Phenoloxidase cascade

Apart from innate, the adaptive immune system in invertebrates specially in insects can be categorised into humoral and cellular branch. Humoral system includes antimicrobial peptides, phenoloxidase and melanisation, reactive oxygen species, hemolymph clotting. Whereas cellular immunity covers phagocytosis, nodulation, encapsulation.

Phenoloxidase cascade is under Phenoloxidase system. It is a major defense system in many invertebrates which includes under humoral immune response. It ultimately leads to melanization of pathogens and damaged tissues. The process of melanization depends on activation of the enzyme phenoloxidase (PO) which is controlled by the prophenoloxidase (proPO) activation system (Amparyup et al., 2013).

During activation of proPO cascade, many other immune reaction are produced, such as cytotoxic, opsonic and encapsulation activities. The phenoxidase cascade plays an important role in invertebrates (especially insects). It involves in three physiologically important processes: immune reactions, sclerotization of the cuticle and wound healing (Sugumaran et al., 2000).

Prophenolxidase (proPO) activation system:

The phenoxidase system begins with the recognition of microbial PAMPs (pathogen-associated molecular patterns) including LPS (gram-negative bacteria), peptidoglycans (gram-positive bacteria) and β-1,3-glucans (fungi). Interaction of PAMPs with PRPs (pattern-recognition proteins) activates a series of serine proteinases and those proteolytically cleave the prophenoloxidase (proPO) zymogen and activate phenoxidase (PO).

During the proPO system activation, reactive intermediates such as quinone-like intermediates, reactive oxygen (ROI) or nitrogen intermediates are produced. These have cytotoxic activity against microorganisms, prevent organism from entering of another pathogen and also assist in wound healing.

Melanization process:

Active PO plays an important role in the initial stages of the melanization process. The PO catalyses a hydroxylation of monophenols (tyrosine) on diphenols and oxidation of diphenols to dichinones. The chinones non-enzymatically change their structure to dopachrome and then dopachrome isomerase changes dopachrome to indole. In the final phase of the process melanin is made by an oxidation and a polymerization. Melanin is rapidly deposited around the pathogen, thereby limiting its ability to damage the host organism. Storage of melanin at the site of damage also prevents further loss of hemolymph.

Long-term or overproduce of substances arising during a PO cascade can lead to tissue damage and cell death at the site of the reaction. For this reason, these reactions are strictly act in regulated manner.

In short, Melanization includes four steps: (1) the recognition of pathogens through specific molecules [e.g., lipopolysacharides (LPSs), LGRP, PGRP]; (2) the recruitment of hemocytes to surround the pathogen by chemoattractant proteins (e.g., plasmatocyte spreading peptide, PSP); (3) melanogenesis within hemocytes; (4) melanin release and pathogen encapsulation.

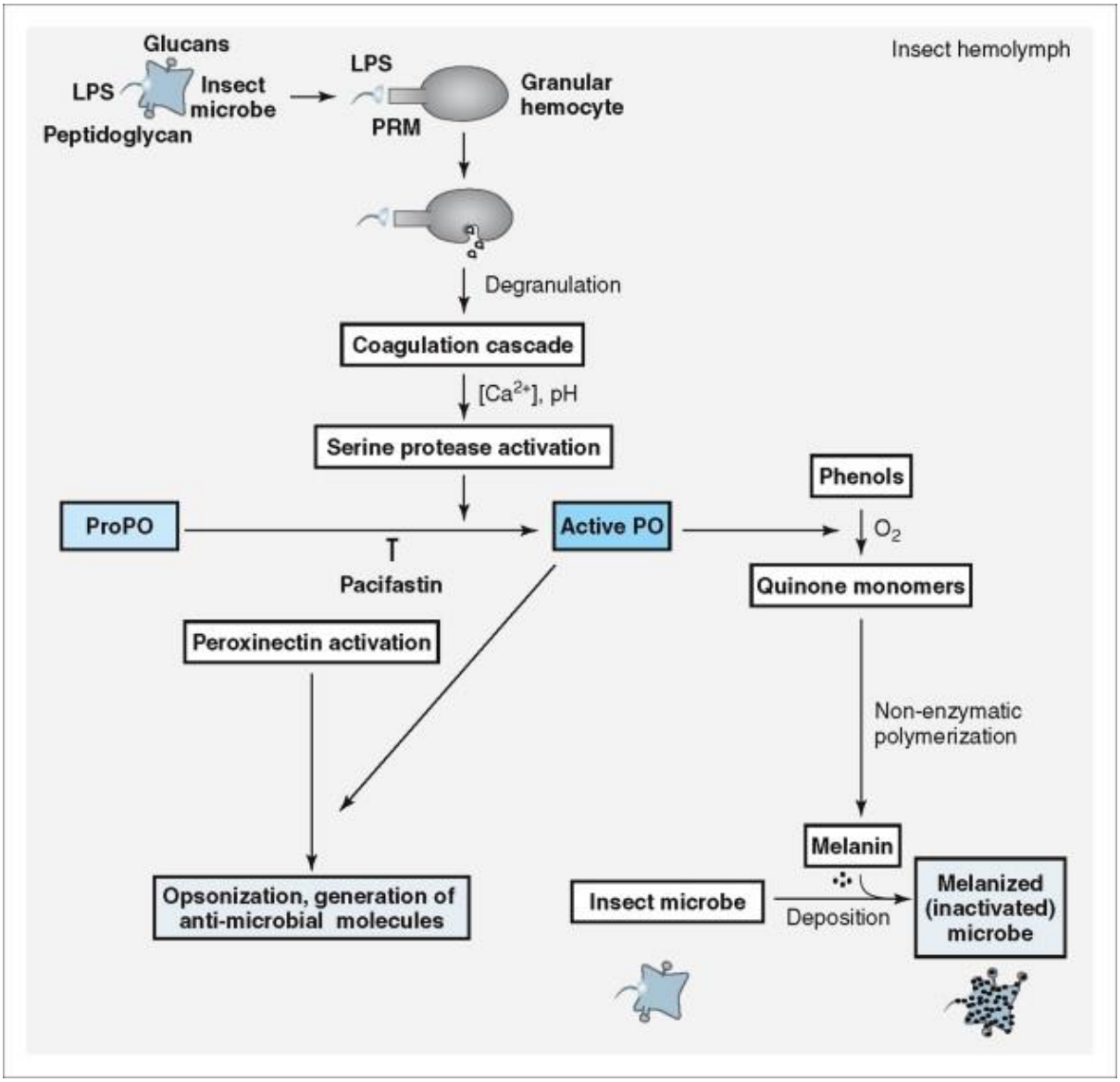

Fig.1: The flow chart showing the Phenoloxidase cascade in insects

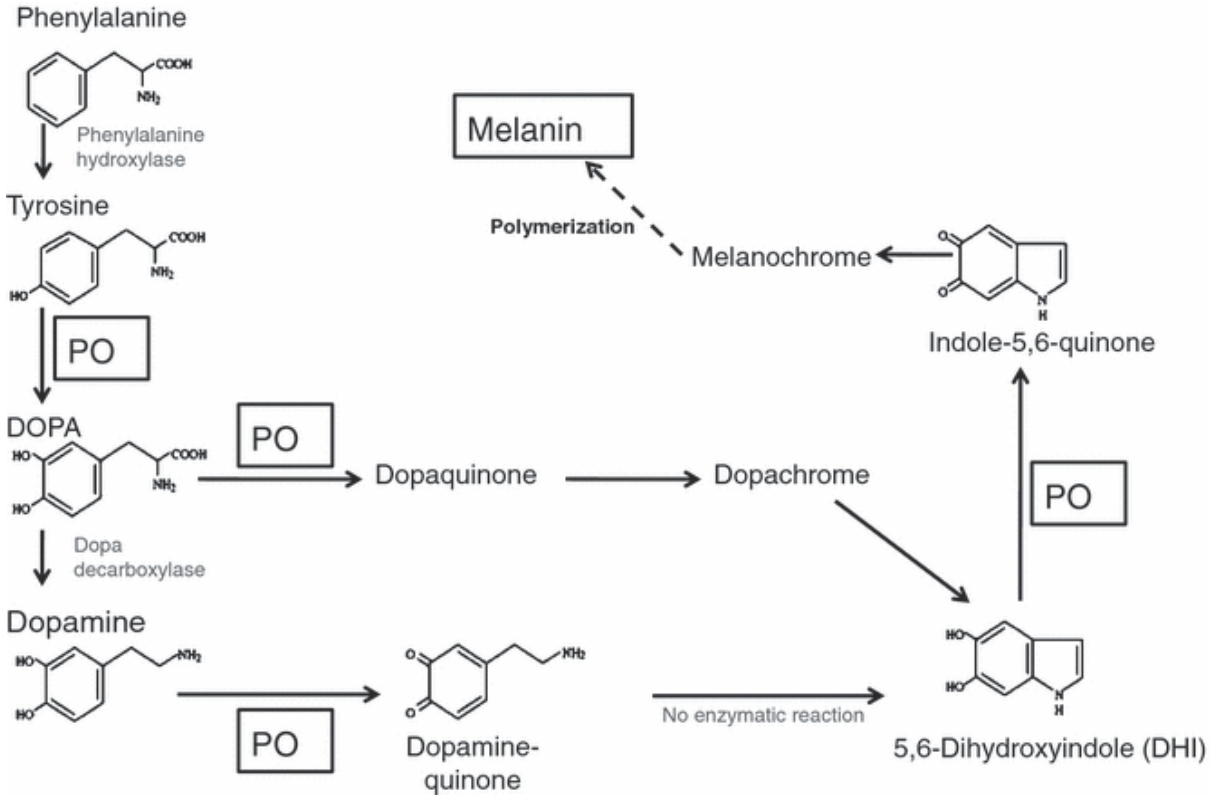

Fig. 2: Phenoloxidase role in melanin synthesis. Phenoloxidase participates in the formation of DOPA from Tyrosine (Tyr). PO also convert DOPA to dopaquinone, and 5,6-dihydroxyindole (DHI) to indole-5,6-quinone. Note the alternative way to obtain DHI from dopamine, in which PO is also involved.

The role of the PO activating system in invertebrate immunity in detail:

ProPO and PO, structures and types:

Phenoloxidase is a member of the tyrosinase group, whose main function is to oxidize phenols. Tyrosinase proteins from different species are diverse in terms of their structural properties, distribution, and cellular location. It has even been suggested that there is no one common tyrosinase protein structure (Mayer, 2006). All tyrosinases, however, have in common a binuclear type 3 copper center within their active site. This copper center is surrounded by three histidine residues (Jaenicke & Decker, 2003). Substrate binding in PO occurs at a site containing a series of hydrophobic amino acids with benzene rings (Nappi & Christensen, 2005). The copper center reacts with dioxygen (O2) and a highly chemically reactive intermediate is obtained by oxidizing the substrate. Phenoloxidases are expressed as inactive zymogens (proPOs) in all insects and are converted to active PO when required. ProPOs are polypeptides that contain two copper atoms per protein molecule, with a total weight of 50–60 and 70–80 kDa in their active and inac

tive forms, respectively (Ashida & Brey, 1997). In arthropods, the proPO amino acid sequences are homologous with hemocyanins (an oxygen-transport protein in invertebrates) and hexamerins (storage proteins). ProPO and hemocyanin contain two well-conserved copper atoms that form an oxygen-binding site, whereas hexamerin lacks copper atoms and therefore does not recruit oxygen. Recent studies support the idea that POs are the ancestral enzyme, that

hemocyanins evolved secondarily, and finally that hexamerins diverged from hemocyanins (Decker & Terwilliger, 2000; Burmester, 2001; Burmester, 2002).

Gene expression of proPO:

Researchers have found two or more proPO genes in most insect species, although there are a few cases, such as the honeybee, with only one proPO gene (Evans et al., 2006). ProPOs are sometimes highly conserved. For example, Drosophila melanogaster Meigen and Tribolium castaneum (Herbst) share two of three proPO genes (Waterhouse et al., 2007). In other groups, there has been a radiation of the proPO gene family – for example, mosquitoes have nine proPO genes in Anopheles gambiae Giles and 10 in Aedes aegypti (L.) (Waterhouse et al., 2007). It is not clear why PO is highly conserved in some clades and diversified in others. One possibility is that a higher number of pathogen species may lead to increased specialization, via a higher gene number in host species (Waterhouse et al., 2007). A second possibility is that a coevolutionary arms race between hosts and pathogens may lead the former to evolve specific regulator mechanisms. In mosquitoes, for example, the same genes that are involved in the melanization process against Plasmodium berghei Vincke and Lips, do not offer any protection against Plasmodium falciparum Welch (Cohuet et al., 2006; Michel et al., 2006). One last explanation is that there is differential expression of proPO in relation to distinct insect life-history stages, so that different genes are involved in the same process, but at different ontogenetic times (Li et al., 2005). Thus, if life history stages are different between host species, different gene numbers may appear.

The site of PO synthesis :

Synthesis of proPO occurs mostly in hemocytes (Figure 4; Cerenius & So¨derha¨ll, 2004) with species-specific variation in relation to hemocyte type. In lepidopterans, a type of hemocyte, known as oenocytoids, functions as the site of proPO synthesis (Jiang et al., 1997). In D. melanogaster, crystal cells, another class of hemocytes present in larval stage, provide a site for proPO synthesis (Rizki et al., 1985; Williams, 2007). In mosquitoes, proPO is synthesized in granular hemocytes and oenocytoids (Hillyer et al., 2003; Castillo et al., 2006). The fat body, however, is definitively not a place where proPO synthesis takes place.

ProPO secretion:

Arthropod proPOs lack a peptide signal for their secretion; therefore the presence of proPO in hemolymph probably results from hemocyte lysis (Kanost & Gorman, 2008). The hemocytes in which proPO synthesis takes place (oenocytoids, crystal cells, and granular hemocytes) vary in robustness. Some sites are extremely fragile, and hemocyte lysis occurs immediately after an injury or infection. In these sites, secretion can occur at a low rate even in the absence of injury or infection, perhaps to maintain standard levels of proPO in hemolymph. As a result, it is difficult to evaluate baseline levels of proPO in insects that have fragile oenocytoids (Ashida & Brey, 1997). In lepidopterans, proPO mostly appears in hemolymph. In other groups such as locusts and cockroaches, proPO appears mainly stored in hemocytes until a pathogen induces its release (Brehe´lin et al., 1989; Durrant et al., 1993).

ProPO-activating system in invertebrates:

ProPO can be activated by several factors. In the laboratory, activation of proPO in hemolymph can occur via detergents, fatty acids, or alcohols. Researchers have activated proPO purified from D. melanogaster by 2-propanol (Asada, 1998). ProPO from Sarcophaga bullata Parker may be activated by the cationic detergent cetyl pyridinium chloride (Xie et al., 2007). In nature, insect proPOs can be activated by amphiphilic lipids such as lysolecithin and perhaps by the presence of damaged cells (Sugumaran & Nellaiappan, 1991). Other natural proPO activators are b-1,3-glucans, lipopolysaccharides, and peptidoglycans. Natural proPO activators have been identified in several insect pathogens such as gram+ bacteria, gram) bacteria, and fungi (Cerenius & So¨derha¨ll, 2004). All of these activation agents could trigger a conformational change in the protein, resulting in an accessible binding site for the substrate (Kanost & Gorman, 2008). Experiments with proPO purified from silkworm identified the presence of a serine protease cascade as an intermediate step between natural proPO activators and the production of active proPO (Ashida et al., 1974; Ashida & Dohke, 1980). Serine proteases are hydrolases with a serine amino acid in their active center (Hedstrom, 2002). Hydrolases include trypsin, chymotrypsin, and subtilisin. Serine proteases cut the polypeptide chain in the carboxyl side of specific amino acids, resulting in peptide bond degradation (Hedstrom, 2002). Studies in the laboratory have generally used chymotrypsin as a proPO activator. This activator has become a useful experimental tool to assay both PO in the hemolymph and total PO in the whole insect (Sugumaran et al., 1985; Saul & Sugumaran, 1987; Adamo, 2004). The first proPO activating serine protease (PAP), also known as prophenoloxidase-activating enzyme (PPAE), to be purified was extracted from cuticle (Aso et al., 1985), and perhaps has a role in proPO activation upon wounding (Kanost & Gorman, 2008). In addition, PAPs have been purified in hemolymph from Manduca sexta L. (Jiang et al., 1998, 2003), Bombyx mori L. (Satoh et al., 1999), Holotrichia diomphalia Bates (Lee et al., 1998), and crayfish Pacifastacus leniusculus (Dana) (Wang et al., 2001). Three types of PAPs have been identified, PAP-1, PAP-2, and PAP-3 (Figure 4). ProPO activating serine proteases can be found before and during an infection depending on the animal and the PAP type. For example, PAP from B. mori is expressed in integument, hemocytes, and salivary glands, but not in the fat body (Satoh et al., 1999). Its putative M. sexta ortholog, PAP-1, is expressed in the larval fat body, tracheae, and nerve tissue, and is up-regulated in the fat body and hemocytes after injection of bacteria (Zou et al., 2005). ProPO activating serine protease-2 expression was detected in the fat body and hemocytes only after M. sexta larvae were injected with bacteria (Jiang et al., 2003). ProPO activating serine protease-3 mRNA is present at a low level in the fat body and hemocytes of nai¨ve M. sexta larvae, and its expression is significantly upregulated in those tissues after injection of bacteria (Jiang et al., 2003). At the prepupal stage, it is highly expressed in integument, the fat body, and hemocytes (Zou & Jiang, 2005). Finally, in some cases, PAPs require the presence of protein cofactors or serine protease homologs (SPH) for their activation (Figure 4; Jiang et al., 1998; Kwon et al., 2000). These SPHs have been found in genomes such as D. melanogaster, A. aegypti, A. gambiae, and Apis mellifera L. (reviewed in Zou et al., 2006; Waterhouse et al., 2007; Kanost & Gorman, 2008).

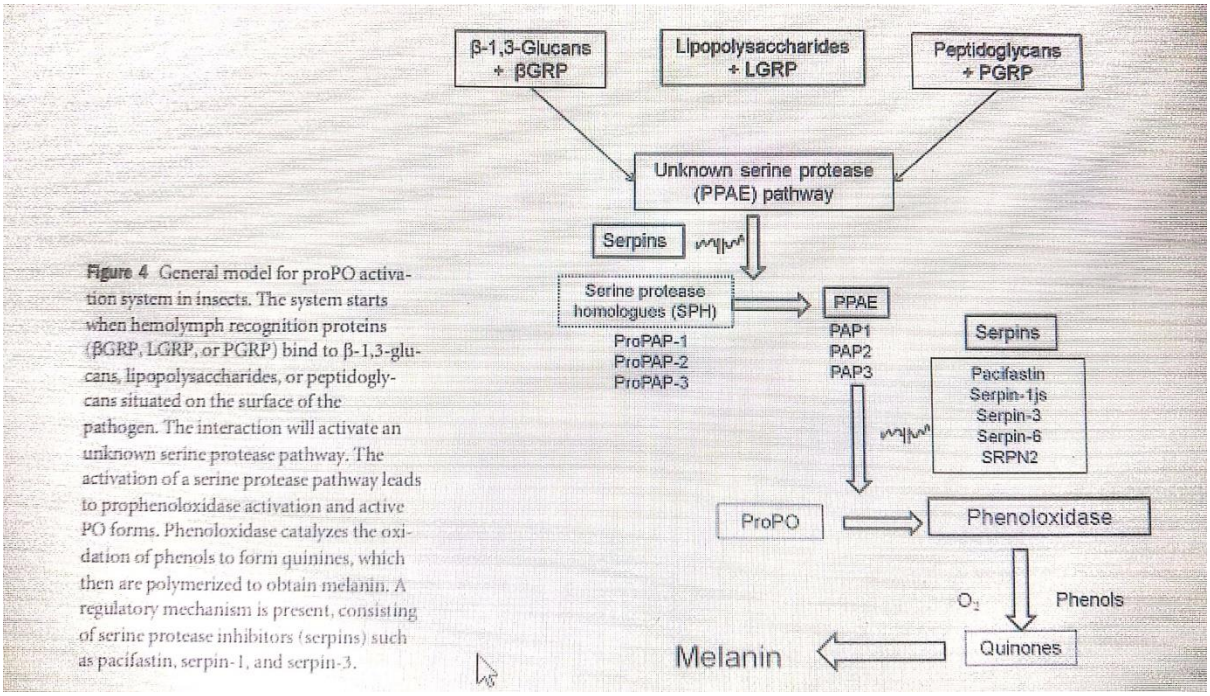

Figure 4 General model for proPO activation system in insects. The system starts when hemolymph recognition proteins (βGRP, LGRP, or PGRP) bind to β-1,3-glucans, lipopolysaccharides, or peptidoglycans situated on the surface of the pathogen. The interaction will activate an unknown serine protease pathway. The activation of a serine protease pathway leads to prophenoloxidase activation and active PO forms. Phenoloxidase catalyzes the oxidation of phenols to form quinines, which then are polymerized to obtain melanin. A regulatory mechanism is present, consisting of serine protease inhibitors (serpins) such as pacifastin, serpin-1, and serpin-3.

PO activity in insect hemolymph:

Mammalian tyrosinases and insect POs catalyze three types of reactions in the melanogenesis process: (1) hydroxylation of a monophenol, (2) oxidation of an o-diphenol and (3) dehydrogenation of a dihydroxyindole (Korner & Pawelek, 1982). However, insect POs and mammalian tyrosinases have different amino acids sequences in their protein structures (van Holde et al., 2001). The insect ability to produce multiple enzymes with similar functions was a major challenge for PO activity studies. For example, laccase can directly oxidize both o- and p-diphenols, but not monophenols (Solomon et al., 1996). Similarly, tyrosine hydroxylase can hydroxylate monophenols, but does not oxidize diphenols. Another enzyme produced by insects, peroxidase, can oxidize both monophenols and diphenols (Nappi & Vass, 1993; Okun 1996; Vie et al., 1999). Later studies have shown that these enzymes have different substrate specificity. In addition, several chemical inhibitors can be used to isolate the enzyme activity specifically (Barrett, 1991). Although insect POs can potentially oxidize monophenol groups, there are only a few isolated POs from S. bullata and M. sexta documented with this function (Chase et al., 2000).

Phenoloxidase substrate:

There are two classes of compounds that have been documented as substrates for insect POs: (1) monophenols, such as tyrosine or tyramine and (2) catechol substrates, including 1,2-dihydroxybenzene (catechol), 4-methyl catechol, DOPA, dopamine, N-acetyldopamine (NADA), N-ß-alanyldopamine (NBAD), N-acetylnonepinephrine (NBANE), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine, 3,4-dihydroxymandelic acid, and 3,4-dihydroxybenzoic acid (Chase et al., 2000). Of these catechols, DOPA has been most frequently used to assay PO activity experimentally. Based on solubility and oxidative properties, dopamine, NADA, or NBAD could be the natural substrates for PO (Sugumaran, 2002). Nonetheless, tyrosine and

most of the diphenols mentioned above have been detected in the hemolymph of one or more insect species, but typically in their non-substrate form. These compounds are stored in hemolymph until needed (Zhao et al., 1995).

Regulatory mechanisms of PO activity:

Phenoloxidases are expressed as inactive zymogens (proPOs) in all insects and are converted to active PO by PPAEs. Nevertheless, PO also produces several molecules such as proteases that could degrade host proteins, cytotoxic quinines, reactive oxygen and nitrogen intermediates. These molecules could harm the host if produced in excess. Therefore, the presence of regulatory mechanisms of PO activity is necessary to produce an optimal response localized to a specific place and for a specific duration (Cerenius & So¨derha¨ll, 2004; Nappi & Christensen, 2005; Kanost & Gorman, 2008). In insect hemolymph, several serine protease inhibitors have been identified that inhibit PO activation (Kanost & Jiang, 1996; Kanost, 1999). One such serine protease inhibitor is pacifastin, which may be obtained from the crayfish P. leniusculus Dana (Hergenhahn et al., 1987; Liang et al., 1997). Other groups of serin proteases inhibitors are serpins (Silverman et al., 2001; Gettins, 2002). Several types of serpins (serpin-1j, serpin-3, serpin-6) have been indentified in M. sexta (Jiang et al., 2003; Kanost, 2007). A serpin-3 ortholog has been found in Hyphantria cunea Drury (Park et al., 2000). Studies in A. gambiae have shown that decreased expression of a serpin-3 ortholog (SRPN2) results in the formation of melanotic pseudotumors and increases lysis of ookynetes. It is likely that all of these effects are caused by excessive synthesis of melanin (Michel et al., 2005). Studies in Musca domestica L. also found a 4 kDa peptide as an efficient PO inhibitor. This peptipe directly inhibits the PO activity rather than PPAEs (Tsukamoto et al., 1992). Finally, studies in M. sexta showed that dopachrome isomerase is also a PO regulator. Because dopachrome isomerase is activated by the insect's own presence of PO, this leads to down-regulation (Sugumaran et al., 2000).