EXPRESSION, PURIFICATION AND CHARACTERIZATION OF PEPTIDOGLYCAN RECOGNITION PROTEIN 1 FROM *MANDUCA SEXTA* (L.)

By

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PROTEIN 1 FROM

MANDUCA SEXTA (L.)

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CHAPTER I

INTRODUCTION

Insects are the most diverse group of animals on earth. They are found in nearly all environments on the planet. For successful colonization in various environments, insects rely on an innate immune system to fight against invading pathogens and parasites. Insects have a well developed defense system which closely resembles the vertebrate innate immune system (Gillespie and Kanost, 1997; Lavine and Strand, 2002). The innate immune system functions by encoding factors for recognition and killing of/or invading microorganisms (Fearon, 1997). The insect immunity includes phagocytosis, nodulation, encapsulation, synthesis of antimicrobial peptides (AMPs), activation of proteolytic cascades that lead to melanization, blood coagulation, and release of stress responsive proteins and molecules which function in opsonization and iron sequestration (Jiravanichpaisal et al, 2006).

Insect immune responses are stimulated by recognizing conserved pathogen-associated molecular patterns (PAMPs) which are unique components of almost all microorganisms (Janeway, 1989). Peptidoglycans, lipopolysaccharides, β-1,3-glucans and β-1,3-mannans act as PAMPs in insects (Gillespie and Kanost, 1997). Lectins, hemolin, lipopolysaccharide-binding protein, Gram-negative bacteria-binding protein,

peptidoglycan recognition proteins (PGRPs), β-1,3-glucan recognition proteins (βGRPs) recognize different PAMPs (Ochai and Ashida, 1999).

Peptidoglycan is a structural component of bacterial cell wall. It is a polymer that contains unbranched glycan strands connected through short peptides. The glycan strands are composed of alternating β-1,4-linked *N*-acetyl glucosamine and *N*-acetyl muramic acid residues. A short peptide chain is attached to the muramic acid residue. The cross-linking occurs between the peptide connected to the glycan strand. Lys- and DAP-type peptidoglycans are the two most common types of peptidoglycans in nature. Lys-type peptidoglycan is mainly found in Gram-positive bacterial cell wall while DAP-type peptidoglycan is mainly found in Gram-negative bacterial cell wall. The difference between these two types of peptidoglycan lies on the third amino acid in the peptide chain connected to the glycan strand. In Lys-type peptidoglycan the third amino acid is a *L*-lysine residue and in DAP-type peptidoglycan *meso*-diaminopimelic acid is found at the third position (Schleifer and Kandler, 1972; Meroueh et al., 2006; Volmer et al., 2008).

Peptidoglycan recognition proteins (PGRPs) are immunity-related proteins involved in recognition of peptidoglycan in bacterial cell wall. PGRPs are conserved from insects to humans. The first PGRP was characterized in the silkworm *Bombyx mori* (Yoshida et al., 1996). The conserved carboxy-terminal PGRP domain is approximately 165 amino acid residues long and homologous to lysozyme of bacteriophage T7 (Yoshida et al., 1996; Werner et al., 2000; Liu et al., 2001; Ochiai and Ashida, 1999; Kang et al., 1998). PGRPs have been identified in several insects. Thirteen PGRP genes have been identified in *Drosophila* (Aggrawal and Silverman, 2007). In *Anopheles gambiae* seven PGRP genes have been identified (Christophides et al., 2002). Also PGRPs have been

identified in the lepidopteran insects (Yoshida et al., 1996; Onoe et al., 2007; Hashimotoa et al., 2007).

Recognition of pathogens (by pathogen recognition molecules) activates cellular and humoral defense responses. Hemocytes function in cell-mediated responses, which include phagocytosis of microorganisms, trapping microorganisms by nodulation and encapsulation.

Humoral defense responses include antimicrobial peptide (AMP) synthesis and melanization (Jiravanichpaisal et al., 2006). The synthesis of AMPs is regulated by Toll and IMD pathways in insects, which leads to the translocation of NF-κB proteins that transcriptionally activates the expression of immunity-related genes (Brennan and Anderson, 2004). The Toll pathway is mainly activated during fungal and Gram-positive bacterial infections, whereas the IMD pathway is activated during Gram-negative bacterial infection (Hetru et al., 2003).

Insects PGRPs function in cell activating, phagocytosis and hydrolysis of peptidoglycan (Werner et al., 2000). Cell-activating PGRPs activate either Toll (*Drosophila* PGRP-SA, PGRP-SD and PGRP-SC1) or IMD (*Drosophila* PGRP-LC) pathways (Li et al., 2007). The Toll pathway is preferentially triggered by Lys-type peptidoglycan and the IMD pathway by DAP-type peptidoglycan (Leulier et al., 2003). Some PGRPs activate the prophenoloxidase (proPO) system (Yoshida et al., 1996; Park et al., 2006). Catalytic PGRPs are known *N*-acetylmuramoyl-*L*-alanine amidases which hydrolyze the lactyl-amide bond between *N*-acetyl muramic acid and L-alanine in the peptide stem. *Drosophila* PGRP-LB, PGRP-SC1B and PGRP-SB1 are *N*-

aetylmuramoyl-*L*-alanine amidases (Kim et al., 2003; Mellroth et al., 2003; Mellroth and Stiener, 2006).

In lepidopteran insects the role of PGRPs in innate immune system has been studied. *Bombyx mori* PGRP binds to *M. luteus* peptidoglycan and activate proPO system (Yoshida et al., 1996). PGRP-A from wild silkworm *Samia cynthia ricini* binds to both Lys- and DAP-type peptidoglycans *in vitro* (Onoe et al., 2007).

Our laboratory works on the humoral immune responses of the lepidopteran insect *Manduca sexta* which is a model organism for insect immune research. In *M. sexta* cDNA of PGRP1 has been isolated from induced fat body by substractive hybridization (Zhu et al., 2003). The expression of PGRP1 is constitutive and induced after a bacterial challenge (Kanost et al., 2004; Yu et al., 2002).

Specific objectives of my research include:~~,~~ 1) study the inducibility and expression of PGRP1 from different tissues, 2) expression and purification of PGRP1 from the baculovirus expression system, and 3) functional analysi~~s~~ of PGRP1 (*i.e*., binding of PGRP1 to peptidoglycan and bacterial cells, role of PGRP1 in proPO cascade in *M. sexta* hemolymph, and antibacterial activity of PGRP1).

CHAPTER II

REVIEW OF LITERATURE

Insect immunity

Insects as all other multicellular organisms possess an efficient immune system against pathogens and parasites (Tzou et al., 2002; Lemaitre and Hoffmann, 2007; Pinheiro and Ellar, 2006; Royet, 2004). Though insects lack an acquired immune system they have a well-developed innate immune system that closely resembles vertebrate innate immune system. Also the integument and gut act as physical barriers for insects. When the foreign entities pass these physical barriers, heomocyte responses are activated and synthesis of antimicrobial peptides by fat body is induced (Gillespie and Kanost, 1997; Lavine et al., 2002).

Physiochemical barriers

Insect cuticle acts as the first physical barrier against invading microorganisms (Brey et al., 1993). The peritrophic membrane (chitinous lining) in the gut and trachea also act as a secondary physical barrier. The low pH in the gut maintained by lysozymes also prevents colonization of microbes (Tzou et al., 2002). In lepidopteran insects an extreme high pH is maintained in the gut (Appel and Maines, 1995).

Recognition

Insects have the ability to distinguish foreign molecules from self molecules, and have evolved a system for recognizing characteristic molecular patterns of microbial polysaccharides (Janeway, 1994). Peptidoglycan unique to bacterial cell walls, lipopolysaccharide from the outer membrane of Gram-negative bacteria, β-1,3-glucans, and β-1,3-mannans from fungal cell walls can be recognized by the insect immune system (Gillespie and Kanost, 1997; Yu et al., 2002; Kanost et al., 2004).

After *Manduca sexta* and *Bombyx mori* larvae are injected with peptidoglycan, the synthesis of hemolymph proteins by the fat body is stimulated as observed after injection of whole bacteria to the larvae (Kanost et al., 1988; Ladendorff and Kanost, 1990; Morishima et al., 1995).

The microbial polysaccharides are recognized by both cell surface receptors and pattern recognition proteins in the plasma. Insects carry several proteins that can serve as pattern recognition proteins. These proteins include lectins, hemolin, lipopolysaccharide-binding protein, Gram-negative bacteria-binding protein, peptidoglycan recognition protein (PGRP), β-1,3-glucan recognition protein (βGRP) (Ochai and Ashida, 1999; Kanost et al., 2004; Jiang, 2008). Binding of foreign molecules by PGRP and βGRP triggers the activation of prophenoloxidase cascade which results in melanization (Ashida and Brey, 1997).

Pepidoglycan

Peptidoglycan (PGN) is a polymer present in the bacterial cell wall. It is the only cell wall polymer common to both Gram-positive and Gram-negative bacteria.

Peptidoglycan polymer contains unbranched glycan strands connected through short peptides. The glycan strands are composed of alternating β-1,4-linked *N*-acetyl glucosamine and *N*-acetyl muramic acid residues (Schleifer and Kandler, 1972). The glycan strands are normally 5 to 10 disaccharides units long in the Gram negative bacteria *E. coli* (Hartz et al., 1990). In the general peptidoglycan structure, the short peptide is composed of L-alanine bound to muramic acid, followed by D-glutamic acid, the γ-carboxyl group of D-glutamic acid is linked to L-diamino acid. And the final alanine residue is attached to the diamino acid (Fig. 1). A peptide unit forms cross-link from ω-amino group of the diamino acid of one of the peptide subunit to the D-Ala carboxyl group of another peptide subunit (Schleifer and Kandler, 1972).

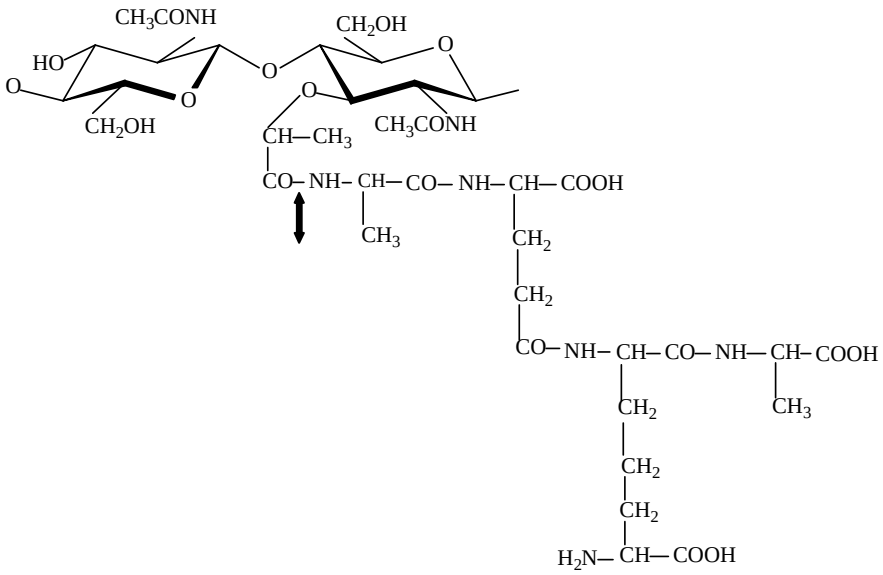


Figure 1. Structure of peptidoglycan monomer of DAP-type peptidoglycan of *E. coli*. The monomer contains the *N*-acetyl glucosamine and *N*-acetyl muramic acid disaccharide and the tetrapeptide subunit connected to the *N*-acetyl muramic acid residue (Vollmer et al., 2008). *N*-acetylmuramoyl-*L*-alanine amidase cleavage site is marked with an arrow ⬍ (Royet and Dziarski, 2007).

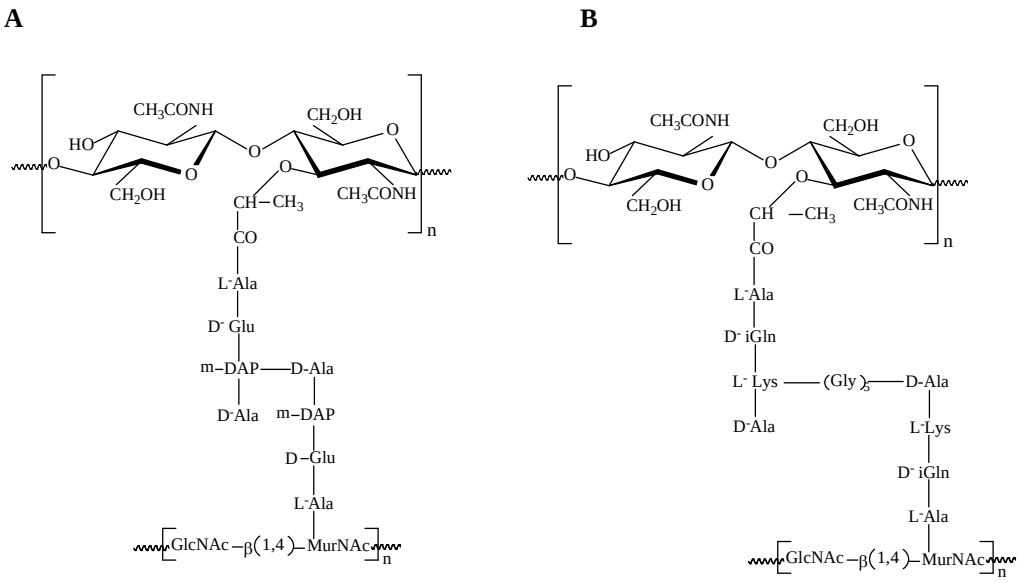


Figure 2. Structures of DAP-type (A) and Lys-type peptidoglycan (B).

The glycan structure in peptidoglycan has a uniform composition in both Gram-positive and Gram-negative bacteria peptidoglycan (Fig.2). The peptide subunit attached to the muramic acid residue shows variation due to different amino acid composition. The muramic acid linking amino acid is usually L-Ala, but in some cases it can be replaced by Gly or L-Ser. The highest variation in the cross-linking subunit occurs at position three, where usually diamino acid is present. Most common diamino acid is *meso*-diaminopimelic acid (m-Dpm) which is present in probably all Gram-negative bacteria and Gram-positive bacterial species belonging to *Bacillaceae*, *Lactobacillaceae*, *Corynebacteriaceae*, and *Propionibacteriaceae* (Schleifer and Kandler, 1972). L-lysine, the second most common amino acid is at the third position of the cross-linking peptide subunit from most other Gram-positive bacteria. Lys-type peptidoglycan is more heterogeneous, due to the variability of inter-peptide. This bridge can be made up of a single amino acid residue or of homo-oligopeptides of up to six residues. In

Staphylococcus aureus the interpeptide bridge contains five glycine residues (Schleifer and Kandler, 1972; Meroueh et al., 2006; Volmer et al., 2008).

In addition to the most common types, DAP- and Lys-type peptidoglycan, there are other types of peptidoglycan which varies in the third position of the peptide subunit. These include L-Orn, L, L-Dpm, *meso*-2, 6-diamino-3-hydroxy-β-pimelic acid (m-hyDpm) and hydroxy-lysine. In some bacterial species the third position diamino acid is not involved in cross-linking. In these peptides cross-linking occurs in D-Glu at position 2. Peptidoglycans can be further modified by amidation of free carboxyl group of D-Glu or *meso*-Dpm and less common *O*-acetylation of *N*-acetyl muramic acid residue. The complete resistance to lysozyme by *Staphylococcus aureus* is due to *O*-acetylation of peptidoglycan (Bera et al., 2005; Schleifer and Kandler, 1972).

The compositions of Gram-positive and Gram-negative bacterial cell walls are different. Major components of Gram-negative bacterial cell wall include lipopolysaccharide and lipo-protein. The peptidoglycan composition is less than 10% of the total cell wall contents. In Gram-positive bacterial cell walls the major component is peptidoglycan, which is about 30-70% of the total cell wall contents. There is high variation in peptidoglycan composition and structure among Gram-positive bacteria. The structure of peptidoglycan is constant among Gram-negative bacteria (Schleifer and Kandler, 1972).

The structure of peptidoglycan brings unique characteristics to bacteria. *N*-acetyl muramic acid in the glycan strand of peptidoglycan is a hexose, only present in bacteria. The presence of D-amino acids is rare in eukaryotic organisms. The alternating D- and L-amino acids in the peptide segment of peptidoglycan are a unique feature to bacteria.

These unique features in bacteria can be utilized by the pattern recognition molecules to recognize bacteria (Mellroth, 2005).

Peptidoglycan recognition proteins (PGRPs)

Peptidoglycan recognition proteins (PGRPs) are a class of immunity related proteins involved in microbe recognition. The first PGRP was characterized in the silk-worm *Bombyx mori* (Yoshida et al., 1996). PGRPs have been identified in insects, mollusks, echinoderms, and vertebrates including mammals. PGRPs are absent in plants and nematodes (Dziarski and Gupta, 2006). PGRPs are expressed in secretory, cytosolic and transmembrane forms, and all these forms contain at least one carboxyl-terminal PGRP domain of approximately 165 amino acid residues which is homologous to lysozyme of bacteriophage T7 (Yoshida et al., 1996; Werner et al., 2000; Liu et al., 2001; Ochiai and Ashida, 1999; Kang et al., 1998).

Insect PGRPs have been classified to short-form or long-form according to its length. In short PGRPs a signal peptide is followed by the PGRP domain. The long PGRPs contain an N-terminal transmembrane with the C-terminal PGRP domain. In *Drosophila* PGRP-SA and PGRP-SD are short secretory PGRPs and PGRP-LC is a long form with a transmembrane domain (Kaneko et al., 2006).

In insects the short PGRPs are secreted into the hemolymph and they are also present in the cuticle, gut, epidermal cells, and fat-body cells. The short PGRPs are either constitutively expressed or induced after an immune challenge. The long membrane-bound PGRPs are mainly expressed in hemocytes. The *Drosophila* PGRP-LE is a long PGRP present in the hemolymph. Long PGRP production is induced following a bacterial

exposure. They can also be induced by purified peptidoglycan (Kang et al., 1998; Werner et al., 2000; Ochiai and Ashida, 1999; Dimopoulos et al., 2002; Christophides et al., 2002).

Two closely spaced cysteine residues in the middle of the PGRP domain which forms a disulfide bond is important for the function of PGRPs (Dziarski and Gupta, 2006). A mutation in one of the two residues (Cys80Tyr) in *Drosphila* PGRP-SA abolishes its ability for activation of the Toll pathway upon binding to Gram-positive peptidoglycan (Michel et al., 2001). Mutation in one of the cysteine residues in human PGLYPR-2 (Cys419Ala) leads to complete loss of its amidase activity (Wang et al., 2003).

Crystal structures of *Drosophila* PGRP-LB, SA and SD, human PGRP-1α and S have been reported (Kim et al., 2003; Reise et al., 2004; Guan et al., 2004a; Guan et al., 2005; Leone et al., 2008). All structures reveal a general fold consisting of several central β-strands and three peripheral α-helices. These proteins have a conserved peptidoglycan binding cleft but differ in N-terminal. PGRPs with catalytic activity have an active site cleft with a Zn^{2+}-binding site. The zinc binding site consists of two histidines, one tyrosine, and one cysteine residue. In catalytic PGRPs Lys128 in T7 lysozyme was replaced with a conserved threonine, which is important in Zn^{2+} binding (Kim et al., 2003). In PGRPs which does not have the amidase activity, the Cys residue important for Zn^{2+} binding is replaced by a serine residue (Mellroth et al., 2003; Wang et al., 2003).

Cellular responses

Insects produce several types of hemocytes that protect the insect from invading microorganisms (Lavine and Strand, 2002). Hemocytes function in recognition, phagocytosis, melanotic encapsulation and cytotoxicity (Cerenius and Söderhäll, 2004; Tzou et al., 2002). Hemocytes also respond to external wounds by participating in clot formation (Lavine and Strand, 2002).

Humoral responses

In insects, the humoral reactions comprise of activation of proteolytic pathways and induced synthesis of immunity related peptides (Hultmark, 1993; Hoffmann, 1995; Meister et al., 1997; Gillespie and Kanost, 1997 and Lehrer and Ganz, 1999). Most commonly produced ones are antibacterial or antifungal peptides that are synthesized mainly in the fat body (Zhu et al., 2003).

Initiation of proPO activation cascade in Manduca sexta

Upon recognition of foreign molecules, prophenoloxidases (proPOs) are activated through a regulated serine protease cascade pathway. Recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) initiates the protease cascade that leads to proPO activation and other immune responses (Ashida and Brey, 1997; Yu et al., 2002). In *Manduca sexta* several PRRs have been identified which bind to PAMPs to activate the proPO cascade. These proteins include immulectin-1 (IML1), IML2, β-1,3-glucan recognition protein-1 (βGRP1), βGRP2, and hemolymph protease 14 (HP14) (Kanost et al., 2004; Ji et al., 2004; Eleftherianos et al., 2006a).

Immunolectins are C-type lectins containing two carbohydrate binding domains. *M. sexta* IML2 stimulates the proPO cascade by binding to lipopolysaccharide from Gram-negative bacteria (Yu et al., 2000; Yu et al., 2006). IML1, less specific than IML2, binds to both Gram-positive and Gram-negative bacteria (Yu et al., 1999). Knockdown of IML2 by RNAi caused great reduction in host resistance against pathogenic bacteria *Photorhabdus asymbiotica* (Eleftherianos et al., 2006a).

β-1,3-glucan recognition protein 1 (βGRP1) and βGRP2 both contain a glucanase-like domain but lack the enzyme activity. Both proteins bind and agglutinate yeasts and bacteria. The proPO system is activated by βGRP1 and βGRP2 through binding to laminarin (Ma and Kanost, 2000; Jiang et al., 2004).

In *M. sexta* two PGRP cDNA clones have been identified by subtractive hybridization, which have identical sequences except to a few differences in the signal peptide (Zhu et al., 2003). The PGRP1 expression is constitutive in naïve larvae and is induced after a bacterial challenge (Kanost et al., 2004; Yu et al., 2002). Supplementation of recombinant PGRP1 to larval plasma did not enhance proPO activation after exposure to *Micrococcus luteus* (Kanost et al., 2004). Knockdown of PGRP1 by RNAi did not have any effect on the cellular immune function (Eleftherianos et al, 2007). However, knocking down PGRP1 expression increased the susceptibility of larvae to *P. asymbiotica* (Eleftherianos et al, 2002a and 2002b).

Hemolymph protease-14 (HP14) became active either by binding to a complex of β-1, 3 glucan and βGRP2 (Wang and Jiang, 2006) or binding to peptidoglycan directly (Ji et al., 2004). Autoactivation of HP14 initiates the proPO cascade via several proteolytic steps (Jiang, 2008).

ProPO cascade

Activation of proPO cascade in insects is a highly regulated process. Recognition of PAMPs, such as peptidoglycans and lipopolysaccharides in bacterial cell wall and β-1, 3-glucan in fungal cell wall, by specific recognition proteins triggers the serine protease pathway (Cerenius et al., 2007). A terminal protease, PAP (proPO activating protease) cleaves proPO to form active phenoloxidase (PO), and PO catalyzes the melanin formation (Cerenius and Söderhäll, 2004).

In *M. sexta* recognition of pathogens leads to the activation of a hemolymph protease cascade. HP14 precursor gets activated by complexing with β-1,3-glucan bound to a βGRP2. Active HP14 activates downstream proHP21. HP21 cleaves PAP2 and PAP3 precursors at a specific peptide bond (Jiang, 2008; Gorman et al., 2007). Two serine protease homologs (SPH1 and SPH2), each containing a protease-like domain at the carboxyl-terminus lacking an active site serine residue, function as a cofactor for proPO activation by a PAP (Yu et al., 2003). In *M. sexta* proPO is activated in the presence of proPO, PAP and SPHs simultaneously (Gupta et al., 2005).

Melanization is a tightly regulated process because excessive melanin formation can also be harmful to host tissues and cells. Serine protease inhibitors of the serpin superfamily play a vital role in regulating melanization, as several critical steps of the proPO cascade including proteolytic cleavage of proPO are controlled by multiple serpins (Cerenius et al., 2007). In *M. sexta* serpin-1J, a variant of serpin-1 gene product inhibits all three PAPs (Jiang et al., 2003a; Gupta et al., 2005). Serpin-4 and serpin-5 regulate hemolymph proteases upstream to the PAPs (Tong and Kanost, 2005; Tong et al., 2005). Serpin-6 inhibits PAP3 in a concentration dependent manner (Wang and Jiang, 2004; Zou

and Jiang, 2005) and it also controls HP8 by forming a covalent complex with the proteases *in vivo*.

Role of PGRPs in proPO cascade

The role of PGRPs in insect proPO cascade has been studied in *Bombyx mori*, *Drosophila melanogaster* and *Tenebrio molitor* (Yoshida et al, 1996; Takhena et al., 2002; Park et al., 2006; Park et al., 2007). The first PGRP was identified in the silkworm, which acts as an entry point for the proPO activation system. Recognition of DAP-type peptidoglycan by *Drosophila* PGRP-LE increases melanization and antimicrobial peptide expression (Takhena et al., 2002). A soluble form of Lys-type peptidoglycan, which contains a long glycan chain with a short peptide stem, binds to PGRP-SA and functions as a potent activator of the *T. molitor* Toll pathway and proPO cascade. *T. molitor* PGRP-SA binds Lys-type peptidoglycan to form clusters, and the clustering is required for activating proPO cascade. Partial digestion of peptidoglycan by lysozyme appears to enhance the clustering of *Tm*PGRP-SA around peptidoglycan and recruitment of Gram-negative bacteria-binding protein (GNBP) and a modular serine protease orthologous to *M. sexta* HP14 (Park et al., 2007). Activation of proPO cascade has been further characterized using a Lys-type peptiodoglycan fragment (T-4P_2), which competitively inhibits melanization stimulated by the natural peptidoglycan. The T-4P_2-coupled column has been used to purify *Tm*PGRP-SA from the hemolymph without activating the proPO cascade. The purified *Tm*PGRP-SA recognizes both Lys-type and DAP-type peptidoglycans but the former is a stronger elicitor than the latter in stimulating melanization (Park et al., 2006).

Signaling pathways and transcriptional activation

Activation of Imd and Toll pathways results in the translocation of NF-κB like factors to the fat body nucleus which induces the transcription of defense related genes (Brennan et al., 2004).

Toll pathway is mainly activated during fungal and Gram-positive bacterial infection in *Drosophila*. Pathway is initiated by proteolytically cleaved form of Späetzle. Activated Späetzle interacts with the extracellular domain of Toll receptor. The receptor-Späetzle complex signals to the ankyrin domain protein Cactus, to dissociate from the NF-κB/Rel protein Dif. Dissociation from its partner causes the exposure of nuclear localization signal (NLS) on Dif which then translocates into the nucleus to initiate transcription of antmicrobial genes (Hetru et al., 2003).

IMD pathway is mainly activated during Gram-negative bacterial infection in *Drosophila*, which regulates the synthesis of antimicrobial peptides including *diptericin, drosocin, cecropins,* and *attacins*. The IMD pathways stimulate the synthesis of antimicrobial peptides through relish an NF-κB/Rel protein. Inactive Relish contains ankyrin repeats that block the NLS (Hoffmann, 2003). After proteolytic cleavage by a caspase the N-terminal fragment of Drosophila Relish which contain a Rel homology domain, transloslocates into the nucleus and initiate transcription of immunity related genes. The C-terminal portion of Relish with ankyrin repeats remains in the cytoplasm after caspase cleavage (Stoven et al., 2000).

Insect Antimicrobial peptides

Synthesis of antimicrobial peptides (AMPs) is an important humoral response in insects. AMPs are small polypeptides less than 150-200 amino acids. Most AMPs are cationic at physiological pH due to high percentage of arginine and lysine residues (Bulet et al., 2004).

In *Drosophila* seven structurally diverse AMP have been identified (Tzou et al., 2000). *Drosomycins* and *metchnikowin* act as antifungal peptides. *Defensin* kill Gram-positive bacteria. *Attacins, cecropins, drosocin* and *diptericins* are active against Gram-negative bacteria. These AMPs function together to inhibit the growth of invading microorganisms in the hemolymph (Hoffmann, 2003).

Functions of Drosophila PGRPs

Insects PGRPs play important roles in the innate immune system, such as recognition, signaling and sometimes effectors (Dziarski and Gupta, 2006). In *Drosophila* 13 PGRP genes encode approximately 17 PGRP proteins through alternative splicing (Aggrawal and Silverman, 2007). *Drosophila* PGRP-SA, PGRP-SD and PGRP-SC1 activate the Toll pathway by Gram-positive bacteria (Michel et al., 2001; Bischoff et al., 2004; Garver et al., 2006). Sensing Gram-positive bacteria by these PGRPs leads to the proteolytic cleavage of Späetzle. The interaction of activated Späetzle with Toll receptor signals the protein Cactus to dissociate from the NF-κB transcription factor Dif. Dissociation from its partner and moving into the nucleus leads to the transcription of drosomycin and the other antimicrobial peptides (Hetru et al., 2003). During Gram-positive bacterial infection *Drosophila* PGRP-SA circulating in the hemolymph forms a

complex with Gram negative bacteria binding protein 1 (GNBP1) that activates the Toll pathway. It has also been suggested that GNBP acts upstream of Späetzle (Pili-Floury et al., 2004). GNBP1 is able to hydrolyze Lys-type peptidoglycan from Gram-positive bacteria; it produces new glycan ends in peptidioglycan, which can be detected by PGRP-SA (Filipe et al., 2005; Wang et al., 2006). *In vivo* RNA interference studies have showed the role of GNBP1 in activation of the Toll pathway during Gram-positive bacterial infection (Pili-Floury et al., 2004). It has been showed that Lys-type peptidoglycan triggers the clustering of PGRP-SA which activates the Toll pathway and melanization cascade by recruiting GNBP1 and a modular serine protease (Park et al., 2007). PGRP-SC1 is involved in phagocytosis of Gram-positive bacteria *Staphylococcus aureus* in addition to sensing Lys-type peptidoglycan for the activation of Toll pathway (Garver et al., 2006).

Gram-negative bacteria and Gram-positive bacilli activate the IMD pathway (Choe et al., 2005; Leulier et al., 2003; Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002; Werner et al., 2003; Kaneko et al., 2004; Stenbak et al., 2004). In *Drosophila* binding of PGRP-LC to peptidoglycan induces receptor multimerization. The receptor multimerization activates the IMD pathway, which leads to the activation of NF-κB transcription factor Relish (Choe at al., 2005). Activated Relish moves into the nucleus and binds to upstream regulatory elements of diptericin and other antimicrobial peptide genes, and induces the immune protein production during Gram-negative bacterial infection (Choe et al., 2002; Gottar et al., 2002; Ramet et al., 2002). The peptidoglycan recognition protein PGRP-LC, activates the IMD pathway by recognizing DAP-type peptidoglycan. This transmembrane protein is actually a mixture of three alternative

splice forms (LC-x, LC-y, and LC-a), all of which have an identical intracellular domain; but differ in the extracellular domain. These isoforms play a unique role in the recognition of Gram-negative bacteria and other microorganisms (Werner et al., 2003). The cytoplasmic domain of PGRP-LC is involved in signal transduction, and its dimerization may be required for the receptor activation (Choe et al., 2005). PGRP-LE also binds to DAP-type peptidoglycan; it functions synergistically with PGRP-LC either upstream or parallel to PGRP-LC during *E.coli* or *Bacillus megabacterium* infection (Takhena et al., 2004). PGRP-LE somehow associates with the prophenoloxidase cascade (Takhena et al., 2002). PGRP-LC is involved in phagocytosis of Gram-negative bacteria (Ramet et al., 2002).

Tracheal cytotoxin (TCT: GlcNAc-1,6-anhydro-MurNAc-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala) is found in the cells of most Gram-negative bacteria. TCT is released constantly during peptidoglycan remodeling (Mengin-Lecreulx and Lemaitre, 2005). TCT is a strong activator of the IMD pathway (Kaneko et al., 2004). In *Drosophila* TCT is recognized by alternative receptors. PGRP-LC which is a surface receptor directly binds to TCT whereas PGRP-LE binds to intracellular TCT (Chang et al., 2006).

Drosophila PGRP-LB, PGRP-SC1B and PGRP-SB1 are *N*-acetylmuramoyl-*L*-alanine amidases, which hydrolyze the bond between the *N*-acetylmuramyl group in the glycan strand and the L-alanine (marked with an arrow Fig.1.) in the stem peptide of peptidoglycan (Kim et al., 2003; Mellroth et al., 2003; Mellroth and Stiener, 2006). Peptidoglycan digested by these enzymes is less immunostimulatory, which is different from lysozyme-digested peptidoglycan that retains its immunostimulatory property (Kim et al., 2003; Leuilier et al., 2003; Mellroth et al., 2003; Kaneko et al., 2004). PGRP-SB1

is an amidase with antibacterial activity prefers DAP-type peptidoglycan, and shows antibacterial activity against *Bacillus megabacterium* (Mellroth and Steiner, 2006). The exact function of catalytic PGRPs in insects is not known. It has been proposed that they may modulate immune responses by scavenging peptidoglycan or act directly as antibacterial factors (Mellroth et al., 2003). *Drosophila* PGRP-LB modulates the IMD pathway during Gram-negative bacterial infection (Zaidman-Remy et al., 2006). *Drosophila* PGRP-SC1 prevents over-activation of the IMD pathway in the gut which is important to prevent larval death and bacteria-induced developmental defects (Bischoff et al., 2006).

PGRP-SA has an L, D-carboxypeptidase activity only against DAP-type peptidoglycans. The Ser158 and His42 residues in the docking groove of PGRP-SA may be involved in the hydrolytic activity. The carboxyl group of the DAP type peptidoglycan interact with the docking groove residues. Ser158 in the docking groove is important for peptidoglycan binding and activation of the Toll pathway (Chang et al., 2004).

PGRPs discriminate Gram-positive and Gram-negative peptidoglycans. The structural difference between the two peptidoglycan types lies in the presence of lysine residue or DAP residue at the third position of the peptide stem. These two residues differ by the presence of a carboxyl-group on their side chain (Fig.2) The carboxy group in DAP-type peptidoglycan is recognized by a conserved arginine residue in the DAP recognizing PGRPs. This Arg residue is conserved among PGRPs recognizing DAP-type peptidoglycan. In PGRP-LE the guanidine group of Arg^{254} charge balances with the carboxyl group of DAP-type peptidoglycan (Lim et al., 2006). For PGRPs recognizing Lys-type peptidoglycan, lysine residue in the third position of the stem peptide is unlikely

to serve as the determinant for discrimination. This may be due to the fact: 1) charge repulsion 2) diverse cross linking patterns are observed in Gram-positive bacterial peptidoglycan (Lim et al., 2006; Schlefifer and Kandler, 1972). It is speculated that the inter-peptide bridges of Lys-type peptidoglycan may be the determinant for PGRPs recognizing Lys-type peptidoglycan (Lim et al., 2006).

Drosophila PGRP-LF is a membrane-bound PGRP which has two extracellular PGRP domains with different affinity for peptidoglycan. The z-domain shows affinity to peptidoglycan of Lys, DAP and ornithine-types but the w-domain only binds to DAP-type peptidoglycan from *E.coli*. PGRP-LF also plays a regulatory role in the immune responses (Persson et al., 2007).

PGRPs in other insects

The first insect PGRP was characterized in the silk-worm *Bombyx mori* (Yoshida et al., 1996). In the mosquito *Anopheles gambiae* seven PGRP genes, four long and three short ones have been identified. Many of the PGRP isoforms are similar in structure to *Drosophia* isoforms. For an example, the exon-intron organization of *A. gambiae* PGRP-LC gene is identical to that of *Drosophila* PGRP-LC and produces three different spliceforms (Christophides et al., 2002).

In the silk-worm (*Bombyx mori*) and mealworm (*Tenebrio molitor*) PGRPs are present in the hemolymph (Yoshida et al., 1996; Park et al., 2006). The silk-worm PGRP activates the prophenoloxidase cascade in the presence of Lys-type peptidoglycan (Yoshida et al., 1996). *Tenebrio molitor* PGRP binds to both Lys-type and DAP-type peptidoglycan to activate the prophenoloxidase cascade (Park et al., 2006).

In the beetle *Holotrichia diomphalia* PGRP binds to peptidoglycan and the fungal cell wall component β-1,3 glucan. Interestingly, it was shown that PGRP binds to β-1,3 glucan for the activation of prophenoloxidase cascade and binding of PGRP to peptidoglycan did not trigger the activation of prophenoloxidase cascade (Lee et al., 2004).

Mammalian PGRPs

In mammals PGRPs have two main functions: antibacterial activity and amidase activity (Dziarski and Gupta, 2006). Mammalian PGLYPR-2 is produced by the liver constitutively and secreted to the blood (Zhang et al., 2005). This is similar to synthesis of insect PGRPs in the fat body and secretion to the hemolymph (Ochiai and Ashida, 1999; Hashimoto et al., 2007; Werner et al., 2000; Dziarski, 2003). In insects, fat body is functionally analogous to mammalian liver (Vierstraete et al., 2003; Gutierrez et al., 2007). Mammalian PGLYPR-2 is also expressed in the intestinal epithelial cells (Dziarski, 2003), similar to expression of insect PGRPs in the gut (Dziarski, 2003; Ochiai and Ashida, 1999; Werner et al., 2000).

Different isoforms in *Drosophila* PGRP-LC are produced by alternative splicing (Werner et al., 2003). Similarly, in some mammals multiple splice forms of PGLYPR-2 are produced. In pigs two isoforms of PGLYPR-2 show differential expression and regulation patterns, but both isoforms have *N*-acetylmuramoyl-*L*-alanine amidases activity (Sang et al., 2005).

The crystallographic structures of mammalian PGLYPR-1 and the carboxy terminal domain of PGLYPR-3 have revealed that these proteins have a ligand binding

groove that binds specifically to peptidoglycan (Guan et al., 2004a; Guan et al., 2005). Insect PGRPs also have ligand binding groove for peptidoglycan binding (Kim et al., 2003: Reiser et al., 2004; Guan et al., 2004b; Chang et al., 2005; Lim et al., 2006; Chang et al., 2006).

Some mammalian PGRPs (carboxy terminal of human PGLYPR-3) have a preference for the binding to Lys-type peptidoglycan over DAP-type peptidoglycan. However human PGLYPR-1 has a high affinity to DAP-type peptidoglycan over Lys-type peptidoglycan (Kumar et al., 2005; Swaminathan et al., 2006). Similarly *Drosophila* PGRP-LC and PGRP-LE have a preference to DAP-type peptidoglycan over Lys-type peptidoglycan (Werner et al., 2003).

Most of the mammalian PGRPs function as bactericidal proteins, in difference to insect PGRPs which very rarely show bactericidal activity. *Drosophila* PGRP-SB1 shows antibacterial activity against *Bacillus megabacterium* (Mellroth and Steiner, 2006). Mammalian PGLYPR-2 is a Zn^{2+}-dependent *N*-acetylmuramoyl-*L*-alanine amidase similar to *Drosophila* PGRP-LB, SC1B and SB-1 (Gelius et al., 2003; Wang et al., 2003; Kim et al., 2003; Mellroth et al., 2003; Mellroth and Stiener, 2006).

CHAPTER III

MATERIALS AND METHODS

Insect rearing, bacterial challenge, and hemolymph collection

M. sexta eggs were purchased from Carolina Biological Supply and larvae were reared on an artificial diet (Dunn and Drake, 1983). Control hemolymph was collected from cut prolegs of day 2, fifth instar larvae. Day 2, fifth instar larvae were injected with formaldehyde-killed *E. coli* (2×10^8 cells/larvae). Hemolymph was collected in the same way from the larvae 6 h, 12 h, and 24 h after the immune challenge. Hemolymph samples from the naïve and induced insects were aliquoted and stored at -80˚C.

Detection of PGRP1 in hemolymph

Control and induced hemolymph samples (6, 12, and 24 h after *E. coli* injection) were analyzed by mixing 2 µl of hemolymph with 6 µl of 20 mM Tris-Cl, pH 8.0, and 4 µl of 5xSDS sample buffer. After incubation at 95˚C for 5 min, 7 µl of the mixture was separated by 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane and reacted with 1:2000 diluted PGRP1 polyclonal antiserum (obtained from Dr. Kanost at Kansas State University). Antibody-antigen complexes were detected using alkaline phosphatase conjugated to goat-anti-rabbit IgG (Biorad) as the secondary antibody.

RT-PCR analysis

Hemocyte and fat body total RNA samples were prepared from the naïve and induced, day 3, fifth instar larvae. Total RNA samples were prepared from muscle, nervous tissue, cuticle, salivary gland, malpighian tubule, and trachea from day 3, fifth instar naïve larvae. The total RNA was extracted using Micro-to-Midi Total RNA Purification System (Invitrogen). The RNA sample (2-4 µg), oligo(dT) (0.5 µg), and dNTPs (1 µl, 10 mM each) were mixed with diethylpyrocarbonate-treated H_2O 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), 5xbuffer (4 µl), 0.1 M dithiothreitol (2 µl), and RNase OUT (1 µl, 40U/µl, Invitrogen) were added to the denatured RNA sample (12 µl) for first strand cDNA synthesis at 37˚C for 50 min. The *M. sexta* ribosomal protein S3 (rpS3) mRNA was used as an internal control to normalize the cDNA samples using specific primers j501 (5´-GCCGTTCTTGCCCTGTT-3´) and j504 (5´CGCGAGTTGACTTCGGT-3´). Primers j297 and j298 (5´-GAACGAAGATCC GATGTCCAGTC-3´) were used to amplify *M. sexta* PGRP1 cDNA under conditions empirically chosen to avoid saturation: 30 cycles of 94˚C, 30 s; 50˚C, 30s; 72˚C, 30s in a multiplex PCR reaction. The relative levels of PGRP1 mRNA in the normalized samples were determined by 1.5% agarose gel electrophoresis.

Expression and purification of M. sexta PGRP1 in insect cells

M. sexta PGRP1 cDNA (obtained from Dr. Kanost at Kansas State University) was amplified using PCR primer j285 (5´-GGAATTCACTGCAACGTCGTC-3´) and j288 (5´-CTCGAGGTCTTTATATTCGGACAC-3´). The PCR product was T/A cloned

into pGEM-T (Promega) and completely verified by DNA sequencing. From the resulting plasmid a 528 bp *Eco*RI-*Xho*I fragment was retrieved by restriction digestion and directionally inserted to the same sites of $pMFH_6$, a plasmid vector modified from pFastBac1 (Lu and Jiang, 2007), to generate the recombinant plasmid ($PGRP1/pMFH_6$). *In vivo* transposition of the expression cassette, selection of bacterial colonies carrying the recombinant bacmid, and isolation of the bacmid DNA were performed according to manufacturer's protocols (Invitrogen Life Technologies). The initial viral stock (V_0) was obtained by transfecting *Spodoptera frugiperda Sf* 9 cells with a bacmid-cellFECTIN mixture, and its titer was improved through serial infections. The V_6 viral stock, containing the highest level of baculovirus, was stored at -70˚C for further experiments. *Sf* 9 cells (at $2.0x10^6$ cells/ml) in 100 ml 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). The cells were removed by centrifugation at 5,000*g* for 10 min. Protein purification was carried out in batches of 100 ml of the conditioned medium. The culture supernatant was mixed with an equal volume of distilled water at 4˚C for 20 min. After centrifugation at 22,100*g* for 20 min, the cleared supernatant (~200 ml) was applied to a dextran sulfate (DS)-Sepharose column (5 ml) (Nakamura et al., 1985) equilibrated with buffer A (10 mM potassium phosphate, pH 6.4, 1 mM benzamidine). Following a washing step with 25 ml of buffer A, bound proteins were eluted with a linear gradient of 0-1.0 M NaCl in buffer A (30 ml). Fractions containing *M. sexta* PGRP1 were pooled and applied onto a Ni^{2+} column (1 ml), equilibrated with buffer B (50 mM potassium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.005% Tween-20). After washing with 5 ml of buffer B, bound

proteins were eluted from the column with a linear gradient of 10-100 mM imidazole in buffer B (20 ml). Finally, tightly bound proteins were eluted with 5 ml of buffer B containing 250 mM imidazole. All the purification steps were carried out at 4˚C. After electrophoretic analysis, PGRP1 fractions were combined and concentrated using Amicon ultracentrifugal 5K MWCO filter device (Millipore). Concentrated protein was buffer exchanged with 20 mM Tris-Cl, pH 7.5, 50 mM NaCl on the same device and stored at -80˚C in aliquots.

Purification of insoluble peptidoglycan from Gram-positive bacteria

Bacterial cells were grown in LB medium (2000 ml) overnight at 37˚C with shaking. The cells, separated from the medium by centrifugation at 2000*g*, 4˚C for 20 min, were resuspended in 100 ml saline (0.85% NaCl) and heated at 100˚C for 20 min. The cells were washed twice with saline, once with water, three times with acetone. Each time 50 ml solution was used for resuspension and removed after centrifugation. The bacterial cells were dried at 37˚C for 8 h (Rosenthal and Dziarski, 1994). Peptidoglyan was extracted from the cells by following a modified protocol described by (Tsuchia et al 1996). Dried cells (10 g) were stirred in 300 ml of 10% trichloroacetic acid (TCA) in a boiling water bath for 20 min. After centrifugation at 10,000*g* for 30 min, the pellet was washed with 250 ml water for three times and once with 250 ml of buffer A (100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$ and 1 mM $CaCl_2$). The resuspension in 100 ml buffer A was incubated with 30 mg of bovine trypsin at 37˚C for 18-24 h with gentle agitation. After trypsin was inactivated with 1 mM PMSF at 37˚C for 30 min, the treated cells were centrifuged at 10,000*g* for 30 min and washed with 200 ml of H_2O for ten times. Each

time the precipitate was completely resuspended by sonication and, after the final wash, lyophilized and stored at -20˚C.

Plate assay of PGRP1 binding to soluble peptidoglycan

Soluble peptidoglycans from *E. coli* or *S. aureus* (InvivoGen) were used to study specific binding. The ligand (2 µg) was applied to each well in a 96-well microplate, air dried overnight at room temperature, and fixed to the well at 60˚C for 30 min. After blocking with 200 µl of 1 mg/ml BSA in TBS at 37˚C for 2 h and washing with 200 µl TBS four times (5 min each), PGRP1 (300 ng) in 50 µl TBS containing 0.1 mg/ ml BSA was added to the wells and incubated at room temperature for 3 h. For competition assay PGRP1 (300 ng) was first incubated with 200 µg of the ligand at room temperature before adding to the well for incubation. Following a washing step with TBS, 100 µl of 1:1000 His-5 monoclonal antibody (Bio-Rad) diluted in TBS containing 0.1 mg/ml BSA was added to the wells and incubated at 37˚C for 2 h. After washing with 200 µl of TBS for four times, 100 µl of 1:1500 goat- anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad) diluted in TBS containing 0.1 mg/ ml BSA was added to the wells and incubated at 37˚C for 2 h. After washing with TBS four times and 0.5 $MgCl_2$, 10 mM diethanolamine once, aliquots of 50 µl of *p*-nitrophernyl 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 on a VersaMax microplate reader (Molecular Devices). BSA was used as a negative control.

Binding of PGRP1 to insoluble peptidoglycan

One mg of insoluble peptidoglycan was mixed with 10 µl (0.3 µg/µl) of PGRP1 and 40 µl of buffer C (20 mM Tris-HCl, pH 8.0, 20 mM NaCl). After incubation for 2 h at 4˚C with mixing, the mixture was centrifuged at 16,000*g* for 15 min. The supernatant was treated with 5x SDS sample buffer and analyzed as unbound fraction. The pellet was washed 3 times with 200 µl of buffer C and boiled with 20 µl of 2xSDS for 5 min to obtain the bound fraction. The unbound (7 µl) and bound (7 µl) samples were separated by 15% SDS-PAGE followed by immunoblot analysis using 1:2000 diluted His-5 antibody as the first antibody and goat-anti-mouse IgG-conjugated to alkaline phosphatase as the second antibody.

Binding of PGRP1 to bacterial cells

A single bacterial colony was grown overnight at 37˚C. Overnight bacterial cultures were subcultured into 4 ml of Luria-Bertani (LB) broth until the OD_{600} was close to 0.8. After centrifugation at 1000*g* and washing with buffer C twice, cells from 4 ml subculture were resuspended in 40 µl of the same buffer. PGRP1 (10 µl, 0.3 µg/µl) was added to the cell suspension and incubated for 2 h at 4˚C with mixing. After centrifugation at 4,000*g* for 15 min, the supernatant was treated with 5xSDS sample buffer and analyzed as unbound fraction. The cell pellet was washed 3 times with 200 µl of buffer C, suspended with 20 µl of 2xSDS buffer, and heated at 95˚C for 5 min to obtain the bound fraction. The unbound (7 µl) and bound (7 µl) protein fractions were analyzed as described above.

Role of PGRP1 in proPO activation in hemolymph from naïve M. sexta larvae

Hemolymph from day 3, fifth instar naïve larvae was centrifuged at 500*g* for 5 min at 4˚C to remove hemocytes. The plasma was diluted ten times with buffer D (20 mM Tris-HCl pH 8.0, 1 mM $CaCl_2$, 0.001% Tween-20). Five µl of diluted sample was mixed with 19 µl of buffer D and incubated at 25˚C for 10 min. PO activity was determined using dopamine as a substrate on a microplate reader (Jiang et al., 2003a). Control hemolymph with low PO activity was selected for the study. Hemolymph was stored in 10 µl aliquots at -80 ˚C.

Five µl of diluted plasma was incubated with purified recombinant PGRP1 (1 µl, 0.2 µg/µl) and different elicitors separately to find out which ones trigger PGRP1-enhanced proPO activation. *Micrococcus luteus*, *Staphylococcus auerus*, *Bacilus megabacterium*, and *Bacillus subtilis* insoluble peptidoglycans (2 µl, 1 mg/ml), *S. auerus* and *E. coli* soluble peptidoglycans (1 µl, 1 mg/ml), curdlan (1 µl, 10 mg/ml), *M. luteus*, *S. auerus*, *B. megabacterium*, *B. subtilis* and *E. coli* cells (1 µl, 2 x 10^5 cells) were tested. The controls were mixtures of diluted hemolymph with buffer, elicitor or PGRP1. The total volume of the test and control mixtures was adjusted to 24 µl with buffer D and incubated at 25˚C for 1 h. PO activity was determined using dopamine as a substrate on a microplate reader (Jiang et al., 2003b).

Antibacterial activity assay

Recombinant PGRP1 was tested against Gram-negative bacteria *S. typhimurium*, *K. pneumonia*, *P. aeruginosa* and *E. coli* and Gram-positive bacteria *M. luteus*, *S. aureus* and *B. megabacterium*. Single bacterial colonies were grown overnight and subcultured

in 4 ml of Trypticase Soy Broth (TSB) for 3-5 h until the bacteria reached mid-log phase. After centrifugation at 1000*g* at 4˚C and washing with 10 mM Tris-HCl, pH 8.0, the cells were suspended in 5% TSB to $5x10^5$ cfu/ml. Aliquots of the diluted cultures (90 µl) were mixed with 10 µl of recombinant PGRP1 (1.2 µg) or 10 mM Tris-HCl, pH 8.0, and cultured at 37˚C for 8 h in a 96-well cell culture plate. Optical density at 600 nm was recorded after 8 h incubation for comparison between the treatment and control groups.

CHAPTER IV

RESULTS AND DISCUSSION

Sequence analysis of PGRP1

M. sexta PGRP1 was initially identified as a pattern recognition receptor in plasma (Yu et al., 2002; Zhu et al., 2003). The open reading frame encodes a 192-residue protein sequence including a 21-residue signal peptide (Fig. 3). No potential *N*- or *O*-linked glycosylation sites are present in the sequence. A BLAST search of mature PGRP1 indicated it is 71%, 69%, 64% and 58% identical to PGRPs from the lepidopteran insects *Antheraea mylitta* (Gandhe et al., 2006), *Samia cynthia ricini* (Onoe at al., 2007), *Trichoplusia ni* (Kang et al., 1998), and *Bombyx mori* (Ochiai and Ashida, 1999) respectively. In PGRP1 the conserved PGRP domain is located between residues 22-164.

Sequence alignment of *M. sexta* PGRP1 with other insect PGRPs revealed that *M. sexta* PGRP1 has a serine residue in the position equivalent to Cys^{130} in T7 lysozyme, which is a common feature of all receptor-type PGRPs. (Fig. 4). This strongly suggests that PGRP1 is not an amidase (Mellroth et al., 2003). (In *M. sexta* PGRP2 and PGRP3, however, Cys is conserved in the position corresponding to Cys^{130} in T7 lysozyme, suggesting that these two proteins are amidases). In addition, Ser^{100} in *M. sexta* PGRP1 is equivalent in position to Arg^{254} of *Drosophila* PGRP-LE, which interacts with the

carboxyl group of DAP-type peptidoglycans (Lim et al., 2006). This Arg residue is conserved in all known *Drosophila* and human PGRPs that recognize DAP-type peptidoglycans, but not always found in PGRPs that bind Lys-type peptidoglycans (Onoe et al., 2007). Arg^{254} of *Drosophila* PGRP-LE corresponds to Arg^{110} and Arg^{115} of *M. sexta* PGRP2 and PGRP3, respectively.

Detection of PGRP1 in M. sexta larval hemolymph

M. sexta PGRP1 was detected in plasma by immunoblot analysis using PGRP1 antibodies. Present at a low level in the naïve plasma, it significantly increased in a time-dependent manner after an immune challenge with *E. coli* (Fig. 5). This is consistent with the data on PGRP1 concentration change after a bacterial challenge (Yu et al., 2002). PGRPs have been detected in plasma of the silkworm (Yoshida et al., 1996), the beetle *Holotrichia diomphalia* (Lee et al., 2004), and the mealworm *Tenebrio molitor* (Park et al., 2006). In *Drosophila* PGRP-SA, PGRP-LE and PGRP-LB are secreted into plasma (Werner et al., 2000; Takhena et al., 2004; Zaidman-Remy et al., 2006).

Inducibility of PGRP1

Semi-quantitative RT-PCR was performed to examine the inducibility of PGRP1 in fat body and hemocytes. *M. sexta* ribosomal protein S3 (rpS3) transcripts were used as an internal control to normalize the cDNA templates. Relative band intensities indicated that PGRP1 was constitutively expressed in the fat body; its level became greatly abundant after an immune challenge (Fig. 6). PGRP1 gene was weakly expressed in

hemocytes from naïve larvae, and its mRNA level also largely increased after the immune challenge.

These results are consistent with the previous report that *M. sexta* PGRP1 gene is constitutively expressed at a low level in fat body of naïve insects and its mRNA level increased after an immune challenge with *E. coli*, *M. luteus, Photorhabdus luminescens* or *P. asymbiotica* (Yu et al., 2002; Eleftherianosa et al, 2006b; Zhu et al., 2003; Eleftherianosa et al., 2006a). Other pattern recognition proteins (*e.g*. *M. sexta* βGRP1, IML2, and IML4) showed similar expression patterns (Ma and Kanost, 2000; Yu et al., 2000 and 2006). *M. sexta* IML1, hemolin and βGRP2 have a different expression pattern; they are produced in the fat body only after an immune challenge (Yu et al., 1999; Wang et al., 2005; Jiang et al., 2004). Hemolin is also expressed in hemocytes and its transcription increased after *E. coli* injection (Eleftherianos et al., 2007).

Other lepidopteran insects show similar expression patterns for PGRPs. In *Bombyx mori* PGRP1 is constitutively expressed in hemocytes and fat body. Its mRNA became more abundant after a bacterial challenge (Ochiai and Ashida, 1999). The expression of PGRP-A in the wild silkworm *Samia cynthia ricini* exhibits the same pattern in fat body and hemocytes (Onoe et al., 2007). *S. cynthia* PGRP-C and PGRP-D transcripts were absent in the naïve larvae, but became much higher in fat body after an immune challenge (Hashimoto et al., 2007).

The inducibility and expression pattern of *Drosophila* PGRPs have been studied. PGRP-SA mRNA is present in the fat body of naïve larvae, but the level did not increase after a bacterial challenge. *Drosophila* PGRP-SB1 and PGRP-SD are mainly expressed in

fat body of induced larvae, whereas PGRP-LA, LC and LD mRNA are present in hemocytes of naïve larvae (Werner et al., 2000).

Expression of PGRP1 in different tissues

Expression of *M. sexta* PGRP1 was studied in the following tissues: malpighian tubule, cuticle, nerve tissue, salivary gland, trachea, muscle, midgut, fat body and hemocytes from naïve larvae by semi-quantitative RT-PCR. PGRP1 was expressed in all tissues tested and transcript levels in fat body and cuticle were higher than the other tissues (Fig. 7). Tissue-specific expression of PGRP has been studied in other insects. *B. mori* PGRP1 is expressed in fat body, hemocytes, and epidermal cells, but not in malpighian tubules, silk gland, or midgut of naïve larvae (Ochiai and Ashida, 1999). *S. cynthia ricini* PGRP-B is constitutively expressed in midgut at a high level in naïve larvae (Hashimoto et al., 2007). In *Drosophila*, PGRP-SC1 and -SC2 are constitutively transcribed in gut; PGRP-LE is also a constitutively expressed but only weakly in gut, hemocytes, and carcass which include all the epidermal layers. PGRP-SA is expressed in fat body and epidermis of naïve larvae (Werner et al., 2000).

Expression and purification of PGRP1 from insect cells

The protein was expressed using the baculovirus expression system. PGRP1 cDNA was cloned to pMHF6 which contains honeybee melittin signal peptide-coding region. The honeybee melittin signal peptide increases the secretion of recombinant proteins (Jarvis et al., 1993). The carboxy-terminal hexahistidine tag facilitates the purification of recombinant protein by affinity chromatography.

The recombinant protein was soluble and secreted into the cell culture medium. After removing the cells from the culture the protein was captured by ion exchange chromatography and eluted from the Dextan Sulfate (DS) column in a small volume. The PGRP1 fractions were affinity purified by Ni-NTA agarose column. The protein was eluted with a linear gradient of imidazole. Fig. 8.A illustrates the purification procedure with comassie blue staning. The immunoblots (Fig. 8.B and 8.C) illustrates the purification procedure using anti-His-5 anibody and PGRP1 antibody respectively. Coomassie blue staining analysis following SDS-polyacrylamide gel electrophoresis indicated that the affinity purified protein was essentially pure (Fig. 8.A). Recombinant PGRP1 run as a 19 kDa band under reducing conditions (Fig. 8.A, 8.B and 8.C).

Binding of PGRP1 to peptidoglycan

In vitro binding assays of *M. sexta* PGRP1 showed, it binds to purified Lys-type peptidoglycan from *M. luteus*, DAP-type soluble peptidoglycan from *E. coli* and amidated DAP-type peptidoglycan from *Bacillus subtilis* and *Bacillus megabacterium*. Recombinant PGRP1 did not show any binding to insoluble and soluble Lys-type peptidoglycan from *S. aureus*. The specific binding of recombinant PGRP1 to *E. coli* (DAP-type) soluble peptidoglycan was confirmed by using a competition assay with ELISA (Fig.9.A). PGRP1 did not show any specific binding to soluble *S. aureus* (Lys-type) soluble peptidoglycan (Fig.9.B). Complete binding of PGRP1 was observed to insoluble peptidoglycan from the bacterial strains *M. luteus* (Fig.10.A), *B. megabacterium* (Fig.12.A) and *B. subtilis* (Fig.13.A). Consistent with ELISA results

recombinant PGRP1 did not show any binding to insoluble peptidoglycan from *S. aureus* (Fig.11.A).

Both mammalian and insect PGRPs have conserved residues for peptidoglycan binding. Sixteen residues have been identified as ligand contacting residues. These residues are invariant in both insect and mammalian PGRPs (Guan et al., 2004b). Five residues which are highly conserved in the ligand binding groove have been identified, these residues in human PGRP-1αC are His-208, His-231, Tyr-242, His-264, and Asn-269. In *M. sexta* PGRP1 His-52, His-75, Ser-86, Pro-108 and Asn-113 are present in the corresponding positions. These conserved residues form a nearly contiguous patch on the floor of binding groove (Guan et al., 2004b). Highly variable residues in the peptidoglycan-binding groove are important in discriminating between Lys- and DAP-type peptidoglycan. The crystal structure of human PGRP-1αC has revealed the Asn-236 and Phe-237 are involved in forming large number of van der Waals bonds with the side chain of lysine (Guan et al., 2004b). Variability of sequences at these two positions may be important for the capability of some PGRPs to discriminate between Lys- and DAP-type peptidoglycan (Michel et al., 2001; Choe et al., 2002; Gottar et al., 2002; Leulier et al., 2003; Werner et al., 2003; Kaneko et al., 2004). In *Drosophila* PGRP-SA which recognizes Lys-type peptidoglycan Asp-96-Phe-97 are the corresponding residues to Asn-236 and Phe-237 of human PGRP-1αC. In contrast to PGRPs recognizing Lys-type peptidoglycan in *Drosophila*, PGRP-LCx and LE which recognize DAP-type peptidoglycan, Gly-Trp are present at the corresponding position (Hoffmann et al., 2003; Kaneko et al., 2004).

In *M. sexta* PGRP1 Asn-80-Tyr-81 are in the corresponding position. In the lepidopteran *Trichoplusia ni* the same residues are found at the corresponding positions. PGRPs from both species recognize both Lys- and DAP-type peptidoglycan (Kang et al., 1998). It has been suggested Arg^{254} in *Drosophila* PGRP-LE interacts with the carboxyl group of DAP-type peptidoglycan (Lim et al., 2006). This arginine residue is conserved in *Drosophila* and human PGRPs which recognize DAP-type peptidoglycan, but not always present in PGRPs that recognize Lys-type peptidoglycan (Onoe et al., 2007). *M. sexta* PGRP1 has a serine residue at the corresponding position of Arg^{254} in *Drosophila* PGRP-LE (marked by a filled arrowhead in Fig. 4). Similar to *M. sexta* PGRP1 in the lepidopteran *Samia cynthia ricini* a serine residue is found at the corresponding position. PGRPs from both species recognize both Lys and DAP-type peptidoglycan (Onoe et al., 2007).

Binding of PGRP to peptidoglycan *in vitro* has been studied in lepidopteran insects. *Bombyx mori* PGRP purified from hemolymph binds to *M. luteus* peptidoglycan (Yoshida et al., 1996). PGRP-A from *Samia cynthia ricini* binds to *M. luteus* (Lys-type) and *B. licheniformis* (DAP-type) peptidoglycans (Onoe et al., 2007). Several *Drosophila* PGRPs bind to both Lys- and DAP-type peptidoglycans include PGRP-LB (Kim et al., 2003), PGRP-SC1B (Mellroth et al., 2003), PGRP-SA (Chang et al., 2004), PGRP-LCx (Mellroth et al., 2005) and PGRP-LF (Persson et al., 2007).

It has been suggested that in lepidopteran insects the DAP or Lysine residue at the third position of the stem peptide may be not critical in recognition of peptidoglycan, but the structure of cross-linking peptide is important for recognition of peptidoglycan (Onoe et al., 2007). This is different to selective recognition of Lys- and DAP-type

peptidoglycans by *Drosophila* and human PGRPs (Leuilier et al., 2003; Swaminathan et al., 2006; Kumar et al., 2005).

Recombinant PGRP1 did not show any binding to either soluble or insoluble *S. aureus* peptidoglycan (Fig.9.B and 11.A). This result is supported by the observation that *M. sexta* PGRP1 in induced hemolymph does not bind to *S. aureus* cells (Ragan, 2008). When recombinant PGRP1 was incubated with *S. aureus* cells a partial binding was observed (Fig.11.B). The partial binding of PGRP1 to *S. aureus* bacterial cells may be due to the homogenous environment in the medium during the binding study, compared to the induced hemolymph which is a complex system with a heterogeneous environment in which no binding of PGRP to *S. aureus* cells has been observed. The binding properties coincide with the proPO activation results of recombinant PGRP1 not triggering proPO activation in control hemolymph by binding to *S. aureus* live cells, insoluble peptidoglycan and soluble peptidoglycan (Fig.16 and 17). Some insect PGRPs *Holotrichia diomphalia* (Lee et al., 2004) *Drosophila* PGRPs SC1B, LB and LF (Mellroth et al., 2003; Kim et al., 2003; Persson et al., 2007) have been shown binding to *S. aureus* peptidoglycan.

When recombinant PGRP1 was incubated with *M. luteus* (Fig. 10.B), *B. megabacterium* (Fig.12.B) and *B. subtilis* (Fig.13.B) cells, only partial binding was observed although a complete binding was observed when purified insoluble peptidoglycan was used from these strains. Recombinant PGRP1 showing higher affinity to peptidoglycan may be due to the less complexity and high accessibility of peptidoglycan compared to cells. This hypothesis is supported by the information from *Samia synthia ricini* PGRP-SA which binds to uncross-linked Lys-type peptidoglycan

from *M. luteus* with a high affinity compared to cross-linked peptidoglycan (Onoe et al., 2007).

ProPO activation

Role of PGRP1 in proPO activation cascade was studied using *M. sexta* control hemolymph. Control hemolymph was incubated with different elicitors and recombinant PGRP1 separately to find which binding triggers proPO activation. When recombinant PGRP1 was added to the control hemolymph without a bacterial elicitor there was an increase in proPO activation (Fig.14). High PGRP1 concentration produced high proPO activity. A sigmoidal curve which is typical for biological processes was obtained when proPO activity was plotted against the PGRP1 concentration. There was a greater increase of proPO activity when PGRP1 was added to the control hemolymph, and with the increase of PGRP1 concentration, proPO activity was increased at a lesser rate and it slowly reaches the asymptote. An increase in melanization has been observed in the absence of microorganisms when *Drosophila* PGRP-LE was expressed in a higher level. PGRP-LE is constitutively present in the hemolymph and is probably involved in the first line of self-defense by recognizing pathogens and transmission of the signals to downstream effectors involved in defense reactions (Takhena et al., 2002). Similar to *Drosophila* PGRP-LE *M. sexta* PGRP1 is a constitutive protein in the hemolymph and it is probably involved in first line of self-defense against invading pathogens. *M. sexta* IML2 which function as pattern recognition molecule enhances melanization (Yu and Kanost, 2004; Ling and Yu, 2006).

When bacterial cells were used as elicitors there was no increase in proPO activation by binding PGRP1 to bacterial cells (Fig.15.B, 16.B, 18.B, 19.B, 20.B) .This was consistent with the reported data that there is no increase in proPO activaton when recombinant PGRP1 was incubated with *M. luteus* cells (Kanost et al., 2004). This may be due to complex structure of cell wall, compared to purified peptidoglycan. Activation of proPO cascade by peptidoglycan but not by bacterial cells coincides with the results of binding studies. The binding was complete with insoluble peptidoglycan but was partial with bacterial cells. There was increase in proPO activation when PGRP1 was incubated with sonicated peptidoglycan from *M. luteus* and *B. megabacterium* and soluble peptidoglycan from *E. coli* (Fig.15.A, 18.A, 20.A). PGRP purified from the hemolymph of *Bombyx mori* activates proPO cascade by binding *M. luteus* peptidoglycan (Yoshida et al., 1996). *Drosophila* PGRP-LC triggers the IMD pathway in the presence of lightly cross-linked (25%) peptidoglycan, but PGRP-LC does not activate the IMD pathway with heavily cross-linked peptidoglycan (75%). This result supports the fact that PGRPs sense degree of cross-linking in peptidoglycan (Kaneko et al., 2005).

It has been reported melanization in *Drosophila* and *Galleria* has increased in the presence of peptidoglycan (Bidla et al., 2008). When *M. sexta* control hemolymph was incubated with sonicated peptidoglycan there was no increase in proPO activation, but it was increased when sonicated peptidoglycan was incubated with recombinant PGRP1. Sonication of peptidoglycan might increase accessibility to peptidoglycan. This hypothesis is supported by the fact that *Samia cynthia ricini* PGRP-A binds to less complex uncross-linked *M. luteus* peptidoglycan with high affinity than to cross-linked peptidoglycan (Onoe et al., 2007). It has been reported when sonicated peptidoglycan

was added to the silk worm plasma there was an increase in proPO activation (Tsuchiya et al., 1996) but this was not observed in *M. sexta* control hemolymph incubated with peptidoglycan.

PGRP1 increased proPO activation by binding to both Lys-type peptidoglycan from *M. luteus* and DAP-type peptidoglycan from *B. megabacterium* and *E. coli*. Similarly PGRP from army meal worm *Tenebrio molitor* activates proPO cascade by binding to both Lys- and DAP-type peptidoglycan (Park et al., 2006). PGRP-LE activates proPO cascade in *Drosophila* larvae by binding DAP-type peptidoglycan (Takhena et al., 2002; Takhena et al., 2004).

Incubation of recombinant *M. sexta* PGRP1 with *S. aureus* cells, insoluble and soluble peptidoglycan did not increase the proPO activation (Fig.16 and 18). There was lowering in PO activity when *S. aureus* cells and insoluble peptidoglycan were incubated with PGRP1 compared to controls with PGRP1 without *S. aureus* cells or insoluble peptidoglycan. Binding experiments showed that recombinant PGRP1 does not bind to *S. aureus* peptidoglycan. The binding characteristic coincides with the proPO activation of PGRP protein with peptidoglycan. The lowering of proPO activation by *S. aureus* cells and peptidoglycan may be due to the complex peptidoglycan structure down regulating the proPO cascade. When soluble *S. aureus* peptidoglycan was incubated with PGRP1 no lowering effect was observed (Fig.17). This is due to the fact peptidoglycan is not complexly cross-linked in the soluble form of peptidoglycan. The cell wall of *S. aureus* is very complex with high degree of cross-linking and it is resistant to lysozyme (Dmitriev et al., 2004; Bera et al., 2005). *S. aureus* cells might be using this complex cell wall structure as a resistance mechanism to host immune responses. *Drosophila* IMD pathway

is activated by *M. luteus* peptidoglycan, but not *S. aureus* (Kaneko et al., 2005). *S. aureus* peptidoglycan is highly cross-linked with 5-glycine cross-bridge, whereas *M. luteus* uses a branched pentapeptide (identical to its stem-peptide) for the cross-bridge (Shokman et al., 1983).

When *Drosophila* and silk worm are infected with live *S. aureus* bacterial cells the insects die within a few days (Needham et al., 2004; Kaito et al., 2002). In silk worm larvae infected with *S. aureus*, it shows a systemic infection of *S. aureus* bacterial cells proliferating in blood, tissues and epithelial surface of the mid gut (Kaito et al., 2002). These results suggest that the insect immune system does not show strong immune responses against *S. aureus* infection. In *Anophels gambiae* it has been shown peptidoglycan from *S. aureus* down regulate PGRPS3 (Christophides et al., 2002). In *Bombyx mori* PGRP genes are weakly induced in the fat body after injection with *S. aureus* compared to *E. coli* and *B. subtilis* infection and some PGRP genes are not induced with *S. aureus* peptidoglycan (Tanaka et al., 2008). But it has been shown that soluble *S. aureus* peptidoglycan is able to stimulate the proPO cascade in silk worm and *Tenebrio molitor* (Kaneko et al., 2004).

PGRP1 did not increase proPO activation by binding to *B. subtilis* peptidoglycan (Fig.19.A) although the protein binds strongly to peptidoglycan from *B. subtilis*. Both *B. subtilis* cells and peptidoglycan function as weak elicitors in *M. sexta* (observed results). This may be due to the fact that *B. subtilis* is a non pathogenic microorganism to insects (Needham et al., 2004).

There was an increase in proPO activation by binding recombinant PGRP1 to DAP-type soluble peptidoglycan from *E. coli* (Fig.20.A). This is consistent with the

specific binding results by ELISA. There was no increase in proPO activation by PGRP1 by binding to *E. coli* cells (Fig.20.B). This may due to the fact that soluble peptidoglycan is more accessible to PGRP1 compared to bacterial cells, and also the fact that in *E. coli* the peptidoglycan layer in the cell wall is not directly exposed to the surface.

In the beetle *Holotrichia diomphalia* PGRP binds to β-1,3-glucan for the activation of prophenoloxidase cascade (Lee et al., 2004). The ability of *M. sexta* PGRP1 to activate proPO cascade by binding to curdlan (soluble β-1,3 glucan) was tested, but there was no increase in proPO activation by binding PGRP1 to curdlan was observed (Fig.21).

Antibacterial activity of PGRP1

Recombinant PGRP1 did not show any antibacterial activity against any of the Gram-positive and Gram-negative bacterial strains tested (Table.4). Only insect PGRP which has shown bactericidal activity is PGRP-SB1 from *Drosophila.* PGRP-SB1 shows antibacterial activity against *Bacillus megabacterium* (Mellroth and Steiner, 2006). In contrast to insect PGRPs, most mammalian PGRPs are bactericidal (Liu et al., 2000; Lu et al., 2006).

CHAPTER V

CONCLUSION

The *in vitro* biochemical analysis of *M. sexta* recombinant PGRP1 showed that it functions as a pattern recognition protein which triggers the humoral cascade of proteases leading to the activation of PPO pathway upon binding to bacterial peptidoglycan. *M. sexta* PGRP1 functions as a common recognition molecule for Lys- and DAP-type peptidoglycan (Table.1).

There was a strong relationship between binding and PGRP1 mediated PPO activation (Table.2 and 3). PGRP1 did not show a complete binding to bacterial cells and when bacterial cells were incubated with recombinant PGRP1 in the control hemolymph there was no PGRP1 mediated PPO activation (Table.2). PGRP1 showed complete binding with bacterial peptidoglycan (except *S. aureus* peptidoglycan) and consistent with this binding, an increase in PPO activation was seen when recombinant PGRP1 was incubated with bacterial peptidoglycan (Table. 2 and 3).

These results illustrate that PGRP1 recognize bacterial peptidoglycan more effectively than bacterial cells. When bacterial cells are inside the insect hemolymph they can be processed by the lysozymes, catalytic PGRPs which have amidase activity and by phagocytosis. Hultmark and Borge-Renberg have proposed a mechanism for processing of bacteria for the recognition by recognition protein. It has suggested the bacteria can be

directly identified by hemocytes which are involved in phagocytosis. Hemocytes have an efficient mechanism for the export of digestion products, such as peptidoglycan fragments, from phagocytosed microorganisms (Hultmark and Borge-Renberg, 2007). This processed peptidoglycan can be recognized by PGRPs to activate the PPO activation and the signaling pathways which regulate the synthesis of antimicrobial peptides.

The inducibility, constitutive expression and role of PGRP1 in the PPO activation suggest that this recognition protein plays an important role in the *M. sexta* immune system.

Bacterial strain	Type of peptidoglycan	Binding of *M. sexta* PGRP1		
		Bacterial Cells	**Insoluble peptidoglycan**	**Soluble peptidoglycan**
M. luteus	Lys-type	partial binding	complete binding	_
S. aureus	Lys-type	partial binding	no binding	no binding
B. megabacterium	DAP-type	partial binding	complete binding	-
B. subtilis	DAP-type	partial binding	complete binding	-
E. coli	DAP-type	partial binding	-	specific binding

Table 1. Binding of *M. sexta* recombinant PGRP1 to bacterial cells and peptidoglycan from different bacterial strains.

		Lys-type peptidoglycan		DAP-type peptidoglycan	
		M. luteus	***S. aureus***	***B. megabacterium***	***B. subtilis***
Bacterial cells	Binding	Partial binding	Partial binding	Partial binding	Partial binding
	PPO activation	No PGRP1 mediated PPO activation	No PGRP1 mediated PPO activation	No PGRP1 mediated PPO activation	No PGRP1 mediated PPO activation
Insoluble peptidoglycan	Binding	Complete binding	No binding	Complete binding	Complete binding
	PPO activation	PGRP1 mediated PPO activation	No PGRP1 mediated PPO activation	PGRP1 mediated PPO activation	No significant PGRP1 mediated PPO activation

Table 2. Relationship between binding of *M. sexta* recombinant PGRP1 to bacterial cells/insoluble peptidoglycan and PGRP1 mediated PPO activation.

		Lys-type peptidoglycan	**DAP-type peptidoglycan**
		S. aureus	***E. coli***
Soluble peptidoglycan	**Binding**	No specific binding	Specific binding
	PPO activation	No PGRP1 mediated PPO activation	PGRP1 mediated PPO activation

Table 3. Relationship between binding of *M. sexta* recombinant PGRP1 to soluble peptidoglycan and PGRP1 mediated PPO activation.

Bacterial strain	Optical density (600 nm)				
	Control	Treatment			Control (medium)
		Replicate 1	Replicate 2	Replicate 3	
S. aureus	0.062	0.074	0.071	0.067	0.039
M.luteus	0.087	0.084	0.083	0.083	0.039
B. megabacterium	0.074	0.093	0.090	0.091	0.039
S. typhimurium	0.032	0.045	0.049	0.052	0.039
K. pneumonia	0.067	0.070	0.075	0.074	0.039
P. aeruginosa	0.046	0.047	0.048	0.054	0.039
E. coli	0.043	0.043	0.045	0.045	0.039

Table 4. Antibacterial activity results of PGRP1.

```
 1      GCAAAATGAAGTTATTTTTGTGTGCATTTTTAGTGCTCGTCGCAAAAACAAGATTCCTTA
              M  K  L  F  L  C  A  F  L  V  L  V  A  K  T  R  F  L
 61     ATGCTGACTGCAACGTGGTCAGTAAAGATGACTGGGACGGTATCACTTCCGTCCACATTG
        N  A  D  C  N  V  V  S  K  D  D  W  D  G  I  T  S  V  H  I
121     AGTACCTTACCCGTCCAATCAAACTGGTCATCATTCAACACACTGACACACCTGGCTGCG
        E  Y  L  T  R  P  I  K  L  V  I  I  Q  H  T  D  T  P  G  C
181     ATACCGACGACGCATGCGCAGCGAGGGTTCGCAGCATTCAGGACTATCACTTGGACACTT
        D  T  D  D  A  C  A  A  R  V  R  S  I  Q  D  Y  H  L  D  T
241     TAAATTACTGGGACATCGGATCTTCGTTCCTGATTGGCGGTAATGGTAAAGTTTACGAAG
        L  N  Y  W  D  I  G  S  S  F  L  I  G  G  N  G  K  V  Y  E
301     GCTCCGGGTGGCTTCACGTGGGCGTGCCCAACTATGCTTACAACCGAAAAGCTATCAAAA
        G  S  G  W  L  H  V  G  V  P  N  Y  A  Y  N  R  K  A  I  K
361     TCACGTTCATCGGAAGCTATAATAGTAAAGAGCCAAACTCCCAACAACTAAATGCTATCA
        I  T  F  I  G  S  Y  N  S  K  E  P  N  S  Q  Q  L  N  A  I
421     AAGCCCTGCTGAAGTGTGGCGTTGACAATGGACATCTATCTTCGGATTACAAAGTCGTGG
        K  A  L  L  K  C  G  V  D  N  G  H  L  S  S  D  Y  K  V  V
481     GCCATCGCCAGCTCTTGGACACCGACAGCCCTGGACGGAAATTATACAACATCATCAGGA
        G  H  R  Q  L  L  D  T  D  S  P  G  R  K  L  Y  N  I  I  R
541     GATGGCCAGAATGGACTAACGATGTGTCCGAATATAAAGACTAATTCTTATTGATTGTTG
        R  W  P  E  W  T  N  D  V  S  E  Y  K  D  *
        TTCAGCTACTTTTGGAGAAGACCATACGTATAAGTTGGTAAACAAATACCAAAGTTCAAA
        TCTGTTATACAATTTTGTCTCAGCAATTTGTATTTCTTTTTTAAGGCAAATTATATATTT
        ACGATTAAAAAAAAATATTTTATTACTTATTGTATCATTATAATGTATATAGCGTCTATT
       GCACTTGATTTCAATTAATTTAATTATTAGCAATAAATAATTGTTAACTATGCAAAAAAA
```

Figure 3. Nucleotide and deduced amino acid sequence of *M. sexta* PGRP1 cDNA. The predicted signal peptide is underlined. Translation stop codon (TAA) is highlighted. Conserved residues involved in active site Zn^{2+}-binding are marked with a box.

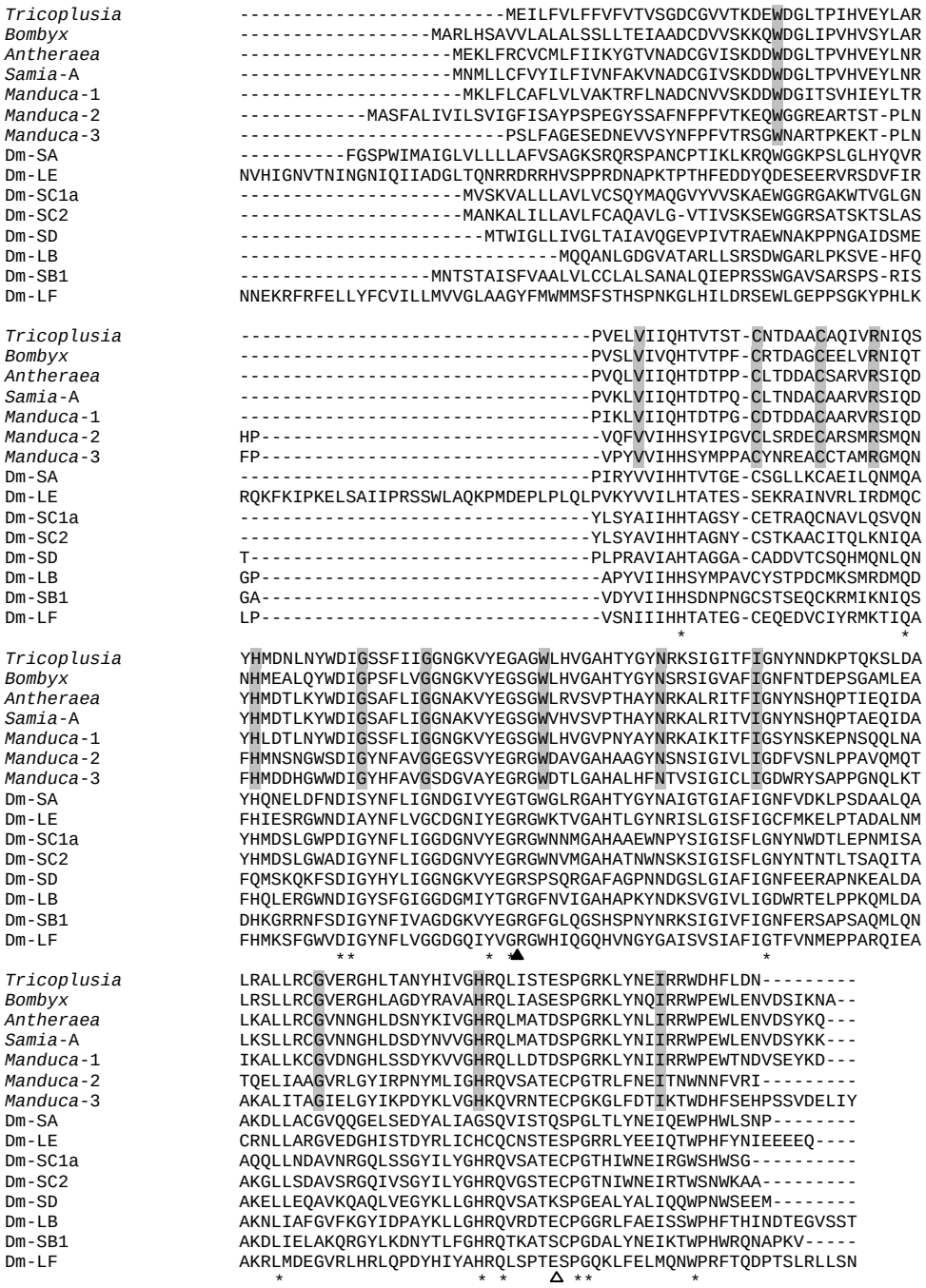


Figure 4. Multiple sequence alignment of PGRP sequences from various insects. Amino acids conserved in lepidopteran PGRPs are shaded. The identical amino acids conserved in lepidopteran and *Drosophila* PGRPs are marked with *. Arginine residue conserved in DAP type peptidoglycan binding PGRP is marked with a filled arrow head (Lim et al., 2006). The Cysteine residue important for amidase activity is marked with an open arrow head (Mellroth et al., 2002; Wang et al., 2003). *Tricoplusia ni* (AAC31820.1), *Bombyx mori* (BAA77209.1), *Antheraea mylitta* (ABG72709.1), *Samia cynthia ricini* (BAF03522.1), *Manduca sexta* 1A (AF413068.1), Dm-SA (NP_572727.1), Dm-LE (NP_573078.1), Dm-SC1a (NP_610407.1), Dm-SC2 (CAD89176.1), Dm-SD (NP_648145.1), Dm-LB (NP_731576.1), Dm-SB1 (NP_648917.1), Dm-LF (NP_648299.3).

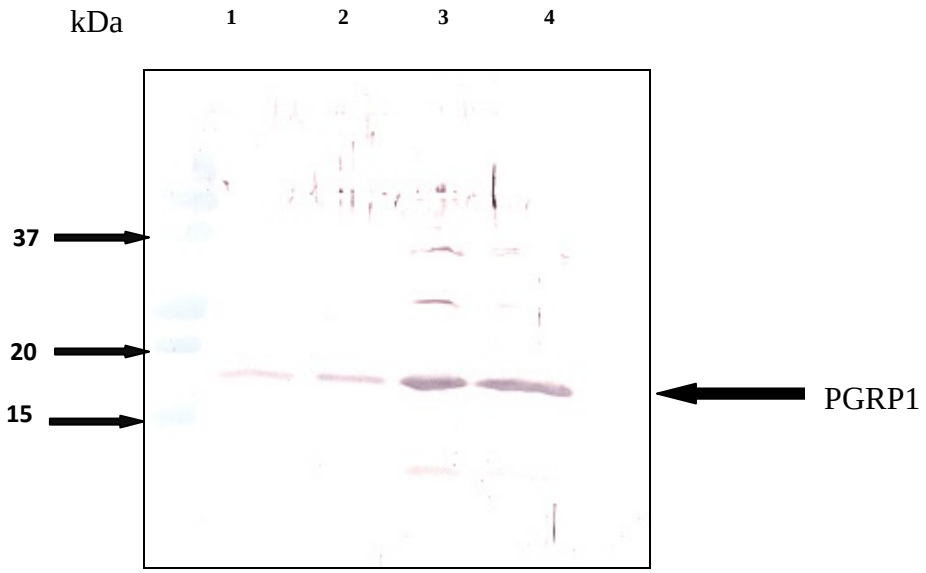


Figure 5. Detection of *M. sexta* PGRP1 in hemolymph. Hemolymph from naïve larvae (lane 1) and hemolymph from larvae injected with *E. coli* after 6 h immune challenge (lane 2), hemolymph from larvae injected with *E. coli* after 12 h immune challenge (lane 3), hemolymph from larvae injected with *E. coli* after 24 h immune challenge (lane 4), immunoblot analysis using PGRP1 first antibody and goat-anti-rabbit IgG conjugated to alkaline phosphatase.

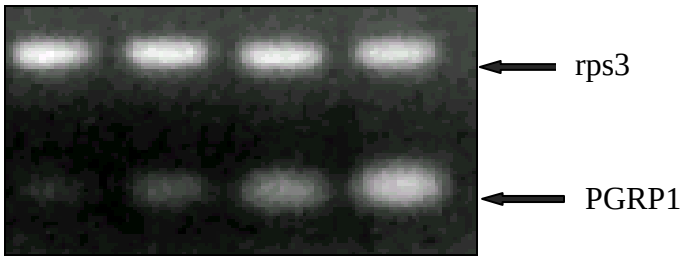


Figure 6. Inducibility of *M. sexta* PGRP1 expression in fat body and hemocytes upon bacterial injection. CH and CF: hemocytes and fat body from the naïve larvae; IH and IF: hemocytes and fat body collected from the larvae at 24h after injection of *E. coli*. *M. sexta* ribosomal protein S3 (rpS3) transcripts were normalized for the analysis.

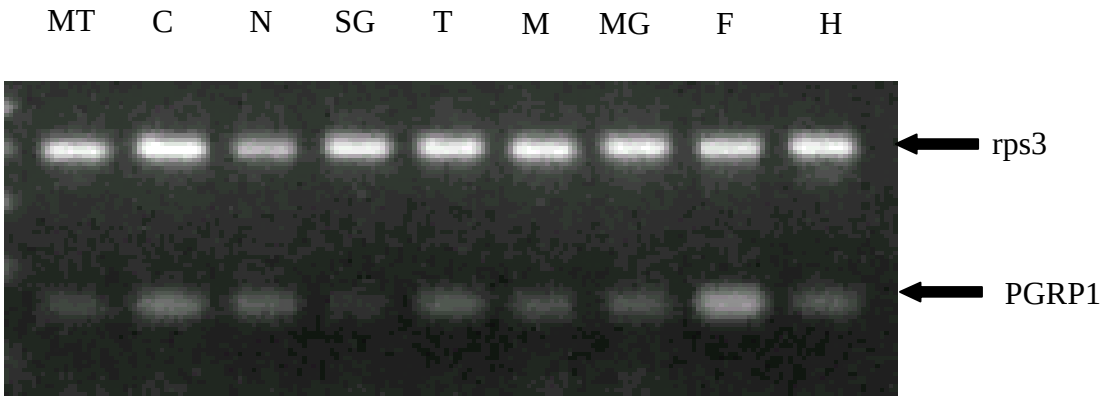


Figure 7. Expression of *M. sexta* PGRP1 in different tissues. Tissues from day 3, 5th instar naïve larvae. MT, Malpighian tubule; C, cuticle; N, nerve tissue; SG, salivary gland; T, trachea; M, muscle; MG, midgut; F, fat body; H, hemocytes. *M. sexta* ribosomal protein S3 (rpS3) transcripts were normalized for the analysis.

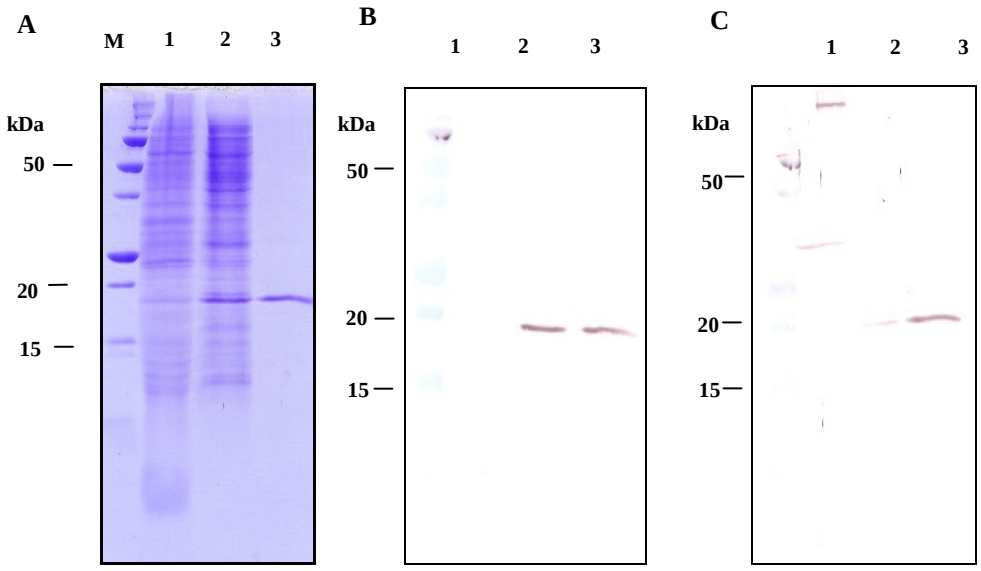


Figure 8. Isolation of *M. sexta* PGRP1 from the baculovirus-infected insect cells. A) 15% SDS-PAGE and Coomassie blue staining; **B)** Immunoblot analysis using His-5 first antibody and goat-anti-mouse IgG conjugated to alkaline phosphatase; **C)** Immunoblot analysis using PGRP1 first antibody and goat-anti-rabbit IgG conjugated to alkaline phosphtase. Conditioned cell culture medium (lane 1, 10 µl), proteins eluted from the dextran sulfate Sepharose column (lane 2, 10 µl), and affinity-purified protein from the Ni-NTA agarose column (lane 3, 10 µl) were separated by SDS-PAGE, along with the molecular weight standards (M).

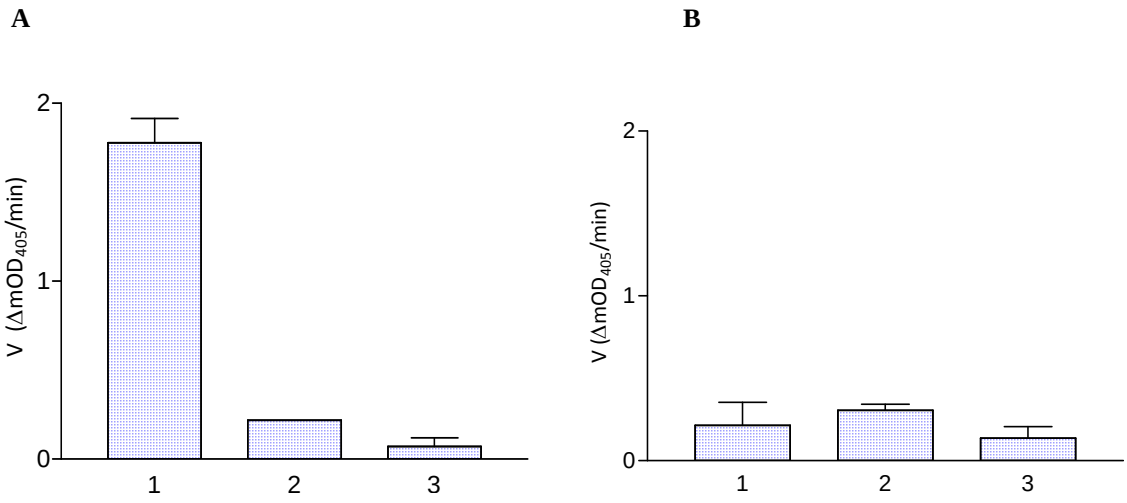


Figure 9. Binding of PGRP1 to soluble peptidoglycan from *E. coli* (A) and *S. aureus* (B). As described in Material and Methods, recombinant PGRP1 was reacted with soluble peptidoglycan immobilized on a 96-well microplate. The binding was detected via ELISA and shown as mean ± SEM (*n*=3). Binding without a competitor (bar 1), with excess soluble peptidoglycan as competitor (bar 2), negative control using BSA (bar 3).

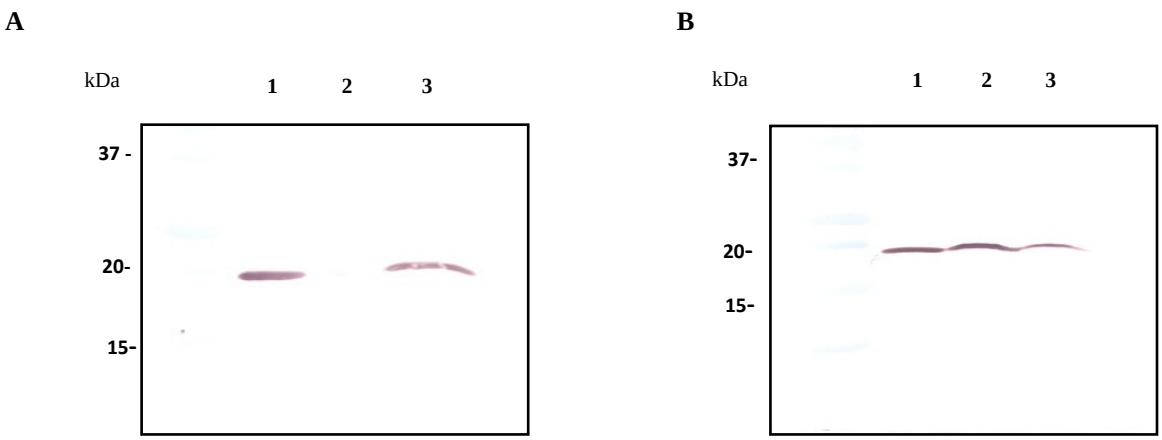


Figure 10. Binding of *M. sexta* PGRP1 to *M. luteus* cells (B) and insoluble peptidoglycan (A). Binding was tested by analyzing the unbound and bound fractions by immunoblotting using His-5 first antibody and goat-anti-mouse IgG conjugated to alkaline phosphatase. Control total protein (lane 1), unbound fraction (lane 2), bound fraction (lane 3).

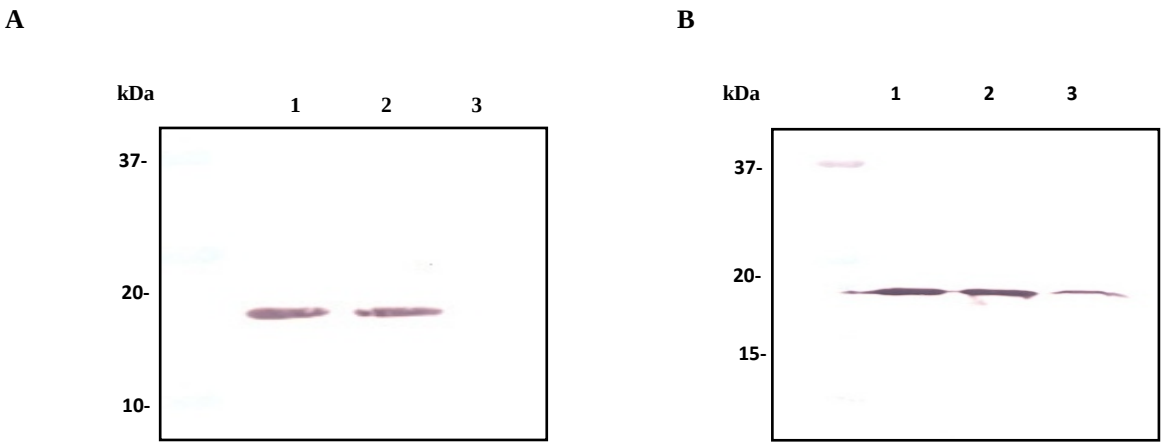


Figure 11. Binding of *M. sexta* PGRP1 to *S. aureus* cells (B) and insoluble peptidoglycan (A). Binding was tested by analyzing unbound and bound fractions by immunoblotting using His-5 first antibody and goat-anti-mouse IgG conjugated to alkaline phosphatase. Control total protein (lane 1), unbound fraction (lane 2), bound fraction (lane 3).

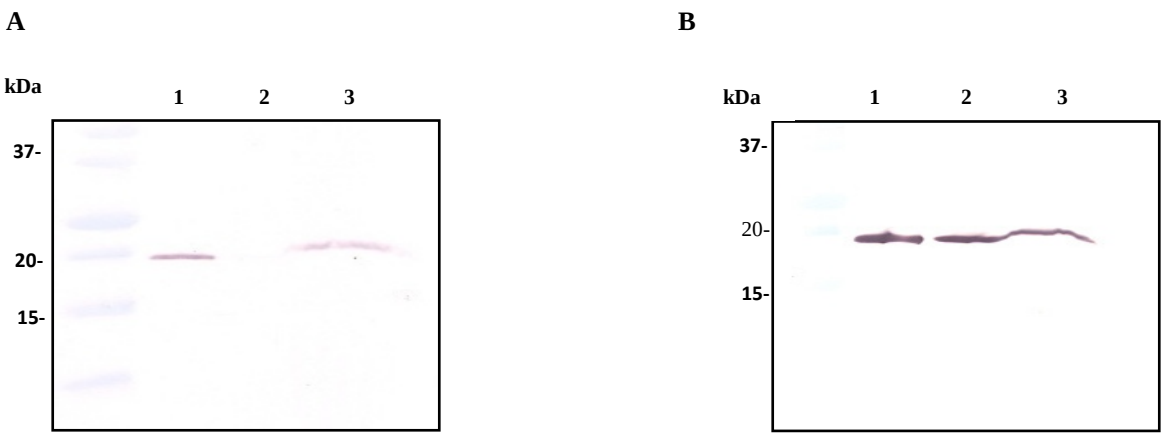


Figure 12. Binding of *M. sexta* PGRP1 to *B. megabacterium* cells (B) and insoluble peptidoglycan (A). Binding was tested by analyzing unbound and bound fractions by immunoblotting using His-5 first antibody and goat-anti-mouse IgG conjugated to alkaline phosphatase. Control total protein (lane 1), unbound fraction (lane 2), bound fraction (lane3).

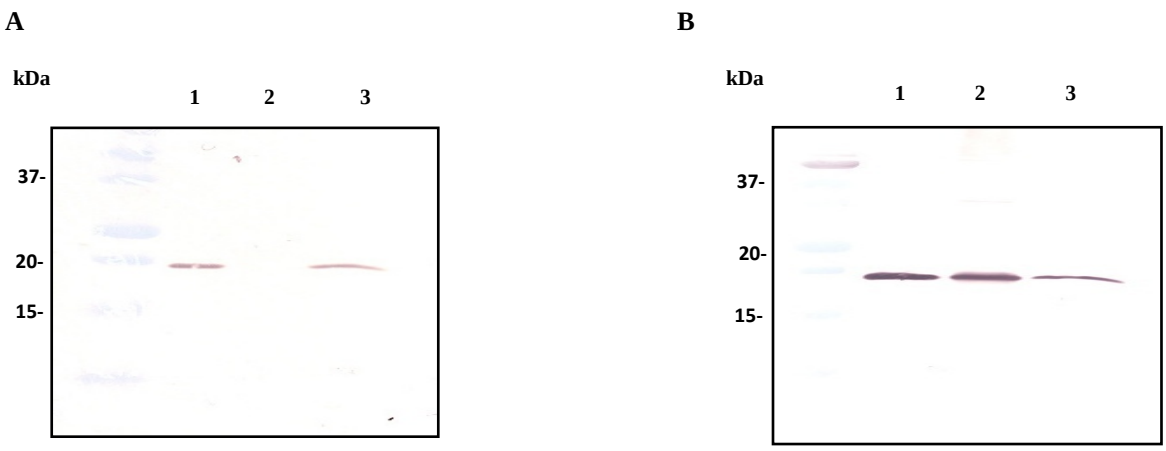


Figure 13. Binding of *M. sexta* PGRP1 to *B. subtilis* cells (B) and insoluble peptidoglycan (A). Binding was tested by analyzing unbound and bound fractions by immunoblotting using His-5 first antibody and goat-anti-mouse IgG conjugated to alkaline phosphatase. Control total protein (lane 1), unbound fraction (lane 2), bound fraction (lane 3).

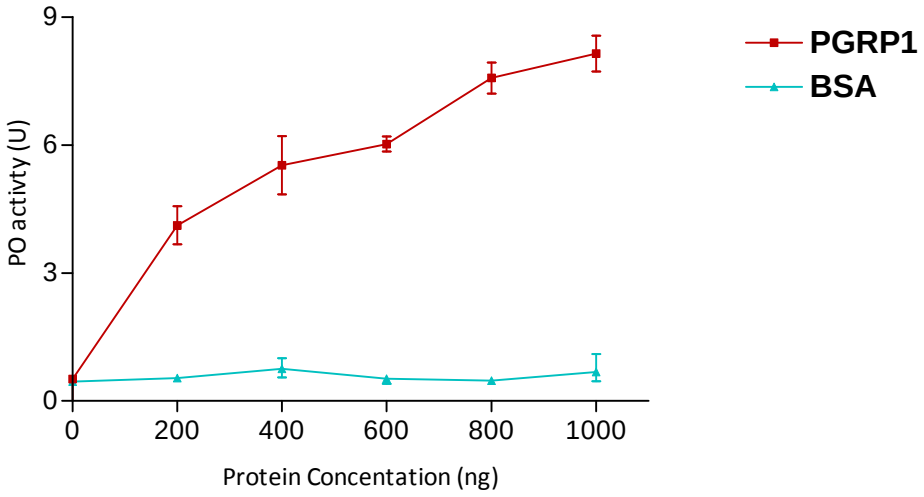


Figure 14. PO activity increase in the control hemolymph after adding *M. sexta* PGRP1.

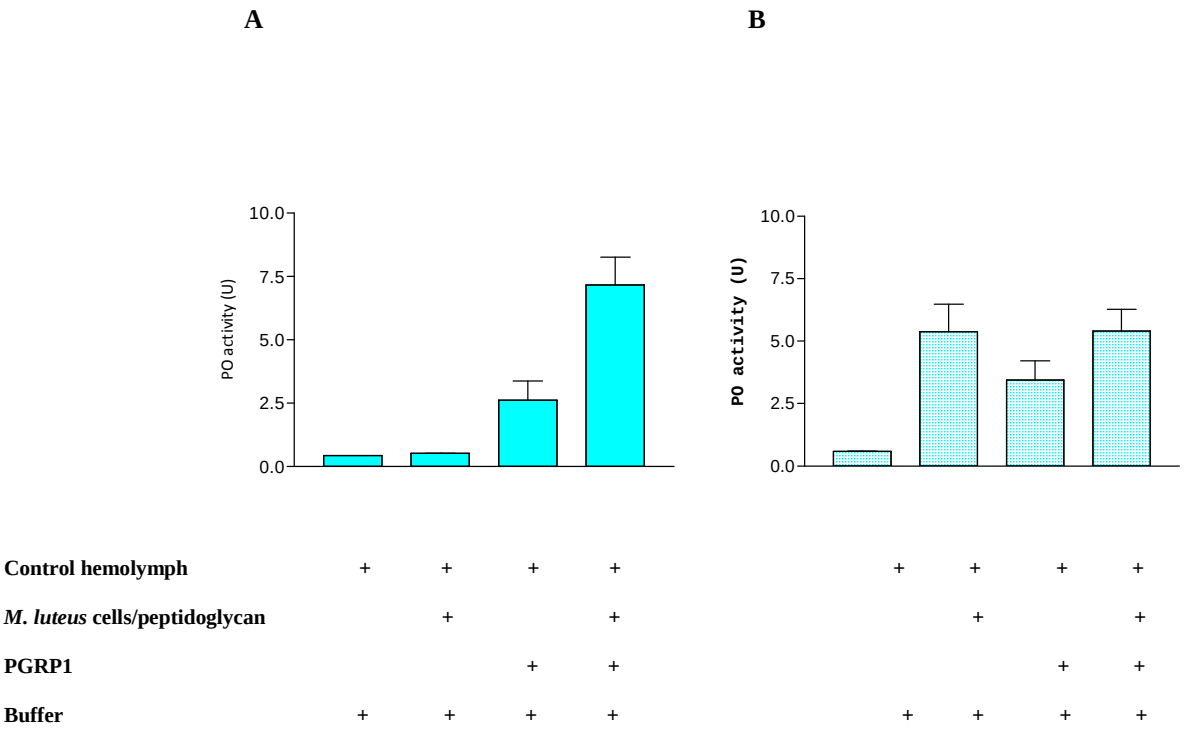


Figure 15. Increase in PPO activation in control hemolymph by *M. sexta* PGRP1 and insoluble peptidoglycan (A) but not by PGRP1 and *M. luteus* cells (B).

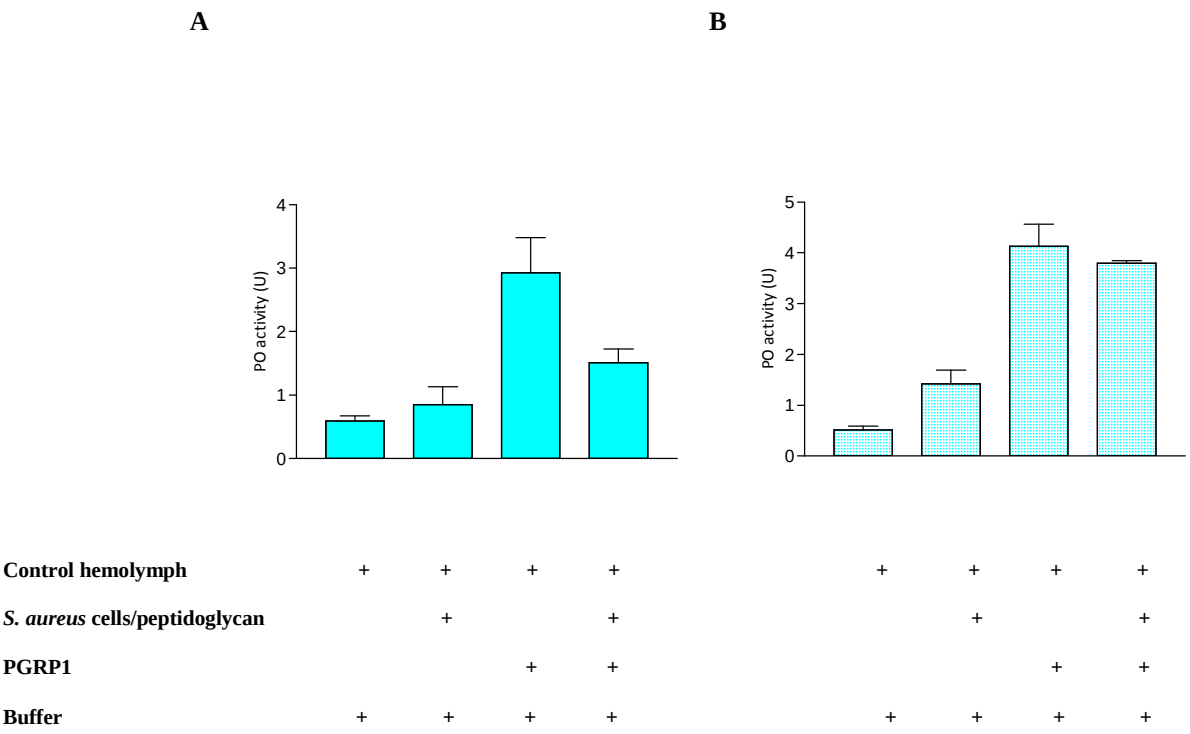
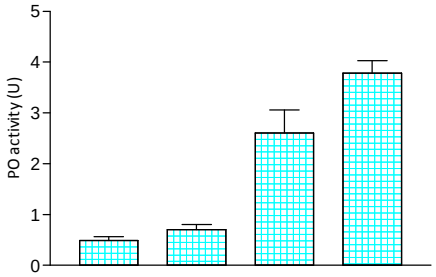


Figure 16. Decrease in PPO activation in control hemolymph by *M. sexta* PGRP1 and insoluble *S. aureus* peptidoglycan (A) and by PGRP1 and *S. aureus* cells (B).



Control hemolymph	+	+	+	+
***S. aureus* s.peptidoglycan**		+		+
PGRP1			+	+
Buffer	+	+	+	+

Figure 17. No significant increase in PPO activation in control hemolymph by *M. sexta* PGRP1 and *S. aureus* soluble peptidoglycan.

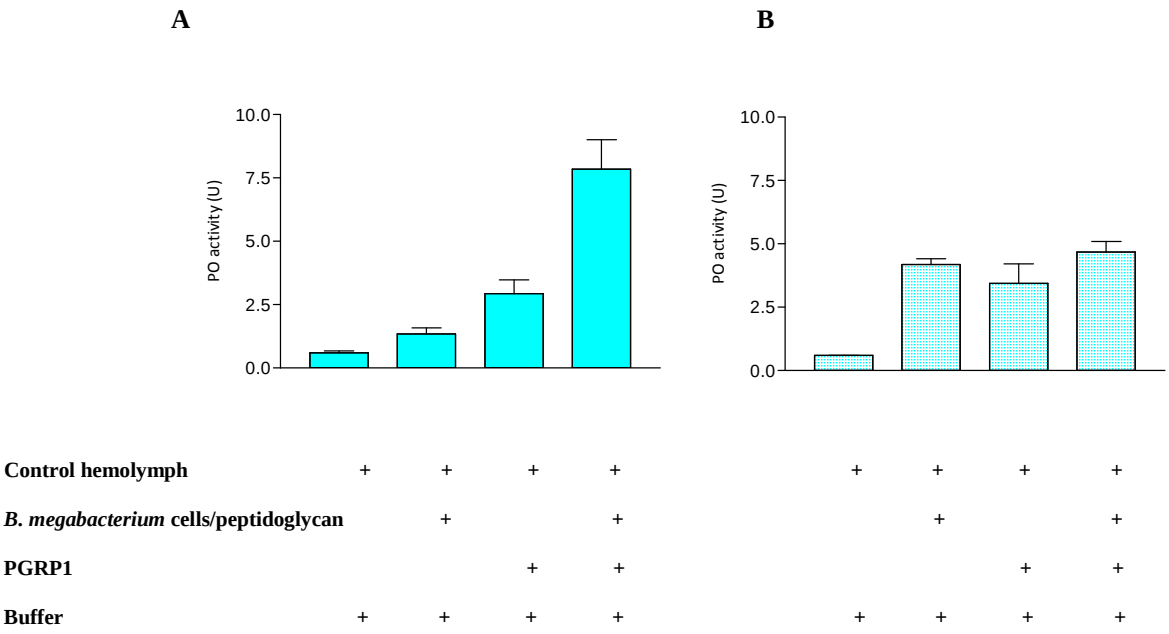


Figure 18. Increase in PPO activation in control hemolymph by *M. sexta* PGRP1 and *B. megabacterium* insoluble peptidoglycan (A) but not by PGRP1 and *B. megabacterium* cells (B).

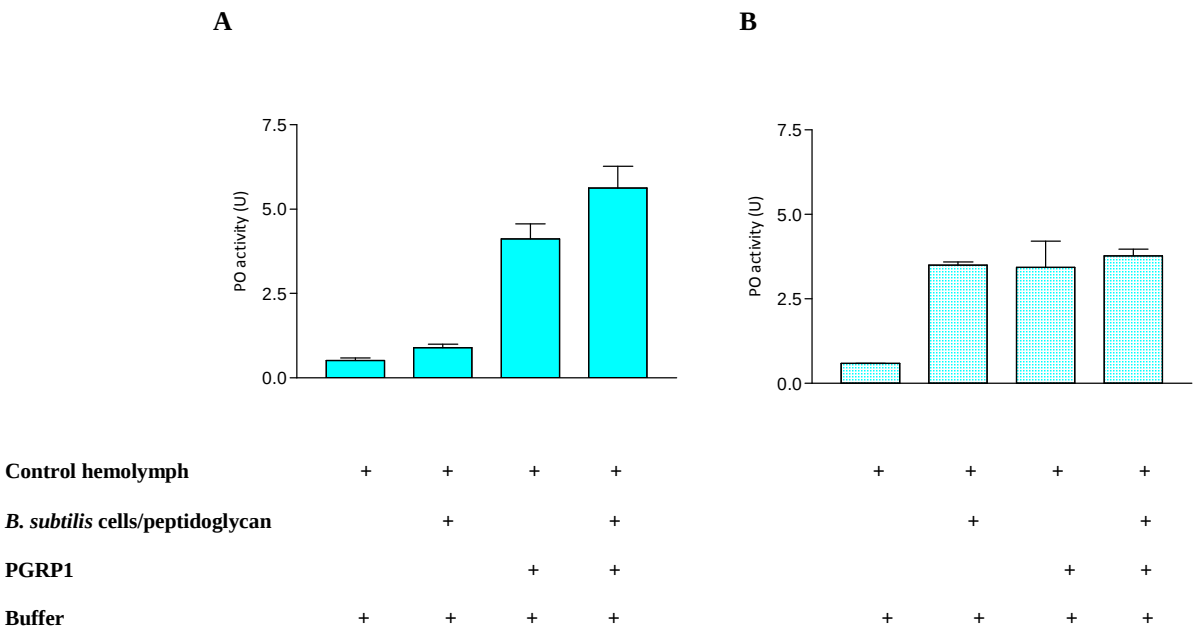


Figure 19. No increase in PPO activation in control hemolymph by *M. sexta* PGRP1 and *B. subtilis* insoluble peptidoglycan (A) or by PGRP1 and *B. subtilis* cells (B).

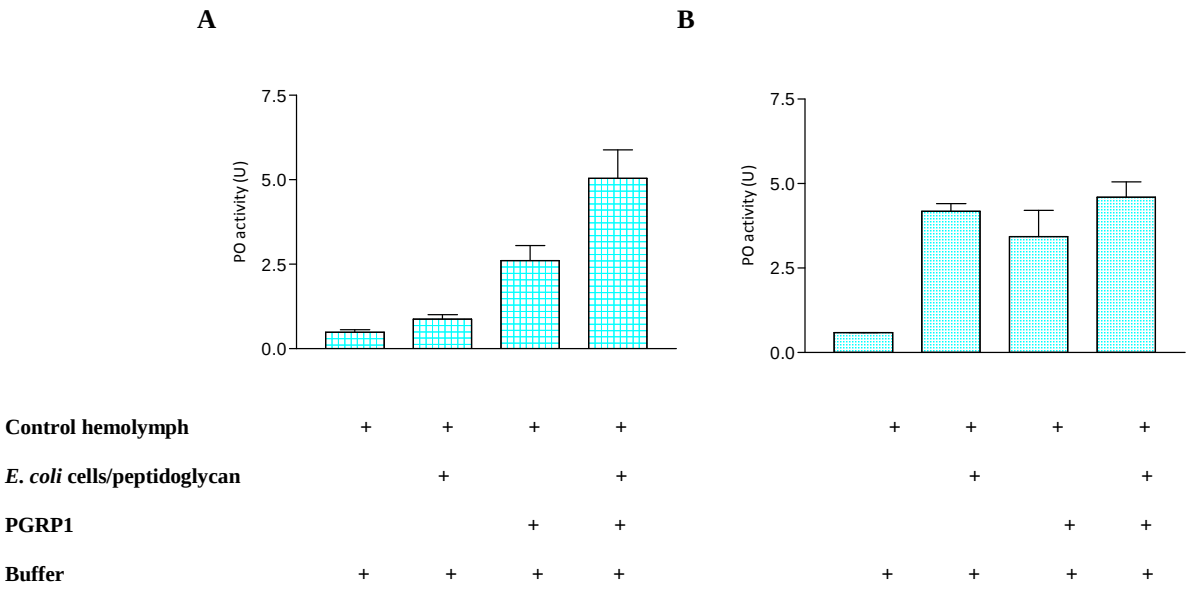


Figure 20. Significant increase in PPO activation in control hemolymph by *M. sexta* PGRP1 and *E. coli* soluble peptidoglycan (A) but not by PGRP1 and *E.coli* cells (B).

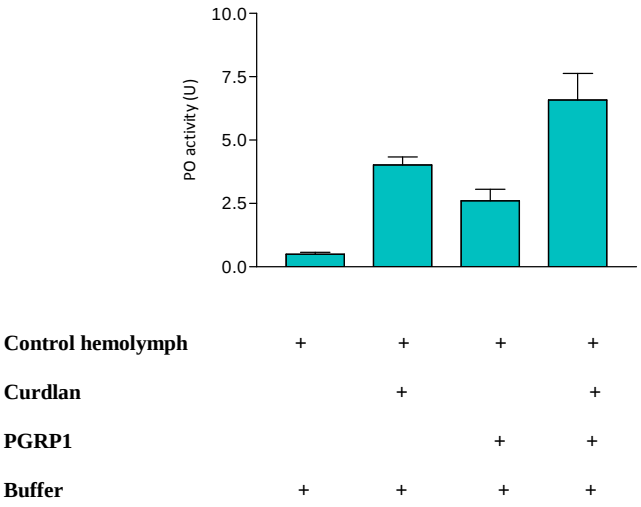


Figure 21. No significant increase in PPO activation in control hemolymph by *M. sexta* PGRP1 and curdlan.

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VITA

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Master of Science

Thesis: EXPRESSION, PURIFICATION AND CHARACTERIZATION OF PEPTIDOGLYCAN RECOGNITION PROTEIN-1 FROM *MANDUCA SEXTA* (L.)

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Title of Study: EXPRESSION, PURIFICATION AND CHARACTERIZATION OF PEPTIDOGLYCAN RECOGNITION PROTEIN-1 FROM *MANDUCA SEXTA* (L.)

Pages in Study: 88 Candidate for the Degree of Master of Science

Major Field: Entomology

Scope and Method of Study:

Findings and Conclusions:

Insects have an effective defense system against invading microbes. Peptidoglycan recognition proteins (PGRPs) in hemolymph detect peptidoglycans (a structural component of bacterial cell walls) and trigger a serine proteinase network that mediates and coordinates various host immune responses. To elucidate its biochemical functions, we have expressed *Manduca sexta* PGRP1 in the baculovirus expression system and purified the protein from the conditioned culture medium. Purified PGRP1 has a molecular mass of 19 kDa.

The recombinant PGRP1 specifically binds to Lys- and DAP-type peptidoglycans from Gram-positive and Gram–negative bacteria, respectively and leads to the proteolytic activation of prophenoloxidase. There was no increase in prophenoloxidase activity by binding recombinant PGRP1 to bacterial cells. Recombinant PGRP1 showed high binding affinity for all polymeric peptidoglycans tested, except for the one from *Staphylococcus aureus*. The recombinant protein showed specific binding to *E. coli* soluble peptidoglycan. Recombinant PGRP1 did not show any antibacterial activity against any of the Gram positive and Gram negative bacterial strains tested.

M. sexta PGRP1 is produced at a low, constitutive level by hemocytes and fat body, and its transcripts become highly abundant in both tissues after larvae are challenged by bacterial injection. PGRP1 is expressed in the all the tissues tested. PGRP1 protein is constitutively present in the hemolymph of naïve larvae, and its level significantly increases after a bacterial injection.

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