INITIATION AND REGULATION OF PROPHENOLOXIDASE ACTIVATION IN TOBACCO HORNWORM, *MANDUCA SEXTA*

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CHAPTER I. INTRODUCTION

Among over 10 million animal species on the earth, insects represent the largest and most diverse group (Dimarcq, 2002). Insects have existed for about 500 million years and, during the evolutionary journey, they have developed a sensitive and effective immune system to recognize and defend themselves against attacks by bacteria, fungi, viruses and parasites. Insects are vectors of a variety of pathogens (e.g., viruses, bacteria, fungi, protozoa, and metazoan parasites), which cause millions of human deaths every year. For instance, *Anopheline* mosquitoes transmit protozoa *Plasmodium* that cause malaria. More than 600 million people in the world, most of which, children in Africa, are facing the threat of death from malaria resulting in about 3000 deaths of children each day (http://www.who.int/mediacentre/events/2006/g8summit/malaria/en/). In order to survive and colonize their hosts, pathogens have to overcome the host insect immune system. Therefore, investigations of insect immune systems will provide information on pathogen-insect interactions and new approaches to block pathogen transmission.

Parasitic wasps (Hymenoptera) lay their eggs in the body cavity of their host insects, typically Lepidoptera. In order to develop successfully, they need to suppress or evade the host defense reactions. Consequently, the parasite-host interaction provides a unique opportunity to understand how parasites manipulate their host immune system and, in other cases, how the host insect immune system recognizes parasites and blocks parasite development (Schmidt et al., 2001).

CHAPTER II. REVIEW OF LITERATURE

Overview of insect immune system

To cope with pathogenninfection, insects have developed several structural barriers and a complex immune system. The first line of defense against pathogens is structural barriers including the hardened outer exoskeleton (Söderhäll and Cerenius, 1998; Theopold et al., 2002), the epithelial barriers (Lehane et al., 1997; Tzou et al., 2000; Lemaitre and Hoffmann, 2007), and the peritrophic matrix of the midgut (Lehane, 1997; Shao et al., 2001). After pathogens penetrate the insects' structural barriers, they may encounter defense reactions of the host. Insect and other invertebrates rely solely on an efficient innate immune system comprising both cellular and humoral responses (Hoffmann and Reichhart, 2002; Tzou et al., 2002; Khush and Lemaitre, 2000; Hultmark, 2003; Dimopoulos, 2003; Leclerc and Reichhart, 2004; Royet, 2004; Pinheiro and Ellar, 2006; Lemaitre and Hoffmann, 2007).

Epithelial tissues such as the gut and trachea not only act as mechanical barriers but also produce antibacterial peptides and reactive oxygen species (ROS) (Lemaitre and Hoffmann, 2007). When the epicuticle of a silkworm larva, *Bombyx mori,* is abraded in the presence of live bacteria or bacteria cell wall components (peptidoglycan, laminarin, or Lipopolysaccharide (LPS)), cecropin transcription is induced in the underlining epithelial cells (Brey et al., 1993). Using a drosomysin-green fluorescent protein reporter gene, Ferrandon et al (1998) showed that the epithelial tissues of *Drosophila* respiratory, digestive and reproductive tracts expressed the antifungal peptide, drosomysin, following an exposure to microbial agents. The cuticular abrasion of *Drosophila* larvae in the presence of bacteria induced *cecropin A1* gene expression in the epithelium (Onfelt et al.,

2001). *Pseudomonas entomophila*, a Gram-negative natural pathogen of *Drosophila*, triggers both local and systemic immune responses, but only the antimicrobial peptide *Diptericin* expression in the gut provides an efficient barrier allowing *Drosophila* to rapidly eliminate most ingested bacteria (Liehl et al., 2006). *Drosophila* nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, dual oxidase (dDuox) is responsible for infection-induced ROS generation. Indispensable for host survival during natural gut infection, dDuox limits the onset of microbial proliferation in the gut. Duox RNAi flies showed a remarkable increase in mortality rate after an oral infection (Ha et al., 2005a). ROS is eliminated by immune-regulated catalase (IRC), a secretory antioxidase protein with catalase activity. Knockdown of *IRC* by RNAi results in higher ROS production and mortality rate after simple ingestion of microbe-contaminated foods, suggesting that IRC-mediated homeostatic redox balance is the most critical host survival strategy during continuous host-microbe interaction in *Drosophila* gut epithelia (Ha et al., 2005b).

Insect cellular defense refers to hemocyte-mediated immune responses, such as phagocytosis, nodulation, and encapsulation (Lavine and Strand, 2002). Phagocytosis is the process of engulfment of entities by an individual cell (Lavine and Strand, 2002). In *M. sexta,* plasmatocytes are major hemocytes involved in phagocytosis of non-self microsphere beads, whereas granulocytes are the hemocytes to ingest self dead cells. Immulectin-2 binds hemocytes to boost phagocytosis with an opsonic activity (Ling and Yu, 2006). Hemolin, a member of the immunoglobulin (Ig) superfamily (Ladendorff and Kanost, 1991), agglutinates bacteria (Yu and Kanost, 2002). Knockdown of hemolin in *M. sexta* markedly reduced the phagocytosis of injected *E. coli* (Eleftherianos et al., 2007), and significantly decreased the ability of the insect to withstand infection when

exposed to pathogenic bacteria *Photorhabdus* (Eleftherianos et al., 2006). In *Drosophila*, phagocytosis is carried out by a major blood cell type, the plasmatocytes (Lanot et al., 2001). Several types of receptor proteins are involved in phagocytosis. These include scavenger receptor family members (SR-CI, e.g.), the EGF-domain protein Eater, thioester-containing proteins (TEPs), and the Ig-domain protein Dscam (Lemaitre and Hoffmann, 2007; Cherry and Silverman, 2006). *Drosophila* SR-CI is a pattern recognition protein (PRP) for both Gram-negative and -positive bacteria; RNAi treatment reduced phagocytosis of heat-killed bacteria by 25% (Ramet et al., 2001). However, *Drosophila* SR-CI only contributes a small fraction of the total phagocytic activity of S2 cells. Subsequent microarray analysis and RNAi experiments revealed that Eater, a single-pass transmembrane receptor containing 32 extracellular EGF-like repeats, is the predominant receptor involved in the recognition, cell-surface binding, and phagocytosis of bacteria in *Drosophila* (Kocks et al., 2005). TEPs resemble to some vertebrate complement factors and to a_2-macroglobulins (a_2M). *Anopheles gambiae* TEP1 binds to bacteria through the thioester bond and is essential for promoting of phagocytosis of Gram-negative bacteria in a mosquito cell line (Levashina et al., 2001). In *Drosophila,* TepII is required for efficient phagocytosis of *E. coli* (but not *C. albicans* or *Staphylococcus aureus*) and TepIII is required for the efficient phagocytosis of *S. aureus* (but not *C. albicans* or *E. coli*) (Stroschein-Stevenson et al., 2006). *Drosophila* Down syndrome cell adhesion molecules (Dscam) are members of an immunoglobulin (Ig)-receptor superfamily comprising of more than 18,000 isoforms generated through alternative splicing. Knockdown of Dscam is shown to impair the hemocyte's capacity of phagocytosis (Watson et al., 2005). *Anopheles gambiae* Dscam family can produce over

31,000 potential alternative splice forms. Challenge with different pathogens produces specific AgDscam splice-form repertoires with different adhesive characteristics. The ability of a hemocyte-like cell line to engulf bacteria is decreased by 55–60% after AgDscam silencing (Dong et al., 2006).

Nodulation is a process of binding and aggregation of bacteria by multiple hemocytes, whereas encapsulation refers to the binding of hemocytes to larger objects such as parasitoids and nematodes (Lavine and Strand, 2002; Jiravanichpaisal et al., 2006). The hemocytes involved in the formation of capsule around the target organisms are granular cells and plasmatocytes in Lepidoptera, and lamellocytes in *Drosophila* (Schmidt et al., 2001; Vass and Nappi, 2001). The encapsulation process involves cell adhesion and melanization (Eslin and Prevost, 2000). Lectins and integrins are two important classes of protein that mediate cell adhesion in immune responses (Vasta et al., 1999). *M. sexta* immunolectins (IML) are C-type lectins serving as humoral pattern recognition receptors (Kanost et al., 2004). *In vitro* encapsulation assays showed that *Manduca* IML-1 and IML-3 enhance cellular encapsulation but not melanization (Ling and Yu, 2006; Yu et al., 2005); IML-2 and IML-4 enhance both encapsulation and melanization (Ling and Yu, 2006; Yu and Kanost, 2004; Yu et al., 2006). Intergin ß1 is constitutively expressed specifically in *Manduca* hemocytes, and RNAi of intergrin ß1 reduced its expression in plasmatocytes and disrupted encapsulation drastically (Levin et al., 2005). In a genome-wide survey on *Drosophila* genes responsive to parasite attack, it was shown that *lectin-24A* and *alphaPS4*, which encodes an intergrin ß subunit, were up-regulated in the parasitized larvae (Wertheim et al., 2005). *Drosophila* Hemese is a small single-pass transmembrane protein with an extracellular N-terminal end that is heavily *O*-

glycosylated (Kurucz et al., 2003). Hemese knockdown enhanced *Drosophila* cellular reaction to the parasitic wasp *Leptopilina boulardi,* indicating that Hemese plays a modulatory role in the activation or recruitment of hemocytes (Kurucz et al., 2003). Changes in adhesiveness and cell shape are essential in cellular immune response. Rac GTPases (Rac) are known to be involved in cellular processes including cytoskeletal organization, regulation of cellular adhesion, cellular polarity, and transcriptional activation (Burridge and Wennerberg, 2004). *Drosophila* Rac1 induces stable actin formation for cellular immune activation, leading to sessile hemocyte release and an increase in the number of circulating hemocytes (Williams et al., 2006); *Drosophila* Rac2 has a critical role in hemocyte spreading and cell-cell contact formation during cellular responses (Williams et al., 2005). In *Rac1* or *Rac2* fly mutants, the proper encapsulation of the eggs of the parasitic wasp *Leptopilina boulardi* was disrupted (Williams et al., 2005; Williams et al., 2006).

Humoral defenses include the production of antimicrobial peptides (AMPs) (Boman, 2003; Bulet et al., 1999; Hetru et al., 2003; Pinheiro and Ellar, 2006; Lemaitre and Hoffmann, 2007), coagulation, and melanization led by protease cascades (Dimopoulos, 2003; Kanost et al., 2004). A key component of insect responses to infection is the synthesis of AMPs by fat bodies and their secretion into hemolymph. Most insect AMPs have low molecular weights (below 5 kDa), a positive net charge at physiological condition, and some of them have amphiphilic a-helices or hairpin-like ß-sheet structures (Bulet et al., 1999). AMPs are usually specific for a certain classes of pathogens, and the killing mechanism relies on distruction of the microbial membrane (Zasloff, 2002). Blood clotting and melanization are immediate responses following a

microbial challenge or septic injury. These two reactions prevent the loss of blood and spread of microbes. During melanization, phenols are converted to quinones. The quinones are toxic to microorganisms and eukaryotic parasitoids, and they polymerize to form melanotic capsules around the parasitoids (Ashida and Brey, 1997; Kanost et al., 2004).

Insect immune responses are triggered by non-self recognition mediated by pattern recognition proteins (PRPs). The interactions between PRPs and conserved structural motifs on the surface of invading microorganisms discriminate different classes of the non-self (Medzhitov and Janeway, 2002). Candidate motifs, or pathogen-associated molecular patterns (PAMPs), include lipopolysaccharide (LPS), peptidoglycan (PG), lipoteichoic acid (LTA) of bacteria, and ß-1,3-glucan of fungi. For example, recognition of Gram-positive bacteria requires the circulating receptors PGRP-SA and GNBP1, or PGRP-SD. PGRP-SA and PGRP-SD to recognize lysine-containing PG, which is produced by Gram-positive bacteria (Leulier et al., 2003). PGRP-SA and GNBP1 are thought to function together as one receptor complex (Michel et al., 2001; Gobert et al., 2003; Pili-Floury et al., 2004). Based on in vitro binding assays using recombinant proteins, a model was proposed in which GNBP1 presents a processed form of PG for sensing by PGRP-SA and that a tripartite interaction between these proteins and PG is able to relay the signal to the serine protease cascade (Wang et al., 2006). PGRP-SD is also required for the detection of other Gram-positive bacterial strains in a GNBP1-independent manner (Bischoff et al., 2004). GNBP3 recognizes ß-1,3-glucan and polysaccharides of the fungal cell wall (Gottar et al., 2006).

Upon recognition, a proteolytic cascade involving serine proteases is activated (Jiang and Kanost, 2000; Kanost et al., 2001, 2004; Kambris et al., 2006). This cascade leads to the activation of intracellular signaling pathways that control AMP gene expression, and other immune responses, such as melanization catalyzed by phenoloxidase (PO) (Tzou et al., 2002). The recognition of pattern molecules from Gram-positive bacteria or fungi by their receptors triggers the proteolytic activation of a serine protease cascade that ultimately processes pro-spätzle into a functional Toll ligand (Levashina et al., 1999; Kambris et al., 2006; Jang et al., 2006). Serpins play critical roles in regulation of this serine protease cascade (Levashina et al., 1999; De Gregorio et al., 2002; Robertson et al., 2003; Ligoxygakis et al., 2002a; Reichhart, 2005) (Fig. 1).

The intracellular signaling pathways transduce the immune response signal from the serine protease cascades to transcription of immune genes. Two major immune signaling pathways are discovered in *Drosophila* (Brennan and Anderson, 2004; Royet et al., 2005). Fungal and Gram-positive bacterial infections stimulate primarily the Toll pathway, whereas Gram-negative bacterial infection stimulates predominately the Imd pathway (Lemaitre et al., 1995; 1996; 1997, Khush et al., 2001). The ligand for Toll is the C-terminal fragment spätzle (Weber et al., 2003). Cleavage activation of spätzle requires the recognition of pathogens and serine protease system. Cleaved spätzle binds to a Leu-rich ectodomain of the transmembrane receptor, Toll. And this binding leads to recruitment of three cytoplasmic proteins, MyD88, Tube and Pelle, to form the signaling complex (Sun et al., 2004). The activated Pelle triggers the phosphorylation and degradation of Cactus, which retains Dorsal and DIF in the cytoplasm under normal condition (Brennan and Anderson, 2004). Once Dorsal and DIF are released from the

complexs with Cactus, they move into the nucleus and activate the expression of AMP genes (Tanji and Ip, 2005).

Infection with Gram-negative bacteria preferentially activates the Imd pathway. Diaminopimelic acid-type PG (DAP-type PG) produced by Gram-negative bacteria is the most potent inducer of the Imd pathway (Leulier et al., 2003). The receptor for DAP-type PG is PGRP-LC (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002b), which functions synergistically with PGRP-LE (Takehana et al., 2004). The Imd pathway has three branches (Tanji and Ip, 2005). TAK1 (transforming growth factor-ß-activated kinase 1) activates IKK complex and leads to the site-specific cleavage activation of Relish (Silverman et al., 2000; Stöven et al., 2003). The second branch, so-called FADD-Dredd pathway, also activates Relish (Elrod-Erickson et al., 2000; Leulier et al., 2000 and 2002; Stöven et al., 2003; Hu and Yang, 2000). The third one is the activation of JNK pathway through TAK1. The JNK pathway regulates the expression of other immune-responsive genes involved in wound healing and stress responses (Boutros et al., 2002; Silverman et al., 2003; Galko and Krasnow, 2004). After proteolytical processing, the N-terminal fragment of Relish enters the nucleus to activate the expression of its target genes (Stöven et al., 2000 and 2003).

Phenoloxidases and their physiological roles

PO system plays a critical role in the fight against pathogens and parasites. PO is involved in blood coagulation, encapsulation and melanization. Compared with the AMP which involves gene expression, PO-catalyzed immune reactions occur as an acute phase response.

Since the identification of prophenoloxidase (proPO) from the silkworm *Bombyx mori* (Ashida, 1971), over 30 proPO proteins or genes have been discovered in different insects (Cerenius and Söderhäll, 2004). PO activities are associated with several physiological processes: sclerotization of insect cuticles, encapsulation and melanization of foreign organisms, wound healing and hemolymph clotting (Ashida and Brey, 1995 and 1998; Gillespie et al., 1997; Sugumaran, 1998; Theopold et al., 2002 and 2004). POs are synthesized as inactive precursors proPOs by insect hemocytes and activated by serine protease, named proPO activating protease (PAP).

POs catalyze the conversion of tyrosine to dopaquinone in two steps: hydroxylation of tyrosine to dopa and the oxidation of dopa to dopaquinone (Nappi and Christensen, 2005; Huang et al., 2005). In silkworm and tobacco hornworm, oenocytoids synthesize proPO (Iwama and Ashida, 1996; Jiang et al., 1997b). There are no signal peptide in nearly all of the known proPOs, and little is known about how these proteins are transported to plasma or cuticle (Theopold et al., 2002).

POs participate in several physiologically critical processes in insects. First, they oxidize *N*-acylcatecholamines to quinones during cuticle sclerotization (Sugumaran, 1998; Lourenco et al., 2005). A recent study in *Aedes aegypti* suggests an essential role of POs in chorion melanization and hardening (Kim et al, 2005).

While hardened cuticles serve as the first line of defense against pathogen invasion (Ashida and Brey, 1995), some organisms can still physically or chemically penetrate this physiochemical barrier and enter the insect body. Under these circumstances, encapsulation and melanization may be initiated to reduce damage caused by the intruders and prevent their development. Due to their involvement in

encapsulation and melanization, POs are considered as essential components of insect immune systems (Söderhäll and Cerenius, 1998 and 2004). *Manduca* granulocytes and spherule cells have proPO on their surface. Agarose beads can be melanized effectively by isolated hemocytes in the presence of cell-free plasma. These observations suggest that hemolymph molecules are involved in the activation of hemocyte surface proPO, which initiate hemocyte melanization, leading to systemic melanization (Ling and Yu, 2005). In an analysis of *A. aegypti* responses to the eukaryotic parasite *Armigeres subalbatus*, increased PO activity was associated with melanotic encapsulation (Guo et al., 1995). During the clearance of Gram-positive bacteria *Micrococcus luteus* by *Anopheles aegypti* hemocytes, PO was detected exclusively in oenocytoids and in many of the melanotic capsules surrounding the bacteria (Hillyer et al., 2003). In *Drosophila*, melanotic encapsulation plays an important role against infection by parasitic wasps (Vass and Nappi, 2000). In the functional study of *Drosophila* serpin27A (a specific inhibitor of proPO activating enzyme), *spn27A* mutant larvae exhibited a more intense melanization reaction at wounded site than the wild-type did (Ligoxygakis et al., 2002a). During invasion of *Leptopilina boulardi*, a parasitic wasp that induces melanotic encapsulation (Russo et al., 1996), the mutant larvae displayed a stronger systemic melanization than the wild-type did (De Gregorio et al., 2002). These results suggest that PO-mediated melanization is involved in the wound healing and in preventing parasite development in *Drosophila*. Nappi et al (2005) reported that injecting Spn27A protein into *Drosophila* larvae remarkably reduced the melanization of *L. boulardi* eggs. This confirms that the proPO pathway can be initiated by parasitization.

POs may participate in wound healing and hemolymph coagulation. The quinones generated by POs could serve as cross-linking agents to seal the wound. In the *Armigeres subalbatus* larvae, melanin deposits were observed at the wounded site by electron microscopy (Lai et al., 2001). Immunocytochemical study demonstrated the presence of PO at the wounded site, but not in the hemolymph clot (Lai et al., 2002). To verify the importance of melanization in wound healing, Ramet et al (2002a) measured the mortality after woulding in *Bc* mutant *Drosophila* larvae, which lacks PO activity and therefore does not produce melanin. The results suggested that PO was crucial in the wound healing. In an attempt to identify the clot proteins of *Galleria mellonella*, proPOs and components of proPO activation cascade were detected in the clot along with components of the coagulation system. This suggested that both systems might work together during clot formation (Li et al., 2002). In a proteomic analysis of *Drosophila* larval hemolymph clot, two POs and a protein similar to proPO activating enzyme were identified (Karlsson et al., 2004). In a whole clot pullout experiment, however, it was observed that aggregation of paramagnetic beads, a mimic of hemolymph coagulation, was comparable or even stronger in *Bc* mutant flies than in the wild-type flies. This implies that, similar to the mosquito *Anopheles subalbatus*, *Drosophila* PO might participate in wound healing and melanization, but not in coagulation (Scherfer et al., 2004). A recent study demonstrated that *Drosophila* PO was not necessary for the preliminary soft clot formation, but was responsible for the clot hardening through cross-linking and melanization (Bidla et al., 2005).

Initiation of proPO activation cascade in *M. sexta*

Upon recognition of non-self, proPOs are activated by limited proteolysis through a serine protease cascade. The first step of activation of the proPO pathway is the recognition of non-self. This process is mediated by PRPs (Ashida and Brey, 1997; Yu et al., 2002b). PRPs recognize PAMPs that are present on the surface of microbes but absent on the host cells (Steiner, 2004). Several groups of PRPs have been identified in insects, which bind to specific PAMPs and trigger proPO activation pathway. In *M. sexta,* such proteins include IML-1, IML-2, ß-1,3-glucan recognition protein-1 (ßGRP1), ßGRP2, and hemolymph proteases 14 (HP14) (Kanost et al., 2004; Ji et al., 2004; Eleftherianos et al., 2006a). Immulectins are calcium-dependent carbohydrate-binding proteins, known as C-type lectins. C-type lectins are involved in pathogen recognition, cellular interactions, and other innate immune responses (Hoffmann et al., 1999; Vasta et al., 1999). Similar to LPS-binding lectins from silkworm (Koizumi et al., 1999) and fall webworm (Shin et al., 2000), *M. sexta* IML-1 and IML-2 contain two carbohydrate recognition domains. IML-1 recognizes Gram-positive and Gram-negative bacteria and yeast (Yu et al., 1999), while IML-2 binds to Gram-negative bacteria only (Yu and Kanost, 2000). The Interaction of IML-1 or IML-2 and LPS stimulate proPO activation in the plasma (Yu et al., 1999; Yu and Kanost, 2000). Knockdown of immunlectin-2 by RNAi increases the rate at which *Photorhabdus*, an insect pathogenic bacteria, kills *M. sexta* (Eleftherianos et al., 2006a). *M. sexta* ßGRP1 (Ma and Kanost, 2000) and ßGRP2 (Jiang et al., 2003) contain a glucanase-like domain at the C-terminal. They bind to ß-1,3-glucan and lipoteichoic acids, and the binding initiates proPO activation system in the larval hemolymph (Ma and Kanost, 2000; Jiang et al., 2004). By subtractive suppression hybridization, two PGRP cDNAs were identified (Zhu et al., 2003a). Their mRNA levels

increased after a bacterial injection (Kanost et al., 2004; Eleftherianos et al., 2006). Introduction of the recombinant PGRPs did not enhance proPO activation in the plasma (Kanost et al., 2004). Although knockdown of PGRP had no effect on cellular immune functions (Eleftherianos et al., 2006b), the treated insects died and had a higher mortality than the control when challenged with *Photorhabdus* (Eleftherianos et al., 2006a; Eleftherianos et al., 2006c). These results suggested that these *M. sexta* PGRPs are associated with humoral immune responses other than the proPO activation pathway (Kanost et al., 2004; Eleftherianos et al., 2006b). A serine protease was identified as pattern recognition receptor, which triggers proPO activation in *M. sexta* (Ji et al., 2004). This protein, designated HP14, is composed of five low density lipoprotein receptor class A repeats, a Sushi domain, a Wonton domain, and a serine protease domain. The expression of HP14 increases significantly after a bacterial challenge. The binding of PG to recombinant proHP14 results in the autoactivation of proHP14, and the activated HP14 initiates proPO activation cascade in the plasma (Ji et al., 2004). The recognition of a fungal cell wall component ß-1,3-glucan by ßGRP2 triggers the autoactivation of proHP14, leading to the initiation of the PO pathway (Wang and Jiang, 2006).

Serine proteases (SPs) and serine protease homologs (SPHs) in insect hemolymph

Serine proteases are involved in many physiological processes such as immune signal transduction in invertebrates (Jiang and Kanost, 2000). Some consist of a C-terminal trypsin-like catalytic domain and one or two clip domains at the N-terminus. The clip domain, which contains three disulfide bonds and resembles a paper clip in the schematic drawing (Muta et al., 1990), is hypothesized to regulate the entire serine protease system (Jiang and Kanost, 2000). Their zymogens are activated by cleavage at

the amino end of the catalytic domains, and the activated catalytic domain remains attached to the clip domain(s) via a disulfide bond (Jiang and Kanost, 2000).

In *Drosophila*, 24 clip-domain SPs and 13 clip-domain SPHs were identified (Ross et al., 2003). Persephone (psh) is the first clip-domain serine protease identified in *Drosophila* to trigger Toll signaling following an immune challenge (Ligoxygakis et al., 2002b). When challenged with an entomopathogenic fungus *Beauveria bassiana*, *psh* mutants exhibited a significant decrease in *drosomysin* transcript level, with a high susceptibility to fungal infections (Ligoxygakis et al., 2002b). Interestingly, it was found that Persephone is processed directly by PR1 protease secreted from the entomopathogenic fungus, leading to the activation of Toll pathway (Gottar et al., 2006). By a large-scale RNAi screen, three SPs and two SPHs involved in Toll pathway in response to Gram-positive bacterial or fungal infection have been identified (Kambris et al., 2006). Spirit, Grass and SPE (Spätzle -processing enzyme) are clip-domain SPs. Grass is activated by GNBP1 and PGRP-SA upon the recognition of Gram-positive PG. Grass then transmits a signal to SPE via Spirit modulated by two SPHs, Spheroide and $Sphinx_{1/2}$. SPE then processes pro-Spätzle to generate the ligand that activates the Toll pathway (Wang et al., 2006). Two clip SPs, MP1 and MP2 were identified to be involved in the *Drosophila* melanization cascade (Tang et al., 2006). MP1 is required to activate melanization in response to both bacterial and fungal infections, whereas MP2 is mainly involved antifungal responses. This also implies the existence of a branched melanization cascade in *Drosophila* (Fig. 1).

The *Anopheles gambiae* genome encodes 41 clip-domain SPs and SPHs (Cristophides et al., 2002). The zymogens of ClipB14 and ClipB15 are synthesized by

hemocytes and secreted into the hemolymph. Functional analysis using RNAi revealed that both ClipB14 and ClipB15 are involved in the defense against Gram-negative bacteria and in the killing of *plasmodium* ookinetes in *A. gambiae.* Studies on parasite melanization demonstrated an additional role for ClipB14 in the PO cascade (Volz et al., 2005). In a systematic investigation of SPs and SPHs roles in *Plasmodium berghei* melanization by *A. gambiae* utilizing an RNAi screen, it has been shown that SPH ClipA8 is essential for parasite melanization but is not involved in parasite killing, whereas SPH ClipA2, A5 and A7 function synergistically to block melanization; ClipB3, B4, B8 and B17 promote melanization (Volz et al., 2006).

In *M. sexta,* 25 SP cDNAs have been cloned (Jiang et al., 2005). This cDNA collection is helpful for the cascade elucidation. First, sequence alignment can provide some clues about their functions. Second, their precursors expressed as recombinant proteins could serve as substrates for isolating their activating enzymes (Wang and Jiang, 2001b; Ji et al., 2003). The third strategy is to study changes in their mRNA and protein levels after an immune challenge, using the cDNAs or antisera raised against the recombinant proteins produced in *E. coli.* The expression profiling may reveal which proteases are involved in the immune responses.

ProPO activation reaction

The last step of this cascade is the proteolysis of proPO by proPO-activating proteases (PAPs), also known as PPAE/PPAF/PPAs (Cerenius and Söderhäll, 2004). Three PAPs have been identified to date in *M. sexta*. PAP-1 has one clip domain and was purified from cuticle (Jiang et al., 1998; Gupta et al., 2005), whereas PAP-2 and PAP-3 containing two clip domains were purified from hemolymph (Jiang et al., 2003a and

2003b). The PAP mRNA levels were up-regulated in the fat body and hemocytes after the larvae were injected with bacteria (Jiang et al., 1998; Jiang et al., 2003a; Jiang et al., 2003b). During the purification of PAP-1, it was noticed that the proPO-activating activity was generated only when two different column fractions were combined. This observation suggests the requirement of a cofactor for proPO activation (Jiang et al., 1998). In the study of PAP-2 and PAP-3, cleaved proPO had little PO activity but, in the presence of SPH-1 and SHP-2, PO activity was generated at a high level and SDS-stable high molecular weight oligomers were formed (Jiang et al., 2003a and 2003b). A similar phenomenon has been observed in other proPO activation systems (Lee et al., 1998a; Lee et al., 2002; Kwon et al., 2000; Kim et al., 2002).

M. sexta SPH-1 and -2 contain a clip domain at the amino-terminus and a serine protease-like domain at the carboxyl-terminus that has no proteolytic activity due to the substitution of active site Ser with Gly (Yu et al., 2003). IML-2, proPO and PAP-1 bind to beads coated with recombinant protease-like domains of SPH-1 and SPH-2, indicating that a complex formed by these proteins might exist in the hemolymph and this complex may restrict the PO-mediated melanization on the pathogen surface through the recognition of pathogen by IML-2. In the analysis of the interactions among proPO, PAP-3 and SPHs, these proteins apparently formed a ternary complex (Wang and Jiang, 2004a). The nature of such molecular interactions and the role of SPHs in the activation of proPO remain to be elucidated.

Regulation of proPO activation and activity

Due to possible cytotoxicity of quinones generated during melanization, the activation of proPO and PO activity are regulated tightly and elaborately (Cerenius and

Söderhäll, 2004). A number of proteins directly regulate PO activity, including PO inhibitors, dopachrome isomerase, and quinone isomerase (Sugumaran 2002). Besides direct regulation of PO activity, serpins regulate proPO activation via specific inhibition to the SPs involved in proPO activation cascade (Kanost et al., 2004). SPHs may also participate in the regulation through unknown ways (Volz et al., 2006). In order to survive and develop in their hosts, endoparasitoids have evolved different strategies to suppress the host immune responses such as PO-mediated melanization (Strand and Peth, 1995; Beckage and Gelman, 2004).

The first phenoloxidase inhibitor (POI) was isolated from the housefly, *Musca domestica* (Tsukamoto et al., 1992). It consists of 38 amino acids with three disulfide bonds. One of the two tyrosine residues, Tyr^{32}, is hydroxylated to dopa, which is crucial for its inhibitory activity (Daquinag et al., 1995). Primary structural analysis showed the disulfide linkage pattern is similar to that in ?-conotoxins from snails and spiders (Daquinag et al., 1999). From *M. sexta* cuticle, a high M_r POI was identified (Sugumaran and Nellaiappan, 2000a). This inhibitor is a heat-labile glycoprotein, with inhibitory activity against insect, plant and fungal POs by forming a detergent-resistant complex (Sugumaran and Nellaiappan, 2000b). Sugumaran et al (2000a and 2000b) found that *M. sexta* quinone isomerase and dopachrome isomerase inhibit PO through complex formation, and the inhibition occurs in a reciprocal way, i.e., PO also inhibits quinone isomerase and dopachrome isomerase.

Serpins play important roles in regulation of proPO activation (Kanost et al., 2004). Serpins are a family of proteins with about 400 amino acid residues typically, and they function as suicide-substrate inhibitors by forming a SDS-resistant complex with

proteases (Gettins, 2002; Elliott et al., 2000). Six serpins have been discovered in *M. sexta,* including serpin-1 (Kanost et al., 1989; Jiang et al., 1994, 1996), serpin-2 (Gan et al., 2001), serpin-3 (Zhu et al., 2003a), serpin-4 (Tong et al., 2005a), serpin-5 (Tong et al., 2005a), and serpin-6 (Wang and Jiang, 2004b, Zou and Jiang, 2005). Serpin-1 gene encodes 12 variants through exclusive use of 12 different versions of exon 9 (Jiang et al., 1994, 1996). One of the serpin-1 variants blocks proPO activation in the plasma by inhibiting PAPs (Jiang and Kanost, 1997). Serpin-3 is an immune responsive serpin, whose level increases remarkably after bacterial challenge (Zhu et al., 2003a). Serpin-3 inhibits PAPs and stops proPO activation efficiently in the plasma (Zhu et al., 2003b). Serpin-4 and serpin-5 are also immune-responsive serpins. Recombinant serpin-4 and serpin-5 can inhibit proPO activation by inhibiting upstream proteases of the cascade, rather than inhibiting PAPs directly (Tong et al., 2005a). Based on further immunoblot and mass spectrometry analysis, sepin-4 is found to inhibit HP1, HP6 and HP21, whereas serpin-5 is an inhibitor of HP1, HP6 and two other unknown proteases. These results imply some of these proteases are involved in the upstream of the proPO system (Tong et al., 2005b). *Drosophila* encodes 29 serpins, one of which, Spn27A, is involved in melanization regulation (De Gregorio et al., 2002; Ligoxygakis et al., 2002a). *Spn27A* loss-of-function mutations lead to a high rate of spontaneous melanization and constitutively elevated PO activity in the hemolymph. Recombinant *A. gambiae* Spn1 and Spn2 bind and inhibit *M. sexta* PAP3 and inhibit proPO activation in *M.sexta* larval plasma (Michel et al., 2006). Depletion of Spn2 from *A. gambiae* increases melanin deposited on Sephadex beads injected into the mosquito body (Michel et al., 2006). It is further shown that Spn2 protects rodent malaria parasite, *Plasmodium berghei,* during

invasion and development on the midgut basal surface (Michel et al., 2005). However, RNAi silencing of Spn2 in *A. gambiae* did not influence the development of field isolates of *Plasmodium falciparum*, human malaria parasite (Michel et al., 2006). Gene silencing of three other genes, LRIM1 (leucine-rich repeat immune protein 1), CTL4 (C-type lectin 4) and CTLMA2 (CTL mannose binding protein 2), increased the rodent parasite survival rate to oocyte stage; by contrast, silencing of the same three genes had no effect on human parasite development (Cohuet et al., 2006). *A. gambiae* Spn6 is involved in *Plasmodium berghei* parasite clearance by inhibiting melanization and/or promoting parasite lysis; interestingly, *A. stephensi* Spn6 is indicated to be involved in the parasite-killing process (Abraham et al., 2006). The different roles of Spn6 in the two mosquito species is considered to be caused by different target serine proteases in the respective species (Abraham et al., 2006). These findings emphasize the importance of studying mosquito immune responses against the pathogens in natural vector-parasite combinations (Michel et al., 2006; Cohuet et al., 2006).

Interaction between parasitoids and host immune systems

For successful development in their hosts, many endoparasitoid wasps actively manipulate their host insect immune responses. The immune suppression is carried out by a venom and polydnaviruses (PDVs) injected by female wasps during oviposition, or by teratocytes derived from the parasitoid embryos (Strand and Peth, 1995; Beckage and Gelman, 2004; Pennacchio and Strand, 2005). The PDV is a family of double-stranded DNA viruses associated with parasitoid wasps, and is divided into two genera, bracoviruses (BVs) and ichnoviruses (IVs), according to their associated wasps, braconidae and ichneumonidae, respectively. In parasitic wasps, the PDV exists as a

provirus intergrated in the genome of the wasp (Fleming and Summers, 1991). The virus replicates specifically in calyx cells of the female ovary and is packaged to form viral particles. During oviposition the wasp injects eggs into the lepidopteran host along with viral particles and secretions from the venom gland. Viral genes are expressed in the host tissues but the viral genome does not replicate in the host. Virus gene products prevent the host immune response from killing the wasp's egg and cause other physiological changes that finally lead the death of the host. The successfully developed wasp larvae emerge from the parasitized host, spin cocoons, and emerge as adults to mate and search for new hosts (Dupuy et al., 200; Webb et al., 2006).

BVs and IVs genomes encode a family of proteins with homology to inhibitor ?B (I?B) proteins from insects and vertebrates (Dupuy et al., 2006; Falabella et al., 2007; Thoetkiattikul et al., 2005). The proteins in this family display short alkyrin domains and lack the regulatory domains for signal-mediated degradation and turnover (Falabella et al., 2007). Two BV I?B-like proteins from *Microplitis demolitor*, H4 and N5, have been shown to suppress the expression of antimicrobial peptides, Attacin and Drosomycin, which are under NF-*k*B regulation through the Toll and Imd pathways (Thoetkiattikul et al., 2005). Immunoprecipitation experiments demonstrated that H4 and N5 bound to the Rel proteins Dif and Relish (Thoetkiattikul et al., 2005). H4 and N5 also inhibited the binding of Dif and Relish to ?B sites in the promoters of the *Drosomycin* and *Cecropin A1* genes. Ank 1, I?B from BVs associated with *Toxoneuron nigriceps* (TnBV), reduced the efficiency of expression of a reporter gene under NF-?B transcriptional control in human HeLa cells (Falabella et al., 2007). In bacterial infected *Heliothis virescens* host parasitized by *T. nigriceps*, NF-?B/Rel failed to enter the nucleus of host hemocytes and

fat body cells (Falabella et al., 2007). These results suggest that I?B proteins prevent NF-?B/Rel factors from entering the nucleus, causing the suppression of the host immune response to the parasites (Falabella et al., 2007; Thoetkiattikul et al., 2005). Glc1.8 gene from *Microplitis demolitor* bracorirus (MdBV) encodes 514 amino acids mucin-like protein characterized by a signal peptide at its N-terminus, an extracellular domain comprised of five 78-amino acid repeats arranged in a tandem array, and a C-terminal hydrophobic domain that encodes a putative anchor sequence (Beck and Strand, 2005). Expression of Glc1.8 caused a loss of adhesion by High Five cells and S2 cells and markedly reduced the ability of these cells to phagocytize bacteria and polystyrene microspheres (Beck and Strand, 2005). Knockdown of Glc1.8 by RNAi rescued the ability of MdBV-infected High Five cells to phagocytize targets (Strand et al., 2006). Collectively, these results indicate that Glc1.8 is a major MdBV pathogenic determinant in the disruption of both adhesion and phagocytosis by the host insect cells (Strand et al., 2006; Beck and Strand, 2005).

In addition to PDVs, fluid from the venom gland is injected into the host during oviposition. Venom proteins protect the eggs from host immune reaction (Webb and Dahlman, 1985), and also affect host physiology and development (Digilio et al., 2000; Ferkovich and Gupta, 1998). In the endoparasitoids that do not carry PDVs, venom is the only factor to regulate the host development and suppress the host immune reactions (Richards and Parkinson, 2000). In some parasitoid systems, PDVs are only effective in conjunction with venom proteins (Asgari, 2006). In the absence of a 14-amino acid peptide, Vn1.5, isolated from *Cotesia rubecula* venom, CrPDV genes are not expressed in host cells and do not cause inactivation of host hemocytes (Zhang et al., 2004a). A

calreticulin-like protein was isolated from *Cotesia rubecula* venom and shown to inhibit hemocyte spreading *in vitro*. This protein also protects beads against the host encapsulation, suggesting that this parasite-specific protein might function as an antagonist competing for binding with the host hemocyte calretulin, which mediates early encapsulation reactions (Zhang et al., 2006).

Melanization and encapsulation of eggs are important ways for resistant hosts to eliminate parasitoids (Carton and Nappi, 2001). POs, key enzymes for melanin synthesis, are potential targets for the parasitoid to regulate. Reduction in PO activity of hosts after parasitism has been documented (Strand and Peth, 1995; Carton and Nappi, 1997; Shelby et al., 2000; Moreau et al., 2003; Asgari et al., 2003). When parasitized by the chneumonid parasitoid *Campoletis sonorensis*, the activity and protein biosynthesis of PO, dopachrome isomerase, and DOPA decarboxylase are inhibited by ichnovirus infection in the Notuid moth, *Heliothis virescens* (Shelby et al., 2000). In a study of *Drosophila* parasitized by *Asobara citri*, the parasitization caused a significant reduction of host hemolymph PO activity, although the count of circulating crystal cells, which are the major carriers of some enzymes involved in PO system, was not statistically different from that of unparasitized larvae; and the authors proposed that the functioning of the host PO system might be impaired (Moreau et al., 2003). A serine protease homologue, Vn50, isolated from the venom of *Cotesia rubecula* exhibits interference to the PO system in its host, *Pieris rapae* (Asgari et al., 2003). Comparative studies using *M. sexta* SPHs, PAP and proPO demonstrated that Vn50 inhibits activation of proPO by PAP, suggesting that the interaction between SPHs and activated PO is impaired (Zhang et al., 2004b). Two groups found that host insect PO system was suppressed by their

ectoparasitoids. Infestation of *Varroa* mites (*Varroa destructor*), the ectoparasitoid of honey bee (*Apis mellifera*) suppresses the expression of host PO gene (Yang and Cox-Foster, 2005). Paralysis of host larvae of Indian meal moth (*Plodia interpunctella*) by its ectoparasitoid *Habrobracon hebetor* causes an increase in PO activity in host hemolymph; subsequent parasitization causes a decrease in PO activity, implying the host PO system is suppressed by the ectoparasitoid (Hartzer et al., 2005).

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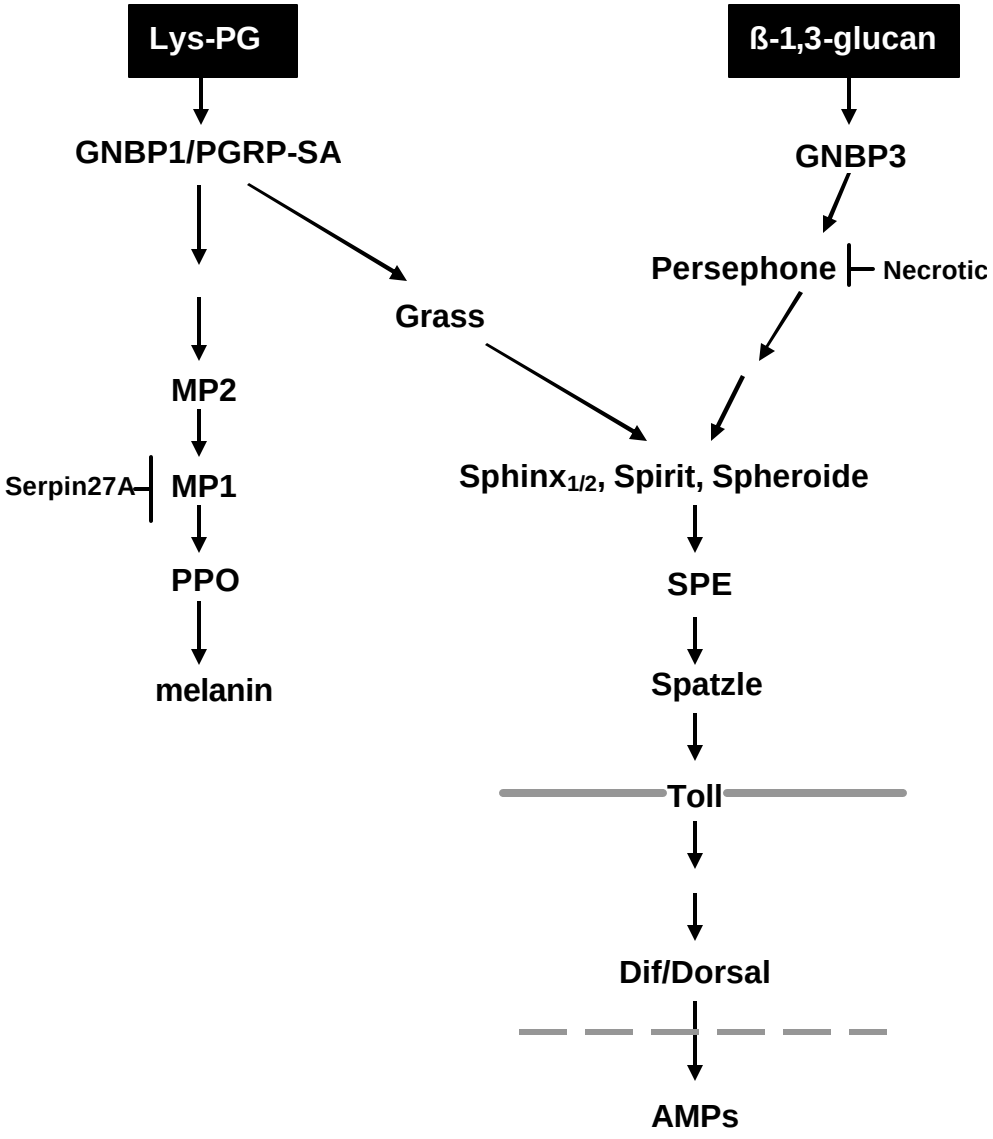

Fig. 1 Serine protease cascades leading to proPO activation and Toll pathway in *Drosophila*.

CHAPTER III

? ETA-1, 3-GLUCAN RECOGNITION PROTEIN-2 (?GRP-2) FROM MANDUCA SEXTA: AN ACUTE-PHASE PROTEIN THAT BINDS ? ETA-1, 3-GLUCAN AND LIPOTEICHOIC ACID TO AGGREGATE FUNGI AND BACTERIA AND STIMULATE PROPHENOLOXIDASE ACTIVATION

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Abstract

We have isolated and characterized a new ß-1,3-glucan recognition protein that is present in *Manduca sexta* cuticle and hemolymph. This 52 kDa protein, designated ßGRP-2, is 57% identical in sequence to ßGRP-1 from larval hemolymph of the same insect. ßGRP-2 differs from ßGRP-1 in its absence in the naive larvae before the wandering stage begins. Transcription of the ßGRP-2 gene was up-regulated in larvae challenged with yeast or bacteria. ßGRP-2 contains a region with sequence similarity to several glucanases but lacks glucanase activity. It aggregates yeasts and bacteria to, perhaps, limit the spread of the invading cells and ensure a localized defense reaction. ßGRP-2 binds laminarin and lipoteichoic acid, but not lipopolysaccharide. Laminarin-triggered prophenoloxidase activation was greatly enhanced in the induced larval hemolymph supplemented with purified ßGRP-2. Complementing other studies on pattern recognition molecules in *M. sexta*, these results indicate that a complex system of protein sensors is an integral component of the insect immune system and that different recognition molecules have overlapping binding specificity and functions.

Abbreviations:

CCF-1, coelomic cytolytic factor-1; Con A, concanavalin A; FITC, fluorescein isothiocyanate; FITC-H6ßGRP-2, recombinant his-tagged ß-1,3-glucan recognition protein-2 labeled with FITC; GBP, ß-1,3-glucan binding protein; GNBP, gram-negative bacteria-binding protein; ßGRP, ß-1,3-glucan recognition protein; HPLC, high performance liquid chromatography; H6ßGRP-2, recombinant his-tagged ß-1,3-glucan recognition protein-2; LGBP, LPS- and ß-1,3-glucan-binding protein; LPS, lipopolysaccharide; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; PAGE, polyacrylamide gel electrophoresis; PAP, prophenoloxidase-activating proteinase; proPO, prophenoloxidase; PO, phenoloxidase; PRP, pattern recognition protein; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecyl sulfate; TBS, Tris buffered saline

Introduction

Innate immunity constitutes the first line of defense against microbial infection in all animals including insects. This system is activated by host proteins that recognize conserved surface determinants of pathogens or parasites, such as lipopolysaccharide (LPS) or peptidoglycans from bacteria and ß-1,3-glucans from fungi (Medzhitov and Janeway, 2002). Upon binding, these pattern recognition proteins (PRPs) stimulate short-term physiological responses mediated by plasma factors and circulating blood cells. Recognition of invading cells also triggers complex signaling pathways that ultimately lead to de novo synthesis of effector molecules such as cytokines and antimicrobial peptides. Extracellular serine proteinase systems have evolved in vertebrates and invertebrates to mediate and coordinate these processes (Krem and Di Cera, 2002 and Jiang and Kanost, 2000). One of these proteinase cascades causes proteolytic activation of prophenoloxidase (proPO), and active phenoloxidase (PO) generates quinones that are intermediates for melanization or sclerotization (Nappi and Vass, 2001 and Sugumaran, 1996). Similar chemical reactions may occur during wound healing and pathogen sequestering.

Several groups of PRPs have been isolated from invertebrates. They bind to cell wall components of the invading microorganisms and initiate defense reactions including hemolymph coagulation and proPO activation (Gillespie et al., 1997, Lavine and Strand, 2002 and Yu et al., 2002). One family of the PRPs is composed of proteins that are similar in sequence to ß-glucanases from bacteria and a sea urchin. Their biological activities appear to be diverse during immune responses. ßGRPs from the tobacco

hornworm, *M. sexta*, and the silkworm, *Bombyx mori*, LPS- and ß-1,3-glucan-binding protein (LGBP) from the crayfish *Pacifastacus leniusculus*, and coelomic cytolytic factor (CCF-1) from the earthworm *Eisenia foetida* are involved in the proPO activation cascade (Ma and Kanost, 2000, Ochiai and Ashida, 2000, Lee et al., 2000 and Bilej et al., 2001). *Drosophila melanogaster* Gram-negative bacteria-binding protein-1 (GNBP-1) enhanced LPS- and ß-1,3-glucan-induced immune gene expression (Kim et al., 2000). The transcription of *Anopheles gambiae* GNBP gene was up-regulated after the malaria mosquito had been infected with *Plasmodium berghei* (Richman et al., 1997).

In this paper, we report the purification, characterization, and cDNA cloning of a new ßGRP from the tobacco hornworm, *Manduca sexta.* Its expression in the fat body is up-regulated in response to immune challenges or certain developmental signals. This protein, designated ßGRP-2, binds ß-1,3-glucan and lipoteichoic acid to aggregate yeast and bacteria. It is also involved in proPO activation.

Materials and methods

Insect rearing and cuticle collection

M. sexta eggs were originally purchased from Carolina Biological Supply, and larvae were reared on an artificial diet (Dunn and Drake, 1983). Pharate pupae with metathoracic brown bars were chilled and dissected to remove hemolymph, gut, and fat body (Jiang et al., 2003). The integuments (and attached muscles) were washed twice in chilled extraction buffer (0.1 M Tris-HCl, 0.002% 1-phenyl-2-thiourea, 1 mM benzamidine, pH 7.5) containing 0.5 M NaCl and stored at – 70 °C.

Isolation of M. sexta ßGRP-2 from cuticular extract

All procedures for purification of ßGRP-2 were carried out at 4 °C. Approximately 250 g frozen cuticles from 75 prepupae were pressed through a meat grinder chilled at -20 °C. The ground tissues were suspended in 300 ml cold extraction buffer containing 0.6 M $(NH_4)_2SO_4$ and homogenized for 1 min in a Waring blender. After centrifugation at 20,000 *g* for 60 min, the supernatant was subjected to ultracentrifugation at 245,000 *g* for 18 h to remove gelatinous compounds and fine particles. Ammonium sulfate saturation of the supernatant was adjusted to 40% (approximately 1.7 M). Precipitated proteins were collected by centrifugation at 20,000 *g* for 30 min and then dissolved in 20 ml of HT buffer (pH 6.8, 20 mM potassium phosphate, 500 mM NaCl). The protein sample was dialyzed against the same buffer (2.0 l for 8 h, twice) and applied to a hydroxylapatite column (2.5 cm i.d.×7 cm, Bio-Rad) equilibrated with HT buffer. After washing with 100 ml of the same buffer, bound proteins were eluted with a linear gradient of 20–150 mM potassium phosphate (pH 6.8), 0.5 M NaCl at a flow rate of 0.4 ml/min for 5 h. Fractions containing ßGRP-2, recognized by the cross-reacting antibodies in *M. sexta* proPO-activating proteinase-1 antiserum (Wang et al., 2001), were pooled and precipitated with ammonium sulfate at 50% saturation. After centrifugation at 20,000*g* for 30 min, the protein pellet was collected and dissolved in 2.0 ml, 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4 and loaded onto a Sephacryl S100-HR column (2.5 cm i.d.×100 cm, Amersham Biosciences) equilibrated with the same buffer. The proteins were separated based on their sizes at a flow rate of 0.4 ml/min and collected at 3.2 ml/fraction. After immunoblot analysis, fractions containing ßGRP-2 were combined, supplemented with $CaCl_2$ and $MgCl_2$ to 1 mM, and

applied to a Concanavalin A-Sepharose 4B column (5.0 ml, Amersham Biosciences) equilibrated with 20 mM Tris-HCl, 0.5 M NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.4. Following a washing step, the bound proteins were eluted with 20 ml of the same buffer containing 0.2 M methyl-a-D-mannopyranoside. The eluted proteins were diluted with four volumes of H_2O, and pH of the solution was adjusted to 7.8. The ßGRP-2 sample was loaded onto an HPLC Mono-Q column (1.0 ml, Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 7.8. After washing, the bound proteins were eluted with a linear gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 1.0 ml/min for 20 min.

Characterization of M. sexta ßGRP-2

The purified protein was subjected to SDS-polyacrylamide gel electrophoresis followed by silver staining (Switzer et al., 1979) or immunoblot analysis using diluted antisera against ßGRP-1 (Ma and Kanost, 2000) and ßGRP-2. A polyclonal antiserum to ßGRP-2 was prepared against the protein purified from the cuticular extract as described before (Wang et al., 2001). The amino-terminal sequence of ßGRP-2 was determined by automated Edman degradation on an Applied Biosystem Model 473 pulse–liquid sequencer. Its molecular weight was measured by MALDI-TOF mass spectrometry (Jiang et al., 2003). The association status of ßGRP-2 was investigated by gel filtration chromatography on a Bio-Silect SEC 250 column (Bio-Rad) equilibrated with 0.1 M sodium phosphate, 0.1 M NaCl, pH 6.9. The column was calibrated with a mixture of molecular weight standards (Bio-Rad).

cDNA cloning and sequence analysis of M. sexta ßGRP-2

The induced *M. sexta* fat body cDNA library in ?ZAP II was screened using the rabbit polyclonal antiserum to ßGRP-2 at 1:1000 dilution, with detection of antibody binding by enzyme-labeled secondary antibody (goat-anti-rabbit IgG conjugated to alkaline phosphatase, Bio-Rad) according to the manufacturer's manual (Stratagene). Positive clones were purified to homogeneity and subcloned by in vivo excision of pBluescript phagemids. Nucleotide sequence of the longest clone was determined using BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) and oligonucleotide primers designed from known sequences. Sequence assembly and database search were performed using MacVector 6.5 (Genetics Computer Group, 1998). Complete sequences were retrieved from GenBank for multiple sequence alignments and phylogenetic analysis using CLUSTALW program (version 1.8) (Thompson et al., 1994). Based on an initial alignment of the full-length sequences, the carboxyl-terminal glucanase-like domain from the 5th residue before the conserved Cys^{137} (ßGRP-2 numbering) to the end of the sequences were selected for further comparison. A Blosum 30 matrix, with a gap penalty of 2 and a K-tuple of 1, was used for each pair-wise alignment. An open gap penalty of 2 and an extension gap penalty of 0.05 were selected for the multiple sequence alignment.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from *M. sexta* larval fat body using the GlassMAX RNA Microisolation Spin Cartridge System. Genomic DNA was removed by DNase I treatment (Invitrogen Life Technologies). One µg of RNA sample was analyzed using

SuperScript One-Step RT-PCR System (Invitrogen Life Technologies) and the following primer pairs: 359 (5' GAT GGA GAG CCT TTA GAC 3') and 360 (5' GTT CCA GGG TTC GTT GC 3') for ßGRP-1; 450 (5' AGA CCT TGC GAA AGA TCC 3') and 451 (5' CGA CAC CTT TGA TGA GTC 3') for ßGRP-2; 501 (5' GCC GTT CTT GCC CTG TT 3') and 504 (5' CGC GAG TTG ACT TCG GT 3') for *M. sexta* ribosomal protein S3. cDNA synthesis and pre-denaturation were performed at 50 °C for 30 min and 94 °C for 2 min, respectively. PCR was carried out by denaturing at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min for a total of 35 cycles. Reactions without reverse transcriptase were performed as negative controls to examine possible contamination of genomic DNA.

Expression and purification of recombinant ßGRP-2

A ßGRP-2 cDNA fragment encoding amino acid residues 1–464 was amplified by PCR and cloned into the *Nco*I and *Pst*I sites of the protein expression vector, H6pQE60 (Lee et al., 1994). Correct insertion and sequence of the resulting plasmid, ßGRP-2/H6pQE60, were examined to confirm that the recombinant ßGRP-2 starts with Met-His-His-His-His-His-His-Ala-Met-Gly-Gln, followed by the mature ßGRP-2 sequence. *E. coli* M15 harboring ßGRP-2/H6pQE60 and pREP4 was cultured in LB medium containing 100 µg/ml ampicillin and induced by 1.0 mM isopropyl-1-thio-ß-D-galactopyranoside (IPTG) when A_{600} reached 0.5–0.7. After 5 h induction, the bacteria from 100 ml culture were harvested by centrifugation and lysed by sonication, and the recombinant protein was purified by Ni^{2+} affinity chromatography in the presence of 8 M urea (Wang et al., 2001). The eluted protein (H_6ßGRP-2) was renatured by dialysis

overnight against two changes of buffer R (2 M urea, 2 mM reduced glutathione, 0.2 mM oxidized glutathione, 5% glycerol, 200 mM NaCl, 2 mM $MgCl_2$, 50 mM sodium phosphate, pH 6.8).

Labeling H_6ßGRP-2 with fluorescein isothiocyanate (FITC)

Purified H_6ßGRP-2 (0.2 mg/ml) in 6.0 ml of buffer R was adjusted to pH 9.0 with NaOH and incubated with 20 mg of fluorescein isothiocyanate (Isomer I, on Celite, Sigma) at room temperature for 4 h. The mixture was then centrifuged at 10,000 *g* for 1 min and FITC-H_6ßGRP-2 in the supernatant was separated from the free label on a Sephadex G-25 column (PD-10, Amersham Biosciences). Purity of the conjugate was confirmed by SDS-PAGE and Coomassie Blue staining.

Binding of FITC-labeled H_6ßGRP-2 to microbial cells

Aliquots of 30 µl of the FITC-labeled recombinant protein (0–0.2 mg/ml) in buffer R were mixed with 30 µl of *Escherichia coli* (5×10^7 cells), *Micrococcus luteus* (5×10^7 cells), or *Saccharomyces cerevisiae* (5×10^6 cells) at 4 °C overnight. The cells were then washed with 1.0 ml of TBS (137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 7.6) and resuspended in 1.0 ml of TBS. Binding of FITC-H_6ßGRP-2 was measured by subjecting the washed cells to flow cytometry analysis using a Becton Dickinson FACScan flow cytometer. For each sample, fluorescence of 10,000 cells was determined. Untreated cells of each kind were used as controls to measure background fluorescence.

Aggregation of microbes by H_6ßGRP-2

An aliquot of 15 µl of H_6ßGRP-2 (0.1 mg/ml) in buffer R was incubated with 15 µl of fluorescein-labeled *S. cerevisiae* (2×10^8 cells/ml), *Staphylococcus aureus* (2×10^8 cells/ml), or *E. coli* (K-12 strain, 2×10^9 cells/ml) (all from Molecular Probes) in TBS at room temperature for 30 min. Samples of the cells were then applied to microscope slides, and degree of the cell aggregation was observed using an Olympus BH-2 fluorescence microscope. Bovine serum albumin at same concentration was used as control.

Plate assay of H_6ßGRP-2 binding to various ligands

Laminarin, lipoteichoic acid, and LPS (all from Sigma) were individually dissolved in H_2O at 0.1 mg/ml. The samples (5–10 µg) were applied to a 96-well microplate and air dried overnight at room temperature. The plate was incubated at 60 °C for 30 min to fix the ligands, and the wells were blocked with 200 µl of 3% dry skim milk in TBS at 37 °C for 1 h. After washing, H_6ßGRP-2 (10–1000 ng) in 100 µl buffer R was added to the wells and incubated at room temperature for 1 h. Following a washing step with TTBS (TBS supplemented with 0.05% Tween 20), 100 µl of 1:500 diluted ßGRP-2 antiserum in TBS containing 1% dry milk was added to the wells. After incubation at 37 °C for 2 h, the wells were washed three times with TTBS and then reacted with 100 µl of 1:3,000 diluted goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad) in TBS containing 1% dry milk at 37 °C for 1 h. Then the wells were rinsed three times with TBS and once with 0.5 M $MgCl_2$, 10 mM diethanolamine. Aliquots of 100 µl of *p*-nitrophenyl phosphate (1.0 mg/ml in 0.5 M $MgCl_2$, 10 mM diethanolamine) were added to the wells

and absorbance at 405 nm was monitored in the kinetic mode by a PowerWave 340 microplate reader (Bio-Tek Instruments, Inc.).

Role of ßGRP-2 in proPO activation in induced M. sexta hemolymph

The day 3 fifth instar larvae were injected with 50 µl, 1 µg/µl *Micrococcus luteus* (Sigma), and hemolymph was collected 24 h later from a cut proleg into 2 volumes of chilled anticoagulant buffer in polypropylene tubes (Ma and Kanost, 2000). After removal of hemocytes by centrifugation at 500*g* for 5 min, the plasma samples (20 µl) were separately incubated with the purified cuticular ßGRP-2 (5 µl, 0.1 mg/ml), laminarin (5 µl, 0.1 mg/ml), lipoteichoic acid (5 µl, 0.1 mg/ml), LPS (5 µl, 0.1 mg/ml), or buffer (5 µl, 0.1M Tris-HCl, 0.1 M NaCl, 1 mM $CaCl_2$, pH7.4). In the control and test groups, 20 µl of buffer or ßGRP-2 was added respectively and incubated on ice for 40 min. PO activity was determined using a microplate reader with dopamine as a substrate (Jiang et al., 2003).

Results

Purification and characterization of native M. sexta ßGRP-2

When the polyclonal antiserum against M. sexta PAP-1 became available, we purified this enzyme from the integuments of M. sexta prepupae (Gupta et al., unpublished data) and found that the antiserum cross-reacted with a 58 kDa protein in the cuticular extract. To test if it had an amino acid sequence similar to a serine proteinase, we developed a scheme to isolate this protein (Fig. 1). It was later designated ßGRP-2 when its cDNA sequence and biochemical properties were known. ßGRP-2 bound to the hydroxylapatite column weakly and eluted soon after the sodium phosphate gradient was

applied. Fractions 33–40 were pooled, concentrated, and resolved by gel filtration chromatography on a Sephacryl S100-HR column. The immunoreactive protein in fractions 38–46 was separated from other proteins that did not bind to Con A. The fractions containing ßGRP-2 were combined and further separated by ion exchange chromatography on an HPLC Mono-Q column. ßGRP-2 is moderately abundant in the integument at this stage—we obtained about 0.5 g pure protein from 75 insects.

The purified protein migrated on an SDS-polyacrylamide gel as a single band under reducing conditions (Fig. 2). It was recognized by antibodies to ßGRP-2 and ßGRP-1. PAP-1 antiserum (lane 5) and its corresponding preimmune serum (data not shown) also reacted with the protein. As determined by MALDI mass spectrometry, ßGRP-2 has a molecular mass of 54,932±55 Da. The purified protein eluted as a single peak at 8.5 min on the HPLC gel filtration column, corresponding to a molecular mass of about 55 kDa. This result indicated that ßGRP-2 was present as a monomer. The amino-terminal 27 residues of the purified protein were determined to be: Arg-Gly-Gly-Pro-Tyr-Lys-Val-Pro-Asp-Ala-Lys-Leu-Glu-Ala-Ile-Tyr-Pro-Lys-Gly-Leu-Arg-Val-Ser-Val-Pro-Asp-Asp.

cDNA cloning and sequence features of ßGRP-2

Using the polyclonal antiserum against ßGRP-2 as a probe, we screened a bacteria-induced *M. sexta* fat body cDNA library and isolated seven positive clones from 1.4×10^5 plaques. This indicated that ßGRP-2 mRNA is moderately abundant in the fat body at 24 h after the insects were injected with bacteria. The longest cDNA contained a short 5′ non-coding region, an open reading frame of 1446 nucleotides, and a long 3′ untranslated sequence of 911 nucleotides (Fig. 3). The open reading frame encodes an amino acid sequence 482-residue long, including an 18-residue signal peptide for

secretion. Beginning at position 19, the first 27 residues of the mature protein perfectly match the sequence determined by Edman degradation. Potential N-linked glycosylation sites are present in the sequence at N^{106}GT and N^{171}WT. The binding of ßGRP-2 to Con A indicated that one or both of the sites are posttranslationally modified. This is also consistent with the fact that the calculated molecular mass of ßGRP-2 (52,490 Da) is around 2400 Da lower than the value determined by MALDI mass spectrometry (Fig. 2). The calculated isoelectric point of ßGRP-2 is 5.8, higher than that of ßGRP-1 (pI=5.1). Perhaps as a result, ßGRP-2 eluted much earlier than ßGRP-1 from the hydroxylapatite column (Fig. 1; [Ma and Kanost, 2000). A putative integrin binding motif (RGD) is located in the middle of the protein (residues 275–277). Four Cys residues at positions 137, 151, 227, and 236, which are well conserved in this family of proteins, probably form two disulfide bonds to maintain the ßGRP-2 structure.

A BLAST search of Genbank and multiple sequence alignment indicated that M. sexta ßGRP-2 was similar in sequence to ßGRP-1 from M. sexta (Ma and Kanost, 2000), ßGRP and Gram-negative bacteria-binding protein (GNBP) from B. mori, ßGRP from Plodia interpunctella and a putative GNBP from the fall webworm Hyphantria cunea (Ochiai and Ashida, 2000, Lee et al., 1996, Fabrick et al., 2003 and Shin et al., 1998). There are at least 3 ßGBP/GNBP genes in the Drosophila melanogaster genome and 7 in the Anopheles gambiae genome (Adams et al., 2000 and Holt et al., 2002).

In addition to these insect proteins, ßGRPs are similar to LGBPs from the freshwater crayfish (*Pacifastacus leniusculus*) and the blue shrimp (*Litopenaeus stylirostris*), ßGBP from the black tiger shrimp (*Penaeus monodon*), and CCF-1 from the

earthworm (*Eisenia foetida*)(Lee et al., 2000, Roux et al., 2002, Sritunyalucksana et al., 2002 and Bilej et al., 2001). These proteins are similar to ßGRP2 mainly in the glucanase-like domain, but their amino-terminal extensions are much shorter than the insect protein.

M. sexta ßGRP-2 lacks three conserved residues (Glu, Asp, Glu) in the active site of ß-1,3-glucanases (Juncosa et al., 1994). This is consistent with the observation that purified ßGRP-2 failed to form a clear zone around the sample well in a curdlan-agarose gel (data not shown).

Expression of the ßGRPs in M. sexta fat body

We performed an RT-PCR experiment to test whether or not the ßGRP mRNAs are inducible in the larval fat body upon microbial challenge. Consistent with the previous Northern blot analysis result (Ma and Kanost, 2000), we observed constitutive, low level of ßGRP-1 expression in this tissue before and after the immune challenge (Fig. 4A). In contrast, the transcription of ßGRP-2 was highly inducible—we did not detect its mRNA in the control larvae without injection or with injection of TBS, but detected its appearance after injection of S. cerevisea, E. coli, or M. luteus. This result demonstrated that ßGRP-2 is an acute-phase protein whose expression was not only induced by a yeast challenge but also by a Gram-negative or Gram-positive bacterial infection.

A similar experiment was carried out to compare mRNA levels of ßGRP-1 and ßGRP-2 in the fat body of M. sexta larvae at different developmental stages starting from

day 0 of fifth instar to day 6 of wandering stage. The mRNA level of ßGRP-1 was relatively constant during this period (Fig. 4B). In contrast, ßGRP-2 transcripts were hardly detected in the fifth instar larval feeding stages but dramatically increased at the start of the wandering stage. As a loading control, M. sexta ribosomal protein S3 did not change in the developmental stages examined.

Since ßGRP-2 binds to Con A (Fig. 1) but ßGRP-1 does not (Ma and Kanost, 2000), we employed lectin affinity chromatography to separate the two proteins in hemolymph samples from naïve, bacteria-injected, and late wandering larvae. Immunoblot analysis indicated that ßGRP-2 was present in plasma of the induced and wandering larvae but absent in the uninduced larvae (Fig. 4C).

Functional analyses of recombinant ßGRP-2

We expressed ßGRP-2 in E. coli as a (His)6-tagged recombinant protein and purified it by affinity chromatography on a Ni2+-NTA column under denaturing conditions. The renatured protein migrated as a single band at 53 kDa on an SDS-polyacrylamide gel (Fig. 2).

To test if ßGRP-2 binds to bacteria or yeast, we labeled the recombinant protein with FITC and incubated it with S. cerevisea, E. coli, and M. luteus as examples of fungi, Gram-negative, and Gram-positive bacteria. After incubation, the microbial cells were washed, and then the fluorescence from the bound FITC-H6ßGRP-2 was quantified by flow cytometry. We observed significant binding of FITC-ßGRP-2 to the tested cells in a

saturable manner (Fig. 5). However, further experiments employing unlabeled ßGRP-2 as a competitor will be required to characterize the specificity of the binding.

The presence of ßGRP-2 at 50 µg/ml caused significant aggregation of S. cerevisiae, E. coli, and S. aureus, whereas bovine serum albumin at the same concentration did not have this effect (Fig. 6). These results indicated that, like ßGRP-1, ßGRP-2 can cause aggregation of invading microorganisms and, perhaps, lead to a more efficient clearance of the microbes by hemocytes.

To better understand mechanisms of the binding and aggregation activities, we investigated what molecules on the microbial surfaces are recognized by ßGRP-2 (Fig. 7). M. sexta ßGRP-2 bound most strongly to laminarin, a ß-1,3-glucan from fungi and then to lipoteichoic acid from Gram-positive bacteria. LPS is a poor ligand of ßGRP-2.

We have also tested involvement of ßGRP-2 during proPO activation and found that laminarin-stimulated proPO activation was greatly enhanced by adding exogenous ßGRP-2 to the induced hemolymph (Fig. 8). Although ßGRP-2 also binds to lipoteichoic acid, the binding failed to trigger proPO activation. LPS did not have any effect on proPO activation.

Discussion

Invertebrates do not have the genetic mechanisms to produce antibodies and T-cell receptors that specifically recognize invading microorganisms. They rely on PRPs to detect common carbohydrate structures on microbial cell surfaces and initiate host defense responses. Therefore, it is essential that multiple PRPs are present in the

circulation, each with distinct binding specificity, to cover a wide spectrum of infectious agents. Accumulating evidence has indicated this to be the case in insects as well as other invertebrates (Yu et al., 2002, Wilson et al., 1999, Christophides et al., 2002 and Iwanaga, 2002). In this work, we have purified from integuments of the tobacco hornworm, M. sexta, a 58 kDa protein that binds to ß-1,3-glucan and lipoteichoic acid. We named this protein ßGRP-2, as it is similar to ßGRP-1, a protein we previously isolated from the hemolymph of M. sexta fifth instar larvae (Ma and Kanost, 2000).

Using the specific antiserum as a probe, we screened a M. sexta fat body cDNA library and isolated cDNA clones encoding ßGRP-2. Its deduced amino acid sequence is 57% identical to those of M. sexta ßGRP-1 (Ma and Kanost, 2000) and B. mori ßGRP (Ochiai and Ashida, 2000). Due to the sequence similarity, the antiserum to ßGRP-1 cross-reacted with ßGRP-2 (Fig. 2), and vice versa (data not shown). On the other hand, the cross-reacting antibodies in the PAP-1 antiserum may not be related to the proteinase at all since its sequence is completely different from ßGBP-2. We note that this cross-reaction was also found with the preimmune serum from the same rabbit.

M. sexta ßGRP-1 and ßGRP-2 are probably composed of two domains. The one at the amino-terminus corresponds to residues 1–102 of the silkworm ßGRP, which is responsible for the specific binding to ß-1,3-glucan (Ochiai and Ashida, 2000). The carboxyl-terminal domain is similar in sequence to ß-1,3- and ß-1,3-1,4-glucanases from bacteria (Juncosa et al., 1994) and to a ß-1,3-glucanase from a sea urchin (Bachman and McClay, 1996). In the earthworm CCF-1 (Bilej et al., 2001), a carbohydrate recognition site was identified in this domain, which is important for its binding to Gram-positive and

Gram-negative bacteria. The ability of ßGRP-2 to cause aggregation of microorganisms suggests that each monomer has more than one binding site, perhaps one in each of its domains. Supporting this speculation is the observation that both domains of the closely related Plodia interpunctella ßGRP (Fig. 9) can bind to laminarin (Fabrick, Baker, and Kanost, unpublished data).

Unlike ßGRP-1, which is produced at a low, constitutive level during all the developmental stages tested, ßGRP-2 transcript appears only after the wandering stage begins. The ßGRP-2 protein was detected in the hemolymph and integument of *M. sexta* wandering stage larvae (Fig. 1 and Fig. 4), suggesting that ßGRP-2 as well as other acute-phase proteins could be important for protecting the insect in a soil environment during pupation.

The *M. sexta* ßGRPs aggregate yeast, Gram-negative, and Gram-positive bacteria. To identify their ligands on the microbial cell surface, we analyzed their binding to laminarin, lipoteichoic acid, and LPS, and found both proteins bind to laminarin significantly (Fig. 7 and data not shown). This demonstrates that the yeast cell wall ß-1,3-glucan is probably responsible for the ßGRP binding and cell–cell association. The ßGRPs also strongly bind to lipoteichoic acid, a cell wall component of Gram-positive bacteria. However, no specific binding was observed with peptidoglycan (data not shown). Neither did we detect a significant binding to LPS. Perhaps, other structures on the surface of Gram-negative bacteria are recognized by the ßGRPs to account for the aggregation activity.

Our previous work showed that native ßGRP-1 can trigger the proPO activation in cell-free hemolymph from naive *M. sexta* larvae (Ma and Kanost, 2000). We also tried to see if purified ßGRP-2 exerts the same function by binding to laminarin in the induced plasma, and indeed detected significant enhancement of proPO activation. Lipoteichoic acid failed to trigger the cascade, suggesting that an "activating" conformation was not induced when lipoteichoic acid bound to ßGRP2. Such a conformation appears to be critical for interacting with other plasma proteins involved in proPO activation or other immune responses.

Multiple sequence alignment of the ßGRPs and GNBPs indicated that this family of proteins is widely spread in invertebrates (Fig. 9). Most of the insect sequences have an amino-terminal extension of about 100 residues. This region is known to be the ß-1,3-glucan-binding site in the silkworm GRP (Ochiai and Ashida, 2000). There was apparently an expansion of this gene family in the *A. gambiae* genome, resulting in seven members, as compared with three in the *D. melanogaster* genome. Five of these *Anopheles* members, lacking the amino-terminal extension, form a tight branch in the phylogenetic tree. This is connected to another branch which includes GBPs/LGBP from crustaceans, a glucanase from a sea urchin, and CCF-1 from an earthworm. Like the mosquito sequences, they only contain the glucanase-like domain responsible for ligand binding.

So far, we have characterized two ßGRPs in *M. sexta.* Both of them are synthesized mainly in fat body, but their expression is controlled differently. They have a similar function in aggregating microorganisms and enhancing proPO activation. How

exactly the insect manages to regulate the two proteins and the physiological significance of the regulation remain interesting questions that should be addressed in the future to better understand the immune system of this insect.

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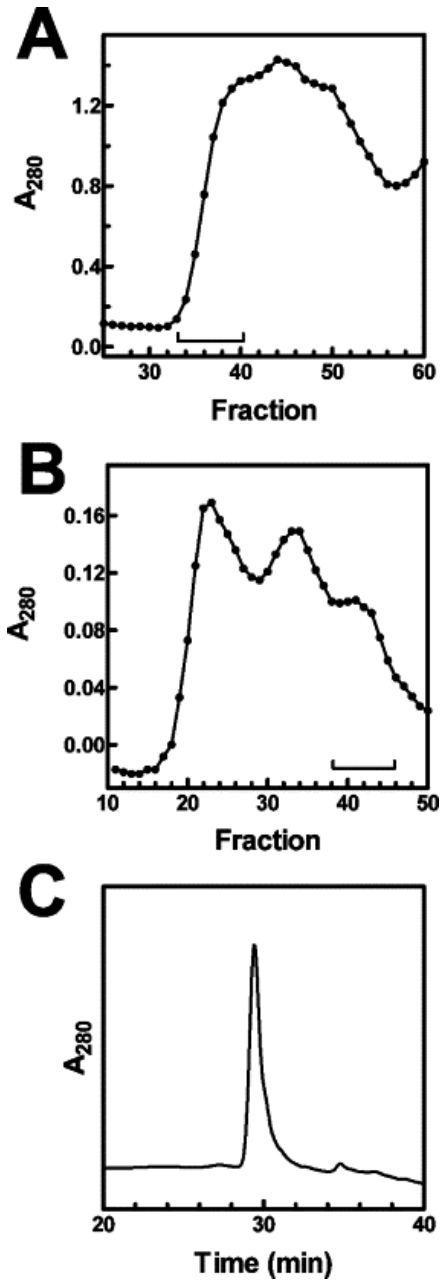

Fig. 1. Purification of ßGRP-2 from integuments of *M. sexta* pharate pupae.

Treated cuticular extract was separated on hydroxylapatite **(A),** Sephacryl S100-HR **(B)**, ConA-Sepharose, and Mono-Q **(C)** columns as described in Materials and methods. ?—?, absorbance at 280 nm. The bar in each panel indicates the fractions containing ßGRP-2 (detected by immunoblot analysis) that were pooled for the next purification step.

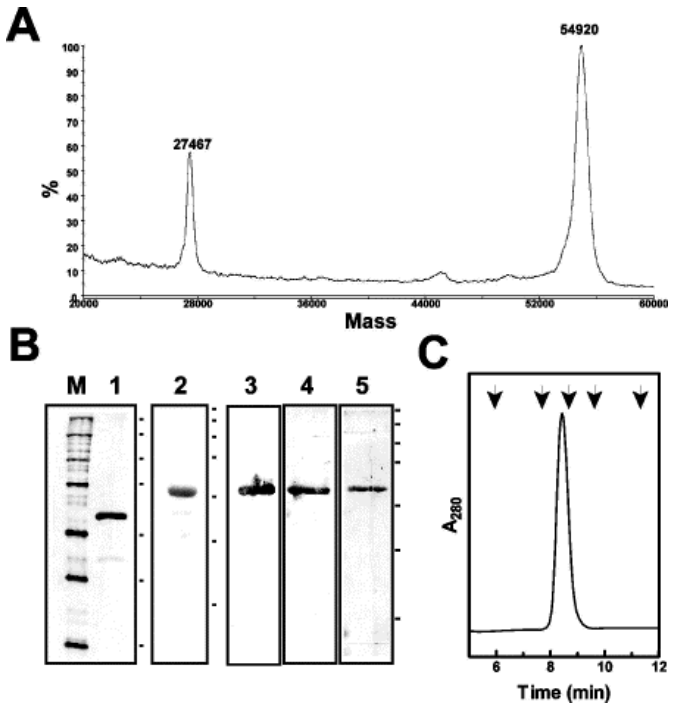

Fig. 2. Characterization of *M. sexta* ßGRP-2.

(A) MALDI-TOF mass spectrometry. A representative strong single-accumulation spectrum for ßGRP-2 is presented. The spectrum was calibrated with a bovine serum albumin standard and subjected to noise removal. **(B)** SDS-PAGE and immunoblot analyses. Lane 1, ßGRP-2 purified from the cuticular extract (40 ng) (silver staining); lane 2, recombinant H_6ßGRP-2 purified by nickel affinity chromatography (0.3 µg) (Commassie Blue staining). Immunoblot analysis of the purified cuticular ßGRP-2 using antisera against ßGRP-2 (lane 3), ßGRP-1 (lane 4), and PAP-1 (lane 5) as the first antibody. Positions of the molecular weight makers (M: 250, 150, 100, 75, 50, 37, 25 kDa) are marked. **(C)** Association status determination. Purified cuticular ßGRP-2 (20 µg) was separated by gel filtration chromatography on the HPLC column. Vertical bars indicate the positions of molecular standards of thyroglobulin (670 kDa), bovine ? globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B12 (1.35 kDa) from left to right.

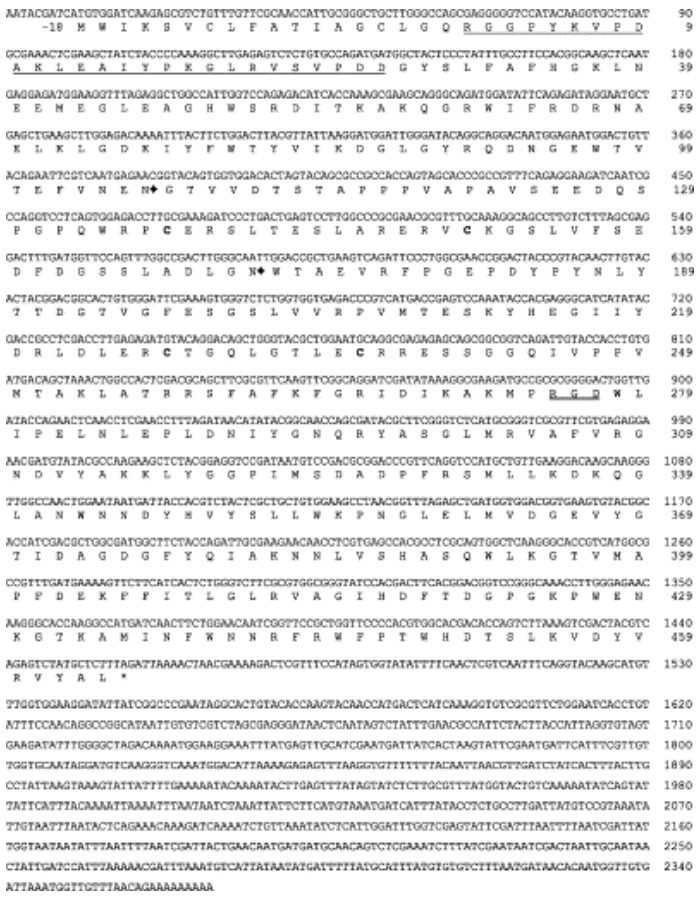

Fig. 3 Nucleotide and deduced amino acid sequences of ßGRP-2

Amino acid residues, shown in one letter abbreviations below the cDNA sequence, are aligned with the second nucleotide of each codon. Residues in the mature protein are assigned positive numbers, and those in the signal peptide are assigned negative numbers. A single underline indicates the amino-terminal sequence of the mature ßGRP-2 determined by Edman degradation. Two potential N-linked glycosylation site are marked with ?. The conserved Cys residues, shown in bold, probably form two disulfide bonds. One putative integrin-binding motif (RGD) is double underlined. The termination codon TAG is marked with an asterisk.

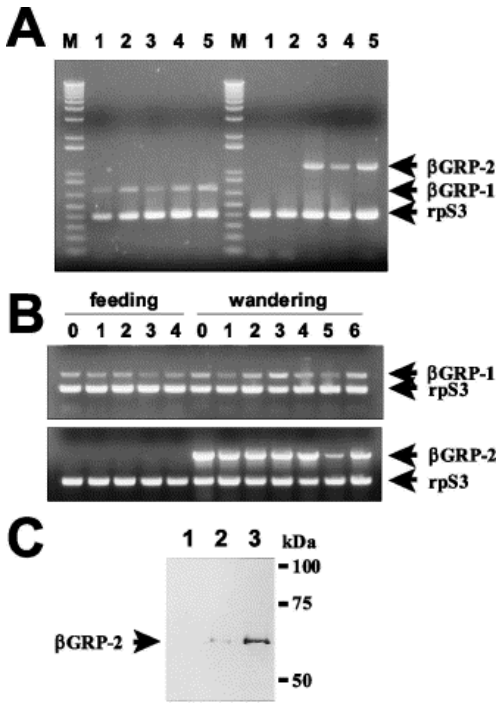

Fig. 4. Expression profiles of *M. sexta* ßGBP-1 and ßGBP-2

In panel **A**, total RNA samples were isolated from fat body of naive larvae (lane 1) and larvae injected with saline (lane 2), *S. cerevisea* (lane 3), *E. coli* (lane 4), and *M. luteus* (lane 5). As described in Materials and methods, RT-PCR was performed using primer pairs specific for ßGRP-1 (left side), ßGRP-2 (right side), and ribosomal protein S3 (both sides). M: DNA size markers. From bottom to top, the sizes of the bands are 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.85, 1, 1.65, 2kb, etc. In panel B, fat body total RNA samples from naïve fifth instar feeding larvae day 0 through day 4 (lane 0 to lane 4) and unchallenged wandering larvae day 0 through day 6 (lane 0 to lane 6) were analyzed similarly. Upper, ßGRP-1; lower, ßGRP-2. The PCR products are marked with arrows at their corresponding sizes. In panel C, hemolymph samples from naïve (lane 1), bacteria-injected (lane 2), or late wandering (lane 3) larvae were subjected to Con A affinity chromatography to separate ßGRP-2 from ßGRP-1. Eluted proteins, equivalent to 2 µl

hemolymph, were analyzed by SDS-PAGE and immunoblotting using 1:2000 diluted ßGRP-2 antiserum as the first antibody.

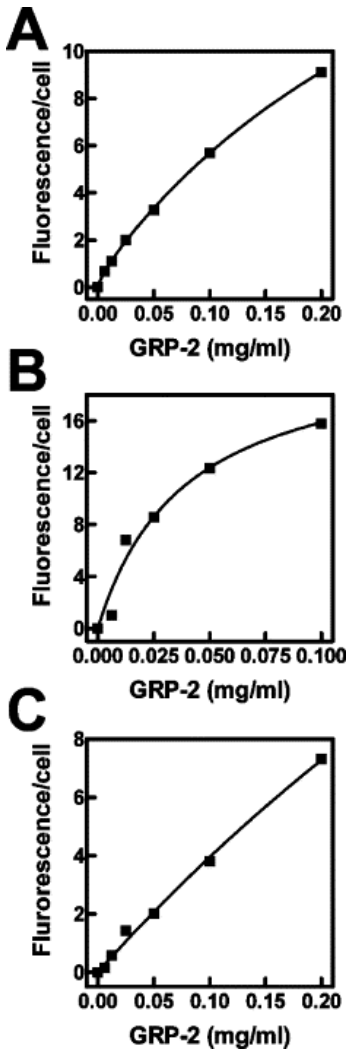

Fig. 5. Concentration-dependent binding of H_6ßGRP-2 to *E. coli*

(A), *M. luteus* **(B)**, and *S. cerevisiae* **(C).** Binding of FITC-labeled H_6ßGRP-2 to the microorganisms was examined by flow cytometry as described in Materials and methods. After incubation with varying amounts of the recombinant protein for 30 min, the washed bacterial or yeast cells (1×10^7) were subjected to fluorescence measurement on a flow cytometer. Each point is the mean fluorescence intensity/cell of 10,000 cells with a standard error of each sample too small to be visible as an error bar. The binding curves represent a one site model with K_d (μg/ml) and r^2: 20.53±1.58 and 0.996 (A); 22.07±3.42 and 0.998 (B), or 46.64±22.52 and 0.968 (C), respectively.

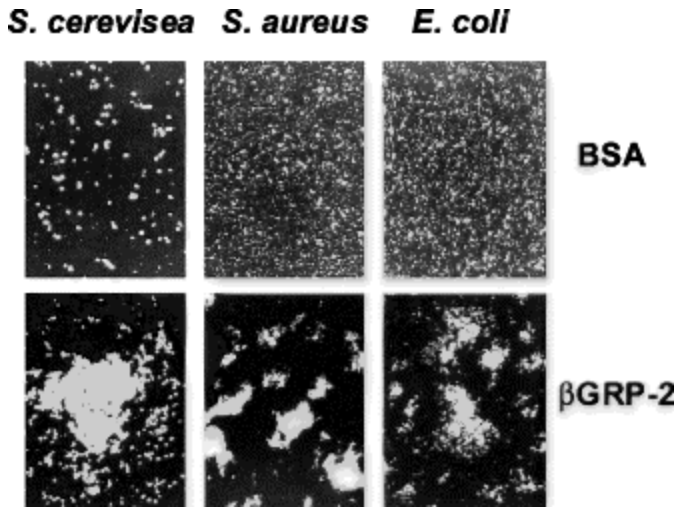

Fig. 6. Aggregation of microorganisms by ßGRP-2.

H_6ßGRP-2 (0.05 mg/ml) was incubated with FITC-labeled *E. coli, S. aureus,* or *S. cerevisiae* at room temperature for 30 min. The microorganisms were incubated similarly with bovine serum albumin (0.05 mg/ml) as a control. The cells were photographed using fluorescence microscopy.

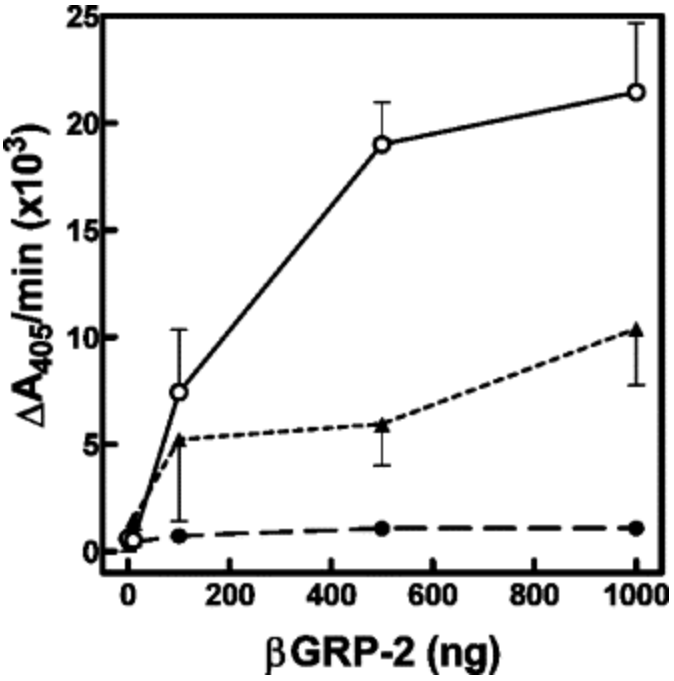

Fig. 7. Association of microbial cell wall components with ßGRP-2.

As described in Materials and methods, recombinant ßGRP-2 was reacted with laminarin (?—?), lipoteichoic acid (▲---▲), and lipopolysaccharide (?---?) immobilized on a 96-well microplate. The binding was detected via an ELISA and shown as mean±SEM (*n*=3).

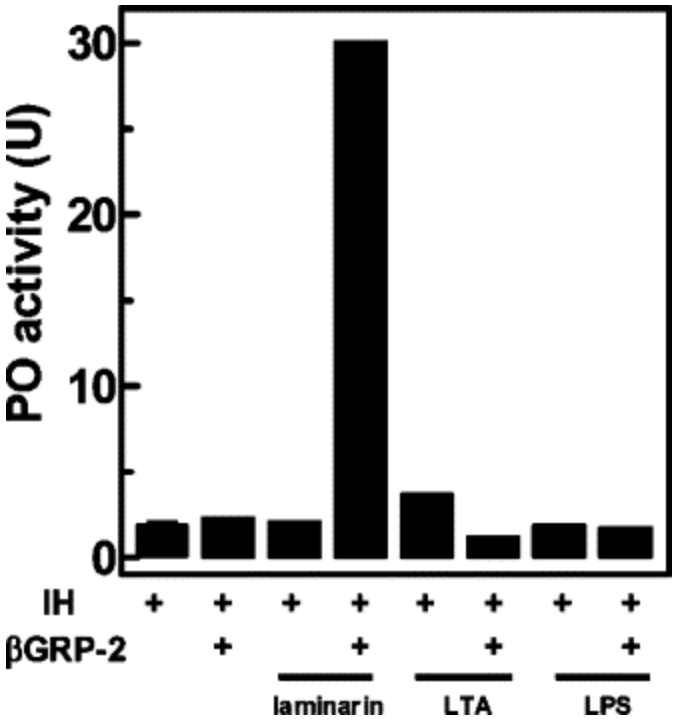

Fig. 8. Roles of H_6ßGRP-2 in proPO activation.

As described in Materials and methods, the induced hemolymph (IH) samples were incubated with laminarin, lipoteichoic acid (LTA), or lipopolysaccharide in the presence or absence of ßGRP-2. PO activities (mean±SEM, *n*=3) were determined and plotted as a bar from each treatment.

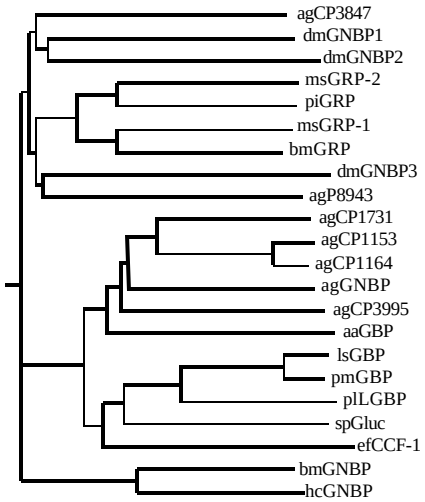

Fig. 9 Phylogenetic relationships among the glucanase-like proteins in invertebrates. A multiple sequence alignment was performed as described under *Experimental Procedures*. Sequences of *Manduca sexta* (ms) ßGRP-1 (AF177982) and ßGRP-2 (AY135522), *Bombyx mori* (bm) ßGRP (AU004243) and GNBP (L38591), *Plodia interpunctala* (pi) ßGRP (AAM95970), *Hyphantria cunea* putative (hc) GNBP (AF023916), *Drosophila melanogaster* (dm) putative GNBP-1 (AAF33849), GNBP-2 (NP_524141) and GNBP-3 (AAF33851), *Anopheles gambiae* (ag) putative GNBP (AJ001042), CP1164 (EEA07705), CP1153 (EAA07707), CP1731 (EAA07723), CP13995 (EAA09015), P8943 (EAA00167), and CP3847 (EAA04713), *Aedes Aegypti* (aa) putative GBP (AFF466594), *Penaeus monodon* (pm) GBP, *Litopenaeus stylirostris* (ls) LGBP (AAM73871); *Pacifastacus leniusculus* (pl) LGBP (CAB65353), *Strongylocentrotus purpuratus* (sp) ß-1,3-glucanase (U49711), *Eisenia foetida* (ef) CCF-1 (AF030028).

CHAPTER IV

EXPRESSION AND ACTIVATION OF *MANDUCA SEXTA* SERINE PROTEINASE HOMOLOG PRECURSORS

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Abstract

Phenoloxidase-catalyzed reactions are crucial to the survival of arthropods after pathogen or parasite infection. In *Manduca sexta*, active phenoloxidase is generated from its precursor by a prophenoloxidase activating proteinase (PAP) in the presence of noncatalytic serine proteinase homologs (SPHs). As terminal components of a proteinase cascade, the PAP and SPHs also require limited proteolysis to be functional. While the processing enzyme of *M. sexta* proPAP-2 and proPAP-3 is known, we are investigating the cleavage activation of proSPH-1 and proSPH-2. Here we report the development of a series of Bac-to-Bac plasmid vectors for co-expression, secretion and affinity purification of proSPH-1 and proSPH-2 in one baculovirus. The purified proteins were characterized and used as substrates in the search of their activating enzymes. Proteolytic processing occurred after the proSPHs had been incubated with hydroxyapatite or gel filtration column fractions. The cleaved proteins were active as a cofactor for prophenoloxidase activation by PAP, and coexistence of SPH-1 and SPH-2 is essential for manifesting the auxiliary effect.

Introduction

With over 430 members listed in MEROPS (http://merops.sanger.ac.uk), chymotrypsin-like serine proteinases constitute the largest family of all peptidases. They participate in vital physiological processes including digestion, development and defense responses (Hedstrom, 2002). At the amino terminus of many arthropod serine proteinases, there is one or two clip domains connected to the carboxyl-terminal catalytic domain through a linker region and a disulfide bond (Jiang and Kanost, 2000). While their proteinase domains all contain a His-Asp-Ser catalytic triad, nonsynonymous substitution(s) lead to the loss of these key residues and enzymatic activity. Indeed, insect genomes encode serine proteinase homologs (SPHs) that are

anticipated to resemble their ancestral enzymes in overall folding (Ross et al., 2003; Zou et al., 2006) and possess functions other than peptide bond hydrolysis. For instance, clip-domain SPHs are essential for generation of active phenoloxidase (PO) in some insects (Kwon et al., 2000; Yu et al., 2004).

Insect POs catalyze the formation of quinones and other reactive intermediates for melanin synthesis, microbe killing, cuticle sclerotization, and wound healing (Ashida and Brey, 1997; Söderhäll and Cerenius, 2004). They are produced as inactive zymogens and proteolytically activated by proPO activating proteinase (PAP, also known as PPAE or PPAF). PAPs have been isolated from several insects and characterized biochemically (Satoh et al., 1999; Jiang et al., 1998, 2003a and 2003b; Lee et al., 1998). In *Manduca sexta*, these enzymes cleave proPO at the correct bond but yield little PO activity. Only when a high M_r complex of SPH-1 and SPH-2 are present at the same time, does PAP generate active PO (Wang and Jiang, 2004; Gupta et al., 2005). In *Holotrichia diomphalia*, proPO are activated by PPAFI (a serine proteinase) and PPAFII (an SPH) (Lee et al., 1998). In *Drosophila melanogaster*, MP1 cuts proMP2 and MP2 activates proPO (Tang et al., 2006; Leclerc et al., 2006). In *Anopheles gambiae*, Clip-B3, -B4, -B8, -B14, and -B17 play a role in the proPO activation pathway (Volz et al., 2005 and 2006; Paskewitz et al., 2006). Clip-A8 (an SPH) is required for parasite melanization whereas Clip-A2, -A5 and -A7 (SPHs) function synergistically to block this process.

While components of the proPO activation system (*e.g.* pathogen recognition proteins, serine proteinases, SPHs, serpins and proPOs) have been elucidated in several insects (Kanost et al., 2004; Christophides et al., 2004; Lee and Iwanaga, 2005), limited information is available about the proteolytic activation of proSPHs: *H. diomphalia* PPAFIII is the only serine proteinase shown to cleave proPPAFII (Kim et al., 2002). In order to explore the proSPH activation branch of *M sexta* proPO activation system, we produced proSPH-1 and proSPH-2 in a baculovirus-

insect cell expression system. The purified proteins, after being cleaved by column fractions of the induced larval plasma, manifested an auxiliary effect in the proPO activation reaction. Coexistence of SPH-1 and SPH-2 is essential for this cofactor activity.

Methods and materials

Modification of pFastBacDual

The plasmid pFastBacDual (Invitrogen Life Technology), which allows co-expression of two genes, was modified for efficient secretion and affinity purification of the recombinant proteins (Fig. 1). As described previously for pMH_F (*i.e.* pMHF6) (Lu and Jiang, 2007), pM_{FBDpH} was constructed by inserting a synthetic DNA fragment into the *Bam*HI-*Eco*RI sites of pFastBacDual. After in-frame insertion of a foreign DNA into the *Eco*RI site of pM_{FBDpH}, the encoded polypeptide is expected to be synthesized under the control of polyhedrin promoter and secreted into the medium using the honeybee melittin signal peptide.

The plasmids pMM_{FBD} and pM_{FBDp10} were constructed as follows: 1) amplification the signal peptide-coding region in pMFH6 using vector-specific primers j023 (5'-TTCCGGATTAT TCATACC, + strand) and j044 (5'-CCATGGATCGATCCCGGGCATAGATGTAAGAAATG, - strand). Primer j044 includes *Nco*I, *Cla*I and *Sma*I restriction sites fused with the reverse complement sequence encoding Ile-Ser-Tyr-Ile-Tyr-Ala, the end of the signal peptide; 2) T/A cloning and sequence verification of the PCR product; 3) Insertion of the *Bam*HI-*Nco*I fragment into pM_{FBDpH} and pFastBacDual digested with *Bbs*I and *Nco*I – in this case *Bbs*I cleavage left an overhang compatible with the *Bam*HI site. The resulting plasmids (pMM_{FBD} and pM_{FBDp10}) (Fig. 1) allows co-expression of two polypeptides under the control of polyhedrin and p10 promoters. In-frame cloning of two different coding regions to the *Eco*RI (pH side) and *Cla*I (p10 side) sites of pMM_{FBD} allows the secretion of corresponding proteins into the culture medium.

Following sequence verification, pMM_{FBD} and pM_{FBDp10} were improved by incorporating a synthetic DNA fragment to the *Kpn*I site: primers j045 (5'-CGGGCCCATCACCATCACCATC ACTAAGTAC, + strand) and j046 (3'-CATGGCCCGGGTAGTGGTAGTGGTAGTGATT-5', - strand) were phosphorylated, annealed, and non-directionally inserted to *Kpn*I-digested and dephosphorylated vectors. After sequence analysis, plasmids containing a single copy of the fragment in the correct orientation (designated $pMMH_{FBDp10}$ and pMH_{FBDp10}, one of the two *Kpn*I sites destructed and one *Apa*I site added in the p10 side) (Fig. 1) were kept. In-frame insertion at the *Apa*I site is required for fusing the hexahistidine tag to the carboxyl-terminus of a target recombinant protein.

The multiple cloning region downstream of the polyhedrin promoter in pMH_{FBDp10} and pFastBacDual was modified by inserting the *Bam*HI-*Hin*dIII fragment of pMH_F (*i.e.* pMFH6) to the same sites to generate $pMHMH_{FBD}$ and pMH_{FBDpH} (Fig. 1). In-frame insertion of one DNA fragment to the *Eco*RI and *Xho*I sites and another to the *Cla*I and *Apa*I sites of $pMHMH_{FBD}$ allows co-expression, secretion, and affinity purification of two proteins.

Construction of recombinant baculoviruses for proSPH-1 and proSPH-2 expression

M. sexta SPH-1 cDNA was amplified using j410 (5'-CTGAATTCAGTCCGAAGATCT, + strand) and j411 (5'-GTCCTCGAGTTCGTAAACCGT, - strand). The PCR product was T/A cloned into pGem-T (Promega) and verified by DNA sequencing. From the resulting plasmid, a 1.2 kb *Eco*RI-*Xho*I fragment was retrieved by partial digestion (because there was an internal *Xho*I site in the cDNA) and inserted into the same sites in $pMHMH_{FBD}$ to generate SPH-1/ $pMHMH_{FBD}$. *M. sexta* SPH-2 cDNA was amplified using j412 (5'-GCTCATCGATCCACTATC GAC, + strand) and j413 (5'-CTGGGCCCCGTAAGTGGAGCT, - strand). After T/A cloning and sequence confirmation, the *Cla*I-*Apa*I fragment was subcloned into the same sites in $pMHMH_{FBD}$ and SPH-1/$pMHMH_{FBD}$ to yield SPH-2/$pMHMH_{FBD}$ and SPH-1&2/$pMHMH_{FBD}$,

respectively. For this cloning step, the parental plasmids were isolated from *E. coli* GM2163 (*F*$^-$, *dam*$^-$, *dcm*$^-$, *hsdR*$^-$, *chloramphenicol*^{r}) so that the *Cla*I site was not methylated.

In vivo transposition of the expression cassette, selection of bacterial colonies carrying the recombinant bacmid, and isolation of bacmid DNA were performed according to manufacturer's protocols (Invitrogen Life Technologies). The initial viral stocks (V_0) for proSPH-1 and proSPH-2 production were separately obtained by transfecting *Spodoptera frugiperda Sf*21 cells with a bacmid DNA–CellFECTIN mixture, and their titers were improved through serial infections. The V_6 viral stock, containing the highest level of baculovirus, was stored at -70°C for further experiments. Expression conditions were optimized as described previously (Ji et al., 2003).

Analysis of proSPH-1 and proSPH-2 expressed individually and mutually

*Sf*21 cells in 5.0 ml insect serum-free medium (Invitrogen Life Technologies) (7.6×10^5 cells/ml, T-25 flask) were infected with 0.5 ml V_3 viral stock at 27°C for 72 h. The recombinant proteins were captured from 0.5 ml of V_4 culture medium using 50 µl of Ni-NTA beads. After washing with 150 µl of buffer A (50 mM sodium phosphate, pH 8.0) containing 0.5 M NaCl for three times, bound proteins were eluted with 50 µl of 0.3 M imidazole in the washing buffer for three times and combined for electrophoretic analysis on native or denatured gels. The proteins were visualized by Coomassie blue staining or immunoblotting using SPH-1 or SPH-2 antibodies.

Large-scale expression and purification of recombinant proSPH-1 and proSPH-2 from Sf21 cells

*Sf*21 cells (at 2.4×10^6 cells/ml) in 1.4 L of insect serum-free medium (Invitrogen Life Technologies) were separately infected with the baculovirus stocks at a multiplicity of infection of 10 and grown at 27°C for 96 h with gentle agitation (100 rpm). After the cells were removed by centrifugation at 5,000×*g* for 10 min, pH of the conditioned medium was adjusted to 8.0 using 1.0 M Tris base. The cell debris and fine particles were spun down by centrifugation at 10,000×*g*, and the supernatant was diluted with 20 mM Tris-HCl, pH 8.0 (buffer A) to a final

volume of 4.2 L and applied to a Q-Sepharose FF column (20 ml bed volume) at a flow rate of 5.0 ml/min. Following a washing step with 100 ml buffer A, bound proteins were eluted from the column with a linear gradient of 0-1.0 M NaCl in 240 ml of buffer A at a flow rate of 1.0 ml/min. The fractions containing proSPH-1 or proSPH-2 were combined and loaded onto a 10 ml Ni^{2+}-NTA agarose column. After washing with 50 ml of 50 mM sodium phosphate, pH 8.0 (buffer B), the bound proteins were eluted with a gradient of 0-0.3 M imidazole in 90 ml of buffer B. Fractions containing proSPH-1 or proSPH-2 were combined, dialyzed against 20 mM Tris–HCl (pH 7.6) and stored at -80°C.

Insect rearing, bacterial challenge, and hemolymph collection

M. sexta eggs were purchased from Carolina Biological Supply, and the larvae were reared on an artificial diet (Dunn and Drake, 1983). Day 2, 5^{th} instar larvae were injected with a mixture of formaldehyde-killed *E. coli* (3×10^7 cells), *M. luteus* (30 µg) and curdlan (30 µg) in 50 µl of H_2O. Hemolymph was directly collected into saturated $(NH_4)_2SO_4$ from cut prolegs of the larvae 24 h after the immune challenge. The final saturation of $(NH_4)_2SO_4$ was adjusted to 50% in order to prevent spontaneous melanization, and the protein suspension was stored in -80°C.

Fractionation of M. sexta induced hemolymph on hydroxyapatite and gel filtration columns

The thawed hemolymph suspension was centrifuged at 22,000×*g* for 20 min, and the pellet was dissolved with 60 ml of buffer C (5 mM sodium phosphate, pH 6.5, 0.5 M NaCl) supplemented with 0.002% 1-phenyl-2-thiourea. After centrifugation, the supernatant was fractionated with 20-50% $(NH_4)_2SO_4$ as described previously (Jiang et al., 2003). This fraction was dissolved in 60 ml of buffer C and dialyzed against the same buffer (1.2 L) overnight. Following centrifugation at 22,000×*g* for 20 min, the protein sample was loaded onto a hydroxyapatite column (2.5 cm *i.d.* × 8 cm) equilibrated with buffer C. After washing with 150 ml of the same buffer, bound proteins were eluted with a linear gradient of 0-125 mM sodium phosphate, pH 6.5 in 200 ml, 0.5 M

NaCl. The fractions (4.0 ml/tube) were collected for the proSPH processing assays. Active fractions were pooled and precipitated with 50% saturated $(NH_4)_2SO_4$. After centrifugation, the pellet was dissolved in 3.0 ml of buffer D (50 mM Tris-HCl, 0.5 M NaCl, pH 7.5) and applied to Sephacryl S-100 column (2.5 cm *i.d.* × 100 cm) equilibrated with the same buffer. The fractions were collected at 6 ml/tube for the activity assays.

Processing of proSPH-1 and proSPH-2 by the column fractions

Column fractions (3 µl) were individually incubated on ice with the purified proSPH-1 (0.140 mg/ml, 2 µl), proSPH-2 (0.120 mg/ml, 2 µl), or both (2 µl + 2 µl) in a total volume of 40 µl, 20 mM Tris-HCl, pH 7.6. After 60 min incubation, the reaction mixtures were treated with 10 µl, 5 × SDS sample buffer at 95°C for 5 min, separated by 10% SDS polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and reacted with 1:5000 diluted SPH-1 or SPH-2 antiserum. The antibody-antigen complex was recognized by goat-anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad), and chemiluminescence emitted from the hydrolysis of SuperSignal West Pico Chemiluminescent substrate (Pierce) was detected by X-ray film exposure and development.

ProPO activation and PO activity assay

PO activity was determined on a microplate reader according to Jiang et al (2003). Briefly, *M. sexta* proPO (1 µl, 40 µg/ml) and a column fraction (3 µl) were incubated on ice with proSPH-1 (2 µl), proSPH-2 (2 µl), or both (2 µl + 2 µl) in a total volume of 40 µl Tris-HCl, pH 7.6. Dopamine (150 µl, 2.0 mM) was added to the reaction mixtures 60 min later and PO activity was measured immediately.

Results and discussion

Construction of a series of expression vectors

In order to facilitate expression, secretion and purification of recombinant proteins in the baculovirus-insect cell expression system, we have developed a series of Bac-to-Bac plasmids based on pFastBacDual. With expanded multiple cloning regions (Fig. 1), one or more of these vectors can be easily selected to meet special requirements of the protein production. For instance, co-expression of two proteins using a single recombinant virus is expected to enhance cell infection and reduce inclusion body formation caused by the lack of partner. Introduction of the honeybee melittin signal peptide may increase the expression of proteins or their constituents which do not contain a secretion peptide. As demonstrated previously (Jarvis et al., 1993), this leader peptide can also be used for producing proteins with an inefficient or incompatible signal peptide. The classical secretory pathway allows the correctly folded and properly modified proteins to enter an oxidized environment, away from the majority of cellular proteins. Moreover, the fusion of carboxyl-terminal hexahistidine tag facilitates the capture of recombinant proteins from a large volume of conditioned medium by affinity chromatography.

Purification and characterization of proSPH-1 and proSPH-2 from the insect cells

We have constructed three plasmids (SPH-1/pMHMH$_{FBD}$, SPH-2/pMHMH$_{FBD}$ and SPH-1&2/pMHMH$_{FBD}$) and generated respective baculoviruses. *Sf*21 infected with these viruses produced proSPH-1, proSPH-2 and both proSPHs at a high level. The secreted proteins were separately isolated from the culture media by nickel affinity chromatography (Fig. 2). The purified proteins migrated to about 60 and 50 kDa positions on the SDS-polyacrylamide gel. Immunoblot analysis showed SPH-2 antibodies weakly reacted with proSPH-1 also. On the native gel, proSPH-2 ran as two defused bands slower than proSPH-1 which showed as a singlet. The co-expressed proteins had the same migration patterns as the individual ones, suggesting there was no strong association between proSPH-1 and proSPH-2 under the experimental conditions. Since proSPH-1 level was unstable during large-scale co-expression tests (data not

shown) and there was no apparent association between the SPH precursors, we chose to produce the two proteins separately and carry out activation assay using one or both of them.

Expression of proSPH-1 and proSPH-2 in insect cells

As shown in Fig. 3 and Fig. 4, the recombinant proteins were soluble and secreted into the cell culture medium. After removing cells, we captured the proSPHs by ion exchange chromatography and eluted them from the Q-Sepharose column in a small volume. The proSPH fractions were pooled and loaded on the Ni-NTA agarose columns. A linear gradient of imidazole was applied to elute the proteins. Silver staining and immunoblot analysis following SDS-polyacrylamide gel electrophoresis indicated that the affinity purified proteins were essentially pure and intact. ProSPH-1 ran as a 60 kDa band whereas proSPH-2 had an apparent M_r of 52 kDa. Heterogeneity in glycosylation may be responsible for the band broadening. While a streak was observed behind the diffused bands of proSPHs, the majority of the proteins migrated deeply into the native polyacrylamide gel, suggesting the precursors were mostly in a low association state. Consistent with that, most of the purified proSPH-1 or proSPH-2 mobilized on the HPLC size exclusion column as a single peak. While proSPH-1 eluted at 9.40 min, corresponding to a calculated M_r of 78.5 kDa, proSPH-2 associated with the column matrix: with an elution time of 13.33 min, its apparent M_r was 6 kDa. These results indicated that the proSPHs probably existed as a monomer.

Detection of a proSPH-1 and proSPH-2 processing activity in the induced plasma

Using the purified proSPHs as substrates, we attempted to isolate their activating enzyme from the induced *M. sexta* hemolymph. After ammonium sulfate fractionation, hydroxyapatite and gel filtration chromatography, we found that certain fractions cleaved proSPH-1 and proSPH-2 (Fig. 5). The processing of proSPH1 was incomplete, and we only detected its 38 kDa proteinase-like domain using SPH-1 antibodies. These column fractions contained PAP-1, hemolymph

proteinase 1 (HP1) and small amounts of other HPs (data not shown). So far, we have not yet been able to show if one or more of these enzymes are responsible for proSPH-1 cleavage. The same column fractions also cleaved proSPH2, yielding a 37 kDa heavy chain and a 16 kDa light chain. While the latter was identical in migration rate to the amino-terminal fragment of SPH-1 from *M. sexta* hemolymph (Wang et al., 2004), the 37 kDa band is slightly larger than the carboxyl-terminal fragment of natural SPH-1 (36 kDa). This small difference may be caused by the carboxyl-terminal histidine tag in the recombinant protein.

Proteolytically processed SPH-1 and SPH-2 precursors as a cofactor for proPO activation

After the hydroxyapatite column fraction and proPO were incubated with one proSPH, we did not detect much PO activity (Fig. 6) even though proSPH-1 was cleaved by the fractions. Similar results were obtained when a Sephacryl S-100 column fraction was used. However, after both proSPH-1 and proSPH-2 were incubated with the column fraction, we observed a large increase in proPO activation. While this increase was likely resulted from cleaved SPH-1 and SPH-2, we could not rule out the possibility that one SPH and the other proSPH (*e.g.* SPH-1 and proSPH-2) have the auxiliary effect. Therefore, we examined if the simultaneous presence of cleaved SPH-1 and SPH-2 is required for this cofactor activity. The inclusion of proSPH-2 to cleaved SPH-1 and PO, or adding proSPH1 to processed SPH-2 and PO, only generated a low level of PO activity (Fig. 7). The processed SPH1 and SPH2 function synergistically to enhance proPO activation.

In the comparison with the complex of SPH-1 and SPH-2 from the hemolymph, we noticed that the cofactor activity generated by proSPH-1 and proSPH-2 cleavage activation was much lower than that of the same amounts of SPHs isolated from the hemolymph (Fig. 8A and 8B). We examined the migration behaviors of processed proSPH-1 and proSPH-2 and found that they had a high mobility on the native polyacrylamide gel (Fig. 8C). In contrast, the plasma SPH complex has a high M_r and migrated slightly into the stacking gel (Wang and Jiang, 2004). We speculate

that the lower cofactor activity from SPH-1 and SPH-2 generated *in vitro* was caused by the failure to form of a higher M_r complexes. Perhaps, certain plasma proteins facilitate the formation of SPH complexes.

In summary, we produced *M. sexta* proSPH1 and proSPH2 using an improved baculovirus-insect cell expression system. The purified proteins were functional as substrates in the search of their activating proteinases. We observed their proteolytic processing by column fractions, and the cleaved SPHs were active as a cofactor for proPO activation. Furthermore, we demonstrated that the coexistence of processed SPH-1 and SPH-2 is required for the auxiliary effect.

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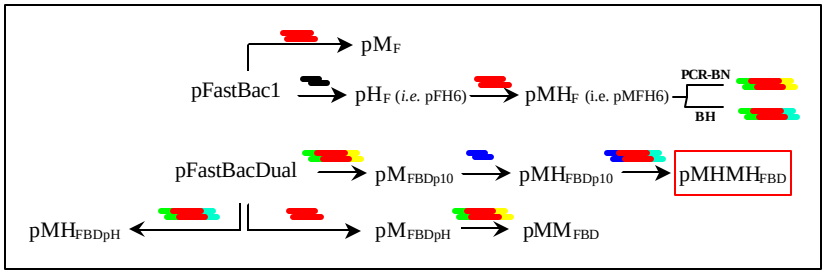
(**A**)



(**B**) **pFastBacDual** Multiple cloning site (MCS) downstream of the polyhedrin (pH) promoter:

```
CCTATAAATATTCCGGATTA TTCATACCGTCCCACCATCG GGCGCGGATCCCGGTCCGAA GCGCGCGGAATTCAAAGGCC TACGTCGACGAGCTCACTAG
          j023                            BamHI   RsrII   BssHII  EcoRI   StuI     SalI  SstI  SpeI
TCGCGGCCGCTTTCGAATCT AGAGCCTGCAGTCTCGACAA GCTTGTCGAGAAGTACTAGA GGATCATAATCAGCCATACC ACATTTGTAGAGGTTTTACT
   NotI     NspV     XbaI  PstI        HindIII                  SV40 poly(A) signal     j024
```

MCS downstream of the p10 promoter:

```
TTAAATAAGAATTATTATCA AATCATTTGTATATTAATTA AAATACTATACTGTAAATTA CATTTTATTTACAATCACTC GACGAAGACTTGATCACCCG
    +1                                                                j047          BbsI        SmaI
GGATCTCGAGCCATGGTGCT AGCAGCTGATGCATAGCATG CGGTACCGGGAGATGGGGGA GGCTAACTGAAACACGGAAG GAGACAATACCGGAAGGAAC
     XhoI   NcoI  NheI  PvuII NsiI  SphI    KpnI            j048              HSV tk poly(A) signal
```

(**C**) **pM$_{FBDpH}$** MCS downstream of the pH promoter (no change in the p10 side, same as (**B**)):

```
TTCATACCGTCCCACCATCG GGCGCGGATCCATGAAATTC TTAGTCAACGTTGCCCTTGT ATCTATGGTCGTATACATTT CTTACATCTATGCCGGAATT
 j023                    BamHI  M  K  F   L  V  N  V  A  L  V   F  M  V  V  Y  I   S  Y  I  Y  A* G  I
                                                                                              EcoRI
CAAAGGCCTACGTCGACGAG CTCACTAGTCGCGGCCGCTT TCGAATCTAGAGCCTGCAGT CTCGACAAGCTTGTCGAGAA GTACTAGAGGATCATAATCA
    StuI    SalI   SstI   SpeI   NotI     NspV  XbaI   PstI        HindIII                SV40 poly(A)
```

(**D**) **pMH$_{FBDpH}$** MCS following the pH promoter (no change in the p10 side, same as (**B**)):

```
TTCATACCGTCCCACCATCG GGCGCGGATCCATGAAATTC TTAGTCAACGTTGCCCTTGT ATCTATGGTCGTATACATTT CTTACATCTATGCCGGAATT
 j023                    BamHI  M  K  F   L  V  N  V  A  L  V   F  M  V  V  Y  I   S  Y  I  Y  A* G  I
                                                                                              EcoRI
CAAAGGCCTACGTCGACGAG CTCACTAGTCGCGGCCGCTT TCGAATCTAGAGCCTGCAGT CTCGAGCATCACCATCACCA TCACTAAGCTTGTCGAGAAG
    StuI    SalI   SstI  SpeI    NotI     NspV  XbaI   PstI   XhoI   H  H  H  H  H   H  * HindIII
TACTAGAGGATCATAATCAG CCATACCACATTTGTAGAGG
       SV40 poly(A) signal     j024
```

(**E**) **pM$_{FBDp10}$** MCS following the p10 promoter (no change in the pH side, same as (**B**)):

```
CATTTTATTTACAATCACTC GACGAAGACTTGATCCATGA AATTCTTAGTCAACGTTGCC CTTGTATCTATGGTCGTATA CATTTCTTACATCTATGCCC
        j047            BbsI        M   K  F  L  V  N  V  A   L  V  F  M  V  V  Y   I  S  Y  I  Y  A*
GGGATCGATCCATGGTGCTA GCAGCTGATGCATAGCATGC GGTACCGGGAGATGGGGGAG GCTAACTGAAACACGGAAGG AGACAATACCGGAAGGAACC
SmaI ClaI NcoI   NheI  PvuII NsiI  SphI    KpnI            j048              HSV tk poly(A) signal
```

(F) pMH$_{FBDp10}$ MCS downstream of the p10 promoter (no change in the pH side, same as (**B**)):

```
CATTTTATTTACAATCACTC GACGAAGACTTGATCCATGA AATTCTTAGTCAACGTTGCC CTTGTATCTATGGTCGTATA CATTTCTTACATCTATGCCC
                      BbsI        M   K  F  L  V  N  V  A   L  V  F  M  V  V  Y   I  S  Y  I  Y  A*
GGGATCGATCCATGGTGCTA GCAGCTGATGCATAGCATGC GGTACCGGGCCCATCACCAT CACCATCACTAAGTACCGGG AGATGGGGGAGGCTAACTGA
SmaI ClaI NcoI   NheI  PvuII NsiI  SphI    KpnI  ApaI                                    j048
R  D  R  S  M                               Y  R  A  H  H  H   H  H  H  *
```

(**G**) **pMHMH$_{FBD}$** MCS downstream of the PH promoter:

```
TTCATACCGTCCCACCATCG GGCGCGGATCCATGAAATTC TTAGTCAACGTTGCCCTTGT ATCTATGGTCGTATACATTT CTTACATCTATGCCGGAATT
 j023                    BamHI  M  K  F   L  V  N  V  A  L  V   F  M  V  V  Y  I   S  Y  I  Y  A* G  I
                                                                                              EcoRI
CAAAGGCCTACGTCGACGAG CTCACTAGTCGCGGCCGCTT TCGAATCTAGAGCCTGCAGT CTCGAGCATCACCATCACCA TCACTAAGCTTGTCGAGAAG
    StuI    SalI   SstI  SpeI    NotI     NspV  XbaI   PstI   XhoI   H  H  H  H  H   H  * HindIII
TACTAGAGGATCATAATCAG CCATACCACATTTGTAGAGG TTTTACTTGCTTTAAAAAAC CTCCCAC
```

MCS downstream of the p10 promoter:

```
CATTTTATTTACAATCACTC GACGAAGACTTGATCCATGA AATTCTTAGTCAACGTTGCC CTTGTATCTATGGTCGTATA CATTTCTTACATCTATGCCC
                      BbsI        M   K  F  L  V  N  V  A   L  V  F  M  V  V  Y   I  S  Y  I  Y  A *
GGGATCGATCCATGGTGCTA GCAGCTGATGCATAGCATGC GGTACCGGGCCCATCACCAT CACCATCACTAAGTACCGGG AGATGGGGGAGGCTAACTGA
SmaI ClaI NcoI   NheI  PvuII NsiI  SphI    KpnI  ApaI                                    j048
R  D  R  S  M                               Y  R  A  H  H  H   H  H  H  *
```

(**H**) **pH$_F$** (previously **pFH6**, modified from pFastBac1, MCS downstream of the pH promoter):

```
AAATATTCCGGATTATTCAT ACCGTCCCACCATCGGGCGC GGATCCCGGTCCGAAGCGCG CGGAATTCAAAGGCCTACGT CGACGAGCTCACTAGTCGCG
          j023                           BamHI Rsr?      BssH?  EcoRI     StuI    SalI   SstI   SpeI
GCCGCTTTCGAATCTAGAGC CTGCAGTCTCGAGCATCACC ATCACCATCACTAAGCTTGT CGAGAAGTACTAGAGGATCA TAATCAGCCATACCACATTT
NotI   NspV  XbaI     PstI   XhoI  H  H   H  H  H  H  * Hind?                SV40 poly(A) signal   j024
```

(**I**) **pMH$_F$** (previously **pMFH6**, modified from pFastBac1, MCS downstream of the pH promoter):

```
AAATATTCCGGATTATTCAT ACCGTCCCACCATCGGGCGC GGATCCATGAAATTCTTAGT CAACGTTGCCCTTGTTTTTA TGGTCGTATACATTTCTTAC
          j023                           BamHI  M  K  F  L  V   N  V  A   L  V  F  M  V  V  Y  I  S  Y
ATCTATGCCGGAATTCAAAG GCCTACGTCGACGAGCTCAC TAGTCGCGGCCGCTTTCGAA TCTAGAGCCTGCAGTCTCGA GCATCACCATCACCATCACT
 I  Y  A* G  I EcoRI StuI   SalI   SstI   SpeI  NotI     NspV  XbaI    PstI   XhoI   H  H  H  H  H  H
AAGCTTGTCGAGAAGTACTA GAGGATCATAATCAGCCATA CCACATTTGTAGAGGTTTTA
* Hind?               SV40 poly(A) signal     j024
```

Fig. 1. Development of a series of Bac-to-Bac plasmid vectors for protein expression in baculovirus-infected insect cells. Cloning scheme (**A**); Sequences of the multiple cloning regions of pFastBacDual (**B**) (Invitrogen Life Technology), pH_F/pFH6 (**H**) (Ji et al., 2003) and pMH_F/pMFH6 (**I**) (Lu and Jiang, 2007) are included for comparison; (**C-G**): these vectors are useful for co-expressing two associating proteins under the control of late promoters (pH and p10) in a single baculovirus. For proteins or domains that lack a signal peptide, efficient secretion is expected from the honeybee melittin signal peptide fused with the protein(s) of interest. In-frame insertion of the coding sequence(s) allows a hexahistidine tag attached to the carboxyl terminus of expressed protein(s) for affinity purification.

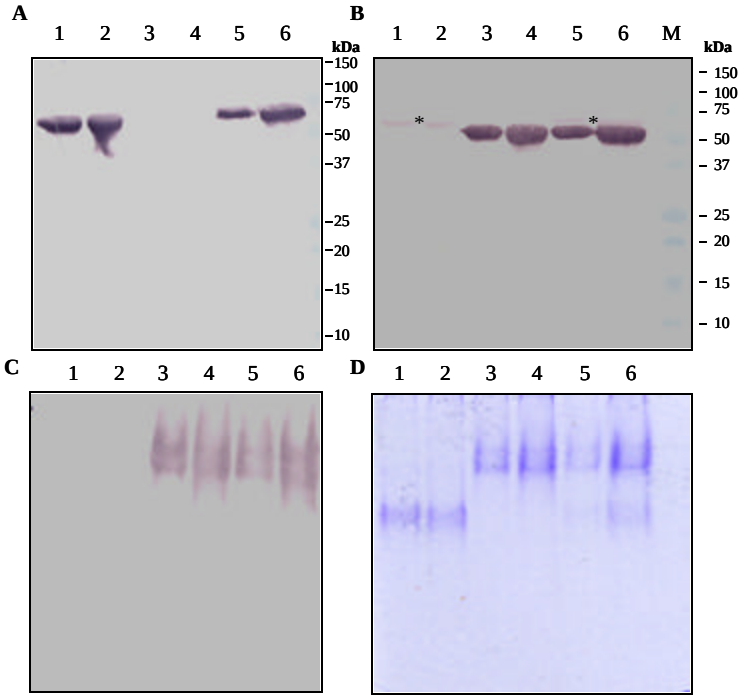


Fig. 2. Expression analysis of proSPH1 and proSPH2 in baculovirus-infected insect cells. Affinity-purified proSPH1, proSPH2 and co-expressed proSPHs were individually separated by 10% SDS-PAGE followed by immunoblotting using SPH-1 (**A**) or SPH-2 (**B**) antibodies. The same protein samples were resolved on 7.5% native polyacrylamide gels and detected using SPH-2 antibodies (**C**) or Coomassie blue staining (**D**). Lanes 1 and 2, proSPH-1 only; lanes 3 and 4, proSPH-2 only; lanes 5 and 6, both proSPHs. SPH-2 antibodies weakly recognize SPH-1 (*).

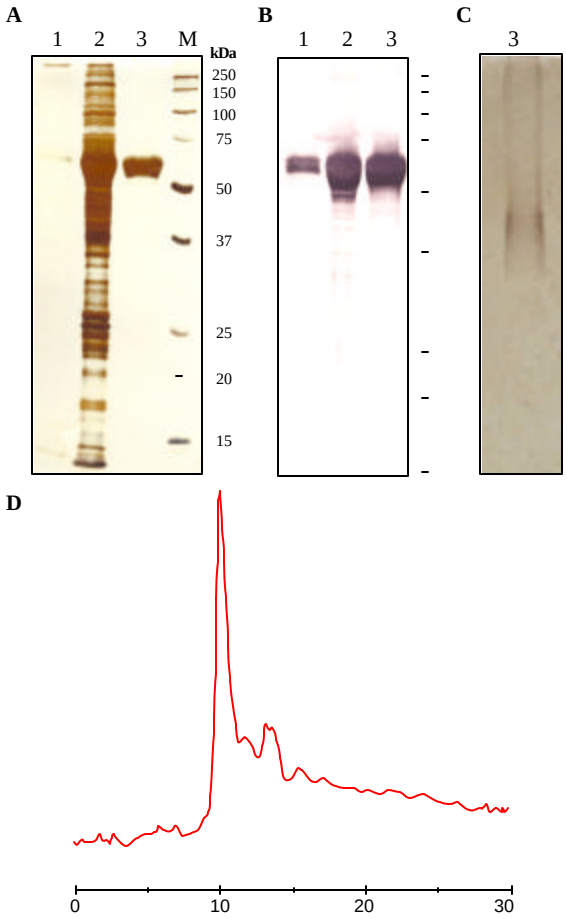


Fig. 3. Isolation of *M. sexta* proSPH-1 from the baculovirus-infected insect cells. (**A**) 10% SDS-PAGE and silver staining. (**B**) Immunoblot analysis using SPH-1 first antibody and goat-anti-rabbit IgG conjugated to alkaline phosphatase. (**C**) 7.5% native PAGE and silver staining. (**D**) Analysis of purified proSPH-1 on an HPLC size exclusion column. 1, conditioned cell culture medium; 2, proteins eluted from the Q-Sepharose column; 3, affinity-purified protein from the Ni-NTA agarose column; M, molecular weight markers.

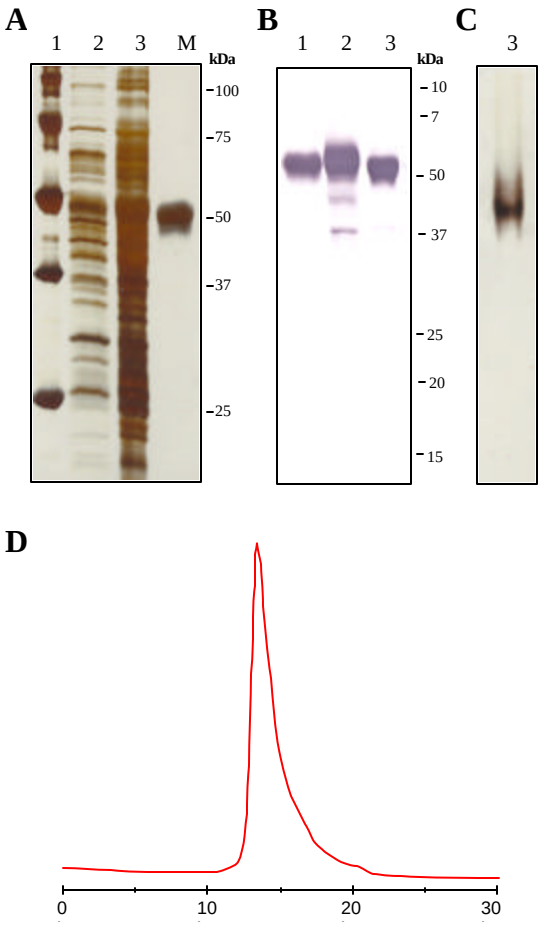


Fig. 4. Isolation of *M. sexta* proSPH-2 from the baculovirus-infected insect cells. (**A**) 10% SDS-PAGE and silver staining. (**B**) Immunoblot analysis using SPH-2 first antibody and goat-anti-rabbit IgG conjugated to alkaline phosphatase. (**C**) 7.5% native PAGE and silver staining. (**D**) Analysis of purified proSPH-2 on an HPLC size exclusion column. 1, conditioned cell culture medium; 2, proteins eluted from the Q-Sepharose column; 3, affinity-purified protein from the Ni-NTA agarose column; M, molecular weight markers.

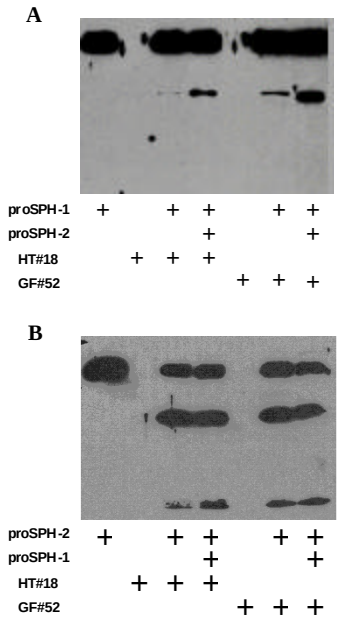


Fig. 5. Processing of the proSPHs by column fractions of the *M. sexta* hemolymph from larvae injected with bacteria. (**A**) Processing of proSPH-1 by the hydroxylapatite (*upper panel*, HT) and Sephacryl S-100 (*lower panel*, GF) column fractions. (**B**) Processing of proSPH-2 by the hydroxylapatite (*left* panel) and Sephacryl S-100 (right panel) column fractions.

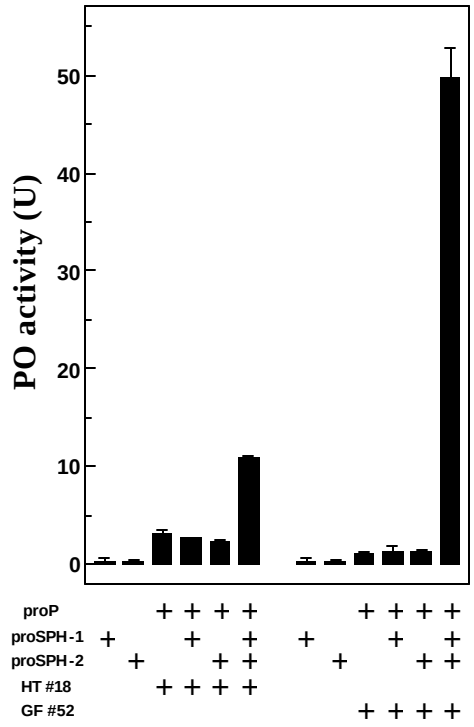


Fig. 6. Relationships between proPO activation and co-presence of proSPH-1 and proSPH-2 in the activation mixture. As described in the Methods and material, proPO and a column fraction were incubated with proSPH-1, proSPH-2, or both for 60 min. The PO activities in the reactions and controls are plotted in the bar graph as mean ± SEM (n = 3).

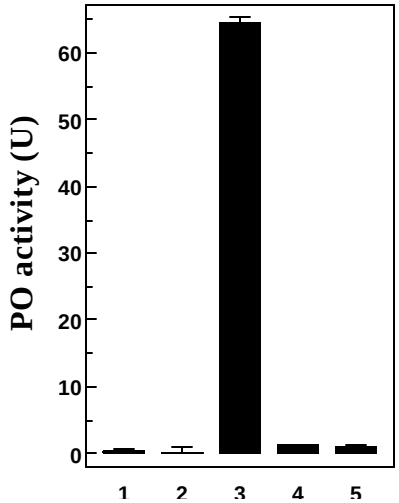


Fig. 7. Requirement of processed SPH-1 and SPH-2 both for the auxiliary effect in proPO activation. As described in the Methods and material, proPO and a gel filtration column fraction (GF#52) were incubated with proSPH-2 (**4**) or proSPH-1 (**5**) for 60 min. PO activities in the control group (**1**, proSPH-1 alone; **2**, proSPH-2 alone; **3**, proSPH-1 and proSPH-2 incubated with proPO and GF #52) and treatment group (**4**, proSPH-1; **5**, proSPH-2.

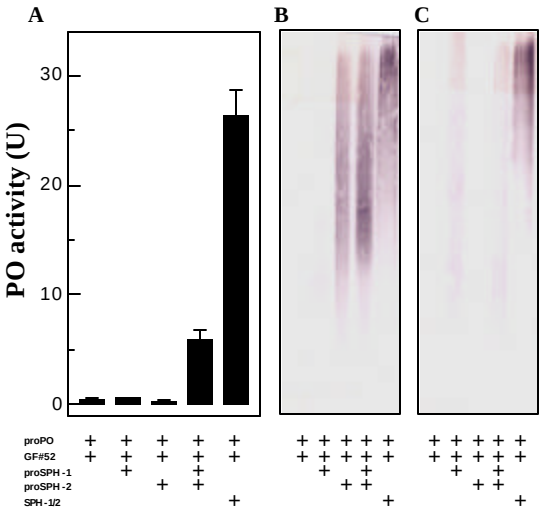


Fig. 8. Comparison of PO cofactor activity and associate satatus of processed proSPHs with SPH1/SPH2 from hemolymph. (**A**). PO activity assay. proSPH-1 (1 µl, 120 ng/µl), proSPH-2 (1 µl, 120 ng/µl), GF # 52 (1 µl), and proPO (1 µl, 40 ng/µl) were incubated on ice for 40 min. Or GF #52 (1 µl), proPO (1 µl, 40 ng/µl), and SPH1/SPH2 (3 µl, 35 ng/µl) were incubated on ice for 40 min. After the incubation, 150 µl of Dopamine (2 mM) was added followed by OD_{470} reading. Mobility of processed proSPH-1 (**B**) and of proSPH-2 (**C**) on 7.5 % native PAGE detected by proSPH-1 and proSPH-2 antisera, respectively.

CHAPTER V

REGULATION OF PHENOLOXIDASE ACTIVITY BY HIGH AND LOW MOLECULAR WEIGHT INHIBITORS FROM THE LARVAL HEMOLYMPH OF *MANDUCA SEXTA*

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Abbreviations:

PO and proPO, phenoloxidase and its precursor; PAP, proPO-activating proteinase; SPH, serine proteinase homolog; POI, phenoloxidase inhibitor; DsbC, *E. coli* protein disulfide isomerase; EST, expressed sequence tag; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; IPTG, isopropyl-ß-D-thiogalactopyranoside; NTA, nitriloacetic acid; PAGE, polyacrylamide gel electrophoresis; PTU, 1-phenyl-2-thiourea; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; CPC, cetylpyridinium chloride

Abstract

Insect phenoloxidases generate quinones and other reactive intermediates to immobilize and kill invading pathogens and parasites. Due to the presumed cytotoxicity of these compounds, phenoloxidase activity and its proteolytic activation have to be regulated as a local, transient reaction against nonself in order to minimize damage to the host tissues and cells. We identified a Manduca sexta cDNA encoding a polypeptide sequence with its carboxyl-terminal 33 residues similar to the housefly phenoloxidase inhibitor (POI). The recombinant POI, secreted into the Escherichia coli periplasmic space along with its fusion partner DsbC, was released by osmotic shock and isolated by nickel affinity chromatography. Following enterokinase digestion and protein separation, the POI was purified to near homogeneity in a soluble form which inhibited M. sexta phenoloxidase at a high concentration. We then produced the inhibitor using a modified baculovirus-insect cell system and isolated the glycoprotein from the conditioned medium. Deglycosylation coupled with inhibition assay revealed that O-glycosylation only moderately increased its inhibitory activity. While this led us to speculate the role of Tyr64 hydroxylation, we were unable to modify the recombinant protein with tyrosine hydroxylase or purify M. sexta POI (Tyr64dopa) from the larval plasma. Instead, we isolated a low Mr, heat-stable compound which strongly inhibited phenoloxidase. The wavelength of maximum absorbance is 257 nm for the inhibitor. These data suggest that the down-regulation of phenoloxidase activity in M. sexta is achieved by two mechanisms at least.

Key words: melanization, insect immunity, hemolymph protein, quinone cytotoxicity

Introduction

Tyrosinase-type phenoloxidases (POs) from insects are copper-containing enzymes that hydroxylate monophenols to o-diphenols (monophenol, oxygen, oxidoreductase, EC1.14.18.1) and oxidize o-diphenols to quinones (o-diphenol, oxygen, oxidoreductase, EC1.10.3.1) (Nappi and Christensen, 2005). Reactive quinones generated by POs polymerize to form eumelanin. Quinones may also crosslink nucleophilic compounds during wound healing and parasite encapsulation (Sugumaran, 1998; Lourenco et al., 2005). A recent study in Aedes aegypti suggested an essential role of POs in chorion melanization and hardening (Kim et al, 2005). While sclerotized cuticles effectively block most pathogens (Ashida and Brey, 1995), this physiochemical barrier is penetrated by others. Under such circumstances, melanization is often initiated in the plasma to reduce damage caused by the intruders and to impede their development. POs may also play a role in hemolymph coagulation, a process closely associated with wound healing (Li et al., 2002). A more recent study demonstrated that Drosophila PO was unnecessary for the preliminary soft clot formation, but was responsible for the clot hardening through crosslinking and melanization (Bidla et al., 2005).

Due to possible cytotoxicity of quinones generated during melanization, proPO activation and PO activity have to be tightly and elaborately regulated (Cerenius and Söderhäll, 2004). POs are synthesized by hemocytes as inactive zymogens. Upon wounding or infection, proPOs are activated via limited proteolysis through a serine proteinase cascade. Serpins regulate proPO activation by specific inhibition of the cascade components (Kanost et al., 2004). POI was first isolated from the housefly Musca domestica (Tsukamoto et al., 1992). It contains 38 amino acid residues stabilized by three

disulfide bonds. One of the two tyrosine residues, Tyr32, is hydroxylated to dopa that is crucial for its inhibitory activity (Daquinag et al., 1995). Sequence comparison indicated the disulfide linkage pattern of POI may be identical to that of ?-conotoxins from snails and spiders (Daquinag et al., 1999). M. sexta cuticle contains a high Mr PO inhibitor (Sugumaran and Nellaiappan, 2000). The heat-labile glycoprotein may inhibit insect, plant and fungal POs by forming detergent-resistant complexes. In addition, M. sexta quinone isomerase may form inactive complexes with POs (Sugumaran et al., 2000). The inhibition is reciprocal: POs also block the isomerase activities. Here, we report the production of M. sexta POI in E. coli and insect cells, and the isolation of a low Mr compound with strong inhibitory activity against M. sexta PO and mushroom tyrosinase.

Materials and methods

Insect rearing and hemolymph collection

M. sexta eggs were purchased from Carolina Biological Supplies, and larvae were reared on an artificial diet (Dunn and Drake, 1983). Hemolymph from the induced larvae was collected and stored according to Jiang et al (2003).

Expression and purification of M. sexta POI in E. coli

M. sexta POI cDNA (GenBank accession number: BE015616), kindly provided by Dr. Robertson at University of Illinois Urbana-Champaign, was amplified using j240 (5'-AGAGCCCGGGCAATTTGATAAGG-3') and j241 (5'-TTGTCGACCTATCCGGAGC-3'). After SrfI and SalI digestion, the amplified cDNA fragment was inserted to the same sites in pET40b (Novagen). The resulting plasmid,

designated POI/pET40b, was sequenced by the dideoxynucleotide termination method to verify the insert and design.

E. coli BL21(DE3) harboring the expression construct was cultured in 2 liter Luria-Bertani medium containing 100 μg/ml ampicillin and induced with 1 mM IPTG when A600 reached 0.7. After 4 h, the bacterial cells were harvested by centrifugation at 5,000×g for 10 min. The pellets were resuspended in 500 ml 20 mM Tris-HCl, 20% sucrose, 1 mM EDTA, pH 8.0 and stirred at room temperature for 10 min. Following centrifugation at 8,000×g for 10 min, the cells were resuspended in 500 ml ice-cold 5 mM MgSO4 and stirred at 4°C for 10 min. The periplasmic fraction was separated from the debris by centrifugation at 10,000×g for 20 min.

The recovered supernatant was applied to a 10 ml Ni2+-NTA agarose column equilibrated in 20 mM Tris-HCl, pH 8.0 at 1 ml/min. After washing, bound proteins were eluted with 30 ml of 250 mM imidazole in the equilibration buffer. The elution fractions were pooled and dialyzed against 20 mM Tris-HCl, 20 mM NaCl, 2 mM CaCl2, pH 7.6 (2.0 L each time for 12 h, twice). Enterokinase (40 U) (Novagen) was added to the dialyzed sample for specific cleavage between POI and its fusion partner DsbC. Following incubation at room temperature for 12 h with gentle agitation, the reaction mixture was loaded onto a Ni2+-NTA column to remove uncleaved fusion protein and DsbC. The flow-through fraction was collected and further separated by reverse phase HPLC on a C18 column (Bio-Rad). The POI peak was collected and lyophilized.

Expression and purification of M .sexta POI from insect cells

The plasmid pFH6 (Ji et al., 2003) was further improved for efficient secretion of recombinant proteins using honeybee melittin signal peptide. Briefly, j551 (5’-

GATCCATGAAATTCTTAGTCAACGTTG CCCTTG TTTTTATGGTCGTATACATTTCTTACATCTATGCCGG) and j552 (3'-GTACTTTAAGAA TCAGTTGCAACGGGAACAAAAATACCAGCATATGTAAAGAATGTAGATACGG CCTT AA) were phosphorylated, annealed, and inserted to the BamHI and EcoRI sites of pFH6 to generate pMFH6. Plasmid DNA from the resulting transformants was verified by sequence analysis to encode Met-Lys-Phe-Leu-Val-Asn-Val-Ala-Leu-Val-Phe-Met-Val-Val-Tyr-Ile-Ser-Tyr-Ile-Tyr-Ala*Gly-Ile, where * denotes the signal peptidase cleavage site. The POI cDNA was amplified by PCR using j274 (5'-AGAATTCTAAGGGACGGAGTTGA-3') and j275 (5'-TCTC GAGTCCGGAGCCAGACAC-3'). After T/A cloning and sequence confirmation, the cDNA fragment was retrieved by EcoRI-XhoI digestion and inserted to the same sites in pMFH6. In vivo transposition of the expression cassette, selection of colonies carrying the recombinant bacmid, and isolation of bacmid DNA were performed according to manufacturer's protocols (Invitrogen Life Technologies). The initial viral stock (V0) was obtained by transfecting Spodoptera frugiperda Sf21 cells with a DNA–CellFECTIN mixture, and its titer was improved through serial infections. The V6 viral stock, containing the highest level of baculovirus, was stored at -70°C for further experiments. Expression conditions were optimized as described previously (Ji et al., 2003).

Sf21 cells (800 ml, 2.0×106/ml) in Ultimate Insect serum-free medium (Invitrogen Life Technologies) were infected with the recombinant baculovirus at a multiplicity of infection of 5 and grown at 27°C for 96 h with gentle agitation (100 rpm). The conditioned cell culture medium was recovered after centrifugation at 5,000×g for 10

min. Following pH adjustment to 7.4 with 1 M Na2HPO4, the medium was applied onto a Ni2+-NTA agarose column (15 ml), equilibrated with 50 mM phosphate buffer, 0.5 M NaCl, 10 mM imidazole, pH 7.4. After washing with 90 ml of the same buffer, bound proteins were eluted from the column with a linear gradient of 10-500 mM imidazole in the phosphate buffer (300 ml). The fractions exhibiting PO inhibitory activity were combined and separated by reverse phase HPLC as described above. Protein peaks were collected manually for the inhibition assay. The POI fractions were combined, lyophilized, and dissolved in 100 µl of 20 mM Tris-HCl, pH 7.5.

Isolation of a low Mr phenoloxidase inhibitor from M. sexta hemolymph

The hemolymph from day 3, 5th instar M. sexta larvae injected with a mixture of formaldehyde-killed E. coli (3 x 107 cells), M. luteus (30 µg), and curdlan (30 µg) in 50 µl of H2O (Jiang et al., 2003) was collected into an equal volume of anticoagulation buffer (Ma and Kanost, 2000). After incubation in a 70°C water bath for 10 min with agitation, the protein sample was chilled on ice and centrifuged at 39,200×g for 20 min. The supernatant was applied to a reverse phase HPLC column equilibrated with 5% acetonitrile, 0.1% trifluoroacetic acid (TFA). A linear gradient of 5-30% acetonitrile in 0.1% TFA was applied to the C18 column at a flow rate of 1.0 ml/min for 30 min with absorbance monitored at 214 nm. Automatically collected fractions (500 µl/tube) were lyophilized and dissolved in 200 µl, 20 mM Tris-HCl, pH 7.5. Fractions with PO inhibitory activity were pooled and further separated on the same column using 10% methanol as the mobile phase. The collected fractions were lyophilized, dissolved in 20 mM Tris-HCl, pH 7.5, and used for PO inhibitory activity assay and other analyses.

Determination of phenoloxidase inhibitory activity

To test the recombinant POI, PO was generated by incubating M. sexta proPO (1.0 µl, 0.3 µg/µl) with proPO-activating proteinase-1 (PAP-1)(2.0 µl, 30 ng/µl), serine proteinase homolog-1 and -2 (SPHs)(2.0 µl, 50 ng/µl), and 20 mM Tris-HCl, pH 7.5 (43 µl) in a 96-well microtiter plate on ice for 30 min (Jiang et al., 2003). The protein inhibitor (2.0 µl, 30, 60, 90, 120, 150, and 180 ng/µl) was then reacted with PO at 0°C for 10 min prior to PO activity assay (Jiang et al., 2003). To test the low Mr inhibitor, M. sexta proPO (3.0 µl, 0.3 µg/µl) or mushroom tyrosinase (3.0 µl, 100 ng/µl) (Sigma) and 20 mM Tris-HCl, pH 7.5 (46 µl) was mixed with 1.0 µl of the compound at different dilutions. The residual activities were measured using dopamine as a substrate (Jiang et al., 2003) in the presence (for PO) or absence (for mushroom tyrosinase) of 0.2% cetylpyridinium chloride (CPC).

Trypsin and chymotrypsin digestion of PO inhibitors

The low Mr inhibitor (3 µl) or recombinant POI (6 µl, 160 ng/µl) from E. coli were incubated with 1 µl of trypsin or chymotrypsin (1.0 mg/ml, Sigma) in a total volume of 30 µl at 37°C for 8 h. After heating at 100°C for 5 min to inactivate the proteinase, 10 µl of the reaction mixtures were separately mixed with 40 µl of induced larval plasma (1:40 diluted in 20 mM Tris-HCl, pH 7.5). Residual PO activity was determined using 100 µl, 2 mM dopamine solution containing 0.2% CPC (Jiang et al., 2003).

2.7 Expression analysis by RT-PCR – The RNA sample (2-4 µg), oligo(dT) (0.5 µg) and dNTPs (1 µl, 10 mM each) were mixed with diethylpyrocarbonate-treated H2O in a final volume of 12 µl, denatured at 65°C for 5 min, and quickly chilled on ice for 3 min. M-MLV reverse transcriptase (1 µl, 200 U/µl, Invitrogen), 5×buffer (4 µl), 0.1 M dithiothreitol (2 µl), and RNase OUT (1 µl, 40 U/µl, Invitrogen) were added to the

denatured RNA sample (12 µl) for cDNA synthesis at 37°C for 50 min. The M. sexta ribosomal protein S3 mRNA was used as an internal control to normalize the cDNA samples in a PCR using primers 501 (5'-GCCGTTCTTGCCCTGTT-3') and 504 (5'-CGCGAGTTGACTTCGGT-3'). POI cDNA fragment was amplified using j218 (5'-AGAAACTTGATAAGGGACG-3') and j219 (5'-ACCTTATTAACCGGAGCCAGACACG-3') under the conditions empirically chosen to avoid saturation. The cycling conditions were 25, 30 or 35 cycles of 94°C for 30 s; 55°C for 30 s; 72°C for 30 s. The relative cDNA levels of POI in the normalized samples were determined by 1% agarose gel electrophoresis.

Results

Expression and purification of M. sexta POI in E. coli

M. sexta POI cDNA was isolated from a male antennal library in an EST project (Robertson et al., 1999). The 572-nucleotide DNA includes a complete open reading frame encoding an 88-residue polypeptide. Following the putative signal peptide, the mature protein is composed of 73 amino acid residues (Fig. 1). While there is no N-linked glycosylation site in the sequence, M. sexta POI is predicted to be modified at Ser11 (Gupta et al., 1999). Multiple sequence alignment indicated that residues 1-40 of the molecule do not align with its homologs from the other insects. The remaining part of M. sexta POI is 33~58% identical in sequence to equivalent regions in the other proteins. This includes six absolutely conserved cysteine residues involved in disulfide bond formation. As determined in Musca domestica POI (Daquinag et al., 1999), the same disulfide linkage pattern (Cys1-Cys4, Cys2-Cys5, and Cys3-Cys6) may also exist in the other proteins to stabilize a common three dimensional structure. Among the highly

conserved residues (Gly47, His53, Asp55, Ser58, Leu62, Tyr64, Lys67 and Val69 in M. sexta POI), Tyr64 may directly interact with the reactive center of its target enzyme, tyrosinase-type phenoloxidases. In the housefly POI, this residue was hydroxylated to become dopa (Daquinag et al., 1995).

For functional analyses, we produced M. sexta POI as a soluble fusion protein using an E. coli expression system. Since the reducing environment of cytoplasm may affect disulfide bond formation, folding and protein association, we selected pET40 as the expression vector for producing M. sexta POI. The fusion partner, DsbC, led the nascent polypeptide to the more oxidative periplasmic space of E. coli and catalyzed isomerization of incorrectly paired Cys residues. Osmotic shock was then applied to release the recombinant protein as well as other periplasmic enzymes. Using the S-tag and 8xHis-tag encoded by the plasmid, we detected and purified the soluble fusion protein by affinity chromatography (Fig. 2). After specific proteolytic digestion at the enterokinase cleavage site, the uncleaved protein and tagged fusion partner were removed by Ni2+-NTA agarose, whereas the liberated POI was further purified by reverse phase HPLC. From 1 liter of the E. coli culture, we obtained approximately 130 µg POI. Automated Edman degradation indicated that its first seven amino acid residues (Ser-Pro-Gly-Asn-Leu-Ile-Arg) were the same as the original design. MALDI mass spectrometry showed that the purified protein had a molecular mass was 8,197.5 Da, 6.7 units smaller than the theoretical value (8204.3 Da) of the recombinant protein (SPGNLIR…RCVSGSG). This suggested that the six cysteine residues formed three disulfide bonds and led to the decrease of 6.0 Da. The inhibitory activity of M. sexta POI further confirmed the correct Cys pairing and protein folding (Fig. 3).

We further tested whether or not the recombinant protein affects the cleavage activation of proPO. There was little difference in the extent of proPO proteolysis (Fig. 3), suggesting that the reduction of PO activity was caused by inhibition of PO activity rather than proPO activation. Nevertheless, the recombinant protein produced in E. coli did not effectively block PO activity: under the experimental conditions, 3.8 μg/ml of POI or a POI:PO molar ratio of =6:1 was required to inhibit 50% of the total activity (Fig. 3). The concentration-dependent inhibition fits well with the one-phase exponential decay ($r^2 = 0.97$), suggesting that PO and POI associated loosely.

Expression and purification of M sexta POI from the insect cells

To examine if the lack of O-glycosylation had led to the low inhibitory activity, we produced M. sexta POI in baculovirus-infected Sf21 cells. We first incorporated a synthetic DNA fragment encoding honeybee melittin signal peptide into pFH6, a modified pFastBac1 containing a coding region for the 6xHis affinity tag in the carboxyl terminus (Ji et al., 2003). Then, the amplified POI cDNA was inserted to pMHF6, yielding POI/pMFH6 and respective baculovirus. From Sf21 cells infected by the virus, the recombinant POI was successfully secreted to the medium and reached a low concentration (~50 μg/L) at 96 h. After affinity chromatography, the active fractions were pooled and further separated by reverse phase HPLC (Fig. 4). The first two protein peaks were pure POI and we obtained approximately 25 μg M. sexta POI from one liter of the conditioned medium.

The molecular mass of POI in peak-1 was determined to be 10,684 Da, larger than the calculated value of the 6×His tagged protein (8,971 Da). The major mass difference of 1,713 Da was probably due to O-linked glycosylation at Ser11. Consistent with that,

O-glycosidase treatment of the POI resulted in an increase in the electrophoretic mobility on the SDS-polyacrylamide gel (Fig. 5). There was a reduction in the PO inhibitory activity accompanied by the removal of sugar moiety. Since the activity decrease was not significant and POI from E. coli had a high IC50 of 1.2 (3.8?) μg/ml (Fig. 2), the glycosylated POI (presumably very similar to that in M. sexta plasma) does not appear to be an effective regulator of PO activity, unless a critical modification is present in the plasma protein but not in the recombinant ones.

Isolation of a low Mr PO inhibitor

Because the housefly POI contained a dopa at the position equivalent to Tyr64, we tested if recombinant tyrosine hydroxylase (a kind gift from Dr. Gorman at Kansas State University) could hydroxylate POI and significantly elevate its activity. Mass spectrometry did not show any mass increase, suggesting that POI was not modified by the hydroxylase. We then attempted but failed to directly isolate POI from the M. sexta hemolymph, partly because antibodies against the recombinant protein from E. coli did not react well with the plasma protein. Finally, we employed the PO inhibition assay to monitor the purification, hoping that the antibodies would better recognize POI after a few fractionation steps. While the target protein was still undetected, we discovered a strong activity against PO and mushroom tyrosinase (Fig. 6). After we separated the active fraction by SDS-PAGE in Tris-Gly-SDS or Tris-Tricine-SDS buffer, there was no band visualized by Coomassie blue or silver staining. Neither did mass analysis reveal any peak with a molecular mass greater than 500 Da. We suspected that this activity came from a small Mr compound hidden in the matrix peaks. To further characterize the inhibitory activity, we separated the pooled active fraction by reverse phase HPLC on the

C18 column using 10% methanol as the mobile phase. While three absorbance peaks were detected, the third one demonstrated a strong inhibitory activity – PO activity decreased from 15.2 to 0.4 U (data not shown?). We further characterized this potent PO inhibitor by proteolysis: trypsin or chymotrypsin digestion did not abolish its inhibitory activity (Fig. 6). On the contrary, the recombinant POI lost its activity after the same treatment. UV spectroscopy revealed a broad peak with a maximum absorption at 257 nm. Consistent with our speculation, this heat-stable colorless compound did not show a significant absorption at 214 or 280 nm, typical for proteinaceous samples.

Expression profile analysis of M. sexta POI by RT-PCR

In order to test whether or not POI transcription is up-regulated after an immune challenge, we analyzed total RNA samples of hemocytes and fat body from M. sexta larvae injected with water or a mixture of microorganisms. POI mRNA was undetected in the hemocyte samples, whereas amplification of the induced fat body cDNA yielded a major PCR product at the expected size. The band intensity was much higher than that of the control (Fig. 7). We also inspected the POI mRNA levels in fat body at different developmental stages and found the transcripts were present in fat body from day 3, 4th instar. The mRNA level declined in the 5th instar and resurged at the early wandering stage. After a decrease in the rest of wandering stage, POI transcript level gradually increased in the pupae.

Discussion

Insect POs are likely involved in melanotic encapsulation, wound healing, and hemolymph coagulation. Their proteolytic activation is under strict regulation, mainly by serine proteinase inhibitors of the serpin family (Kanost et al., 2004). Active POs are also

regulated by different means (Sugumaran, 2002; Tsukamoto et al., 1992). Based on the sequence similarity, we identified a potential POI from M. sexta and produced it in E. coli for activity analysis (Fig. 1). To ensure formation of the correct disulfide bonds, we expressed the POI as a fusion protein with DsbC, which acts as a chaperone to improve the folding of proteins with complex disulfide linkages (Jurado et al., 2002; Maskos et al., 2003). We then raised a rabbit polyclonal antiserum against the purified POI that inhibited M. sexta PO (Fig. 2 and Fig. 3). However, the antiserum failed to detect the natural protein from M. sexta, suggesting that the hemolymph POI was present at a low concentration and/or in a considerably modified form.

Hence, we expressed the POI in Sf21 cells in order to test the role of glycosylation and to obtain sufficient amount of the glycoprotein for preparing another polyclonal antiserum. The yield of recombinant POI in insect cells was low (25 μg/L) and the protein differed in glycosylation extent (Fig. 4 and unpublished results). Upon removal of the carbohydrate moiety, the PO inhibitory activity decreased (Fig. 5), implying that the glycosylation enhances the interaction between PO and POI.

At its estimated concentration in the larval hemolymph, even the glycosylated POI is not expected to play a major role in the regulation of PO. This is consistent with the finding that POI knockdown did not lead to a faster or more extensive melanization of Sephadex beads injected to the mosquitoes (Shi et al., 2006). To test if a posttranslational modification (e.g. Try64 to dopa) occurring in vivo could greatly enhance its inhibitory activity, we attempted but failed to isolate the natural POI from the M. sexta hemolymph for structural and activity comparison.

Instead, we isolated a low Mr compound with potent inhibitory activity against the PO and mushroom tyrosinase (Fig. 6). Based on the initial results, we suggest that this compound contains a phenyl ring which strongly absorbs at 250-260 nm. Further chemical analyses are largely limited by the sample quantity. Despite an unknown structure, the compound represents the first endogenous low Mr PO inhibitor isolated from an insect. Previous study showed that POs from Coleopteran and Lepidopteran insects were strongly inhibited by a fungi metabolite (Dowd, 1999). Produced by the entomopathogens Aspergillus and Penicillium species, kojic acid blocks the PO activities and protects the fungi from the host defense response. In contrast, the small inhibitor described herein perhaps minimizes damage of PO-generated compounds to host tissues/cells.

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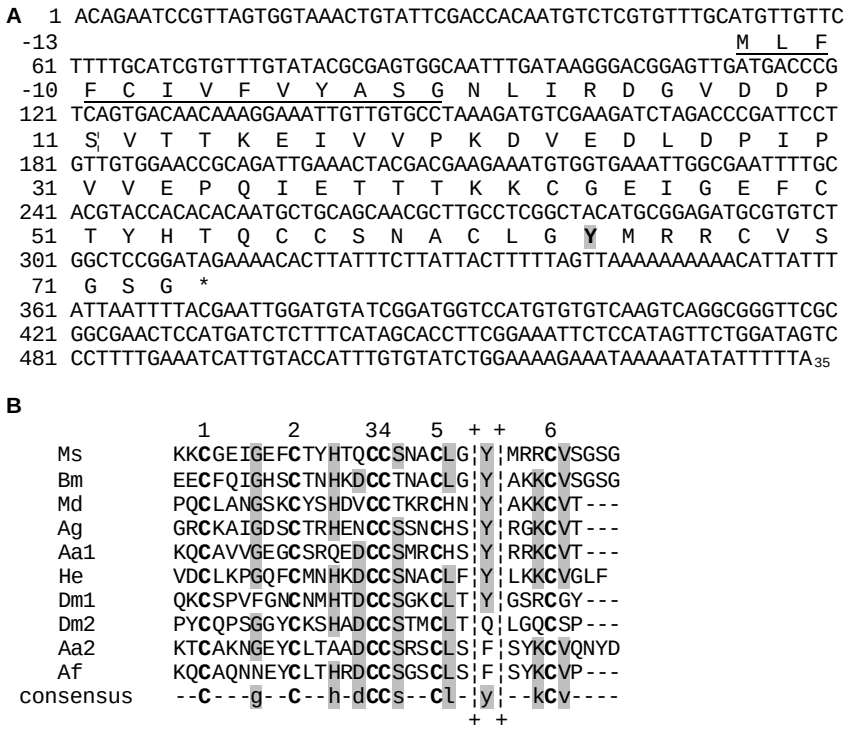

Fig. 1 *M. sexta* POI and its comparison with homologous sequences from other insects.

(A) cDNA and deduced amino acid sequence. The predicted signal peptide is underlined, and the *O*-linked glycosylation site is marked "ˈ". Shaded Tyr^{64} corresponds to dopa^{32} in the housefly POI, which is considered to be critical for its activity. **(B)** Alignment of the disulfide-knotted regions in homologous molecules. Ms, *M. sexta* (BE015616); Bm, *Bombyx mori* (BY939736); Md, *M. domestica* (AAB33998); Ag, *Anopheles gambiae* (CD747521); Aa1 and 2, *Aedes aegypti* (EB099073 and EB102275, respectively); He, *Heliconius ethilla* (DT668523.3); Dm1 and 2, *Drosophila melanogaster* (BK002735 and BK002734, respectively). Six Cys residues that form three disulfide bonds (Cys_1-Cys_4, Cys_2-Cys_5, and Cys_3-Cys_6) in *M. domestica* POI (Daquinag et al., 1999) are conserved in all of the sequences. The highly similar residues (Gly, His, Asp, Ser, Leu, Tyr, Lys, Val) are shaded, and the conserved Tyr is boxed.

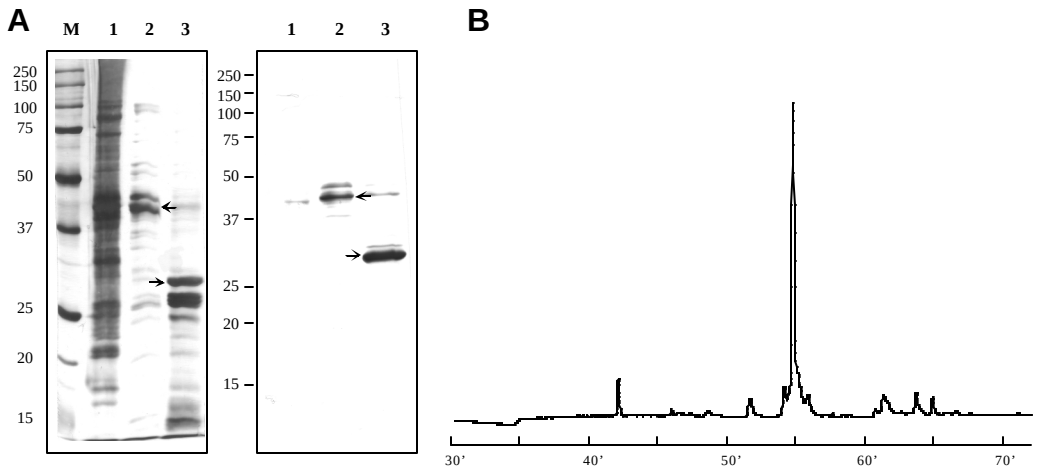

Fig. 2 Isolation of *M. sexta* POI from *E. coli*.

(A) SDS-PAGE and immunoblot analysis of the DsbC-POI fusion protein in the periplasmic extract (lane 1), Ni-NTA elution fractions (lane 2) and enterokinase digest (lane 3). *Left* panel, silver staining; *right* panel, immunoblot analysis using S-protein antibodies. Positions and sizes of the M_r markers are indicated. The fusion protein and DsbC are marked by arrows. **(B)** Purification of recombinant POI by reverse phase HPLC on a Bio-Rad Hi-Pore RP-318 column. Flow rate: 1.0 ml/min; buffer A: 0.1% TFA in 5% acetonitrile; buffer B: 0.1% TFA in 95% acetonitrile; gradient: 0-75% B in 75 min; UV detection: 214 nm.

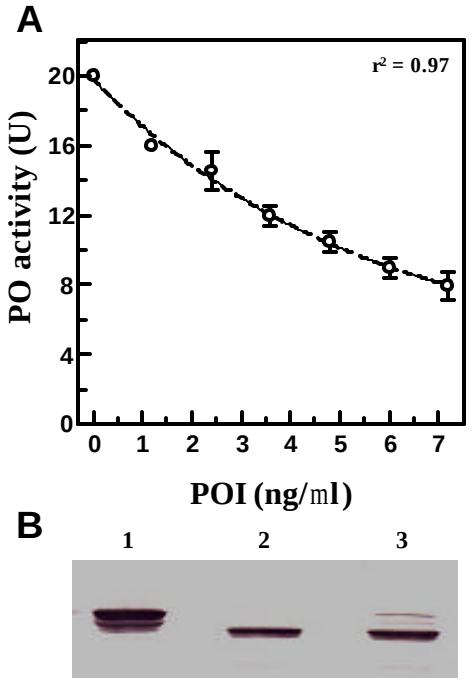

Fig. 3 Inhibition of *M. sexta* PO by recombinant POI from *E. coli*.

(A) Concentration-dependent inhibition by the purified protein. **(B)** Effect of POI on proPO activation. Lane 1, proPO (with a small amount of PO cleaved after prolonged storage); lane 2, PO activated by PAP-2 and SPHs; lane 3, PO activated by PAP-2 and SPHs in the presence of recombinant POI (60 ng/µl).

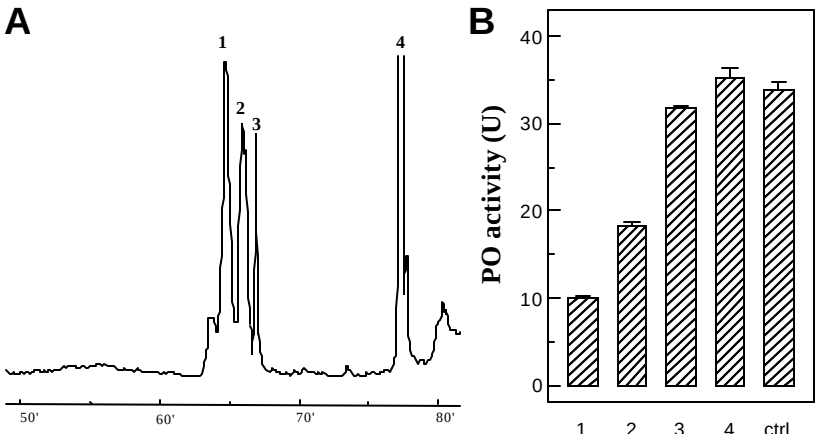

Fig. 4 Purification of recombinant POI produced in baculovirus-infected *Sf*21 cells. The recombinant POI from insect cells were enriched and purified by affinity chromatography on a Ni-NTA column. As described in Fig. 1, eluted proteins were further separated by reverse phase HPLC **(A)**. The gradient was 0-40% B in 40 minutes. **(B)** Inhibitory activity.

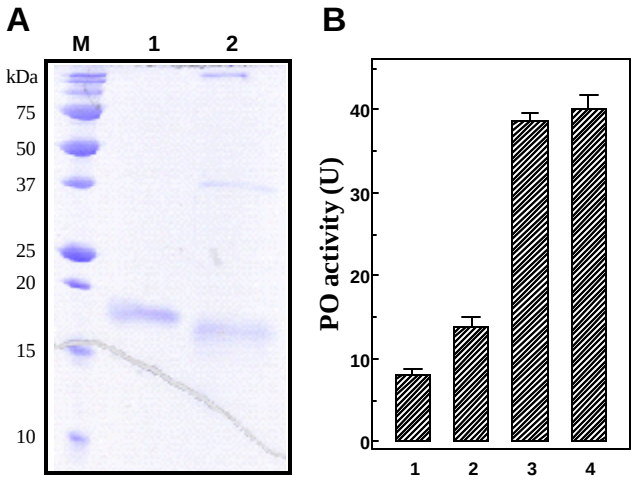

Fig. 5 Detection of *O*-linked glycosylation in *M. sexta* POI from *Sf*21 cells.

(A) SDS-PAGE analysis of the recombinant POI before (lane 1) and after (lane 2) deglycosylation. **(B)** Effect of glycosylation on POI activity. 1, purified POI; 2, purified POI treated with *O*-glycosidase; 3 and 4, *O*-glycosidase and buffer controls.

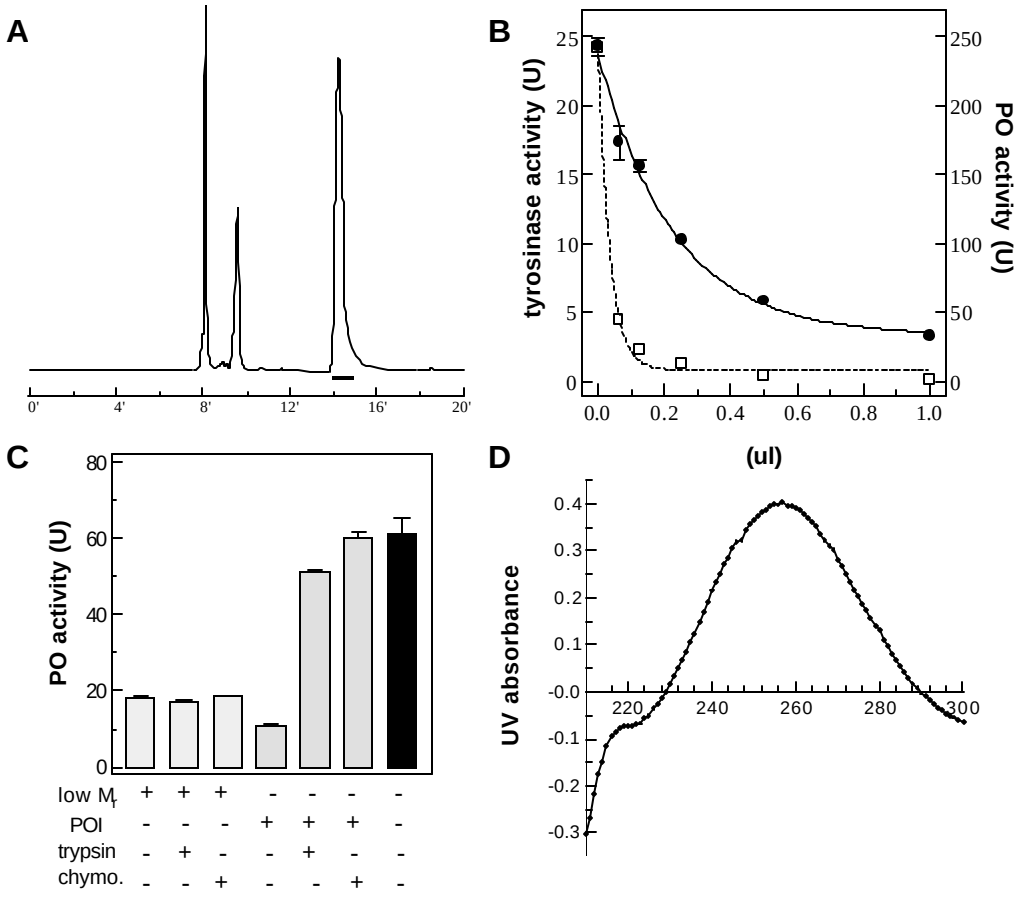

Fig. 6 Isolation of a low M_r PO inhibitor from the larval hemolymph.

(A) Elution profile. Column: Bio-Rad Hi-Pore reverse phase column RP-318; flow rate: 1.0 ml/min; mobile phase: 10 % methanol; UV detection: 214 nm. **(B)** Inhibition of mushroom tyrosinase (*left* axis, ? - - ?) and *M. sexta* PO (*right* axis, ? - - ?) by the low M_r compound. **(C)** Effect of proteinase treatment on the

recombinant POI and low M_r inhibitor. **(D)** UV absorption spectrum. The low M_r inhibitor was scanned between 210 and 300 nm on a Beckman DU-520 UV/vis spectrophotometer using pH 7.5, 20 mM Tris-HCl as blank.

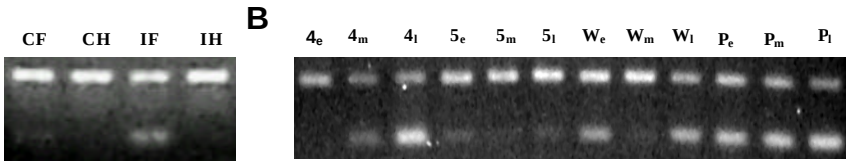

Fig. 7 Expression profiles of *M. sexta* POI in fat body at different immune states or development stages.

(A) CF and CH, fat body and hemocytes from the naïve larvae; IF and IH, fat body and hemocytes from the larvae injected with bacteria 24 h before. **(B)** 4_e, 4^{th} instar day 1; 4_m, 4^{th} instar day 3; 4_l, 4^{th} instar day 5; 5_e, 5^{th} instar day 1, 5_m, 5^{th} instar day 3; 5_l, 5^{th} instar day 5, W_e, W_m and W_l, early, middle and late wandering stage; P_e, P_m and P_l: early, middle and late pupal stage.

CHAPTER VI

NEGATIVE REGULATION OF PROPHENOLOXIDASE (PROPO) ACTIVATION BY A CLIP-DOMAIN SERINE PROTEINASE HOMOLOG (SPH) FROM ENDOPARASITOID VENOM

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Abstract

Most parasitic wasps inject maternal factors into the host hemocoel to suppress the host immune system and ensure successful development of their progeny. Melanization is one of the insect defence mechanisms against intruding pathogens or parasites. We previously isolated from the venom of *Cotesia rubecula* a 50 kDa protein that blocked melanization in the hemolymph of its host, *Pieris rapae* (Asgari et al, 2003). This protein, designated Vn50, is a serine proteinase homolog (SPH) containing an amino-terminal clip domain. In this work, we demonstrated that recombinant Vn50 bound *P. rapae* hemolymph components that were recognized by antisera to *Tenebrio molitor* prophenoloxidase (proPO) and *Manduca sexta* proPO-activating proteinase (PAP). Vn50 is stable in the host hemolymph – it remained intact for at least 72 h after parasitization. Using *M. sexta* as a model system, we found that Vn50 efficiently down-regulated proPO activation mediated by *M. sexta* PAP-1, SPH-1, and SPH-2. Vn50 did not inhibit active phenoloxidase (PO) or PAP-1, but it significantly reduced the proteolysis of proPO. If recombinant Vn50 binds *P. rapae* proPO and PAP (as suggested by the antibody reactions), it is likely that the molecular interactions among *M. sexta* proPO, PAP-1, and SPHs were impaired by this venom protein. A similar strategy might be employed by *C. rubecula* to negatively impact the proPO activation reaction in its natural host.

Abbreviations:

PAGE, polyacrylamide gel electrophoresis; proPO and PO, prophenoloxidase and phenoloxidase; PAP, proPO-activating proteinase; SPH, serine proteinase homolog; PDV, polydnavirus; SDS, sodium dodecyl sulfate; TBS, Tris buffered saline; PBS, phosphate buffered saline; PTU, 1-phenyl-2-thiourea.

Introduction

Introduction of maternal factors into the body cavity of their host insects is a common strategy evolved in endoparasitic wasps to manipulate host physiology for their own benefits. These include viruses or virus-like particles, such as polydnaviruses (PDVs), and venom fluid. PDVs, produced in the calyx region of the female reproductive organ, are essential for successful development of the parasitoids inside the host (Edson et al., 1981; Fleming and Summers, 1991). They disrupt the host cellular immune system and inhibit formation of hemocyte capsules around the parasitoid eggs. PDVs are accompanied by venom proteins in most parasitoid-host systems, and in some cases are only effective when injected together with venom. Venom proteins are probably involved in uncoating of PDVs *in vitro* and virus persistence *in vivo* (Stoltz et al., 1988). In many instances, it has been shown that venom enhances the effects of PDVs (Kitano, 1986; Tanaka, 1987) or might provide protection for the eggs during the period between oviposition and expression of PDV genes (Webb and Luckhart, 1994). In addition to their synergistic effects together with PDVs, venom components affect host physiology and development (Digilio et al., 2000; Gupta and Ferkovich, 1998). In other endoparasitoids that do not produce PDVs, venom becomes the only factor in suppression or regulation of the host immune system (Richards and Parkinson, 2000).

Inhibition of melanization following parasitism has been reported from several systems. Melanin formation involves the proteolytic activation of proPO, which leads to the generation of melanin and other toxic phenolic compounds. This reaction is considered as a vital defence mechanism mounted against intruding organisms (Ashida and Brey, 1998; Vass and Nappi, 2000). This process is impaired to various extents in

several host-parasitoid systems. However, the molecular mechanism behind this suppression is unknown. In a recent study, we isolated a 50 kDa protein from the venom of *C. rubecula*, a Braconid parasitic wasp, which blocked hemolymph melanization of its host, *P. rapae* (Asgari et al., 2003). This protein is similar in sequence and domain structure to SPHs from various insects (Kwon et al., 1999; Lee et al., 2002; Yu et al., 2003). Many SPHs consist of a clip domain at the amino terminus and a serine proteinase-like domain at the carboxyl terminus (Ross et al., 2003). Since the residues essential for catalytic activity are missing in these proteins, SPHs do not have proteolytic activity. Recent study indicated that *M. sexta* SPH-1 and SPH-2 mediate proPO activation in conjunction with other components from the hemolymph (Yu et al., 2003). Although cleaved, their clip and proteinase-like domains remain attached by an interchain disulfide bond.

In this complementary study, we show that Vn50 is not cleaved after introduction into the host hemolymph and is stable for a long period of time after parasitization. The recombinant Vn50 may specifically interact with proPO and PAP in *P. rapae* hemolymph. Vn50 reduces proteolytic activation of proPO by interfering with the molecular interactions among *M. sexta* proPO, PAP-1, SPH-1, and SPH-2.

Materials and methods

Insects and isolation of hemolymph

The parasitoid *C. rubecula* and its host *P. rapae* were maintained at 25°C on a 14h light:10h dark photoperiod. *P. rapae* larvae were reared on cabbage plants. Adult wasps were fed with honey-water solution. Hemolymph (ca. 80µl) was collected from

five 4th instar *P. rapae* larvae by surface sterilizing in 70% ethanol and bleeding from a proleg into ice-cold phosphate buffered saline (PBS, 200µl) containing a few crystals of 1-phenyl-2-thiourea (PTU). To obtain the plasma, hemolymph was centrifuged at 800 × *g* for 5 min and the supernatant was transferred into a fresh tube.

Preparation of C. rubecula Vn50, M. sexta proPO, PAP-1, and SPHs

Venom proteins were isolated from the female wasps and fractionated by reversed phase HPLC to purify Vn50 (Asgari et al., 2003). Recombinant Vn50 was expressed in *E. coli* as an insoluble protein and purified by nickel affinity chromatography under the denaturing conditions. *M. sexta* proPO was isolated from the larval hemolymph as described previously (Jiang et al., 1997). Activated recombinant PAP-1 (Yu et al., 2003) was separated from other proteins under the conditions used for proPAP-1 purification (Wang et al., 2001). *M. sexta* SPH-1 and SPH-2, which co-purified with immulectin-2, were purified from hemolymph of bacteria-induced insects according to Yu et al (2003).

Production of anti-Vn50 antibodies

Purified recombinant Vn50 expressed in *Escherichia coli* (Asgari et al., 2003) was run on preparative 10% SDS-PAGE gels, stained in 0.05% Coomassie blue R-250 prepared in H_2O, and destained with several changes of distilled water. Vn50 corresponding band was excised from the gels, squashed into fine pieces in PBS, and injected into a rabbit (ca. 5 µg/injection). Two subsequent booster injections were carried out in 2-week intervals, four weeks after the initial injection. The first and booster injections contained complete and incomplete Freud's adjuvants, respectively. Serum was obtained one week after the last injection. Production of anti-Vn50

antibodies was confirmed by Western blots containing the recombinant Vn50 and the total venom from *C. rubecula* using 1:5000 dilution of the antiserum.

Stability of C. rubecula Vn50 in P. rapae hemolymph

Hemolymph was collected from third instar *P. rapae* larvae (ca. 20 µl) at various times following parasitization by *C. rubecula,* as described above. Samples were analyzed by Western blot analysis using anti-Vn50 antibodies.

Identification of hemolymph proteins bound to Vn50

Binding of *P. rapae* hemolymph proteins to Vn50 was examined as described previously with minor modifications (Yu et al., 2003). Briefly, washed Ni-NTA agarose beads (Qiagen, 0.1 ml) were coated with renatured 6xHis-tagged recombinant Vn50 or bovine serum albumin (BSA) in Tris buffered saline (TBS) for 60 min at room temperature. Beads were washed with TBS to remove unbound proteins and incubated with 0.5 ml plasma-PBS containing PTU from four *P. rapae* larvae. The incubation was carried out at room temperature for 1 h with shaking. After washing with TBS, nonspecifically bound proteins were eluted with 1.0 M NaCl. SDS sample buffer was then added to the resuspended beads in TBS and treated at 95°C for 5 min. After centrifugation, the supernatant was subjected to 12% SDS-PAGE (Laemmli, 1970) and immunoblot analysis. Blots were probed with various antiserum against *C. rubecula* Vn50 (1:5000 dilution) (Asgari et al., 2003), *M. sexta* PAP-1 (1:2000 dilution) (Wang et al., 2001), or *Tenebrio molitor* proPO (1:2000 dilution) (a kind gift from Prof. Bok Lee at Pusan National University, Korea). Alkaline phosphatase-conjugated anti-rabbit IgGs (Sigma) were used as secondary antibodies.

Quantification of Vn50 injected into P. rapae larvae

Comparative Western blot analysis was used to determine the amount of Vn50 injected into a host larva at various times after parasitization. Three larvae were used for each time point. Recombinant Vn50 was produced in bacteria and purified as described (Asgari et al., 2003). The recombinant protein concentration was measured using Bio-Rad Protein Assay based on the method of Bradford. Cell-free hemolymph (see above) was collected from the parasitized larvae, and a fixed volume of 20 µl of each sample was run on Western blots together with known concentrations of recombinant Vn50. Intensity of bands was compared to estimate the amount of Vn50 injected into the caterpillars.

Down regulation of M. sexta proPO activation by C. rubecula Vn50

M. sexta proPO (10 µl, 10 ng/µl), PAP-1 (2 µl, 30 ng/µl), and SPHs (2 µl, 50 ng/µl) were mixed with 10 µl of reaction buffer (20 mM Tris-HCl, pH 7.5) in the wells of a flat-bottom microtiter plate. After 2 µl of Vn50 at different concentrations was added to the wells, the reaction mixtures were incubated on ice for 40 min. PO activity was measured using dopamine as the substrate and a microplate reader (Jiang et al., 2003).

To examine a possible effect of *C. rubecula* Vn50 on PO activity, *M. sexta* proPO, PAP-1, SPHs, and buffer were incubated in the wells of a microplate for 40 min on ice as described above. After 2 µl of Vn50 (12 ng/µl) or buffer (20 mM Tris-HCl, pH 7.5) was added, PO activity in the reaction mixtures was determined 10 min later (Jiang et al., 2003).

To test if *C. rubecula* Vn50 directly inhibits a PAP, *M. sexta* PAP-1 (5 µl, 30 ng/µl) was incubated with Vn50 (2 µl, 10 ng/µl) or buffer (2 µl) on ice for 10 min. The amidase activity of PAP-1 was measured using acetyl-Ile-Glu-Ala-Arg-*p*NA as a substrate (Jiang et al., 2003).

Effect of C. rubecula Vn50 on proPO cleavage

For understanding how Vn50 may affect proPO cleavage, proPO activation was performed in the absence or presence of Vn50 (2 µl, 5 ng/µl) under the conditions described above. After incubation on ice for 40 min, 10 µl of the reaction mixtures as well as the negative controls were subjected to Western blot analysis using 1:2000 diluted proPO antiserum as the first antibody.

Results and discussion

In our previous paper (Asgari et al., 2003), we showed that the most abundant component in *C. rubecula* venom was a 50 kDa protein (Vn50), which is similar in sequence to arthropod SPHs and inhibits melanization of hemolymph from the host *P. rapae*. Many of these proteins consist of two domains: an amino-terminal clip domain and a carboxyl-terminal proteinase-like domain (Ross et al., 2003). Like many clip-domain serine proteinases from arthropods (Jiang and Kanost, 2000), *M. sexta* SPH-1 and SPH-2, as well as *Holotrichia diomphalia* proPO-activating factor II, are cleaved at a certain position but the two domains remain attached by a disulfide bond (Kwon et al., 1999; Lee et al., 2002; Yu et al., 2003). These three SPHs function as co-factors for PAPs. Because the conserved Ser residue at the active site of their proteinase-like domains is changed to Gly or other residues, all SPHs (including Vn50) are not expected to have any proteolytic activity.

Status of Vn50 in the host hemolymph following parasitization

The clip-domain serine proteinases are produced as zymogens and cleaved at a specific location between the clip domain and the proteolytic domain (Jiang and Kanost, 2000). The cleavage is necessary for activation of these enzymes. Clip-domain SPHs, with a similar domain structure, were also cleaved (Yu et al., 2003, Lee et al., 2002). Assuming that the cleavage is also necessary for SPHs to exert their function as enhancers/mediators for proPO activation, we investigated the status of Vn50 after it was injected into the host hemolymph to block melanization. At 2 h after parasitization with *C. rubecula*, we analysed the plasma sample from *P. rapae* larvae by SDS-PAGE and Western blotting under reducing and non-reducing conditions. A 50 kDa immunoreactive band was detected in the parasitized hemolymph and positive control of venom, but not in the non-parasitized hemolymph (Fig. 1). There was only a small size difference in Vn50 under the two conditions. The predicted molecular mass for secreted Vn50, based on its deduced amino acid sequence, is 40.6 kDa. The difference between the calculated and observed sizes has been shown to be due to glycosylation (Asgari et al., 2003). Based on the other SPHs which were characterized biochemically, we predict that the conserved cleavage site in Vn50 would be located after Arg^{127} residue. In other words, if the protein is cleaved, it should result in an 11.8 kDa clip domain and a 28.9 kDa proteinase-like domain under the reducing conditions. Our observation, however, indicates that Vn50 remained intact even in the presence of ß-mercaptoethanol. In fact, the protein ran slightly slower under this condition than under the non-reducing conditions. The change in electrophoretic mobility is likely caused by the breakdown of intramolecular disulfide bonds (Fig. 1). As a control, venom fluid was analysed under

the same conditions and found to be identical to the samples from the cell-free hemolymph in terms of the sizes of the immunoreactive bands.

We demonstrated that intact Vn50 suppresses the melanization reaction in the host hemolymph (Asgari et al., 2003). To maintain this status, Vn50 must remain at a certain level in the circulation as an uncleaved proSPH until the host immune system is suppressed by other maternal components such as the PDVs. Therefore, we analysed the persistence of Vn50 in the host hemolymph after parasitization. In our analysis in which we analysed plasma samples from *P. rapae* larvae up to 96 h after parasitization under reducing condition, the intact protein was still detected at a significant level at 72 h following parasitization (Fig. 2; see 3.3). The low turnover indicates that Vn50 could be resistant to degradation by host proteinases. Under this condition, the developing parasitoid eggs are protected from melanotic encapsulation for a prolonged period of time.

While Vn50 is maintained in an active form to block melanization, it is not clear how this proSPH exerts its function. Detection of Vn50-binding proteins in the host hemolymph could provide some useful cues on its mode of action.

Identification of plasma proteins bound to Vn50

In a model that was recently proposed based on experimental evidence, SPHs mediate proPO activation by directly interacting with proPO, PAP-1, and immulectin-2 (Yu et al., 2003). Although the exact mechanism is not understood, two scenarios were envisaged: 1) SPHs bring proPO into a correct spatial orientation or 2) the interaction among the proteins might confer a conformational change in proPO to facilitate its activation by the PAP. To find out whether proPO and PAP in *P. rapae* hemolymph

interact with Vn50, recombinant 6×His-tagged Vn50 was used to coat nickel agarose beads. After incubation with the plasma, washing, and elution with high salt buffer, proteins bound to the beads were separated by SDS-PAGE and subjected to Western blot analysis. Two immunoreactive bands, recognized by the antibodies against *M. sexta* PAP-1, may represent *P. rapae* proPAP (46 kDa) and its catalytic domain (35 kDa) (Fig. 3). Similarly, the antibodies against *T. molitor* proPO reacted with two Vn50-binding proteins at 90 and 62 kDa. Based on the typical size of insect proPO, we suggest that they may correspond to zymogen and a cleaved form of proPO in *P. rapae* hemolymph. The antibodies also recognized the respective proteins in *P. rapae* hemolymph, confirming that proteins bound to the beads came from the larval hemolymph. Similar observations were made in *M. sexta* SPH-1, whose proteinase-like domain binds to proPO, PAP-1, and immulectin-2 (Yu et al., 2003). In the negative control, none of these proteins bound to BSA-coated nickel agarose beads (Fig. 3). These results suggest that Vn50 may interact with one or more of proteins involved in melanization. Further experiments are needed to examine which components bound to Vn50 directly and which ones were pulled down simply because of their association with the Vn50-interacting proteins.

Regulation of M. sexta proPO activation by C. rubecula Vn50

Knowing that Vn50 may interact with proPO and PAP in the host hemolymph, we wanted to test if the parasitoid SPH blocks melanization by interfering with the proPO activation reaction. Since *P. rapae* proPO, PAP, and a cofactor (perhaps) are unavailable at this moment, we examined whether Vn50 might negatively impact the activation reaction involving *M. sexta* proPO, PAP-1, SPH-1, and SPH-2. In the presence of Vn50,

proPO activation was greatly reduced (Fig. 4A), even though *M. sexta* is not a host of *C. rubecula*. This effect was found to be concentration-dependent, with 50% of the proPO activation occurring at a Vn50 concentration of 0.4 μg/ml (Fig. 4B). We measured the amount of Vn50 injected by *C. rubecula* into *P. rapae* caterpillars at various times after parasitization by comparative Western blot analysis using recombinant Vn50 as reference. The data (Fig. 5) indicated that the concentration of Vn50 in the host hemolymph at 20 min after parasitization was much higher than what is needed for reducing 50% of the proPO activation *in vitro* (Fig. 4B). While the concentration gradually decreased, Vn50 remained at a significant level up until 72 h after parasitization.

Regulatory mechanism of Vn50 in proPO activation

To understand the mechanism of this down-regulation, we tested if Vn50 directly inhibits PO activity generated by PAP-1 and its cofactor. The result indicated that once PO is activated, supplementation of the reaction mixture with Vn50 did not cause a major change in the oxidase activity (Fig. 6A). In other words, the clip-domain SPH does not inhibit PO. We then examined the effect of Vn50 on PAP-1's amidase activity. There was no decrease in hydrolysis of acetyl-Ile-Glu-Ala-Arg-*p*NA after *C. rubecula* Vn50 was added (Fig. 6B), indicating that the clip-domain SPH does not inhibit the serine proteinase activity of PAP-1. We also studied the cleavage of *M. sexta* proPO by PAP-1 and its cofactor in the presence or absence of Vn50. Western blot analysis demonstrated that when Vn50 was introduced into the reaction mixture, amount of the 74 kDa cleavage product of proPO was significantly decreased (Fig. 6C). This was consistent with the decrease in PO activity from 9.79 units to 3.07 units. Our recent investigation indicated

that proPO, PAP-1, and its cofactor (SPH-1 and SPH-2) may form a ternary complex (Gupta et al., unpublished data). Taken together, these results suggested that Vn50 may have disturbed the formation of the complex among *M. sexta* proPO, PAP-1, and the cofactor. Due to the sequence similarity between Vn50 and *M. sexta* SPHs (Asgari et al., 2003), we hypothesize that Vn50 may interact with PAP-1 and proPO stronger than or different from the cofactor does. To test this hypothesis, we plan to examine if uncleaved Vn50 competes with the SPHs in binding to proPO and PAP-1. Cleaved Vn50 will also be generated to test if it functions properly as a PAP cofactor.

There are a variety of strategies developed by parasitic wasps to overcome their host immune responses during the co-evolution process (Strand and Pech, 1995; Lavine and Beckage, 1995; Carton and Nappi, 1997). Playing a critical role in preventing endoparasitoid eggs from development, melanization in the host hemolymph has been targeted by many parasitoids. For instance, PO activity in *M. sexta* hemolymph was reduced by PDVs (Beckage et al., 1990). The titer of proPO decreased significantly in the larvae of *Heliothis virescens* after parasitisation by Ichnemonid parasitoid *Campoletis sonorensis* (Shelby et al., 2000). Recently, Moreau et al. (2003) showed that PO activity in the hemolymph was greatly reduced after *Drosophila melanogaster* was parasitized by *Asobara citri* even though the number of circulating crystal cells did not change significantly – crystal cells are considered as the major carriers of some enzymes of the PO system. The authors suggested that functioning of the host PO system was disrupted. In our previous research, we demonstrated that Vn50 blocked the melanization process. To understand its mechanism, we employed purified *M. sexta* proPO, PAP-1, and SPHs as an *in vitro* system to test the possible role of Vn50 in regulating the proPO activation

reaction. While molecular details are still lacking, our results strongly suggest that interactions among the substrate, proteinase, and cofactor were impaired by Vn50. To our best knowledge, this is the first report indicating that the proPO activation step is affected by a venom protein. Further binding and comparative studies should allow us to gain insights on the mechanism of proPO activation and its regulation.

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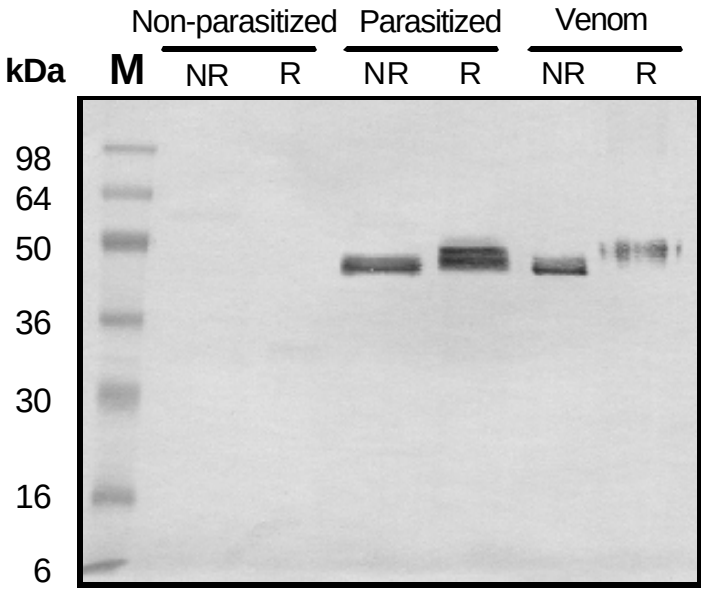

Fig. 1 Status of Vn50 in *P. rapae* hemolymph following parasitization

Cell-free hemolymph was collected from non-parasitised or parasitised (2 h) larvae and analysed on a Western blot under reducing (R) and non-reducing (NR) conditions. The blot was probed with Vn50 antibodies. Vn50 was not detected in the naïve caterpillars but in parasitised ones. The result also indicated that the protein was not cleaved into the clip and proteinase-like domains. Molecular masses of the protein standards (M) are marked on the left.

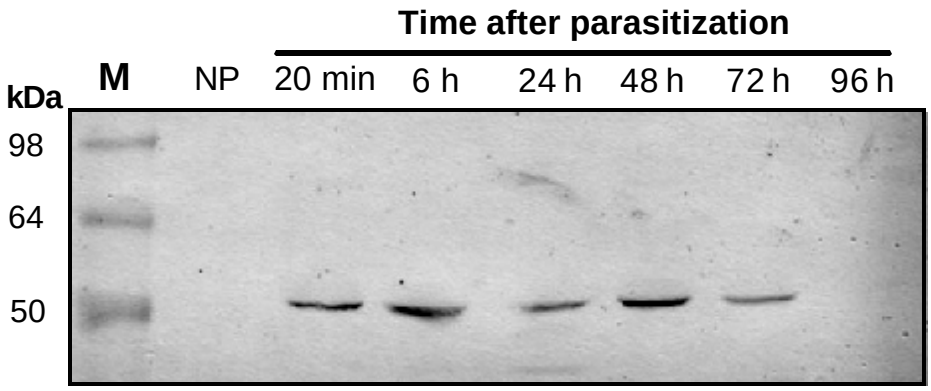

Fig. 2 Stability of Vn50 in the host hemolymph

Western blot analysis of cell-free hemolymph samples from third instar *P. rapae* larvae at various times after parasitization showed that the protein is not degraded by host proteinases and persists for at least 72 h. In each well, hemolymph (ca. 20 µl) from each parasitized larva was loaded. The blot was probed with anti-Vn50 antibodies. NP: hemolymph from non-parasitised larvae. Sizes of the molecular weight standards (M) are indicated on the left.

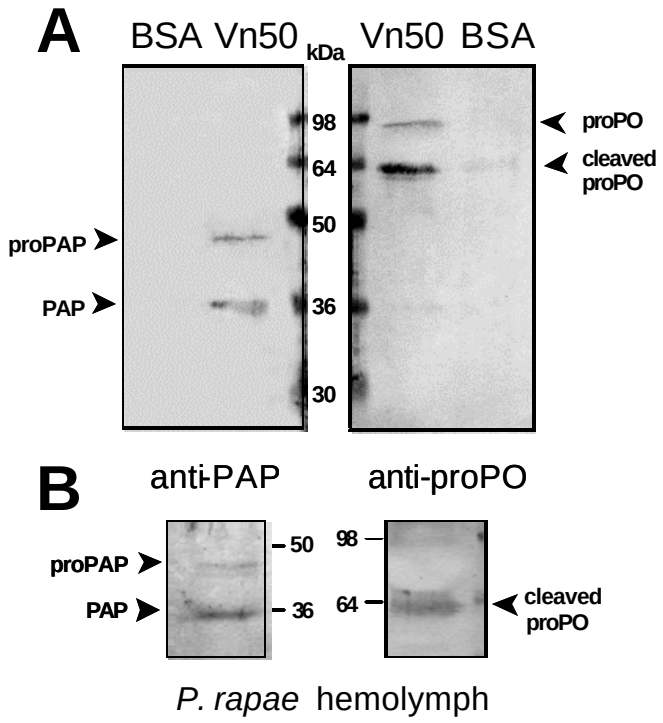

Fig. 3 Interaction of Vn50 with host hemolymph components

(A) Cell-free hemolymph from *P. rapae* larvae was incubated with beads coated with bovine serum albumin (BSA, control) or recombinant Vn50 and washed with buffers and salt as described in Materials and methods. Proteins bound to the beads were then analysed by SDS-PAGE and Western blotting, which were probed with PAP-1 (left panel) or proPO (right panel) antibodies. Both antibodies recognized proteins bound only to the beads that were coated with Vn50. Their sizes are consistent with those of precursor and cleaved forms of PAP and PO. However, further research is necessary to confirm their identities. This indicates that Vn50 may specifically interact with components of the proPO activation cascade. Sizes of the molecular weight markers are indicated. **(B)** The anti-proPO and anti-PAP-1 antibodies recognized the corresponding proteins in *P. rapae* hemolymph.

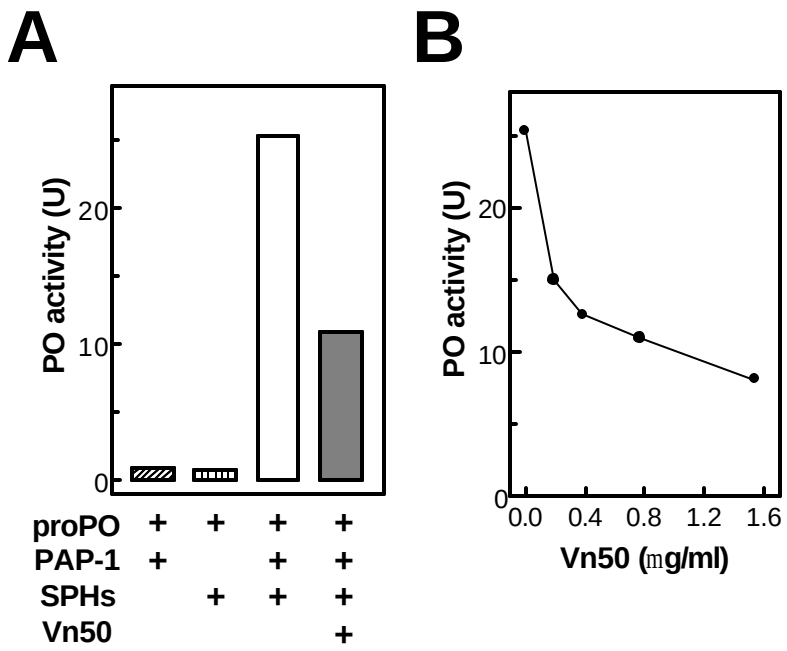

Fig. 4 Down-regulation of proPO activation by Vn50

(**A**) Reduction of proPO activation. Two microliters of Vn50 (10 ng/µl) or buffer was incubated with proPO, PAP-1 and SPHs for 40 min as described in Materials and methods. PO activity was determined using 150 µl of 2 mM dopamine and a microplate reader. The negative controls were proPO incubated with PAP-1 or SPHs only. (**B**) Concentration-dependence. Two microliters of Vn50 at different concentrations were reacted with proPO, PAP-1 and SPHs under the same conditions. PO activity generated in the mixtures was measured and plotted against the final concentrations of Vn50 in the reaction mixtures.

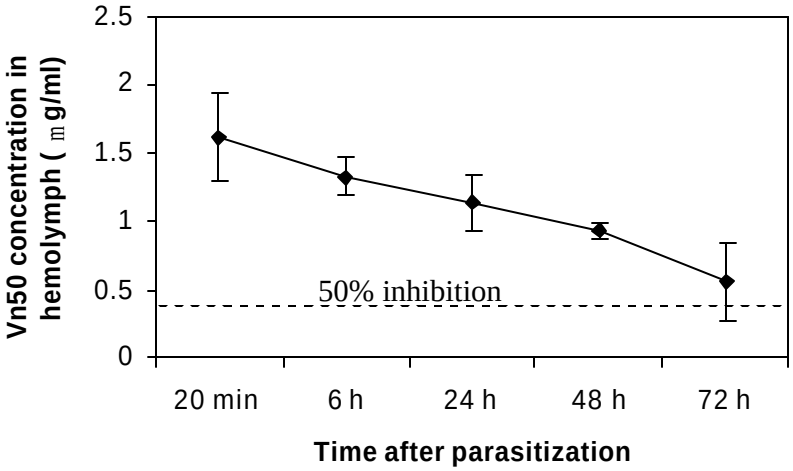

Fig. 5 Quantification of Vn50 in *P. rapae* hemolymph following paraistization

Concentration of Vn50 in *P. rapae* larval hemolymph was estimated at various times after parasitization by comparative Western blotting (Materials and methods). Three larvae were used for each time point. Dotted line represents the Vn50 concentration at which 50% reduction of proPO activation occurred *in vitro* (Fig. 4B). Bars indicate standard errors of the means.

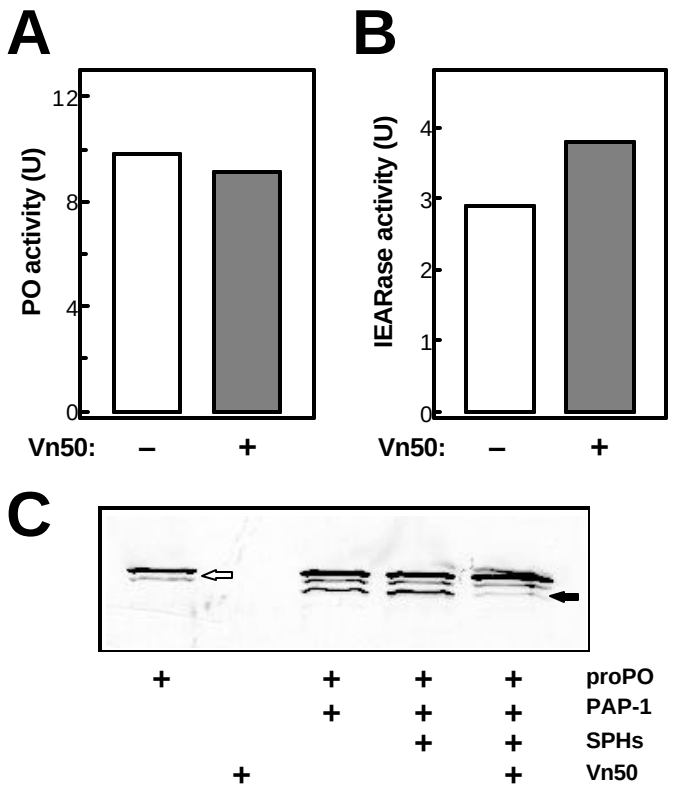

Fig. 6 Mechanistic analysis of the proPO activation regulation by Vn50

(**A**) Effect of Vn50 on active PO. To test if Vn50 directly inhibit PO activity, *M. sexta* proPO, PAP-1 and SPHs were first incubated on ice for 40 min before buffer (open bar) or Vn50 (2 µl, 12 ng/µl)(closed bar) was added. PO activity was measured 10 min later and plotted in the bar graph. (**B**) Effect of Vn50 on the proteolytic activity of PAP-1. Purified PAP-1 was mixed with 2 µl of buffer (open bar) or Vn50 (10 ng/µl) (closed bar), incubated on ice for 10 min. The amidase activity was determined using IEAR*p*NA as a substrate as described in the Methods and Materials. (**C**) Effect of Vn50 on proPO cleavage by PAP-1. Vn50 or buffer was incubated with proPO, PAP-1 and SPHs on ice for 40 min. The reaction mixtures were separated by 12% SDS-PAGE and subjected to Western blot analysis using proPO antibodies. The open arrow indicates *M. sexta* proPO

polypeptide-1 and -2, whereas the closed arrow marks a doublet representing a lower molecular weight, processed form consistent with the size expected for active PO (Jiang et al., 1997).

CHAPTER VII

SUMMARY

Melanization is insect acute-phase defense response to invading bacteria, fungi, protozoa, and endoparasitoids. Phenoloxidase (PO) participates in multiple steps of the melenization reaction. Produced as an inactive zymogen, prophenoloxidase (proPO), it is activated by a serine proteinase cascade upon recognition of the invaders. In *Manduca sexta,* the final step involves a proPO activating protease and a serine proteinase homolog (SPH) complex. After the proPO is activated, its activity is tightly regulated.

I purified a ß -1,3-glucan Recognition Protein 2 (ßGRP2) from *Manduca* cuticle extract. ßGRP2 specifically binds to laminarin, a soluble form of ß -1,3-glucan. This binding is linked with proPO activation. Based on this and other evidence, we conclude that ßGRP2 functions as a pattern recognition receptor for proPO activation in *Manduca*.

Using recombinant proSPH1 and proSPH2 as substrates, I attempted to purify their activating enzymes. I observed cleavage of proSPH1 and proSPH2 by plasma fractions, and detected PO cofactor activity from the processed SPHs. More importantly, I found that the copresence of SPH1 and SPH2 is necessary for manifesting the cofactor activity.

I expressed a *Manduca* PO inhibitor in *E. coli* and insect cells. The recombinant peptides moderately inhibit PO. My effort to isolate the natural inhibitor from the hemolymph was unsuccessful. Instead, I found a low molecular weight chemical with strong inhibitory activity to *Manduca* PO and mushroom tyrosinase.

A clip-domain SPH, Vn50, from the endoparasitoid wasp *Cotesia rubecula,* venom, was found to inhibit PO from its host insect *Pieris rapae*. I used *Manduca* proPO system

to study its inhibitory mechanism. I found that Vn50 down-regulated proPO activation by disrupting the protein-protein interactions among proPO, PAP, and SPH1/SPH2.

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Scope and Method of Study:

Findings and Conclusions:

Melanization is insect acute-phase defense response to invading bacteria, fungi, protozoa, and endoparasitoids. Phenoloxidase (PO) participates in multiple steps of the melenization reaction. Produced as an inactive zymogen, prophenoloxidase (proPO), it is activated by a serine proteinase cascade upon recognition of the invaders. In *Manduca sexta,* the final step involves a proPO activating protease and a serine proteinase homolog (SPH) complex. After the proPO is activated, its activity is tightly regulated.

I purified a ß -1,3-glucan Recognition Protein 2 (ßGRP2) from *Manduca* cuticle extract. ßGRP2 specifically binds to laminarin, a soluble form of ß -1,3-glucan. This binding is linked with proPO activation. Based on this and other evidence, we conclude that ßGRP2 functions as a pattern recognition receptor for proPO activation in *Manduca*.

Using recombinant proSPH1 and proSPH2 as substrates, I attempted to purify their activating enzymes. I observed cleavage of proSPH1 and proSPH2 by plasma fractions, and detected PO cofactor activity from the processed SPHs. More importantly, I found that the copresence of SPH1 and SPH2 is necessary for manifesting the cofactor activity.

I expressed a *Manduca* PO inhibitor in *E. coli* and insect cells. The recombinant peptides moderately inhibit PO. My effort to isolate the natural inhibitor from the hemolymph was unsuccessful. Instead, I found a low molecular weight chemical with strong inhibitory activity to *Manduca* PO and mushroom tyrosinase.

A clip-domain SPH, Vn50, from the endoparasitoid wasp *Cotesia rubecula,* venom, was found to inhibit PO from its host insect *Pieris rapae*. I used *Manduca* proPO system to study its inhibitory mechanism. I found that Vn50 down-regulated proPO activation by disrupting the protein-protein interactions among proPO, PAP, and SPH1/SPH2.

ADVISER'S APPROVAL: Haobo Jiang