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Biochemistry of Insect Immune System

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Abstract: ***The multicellular organisms have been encountered by a diverse array of pathogens. In response to the foreign invaders, the insects has been reported to develop an immune system which is basically the interaction between the virulence of the pathogen and the defending capacity of the host insects. The immunity system in insects may be divided into basically innate and adaptive type immunity, but in insects only innate immunity is functional and the adaptive immunity is being absent in insects unlike mammals. Furthermore, the innate immunity is divided into cellular and humoral immunity in insects. Cellular immunity is being imparted by various haemocytes such as, plasmatocytes, granulocytes and oenocytoids and the humoral immunity is provided by various Anti Microbial Peptides (AMPs) which are produced by fatbodies. Behavioural immunity includes the avoidance and antiseptic behavior by the host insects towards the pathogens or the products of pathogens. The insect has to overcome a series of barriers before reaching the haemocoel. The cuticle, trachea and midgut act as major site for invasion by the nonself microbes. The cuticle or integument is the outermost layer for taget. It is chemically composed of chitins which are crosslinked with various types of proteins. Integument is the primary target for fungi on which the fungal spore adhere and germinate. After overcoming the morphological external barriers in insects the pathogen has to gain access to haemocel by overcoming the physiological immunity in insects. Physiological immunity basically comprises of the cellular immunity and the humoral immunity. For the activation of physiological immunity in insects, the identification of nonself is the most important. Recognition of nonself occurs by the help of fat body cells, hemocytes, midgut epithelium and cuticular epithelium. Thus different biochemical pathways such as IMD, TOLL, JAK-STAT are activated in response to the nonself invaders. The study if immunity in insects can help in better understanding and effective utilization of entomopathogens for the control of insects.***

Keywords: Immunity, cellular immunity, humoral immunity, haemocytes, behavioural immunity, antimicrobial peptides (AMPs), nonself, entomopathogens

1. Introduction

The multicellular organisms have been encountered by a diverse array of pathogens (Roaff and Reynolds, 2009). Thus the insects have been reported to develop an immune system which is basically the interaction between the virulence of the pathogen and the defending capacity of the host insects (Beckage, 2007). The immunity system in insects is divided into basically innate and adaptive type immunity, but in insects only innate immunity is functional and the adaptive immunity is being absent in insects unlike mammals (Hoffmann, 1995). Furthermore, the innate immunity is divided into cellular and humoral immunity in insects. Cellular immunity is being imparted by plasmatocytes, granulocytes and oenocytoids and the humoral immunity is provided by various Anti Microbial Peptides (AMPs) which are produced by fatbodies (Lavine and Strand, 2002).

2. Behavioural Immunity in Insects

Behavioural immunity includes the avoidance and antiseptic behavior by the host insects towards the pathogens or the products of pathogens (Alma *et al.*, 2010). The insect tries to avoid contact with the pathogen infected surfaces. The omnivorous pirate bug, *Anthocoris nemorum* avoids contact with the plants treated with *Beauveria bassiana (*Meyling and Pell, 2006). Furthermore, the gypsy moth, *Lymantria dispar* can detect the cadaver and foliage infected with NPV (Nuclear Polyhedrosis Virus) occlusion bodies and avoids the contact with it (Parker *et al.*, 2010). Apart from this type of direct avoidance, insects also have a tendency to avoid indirectly i.e. avoiding the toxins produced by the pathogens. The beet army worm, *Spodoptera frugiperda* avoids a diet containing Cry1Ac toxins (Berdegue *et al.*, 1996). Basically the detection of a pathogen or pathogenic entity is depending upon the selection pressure exerted by the pathogen or the pathogenic entities (Thompson *et al.*, 2007).

Apart from avoidance behavior, the insects also exhibit the antiseptic behavior. Grooming is the simplest type of antiseptic behavior observed till date (Evans *et al.*, 2006). In grooming, insects remove the pathogens from the external body surfaces. Some of the insects have been reported to replace the physiological immunity with the social immunity. Social immunity mostly observed in social insects. Grooming is considered as a form of social immunity, which can be further divided into autogrooming and allogrooming in which the insect may groom itself or the nestmates respectively (Cremer *et al.*, 2007). Hygienic behavior is another type of social immunity. The dead and diseased counterparts are removed by hygienic bees when the bees are infected by American foul brood and the chalk brood disease caused by *Paenibacillus larvae* and *Ascophaera apis* respectively (Evans and Spivak, 2010). The detection of infected larva is carried out by presence of chemical phenethyl acetate (Swanson *et al.*, 2009). Sometimes the healthy larva having traces of the chemical are also misidentified and discarded out of the hive by the hygienic workers. Another form of social immunity is necrophagy (Evans and Spivak, 2010), in which the healthy ants separate the infected counterparts in the isolated chamber of the nest and maintain physical isolation. Coating of antibiotic substances is also the extreme form of immunity reported till date (Ruepell *et al.*, 2010). Honey bees coat the bee hive with propolis which is having antimicrobial property.

Morphological Barriers to Infection

The insect has to overcome a series of barriers before reaching the haemocoel. The cuticle, trachea and midgut act as major site for invasion by the nonself microbes

(Chapman, 1998). The cuticle or integument is the outermost layer for taget. It is chemically composed of chitin which is crosslinked with various types of proteins. Integument is the primary target for fungi on which the fungal spores adhere and germinate (Moussain, 2010). Furthermore, some antimicrobial glandular secretions of the cuticle are also helpful in imparting immunity to insects. The ants of Formicidae family produce some antibiotic secretions from the thoracic metapleural glands (Poulsen *et al.*, 2003). Many of the pathogens such as bacteria, viruses, protozoans can not get access to the haemocel directly through cuticle rather they enter per os i.e. through mouth. For the survival of nonself microbes inside insect body some factors are crucial such as, pH of the gut, chemical composition and resident microbial composition (Haider *et al.*, 1996). If the microbes find the gut environment suitable, then the peritrophic membrane is the next barrier followed by the cellular epidermal layer and an acellular basement membrane (Lehane, 1997). The midgut is devoid of cuticle, thus acting as the major site of target for the pathogen attack. Apart from this, the tracheal system, is also acting as the site of invasion by the pathogen in a manner similar to midgut.

Physiological Immunity in Insects

After overcoming the morphological external barriers in insects the pathogen has to gain access to haemocel by overcoming the physiological immunity in insects. Physiological immunity basically comprises of the cellular immunity and the humoral immunity (Hoffmann, 1995; Strand, 2008). Cellular immunity is the involvement of plasmatocytes, granulocytes and oenocytoids for the phagocytosis and encapsulation processes, whereas the humoral immunity involves the identification of nonself through the PAMPs (Pathogen Associated Molecular Pattern) by the PRR (Pattern Recognition Receptors) present in insects (Hoffmann, 2003). Most of the studies on physiological immune response is conducted on *Drosophila melanogaster* and *Anopheles gambiae* due to wider availability of genetic tools and complete information of genomic sequences in these two species (Kanost and Nardi, 2010). In insect defence the identification of altered-self and nonself from self is the most crucial step.

Identification of Altered Self and Nonself from Self

Recognition of nonself occurs by the help of fat body cells, hemocytes, midgut epithelium and cuticular epithelium. Insects use the basement membrane as an identification tool for the nonself and a differentiating agent from the self (Royet, 2004). When the insect's body cells identify the invading microorganisms, the epithelial cells in the body secrete various PRPs (Pattern Recognition Proteins). PRPs are of two types i.e. some may be released into the hemolymph whereas; others are attached to the cells that produce them. Major functions (s) of the PRPs are identification of the carbohydrate or carbohydrate- peptide linkages present in the microbial cell wall (Brennan and Anderson, 2004). The patterns that are typically associated with the microbial structures are known as PAMPs (Pathogen Associated Molecular Pattern). Thus the PAMPs identified in fungi are beta- 1, 3- glucans and lipopolysaccharides and peptidoglycans in the bacterial cell wall (Lavine and Strand, 2002). When the PRRs mark the PAMPs these are marked for destruction by the haemocytes in the open circulatory system of the invading insects. Some of the C type lectin, immunolectin, Tep protein also acts as identifying tool for the nonself recognition. C type lectin donot recognise the proteins rather they bind to the carbohydrates (Govind, 2008). Sometimes haemocytes can also function as PRRs when they get themselves attached to the foreign targets. Some granulocytes sometimes produce PRRs i.e. lacunin and haemocytin (Lavine and Strand, 2002).

Cellular Immunity in Insects

The insect hemolymph contains various mesodermal, amoeboid and nucleated cells are called as haemocytes. Arnold (1974) has classified the hemocytes into following 9 categories such as prohemocytes, plasmatocytes, granulocytes, adipohaemocytes, coagulocytes, spherule cells, oenocytoids, podocytes and nematocytes (Sonawane and More, 1993). Prohaemocytes are the progenitor of other types of haemocytes, with larger nuclei, granular basophilic cytoplasm whereas; plasmatocytes are polymorphic with large nuclei and exhibiting phagocytic behavior (Carton *et al.*, 2008). Out of the forementioned haemocytes, only plasmatocytes and granulocytes are useful for imparting cellular immunity in insects by involving in phagocytosis, encapsulation and nodule formation. Granular haemocytes are compact cells containing oval or spherical shaped small nuclei and large cytoplasm with acidophilic granules (Strand *et al*., 1992). These are non motile, amoeboid but are of wide occurance. The haemocytes can be differentiated into primary haemocytes and secondary haemocytes. The primary haemocytes are the prohaemocytes which give rise to other forms of haemocytes. The plasmatocytes are the major players in the phagocytic processes (Figueiredo *et al*., 2006).

Phagocytosis

In phagocytosis, the plasmatocytes have acidophilic pH and phosphatase activity, the randomic contact of phagocytes (plasmatocytes and granulocyes) with the foreign bodies in the haemolymph, lead to extension of pseudopodia (Browne *et al.*, 2013). The foreign objects contain the receptor molecules which bind to the appropriate binding site of the haemocyte surface leading to the adhesion of the foreign particles in the haemolymph (Rosales, 2005). Apart from, plasmatocytes, some of the adipohaemocytes are also useful in the phagocytic processes (Castillo *et al.*, 2006). The phagocytosis is carried out by the formation of pinocytic vesicles, or by engulfing the foreign bodies with pseudopodia and finally the autolysed cells are removed (Lamprou *et al*., 2007). The phagocytic process involves chemotaxis, activation of receptors on the phagocytic plasma membrane, attachment of the micro-organism to the membrane receptors (Franc *et al*., 1996), engulfment by extension of the phagocytes (pseudopodia), formation of the phagosome followed by the final killing and digestion of the foreign invaders (Manaka et al., 2004; Vlisidou *et al.*, 2009; Kocks *et al.*, 2005).

Encapsulation

The encapsulation is a process of defense against large metazoan parasites. It is carried out mainly by plasmatocytes but apart from the plasmatocytes, lamellocytes, oenocytoids and spherule cells also helps in the process of encapsulation

(Ling and Yu, 2009). Aggregation of large number of haemocytes occure around the foreign body to form a rigid capsule. Finally the rigid capsule is being cemented by intracellular substances such as mucopolysaccharides and the melanization occurs through the help of oenocytoids (Carton *et al.*, 2009). Due to complete encapsulation, the foreign body is deprived of oxygen and the death occurs inside the capsule (Rosales, 2007). The cellular envelope in encapsulation is composed of 20-40 layers of extremely flattened haemocytes, which are attached freely to one another with the help of microtubules and desmosomes (Zhuang *et al.*, 2008). The intracellular cells between the cells are being filled with the electron dense material. When the invaders are too big to be phagocytised or to form nodules they are encapsulated. The encapsulation process involves detection and recognition of the microbial inoculums by the plasmatocytes, proliferation and activation of lamellocytes, formation of capsule and the final step I.e. production of ROS (Reactive Oxygen Species) and toxic intermediates of melanin cycle (Irving *et al.*, 2005).

Nodule Formation

Larger objects such as eggs of some parasitoids are enclosed in a nodule formed by the aggregation of haemocytes usually followed by melanization. Melanization involves the action of phenoloxidase (PO) on phenolic compounds (Tyrosine and Dihydroxyphenylalanine) to produce quinines that ultimately autopolymerise to form melanin (Fujimoto *et al.*, 1993; Chase *et al.*, 2000). Activation of the inactive form of phenoloxidase is the prime mode of defense against the bacterial and fungal invasion (Cerenius and Soerhall, 2004). An inactive form of phenoloxidase i.e. prophnoloxidase is activated by an array of serine proteases upon immune challenge.

Coagulation

It is another form of immunity or defense in insects. It is of 3 types, in Type-I the immediate rupture of hyaline haemocytes occure followed by formation of coagulation islands (Gregoire, 1951). This is the predominant type of coagulation in Hemiptera, some Coleoptera and Hymenoptera, some Homoptera, Neuroptera, Mecoptera and Trichoptera. In Type –II coagulation, the coagulation islands are absent, and instead of this pseudopodial meshworks develop, this meshwork gradually expands and traps other types of haemocytes. This type is found in Carabidae, Scarabidae and Odonata. In Type-III, there is a combination of Tyoe –I and Type –II, and found in Homoptera, many Coleoptera and Hymenoptera

The Inducible Humoral Response

The humoral immune response mainly targets at the production of Anti Microbial Peptides (AMPs). Upon microbial invasion, a series of small peptides, the AMPs are produced mainly by the fatbody cells and released into the hemolymph. The first identified antimicrobial protein of insects was the lysozyme from *Galleria mellonella*. AMPs are rapidly synthesized during systemic infection, and paused immediately as the invading microbes are destroyed. The AMPs may be antibacterial, antiviral or antibacterial. Cataionic AMPs bind to anaionic bacterial surfaces thus induce a series of processes, i.e. break down bacterial integrity by creating holes and finally the bacterial contents leak out. Another mode of action of AMPs includes depolarization of the bacterial membrane (Wasterhoff *et al.*, 1989) and induction of hydrolase enzyme that degrade bacterial cell wall (Bierbaum and Stahl, 1987). According to the Shai –matzuaki-Huang model (Matzuaki *et al.*, 1999, Yang *et al.*, 2000) the interaction of AMPs with bacterial membrane leads to displacement of lipid membrane and alteration of membrane structure. Structurally the AMPs can be classified into 3 types. The first groups of molecules have intramolecular disulphide bonds that form hairpin beta sheets; second group has peptides that form amphipathic alpha helices, followed by the third group which has disproportionate amount of proline and/or glycine residues. For example cercopin are group of peptides which are confined to the cell membranes whereas, lysozymes are omnipresent throughout the insect tissues.

Antimicrobial Peptides

Through biochemical analysis, seven groups of AMPs have been discovered in the hemolymph of the fruit-fly *D. melanogaster* and other Diptera. The present group of AMPs can be divided again into 3 families based on the biological target of microorganisms for example, against Gram-positive bacteria, there are defensins. Against Gram-negative bacteria, there are cecropins, drosocin, attacins, and diptericin. Against fungi, there are drosomycin and metchnikowin. Defensin were chemically characterized and observed to have three to four stabilizing intramolecular disulphide bonds (Bulet and Stocklin, 2005). Insect defensins can be categorized into 2 types such as one with peptides presenting α-helix/β-sheet and the other type is having triple stranded antiparallel β-sheets. Defensins posses antifungal and antibacterial activity in insects (Steiner *et al.*, 1981).

Cecropins are another indueable group of AMPs having a number of small basic peptides of about 31–37 amino acid residues with an amphipathic α-helix conformation (Tanaka *et al.*, 2008). It was the first amphipathic AMP isolated from the hemolymph of the silkworm *Hyalophora cecropia* and was named cecropin. The mode of action of cecropin includes the damage to pathogen cell membranes by inhibition of proline uptake (Dai *et al.*, 2008). Moricins are also another group of amphipathic α-helical AMPs found first in the silkworm *B. mori*. Apart from Bombyx mori, in *G. mellonella* also eight moricin homologs are reported to have activity against bacterial as well as against yeast and filamentous fungi. Drosocin is a cationic antimicrobial peptide from *D. melanogaster* having a threonine residue (Gobbo *et al.*, 2002). Attacins are glycine-rich 20 kDa AMPs which were originally isolated from the hemolymph of *H. cecropia (*Engstom *et al.*, 1984). Two attacin isoforms, one acid and one basic, have been cloned from *H. cecropia* and they induce an increase of permeability of the outer-membrane of bacteria, binding mainly to lipopolysaccharide (LPS) (Bang *et al.*, 2002). Thus the basic attacin is more effective than the acidic attacin against E. coli. Apartfrom this attacin also acts as an inhibitor of the protein synthesis in the outer membrane of the bacteria (Hwang and Kim, 2011). Furthermore, the gloverins are also observed with antifungal and antiviral activities. Diptericin is an AMP which is rich in glycine in response to bacterial injury (Imler and Bulet, 2005). It is having molecular weight

of 8.6 kDa. It functions by the disruption of cytoplasmic membrane. Diptericin has also been reported to be involved in the protection from oxidative stress (Zhao *et al.*, 2011). It has been involved in antioxidant enzyme activities in insects like Drosophila melanogaster. Drosomycin is observed to have antifungal activities but the antibacterial activity is observed to be absent. Drosomycin belongs to the cysteine-stabilized α-helical and β-sheet (CSαβ) superfamily and is composed of an α-helix and a three-stranded β-sheet stabilized by four disulphide bridges (Gao and Zhu, 2016). Drosomycin is only active against some filamentous fungi (Fehlbaum *et al.*, 1994). Metchnikowin is 26 residue proline rich peptide which is expressed in D. melanogaster in response to infection (Levashina *et al*., 1995). It is effective both against fungal and bacterial infection (Rahnamaeian *et al.*, 2009). Transgenic barley plant has been created by expressing the metchnikowin gene which was observed resistant to several ascomycetes fungi which include Fusarium head blight and powdery mildew.

Through various signaling pathways, the forementioned AMPs are produced after recognition of the PAMPs by the PRRs. Thus in insects, the signaling pathways involved in humoral immune responses have been best described in *D. melanogaster*. The humoral immune responses mainly involve the release of AMPs by the fat-body upon identification of the nonself, via the Toll (Vallane *et al*., 2011; Lindsay and Wasserman, 2013), the immune deficiency (Imd) (Kleino and Silverman, 2014; Myllymaki *et al.*, 2014), and the JAK-STAT (Myllymaki and Ramet, 2014) pathways. Gram-positive bacteria and fungi predominantly induce the Toll signaling pathway, whereas Gram-negative bacteria activate the Imd pathway. The major mechanism of antiviral defense is the RNA interference (RNAi) pathway that recognizes virus-derived double-stranded RNA (dsRNA) to produce small, interfering RNAs (siRNAs). Furthermore, in addition to providing antibacterial immunity, the Toll and Imd signaling pathways have been also reported to be involved in antivirus responses. Apart from producing AMPs, these pathways have been reported to induce the production of particular sets of genes that are distinct from the genes induced by bacteria or fungi, based on the virus involved. Another well studied mechanism is autophagy, which was reported to be independent of the Toll, Imd, or JAK-STAT pathways (Nakamoto et al., 2012). Autophagy may be defined as the process, by which double-membrane vesicles named autophagosomes are formed inside cells (Shelly et al., 2009). These vesicles are formed with newly synthesized membranes that incorporate large cytoplasmic components including damaged organelles or protein aggregates. Then, the autophagosome fuses with lysosomes and degrades its content (Kuma and Mizushima, 2010).

3. Conclusion

The insects, being multicellular organisms are the target of various microbial pathogens, thus upon microbial challenge the insect defense mechanism gets activated. The typical immune system of insects comprises of identification of nonself which is followed by various cellular defensive activities such as phagocytosis, encapsulation, nodule formation and coagulation. The cuticle being the first line of defense, when surpassed by the foreign invader, the humoral defense system gets activated. Typical humoral defense system includes the production of various AMPs upon immune challenge. Thus the elaborative study on immune system can be utilized for the control of insects by down regulating the immune related genes and the manipulation of the various immune pathways. This approach can be better utilized in an ecofriendly manner to overcome the resistance, residue and replacement related problems. Furthermore, this study can help in better understanding of the effective utilization of entomopathogens by overcoming the immunity of targeted insects.

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