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Antimicrobial proteins: From old proteins, new tricks

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ABSTRACT

This review describes the main types of antimicrobial peptides (AMPs) synthesised by crustaceans, primarily those identified in shrimp, crayfish, crab and lobster. It includes an overview of their range of microbicidal activities and the current landscape of our understanding of their gene expression patterns in different body tissues. It further summarises how their expression might change following various types of immune challenges. The review further considers proteins or protein fragments from crustaceans that have antimicrobial properties but are more usually associated with other biological functions, or are derived from such proteins. It discusses how these unconventional AMPs might be generated at, or delivered to, sites of infection and how they might contribute to crustacean host defence *in vivo*. It also highlights recent work that is starting to reveal the extent of multi-functionality displayed by some decapod AMPs, particularly their participation in other aspects of host protection. Examples of such activities include proteinase inhibition, phagocytosis, antiviral activity and haematopoiesis.

1. Introduction

Conventional antimicrobial peptides are small (usually <10 kDa or <100 amino acids) cationic and amphipathic proteins that kill a broad spectrum of micro-organisms in a stoichiometric manner. It has now been nearly 40 years since defensins, cecropins and magainins were independently isolated from mammals, insects and amphibians by Bob Lehrer, Hans Boman and Michael Zasloff, respectively. Since then thousands of antimicrobial peptides (AMPs) have been reported by other workers, with over 5500 listed on protein, gene or AMP databases. Indeed at least 13 AMP-dedicated databases have been created since 2002, with, to the best of our knowledge at the time of writing, the most recent being LAMP (Zhao et al., 2013a). However, different databases have been compiled for different purposes and search criteria, so do not necessarily list every AMP discovered. What these databases do show is that AMPs are expressed in the blood, mucosa and other body tissues of a very wide range of taxa, from simple protists and acoelomate invertebrates to mammals, with some also identified in plants, yeasts or fungi (http://aps.unmc.edu/AP/main.php). Clearly, AMPs are no longer the new kids on the immunology block but are now fully recognised potent effectors against infection and, as such, are a key part of innate inflammatory and mucosal defences. We can therefore regard AMPs as established and evolutionarily very old defence proteins.

As review articles have already been published that describe the various AMP families in crustaceans and other invertebrate taxa (Smith et al., 2008, 2010; Sperstad et al., 2011; Tassanakajon et al., 2011, 2015; Song and Li, 2014), the present article is concerned with considering recent advances in our knowledge of crustacean AMPs (*i.e.* over the last 5 years) but places this within the context of established knowledge. It focuses primarily, but not exclusively, on decapods, as this group of crustaceans are a major focus of research on account of their economic importance in aquaculture. There is an ever growing problem of disease in shrimp aquaculture and the farming of other comestible decapod species, mainly through increased intensification in the farming methods and expansion of global markets for brood-stock and seed. This, of course, is the major driver for much of the funding that underpins research, so it is not surprising that antimicrobial peptides and the factors that regulate their transcription and synthesis are amongst the most frequently studied immune proteins in these decapods.

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This article will, first, give an overview of the range of AMPs produced by crustaceans, followed by a survey of the current landscape in our understanding of their antimicrobial activities and patterns of expression. It will then discuss new research that reveals some quirky aspects of their biological roles and participation in host defence.

2. AMPs in crustaceans: a brief survey

Following the purification of the first crustacean AMPs, the proline-rich peptide, Bac-C, isolated from the haemocytes of the shore crab, *Carcinus maenas* (Schnapp et al., 1996) and the penaeidins from the shrimp, *Litopenaeus vannamei* (Destoumieux et al., 1997), several families are now known to exist (Tables 1 and 2), with numerous individual, novel AMPs also recorded (Table 3). In terms of the number of peptides known, the families of crustins and penaeidins are the most prominent, so are commonly regarded as the two 'main' groups for the Crustacea.

2.1. Crustins

The largest family of crustacean AMPs is the crustin group, of which more than 50 such proteins are now reported in the literature or lodged on databases. The first crustin to be discovered was purified from *C. maenas* haemolymph (Relf et al., 1999) and was only later designated as 'carcinin' by Brockton et al. (2007). The term crustin was coined following the discovery of highly similar proteins in shrimp (Bartlett et al., 2002). Crustins have since been found to occur across many decapod taxa (Tables 1 and 2), with crustin-like sequences also detected in amphipods, branchiopods and copepods (see review by Smith et al. (2008)). Remarkably, crustin-like sequences have even been found in ants (Zhang and Zhu, 2012), showing that they are not necessarily confined to decapods, or even the crustacean group as whole. Crustins are constitutively expressed, cationic, cysteine-rich AMPs (~7–14 kDa), containing a signal sequence and usually 1, or occasionally 2, whey acidic protein (WAP) domains (Smith et al., 2008; Li et al., 2012). WAP domains are characterised by a conserved arrangement of 8 cysteines that form a 4-disulphide core at the C terminus. The number of WAP domains, together with the presence or absence of other cys-rich or gly-rich domains, is the basis of crustin classification into four main types, namely I–IV (Table 1; Smith, 2011). Crustins act primarily against Gram-positive bacteria, although some have also been reported to kill Gram-negatives (Table 2; Li et al., 2012). In general, Gram-positive bacteria are killed at relatively high MIC values compared with some other invertebrate AMPs, while killing of Gram negatives is achieved at quite low MIC levels (Table 2). For example, a Type III crustin from the shrimp, *Penaeus monodon*, is active against Gram-negative bacteria with an MIC value of <5 μM (Amparyup et al., 2008a).

2.2. Penaeidins

The penaeidins are the second largest group of crustacean AMPs with some 40 types discovered from at least 8 shrimp species so far (reviewed by Tassanakajon et al., 2011; Song and Li, 2014). Penaeidins were initially identified from the shrimp, *L. vannamei,* (Destoumieux et al., 1997) but have since been found in several other shrimp species including: *Fenneropenaeus chinensis*, *Fenneropenaeus indicus*, *Litopenaeus stylirostris*, *Litopenaeus schmitti*, *Litopenaeus setiferus*, *Marsupenaeus japonicus* and *P. monodon* (Tassanakajon et al., 2011; Shanthi and Vaseeharan, 2012). Unlike crustins, penaeidins have yet to be identified outside the penaeid group. The encoded proteins comprise a signal sequence, and two domains: a long (*ca.* 21–28 amino acid) proline–arginine rich domain at the N terminus and a compact, cysteine-rich (typically six cysteine residues) domain at the C terminus. Currently, penaeidins are the only crustacean AMP family to have had a universally agreed classification and nomenclature scheme based on amino-acid similarity (Gueguen et al., 2006). This was organized as a database called 'Penbase' and hosted at http://www.penbase.immunoaqua.com, although the site no longer appears to be active. Four classes of penaeidins are recognised (Tables 1 and 2), with their spectra of activities mainly against Gram-positive bacteria and fungi. More strongly antimicrobial than crustins in terms of their MIC (Table 2), penaeidins also possess chitin-binding activity, which is thought to account for their antifungal properties and confers antimicrobial protection to the shrimp carapace (Destoumieux et al., 2000).

2.3. Anti-lipopolysaccharide factors

Anti-lipopolysaccharide factors (ALFs) were originally purified from the amoebocytes of the chelicerate horseshoe crabs, *Limulus polyphemus* and *Tachypleus tridentatus* (Tanaka et al., 1982; Ohashi et al., 1982) and only discovered from signature sequences in EST libraries of shrimp *L. vannamei* and *L. setiferus* much later (Gross et al., 2001). Originally identified as binding proteins in horseshoe crabs, they are now known to also have antimicrobial activity and are, at present, routinely considered as AMPs (Tassanakajon et al., 2011). The ALF family comprises five groups, defined by their amino acid sequences, the characteristics of their lipopolysaccharide binding sites and their predicted pI values. Group A contains both anionic and cationic peptides whereas Groups B and C have only cationic ones, but differ in the number of introns in the genes encoding them, as well as their tissue distribution. Group D differs from the other groups in as much as it contains only strongly anionic peptides. The fifth, Group E, is another cationic group recently identified from the transcriptome of the haemocytes and hepatopancreas from the kuruma shrimp, *Marsupenaeus japonicus*, and confirmed as separate to Groups A–D by sequence alignments and phylogenetic analyses (Jiang et al., 2015). While ALFs in general have potent, broad-spectrum antimicrobial activities (Table 2), there are some differences between these groups. For example, Groups A–C and E have strong antibacterial and binding properties (Jiang et al., 2015) whereas Group D has only weak killing and binding properties. The biological role(s) played by Group D ALFs has yet to be elucidated (Rosa et al., 2013).

2.4. Lysozymes

Although not fitting the traditional definition of an AMP, lysozyme is widely considered to be an antimicrobial protein. It is a muramidase that primarily exerts its effects on target bacteria by cleaving the β 1,4-glycosidic bonds in the peptidoglycan of bacterial cell walls. Lysozyme activities have been observed and quantified in the immune cells of many animal species, although its investigation in crustaceans has been relatively limited compared with other taxa. As with crustins, penaeidins and ALFs, knowledge of lysozyme in crustaceans has come largely from cDNA or EST data coupled with recombinant protein assays (*e.g.* Pan et al., 2010; Supungul et al., 2010).

Of three main types of lysozyme recognised across animal taxa: namely the c- (chicken), g- (goose) and i- (invertebrate) types (reviewed by Callewaert and Michiels, 2010), only c- and i- types have been found in crustaceans (Supungul et al., 2010; Kaizu et al., 2011; Peregrino-Uriarte et al., 2012). These types are notable for also killing some Gram-negative bacteria (Mai and Hu, 2009; Supungul et al., 2010), with the activity, at least in c-types, attributed to the presence of a hydrophobic region in the molecule