cysteine-rich AMP group) were originally isolated from the hemocytes of *T. tridentatus* (Nakamura et al., 1988), and several isoforms have since been characterized in the related species *Limulus polyphemus,* in which they were referred to as polyphemusins (Miyata et al., 1989). These peptides are 17 to 18 amino acids long, including 4 cysteine residues that form 2 disulfide bonds resulting in a hairpin-like β-sheet structure (Iwanaga et al., 1994). Tachyplesins form a complex with bacterial cell wall components (e.g., LPS) and exhibit a broad range of activity against bacteria and fungi (Nakamura et al., 1988). Tachyplesins have been shown to interact directly with hemocyanin, conferrring a phenoloxidase function to the hemocyanin (Nagai et al., 2001).

## Proline-rich Antimicrobial Peptides

Several proline-rich AMPs have been isolated from a variety of insects (for review, see Hétru et al., 1998; Bulet et al., 1999). The proline residues, which comprise over 25% of the amino acid composition, are often found associated with basic residues (e.g., lysine and arginine) in doublets and triplets, particularly in a Pro-Arg-Pro motif. This class of peptides can be divided into 2 groups, the unsubstituted and the *O*-glycosylated peptides.

Unsubstituted proline-rich AMPs are represented by apidaecins, abaecins, metalnikowins, and the metchnikowins. The apidaecins are a homogeneous family of 16 to 20 residue peptides found only in Hymenoptera (Casteels

et al., 1989, 1994). The largest proline-rich antibacterial peptides to have been isolated in insects are the abaecins from the honeybee *Apis mellifera* (34 amino acids; Casteels et al., 1990) and more recently from the bumblebee *Bombus pascuorum* (39 amino acids; Rees et al., 1997). The 2 peptides share approximately 54% primary sequence identity; however, the honeybee abaecin does not possess the Pro-Arg-Pro motif common among the proline-rich peptides. The abaecins from Hymenoptera have structural similarities with the metchnikowins from the fruitfly *D. melanogaster* (Levashina et al., 1995). Two forms of the metchnikowins have been isolated thus far, and both are composed of 26 amino acids with 7 proline residues and bear the common Pro-Arg-Pro motif.

Unlike the unsubstituted peptides, some proline-rich AMPs contain an *O*-glycosylated substitution of threonine residues that appears to affect the activity of the peptides. Drosocin, a 19-residue peptide isolated from *D. melanogaster,* contains the common triplet Pro-Arg-Pro motif as well as a threonine residue *O*-glycosylated by *N*-acetylgalactosamine-galactose (Bulet et al., 1993). Additional *O*-glycosylated AMPs have been characterized from 3 other insect species, pyrrhocoricin from the bug *Pyrrhocoris apterus* (Cociancich et al., 1994b), lebocins from the silkworm *Bombyx mori* (Hara and Yamakawa, 1995b), and formaecins from the bulldog ant *Myrmecia gulosa* (Mackintosh et al., 1998b).

Most of the proline-rich AMPs are active against gram-negative bacteria. However, abaecins are active against both gram-negative and gram-positive bacteria, while metchnikowins only affect the growth of *Micrococcus luteus* and the filamentous fungus *Neurospora crassa* (Levashina et al., 1995). In addition, the *O*-glycosylated peptides, drosocin and pyrrhocoricin, are active against a few gram-positive bacteria. Unlike the rapid activity of defensins and cecropins, the activity of apidaecin and drosocin is slow and appears to involve stereospecific recognition of a chiral cellular target (Casteels and Tempest, 1994; Bulet et al., 1996). Similar activities are seen in the apidaecin-like metalnikowins, 15 to 17 amino acid residue peptides isolated from 2 different hemipteran species, the shield bugs *Palomena prasina* (Chernysh et al., 1996) and *Podisus maculiventris* (Fehlbaum et al., 1996).

## Glycine-rich Antimicrobial Peptides

Several glycine-rich AMPs have been isolated from various insect species (for review, see Hétru et al., 1998). These include, but are not limited to, attacins, diptericins, coleoptericin, holotrocin II and III, sarcotoxin II, rhinocerosin, and gloverins. Coleoptericin, isolated from coleopteran insects is a 74-residue protein (Bulet et al., 1991). Coleoptericin and holotricin II, a 72-residue protein (Lee et al., 1994), share 39.2% sequence identity and rapidly kill gram-negative bacteria. A similar glycine-rich antibacterial peptide, designated rhinocerosin, has been isolated recently from another coleopteran, the coconut rhinoceros beetle *Oryctes rhinocerus* and showed 77.8% and 44.6% deduced amino acid identity to holotricin II and coleoptericin, respectively (Yang et al., 1998). However, unlike its counterparts, rhinocerosin strongly inhibited the growth of gram-positive bacteria. Isolated from the same species as cecropins, 6 attacins were first found in the *H. cecropia* moth (Hultmark et al., 1983). Whereas cecropins act on a variety of gram-positive and gram-negative bacteria, attacins are effective against only a few gram-negative bacteria. Gloverins are another group of glycine-rich peptides isolated from the giant silk moth *H. gloveri* (Axen et al., 1997) and the old world bollworm *Helicoverpa armigera* (Mackintosh et al., 1998a). These peptides have a mass of 14-kDa, containing 18% glycine residues, and are only effective against gram-negative bacteria. Gloverins may act in a mode similar to attacins (Carlsson et al., 1991) by inhibiting the synthesis of outer membrane proteins, thus increasing the permeability of the outer membrane (Axen et al., 1997).

## Antimicrobial Proteins in Crustaceans

In contrast to the vast number of AMPs isolated from the terrestrial arthropods, few have been characterized in crustaceans. However, there is no lack of evidence that antimicrobial activity exists in crustacea, and antibacterial activity has been demonstrated in the hemocytes of the blue crab *Callinectes sapidus* (Noga et al., 1996b), the squat lobster *Galather strigosa,* the Norway lobster *Nephrops norvegicus,* the common shrimp *Crangon crangon,* and the giant Antarctic isopod *Glyptonotus antarcticus* (Chisholm and Smith, 1995). Antibacterial activity has also been seen in the plasma of the spiny lobsters *Panulirus argus* (Evans et al., 1968) and *P. interruptus* (Evans et al., 1969), the penaeid shrimps *penaeus monodon* (Adams, 1991) and *L. setiferus* (Noga et al., 1996a), and in the hepatopancreas of the American lobster *Homarus americanus* (Stewart and Zwicker, 1972). While these species displayed activity mainly against gram-negative

bacteria, Chisholm and Smith (1992) found that hemolymph from the shore crab *Carcinus maenas* exhibited potent antibacterial activity against both gram-negative and gram-positive bacteria. They determined that the activity was localized in the granular hemocytes, heat-stable, not dependent upon divalent cations, and nonlytic. In addition to antibacterial activities, crustaceans have recently been shown to express antiviral activity. Pan et al., (2000) found that tissue extracts of blue crab *C. sapidus,* the white shrimp *L. setiferus,* and the crayfish *Procambarus clarkii* contained antiviral activities that inhibit a variety of DNA and RNA viruses.

Surprisingly, even though evidence of antimicrobial activity has existed for many years, characterization of the first antibacterial peptide in crustaceans did not occur until 1996 when Schnapp et al. (1996) isolated and partially sequenced a proline-rich antibacterial peptide from the shore crab *C. maenas.* This 6.5-kDa peptide exhibited activity against both gram-negative and gram-positive bacteria. Partial amino-terminal amino acid sequence analysis also showed that the peptide shares more than 60% identity with bactenecin-7, an antimicrobial peptide from bovine neutrophils (Schnapp et al., 1996). Furthermore, the 6.5-kDa peptide contains the triplet Pro-Arg-Pro motif commonly seen in other proline-rich AMPs described above.

Next characterized was the penaeidin family of AMPs from the Pacific white shrimp *L. vannamei* (Destoumieux et al., 1997). These unique AMPs contain both a proline-rich amino-terminal domain and a carboxy-terminal domain containing 6 cysteines, which form 3 intramolecular disulfide bridges. The proline-rich domain shows striking similarities to the 6.5-kDa peptide from *C. maenas* and insect proline-rich antimicrobial peptides, including the presence of the Pro-Arg-Pro motif. However, in contrast to the insect proline-rich peptides, the penaeidins do not display strong activity against gram-negative bacteria (Bachère et al., 2000). Furthermore, the cysteine-rich carboxy-terminal region also exhibits similarity to other insect cysteine-rich peptides, including those that demonstrate antifungal activity (Bachère et al., 2000). This domain may provide the penaeidins with their broad-spectrum activity against filamentous fungi (Destoumieux et al., 1999). Recently, cDNA clones of new penaeidin isoforms have been isolated from the hemocytes of *L. vannamei* and *L. setiferus* (Gross et al., 2001).

Callinectin, a partially sequenced 3.7-kDa basic AMP, was isolated from the hemocytes of *C. sapidus* (Khoo et al., 1999). This peptide demonstrated activity against *E. coli* and possessed a proline-rich amino-terminal region. However, the proline residues are not arranged in the typical motifs seen in other proline-rich AMPs, and the peptide does not show significant homology with any other known peptides (Khoo et al., 1999).

An 11.5-kDa antibacterial peptide (GenBank Accession number AJ237947) has been isolated from the granular hemocytes of the shore crab *C. maenas* (Relf et al., 1999). This 84 amino acid residue peptide was shown to be cationic and hydrophobic. Interestingly, this 11.5-kDa antibacterial peptide exhibits specific activity for only gram-positive marine or salt-tolerant bacteria (*Planococcus citreus, P. kocurii, M. luteus,* and *Aerococus viridians;* (Relf et al., 1999).

As a result of an expressed sequence tag (EST) analysis of the hemocytes of the penaeid shrimps *L. vannamei* and *L. setiferus,* cDNA clones were isolated that showed sequence identity with the 11.5-kDa antibacterial peptide (ABP) isolated from *C. maenas* (Gross et al., 2001). The full nucleotide sequences have been determined for several isoforms that appear in both shrimp species, and we are terming this new family of AMPs and related molecules, the *crustins*. Like the 11.5-kDa ABP from *C. maenas* (crustin *Cm*1), crustins from shrimp show no homology with other known antibacterial peptides, but do possess sequence identity with members of the whey acidic protein (WAP) family of proteinase inhibitory proteins. Crustins may thus represent a family of peptides with antimicrobial proteinase inhibitory activity present in the order Decapoda.

## Materials and Methods

### Libraries, Cloning, and and Sequencing

Construction of the hemocyte cDNA libraries of *L. vannamei* and *L. setiferus* based on polymerase chain reaction (PCR), from which shrimp crustins were identified by EST analysis, has been described previously (Gross et al., 2001). Complete sequences of full-length *L. vannamei* and *L. setiferus* crustin clones were obtained using vector-specific primers (Gross et al., 2001) and internal primers (Figure 1). A second library constructed using non-PCR-based methods ($\lambda$ZAP Express, Stratagene, La Jolla, Calif.) was constructed from *L. vannamei* hemocytes, according to the manufacturer's instructions, to verify the sequences of the

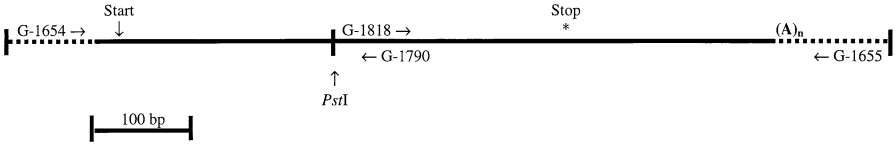


**Figure 1.** Sequencing and probe construction strategy for the crustin nucleotide sequence of *L. vannamei:* (—), clone sequence; (•••), vector sequences. Primers used to sequence and construct crustin probes denoted are: G-1654=5′-AGCTCCGAGATCTGGACGAGC-3′ (Forward, pTriplEx2 vector, pos. 487); G-1790 = 5′-TGGTGCCAACAGGTGTCTCTGG-3′ (Reverse, internal, pos. 298); and G-1818 = 5′-TATTGGTGCAAGACTCCGG-3′ (Forward, internal, pos. 244); and G-1655 = 5′-TAATACGACTCACTATAGGGC-3′ (Reverse, pTriplEx2 vector, pos.

crustin clones. The library was screened with the full-length crustin *Lv*1 probe constructed by PCR amplification of the *L. vannamei* clone PvB 031 (GenBank BE188323) using primers specific for the flanking pTriplEx2 plasmid (Figure 1). Hybridizing clones were plaque purified and converted to plasmid and sequenced using the primers shown in Figure 1.

### Sequence Analysis

Sequences were aligned using the application MegAlign within the DNAStar (Madison, Wis.) suite of programs.

### Genomic DNA Preparation

Fresh tail muscle tissue, taken from 2 *L. vannamei* individuals (1 and 2), was snap frozen in liquid nitrogen and ground to a powder. Tissue powder was dissolved at 55°C for 1 hour in 10 ml of lysis buffer (0.1 M EDTA, 1.0% Sodium dodecylsulfate [SDS], 50 mM Tris-HCl, pH 8.0, 100 μg/ml Proteinase K) for every 1 g of muscle tissue. Additional buffer was added as necessary to accomplish solubilization with overnight incubation at 53°C. Genomic DNA from individual 1 was prepared by adding saturated NaCl (1 ml per 3.5 ml of lysate), followed by centrifugation at 2500 *g* for 15 minutes 4°C to remove precipitated material. The genomic DNA was then precipitated from the supernatant with 3 vol of 70% ethanol. Genomic DNA from individual 2 was prepared by precipitating the lysate with one part 10% cetyltrimethylammonium bromide (CTAB) in 0.7 M NaCl at room temperature for 5 minutes then centrifuging at 2500 *g* for 15 minutes at 4°C. Supernatants were collected and extracted with 1 vol of phenol–chloroform–isoamyl alcohol (25:24:1) on ice with occasional mixing for 1 hour and centrifuged at 3500 *g* for 15 minutes at 4°C. Following extraction of the supernatant with 1 vol of chloroform–isoamyl alcohol (24:1), DNA was precipitated with 3 vol of 70% ethanol. Genomic DNA was redissolved in 0.5 to 1.0 ml of TE (Tris-HCl, pH 7.5, 10 mM EDTA) at 4°C. DNA concentration was assessed by UV spectrophotometry and agarose gel electrophoresis.

### Southern Blot Analysis

Genomi DNA (10 μg) was digested with 50 to 100 U of *Bam*HI, *Ava*II, and a combination of *Bam*HI and *Hin*dIII at 37°C overnight, electrophoresed on 0.8% agarose gels, and transferred to a nylon membrane (Nytran, Schleicher & Schuell, Keene, N.H), using an alkaline transfer procedure. Nylon filters with bound DNA were UV-crosslinked before drying. Membrane prehybridization, probe labeling, hybridization, washes, and autoradiography were performed using standard protocols (Sambrook et al., 1989). The full-length crustin *Lv*1 probe was prepared by PCR as described above from the *L. vannamei* clone PvB 031. A probe containing the amino-terminal repetitive leader sequence (approx. 350 bases) and one containing the carboxy-terminal unique sequence (approx. 550 bases) were constructed by digesting the full-length clone with *Pst*I (Figure 1).

## Results and Discussion

The EST screening of the *L. vannamei* and *L. setiferus* hemocyte cDNA library prepared by Gross et al., (2001) yielded 14 and 7 crustin clones, respectively. Complete sequencing of each clone on both strands indicated that 3 unique isoforms existed in the *L. vannamei* hemocyte cDNA library (GenBank AF430071, AF430072, and AF430073) and an additional 3 unique sequences were noted in the *L. setiferus* hemocyte cDNA library (AF430077, AF430078, and AF430079). These clones exhibited strong similarity (greater than 40% identity at the inferred amino acid level) to the previously described

crustin *Cm*1 (11.5-kDa ABP) from the shore crab *C. maenas* (Relf et al., 1999).

## Crustin Clones from *L. vannamei*

The full crustin *Lv*l nucleotide and inferred amino acid sequence of the predominant isoform isolated from the original cDNA library of *L. vannamei* (PvB031; GenBank AF430071) is shown in Figure 2. A putative signal sequence at the amino-terminus ends with a possible cleavage site carboxy-terminal to the $Gly_{25}$ residue (von Heijne, 1986). Following the putative signal sequence is a glycine-rich repeat region of approximately 50 amino acid residues. Hydropathy analysis (Figure 3) shows the leader sequence and glycine-rich repeat region to be hydrophobic in nature. Carboxy-terminal to this repeat region is a Pro/Cys-rich region, which possesses 12 Cys residues that may participate in the formation of 6 disulfide bridges (Figure 2). There are no obvious proteolytic cleavage sites between the amino-terminal repeat region and the carboxy-terminal unique region based on those methods conventionally used to predict such sites (Schwartz, 1986; Devi, 1991; Hosaka et al., 1991). However, there are 2 locations within the carboxy-terminal region that may be possible sites for proline-directed arginine cleavage (Figure 2). The first cleavage site is located amino-terminal to the Arg-Pro doublet at residue 115, while the second is located carboxy-terminal to the Pro-Arg doublet at residue 120.

Full sequencing of the crustin EST clones from *L. vannamei* revealed inferred amino acid sequences that were significantly longer (by 63 to 75 residues) than that found in *C. maenas,* giving a calculated mass of approximately 16.3-kDa as opposed to 11.5-kDa (Figure 4). Unlike the 11.5-kDa ABP of *C. maenas* (crustin *Cm*1), the *L. vannamei* crustin clones contained an amino-terminal glycine-rich repeat region, consisting predominantly of the VGGGLG motif and spanning 40 to 50 amino acid residues. The area of greatest identity between the sequences isolated from *L. vannamei* and that from *C. maenas* was located among the last 80 residues of the carboxy-terminal region (Figure 4). Little diversity was present within the 14 clones from the *L. vannamei* hemocyte cDNA library, with three variants being identifiable (Figure 4). Most of the variation is due to single base substitutions, while one clone, crustin *Lv*3 (PvB 568; GenBank AF430073), was found to have a 12 amino acid deletion within the glycine-rich repeat region.

To address the potential of introduced PCR error in sequences isolated from the original cDNA library, a second

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                                                    GGGGACCACTAGCTTGTACTGGAGGCAACC  -1

ATGAAGGGCATCAAGGCGGTGATTCTGTGCGGCCTCTTTACGGCGGTTTTGGCTGGCAAG  60
 M  K  G  I  K  A  V  I  L  C  G  L  F  T  A  V  L  A  G  K
                                10                          20

TTTCGCGGCTTCGGACAGCCATTTGGAGGTCTGGGTGGTCCAGGAGGCGGTGTAGGTGTT 120
 F  R  G  F  G  Q  P  F  G  G  L  G  G  P  G  G  G  V  G  V
                                30                          40

GGTGGTGGTTTCCCCGGAGGCGGTTTAGGTGTAGGTGGCGGTCTTGGTGTAGGTGGCGGT 180
 G  G  G  F  P  G  G  G  L  G  V  G  G  G  L  G  V  G  G  G
                                50                          60

CTTGGTGTGGGTGGCGGTCTTGGTGTAGGTGGCGGTCTTGGAACTGGCACAAGCGACTGC 240
 L  G  V  G  G  G  L  G  V  G  G  G  L  G  T  G  T  S  D  C
                                70                          80

AGGTATTGGTGCAAGACTCCGGAGGGTCAAGCCTACTGCTGCGAGTCGGCCCACGAACCA 300
 R  Y  W  C  K  T  P  E  G  Q  A  Y  C  C  E  S  A  H  E  P
                                90                         100

GAGACACCTGTTGGCACCAAGCCACTCGACTGCCCACAAGTCCGTCCCACATGCCCACGT 360
 E  T  P  V  G  T  K  P  L  D  C  P  Q  V  R  P  T  C  P  R
                               110                         120↑

TTCCATGGGCCCCCTACAACCTGTTCCAACGACTACAAGTGTGCTGGCCTCGATAAGTGT 420
 F  H  G  P  P  T  T  C  S  N  D  Y  K  C  A  G  L  D  K  C
                               130                         140

TGCTTCGACAGGTGTTTGGGAGAACACGTGTGCAAGCCTCCCTCATTCTTCGGATCGCAG 480
 C  F  D  R  C  L  G  E  H  V  C  K  P  P  S  F  F  G  S  Q
                               150                         160

GTTTTCGGATGAAGGATAAGCACGAAAGAATTTGAAAGGATGAAGAGAAAGAAGAAAAGA 540
 V  F  G ***
     163

CCATCTGAAGAACGACCGATGTTTTGGAATTTGACTGAAAAAAGAAAGAAAAACAGGGAA 600
TTCTTTCTTTCTGTAGGATTTATCTGATTACCATGATTTTTTTTATTTGTGAATTAGACT 660
ATTCTTCTGTCAAAAGAAACTTATAGGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  717
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**Figure 2.** Complete nucleotide and amino acid sequence of crustin *Lv*1 (PvB 031; GenBank AF430071), the predominant crustin clone isolated from the first *L. vannamei* hemocyte cDNA library. Italicized amino acid residues indicate putative signal/leader sequence. Cysteine residues that may participate in the formation of intramolecular disulfide bonds are in boldface. Potential proline-directed arginine cleavage sites are underlined and indicated with arrows (↑) and *** indicates stop codon.

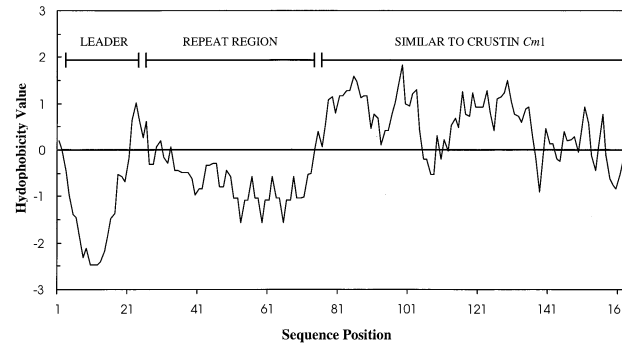


**Figure 3.** Hydropathy profile of crustin *Lv*1, representative of the predominant crustin sequence isolated from the PCR-based *L. vannamei* hemocyte cDNA library. Method used as described by Kyte and Doolittle (1982).

*L. vannamei* hemocyte cDNA library was constructed using *E. coli* DNA *pol* I. The resulting cDNA library was probed with the full-length *L. vannamei* crustin *Lv*1 sequence. Complete sequencing of the clones obtained from this screen yielded an additional 3 unique clones (GenBank

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crustin Lv1    1  MKGIKAVILCGLFTAVLAGKFRGFGQPFGGLGGPGGGVGVGGGFPGGGLGVGGGLGVGGGLGVGGGLGVGGGLG------TG  76
crustin Lv2    1  MKGIKAVILCGLFTAVLAGKFRGFGRPFGGLGGPGGGVGVGGGFPGGGLGVGGGLGVGGGLGVGGGLGVGGGLG------TG  76
crustin Lv3    1  MKGIKAVILCGLFTAVLAGKFRGFGRPFGGLGGPGGGVGVGGGFPGGGLGVGGGLGVGGGLG----------------TG  64
crustin Lv4    1  MKGIKAVILCGLFTAVLAGKFRGFGQPFGGLGGPGGGVGVGGGFPGGGLGVGGGLGVGGGLGVGGGLGVGGGLG------TG  76
crustin Lv5    1  MKGIKAVILCGLFTAVLAGKFRGFGQPFGGLGGPGGSVGVGGGFPGGGLGVGGGLGVGGGLGVGGGLGVGGGLG------TG  76
crustin Lv6    1  MKGIKAVILCGLFTAVLAGKFRGFGQPFGGLGGPGGGVGVGGGFPGGGLGVGGGLGVGGGLGVGGGLGVGGGLGVGGGLGTG  82

crustin Cm1    1  NKDCKYWCKDNLGLNYCCGQPGVTYPPFTKKHLGRCPAVRDTCTGVRTQLPTYCPHDGACQFRSKCCYDTCLKHHVCKTAEYPY.  84
crustin Lv1   77  TSDCRYWCKTPEGQAYCCESAHEPETPVGTKPLD-CPQVRPTCPRFH-GPPTTCSNDYKCAGLDKCCFDRCLGEHVCKPPSFFGSQVFG.  163
crustin Lv2   77  TSDYRYWCKTPEGQAYCCESAHEPETPVGTKPLD-CPQVRPTCPRFH-GPPTTCSNDYKCAGLDKCCFDRCLGEHVCKPPSFFGSQVFG.  163
crustin Lv3   65  TSDCRYWCKTPEGQAYCCESAHEPETPVGTKPLD-CPQVRPTCPRFH-GPPTTCSNDYKCAGLDKCCFDRCLGEHVCKPPSFFGSQVFG.  151
crustin Lv4   77  TSDCRYWCKTPEGQAYCCESAHEPETPVGTKILD-CPQVRPTCPRFH-GPPTTCSNDYKCAGLDKCCFDRCLGEHVCKPPSFFGSQVFG.  163
crustin Lv5   77  TSDCRYWCKTPEGQAYCCESAHEPETPVGTKPLD-CPQVRPTCPRFH-GPPTTCSNDYKCAGLDKCCFDRCLGEHVCKPPSFFGSQVFG.  163
crustin Lv6   83  TSDCRYWCKTPEGQAYCCESAHEPETPVGTKILD-CPQVRPTCPRFH-GPPTTCSNDYKCAGLDKCCFDRCLGEHVCKPPSFFGSQVFG.  169
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**Figure 4.** Alignments of inferred amino acid sequences, comparing the major crustin isoforms isolated from the *L. vannamei* hemocyte EST library (PvB; crustin *Lv*1–*Lv*3) and the *L. vannamei* non-PCR-based hemocyte library (ZAP; crustin *Lv*4–*Lv*6) with the crustin *Cm*1 sequence reported from *C. maenas* (Relf et al., 1999). Gaps were added to optimize alignments. Residues in boldface identify substitutions. Underlined residues are identical to those of crustin *Cm*1.

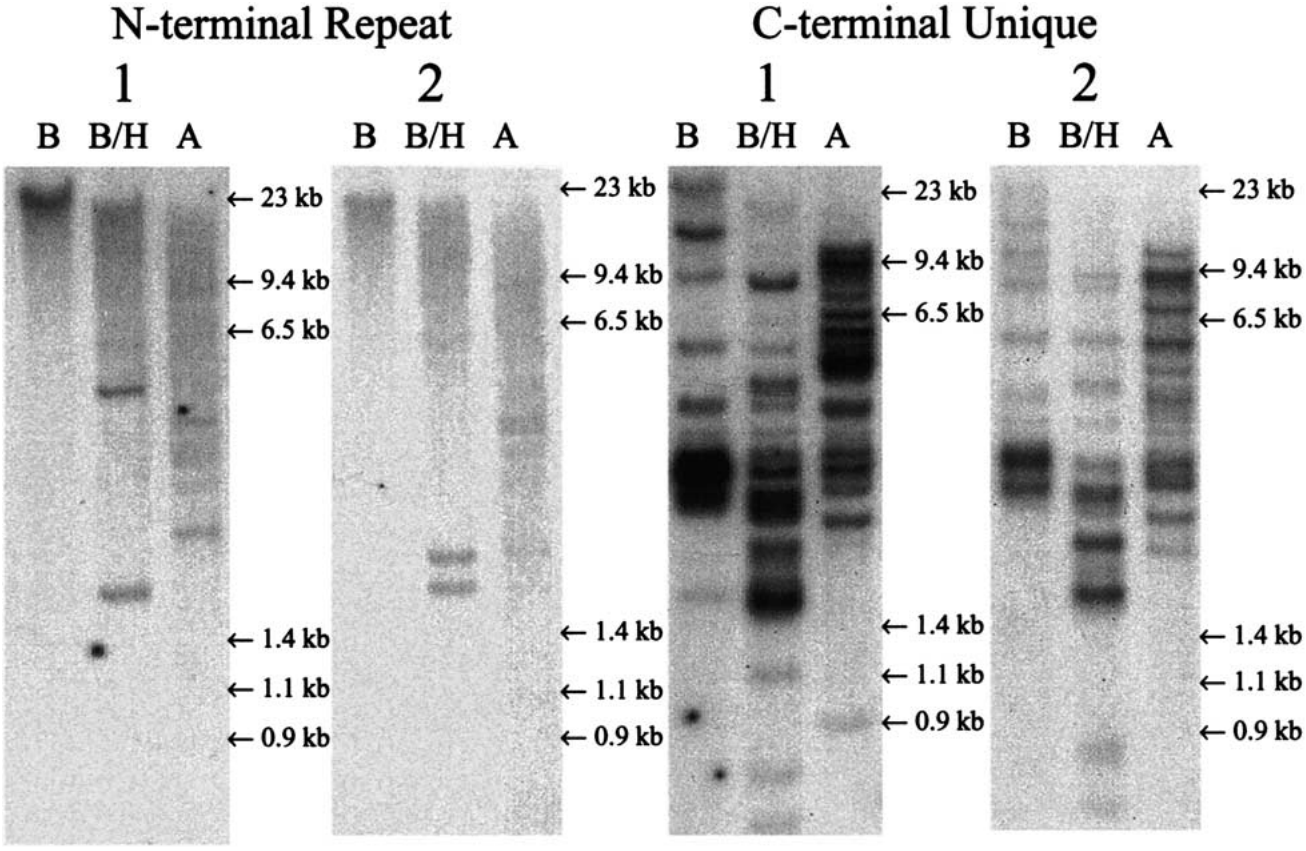


**Figure 5.** Southern blot analysis of *L. vannamei* genomic DNA. Genomic DNA was digested with *Bam*HI (lane B), mix of *Bam*HI and *Hind*III (lane B/H), and *Ava*II (lane A). Each set of blots represents a different individual (1 and 2). See text for probe construction details.

AF430074, AF430075, and AF430076). Hybridizing clones were isolated, sequenced, and compared with those isolated from the original cDNA library (Figure 4). In comparing the 2 sets of sequences, it is clear that at least some of the variations in amino-terminal repetitive region length cannot be due to intramolecular polymerase jumping (Viswanathan et al., 1999) but rather reflect underlying genetic variation within the individual organism. The data from the second cDNA library suggest (but do not prove) that certain substitutions (in 2 of the 14) *L. vannamei* crustin clones (e.g., C80Y in crustin *Lv*2 and Q26R in both crustin *Lv*2 and crustin *Lv*3) may be PCR-introduced.

Southern blot analysis (Figure 5) was performed using the full-length sequence and the 2 portions of the sequence produced by *Pst*I digestion (Figure 1) as probes to estimate crustin gene copy number in the *L. vannamei* genome. Whereas the unique carboxy-terminal region produced a complex banding pattern similar to that of the full-length

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crustin Lv1   1  MKGIKAVILCGLFTAVLAGKFRGFGQPFGGLGGPGGGVGVG-GGFPGGGLG--VGGGLG--VGGGLG-VGGGLG-VGGGLG----------------  76
crustin Ls1   1  MKGIKAVILCGLFTAVLAGKYRGFGQPLGGLGVPGGGVGVGVGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGGGLGG  98
crustin Ls2   1  MKGL-GVILCCVL-AV----------------VPAHAGPGGF----------------PGGVPGRFPS----------------------A  35
crustin Ls3   1  MKGL-GVILCCVL-AV----------------VPAHAGPGGFSGGVPGGFPGGRPGGFPGGVPGGFPS----------------------A  51

crustin Lv1  77  -TGTSDCRYWCKTPEGQAYCCESAHEPETPVGTKPLDCPQVRPTCP--RFHGPPTTCSNDYKCAGLDKCCFDRCLGEHVCKPPSFFGSQVFG.  163
crustin Ls1  99  SHGTSDCRYWCKTPEGQAYCCESAHEPETPVGTKPLDCPQVRPTCP--RFHGPPTTCSNDYKCAGLDKCCFDRCLGEHVCKPPSFFGQQIFG.  188
crustin Ls2  36  TAPPATCRRWCKTPENQAYCCETIFEPEAPVGTKPLDCPQVRPTCP--RFHGPPVTCSSDYKCGGVDKCCFDRCLGEHVCKPPSF-YSQ-FP.  123
crustin Ls3  52  TAPPATCRRWCKTPENQAYCCETIFEPEAPVGTKPLDCPQVRPTCPPTRFGGRPVTCSSDYKCGGLDKCCFDRCLGEHVCKPPSF-YSQ-FR.  141
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**Figure 6.** Alignments of inferred amino acid sequences, comparing the major crustin isoforms isolated from the *L. setiferus* hemocyte EST library with crustin *Lv*1 from the *L. vannamei* hemocyte EST library. Gaps were added to optimize alignments. Residues in boldface identify substitutions. Underlined residues are identical to those of the crustin

probe (data not shown), the repetitive amino-terminal region produced only 2 major bands when the genomic DNA was digested with *Bam*HI and *Hind*III (Figure 5). In addition, the banding patterns of this digest differed between 2 individuals, indicative of allelic polymorphism. These results show that a large family of sequences related to the crustins is present in the *L. vannamei* genome, that the majority of these sequences do not include the sequence encoding the amino-terminal portion of the crustin, and that full-length crustin, characterized as cDNA, is encoded by one or possibly two genes displaying allelic polymorphism. Antimicrobial peptides are typically encoded by single-copy genes (Hancock and Diamond, 2000), as demonstrated in human defensins (Sparkes et al., 1989; Palfree et al., 1993; Bevins et al., 1996; Liu et al., 1997). The appearance of multiple copies of sequences related to the unique carboxy-terminal region may represent multiple pseudogenes, which would thus remain unexpressed in the organism. Pseudogenes have been encountered while cloning the human defensin genes (Jones and Bevins, 1992; Ganz, 1994), but whether the banding patterns seen with the carboxy-terminal probe are due to pseudogenes or to additional exons of the expressed gene or genes is not known. Introns have been shown previously to be present in other AMP genes (Linzmeier et al., 1993; Zhao et al., 1995; Dushay et al., 2000), but definitive answers will require characterization of the crustin locus at the genomic level.

## Crustins from *L. setiferus*

Full sequencing of the 7 crustin clones isolated from *L. setiferus* showed the presence of 3 distinctly different crustin isoforms (crustin *Ls*1–*Ls*3 in Figure 6). These isoforms differed from each other, and from the *L. vannamei* crustins, primarily in the length and sequence composition of their repeat regions (Figure 7). Despite these variations, overall similarity between *L. setiferus* crustin sequences and crustin *Cm*1 from *C. maenas* was comparable to that seen with the *L. vannamei* crustin sequences.

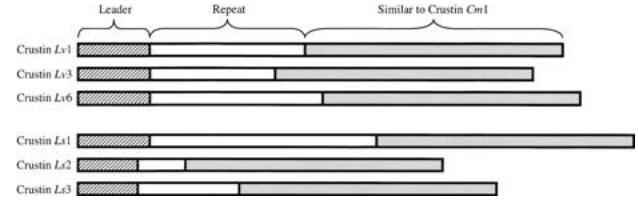


**Figure 7.** Variations seen in the inferred amino acid sequences of crustin clones isolated from the primary, PCR-based (Clontech, Palo Alto, Calif.) *L. vannamei* hemocyte library (crustin *Lv*1 and *Lv*3) and the secondary, non-PCR-based (λZAP, Stratagene) library (crustin *Lv*6) and those isolated from the PCR-based L. *setiferus* hemocyte library (crustin *Ls*1-*Ls*3).

## Potential Functions of Crustin Peptides

The portion of the litopenaeid crustins sharing similarity to the crab sequence has putative (but as yet unproven) antimicrobial activity, but the function of the amino-terminal repeat region of litopenaeid crustins remains in question. The native crustin *Cm*1 found in *C. maenas* does not possess such a region. However, the published sequence for crustin *Cm*1 does not represent the full length of the coding region, lacking the requisite methionine start codon. In contrast, the crustin cDNA clones isolated from *L. setiferus* and *L. vannamei* appear to be complete, containing 3′ and 5′ untranslated regions and appropriate in-frame start and stop codons. Originally isolated as a granular hemocyte protein extract, crustin *Cm*1 cDNA was only partially sequenced following isolation by reverse transcriptase PCR using degenerate primers. Thus, the full (as yet uncharacterized) crustin *Cm*1 message may encode a longer prepro form of a mature functional crustin *Cm*1 in *C. maenas.*

The hydrophobic nature of the amino-terminal sequence provokes speculation about a number of possible functions that it may play before being modified (if indeed such modification occurs) in the native protein. One likely function could be that these 25 residues act as

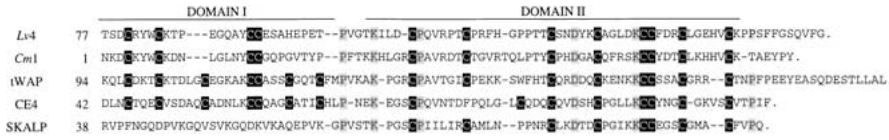


**Figure 8.** Alignment of the inferred carboxy-terminal region of crustin *Lv*4 (GenBank AF430074), with the crustin *Cm*1 (AJ237947) amino acid sequence as well as other putative and known proteinase inhibitors; tWAP (Tammar whey acidic protein, AJ005356), CE4 (canine epididymal secretory protein, S77395), SKALP (skin-derived antileukoproteinase, S58717). 4-DSC domain conserved cysteine residues are shown in white type with a black background. Other conserved residues are shown on gray background.

a signal sequence that directs the nascent polypeptide from the ribosome to the endoplasmic reticulum membrane, initiating transport across the membrane. A second possibility is that the glycine-rich repeat region protects the shrimp hemocytes from the toxic effects of the functional protein prior to activation. Once stimulated by a pathogen, the cell could cleave the unmodified protein that is stored in the granules, thereby activating it, and degranulate, releasing the functional protein into the extracellular space. Although no obvious proteolytic cleavage site can be recognized between the hydrophobic repeat region and the unique carboxy-terminal region, this does not preclude its processing by a precursor convertase (Seidah et al., 1998). An interesting comparison can be made between crustins and tachyplesins, which are synthesized as a 77-residue preproprotein with a carboxy-terminal acidic amino acid cluster. This region is proposed to stabilize the precursor molecule during processing by interacting with a cationic region of the mature peptide (Shigenaga et al., 1990). A third alternative is that crustins are integral membrane proteins, and the hydrophobic repeat region could act to anchor the protein within the membrane bilayer. Finally, the repeat region could be a consensus sequence for a functionally related group of peptides, or a "postage stamp" for sequestration in a specific vesicle.

In addition to their homology with crustin *Cm*1, litopenaeid shrimp crustins posses a conserved sequence shared by proteins that have confirmed or putative proteinase inhibitory activity (Figure 8). Some examples of molecules that share this conserved motif are skin-derived antileukoproteinase (SKALP), or elafin (Wiedow et al., 1990), which is a low molecular weight elastase and proteinase-3 inhibitor found in the suprabasal differentiated keratinocytes of psoriatic epidermis (Chang et al., 1990; Schalkwijk et al., 1990, 1991, 1993). CE4 is a major epididymis-specific protein precursor, found in dogs, and shows structural homology with its human counterpart (Ellerbrock et al., 1994). WAP, a major whey protein in the milk of the mouse, rat, rabbit, camel, and pig also bears this conserved motif (Ranganathan et al., 1999). All of these peptides (including the 11.5-kDa ABP) contain at least one 4-disulfide core (4-DSC) domain, comprising approximately 50 amino acids and including 8 cysteine residues in a conserved arrangement (see Domain II, Figure 8). The rigidity of multiple disulfide bonds helps to hold a reactive site in the correct conformation needed for inhibition (Kanost, 1999). Eighty-four 4-DSC domain sequences, derived from WAP and other proteins containing either confirmed or putative proteinase inhibitory activity, including an equine neutrophil antimicrobial peptide (Couto et al., 1993), were aligned and show the characteristic conservation of cysteine residues arranged in the following pattern:

$$C_1\text{-}(Xn)\text{-}C_2\text{-}(Xn)\text{-}C_3\text{-}(X5)\text{-}C_4\text{-}(X5)\text{-}C_5\text{-}C_6\text{-}(X3\text{-}5)\text{-}C_7\text{-}(X3\text{-}4)\text{-}C_8,$$

where X is any amino acid residue, and Xn is a stretch of n residues (Ranganathan et al., 1999). Our clones follow this pattern exactly except for 1 extra residue (for a total of 5) between $C_7$ and $C_8$ (i.e., $C_7$ X5 $C_8$). Several other consensus sequences also appear significant in the recognition of the 4-DSC domain:(1) the consensus **KXGXCP** containing $C_1$; (2) a conserved aspartate (**D**) residue between $C_3$ and $C_4$ (3) **KCC** with $C_5$ and $C_6$; and (4) **C**XX**P** with $C_8$ (Ranganathan et al., 1999). The only deviation seen in our clones is the substitution of a leucine (**L**) residue for the glycine (**G**) residue in **KXGXCP** in the $C_1$ peptide.

In WAP proteins the consensus pattern described above characterizes the second domain (Domain II) of a 2 or 3 domain sequence, all of which bear 8 cysteine residues (Ranganathan et al., 1999; Simpson et al., 2000). However, Domain II of the WAP proteins appears more conserved than Domain I (Simpson et al., 1998). Domain II also demonstrates surface electrostatic potentials similar to the functional human mucous proteinase inhibitor

domain, whereas Domain I may be nonfunctional (Ranganathan et al., 1999). Our clones and the 11.5-kDa ABP appear to contain the complete Domain II starting with the variation of the **KXGXCP** consensus, but only 4 of the last 6 cysteine residues of Domain I (Figure 8). This supports the hypothesis that our clones represent preprotranscripts of a much smaller mature peptide. Posttranslational modifications could include cleaving the protein at or near the $Lys_{107}$ residue to form an active mature peptide (approx. 57 residues) from the carboxy-terminal region.

Proteinase inhibitory activity, like antimicrobial activity, can have a number of beneficial immune-related properties in the crustacean defense system (for review, see Kanost and Jiang, 1996; Kanost, 1999). Proteinase inhibitors in the hemolymph and the cuticle may defend the host against microbial proteinases (Eguchi et al., 1993; Jiang and Kanost, 1997) and fungal infection (Polanowski et al., 1997). Proteinase inhibitors are also necessary in the regulation of many immune-related cascades such as hemolymph coagulation and the pPO cascade. The best understood invertebrate proteinase cascade system is the hemolymph coagulation system of the horseshoe crab (Iwanaga, 1993). Four serine proteinases (factor C, factor B, factor G, and clotting enzyme) are involved in the formation of a coagulin clot when activated by either bacterial endotoxin or by β-1,3-glucans. LICI-1 of the horseshoe crab has been shown to inhibit factor C, the endotoxin-triggered proteinase (Miura et al., 1994). While LICI-2 also inhibits factor C, it more efficiently inhibits the clotting enzyme (Miura et al., 1995). Two serine proteinases have been identified that are required for activation of the pPO cascade (Ashida and Brey, 1997). Several proteinase inhibitors from hemolymph inhibit pPO activation. Pacifastin, a 155-kDa trypsin inhibitor (Hergenhahn et al., 1987), and α-macroglobulin (Aspan et al., 1990), both isolated from the crayfish *P. leniusculus,* inhibit the crayfish pPO activating enzyme. Furthermore, several proteinases have been characterized that have not yet been assigned a physiologic function, implying the existence of a complex system of proteinases and inhibitors in crustacean hemolymph (Kanost, 1999).

## Conclusions

Antimicrobial peptides are a major component of the innate immune defense system in arthropods, providing an immediate and usually rapid response to invading microorganisms. Of those AMPs characterized in arthropods, most have been isolated from insects and many share common features, which implies wide distribution across species. To date, few AMPs have been isolated from crustaceans. Given the wide variety of AMPs isolated from insects, it seems likely that crustaceans also possess a wide variety of AMPs, most of which have yet to be discovered.

We have identified crustins from hemocyte cDNA libraries of the Pacific white shrimp *L. vannamei* and the Atlantic white shrimp *L. setiferus.* These encode a protein homologue to the crustin *Cm*1 previously isolated from the shore crab *C. maenas* (Relf et al., 1999). The crustin cDNA encodes a protein with a leader sequence, a hydrophobic repeat-rich amino-terminal region, and a unique carboxy-terminal region similar to the 11.5-kDa ABP. These crustins also contain a motif in common with several known or putative proteinase inhibitors of the WAP family. The sequence encoding the amino-terminus is represented in low copy number in the genome of *L. vannamei,* while the sequence encoding the carboxy-terminus is present in high copy number. The transcripts may encode a prepro form of a mature, native protein. In addition, recently an EST was deposited in the dbEST database of GenBank (AW618962) from another penaeid species, the Black Tiger prawn *P. monodon,* which appears to have homology to the crustins, adding further weight to the suggestion that crustin AMPs are widely distributed.

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