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Aquaculture 191 (2000) 71–88

Aquaculture

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Penaeidins, antimicrobial peptides of shrimp: a comparison with other effectors of innate immunity

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Received 1 March 2000; accepted 9 May 2000

Abstract

The production of antimicrobial peptides is a first-line host defense mechanism of innate immunity. However, in spite of the importance of infectious diseases in crustaceans, few molecules displaying antimicrobial activities have been fully characterized in these invertebrates. This paper presents the recent findings on the identification of a family of antimicrobial peptides, named penaeidins, in the shrimp *Penaeus vannamei*. The penaeidins are original, 5.5 to 6.6 kDa peptides which combine a proline-rich amino-terminal domain and a carboxyl-domain containing six cysteines engaged in three disulfide bridges. These two domains are respectively compared, structurally and biologically, with other molecules hitherto characterized in a wide range of living organisms, from plants to invertebrates and vertebrates.

Keywords: Crustacean; Penaeid; Shrimp; Innate immunity; Penaeidins; Antimicrobial peptides; Chitin-binding

1. Introduction

In crustaceans, the development of infectious diseases is particularly reported from penaeid shrimp since they are subject to intensive aquaculture production, the main causative agents encountered being viruses, fungi and bacteria.

Viral diseases are probably still underestimated in crustaceans, but nevertheless, they emerge as being responsible for serious enzootics (or massive pandemics), on a regional

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0044-8486/00/$ - see front matter
PII: S0044-8486(00)00419-1

scale in shrimp-farming countries. The principal viral agents known belong to the families of (i) Parvo-like-viridae with Infectious Hypodermal and Haematopoietic Necrosis Virus (IHHNV); (ii) Picornaviridae with the Taura syndrome virus or the Yellow Head Virus group (YHV), which seriously affects the Indo-Pacific shrimp industry (for review, see Lightner et al., 1997); and (iii) Baculoviridae, which is the most important group of viruses known from different decapod crustaceans and arthropods in general (Anderson and Prior, 1992).

Bacteria, both gram-positive and gram-negative, are also etiological agents responsible for severe diseases in crustaceans. A gram-positive tetracoccus bacterium, *Aerococcus viridans* (originally *Gaffkya homari*), has been reported as responsible for fatal septicemia in the lobster, *Homarus americanus*, and has been found in shrimps and crabs (Newman and Feng, 1982). Avirulent *A. viridans*-like strains have also been described in crustaceans (Wiik et al., 1986). As for marine fish and shellfish, the Vibrionaceae gram-negative bacteria indubitably represent the most harmful pathogenic bacteria to both larvae and juvenile shrimp. Different species, such as *Vibrio harveyi* or *V. vulnificus* (Song and Lee, 1993), *V. damsela* (Song et al., 1993) or *V. penaeicidae* (Ishimaru et al., 1995), are regularly reported in different shrimp species, *Penaeus monodon*, *P. vannamei*, *P. stylirostris* or *P. japonicus*.

Finally, fungi form potential pathogens for crustaceans in aquaculture and particularly for stressed or immunodeficient animals (Söderhäll et al., 1993). The filamentous fungus, *Lagenidium* sp., can be encountered in different decapod species. It particularly affects the larval stages of shrimp and lobster, and may cause high levels of mortalities (Crisp and Bland, 1989). Moreover, fungi in the genus *Fusarium* are also reported to be responsible for epizootics both in freshwater or marine crustaceans. For example, *Fusarium solani* has been shown to be involved in gill and cuticular infections in shrimp, lobster and crayfish, leading to high mortalities (Burns et al., 1979; Chinain and Vey, 1988).

With the amount of data collected from pathological studies, it appears that many pathogens, either viruses, bacteria or fungi, can be equally encountered in different crustaceans without an apparent species specificity. Along this line, crustaceans could be vectors for infections, as has been suggested with the isolation from the crayfish, *Astacus leptodactylus*, of *Saprolegnia parasitica*, a fungus pathogenic for fish. Since it is not affected by this fungus, it was assumed that the crayfish may serve as a vector for the fish pathogen (Söderhäll et al., 1991).

Whereas, numerous works are devoted to pathological studies in crustaceans, investigations on the host responses to pathogens remain relatively limited, probably due to experimental difficulties. Moreover, while cellular and humoral immune mechanisms have been studied in crustaceans, the molecular characterization of antimicrobial effectors remains poorly investigated.

2. Immunity and antimicrobial effectors

Present knowledge of crustacean defense systems mainly concerns hemocyte activities such as phagocytosis and encapsulation, the hemolymph clotting reaction (Bachère

et al., 1995), and most of all, the prophenoloxidase activating cascade which has been particularly well-studied in crayfish (as reviewed in Söderhäll et al., 1996). Briefly, the latter defense system leads to the production of quinones and intermediates in the biosynthesis of melanin. These compounds are associated with encapsulation of foreign organisms as well as with the production, by phagocytes, of reactive oxygen intermediates (Nappi and Vass, 1993). In crayfish, melanin was shown to display fungistatic properties, by acting as an inhibitor of both growth and proteinase activity of the fungus, *Aphanomyces astaci* (Söderhäll and Ajaxon, 1982).

In crustaceans, hemolymph antimicrobial activities have been demonstrated but few molecules have been characterized. Tentatively, antiviral factors were searched for in *Callinectes sapidus*, using bacteriophages and poliovirus, plasmatic neutralizing factors were evidenced (McCumber et al., 1979). However, the progress of such research is greatly limited by the lack of crustacean viral models and of culture cell line systems necessary for virus titration and quantification. For these reasons, until now, antibacterial activities have attracted more attention. In lobsters, the bactericidal activity of hemolymph was demonstrated against gram-negative bacteria but no activity has been shown against the pathogenic, gram-positive, *A. viridans* (Mori and Stewart, 1978). More recently, microbicidal capacity of crustacean hemolymph and hemocytes has been reinvestigated. In the shrimp, *P. monodon*, bactericidal activity and increased hemagglutinating activity were detected in the plasma after exposure to the gram-negative bacteria, *V. alginolyticus* (Adams, 1991). Simultaneously, anti-gram-positive and gram-negative activities were found to reside exclusively in the granular hemocytes of the shore crab, *Carcinus maenas* (Chisholm and Smith, 1992). Such hemocytic antibacterial properties were further analysed in different decapods (Chisholm and Smith, 1995), and the purification of the effectors responsible for this activity in *C. maenas*, led, for the first time in crustaceans, to the evidence of peptidic molecules. Three constitutive hemocytic molecules were isolated and one of them, a 6.5-kDa peptide, was partially characterized (Schnapp et al., 1996). At the same time, research on the shrimp, *P. vannamei*, allowed full characterization of three members of a new family of antimicrobial peptides which were named as the penaeidins (Destoumieux et al., 1997).

3. Antimicrobial peptides

While only recently demonstrated in the shrimp and crab, the production of antimicrobial peptides is a widespread mechanism of host defense in the living kingdom, present from bacteria, protozoans, invertebrates to vertebrates and in plants. These effectors of innate immunity were initially characterized in insects and are small and cationic molecules. For convenience, the antimicrobial peptides have been grouped into four distinct families according to common features of primary and secondary structures (for review, see Hétru et al., 1998). The two main classes correspond to: (i) linear peptides forming amphipathic α-helices which are devoid of cysteine residues, (ii) cysteine-rich peptides with intramolecular disulfide bridges (from one to six). These two families, respectively, represented by cecropins and defensins, are relatively homogenous compared to the two other families of linear peptides which group molecules rather

differently: (iii) the proline-rich peptides such as the apidaecins or drosocin, and (iv) the glycine-rich peptides or polypeptides (9–30 kDa) such as attacins, diptericins or sarcotoxins II (as detailed by Hétru et al., 1998).

3.1. Penaeidins

The three antimicrobial peptides characterized in *P. vannamei* were purified from the plasma and the hemocytes of shrimp collected from intensive shrimp farms and in which immune response had not been experimentally induced. The fully characterized molecules are highly homologous and they were named penaeidins (Pen-1, -2 and -3), after the genus *Penaeus*. They are composed of 50 and 62 amino acid residues for Pen-1 and Pen-2, and for Pen-3, respectively. They are highly cationic with a p*I* of 9.34 for Pen-1 and -2 and 9.84 for Pen-3 (Destoumieux et al., 1997). The penaeidins were purified from a hemocyte organelle-rich fraction and further cloned from a hemocyte cDNA library. Therefore, the hemocytes were found to be a site of production and storage for these peptides. Thus, as suggested by their presence in the plasma of the animals used for the initial work of purification, the penaeidins could be secreted or released from hemocytes by degranulation into the blood upon immune response stimulation. Although the shrimps used in these experiments have not been experimentally immune-challenged, it can be assumed that, under the intense conditions of stress generated by their harvest, some hemocyte activation has occurred leading to the release of the penaeidins into the blood circulation.

The antimicrobial activity spectrum of penaeidins, established with yeast-expressed recombinant peptides, is rather large with antibacterial and antifungal properties (Destoumieux et al., 1999). The antibacterial activity is predominantly directed against gram-positive bacteria with different specificities in their mode of action, depending of the bacterial strain considered. Indeed, a bactericidal effect of the penaeidins has been observed against the bacteria, *Bacillus megaterium*, whereas, the peptides display a bacteriostatic effect on *Micrococcus luteus*, or a slow bactericidal effect on the crustacean pathogenic strain, *A. viridans*. In the experimental conditions used, the penaeidins had no effect on gram-negative bacteria such as the Vibrionaceae (Destoumieux et al., 1999). On the contrary, the peptides inhibit the growth of a large range of filamentous fungi, including *F. oxysporum*, pathogenic for shrimp. At a concentration lower than the minimum inhibitory concentration value (MIC < 5 μM), penaeidins cause reduced growth and elongation of the fungal hyphae leading to abnormal morphology, while at higher concentrations (10 μM), the peptides have a fungicidal effect on the *Fusarium* spores as no germination occurs after replacement of the culture medium by penaeidin-free broth.

The overall structure of the shrimp antimicrobial peptides is quite unique among the families already known, and this originality has led to the distinction of the new family of penaeidins. The penaeidins are composed of, first, a NH_2-terminal proline-rich domain and a COOH-terminal domain containing six cysteine residues engaged in the formation of three intramolecular disulfide bridges (Destoumieux et al., 1997) (Fig. 1). The two structural domains are highly conserved between the three penaeidins but Pen-3a differs by the presence of a medial sequence (six amino acids), linking together

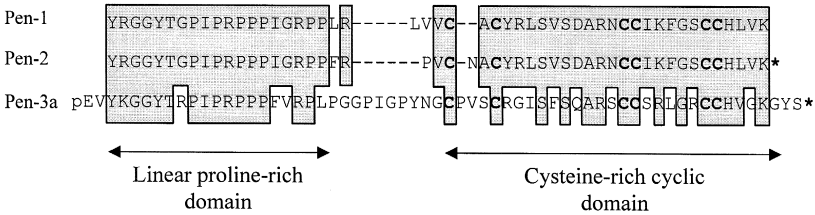

Fig. 1. Sequence comparison of Pen-1, -2 and -3 from *P. vannamei*. The full sequences of the penaeidins were aligned and gaps were introduced to optimize the alignment. Cysteines are in boldface, identical residues and conservative replacements are shaded. The asterisk indicates a C-terminal α-amide, and *pE* stands for pyroglutamic acid.

the two conserved domains. Another feature of the penaeidin family is that the peptides are post-translationally modified by NH_2-terminal cyclisation of a glutamine residue (formation of a pyroglutamic acid) in Pen-3a, and by a COOH-terminal amidation involving the elimination of a glycine residue in Pen-2 and Pen-3. Such a COOH-terminal amidation is also present in other antimicrobial peptides isolated from marine invertebrates, such as tachyplesins, 17–18-residue peptides with two disulfide bridges, found in limulus hemocytes (*Tachypleus tridentatus*) (Nakamura et al., 1988), clavanins, which represent a family of α-helical 23 amino acid peptides isolated from the hemocytes of a solitary tunicate, *Styela clava* (Lee et al., 1997) and a defensin characterized in the mussel *Mytilus galloprovincialis* (Mitta, pers. comm.). This post-translational modification is also a common feature of peptides forming amphipathic α-helices: most of the insect cecropins but not the pig cecropin (reviewed in Hétru et al., 1998), and the bombinin-like peptide family characterized from skin secretions of the toad, *Bombina orientalis* (Gibson et al., 1991). The role and importance of this modification are not clearly known. Introduced in a magainin analogue, a COOH-terminal amidation has been shown to result in enhanced α-helical structure and antimicrobial activity compared to the original magainins which have a free carboxyl group (Chen et al., 1988). Conversely, the replacement of the α-amide of the original polyphemusin (from *Limulus polyphemus*) by a COOH-terminal glycine does not affect the activity of the peptide (Pierce et al., 1997). Similarly, the non-amidation of the COOH-terminus in recombinant penaeidins has no effect on the antifungal activity, whereas, it would result in a slight reduction of their antibacterial activity (Destoumieux et al., 1999).

3.2. The proline-rich region of penaeidins

The NH_2-terminal proline-rich domain of the penaeidins presents some similarities with antimicrobial peptides already described in other invertebrates and in vertebrates. The penaeidin NH_2-terminal domain (21 residues) contains four arginine residues and a relatively high number of proline residues (7–9) with some of them involved in a conserved Pro-Arg-Pro motif (see Fig. 1). Such triplets are present and repeated in different closely related members of the proline-rich antimicrobial peptide family from insects (for a review in Hétru et al., 1998) (Fig. 2). For instance, the motif is found in

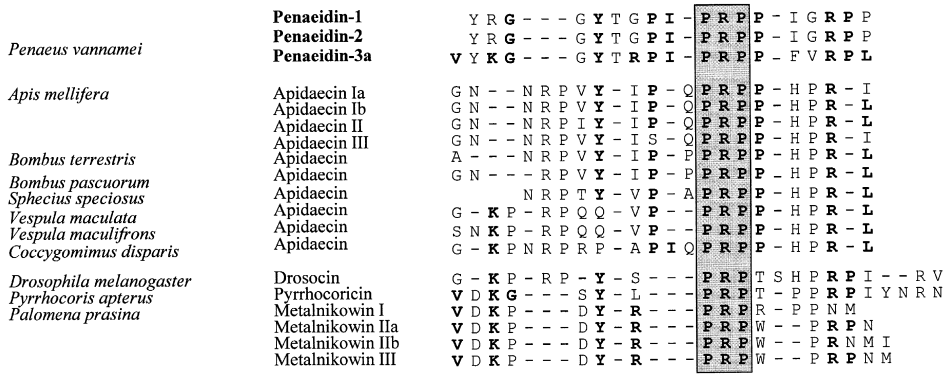

Fig. 2. Sequence comparison of penaeidin NH_2-terminal domain with proline-rich antimicrobial peptides from insects (reviewed in Hétru et al., 1998). The conservative triplet P–R–P also present in penaeidins is shaded and identical residues are bolded. Bars indicate gaps introduced to optimize the alignment.

the apidaecin sequence. This homogeneous family of 16–20-residue peptides were isolated from the honey bee, *Apis mellifera*, and the different isoforms can have distinct activity spectra against exclusively gram-negative bacteria (Casteels et al., 1989; Casteels and Tempst, 1994). It has been shown that apidaecins would not act by a lytic mechanism on the bacterial membranes but by a stereospecific recognition mechanism involving a protein target. Similar activities are shared by the metalnikowins (26-residue peptides) from the hemipteran, *Palomena prasina* (Chernysh et al., 1996), the pyrrhocoricin, a 20-residue peptide isolated in the bug, *Phyrrhocoris apterus* (Cociancich et al., 1994), and the drosocin, a *Drosophila*, 19-residue peptide which displays a slow bactericidal effect (Bulet et al., 1993) through a stereospecific mechanism (Bulet et al., 1996). Another remarkable feature among some of these proline-rich peptides is an *O*-glycosylated substitution on threonine residues which could be involved in the activity of the peptides. This is the case for drosocin, for which the level of activity has been shown to be significantly reduced in the absence of the disaccharidic substitution (*N*-acetylgalactosamine-galactose) (Bulet et al., 1996). However, such a glycosylation does not appear necessary for penaeidin biological activity. Indeed, no saccharide substitution has been observed in the native peptides, whereas they show, in their sequence, a threonine residue at a potential *O*-glycosylation site. Finally, in contrast to the insect proline-rich peptides, the penaeidins do not display anti-gram-negative activity.

Some sequence similarities can also be evidenced between the proline-rich domain of the penaeidins and other families of antimicrobial peptides also found in blood cells from various origins. First of all, homologies are shown with the NH_2-terminal sequence of a partially characterized 6.5 kDa peptide isolated from the hemocytes of the crab, *C. maenas* (Fig. 3(a)). The chromatographic fractions containing this peptide showed an activity against various gram-positive and gram-negative bacteria (Schnapp et al., 1996). Although preliminary, these sequence similarities would suggest that the peptides isolated in the crab and in the shrimp could be members of the same family. Moreover,

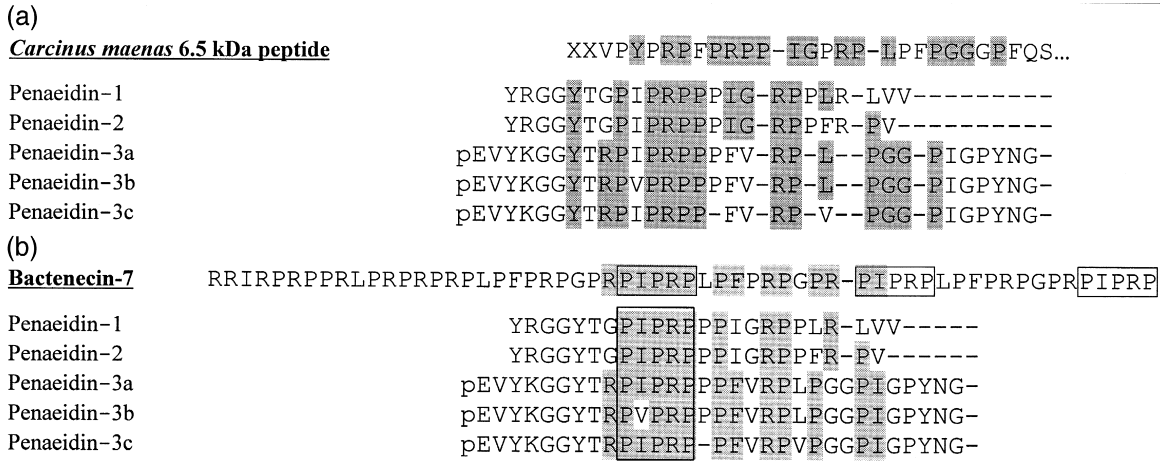

Fig. 3. Sequence comparison of the NH_2-terminal proline-rich domain of different penaeidin isoforms, (a) with the NH_2-terminal sequence of *C. maenas*, a 6.5-kDa peptide (Schnapp et al., 1996), and (b) with Bac-7 from bovine neutrophils (Gennaro et al., 1989). Bars indicate gaps introduced to optimize the alignment. Identical amino acids and conservative replacements are shaded and the PIPRP motif repeated three times in Bac-7 is boxed. Unidentified amino acids are represented by *X*.

the penaeidins contain a Pro-Ile-Pro-Arg-Pro motif which is repeated three times in bactenecin-7, a mammalian proline and arginine-rich peptide (Fig. 3(b)). The bactenecins, Bac-5 and Bac-7, contain 42 and 59 amino acid residues, respectively, and were extracted from bovine neutrophils (Gennaro et al., 1989). These antimicrobial substances are stored as non-cidal precursors in the large granules of the resting neutrophils. The maturation of bactenecins, leading to peptides with antimicrobial properties, is induced in stimulated neutrophils and during the degranulation process (Zanetti et al., 1991). Bactenecins are active against various gram-positive and gram-negative bacteria and Bac-7 has been shown to inactivate human herpes simplex virus types 1 and 2. The Bac-7 sequence homologous to the penaeidin proline-rich region does not display activity but presents a capability to bind membranes by electrostatic interaction (Tani et al., 1995). Interestingly, we noticed that no antimicrobial activity was observed for a synthetic sequence corresponding to the penaeidin proline-rich region (Destoumieux et al., 1999). From the accumulated data, we can hypothesize that the proline-rich domain of the penaeidins could be involved in recognition or interaction processes of the peptide with the membrane of target microorganisms.

3.3. The cysteine-rich region of penaeidins

The COOH-terminal domain of penaeidins is characterized by the presence of six cysteine residues engaged in the formation of three intramolecular disulfide bridges, a feature initially attributed to peptides classified as defensins. In fact, several groups of defensins exist, all differing by the spacing and arrangement of their six cysteine residues, the classification of peptides in this group is in continuous revision with the discovery of new molecules. In this respect, by the placement of their six cysteines, four of them being organized in doublets (Fig. 1), the penaeidins differ from all of these peptides.

3.3.1. Antimicrobial peptides with six cysteines

The defensin group is the most important in terms of number of molecules already characterized. They seem to have a large distribution within different phyla, including plants, insects, other arthropods and vertebrates (for review, see Dimarcq et al., 1998).

3.3.1.1. Vertebrate defensins. Today, two branches are distinguished in the vertebrate defensin family (reviewed in Ganz and Lehrer, 1998), according to the differences in their primary sequences and in their unique consensus and cysteine pairing. However, as a common feature in the cysteine arrangement, the two groups, namely α- and β-defensins, bear a cysteine-doublet in the COOH-terminus, a position also observed in penaeidins, for one of the two doublets. The α-defensins, which contain 29–35 amino acid residues, are stored in neutrophil granules, alveolar macrophages and intestinal Paneth cells. These peptides present antimicrobial activity against gram-positive and gram-negative bacteria, against mycobacteria, many fungi, parasites, eukaryotic cells and against some enveloped viruses (reviewed in Lehrer et al., 1993). Most of the β-defensins characterized are produced by circulating phagocytic cells (Selsted et al., 1993), but they represent an emerging family of peptides particularly prominent at

mucosal epithelial sites in mammals, such as the tracheal antimicrobial peptide (TAP) from bovine respiratory mucosa (Diamond et al., 1991) or the lingual antimicrobial peptide (LAP) isolated from bovine tongue (Schonwetter et al., 1995). Unlike the α-defensins, epithelial β-defensins are not stored in granules and their production (gene transcription) is stimulated by bacteria or bacterial products acting at sites of inflammation (Schonwetter et al., 1995; Diamond et al., 1996). β-defensins present activity against gram-positive and gram-negative bacteria. Finally, different properties of defensins are now described in vertebrates, including the ability to serve as signaling molecules. Human defensins have been shown to be chemotactic for monocytes and polymorphonuclear leukocytes as well as for murine and human T-lymphocytes (Territo et al., 1989; Chertov et al., 1996).

3.3.1.2. Invertebrate defensins. To date, about 35 members of the defensin superfamily have been characterized from various arthropod sources. They are found in all the insect orders (for a review in Bulet et al., 1999), if we include the recently identified heliomicin, an antifungal defensin from the lepidopteran, *Heliothis virescens* (Lamberty et al., 1999). As for all the antimicrobial peptides characterized in insects, the synthesis of insect defensins is rapidly and transiently induced, upon injury, in the fat body tissue from which they are released in the blood stream. Interestingly, they can also be produced by certain blood cells, epithelial gut cells and by salivary glands (Engström, 1998). The defensins are occasionally active against gram-negative bacteria or viruses but they are mainly active against gram-positive bacteria, an activity also found predominantly in penaeidins. Insect defensins generally display a rapid bactericidal effect (within a minute), associated with a membranolytic process involving pore formation (Cociancich et al., 1993a). The insect defensins are characterized by a cysteine stabilized αβ motif (CSαβ), which stabilizes an α-helix on one strand of the β-sheet through two disulfide bridges (Cornet et al., 1995). This CSαβ motif is also found in scorpion toxins (Bontems et al., 1991), and in plant defensins (Broekaert et al., 1995). This structure differentiates the insect defensin from the two families of vertebrate defensin (α and β), which lack an α-helix and consist of β-sheets only (Hill et al., 1991) (Fig. 4).

Other molecules with the signature of insect defensins have been characterized in chelicerates: the scorpions, *Leiurus quiquestriatus* (Cociancich et al., 1993b) and *Androctonus australis* (Ehret-Sabatier et al., 1996), and recently in bivalve molluscs, *M. edulis* (Charlet et al., 1996) and *M. galloprovincialis* (Hubert et al., 1996). Strikingly, the *M. galloprovincialis* 38-residue peptide named, MGD1, though presenting the signature of insect defensins, contains eight cysteine residues. This characteristic — presence of eight cysteine residues — is also found in mytilins and myticin, novel types of cysteine-rich peptides isolated from *M. edulis* (Charlet et al., 1996) and *M. galloprovincialis* (Mitta et al., 1999).

3.3.2. Peptides with eight cysteines / antifungal peptides

3.3.2.1. Drosomycin. Drosomycin is a 44-residue peptide, containing eight cysteine residues engaged in four intramolecular disulfide bridges (Fehlbaum et al., 1994). This

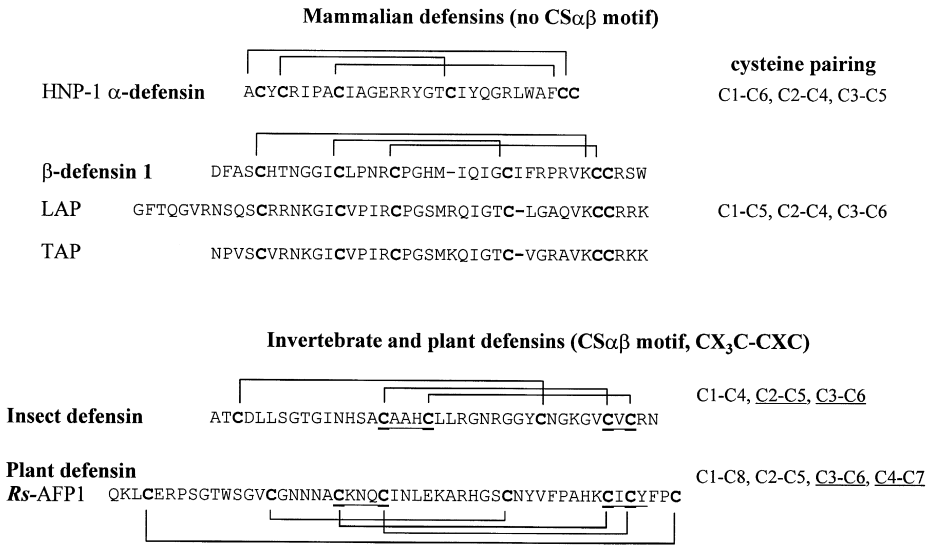

Fig. 4. Sequences of representatives of mammalian, plant and insect defensins. The cysteine residues are in bold characters and the disulfide bridges are represented by broken lines. In addition, the cysteine pairing is indicated in order to point out the differences existing between the different classes of defensins. The CSαβ motif is underlined. HNP-1 is an α-defensin isolated from human neutrophils (Selsted et al., 1985), the β-defensin-1 from bovine neutrophils (Selsted et al., 1993), TAP (Diamond et al., 1991) and LAP (Schonwetter et al., 1995). The plant defensin, Rs-AFP_1, has been isolated from radish seeds (Terras et al., 1992). The insect defense sequence presented is the defensin A from the diptera, *Phormia terranovae* (Lambert et al., 1989).

inducible peptide, secreted by the fat body into the insect hemolymph, shows significant homologies with plant defensins (Broekaert et al., 1995). Besides structural similitude and 34% identities in amino acid sequences (Fig. 5), drosomycin and the peptides isolated from the plant, *Raphanus sativus* (Brasicaceae) (Terras et al., 1992), share a potent antifungal activity with no antibacterial activity. Drosomycin has a broad activity

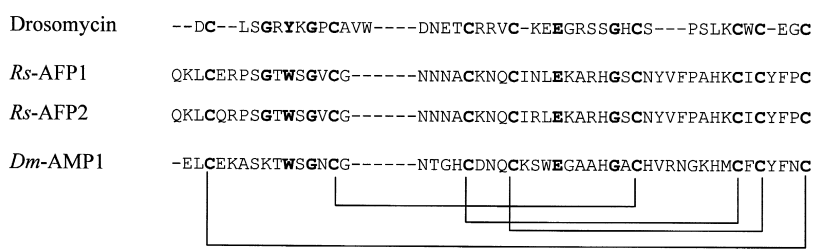

Fig. 5. Alignment of amino acid sequences of antifungal peptides from insects and representatives from plants with three and four disulfide bridges. The conservative residues, including the cysteines, are in bold characters. The disulfide bridge structure is indicated by broken lines. Drosomycin was isolated from *Drosophila melanogaster* (Fehlbaum et al., 1994), Rs-AFP_1 and $_2$ were from the radish seeds of *R. sativus* (Terras et al., 1992), and Dm-AMP_1 from dahlia seeds (Broekaert et al., 1995).

spectrum against filamentous fungi with an inhibitory effect on spore germination and hyphal growth, leading to morphological abnormalities of the hyphae (Fehlbaum et al., 1996). A similar mode of action against fungi has been observed with penaeidins (Destoumieux et al., 1999).

3.3.2.2. Plant defensins. Plant defensins form a group of cysteine-rich peptides consisting of 45–54 residues with four intramolecular disulfide bridges. They were initially isolated from *R. sativus* (Terras et al., 1992), and then from different plant species and various tissues, seeds, flowers and injured leaves. The arrangement of the cysteine residues is highly conserved among the plant defensins and they bear a CS$\alpha\beta$ motif (as reviewed in Broekaert et al., 1995), which has been defined for insect defensins (see above, Fig. 4). While most of the plant defensins isolated exhibit antifungal activity, they are divided into two groups according to differential antifungal properties. The first group, which contains the radish seed defensin, Rs-AFP_2, causes growth inhibition of fungal hyphae with resulting morphological abnormalities and distortions. This effect is also observed, as previously mentioned, for the insect drosomycin and for the penaeidins. The second group inhibits fungal growth but without evident morphological changes, that is the case of a peptide isolated from *Dalhia merckii*, Dm-AMP_1 (Osborn et al., 1995). In contrast with insect and vertebrate defensins which exhibit membrane permeability, the plant defensins would interact with the fungal membrane through a receptor. This interaction, either directly or indirectly affecting the generation of ion fluxes on the fungal membrane, could be directly involved in the antifungal activity and mode of action of the peptides (Thevissen et al., 1997).

3.3.3. Chimeric-like structure of the penaeidins and other effectors

A composition similar to that observed for penaeidins combining two distinct domains has been observed, in a few cases, in antimicrobial peptides.

3.3.3.1. The diptericins. This characteristic is present in diptericins, 9-kDa inducible, antibacterial polypeptides isolated from different dipterans, *Phormia* (Dimarcq et al., 1988), *Drosophila* (Wicker et al., 1990) and *Sarcophaga* (Ishikawa et al., 1992). The diptericins consist of a main COOH-terminal, glycine-rich domain, 67-residue long, preceded by a short, proline-rich domain (15 residues). This NH_2-terminal proline-rich domain shows sequence similarities with the family of proline-rich antimicrobial peptides, e.g. drosocin and pyrrhocoricin. As for these short proline-rich peptides, *Phormia* diptericin carries two *O*-substitutions on threonine residues, one in the NH_2-terminal proline-rich domain and the other one in the COOH-terminal glycine-rich domain. These post-translational modifications have been shown to be essential for the full antibacterial activity of the polypeptide (Bulet et al., 1995). Conversely, the *Sarcophaga* diptericin is not *O*-glycosylated (Ishikawa et al., 1992).

3.3.3.2. The big defensin. The existence of two distinct functional domains is a striking characteristic of the big defensin, a 79-residue antimicrobial peptide present in the granules of horseshoe crab hemocytes (Saito et al., 1995). The big defensin inhibits the growth of gram-positive and gram-negative bacteria but also fungi such as the yeast-like

fungus, *Candida albicans*. The peptide is composed of: (i) a hydrophobic NH_2-terminal portion (35 residues), with no homology to any other peptide, and (ii) a cationic cysteine-rich COOH-terminal portion (37 residues). It was named big defensin because this COOH-terminal has a sequence similar to vertebrate defensins and a cysteine arrangement similar to that of the β-defensins from bovine neutrophils (Saito et al., 1995). To prove that big defensin is a chimeric or hybrid antimicrobial peptide, the NH_2-terminal extension can be separated by trypsin digestion from the COOH-terminal portion and it has also been shown that each of them presents different antimicrobial activities. The NH_2-terminal hydrophobic portion was found to be effective against gram-positive bacteria, whereas the COOH-terminal portion appeared to be active against gram-negative bacteria (Saito et al., 1995).

3.3.3.3. Chimeric plant proteins. Several molecules isolated from plants but not known as antimicrobial proteins were shown to have a chimeric structure. Two families of plant proteins, similar to the penaeidins, with a proline-rich domain at their NH_2 terminus and a cysteine-rich domain at the COOH-terminal end, were found: (i) the tobacco extracellular matrix proteins, cysteine-rich extensin-like protein (CELP) (Wu et al., 1993), that have a proline-rich extensin-like domain and a cysteine-rich domain with a highly charged COOH-terminus; and (ii) the potato lectins that have an extensin domain rich in glycosylated hydroxyproline residues, fused with a lectin domain. The latter consists of a cysteine-rich domain containing partial conservation of a repeated motif common to several chitin-binding proteins of the hevein family, including the wheat germ agglutinin (WGA) (Allen et al., 1996).

According to database searches, the penaeidin NH_2-terminal domain presents homologies with that of the CELP and some similarities with extensins (Fig. 6). Moreover, Pen-3 contains the Val–Tyr–Lys motif which constitutes, in extensins, a functional site for reactions of adhesion or intermolecular cross-links involved in cell wall assembly and extension (Kieliszewski and Lamport, 1994). In addition, some homologies are observed between the COOH-terminal cysteine-rich domain of the penaeidins and a conserved motif common to some plant chitin-binding proteins (as reviewed in Raikhel et al., 1993), including chitinases, lectins (Mirelman et al., 1975), antifungal peptides

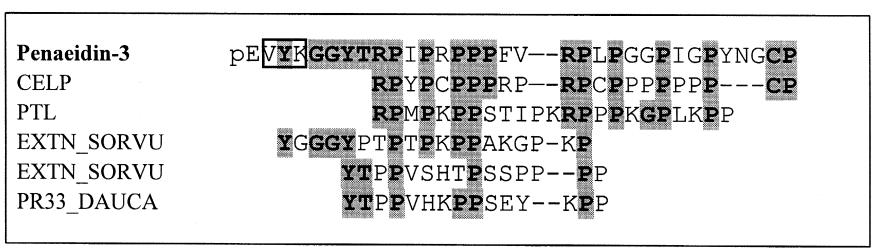

Fig. 6. Comparison of the NH_2-terminal domain of Pen-3 with different plant extensins showing some sequence similarities. Conservative replacements of identical amino acids are shaded. The motif VYK involved in intermolecular cross-links in extensins is boxed. CELP from *Nicotiana tabacum* (Wu et al., 1993); PTL1 'extensin-like protein' from *Antirrhinum majus* (Baldwin et al., 1992); EXTN_SORVU, extensin from *Sorghum bicolor* (Raz et al., 1991); PR33_DAUCA, extensin from *Daucus carota* (Chen and Varner, 1985).

and proteins such as heveins (Koo et al., 1998) (Fig. 7(A)). Partial conservation of this motif has also been reported in the antimicrobial protein-2 from the plant *Amaranthus caudatus* (Ac-AMP_2) (Broekaert et al., 1992), which contains six cysteine residues, as with the penaeidins, and displays chitin-binding properties. Such a partial conservation is also encountered in tachycitin, a COOH-terminally amidated antimicrobial peptide composed of 73 amino acids, including 10 cysteines (Fig. 7(B)). Tachycitin, purified from the small granules of the horseshoe crab, *T. tridentatus* hemocytes, is co-localized with the big defensin and tachyplesins (Kawabata et al., 1996). Tachycitin inhibits the growth of gram-positive and gram-negative bacteria and fungi. Moreover, it displays significant binding to LPS, a chitin-binding ability and it agglutinates some bacteria such as *Escherichia coli* and *Enterococcus hirae*.

It is considered that the structure of the chimeric proteins, such as potato lectins or CELP, indicates that they are multifunctional (Wu et al., 1993), as observed to some extent in the horseshoe crab big defensin and as assumed for the penaeidins as well. Thus, the CELP would be concomitantly involved in plant growth, development and defense. Similarly, the penaeidins would exhibit antifungal and antibacterial activities together with a chitin-binding and/or agglutinating capability. To go further, studies are currently being carried out to determine if the penaeidins are also true chimeric peptides with distinct domains and, consequently, to analyse which functional role and property these two domains have, respectively. By their chitin-binding properties, one can speculate that the penaeidins could be involved, both in antimicrobial defense and in wound healing and synthesis of chitin, thus, displaying a dual function determinant for the survival of the shrimp, particularly those exposed to microbial infections during the moulting process.

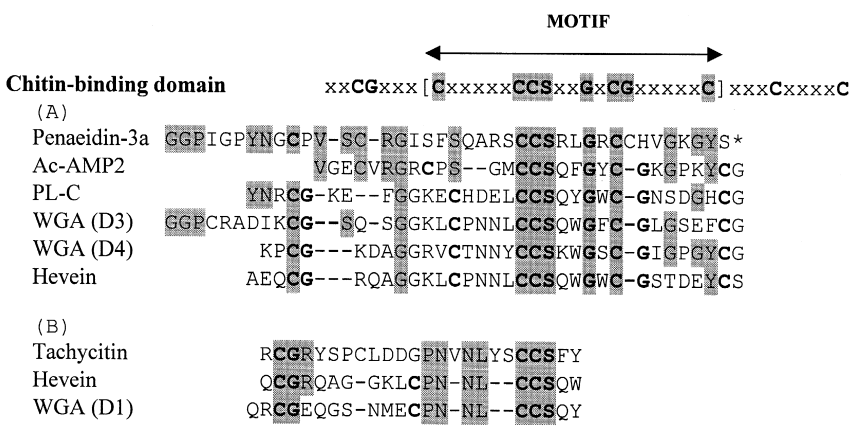

Fig. 7. (A) Comparison of the COOH-terminal amino acid sequence of penaeidin with chitin-binding domain of plant lectins and antifungal peptides. (B) Aligned amino acid sequences of chitin-binding domains of tachycitin and plant proteins. The amino acids belonging to the chitin-binding domain are in bold type and those belonging to the consensus pattern are shaded, as well as the conservative replacements of identical amino acids. WGA and hevein, antifungal peptide from *Hevea brasiliensis* (in Raikhel et al., 1993); PL-C, *Phytolacca americana*, lectin-C (Yamaguchi et al., 1995); Ac-AMP_2, *A. caudatus*, antimicrobial peptide (Broekaert et al., 1992); tachycitin, antimicrobial peptide from *T. tridentatus* (Kawabata et al., 1996).

4. Concluding remarks

Antimicrobial peptides are paramount substances of the immune defense reactions developed by living organisms to fight infection by microorganisms. It is interesting to notice that even if the structures are varied, some common features allow classification of these compounds into broad families. However, the recent discovery of the penaeidins suggests that this classification has to be flexible, in order to accommodate other chimeric-like molecules, such as the horseshoe crab big defensin. Finally, the functional significance of the combination of two distinct structural domains in a single molecule is not yet fully understood. So, the penaeidins which are present in different tissues and locations in the shrimp body, by the presence of hemocytes, could combine antimicrobial and chitin-binding properties that may be important in interactions between immune function and developmental function through the synthesis of exoskeleton in shrimp. These possible multifunctional properties of antimicrobial peptides represent an important new area to be investigated. Any progress in this field would contribute to a better understanding of the penaeid shrimp physiology and of their capacity to respond to pathological injuries.

Acknowledgements

This work is supported by the Institut Français de Recherche et d'Exploitation de la Mer (IFREMER), Centre National de la Recherche Scientifique (CNRS) and the Université de Montpellier 2. It is also part of a collaborative project supported by the European Commission, DG XII, in the programme, International Cooperation with Developing Countries, INCO-DC: Contract no. IC18CT970209 (Shrimp Immunity and Disease Control).

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