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Antimicrobial Peptides (AMPs) and their Role in Insect Immunity : A Review

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ABSTRACT

Insects have developed a powerful and diversified immunity to defend themselves against bacteria, fungi, viruses and other entomopathogens. The insect immunity involves both cellular and humoral reactions that include phagocytosis, capsule and nodule formation, coagulation, melanisation and induction of antimicrobial peptides depending on the number, size and type of microorganisms. Humoral response is exclusively protein mediated and in which anti-microbial peptides (AMPs) play a major role. Invading pathogens are recognised by recognition receptors (PRRS) while they themselves are called as Pathogen associated molecular patterns (PAMPs) and a defence cascade is produced. The AMPs have been reported both constitutively and produced on induction by invading microbes. They are synthesized in fat bodies and a few cell types of the haemolymph and released into the haemolymph in quantities ranging from 1 to 10 micro litres. Two different signal transduction pathways, Toll and Immuno-deficiency (Imd) pathways involved in the activation of AMPs synthesis. The Toll pathway activates the expression of the gene encoding the antifungal peptide drosomycin through a cascade of events. Toll-activation can be observed in response to either fungal infection or infection with Gram-positive bacteria. In contrast, the Imd pathway was found to be involved in the resistance of insects to Gram-negative infections. These mechanisms have tremendous roles to play in entomological sciences as well as in host pathogen interactions e.g., boosting immunity of beneficial insects, manipulating immunity of mosquitoes against *Plasmodium,* silencing of genes to hinder eicasinoid biosynthesis etc. The long-term significance of research in this arealies in developing new pest management technologies to contribute to food security in a world with rapidly growing human population.

Keywords : Antimicrobial peptides, Insect as antimicrobial peptides, Biological functions, Insect amps Gene regulation

Introduction :

Insects, like any other organisms on the earth, are subjected to diseases caused weaken it. Insects defend themselves against these microbes in two ways. Firstly, the chitinous cuticle in conjunction with

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the respiratory system and the intima, provide as major physical barriers. Secondly, the insect blood plays a major role in healing the external wounds as well as destroying the invading organisms within the body. Interestingly, along with the passive immunity, they also have the evidence of acquired immunity. There can be free-floating cells, the hemocytes, in the haemolymph. They play a major role in the insect immune system. Defence system of insects depend on cascades of innate reactions. Upon injury, the proteolytic cascades begin which lead to localized blood clotting and melanisation. The latter process involves the production of reactive oxygen intermediates; the phagocytosis and the encapsulation of bacteria and larger parasites; synthesis of a battery of effective antimicrobial peptides/polypeptides by the fat body which are released into the blood stream where they act synergistically to kill the invading microorganisms (Lei *et al.,* 2019). The insect host defence system has much similarity with the mammalian acute phase response, especially at the level of gene expression, where cis-regulatory and trans inducible activators appear to play key functions (Hoffmann, 1995). Much of the work have been done on the model insect, *Drosophila melanogaster* which provides a unique opportunity to have insights on differentiation of the primordial immune system and it has contributed a lot to our understanding of the innate immune response in the vertebrates (Buchon *et al.,* 2014). However, eusocial insects additionally have a collection of behavioural and chemical defences and in the case of the ant, metapleural gland secretions over their cuticle prevent the pathogens to settle on (Konrad *et al.,* 2012).

Antimicrobial peptides :

Most living organisms are constantly exposed to harmful pathogens through contact, ingestion and inhalation. The survival of such organisms in a microbe thriving environment depends on a network of host defence mechanisms involving various components (Alberts *et al.,* 2002). Often during microbial invasion, the first line of defence is the innate mechanisms of immunity which is followed by acquired responses involving the activation of T and B cells against specific pathogens. In contrast to the later mechanism, endogenous peptides, which are constitutively induced (in a few cases), provides a fast and effective means of protection against the pathogens. This group of peptides are termed ‘antimicrobial peptides’ (AMPs) and they constitute a primitive immune defence mechanism and are found in a wide range of eukaryotic organisms, from humans to plants to arthropods including insects (Shizuo *et al.,* 2006).

Insects as sources of antimicrobial peptides :

Insects demonstrate a remarkable evolutionary success that can be attributed to a variety of reasons, among which the antibacterial defence reactions play an important role. These creatures are continuously exposed to potential pathogens and parasites. However, only a few interactions result in infection (Hoffmann, 1997). The great diversity of insects achieved only because of low extinction rates compared to other animal groups. The class “Insecta” contains more species than any other class of animals. Insects mainly rely on innate immunity as far as their defence mechanism is concerned. Comparing the size of the DNA required to produce one immunoglobulin (Ig) molecule with that of 20 peptides, including all theenzymes, it can be said that the innate immunity appears to be 100 times more efficient than adaptive form of immunity (Nicholson, 2016). Antimicrobial peptides can be detected in insect haemolymph as early as 2 hours after injury. A single insect produces approximately 10-15 peptide antibiotics and each peptide exhibit completely different activity spectrum than the others (Hancock *et al.,* 1999). The first ever insect antimicrobial peptide named cecropin was identified from the pupal hemolymph of the *Hyalophora cecropia* (Steiner *et al.,* 1981). Since then, more than hundreds of such peptides have been identified in various insects. These AMPs can be classified to various families (such as cecropins, ponericins, defensins, lebocins, drosocin, Metchnikowin, gloverins, diptericins and attacins) according to their structure or function. Cecropins are active against fungi, Gram-negative as well as Gram-positive bacteria and they are cationic peptides of 31–39 amino acid residues (Yi *et al.,* 2014). *Drosophila melanogaster* has served as a model organism for the study of immune mechanisms. Injury in this insect rapidly upregulates the genes encoding AMPs in the fat body and produce a cascade of peptides namely drosomycin, cecropins, diptericin, drosocin, attacin and metchnikowin (Bulet *et al.,* 1999). The first and last one has antifungal properties while others exhibit antibacterial properties (Hoffman *et al.,* 1999). Diptericins are mostly expressed in the midgut of the insect during systemic infections. Ponericins have been isolated from the venom of the ant, *Pachycondyla goeldii*. They are classified into ponericins G, W and L based on their primary structures (Orivel *et al.,* 2001). Insect defensins were initially isolated from cell cultures of the flesh fly, *Sarcophaga peregrina* and from diseased larvae of the black brown fly, *Phormia terranovae*. In holometabolous insects, AMPs are transiently and rapidly synthesised by the fat body and by other epithelia. Fat body induced production of AMPs allows them to get secreted into the haemolymph, from where they easily diffuse to perform throughout the entire body. Most of the AMPs are produced copiously, reaching high micromolar concentrations. However, in heterometabolous insects, AMPs are produced by haemocytesand secreted into the haemolymph upon infection.

Structure of insect antimicrobial peptides (AMPS) :

On the basis of their sequence and secondary structural features - three broad categories

1. Peptides with á -helix and two to four disulphide bonds (e.g., defensins)
2. Peptides with hairpin-like ß-sheet structure (e.g., thanatin)
3. Peptides with a triple-stranded antiparallel ß-sheets (e.g., Proline rich peptides)

Peptides with á-helix and two to four disulphide bonds :

The most abundant family of linear AMPs of insect origin are the cecropins including sarcotoxins, hyphancin, enbocin and spodopsin, as well as cecropin-like molecules totalling more than 60 described sequences.

Cecropins :

Cecropins were the first animal inducible AMPs to be isolated and fully characterized (Hultmark *et al.,* 1980). The first insect cecropin was isolated from the blood of infected diapausing pupae of the moth *Hyalophora cecropia*. Apart from that, expression of these peptides has been documented in several other phylogenetically higher insect orders of Diptera and Lepidoptera. Comparison of the cecropin sequences reveal that dipteran cecropins form a particularly homologous group with more than 70% identity in their amino acid composition. However, cecropins from Lepidoptera present more variation in their primary structure. Most insect cecropins have the presence of a tryptophan residue at position 1 or 2. However, mosquito cecropins isolated from *Aedes sp. lack* tryptophan residue cell line (Lowenberger *et al.,* 1999). This absence of tryptophan residue within the N-terminal domain have also been was also reported from *Bombyx mori*. *Anopheles* cecropin without the tryptophan residue has been seen to be more efficient against Gram-positive bacteria than *Drosophila* cecropin having a tryptophan residue in position 2 (Vizioli *et al.,* 2000). Increased number of positive charges and absence of tryptophan may be the reason for the differences in the observed antimicrobial activity. Comparison of amidated andnon-amidated cecropins shows a higher efficacy for the amidated version (Efimova *et al.,* 2018). A group of cecropin-related peptides, ponericins, have been isolated from venom glands of the ant *Pachycondylas goeldii*. Sequence similarities exist between cecropins and ponericins G (60%), and between ponericins W and melittin (70%), a helical toxin isolated from the venom of *Apis mellifera* (Hymenoptera). Melittin, the signature peptide for haemolytic activity to mammalian cells, also exhibits some antimicrobial properties (Lee *et al.,* 2015). Additionally, helical AMPs have been isolated from the medfly *Cerratitis capitata*. Cecropins like Ceratotoxins, stomoxyn and spinigerin are constitutively present in the secretion of female accessory reproductive glands, in the anterior midgut and in the hemocytes, respectively. The á-helical linear AMPs of insect origin are mostly active against bacteria, with higher efficacy on Gram-negative than on Gram-positive strains. It has been seen during antibacterial activity screening that they had no haemolytic activity when tested in the concentration range. Interestingly, recent reports established that cecropins and cecropin-like peptides might also affect the growth of filamentous fungi including human and plant pathogens (i.e., *Aspergillus* spp. and *Fusarium* spp.), and yeasts. Stomoxyn, which has a broad-spectrum activity, also exhibits trypanolytic activity (Chowdhary *et al.,* 2016).The solution conformation of a cecropin was first demonstrated on *Hyalophora* cecropin A by circular dichroism (CD) spectroscopy. CD studies also reveal that cecropins are unordered in aqueous solution. However, in hydrophobic conditions, they undergo a stable á-helical shape. The structure of Cecropin A has been further characterized by spectroscopy images inhexafluoropropanol mixtures, a secondary structure promoting solvent combination. This peptide has a long N-terminal, basic, amphipatic a-helix (residues 5 to 21) and a shorter and more hydrophobic C-terminal helix (residues 24 to 37), linked by a hinge region (Lee *et al.,* 2013). Activity relationship studies outline the significance of different parameters that may influence the activity of the á-helical AMPs *viz.,* charge, helicity, size, hydrophobic moment and hydrophobicity. Efforts were devoted to improve the therapeutic potential of cecropins by combining fragments of different AMPs forming á-helices (cecropins and magainins) or a fragment of *Hyalophora* cecropin A with melittin (Wei *et al.,* 2016).

Defensins :

Defensins have been found in almost all insect species investigated till date. These are of small, cationic peptides and include compounds like phormicins, sapecins, royalisin and spodoptericin. Defensins, were first reported from cell cultures of the flesh fly *Sarcophaga peregrina* and from experimentally injured larvae of the black blowfly, *Phormia terranovae* (Lambert *et al.,* 1989). More than 60 defensins have been isolated from insects belonging to phylogenetically recent orders (Diptera, Lepidoptera, Coleoptera, Hymenoptera) and to the ancient order of Odonata (dragonfly). Surprisingly, the primary structure of the dragonfly defensin, with over 75% identity, is closely related to the mollusc and scorpion defensins. Comparison of the peptide sequences revealed 70 to almost 100% homology among the group of dipteran defensins and indicated that these defensins have more than 35% similarity to the defensin from the dragonfly. This is particularly interesting as dragonflies evolved about 100 million years before the emergence of the dipteran insects. Defensins like phormicin, sapecin, and lucifensin only differ by individual amino acids and thus it is not a matter of surprise that their three-dimensional structures are highly similar (Nygaard *et al.,* 2012).

Hairpin-like â-sheet peptide :

Among the group of insect AMPs containing an even number of cysteine residues, thanatin is the smallest one containing only 21 amino acids including two cysteine residues. Thanatin, with noticeable sequence similarities to frog AMPs of the brevinin family, has been isolated from the spined soldier bug *Podisus maculiventris*. Thanatin has a broad spectrum of activity, killing (at mM concentrations) both Gram-positive and Gram-negative bacteria, filamentous fungi and yeasts without measurable toxicity for red blood cells. A recent study by Pagès and co-workers on the activity of thanatin against multidrug resistant bacteria isolated from hospitalized patients (*Enterobacter aerogenes* and *Klepsiella pneumonia*) evidenced that the activity of this peptide depends on the size of the lipopolys saccharide/endotoxin (LPS), a component of the outer membrane of Gram-negative bacterial cell wall. The authors suggested that the accessibility of thanatin to an internal target is improved when the size of LPS is decreased. SAR studies established that an all-D enantiomer is ineffective against Gram-negative bacteria, but exhibits the same level of activity as the natural L peptide on fungi. The *in vitro* efficacy of all-D thanatin against Gram-positive bacteria is strain dependent. This suggests that for killing different types of microorganisms, thanatin uses different mechanisms of action, involving a stereospecific interaction with a bacterial target.

Additional studies on thanatin included activity measurements of N- and/ or C terminally truncated forms, or analogues containing amino acid substitutions within the disulfide loop, and functional mapping by random mutagenesis in *E. coli* combined with the determination of the solution structure by NMR spectroscopy and molecular dynamics calculations. It appears that (i) elimination of the C-terminal methionine residue diminishes the efficacy against Gram-negative bacteria, and removal of the last two residues completely abolishes the activity; (ii) the deletion of the five N-terminal residues retains activity against some Gramnegative bacteria, while the removal of two additional residues is deleterious for the activity; (iii) the deletion of amino acids within the disulfide loop increases the efficacy against Gram-positive strains, but an insertion of an alanine residue to the disulfide loop decreases the activity against all types of bacteria; and (iv) maintenance a hydrogen bonding network within the C-terminal loop linking the two antiparallel b-strands is needed for the activity. The 3D structure of thanatin has been elucidated by 1H 2D NMR spectroscopy and molecular modelling. The peptide adopts a well-defined, two-stranded â-sheet structure, stabilized by the intramolecular disulfide bridge, delineating a box extended by a rather flexible N-terminal tail. While the central part of the molecule is hydrophobic, the outside sites (loop and N-terminal tail) are rich in hydrophilic residues. Thanatin appears to be a good candidate for the development of novel therapeutic agents against multidrug resistant bacteria because this peptide (i) is devoid of toxicity toward eukaryotic cells, (ii) kills bacteria including those featuring multidrug resistant phenotypes, (iii) acts on bacteria through different modes of action, and (iv) is short enough to facilitate SAR studies.

Linear proline-rich AMPS :

The proline-rich peptides are linear molecules and are made up from 14-39 amino acids (the two extremes are 14 residues for the apidaecin from the baldfaced hornet and 39 residues for abaecin from a bumblebee). They are classified in two sub-families: short-chain (less than 20 residues) and long-chain peptides (more than 20 amino acids). They have been isolated from bees and wasps (abaecins, apidaecins), from ants (formaecins), from the *D. melanogaster* (drosocins and metchnikowins), from the silkworm *Bombyx mori,* the cabbage looper moth *Trichoplusia ni* (lebocins) and from bugs (pyrrhocoricin, metalnikowins).

Biological functions of insect AMPS :

Recognition of pathogen by AMPs :

The first and possibly the most essential part of any immune response is the recognition of non-self-molecules. The recognition of pathogens relies on the difference in the surface patterns between harmful microbes and the insects' own cells; these patterns are called the pathogen associated molecular patterns (PAMPs). Typical PAMPs include lipopolysaccharides (LPS), from gram negative bacteria such as *Escherichia coli,* and peptidoglycan, from gram positive bacteria such as *Bacillus subtilis*. It has been known for some time that the recognition of PAMPs and the triggering of the cascade involve the cells of the insect's fat body. Exciting new research now suggests that the haemocytes may be essential in recognising and breaking down the bacteria. Components of the bacterial cell wall or cytokines released by the haemocytes are then thought to stimulate the fat body to release antimicrobial peptides. Once the pathogen has been detected by the recognition proteins, signalling cascades led to the release of antimicrobial peptides. The primary site of release is the insect's fat body. However, other tissues such as haemocytes, epithelial tissues, malpighian tubules, reproductive organs and the salivary glands can also be important. It has also been shown that in addition to its role as a physical barrier to pathogens, the cuticle may be actively involved in the production of antimicrobial peptides. Antimicrobial peptides have a powerful bactericidal effect in contrast to synthetic antibiotics which are typically bacteriostatic. The exact mode of action is not fully understood and is likely to vary with different peptides and with the pathogen that is being targeted. However, in general it seems that the damage caused by antimicrobial peptides is focused on the pathogen cell membrane. It is remarkable that these peptides cause such devastating effects in the pathogens that they target yet are generally not toxic to the insects' own cells. The mechanism behind this specificity is also not fully understood, but one likely explanation relates to the charge on the outer membrane. As well as antimicrobial peptides having an overall net charge, they are amphipathic, i.e., they have a hydrophilic and a hydrophobic end. These properties allow the peptides to disrupt phospholipid cell membranes. A general mode of action has been proposed whereby antimicrobial peptides form pores or channels through the cell membrane. This would result in free passage of ions destroying the membrane potential, leading to the prevention of cellular metabolism and the death of the cell. However, a completely different mechanism has been demonstrated for one particular antimicrobial peptide. Pyrrhocoricin isolated from *Drosophila melanogaster* interferes with the action of the *Escherichia coli* DnaK heat shock protein. Without this protein many essential enzymes are destroyed, resulting in death of the bacteria.

Mode of action of insect AMPs :

Although the exact mode of action of AMPs has always been a controversy, there is a consensus that these peptides selectively disrupt the cell membranes and the amphipathic structural arrangement of the peptides is believed to play an important role in this mechanism. The phospholipids head group charge on cell membranes and peptide charge distribution appears to play an important role in the peptide membrane interactions. The following models have been proposed.

a. The barrel-stave model of AMPs :

The peptide monomers bind to the cell membrane in á-helical confirmation. This is followed by the localization of more peptide molecules on the cell membrane after which the peptide helices insert themselves into the hydrophobic core of the membrane. Progressive recruitment of additional monomers increases the pore size causing leakage of cytoplasmic materialand hence there is death of the cell.

b. The carpet model of AMPs :

According to the model the peptides are in contact with the phospholipid head group throughout the entire process of membrane permeation and there is preferential binding of the peptide monomers to the phospholipid head groups. This is followed by the alignment of the peptide monomers on the membrane surface such that the hydrophilic residues face the phospholipid head groups and later the peptides reorient themselves towards the hydrophobic core of the membrane followed by the disintegration of the membrane due to disruption of bilayer curvature.

c. The toroidal model of AMPs :

In this model the attached peptides aggregate and induce the lipid mono layers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the lipid head groups.

Insect AMPs gene regulation :

Insect immune response gets activated by both cellular and humoral activities. Microbes are detected by host proteins, like the peptidoglycan recognition protein (PGRP), and the gram-negative binding protein (GNBP) (Kong *et al.,* 2018). Serine proteases are involved in the extra cellular events that activate the phenol oxidase (PO) cascades, leading to wound healing and melanin formation. Serine proteases may also be involved in formationof ligand(s) that bind to cell surface receptors and activate signal transduction pathway(s). Transcription factors of the Rel and STAT family are cytoplasmic proteins that enter the nucleus upon immune challenge, and have been shown to be important regulators of AMP gene activation, and of proliferation and differentiation of hemocytes. The Drosophila GATA factor serpent is required for the differentiation of hemocytes and fat body, as well as for the expression of AMP genes in larval fatbody (Tingvall *et al.,* 2001). Genetic analyses in Drosophila have in addition to the Rel-related pathways, identified a pathway which requires theimmune deficiency (imd) gene. A large number of immune response deficiency (ird) mutants have been isolated, which inter-fere with nuclear translocation of Rel proteins and with the activation of AMP genes.

The Toll pathway :

The activation of the Toll signalling pathway is dependent on a cleaved form of the cystine-knot cytokine like protein Spaetzle. A dimeric form of the mature Spaetzle binds to the ecto domain of two Toll receptors. The intra cytoplasmic domain of Toll then interacts with three proteins including Pelle, which contains a serine-threonine kinase domain. This process results in the activation of either a dorsal-related immunity factor (DIF) in adults or Dorsal and/or DIF in larvae, both factors being closely related to NF-kB proteins. The final result of this signalling is the dissociation of these proteins from the phosphorylated Cactus protein. Dif and/or Dorsal then translocate to the nucleus of immune cells where they activate the target immune genes such as *drosomycin.*

The Imd pathway :

This pathway is predominantly implicated in the regulation of the genes encoding anti Gram-negatives AMPs, including the cecropins, drosocin, diptericin (a glycine-rich AMP) and attacins. The activation of the genes encoding these AMPs involves a NF-kB protein (Relish) that is cleaved into two parts, together with the nuclear translocation of the N-terminal cleaved-domain. Ultimately promoters of the above-mentioned antimicrobial genes are activated in the nucleus. In cases when the sequence of events downstream of Imd was not fully dissected, genetic data suggested the involvement of a series of activation partners including a MAP kinase.

Conclusion :

The innate defense of insects starts with the initiation of two important proteolyticcascades, which then lead to localized blood clotting and melanization at the site of injury. It is then followed by the phagocytosis of microorganisms by specialized cells or by formation of capsules around larger sized parasites by certain blood cells. Concomitantly, in the fat body there is synthesis of a battery of potent and cationic antibacterial and antifungal peptides. These peptides are released into the hemolymph and eventually they kill the invading microorganisms. Recognition of infectious molecules is mediated by proteins that bind preferentially to bacterial or fungal cell wall components such as polysaccharides, peptidoglycan, glucans etc. Recognition can probably also happen due to polyanions and cell debris released at the wound site. These recognition proteins, known as pattern recognition receptors, wait for the structural and functional characterization. Our present understanding is that there is no clonal distribution of these receptors and that the arthropod host defense system lacks any specificity and memory. The gene expression during the immune response shares essential characteristics with that of the acute phase response of vertebrates and it is possible thatmany of the regulatory pathways are similar in between insects and vertebrates. The genetics of many insects provide opportunity to study the development and the differentiation of a immune system and researchers in the field may expect that the study of insect immunity will contribute significantly to our insights on the innate immune response in general.

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