ROLES OF *MANDUCA SEXTA* PEPTIDOGLYCAN RECOGNITION PROTEINS IN BACTERIAL SENSING AND PROPHENOLOXIDASE ACTIVATION

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
May, 2021

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ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my advisor Prof. Haobo Jiang for the continuous support and guidance given throughout my Masters study and related research, for his patience, motivation, and immense knowledge that made it possible for me to complete this journey. I could not have done it without you. I would like to thank my committee members Dr. Jose Soulages and Dr. Xia lei for providing me the resources and selfless assistance throughout my masters' study. Besides them, I would like to thank my previous supervisors, Prof. Nadira Karunaweera and Prof. Hira Nakhasi for their insightful comments and encouragement, but also for the inspiration for me to widen my research from various perspectives.

I thank Ms. Yang Wang and my fellow lab mates, Miao, Qiao Jin and Tisheng for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we had in the last two years. Also, I would like to thank Dr, Udaya De Silva, Rupi and my friends in Stillwater for all the help and companionship provided.

A special thank goes out to my beloved husband and my daughter who has provided me with immense moral and emotional support throughout this research and throughout the decade that we have been together. My special gratitude goes to Dr. John Gustafson and all the faculty, staff members and my fellow students at Department of Biochemistry and Molecular Biology, for the wisdom and guidance provided, that kept me rolling through difficult times. Last but not least, I would like to thank my parents, parent in-laws, my brother and sister for the support given that made it possible to complete this journey.

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Date of Degree: MAY, 2021

Title of Study: ROLES OF *MANDUCA SEXTA* PEPTIDOGLYCAN RECOGNITION PROTEINS IN BACTERIAL SENSING AND PROPHENOLOXIDASE ACTIVATION

Major Field: BIOCHEMISTRY AND MOLECULAR BIOLOGY

Abstract:

Insects, rely on their innate immune system as the first line of defense against invading microorganisms. Innate immunity is mediated by germline-encoded pattern recognition receptors (PRRs), such as TLRs, NOD-like receptors, and peptidoglycan (PGN) recognition proteins (PGRPs). These receptors induce interactions with pathogen-associated molecular patterns, to initiate innate immune responses by activating pathways that regulate the expression of antimicrobial peptides (AMPs). PGRPs are one of the most important types of PRRs found in insects and were first discovered in the hemolymph of silkworms as proteins that bind bacterial PGN and activate the prophenoloxidase (proPO) pathway to initiate melanization, which is an antimicrobial host defense mechanism in insects. The differential recognition of diaminopimelic acid (DAP) and Lys-type PGs is in fact common across the PGRP family.

Previous studies on *Drosophila melanogaster* showed that increase in spontaneous melanization was observed when PGRP-LE was over-expressed *and Drosophila* PGRPs prefer Lys-type PGNs than DAP type PGNs. However, specific recognition of different types of peptidoglycans by PGRPs and also how they regulate the proPO system are not yet fully understood in *Manduca sexta.* Therefore, this study is focused on determining the role of *M. sexta* PGRPs in bacterial recognition and proPO activation, and elaboration of their specificity towards different bacteria. Recombinant MsPGRPs were expressed in a baculovirus expression system and purified. To gain functional insights into the recognition of PGN and the activation of proPO pathways by MsPGRPs (MsPGRP 2, 3, 4, 5, 12 ecto and 13), binding specificity and proPO levels were analyzed in this study.

Taken together, our results from ELISA, pull-down assays with PGNs and live bacteria, and PO activity assays suggest that MsPGRPs 2-5 and 13 are positive regulators of the proPO activation system. They preferentially recognize DAP-type PGNs over Lys-type PGNs. Although the recent progress has brought us closer to understanding the role of *M. sexta* PGRPs in bacterial sensing and proPO activation, the precise mechanism of MsPGRP-PGN specific binding that leads to synergistic enhancement in the proteolytic activation of proPO in plasma need further investigations.

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CHAPTER I

INTRODUCTION

Biochemistry behind insect immune system has been a hot topic for several decades. Contribution of insect biochemistry and genetics to human research is significant in many ways. Simpler than vertebrates, insects provide a lead for the progress of knowledge to bridge existing knowledge gaps on fundamental aspects of biochemistry of immune system and ethical guidelines are less stringent for insect research (Arrese and Soulages, 2010; Didham et al., 2019). For example, *Drosophila* is used as a model system to study molecular mechanisms of human diseases (Pandey and Nichols, 2011). Such studies are facilitated by genetic manipulation and directed to discover new regulators of lipid metabolism in the case of obesity research. The adverse effects of insect pests on humans range from competing for food to transmission of vector-borne diseases such as malaria, leishmaniasis, filarisis and yellow fever etc. Anthropogenic environmental changes might be acting as anchors, assisting the spread of the insect pests and their breeding habitats.

Insects are constantly exposed to pathogenic microorganisms and mainly rely on their innate immunity as the first line of defense against the invading microbes (Wang et al., 2019). Recognition and distinction of invaders from host cells is critical in a successful immune response (Kang et al., 1998; Kanost et al., 2004). Two arms of the innate

immunity are cellular and humoral responses. The former includes phagocytosis, nodule formation and encapsulation, mediated by insect hemocytes. The latter consists of antimicrobial peptide production through the Toll and immune deficiency (Imd) pathways, melanization via prophenoloxidase (proPO) activation, and hemolymph coagulation (Leulier et al., 2003). Insect humoral immune responses are mediated by proteins in body fluids such as hemolymph (Gillespie and Kanost, 1997; Kaneko et al., 2005, 2004)

Humoral responses occur mainly through pattern recognition receptors (PRRs), which recognizes pathogen associated molecular patterns (PAMPs) that are absent in the host. These PAMPs include lipopolysaccharides (LPS) of Gram-negative bacteria, lipoteichoic acid (LTA) of Gram-positive bacteria, peptidoglycans (PGN) of the two types of bacteria, β-1,3-glucans of fungi, and nucleic acids of microbes (Akira et al., 2006; Medzhitov and Janeway, 2002). The proPO activation is remarkably sensitive to specific recognition of bacterial PGNs. Pathways leading to AMP expression have been extensively studied in *Drosophila melanogaster* and *Manduca sexta* (Choe et al., 2002; Myllymäki et al., 2014; Royet et al., 2011; Yu et al., 2002).

Insect peptidoglycan recognition proteins (PGRPs) play vital roles as PRRs in recognition and distinction of invading pathogen and activation of intracellular signaling pathways (Dziarski and Gupta, 2006). Some PGRPs are involved in activating the proPO cascade and inducing phagocytosis. The association of PGRPs with a broad spectrum of activities inside cells and on cell surface of tissues makes them an effective surveillance

toward PGNs (Choe et al., 2002; Kang et al., 1998; Wang et al., 2019; Yu et al., 2002). Their study remains a subject of interest to many scientists, many years after the silkworm PGRP was discovered as the first PRR (Yoshida et al., 1996) . PGRPs are evolutionary conserved, and their gene duplication and sequence divergence have yielded new functions worthy of further investigation (Kang et al., 1998).

Spontaneous melanization occurs when *Drosophila* PGRP-LE and *M. sexta* PGRP1 were over-expressed (Sumathipala and Jiang, 2010; Takehana et al., 2004; Yu et al., 2002; Zhu et al., 2003). While the role of PGRPs in bacterial recognition and inducing immune responses is well recognized in *D. melanogaster* and some other insect species, such a role has not yet been experimentally established for PGRPs beyond PGRP1 in *M. sexta*. Thirteen PGRP genes were identified in the *M. sexta* genome (Hu et al., 2019; Yu et al., 2002; Zhang et al., 2015). The phylogenetic analysis of *Manduca* and *Drosophila* PGRPs showed that *Manduca* PGRP1, 5–7, 9, 13 are similar to *Drosophila* PGRP-SA, whereas *Manduca* PGRP2–4 are similar to *Drosophila* PGRP-SB/SC/SD. *M. sexta* PGRP8 is orthologous to *Drosophila* PGRP-LD, while PGRP10 and PGRP11 are 2:1 orthologous to *Drosophila* PGRP-LA. *Manduca* PGRP12A/B may correspond to *Drosophila* PGRP-LC/LE (Zhang et al., 2015). Sequence comparison of *Manduca* PGRPs showed that *Manduca* PGRP13 contains a low molecular weight lipoprotein-11 domain. Such domain structure has not been associated with PGRP domain in other lepidopteran insects. *M. sexta* PGRP2–4 contain key residues (H, Y, H, T, C) at conserved positions for amidase activity.

On the basis of the transcriptome and proteome data, PGRP1–3, and 5 are up-regulated upon microbial challenge (Zhang et al., 2015). *Manduca* PGRP1 participates in the proPO activation system in a Ca^{2+}-dependent manner. It preferably binds to DAP-PGN rather than Lys-PGN (Sumathipala and Jiang, 2010; Wang and Jinag, 2017)(Hu et al., 2019). However, specific recognition of different types of PGNs by the other PGRPs and how they may regulate the proPO system are not yet reported in *M. sexta*. Consequently, my current study is focused on determining binding specificity of some other *M. sexta* PGRPs towards PGNs from different bacteria and their possible roles in proPO activation.

CHAPTER II

REVIEW OF LITERATURE

2.1 Overview of insect immune system

Insects are exposed to various entomopathogenic microorganisms including bacteria, fungi, viruses, and parasites. Due to their efficient system of biological defense, only few cause infection (Gillespie and Kanost, 1997). During evolution, insects have developed lines of defense against microbial infection, including physical barriers and innate immunity. Physical barriers such as cuticle, midgut, and trachea are hard to penetrate; some hemolymph proteins and hemocytes mediate humoral and cellular responses, respectively (Gillespie and Kanost, 1997; Tsakas and Marmaras, 2010; Wang et al., 2019). Compared with immunoglobulins and T-cell receptors in mammals, insect innate immunity is less specific and has no memory (Tsakas and Marmaras, 2010). Recent studies showed that the immune system of insects is more robust and specific than contemplated previously (Cooper and Eleftherianos, 2017; Sheehan et al., 2020). Insect innate immunity plays an important role in preventing infectious diseases and maintaining homeostasis (Sheehan et al., 2018).

Two arms of the innate immune system are cellular and humoral responses. After microbes overcome the physical barrier of insects, humoral and cellular defense responses are often activated. Since the two types of reactions share an extracellular

signaling system upon infection, the distinction between humoral and cellular responses is, up to a point, artificial (Tsakas and Marmaras, 2010).

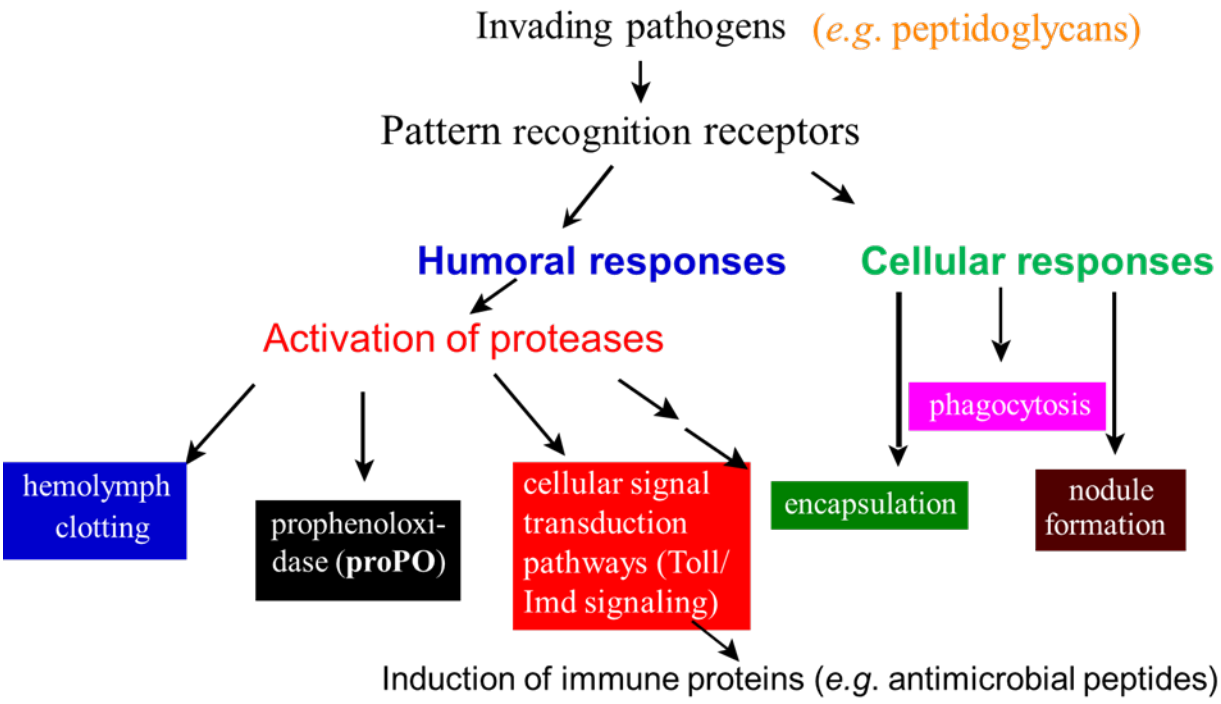


Figure 1: Overview of the antimicrobial defense of insects

2.2 Insect cellular responses

Hemocyte responses include phagocytosis, nodule formation, and encapsulation. Insect hemocytes and human neutrophils and macrophages are similar structurally and functionally (Akira et al., 2006; Kanost et al., 2004; Sheehan et al., 2018). Cellular responses occur immediately after a pathogen invasion of hemocoel. Previous studies on *Drosophila* larvae showed a decrease in circulating plasmatocytes during infection. In

adults, removal of phagocytic hemocytes leads to increased susceptibility to various pathogenic infection (Manachini et al., 2011; Wang et al., 2010).

Several types of hemocytes has been identified in insects based on cell morphology, including granulocyte (or lamellocyte in *Drosophila*), oenocytoid (or crystal cells in *Drosophila*), prohemocyte, spherulocytes, and plasmatocytes (Rosales, 2017). Types of hemocytes and hemocyte responses vary species to species (Akira et al., 2006; Meister, 2004). Not all of these hemocyte types are present in all insect species. Granulocytes and plasmatocytes have the ability of adhesion and phagocytosis, while oenocytoids produce prophenoloxidase (proPO) (Rosales, 2017). Phagocytosis is a potent immune response. Upon invasion of insect hemocoel by pathogenic microbes, plasmatocytes and granular cells recognize, engulf, and entrap them in hemocyte aggregates known as nodules, and finally destroy them.

Previous studies on insect immune system have observed melanization in those nodules and other sites of infection, which happens via the activation of proPO by proteinases present in the insect hemolymph (Kanost et al., 2004; Nazario-Toole and Wu, 2017; Tsakas and Marmaras, 2010). Encapsulation occurs when hemocytes encounter larger targets, such as parasites, protozoa, nematodes, and parasitoid wasp eggs. Encapsulation is limited to invertebrates. During this process, hemocytes form a capsule around a target at first and then kill the target within the capsule (Meister, 2004).

2.3 Insect humoral responses

Insect humoral defense responses occur in minutes to hours post infection. They are mainly composed of three immune reactions: antimicrobial peptide production through the Toll and Imd pathways, melanization through proPO activation pathway, and hemolymph coagulation. These responses are mediated by proteins in body fluids, mainly hemolymph (Augustin and Bosch, 2010; Gillespie and Kanost, 1997; Tsakas and Marmaras, 2010).

2.3.1 Hemolymph coagulation or clotting

Open wound is an ideal site for microorganisms to enter their host circulatory system and, thus, bears a great risk for systemic infection. To prevent invasion and propagation of invading pathogens, wound sealing has to be a rapid process to reduce blood loss. Hence, reestablishment of tissue integrity via hemolymph clotting is a crucial part of the innate immune system (Aprelev et al., 2019; Loof et al., 2011; Schmid et al., 2019). In contrast to the closed circulatory system in vertebrates, which requires delicate balance between thrombosis and fibrinolysis, the open circulatory system in insects may allow blood to clot more rapidly to minimize fluid loss (Dushay, 2009; Loof et al., 2011; Manachini et al., 2011; Schmid et al., 2019). Hemolymph coagulation forms an insoluble matrix to plug the wound, maintain the hydrostatic skeleton of small insects prone to dehydration, entrap microbes at wound sites to ward off infection (Dushay, 2009).

The process of coagulation differs from species to species and also varies in life stages of the same species. Some similarities have been observed in different species as well. The

evolution of a clotting system in insects under different environmental, physiological, and immunological pressures is still poorly understood (Dushay, 2009; Loof et al., 2011; Manachini et al., 2011; Schmid et al., 2019). Hemolymph coagulation has been studied using model insect *Drosophila melanogaster* in the past two decades. Recent microscopic and genetic studies revealed multiple signals from the wound, which coordinate the cellular responses. Interactions between soluble hemolymph proteins (*e.g*. lipid carrier lipophorin) and hemocyte-released components (*e.g*. hemocytin) participate in the formation of hemolymph clot, whereas enzymes such as transglutaminase may crosslink clot proteins (Theopold et al., 2002, 2004; Wang et al., 2010). Insect transglutaminase is found to be involved in clotting at an initial stage (Schmid et al., 2019; Sheehan et al., 2018). Wound leaking in some species triggers humoral reactions to cause self-assembly of lipids and proteins that lead to formation of fibrin-like threads (Bidla et al., 2005; Dziedziech et al., 2020).

After the formation of such clot in most insects, eventual crosslinking produces hard, mature clot through melanization, indicating the presence of phenoloxidase (PO), PO2 in *Drosophila* proPO2 (Scherfer et al., 2004). POs play different roles in insect immunity including killing of invading pathogens, clot formation, cross-linking during wound closure and wound healing (Bidla et al., 2005; Loof et al., 2011; Zhao et al., 2011, 2007). Several steps in the process of coagulation in *D. melanogaster* are wound plugging, scab formation, and epidermal cell mobilization at the site of wounds, which happen at different time scales. In insects with high hemocyte density, such as *M. sexta* larvae,

coordinated cell aggregation and humoral reactions suggest a collective conduct (Aprelev et al., 2019).

2.3.2 Antimicrobial peptide production

Antimicrobial peptides (AMPs) are multifunctional peptides involved in insect humoral immune response. Produced mainly in the fat body and released into the circulation (Yu et al., 2010). AMPs have a wide range of antibacterial, antifungal, and antiviral activities. AMPs kill pathogenic microbes by physically damaging the microbial cell membrane and may not cause resistance in bacteria like antibiotics do. AMPs have several mechanisms for their activity, such as interfering microbial metabolism and disrupting membrane to facilitate the entrance of antibiotics into their cytoplasm. Recent studies have found that electrostatic or hydrophobic interactions between AMPs and microbial cell membrane are dependent on lipid composition of the membrane (Wu et al., 2018). Based on amino acid sequence and structures, insect AMPs can be categorized into three groups, 1) linear peptides with α-helical structures that lack cysteine residues, such as cecropins and moricins, 2) compact structures stabilized by disulfide bonds, such as defensins, drosomycins and gallerimycins, 3) peptides rich in proline and/or glycine residues (Rosales, 2017; Sheehan et al., 2018; Tsakas and Marmaras, 2010). In insects several AMPs including cecropins, drosocin, attacins, diptericins, defensins, ponericins, drosomycin, and metchnikowin are well studied (Rosales, 2017).

In insects, Toll and Imd pathways are the main signaling pathways to regulate AMP production (Wu et al., 2018). The spectra of AMPs vary. For instance, Gly/Pro-rich

AMPs are mostly active against Gram-negative bacteria. Defensins are effective against Gram-positive bacteria. Cecropins are active against both Gram-positive and -negative bacteria (Sheehan et al., 2018). In *Drosophila*, infections by Gram-positive bacteria and fungi activate the Toll pathway to produce AMPs, whereas infection by Gram-negative bacteria triggers the Imd pathway to produce AMPs (Dziarski and Gupta, 2018; Gillespie and Kanost, 1997).

Both Toll and Imd pathways are initiated by PGRPs upon recognition of invading pathogens, and finally induce AMP production via conserved NF-κB signaling cascades (Akira et al., 2006). According to recent literature, there is some overlap between the two pathways in response to Gram-positive or Gram-negative bacteria (Horng and Medzhitov, 2001; Mellroth et al., 2005; Nishide et al., 2019; Yokoi et al., 2012). In some conditions, *Drosophila* PGRP-SD can recognize Gram-negative bacteria and activate Toll pathway (Leone et al., 2008). In addition, Gram-positive *Bacillus subtilis* with DAP-type PGN activate the Imd pathway rather than Toll pathway (Horng and Medzhitov, 2001; Wang et al., 2019; Yokoi et al., 2012). While, *Drosophila* PGRP-SA has selective affinity for different PGNs, PGRP-LCx has affinity for both Lys and DAP-PGNs (Mellroth et al., 2005). Antimicrobial effectors then kill the invading pathogens and, after an appropriate level of immune responses is reached, their production is down regulated.

Pattern-recognition by PGRPs initiates a serine protease cascade that leads to activation of Spätzle-processing enzyme (SPE), which in turn cleaves proSpätzle (an inactive precursor) to generate an active cytokine, Spätzle. The mature Spätzle acts as a ligand for

the Toll receptor to initiate a signaling pathway that leads to activation of the transcription factors Dif and Dorsal to initiate transcription of AMP genes (Duneau et al., 2017; Issa et al., 2018; Rahimi et al., 2016). Basic components of the Toll pathway are transmembrane receptor Toll, which contains extracellular leucine rich repeats, and intracellular adaptors such as Tube and MyD88 (Gobert et al., 2003; Kang et al., 1998; Tsakas and Marmaras, 2010). Insect Toll receptors do not directly bind to pathogens or pathogen-derived compounds and are activated by Spätzle. In contrast, PGRP-LC and PGRP-LE are the upstream PRRs of *Drosophila* Imd proteins. Downstream molecules of Imd pathway are dFADD, Dredd (a caspase homolog), dTAK1, dIKK complexes, and Relish, another NF-kB (Aggarwal and Silverman, 2008). Activated Relish enters the cell nucleus to enhance the expression of AMPs (Swaminathan et al., 2006; Zhao et al., 2018).

2.3.3 Melanization through prophenoloxidase (proPO) activation pathway

In insects, melanization plays a vital role in various physiological processes including antimicrobial activity, wound healing, and cuticle tanning (Sheehan et al., 2018). Melanization has a wide range of anti-pathogenic effect on parasites, bacteria, fungi, and viruses. In insects, pathogen recognition by PRRs initiates an extracellular serine protease cascade, which finally activate proPO to catalyze the oxidation of phenols to quinones to produce melanin and activate cytokines (*e.g.* Spätzle, stress responsive peptides). These cytokines then activate intracellular signaling pathways to express immunity-related genes and cellular defense reactions including phagocytosis, encapsulation, and others to kill and sequester the infectious agents (Bidla et al., 2005;

Kanost et al., 2004; Laughton and Siva-Jothy, 2011; Yu et al., 2002). Previous literature states that the anti-pathogenic activities are coordinated by interactions between melanization and some immune responses (Bidla et al., 2005; Binggeli et al., 2014; Kanost et al., 2004).

In *M. sexta*, pathogen recognition by PRRs causes auto-activation of hemolymph serine protease-14 (HP14) precursor. The initiator HP14 then activates proHP21, HP21 activates HP5 and proPO activating protease 3 (PAP3) precursors. HP6, activated by HP5, further activates PAP1 and HP8 precursors to induce the Toll pathway, since HP8 is an activating enzyme of Spätzle-1, which is a ligand of Toll receptor. PAP1–3 generate active PO in the presence of a cofactor composed of serine protease homolog-1 and -2 to generate active intermediates to kill invading pathogens (Bidla et al., 2005; Kanost et al., 2004; Sheehan et al., 2018; Y. Wang et al., 2020).

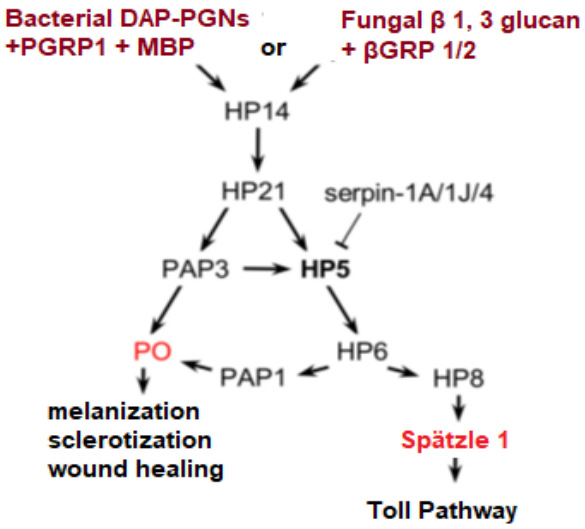


Figure 2: A simplified model of the serine protease network in *M. sexta*, adapted from (Y. Wang et al., 2020).

Enzymatic reactions of active PO that generate toxic compounds have been extensively studied over the past two decades. Briefly, PO hydroxylates monophenols to form o-diphenols and then oxidize the latter to o-quinones. These quinones then polymerized to form melanin (Binggeli et al., 2014; Kanost et al., 2004). For instance, active PO catalyzes the conversion of L-tyrosine to L-dihydroxyphenylalanine (L-DOPA). After DOPA oxidation, decarboxylation, and nucleophilic substitution, 5,6-dihydroxyindole (DHI) is produced. Oxidation and polymerization of DHI produce DHI-eumelanin. PO involves not only in melanization but also sclerotization in some species.

PO catalyze the formation of dopamine quinone using dopamine as a substrate, which cyclizes non-enzymatically to produce DHI (Laughton and Siva-Jothy, 2011; Zhao et al., 2011, 2007). Dopamine can also be N-acetylated to produce N-acetyldopamine (NADA) or N-alanylated to produce N-β-alanyldopamine (NBAD) using acetyl/β-alanyl transferases. Both NADA and NBAD are transported to insect cuticles during pupation and converted to oxidative intermediates for crosslinking of cuticle proteins and chitins, *i.e.* cuticle sclerotization, which is mainly catalyzed by laccase (Wang et al., 2010; Zhao et al., 2011, 2007).

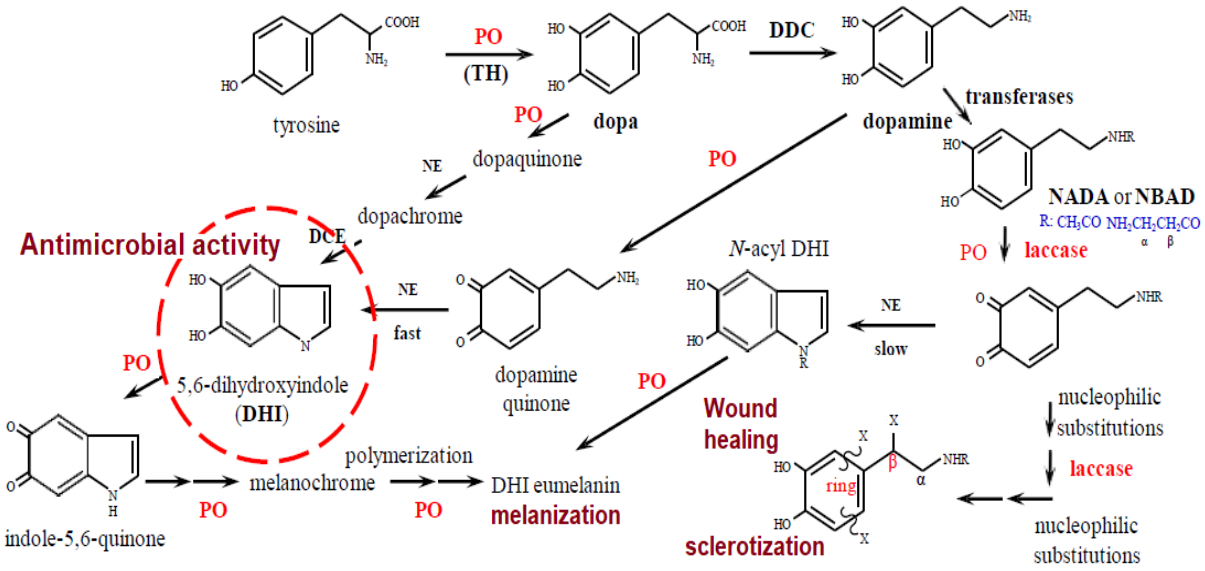


Figure 3: Mechanisms and physiological functions of PO-mediated reactions in insects and crustaceans, adapted from (Zhao et al., 2007).

Evidence supports that DHI has a broad-spectrum of antimicrobial activities, kills wasp embryos, kills insect hemocytes, and may also cause damage of host tissues (Zhao et al., 2011, 2007). Hence, melanization needs to be tightly regulated as a local, transient reaction against non-self. Various serpins negatively regulate the serine protease cascade for focused responses by inhibiting proPO activation and thereafter PO activity (Binggeli et al., 2014; Kanost et al., 2004). Serpins have been extensively studied in *M. sexta*, *D. melanogaster*, and some other insects (Kanost and Jiang, 2015; Meekins et al., 2018; Suwanchaichinda et al., 2013; Y. Wang et al., 2020). One recent example is that *M. sexta* HP5 is inhibited by multiple serpins (1A, 1J, and 4) in hemolymph (Y. Wang et al., 2020).

2.4 Recognition and distinction of invading microorganism

The recognition and distinction of invading microbes from the host cells is an essential step in successful immune responses. Telling apart self from non-self must happen first (Kang et al., 1998; Kanost et al., 2004). The activation of humoral responses occurs mainly through PRRs, which recognizes PAMPs that present only in pathogens but not in the host. PAMPs include lipopolysaccharides (LPS) of Gram-negative bacteria, lipoteichoic acids (LTAs) of Gram-positive bacteria, peptidoglycans (PGNs) of walled bacteria, β-1,3-glucans of fungi, and nucleic acids of bacteria and viruses (Akira et al., 2006; Medzhitov and Janeway, 2002). Although, LPS is a potent immune stimulator, studies showed that it cannot stimulate Imd pathway in *Drosophila melanogaster* (Kaneko et al., 2004; Leulier et al., 2003).

In comparison, PGNs stimulate multiple immune reactions in *Drosophila* (Akira et al., 2006; Swaminathan et al., 2006). PGNs are essential cell wall components of almost all walled bacteria, providing the host immune system an advantage for detecting invading bacteria. PGN are polymers of β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) cross-linked by short stem peptides. The amino acid composition of this stem peptides and the linkage between stem peptides vary from species to species, whereas the glycan chain is relatively conserved in all bacteria. Most of Gram-positive bacteria have lysine residue (Lys-type PGN) at the third position of the stem peptide, whereas some Gram-positive bacteria such as the *Bacillus* species and many Gram-negative bacteria replace lysine residue with *meso*-diaminopimelic acid (DAP) in their PGNs (Vollmer et al., 2008).

2.5 Pattern Recognition Receptors

Innate immunity is mediated by germline-encoded PRRs, such as NOD-like receptors and PGRPs (Dziarski and Gupta, 2006; Gillespie and Kanost, 1997; Kanost et al., 2004). These receptors interact with PAMPs to initiate innate immune responses by activating pathways that regulate AMP expression.

2.5.1 Peptidoglycan recognition proteins in insects

PGRPs are one of the most important types of PRRs found in insects and were first discovered in the hemolymph of silkworms as proteins that bind bacterial PGN and activate the proPO pathway to initiate melanization, an antimicrobial defense mechanism of insects (Yoshida et al., 1996). The discovery of PGRPs significantly contributed to scientific progress in the field of immunobiology. PGRPs conserved from insects, mollusks and mammals, which recognize PGN in bacterial cell wall, and function in antibacterial immunity and inflammation (Ramirez et al., 2020). PGRPs are evolutionary conserved but number and type of PGRPs vary from species to species. In *D. melanogaster*, there are 13 PGRP genes encoding 19 proteins (Royet et al., 2011). The silkworm *Bombyx mori* has 12 PGRP genes (Kayalvizhi and Antony, 2011; Tanaka et al., 2008). There are 7 PGRP genes in the yellow fever mosquito *Aedes aegypti* and 13 in *M. sexta* (Dziarski and Gupta, 2006; Wang et al., 2019; Zhang et al., 2015).

PGRPs can be categorized into several types based on their function (catalytic or sensor) and on their transcript length (short or long, PGRP-S/L) (Dziarski and Gupta, 2018, 2006). The short forms have an N-terminal signal peptide leading them to hemolymph.

The long forms can be transmembrane or secretory PGRPs (Steiner, 2004; Werner et al., 2000). The main sites of PGRP expression are tissues involved in insect immune responses. PGRP-Ss, present in cell-free hemolymph, are synthesized in fat body, hemocytes, and epidermal cells in the midgut, whereas PGRP-Ls are mainly expressed in hemocytes (Dziarski and Gupta, 2006). PGRP-S expression is up-regulated in response to bacterial infection, whereas PGRP-L are mostly constitutive proteins (Werner et al., 2000; Yu et al., 2002).

Some PGRPs with the catalytic residues hydrolyze peptidoglycans prior to immunogenic cascade and act as non-immunogenic molecule. For example, in *Drosophila*, six PGRPs (-LB, -SB1, -SB2, -SC1a, -SC1b, and -SC2) have catalytic activity (Mellroth and Steiner, 2006). This may protect the host immune system from over activation by non-pathogenic organisms. Only the number of bacteria exceeding this catalytic activity of PGRPs facilitates the activation of signaling pathway (Dziarski and Gupta, 2018). The catalytic PGRPs contain conserved residues for Zn^{2+} binding, required for the amidase activity (Reiser et al., 2004). On the other hand, sensor PGRPs bind to PGNs and activate immune responses via Toll and Imd pathways, but do not hydrolyze PGNs due to the lack of Zn ion binding residues such as Cys that is needed for the enzyme activity (Dziarski and Gupta, 2018, 2006; Royet et al., 2011). However, *Drosophila* PGRP-LB has both sensory and catalytic activity that control the level of the host immune responses during microbial infections (Zaidman-Rémy et al., 2006).

The function of PGRPs in *Drosophila* and some other insects have been well studied

(Akira et al., 2006; Basbous et al., 2011; Binggeli et al., 2014; Dziarski and Gupta, 2018; Gillespie and Kanost, 1997; Saul and Sugumaran, 1986; Sugumaran et al., 2006). The gene duplication and sequence divergence of PGRPs allow recognition of structurally diverse PGNs to activate different immune pathways (*e.g*. Toll or Imd), induce proteolytic cascades that generate antimicrobial products via PO or Spätzle, induce phagocytosis, and hydrolyze peptidoglycans to finally protect insects from bacterial infection.

In *Drosophila*, Gram-positive bacterial and fungal infection stimulates the Toll pathway, while Gram-negative bacterial infection stimulates the Imd pathway (Gillespie and Kanost, 1997; Swaminathan et al., 2006). However, crosstalk between Toll and Imd pathways has been observed (Wang et al., 2019; Zhang et al., 2019; Zhao et al., 2011, 2018). Previous biochemical work have observed crosstalk between Toll and Imd Pathways in *Drosophila* , facilitated by the interaction of FADD with IMD, Dredd, and MyD88 (Mellroth et al., 2005). Some insect PGRPs take part in proPO activation that leads to melanization through the activation of extracellular serine protease cascade (Binggeli et al., 2014; Dunn and Drake, 1983; Dziarski and Gupta, 2006).

Individual PGRPs show preferences for different types of PGNs. The variability in PGRP sequences has given rise to their specificity. In *Drosophila*, PGRP-SA in hemolymph binds to Lys-type PGNs and, together with PGRP-SD and Gram-negative binding protein-1 (GNBP1), activates the Toll pathway (Gobert et al., 2003; Pili-Floury et al., 2004). After polymeric DAP-PGN binds to PGRP-LCx homodimer (DAP-type polymeric

PGN) or after monomeric DAP-PGN binds to the LCx-LCa heterodimer, the Imd pathway is activated to produce AMPs in *Drosophila* (Kaneko et al., 2004). While PGRP-LCx prefers DAP-PGN, it can also recognize Lys-PGN to a lesser extent (Capo et al., 2016; Choe et al., 2002). PGRP-LE can bind both polymeric and monomeric DAP-type PGN to activate the Imd pathway in two ways (Kaneko et al., 2006): 1) the extracellular PGRP-LE activates through PGRP-LC, possibly by forming a PGRP-LE and -LC complex (Takehana et al., 2004, 2002), 2) intracellular PGRP-LE activates through interaction with the Imd adaptor protein (Kaneko et al., 2006; Takehana et al., 2004, 2002; Yano et al., 2008). In contrast, PGRP-LF inhibits the Imd pathway by binding to PGRP-LCx but not to peptidoglycan and, thus, prevents the formation of a PGRP-LC active dimer (Chevée et al., 2019).

Some PGRPs bind to bacterial PGN to activate the proPO pathway, which promotes wound healing and melanization. In *Drosophila*, PGRP-LE binds to DAP-type PGN and activate zymogenic proPO into active PO. Then through a series of reactions the active PO will produce melanin to encapsulate the pathogen (Gillespie and Kanost, 1997; Söderhäll et al., 2013; Takehana et al., 2002; Tsakas and Marmaras, 2010; Wang et al., 2019). In *Helicoverpa armigera*, association of PGRP-A with Lys- and DAP-type PGN triggers the proPO activation and participate in the melanization of nodules and capsules (Li et al., 2015). In *B. mori*, PGRP-S5 plays multiple roles, as a receptor for activation of the proPO pathway, as a negative regulator for the Imd pathway, and as a bacteriocide (Chen et al., 2016).

2.6 *Manduca sexta* (Tobacco hornworm)

M. sexta represents a large group of pest insects in the order of Lepidoptera, and is often used as a model organism to study the insect biochemical pathways and biochemistry of insect immunity, due to their ease of rearing and large size that makes it collect a large volume of hemolymph. The life cycle of *M. sexta* consists of four stages: embyo, five larval instars, pupa, and adult. *M. sexta* is less susceptible to a variety of pathogenic bacteria, compared to other lepidopterans, possibly be due to the high level of hemocytes and detoxification ability (Dean et al., 2004; Koenig et al., 2015; Pauchet et al., 2010). Expanding the knowledge of the immunobiochemistry of lepidopteran insects may be beneficial to the development of novel strategies for management of agricultural pests and disease vectors and could be used to understand human health related problems.

CHAPTER III

METHODOLOGY

3.1 Insect rearing and plasma collection

M. sexta eggs were purchased from Carolina Biological Supply and larvae were reared on an artificial diet as described previously (Dunn and Drake, 1983). Prolegs of naïve fifth instar larvae at day 2 were cut to collect hemolymph. Plasma samples from both naïve (i.e. control) and immune challenged insects were separated from hemocytes by centrifugation at 5000×*g* for 4 min, aliquoted, and stored at -80°C for later use.

3.2 cDNA cloning and construction of expression plasmid for *M. sexta* PGRP3, PGRP4, PGRP12ecto, PGRP13FL, PGRP13N and PGRP13C domains

cDNA fragments of *M. sexta* PGRP3, PGRP4, and PGRP12 ectodomain were amplified from a cDNA pool of induced fat body. cDNA fragments of full-length, N-and C-terminal domains of MsPGRP13 were amplified from a cDNA pool of nervous tissue. Primers were designed to have *Eco*RI and *Nde*I restriction sites at 5' end of the forward primers (FP) and *Hin*dIII and *Xho*I sites at 5' end of the reverse primers (RP). For PGRP3, FP j287 (5'-GGAATTCTTCCATCATTATTTGCA) and RP j1490 (5'-CTCGAGAGTGGTATTATTTCTGCG); for PGRP4, FP j1491 (5'-GAATTCGACCT AACTTTCACAGTG) and RP j1492 (5'-CTCGAGTGTCTTTTTAATTTTGTCGA); for

PGRP12ecto, FP j1475 (GAATTCATATGGATTCAACAAGAGATGACA) and RP j1476 (5'-AAGCTTACTCGAGTGTTTTTGAGACCATCTC); for PGRP13FL, FP j429 (5'GAATTCATATGGATTGTGACGTAATCGATAAG) and RP j430 (5'-AAGCTTA CTCGAGATGAAAGATGCGCCAAC); for PGRP13N, FP j429 (5'-GAATTCATATGG ATTGTGACGTAATCGATAAG) and RP j431 (5'-AAGCTTACTCGAGCCATTGAGG CCGT); for PGRP13C, FP j432 (5'GAATTCCTCAATGGATAGAAAAC) and RP j430 (5'-AAGCTTACTCGAGATGAAAGATGCGCCAAC). Following T/A cloning of the amplified products into pGEM-T vector (Promega) and sequence validation of the recombinant plasmids, the insert was retrieved by *EcoRI-XhoI* double digestion and subcloned into the same sites of pMFH6 (Lu and Jiang, 2008).

3.3 Generation of baculovirus and infection of insect cells for the expression of *M. sexta* PGRP3, 4, 12ecto, 13FL, 13N and 13C.

Baculoviruses were generated by *in vivo* transposition of the pMFH6 expression cassette with $DH10_{bac}$. White bacterial colonies were used to isolate bacmids DNA according to the manufacturer's instructions (Life Technologies). Correct bacmids were confirmed by PCR using a vector-specific reverse primer (j030) and the forward primers of corresponding cDNA fragments. Then these bacmid DNA samples were used to transfect *Spodoptera frugiperda Sf9* cells with the DNA-Cellfectin mixture in a conditioned medium, to obtain initial viral stock (V_0), as previously described (Y.Wang et al., 2011). Gradual increase in viral titer and protein expression was achieved through serial infections. Large-scale infection of *Sf9* insect cells (2×10^6 per ml) in 900 ml Sf-900 II serum-free medium (Life Technologies) was performed with a viral stock at a

multiplicity of infection of 10 as previously described (Y.Wang et al., 2011). After incubation of the infected cells at 27°C for 72−84 h with gentle agitation (150 rpm), cells were pelleted by centrifugation at 5000×*g* for 20 min at 4°C and supernatants were used for protein purification.

3.4 Multiple sequence alignment of *M. sexta* PGRPs with their homologs in other organisms

In order to study the sequence similarities and differences between the PGRPs, a multiple sequence alignment was performed using UPGMA clustering method in MEGA-X with a gap opening penalty of -2.9 and gap extension penalty of 0.00 (Kumar et al., 2018).

3.5 Purification of *M. sexta* PGRPs 2−5, 12ecto, 13FL, 13N, and 13 C

Supernatants of the insect cell cultures were mixed with equal volume of H_2O containing 1 mM benzamidine. After pH adjustment to 6.4, the mixture was then centrifuged at 22100×*g* for 20 min at 4°C and loaded onto a 40 ml column of dextran sulfate-Sepharose CL-6B (DS) equilibrated in buffer A (10 mM potassium phosphate, 1 mM benzamidine, 0.01% Tween 20, pH 6.4). Next the column was washed with 5 volumes of buffer A (200 ml). Proteins bound to the DS column were eluted with a linear gradient of 0−1.0 M NaCl in buffer A and collected using fraction collector at 4.5 ml/tube/3 min. Collected column fractions were analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining or immunoblot analysis using 1:1000 diluted, affinity-purified rabbit anti-$(His)_6$ IgG as a primary

antibody and goat-anti-mouse IgG as a secondary antibody (Sumathipala and Jiang, 2010; Y.Wang et al., 2011).

Based on the SDS-PAGE analysis, column fractions containing the target protein and least amount of other proteins were combined and pH was adjusted to 7. Then the pooled DS fractions were loaded onto a pre-equilibrated 2-ml Ni^{2+}-NTA agarose column with 10 mM imidazole in buffer B (50 mM Tris, pH7.5, 300 mM NaCl, 0.005% Tween 20, 5% glycerol, 1 mM benzamidine or 0.5 mM benzamidine and 0.5 mM 4-amino-benzamidine, and 10 mM imidazole, pH 8.0). Bound proteins were eluted using linear gradient of 10-100 mM imidazole in 20 ml buffer A followed by 250 mM imidazole in buffer A at 1 ml/tube/2 min. Eluted protein fractions were analyzed as described above. All the steps of purification were performed at 4°C. Finally, the desired protein fractions were combined and concentrated using Amicon ultracentrifugal 10K MWCO filter device (Millipore). Concentrated proteins were buffer exchanged with 20 mM Tris-Cl, pH 7.5, 50 mM NaCl on the same device to have final concentration of 1 mg/ml of protein. Proteins were aliquoted and stored at -80°C until further use.

3.6 Elicitor-independent proPO activation by *M. sexta* PGRPs at different concentrations

Hemolymph collected from day 2, fifth star naïve *Manduca* larvae, were diluted (1:10) with buffer E (20 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 0.001% Tween-20). Then 5 μl of diluted hemolymph were incubated with purified PGRPs (0, 200, 400, 600, 800, 1000 ng) or BSA (0, 200, 400, 600, 800, 1000 ng) as a negative control, in 0.001%

Tween-20, 1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.5, in a final volume of 24 µl. PO activity was determined after 60 min using dopamine as a substrate on a 96-well microplate reader. Absorbance was monitored at 470 nm in the kinetic mode, and plotted as mean ± SEM (n = 3) against amount of PGRPs added (Sumathipala and Jiang, 2010; Y.Wang et al., 2011).

3.7 Elicitor-dependent proPO activation by *M. sexta* PGRPs

Insoluble PGNs from *M. luteus, S. aureus, B. megaterium* and *B. subtilis* (2 µg) (Sumathipala and Jiang, 2010) and soluble PGNs (2 µg) from *E. coli* ((InvivoGen) were used as elicitors for this experiment. Hemolymph collected from day 2, fifth star naïve *Manduca* larvae, were diluted (1:10) with buffer E. To test the effect of PGRP-elicitor interaction on proPO activation, five µl of diluted plasma was incubated with 15 µl of the buffer (#1), 2.0 µg of elicitor in buffer (#2), 200 ng of PGRP in buffer (#3), or both 2.0 µg elicitor and 200 ng of PGRP in buffer (#4). The total volume of the four mixtures in each elicitor group was adjusted to 20 µl with buffer. The reaction mixtures were incubated at room temperature for 1 hr prior to the PO activity assay. The enzyme activities were plotted as mean ± SEM (n=3) in bar graphs, along with those of the controls (#1, plasma only; #2, plasma and elicitor; #3, plasma and PGRP). Interaction of plasma factors with elicitor (#2 - #1) and also the presence of plasma with PGRPs (#3 - #1) led to proPO activation. Therefore, an interaction of elicitor with PGRPs in plasma (#4 - #1) was expected to increase proPO activation to a level significantly higher than the sum of the two components [(#2 - #1) + (#3 - #1)]. Hence, PO activities of #4 and (#2

+ #3 - #1) were directly compared using unpaired t-test to reveal possible synergistic enhancement caused by elicitor-PGRP interaction.

3.8 Enzyme-linked immunosorbent assay (ELISA) of *M. sexta* PGRP binding to soluble peptidoglycans

Soluble PGNs from *E. coli* DAP-PGN and *S. aureus* Lys-PGN (InvivoGen) were separately used as ligands to measure total and specific binding to *M. sexta* PGRP3, 4, 12ecto, 13FL, 13N, and 13C as previously described (Sumathipala and Jiang, 2010; Y.Wang et al., 2011). Two μg each PGN per well (50 μl, 40 ng/μl) was added to 96-well microplate and air dried overnight under the room temperature. Then, PGNs were fixed by incubating the plates at 60°C for 30 min, followed by blocking with 200 μl of 1 mg/ml bovine serum albumin (BSA) in Tris buffered saline (TBS: 137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 7.6) at 37°C for 2 h. After washing the plate three times with TBS, diluted PGRP samples (300 ng in 50 μl TBS containing 0.1 mg/ml BSA) were added to the wells (50 μl/well) and the plate was incubated at room temperature for 3 h and further processed to get total binding.

To test specific binding between the PGRPs and PGNs, a competition experiment was performed. Aliquots of the PGRPs (200 ng in 1 μl) were pre-incubated with 20 μg of PGNs in 50 μl TBS with 0.1 mg/ml BSA for 1 h at room temperature. Following BSA blocking of the plate wells coated with PGNs (2 μg), the pre-incubation mixtures (with the corresponding PGNs) were added to the wells and incubated for 3 h at room temperature. Following a washing step with 200 μl TBS for 4 times, 100 μl of 1:1000

diluted anti-$(His)_5$ monoclonal antibody in TBS containing 0.1 mg/ml BSA was added and incubated for 2 h at 37°C. Unbound anti-$(His)_5$ antibody molecules were removed by washing with 200 µl TBS for 4 times, followed by adding 100 µl of 1:2000 diluted goat anti-mouse IgG conjugated to alkaline phosphatase (AP) (Bio-Rad) in TBS containing 0.1 mg/ml BSA. Plates were incubated at room temperature overnight. Then unbound samples were washed four times with 200 µl TBS followed by adding 50 µl of 1.0 mg/ml *p*-nitrophenyl phosphate in 0.5 M $MgCl_2$, 10 mM diethanolamine. Plates were incubated at room temperature for 20 min and absorbance was measured at 405 nm in the kinetic mode on a microplate reader. One unit of AP activity is defined as the amount of enzyme causing an increase of 0.001 absorbance unit per minute.

3.9 Binding of *M. sexta* PGRPs to insoluble peptidoglycans

One mg each of the insoluble PGNs from *B. megaterium*, *B. subtilis*, *S. aureus* and *M. luteus* was separately mixed with 0.2 µg each of the PGRPs in 50 µl of buffer D (20 mM Tris-HCl, pH 8.0, 20 mM NaCl). The mixture was incubated at 4 ˚C for 2 h with mixing. Then 10 µl of the total mixture was mixed with 2.0 µl 5×SDS sample buffer and analyzed as total fraction. The rest of the reaction mixture (40 µl) was centrifuged at 6000g for 15 min. After the centrifugation of this mixture, 10 µl of supernatant was mixed with 2.0 µl 5×SDS sample buffer and analyzed as unbound fraction. The pellet was washed 3 times with 200 µl of buffer D each and then mixed with 20 µl of 2×SDS sample buffer (total volume: about 40 µl) and analyzed as bound fraction. The total, unbound, and bound samples (10,10,5 µl) were heated at 95˚C for 5 min and were separated by 15% SDS-PAGE followed by immunoblot analysis using 1:1000 diluted anti-$(His)_5$ monoclonal

antibody (Bio-Rad) and goat-anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad).

3.10 Binding of the *M. sexta* PGRPs to microbial cells

Single bacterial cultures of *B. megaterium, B. subtilis, S. aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella typhimurium, E. coli* and *M. luteus* were used in this experiment. Bacterial cultures were grown in 3 ml LB medium at 37 °C until the OD_{600} was close to 0.5 and centrifuged at 10,000×*g* for 3 min to obtain cell pellet, followed by washing the pellet twice with 200 ml of buffer D (20 mM Tris-HCl, pH 8.0, 20 mM NaCl). Cell pellets were resuspended in 40 µl of buffer. Then 10 µl of the purified PGRPs (3 µg) were added separately to each cell suspension and incubated for 2 h at 4 °C with mixing. Then 10 µl of the total mixture was mixed with 5×SDS buffer and analyzed as total fraction. The rest of the reaction mixture (40 µl) was centrifuged at 6000×*g* for 15 min. After the centrifugation of this mixture, supernatant (10 µl) was mixed with 5×SDS buffer and analyzed as unbound fraction. The pellet was washed 3 times with 200 µl of the buffer each and mixed with 20 µl of 2×SDS bufferand analyzed as bound fraction. The total, unbound, and bound samples were heated at 95˚C for 5 minutes and subjected to 15% SDS-PAGE and immunoblot analysis using 1:1000 diluted anti-$(His)_5$ monoclonal antibody and goat-anti-mouse IgG conjugated to alkaline phosphatase.

CHAPTER IV

RESULTS

4.1 Multiple sequence alignment of *M. sexta* PGRPs

Multiple sequence alignment was performed to compare sequences of PGRPs from *M. sexta*, other insects and human, as well as T7 lysozyme (Fig. 4). MsPGRP2, 3, 4, 5, 12 and 13 are >34% identical and >52% similar to MsPGRP1. MsPGRP2, 3 and 4 have four of the five catalytic residues (His18, Tyr47, His123, Lys129, Cys131, T7 lysozyme numbering) identical to T7 lysozyme, with an exception of Thr substituting Lys129. These five residues in T7 lysozyme interact with a catalytic zinc ion. Manduca PGRP 2-4 have the five residues identical to *Drosophila* PGRP-SB and -SC, which have the amidase activity (Steiner, 2004). MsPGRP1, 5, 12, and 13 lack 2−3 of these residues, suggesting that are PGN receptors but not enzymes.

PGN binding sites of DmPGRP-SB contains His-Thr/Ser (H-S/T), Asn-Phe (N-F), Arg (R), Asn (N), His (H) and Cys (C). However, DmPGRP-SA and SD contain Asp-Phe (DF) and Lys-Phe (KF) at the corresponding positions of NF respectively. In contrast, DmPGRP-LC and LE have Gly-Trp (GW) rather than NF. Similar NF residues at the corresponding positions were observed in MsPGRP5. However, these residues

were replaced by NY, QW and KF in MsPGRP 1, 12 and 13 respectively. Similar to DmPGRP-LC and LE, MsPGRP2,3 and 4 contain Gly-Trp (GW) instead of NF.

In addition, Arg254 residue is highly conserved in DAP-type PGRPs, including DmPGRP-LE, LB, LC and SD. This Arg residue, is not found in DmPGRP-SA which recognize Lys-PGNs. Since Arg has a positively charged side chain that could interact with the carboxyl group of DAP-PGN, it is believed to be responsible for DAP-PG recognition (Hu et al., 2019; Leone et al., 2008; Lim et al., 2006). MsPGRP2, 3, 4 and 12 contain Arg at the corresponding position, while it is replaced by Ser in MsPGRP1 and Val in MsPGRP5 and 13.

4.2 Construction, expression and purification of recombinant *M. sexta* PGRP 2-5, 12 ecto and 13 full, 13N and 13 C domains

cDNA fragments of the PGRPs were PCR amplified and sequence verified, before cloning into pMFH6, followed by expression in baculovirus infected Sf9 cell cultures. This system allows secretion of the mature proteins into the medium and eliminated contamination of bacterial components that may interfere with functional assays including proPO activation assay. All the recombinant PGRPs with a C-terminal hexahistidine tag were purified by cation exchange chromatography on a dextran sulfate-Sepharose column and further isolated by affinity chromatography on a Ni-NTA agarose column. From one liter of conditioned Sf9 medium for each protein, we obtained 2, 3.08, 2.4, 1.5, 1.5, 3.04, 1 and 3.28 mg of MsPGRPs 2−5, 12ecto, 13FL, 13N and 13C. In the SDS-PAGE analysis, the purified recombinant MsPGRPs 2−5,

13FL, 13N and 13C migrated as a single band at 19, 24, 21, 19, 48, 15 and 27 kDa positions close to their calculated masses (20,770, 25230, 22,312, 20,706, 51,451, 15,152 and 26,799 Da).

MsPGRP12ecto domain mainly migrated as a doublet at 24 kDa position. All purified recombinant MsPGRPs were recognized by anti-(His)5 antibody (Fig. 5).

4.3 Elicitor-independent proPO activation by MsPGRP 1-5, 12 ecto, 13Fl, 13N and C terminal domains at different concentrations

In order to test the role of *M. sexta* PGRPs in proPO activation, purified recombinant MsPGRPs were incubated in concentration dependent manner only with diluted hemolymph plasma from naïve larvae (Fig 6). Addition of MsPGRPs enhanced proPO activation with the increase of their concentrations, even in the absence of microbial elicitor. The MsPGRP1-triggered proPO activation (Sumathipala & Jiang, 2010) was used as a positive control to validate the reproducibility of protocol. As concentration of MsPGRPs increase, proPO activation increase. For MsPGRP5, 13Fl and 13N proPO activation reached the peak values of 15.4, 10.6 and 10.3 U at the PGRP concentration of 0.1 mg/ml. For MsPGRPs 1-4, proPO activation appeared to increase and level out towards a maximum, with the increase of recombinant MsPGRP 1-4 concentrations (Fig. 6). Maximum values of PO activity with MsPGRPs 1-4 were 8.7, 10.5, 13.2, and 11.7 U, respectively.

The elicitor-independent proPO activation did not occur until 600 ng of PGRP12ecto was added. The domain was truncated from the transmembrane receptor for use as a control. The true negative control of BSA did not cause any PO activity increase. However, for MsPGRP 12 ecto domain I did not see any increased proPO activity until 600 ng and even after that the increase was low. This may be due to the fact that the PGRP 12 being transmembrane receptor. I used BSA as a negative control and did not observe such response in proPO activation after adding BSA to the plasma in concentration dependent manner. Similarly, I did not see any increased proPO activity with MsPGRP 13 C.

4.4 Elicitor-dependent proPO activation by *M. sexta* PGRPs 3FL, 4, 12ecto, 13FL, 13N, and 13C

I further tested effects of the PGRPs on proPO activation in the presence of *M. luteus* and *S. aureus* Lys-type PGNs and *E. coli*, *B. megaterium* and *B subtilis* DAP-type PGNs (Fig. 7). Since the elicitor-dependent proPO activation by MsPGRPs 2, 3short, and 5 were already tested by a previous lab member, I started with MsPGRPs 3FL, 4, 12ecto, 13FL, 13N and 13C. Diluted plasma was incubated one hour at room temperature with buffer, PGNs, PGRPs or both PGNs and PGRPs. In both samples of plasma alone (#1) and the mixture of PGRP with plasma (#2), low levels of PO activity were observed. Increased levels of proPO activation in plasma were observed after PGRP (#3) and PGRPs with PGNs (#4) had been added.

The observed PO activity increases with the combination of PGRPs (3FL, 4, 13FL and 13N) with DAP-PGNs were significantly higher than with Lys-PGNs (Fig7). A significantly high PO activity was observed for a combination of MsPGRP3, plasma and PGNs from *E. coli, B. megaterium* and *B. subtilis*. For the combination of PGRP3 with *M. luteus* PG, the increased PO activity was not significant. With *S. aureus* PG, PO activity of the plasma was significantly lower than when PGRP3FL added. Similar results were obtained for PGRP13FL and 13N, in which a significantly higher PO activities were observed only with a combination of the PGRPs, plasma, and DAP-PGNs but not with Lys-PGNs.

However, with Lys-PGs from *S. aureus* and *M. luteus*, PO activity of the plasma was significantly lower than when PGRP 13FL added. In case of the PGRP4, significantly higher PO activity was observed with DAP-PGNs (*E. coli, B. megaterium* and *B. subtilis*) and Lys-PGNs (*M. luteus*). Neither PGRP12ecto nor PGRP13C significantly increased PO activity in plasma in the presence of DAP- and Lys-PGNs. Surprisingly, none of these PGRPs increased PO activity in plasma by *S. aureus* PGN. The synergistic effects on proPO activation by the PGRPs 3FL, 4, 13FL, and 13N in the presence of DAP-PGNs were likely caused by specific interactions with these PGNs, whereas the lack of synergism with Lys-PGNs coincided with incomplete binding of the PGRPs to these PGNs (see below).

4.5 ELISA-based plate assay of the PGRP bindings to *E. coli* and *S. aureus* peptidoglycans

In order to elucidate binding specificity of the purified PGRPs to bacterial PGNs, we performed ELISA to examine total and specific bindings (Fig. 8). PGNs from *E. coli* (DAP-tye) and *S. aureus* (Lys-type) and *M. sexta* PGRPs 2, 3FL, 3s, 4, 5, 12ecto, 13FL, 13N, and 13C were tested. Low alkaline phosphatase activities (<4 U) indicated low total binding of the PGRPs with *S. aureus* PGN. In comparison with the negative control of BSA, some concentration-dependent bindings were observed for PGRP3FL, 13FL and 13N with *S. aureus* Lys-PGN.

After the PGRPs had been pre-incubated with excess amount of *S. aureus* Lys-PGN, the mixtures were subjected to the binding assay. If their bindings to the PGNs was specific during the preincubation, no PGRPs would be left for interacting with the immobilized PGNs. In contrast, nonspecific binding is unsaturable. As observed in competition experiment, the bindings of PGRPs 2, 3FL, 3s, 5 and 13 with Lys-PGN of *S. aureus* were low and nonspecific in most cases. However, significant decreases in the PGRP4, PGRP12ecto, and PGRP13N binding were detected after competition with *S. aureus* PGN, except for 400ng of PGRP4 and 300 ng of PGRP13N (Fig. 8).

When the same experiment was done using *E. coli* DAP-PGN, specific bindings were observed for *M. sexta* PGRPs 2-5, 12ecto, 13FL and 13N (Fig. 8). All of these PGRPs showed much higher binding than the BSA control. The specific interaction of *E. coli* DAP-PG with PGRP12ecto at the concentration of 200 ng caused a decrease in the

enzyme activity but the *p* value was 0.07. Taken together, the ELISA support high and specific binding of all but PGRP13C to *E. coli* DAP-PGNs and low and nonspecific binding of PGRPs 2, 3FL, 3S, 5, 13FL, and 13C to *S. aureus* PGN.

4.6 Binding of *M. sexta* PGRPs 2-4, 12ecto, 13FL, 13N, and 13C to insoluble peptidoglycans

To further test binding specificity of MsPGRPs, pulldown assays were performed for each of the seven PGRPs and insoluble peptidoglycans from *B. megaterium* (DAP-type), *B. subtilis* (DAP-type), *M. luteus* (Lys-type), and *S. aureus* (Lys-type) (Fig. 9). All of the PGRPs, except for PGRP13C, showed specific binding with DAP-PGN from *B. megaterium*. However, PGRPs 3FL, 3s, 4, 13FL and 13N terminal domain displayed more binding with *B. subtilis* PGN than PGRPs 2 and 12ecto did. were more specificity than PGRP 2 and 12 ecto domain which showed partial bindings. The PGRP13C, a lipoprotein-11 domain, did not bind to any PGNs.

The PGRP4 completely bound to *M. luteus* Lys-PG whereas PGRP2 showed partial binding (Fig. 9). In contrast, the PGRPs 3FL, 12ecto, 13FL, and 13N did not bind the Lys-PG at all. In case of *S. aureus* Lys-PGN, PGRP12ecto displayed partial binding but none of the other proteins bound. Again, the pulldown assay indicated that the *M. sexta* PGRPs recognized DAP-PGNs better than Lys-PGNs in an order of Bm > Bs > Ml > Sa. *Manduca* PGRP 2 showed binding preference for PGNs in the order of Bm > Bs, Ml > Sa, whereas PGRP 3FL showed binding preference for PGNs in the order of Bm, Bs > Ml, Sa. *Manduca* PGRP 4 and 13N terminal domain showed

binding preference for PGNs in the order of Bm, Bs > Ml > Sa. PGRP 12 e showed binding preference for PGNs in the order of Bm > Bs > Sa > Ml. *Manduca* PGRP 13FL showed binding preference for PGNs in the order of Bm, Bs > Sa > Ml.

4.7 Binding of *M. sexta* PGRPs 2-4, 12ecto, 13FL, 13N, and 13C to microbial cells

Bindings of PGRPs to bacteria and PGNs may differ greatly, due to the presence of other cell wall components such as techoic acids, lipoteichoic acids, and proteins. PGN layers of Gram-negative (G-) bacteria are covered by lipopolysaccharides embedded in lipid bilayer of the outer membrane. To further examine the process of bacteria recognition, we incubated the purified PGRPs with eight live bacteria (Fig. 10), *B. megaterium* (G+, DAP-PGN), *B. subtilis* (G+, DAP-type), *E. coli* (G-, DAP-type), *K. pneumoniae* (G-, DAP-type), *S. typhimurium* (G-, DAP-type), *P. aeruginosa* (G-, DAP-type), *M. luteus* (G+, Lys-type), and *S. aureus* (G+, Lys-type).

In agreement with the results obtain from the pulldown assays with PGNs, the PGRPs 2, 3FL, 3s, 4, 13FL, and 13N showed complete bindings with *B. megaterium*, whereas the PGRP12ecto had partial binding (Fig. 10). With *B. subtilis,* PGRPs 4, 13FL and 13N displayed near complete bindings, while the PGRPs 2, 3FL and 12ecto showed partial bindings. PGRP13 C terminal domain has no binding with *B. subtilis* and other seven bacteria. Only PGRP3FL showed inconsistency in binding with *B. subtilis* cells and PGNs. No binding was observed for *S. typhimurium*, *P. aeruginosa* and *S. aureus* by the PGRPs 3FL, 4, 13FL, 13N and 13C. Partial bindings of the

bacteria were observed for PGRP12ecto. These results are consistent with the PGN bindings (Fig. 10).

With *M. luteus*, the PGRPs 2, 4 and 12ecto showed partial binding while the other four did not bind. This is consistent with the PGN binding data of the PGRPs 2, 3, 13FL, 13N and 13C, but not PGRP4 or PGRP12ecto (Fig. 9). After the proteins had been incubated with *K. pneumoniae* cells, complete binding of the PGRP2 occurred, partial bindings were observed for the PGRPs 12ecto, 13FL, and 13N, no bindings were found for PGRPs 3FL, 4 or 13C. None of the PGRPs showed complete binding with *E. coli* cells. *M. sexta* PGRPs 2, 3FL, and 13FL showed more binding than PGRP4 or 12ecto. Little binding occurred between *E. coli* cells and PGRPs 4, 12ecto, or 13C.

Again, the pulldown assay with whole bacteria indicated that the *M. sexta* PGRPs recognized Gram-positive bacteria with DAP-PGNs better than Gram-positive bacteria with Lys-PGNs in an order of Bm > Bs > Ml > Sa. Gram-negative bacteria showed low binding compared to Gram-positive bacteria. *Manduca* PGRP 2 showed binding preference for Gram-positive bacteria in the order of Bm > Bs > Ml > Sa, whereas for Gram-negative bacteria the order was Kp > Ec > St, Pa. *Manduca* PGRP 3FL showed binding preference for Gram-positive bacteria in the order of Bm > Bs, Ml > Sa, whereas for Gram-negative bacteria the order was Ec > Kp, St, Pa. *Manduca* PGRP 4 showed binding preference for Gram-positive bacteria in the order of Bm > Bs > Ml > Sa, whereas for Gram-negative bacteria the order was Ec > Kp, St > Pa. *Manduca* PGRP 12 ectodomain showed similar binding preference for all of the Gram-positive bacteria used in this experiment, whereas for Gram-negative bacteria the order was Kp,

St, Pa > Ec. *Manduca* PGRP 13 FL and 13 N terminal domains showed similar binding preference for Gram-positive bacteria in the order of Bm, Bs > Ml, Sa. However, they showed different binding preferences for Gram-negative bacteria. In case of PGRP 13 FL the order of binding preference was Ec > Kp > Pa, St, where as for PGRP 13 N it was Kp > Ec, Pa, St.

CHAPTER V

DISCUSSION

Biochemistry of insect immune responses has been a hot topic for several decades. Insect pests are responsible for major damages by destroying crops or transmitting vector-borne diseases. *M. sexta* represents a large group of agricultural pests in the order of Lepidoptera and has a low susceptibility to a variety of pathogenic bacteria (Cooper and Eleftherianos, 2017). Better understanding the lepidopteran insect immune system may be beneficial to the development of novel strategies for management of agricultural pests and disease vectors and also could be used to understand human health related problems.

During evolution, insects have developed several lines of defense against microbial infection. Innate immune system is the main defense against invading pathogens. In insects, Toll, IMD and proPO activation are the main signaling pathways to eliminate invading pathogens (Sheehan et al., 2018; Tsakas and Marmaras, 2010). The activation of these pathways occurs mainly through PRRs, which recognizes PAMPs, such as lipopolysaccharide, peptidoglycan and lipophosphoric acid (Tsakas and Marmaras, 2010; Wu et al., 2018). The recognition of invading microbes and distinction from the host are mediated by PRRs such as PGRPs.

The interactions between PGRPs and PGNs on the surface of invading microbes trigger some intracellular signaling pathways through an integrated serine protease network (Akira et al., 2006; Dziarski and Gupta, 2018; Yu et al., 2002). Although the importance of PGRPs in detecting bacteria and promoting immunity is well recognized in *D. melanogaster*, such a role has not yet been experimentally established for PGRPs as a system in *M. sexta*. This study is focused on determining the roles of *M. sexta* PGRPs in proPO activation and specificity towards different PGNs and bacteria.

5.1. Structural basis and evolutionary relationships of the PGRPs in *M. sexta*

PGRPs, conserved from insects to mammals, are a superfamily of ubiquitous proteins that recognize PGNs to initiate innate immune signaling (Kang et al., 1998; Leulier et al., 2003; Steiner, 2004). Thirteen PGRP genes have been identified in the *M. sexta* genome. In addition to the recognition domain, PGRP13 has a lipoprotein-11 domain, which is not found in other insects (Zhang et al., 2015). According to the phylogenetic tree of *M. sexta* and *D. melanogaster* PGRPs, *Manduca* PGRPs 1, 5-7, 9, and 13 are close to *Drosophila* PGRP-SA, PGRP2–4 to -SB/SC/SD, PGRP8 to -LD, PGRP10/11 to -LA, and PGRP12A/B to -LC/LE. *M. sexta* PGRPs 2-4 contain the key residues (His, Tyr, His, Thr, and Cys) for the amidase activity that cleaves the amide bond between lactyl and L-alanine residues in the stem peptide of PGNs. This cleavage can break down PGNs to attenuate host immunity. While the Lys residue in T7 lysozyme is required for the amidase activity is replaced by Thr in the *Manduca* and *Drosophila* PGRPs, the enzyme activity remains in PGRP-SB and -SC (Steiner, 2004).

M. sexta PGRP12 has two splicing variants, 12A and 12B (Hu et al., 2019; Zhang et al., 2015).

The crystal structure of *Manduca* PGRP1 (Hu et al., 2019) showed that the overall folding is closely similar to human and *Drosophila* PGRPs and several differences are also present (Hu et al., 2019; Zhang et al., 2015). Similar observations were made in the multiple sequence alignment of *Manduca* and *Drosophila* PGRPs (Fig. 4). Sequence variations between *Manduca* and *Drosophila* PGRPs at certain positions of PGN binding sites have some effect on their ability to recognize PGNs but largely affect the distinction of Lys- and DAP-PGNs (Charroux et al., 2009; Royet and Dziarski, 2007; Steiner, 2004). For example, *Manduca* PGRP1 contains Asn^{96} and Tyr^{97} (NY) and recognizes DAP-PGNs. In contrast, *Drosophila* PGRP-SA which is orthologous to MsPGRP1 contains Asp and Phe (DF) at the same positions, prefers Lys-PGNs over DAP-PGNs (Fig 4).

5.2. *M. sexta* PGRPs and melanization

ProPO activation is one of the defense responses in insects. In order to check the connection between PGRPs and proPO activation cascade, elicitor-independent PO activity increase was tested using the PGRPs in a range of concentrations. Addition of MsPGRPs alone enhanced proPO activation in concentration dependent manner, confirming a correlation between PGRPs (2-5 and 13) and proPO activation. Similar results were observed for MsPGRP1 (Sumathipala and Jiang, 2010) and DmPGRP-LE (Sumathipala and Jiang, 2010; Takehana et al., 2002). These findings support the

hypothesis that increased concentrations of PGRPs in plasma may have triggered self-association, that favors spontaneous melanization by interacting with other components of the proPO activation cascade (Y.Wang et al., 2011). However, for PGRP3s and 12ecto, we did not detect much increased PO activity until 400-600 ng and the enhancement was much lower in the range of 600-1000 ng. Their C-terminal truncation is responsible for the changes.

While the non-physiological increases of spontaneous melanization supported the involvement of PGRPs 1-5 and 13 in proPO activation, a more direct connection was tested using microbial elicitors. When compared to the plasma only (#1), adding small amounts of PGNs did not cause major PO activity increase in the diluted plasma (#2). The increases caused by a low level of the purified PGRPs (#3) were higher. Addition of the PGRPs (3, 4, 13FL, 13N) and DAP-PGNs to 1:10 diluted larval plasma caused significant increased PO activity (#4). In contrast, the Lys-PGNs did not cause statistically significant increase with the PGRPs under the assay conditions except for PGRP4, which shows synergistic enhancement in proPO activation with *M. luteus* Lys-PGNs. The synergistic effect on proPO activation by the exogenous PGRPs in the presence of DAP-PGNs in plasma was likely caused by specific interactions with these DAP-PGNs, whereas lack of synergism with Lys-PGNs coincided with the incomplete binding of MsPGRPs to these PGNs. On the other hand, MsPGRPs 12ecto and 13C did not cause significant increased PO activity, may be due to the fact that MsPGRP12 being a transmembrane receptor and 13C being a lipoprotein domain that do not interact with PGNs.

Previous studies on Lepidoptera hemolymph have revealed a constant low PO activity. However a significant increase of PO level was observed after an injury and/or infection (Kanost et al., 2004; Shrestha and Kim, 2009, 2008; Q. Wang et al., 2020; Yu et al., 2002). In physiological conditions, a low, constitutive level of PGRPs are present in hemolymph. Upon a recognition of invading pathogens, PGRPs may form clusters on pathogen surface and transmit the invasion signal to other molecules to trigger the immune system including proPO activation (Park et al., 2007).

5.3. *M. sexta* PGRP binding patterns affected by bacterial surface structures

The biochemical analysis of proPO activation provided evidence that the PGRPs sense DAP-PGNs better than Lys-PGNs and specific binding between DAP-PGNs and MsPGRPs synergistically induce the proPO activation cascade. To test the role of binding specificity on immunological function, ELISA and pulldown assays using both PGNs and microbial cells were performed. In agreement with the ELISA data, MsPGRPs 2-4, and 13 showed specific binding with DAP-PGN from *E. coli* not with *S. aureus* Lys-PGN. In case of *M. luteus*, we observed binding and proPO activation with MsPGRP4, which is similar to the results observed for *M. sexta* PGRP1 (Sumathipala and Jiang, 2010). However, MsPGRPs 3, 12ecto and 13 were not able to bind and increase proPO activation with *M. luteus* in plasma. Sequence variations in PGN binding sites likely have impacted the differential binding of MsPGRPs.

All of the MsPGRPs used in this experiment, specifically bound to DAP-PGNs from both *B. megaterium* and *B. subtilis,* and synergistically enhanced proPO activation. The complete binding shown by *B. megaterium* PGNs in contrast to *B. subtilis*', may be due to the low cross-linking exhibited by *B. megaterium* PGNs and the glucosamine deacetylation that occurs in *B. subtilis* PGNs (Atrih et al., 1999). The differences in melanization stimulation, attributed to the *B. megaterium* and *B. subtilis* PGNs may also stem from these structural differences. The results observed in ELISA agreed well with those of the pull-down tests using the purified PGRPs and PGNs from *E. coli* and *S. aureus*. On the other hand, MsPGRP12ecto showed partial binding with *S. aureus* PGN, even though it didn't increase PO activity. We found that the extracellular PGRP domain of PGRP12 had affinity for all types of PGNs.

Although the polysaccharide chain in PGN is conserved in all bacteria, the stem peptides vary in amino acid composition (Vollmer et al., 2008). Hence, the structural changes in the stem peptides of different bacteria such as degree of cross-linking, glycan strand modifications, as well as the type of MsPGRPs may also be responsible for the differential binding and initiation of the immune pathways (Royet and Dziarski, 2007; Swaminathan et al., 2006). Even though *S. aureus* is a pathogenic species, lack or low level of virulence have also been observed due to the strain diversity of *S. aureus* in some *C. elegans* experiments (García-Lara et al., 2005; Sifri et al., 2003). *S. aureus* usually have a pentaglycine cross bridge in between 3rd L-Lys and 4th D-Ala in the stem peptide and the glycine content differs from strain to strain (Vollmer et al., 2008). Hence, this highly crosslinked pentaglycine bridge will prevent sensing and binding of

S. aureus PGNs by MsPGRPs. The lack of binding also coincided with the result that PGRP did not further enhance proPO activation triggered by *S. aureus* and *M. luteus*.

Binding specificity of MsPGRPs were further tested with live bacterial cells, since the bacterial cell wall may interfere the PGN bindings by MsPGRPs. Gram-positive bacteria contain a thick PGN layer which is exposed to outside environment, whereas Gram-negative bacteria contain relatively thin PGN layer covered by the outer membrane. This phenomenon supports the findings that the MsPGRPs showed partial or no binding to the live cells of Gram-negative *E. coli*, *S. typhimurium*, *K. pneumonia*, and *P. aeruginosa*. This suggests outer membrane of these bacteria might have prevented the interactions between their PGNs and MsPGRPs. Current understanding on the mechanism and structural moieties (*e.g*. lipopolysaccharides, proteins) are involved in the observed PGRP associations remain unclear and need further investigations.

CHAPTER VI

CONCLUSION

Upon pathogen invasion, a battle occurs between the microbe trying to establish an infection and host defense to prevent the infection. Virulence index of the pathogen, host immune responses and other host factors determine the outcome of this battle. PGRPs are one of the immune surveillance protein sets present in *Manduca* hemolymph that recognize bacterial peptidoglycans and transmit signal to trigger immune responses. Taken together, our results from ELISA, pull-down assays with PGNs and live bacteria, and PO activity assays suggest that MsPGRPs 1-5 and 13 are positive regulators of the proPO activation system. They preferentially recognize DAP-type PGNs over Lys-type PGNs. Our results suggest that *Manduca sexta* PGRPs are tailored towards DAP-PGNs rather than Lys-PGNs. Although the recent progress has brought us closer to understanding the role of *M. sexta* PGRPs in bacterial sensing and proPO activation, the precise mechanism of MsPGRP-PGN specific binding that leads to synergistic enhancement in the proteolytic activation of proPO in plasma need further investigations.

The findings of my research have expanded the current understanding of MsPGRPs in bacterial sensing and proPO activation in *Manduca sexta*, paving the way for further

explorations to better translate the host-pathogen interactions and bridge current knowledge gaps in pathogen infections. With the global trend of being continuously challenged by emerging and reemerging infectious diseases and classical infections, an improved understanding of insect innate immune system is important for developing improved disease diagnostics, interventional strategies, or novel vaccines.

FIGURES

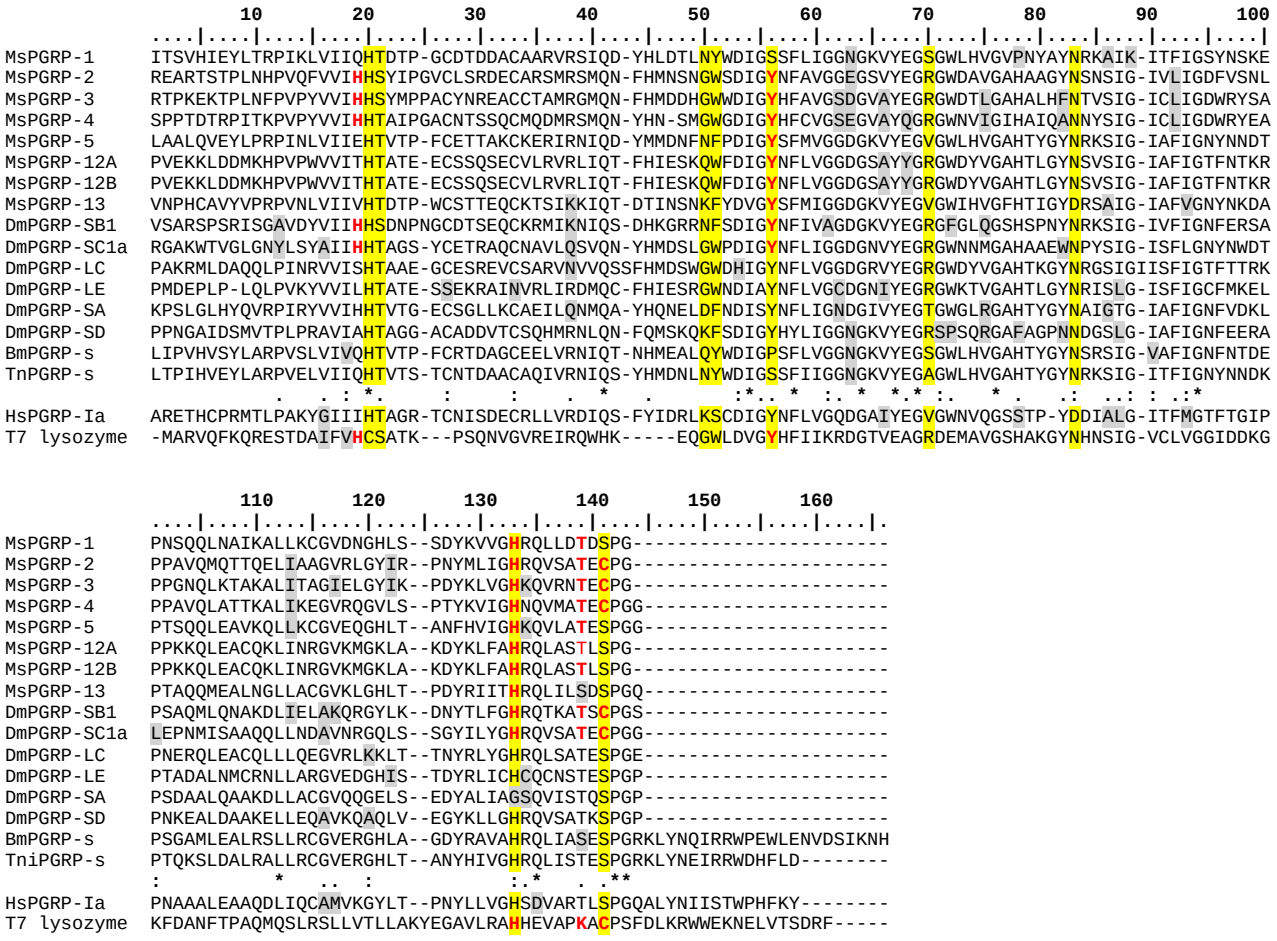
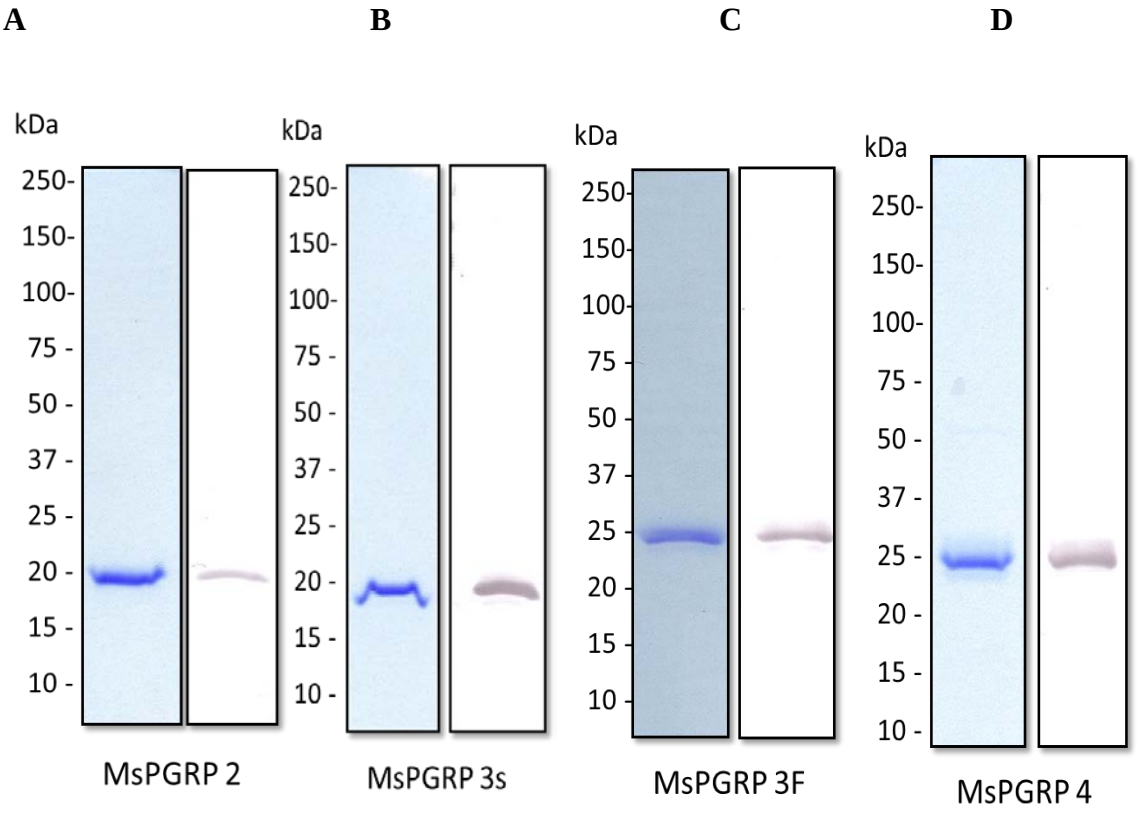
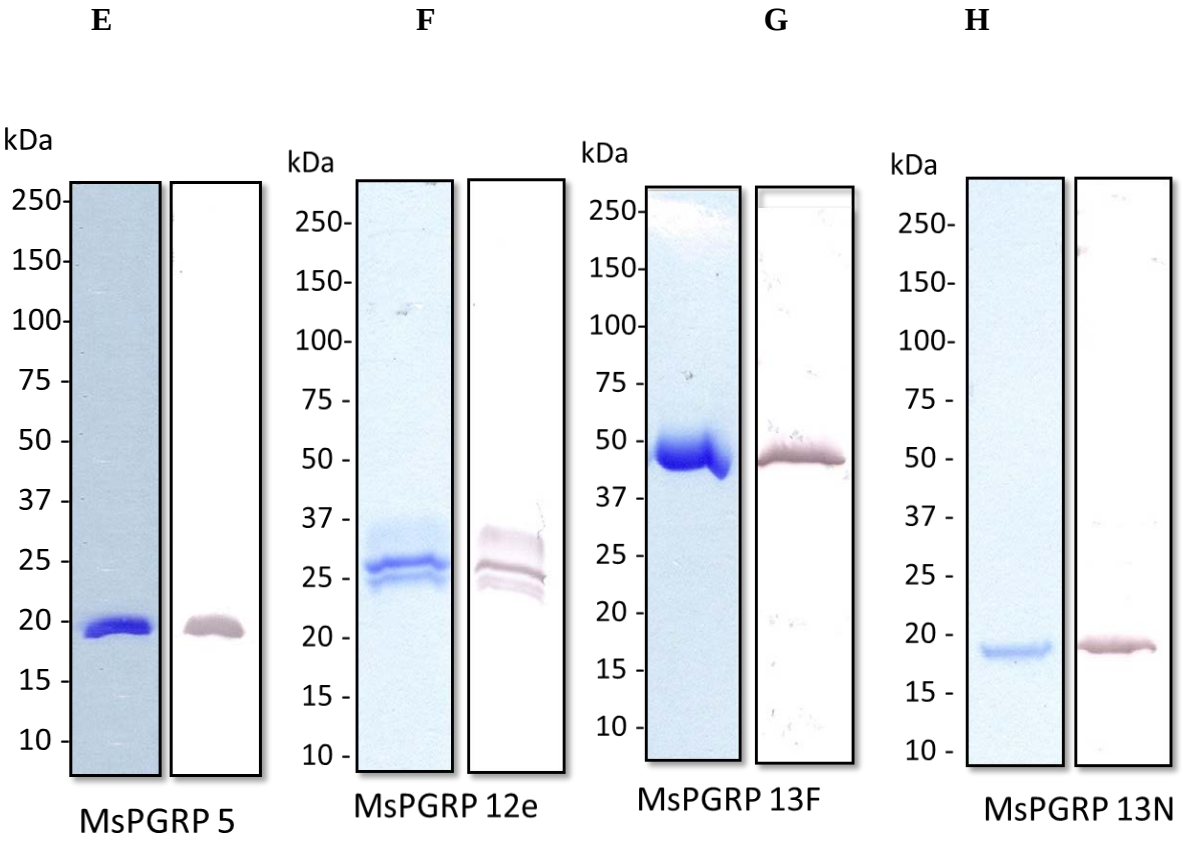


Fig. 4. Multiple sequence alignment of PG-binding domains in insect and human PGRPs with T7 lysozyme. Amino acid sequences of the domains in *M. sexta* PGRP1, **2**, **3**, **4** , 5, 12 and 13 and in *D. melanogaster* PGRP-SB1 (DmPGRP-**SB1**), PGRP-SC1a (DmPGRP-**SC1a**), PGRP-LE (DmPGRP-LE), PGRP-SA (DmPGRP-SA), PGRP-SD (DmPGRP-SD), *B. mori* PGRP1 (BmPGRP-S), *T. ni* PGRP (TniPGRP-S), *Homo sapiens* PGRP1αC (HsPGRP-1a) and **T7** lysozyme are aligned.

amino acids in bold red are shown or predicted to be *N*-acetylmuramoyl-L-alanine amidases with the conserved His^{18}, Tyr^{47}, His^{123}, K^{129}/T and C^{131} all present for binding the catalytic zinc ion in T7 lysozyme as well as the five insect PGRPs (three from *Manduca* and two from *Drosophila*). Numbers on the *right* indicate positions of the residues in the entire PGRP sequences. For the sixteen insect PGRPs, positions with 70, 90 and 100% identities are marked with ".", ":" and "*", respectively, and residues different from the consensus are shaded *gray*. Residues highlighted *yellow* represent the conserved residues for PGN binding.





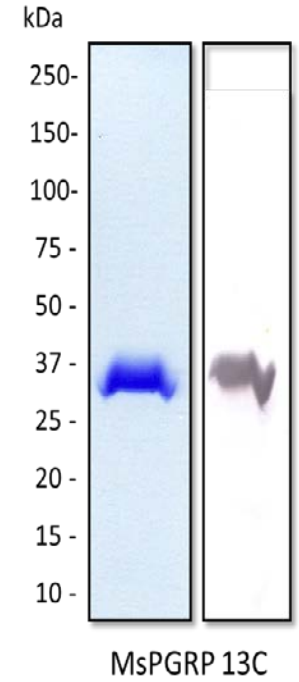


Fig. 5: The *M. sexta* PGRPs purified from the baculovirus-infected Sf9 cell cultures.

Purified PGRPs were analyzed by 12% SDS-PAGE. Left panel, Coomassie blue staining; right panel, immunoblot analysis using anti-$(His)_5$ as the first antibody, along with pre-stained molecular weight standards (M) with their sizes indicated on the left.

The purified recombinant MsPGRPs 2−5, 13 were migrated as a single band at 19, 24, 21, 19, 48, 15 and 27 kDa positions close to their calculated masses (20,770, 25230, 22,312, 20,706 and 51,451 Da). MsPGRP12ecto migrated as a double band at 24 kDa position.

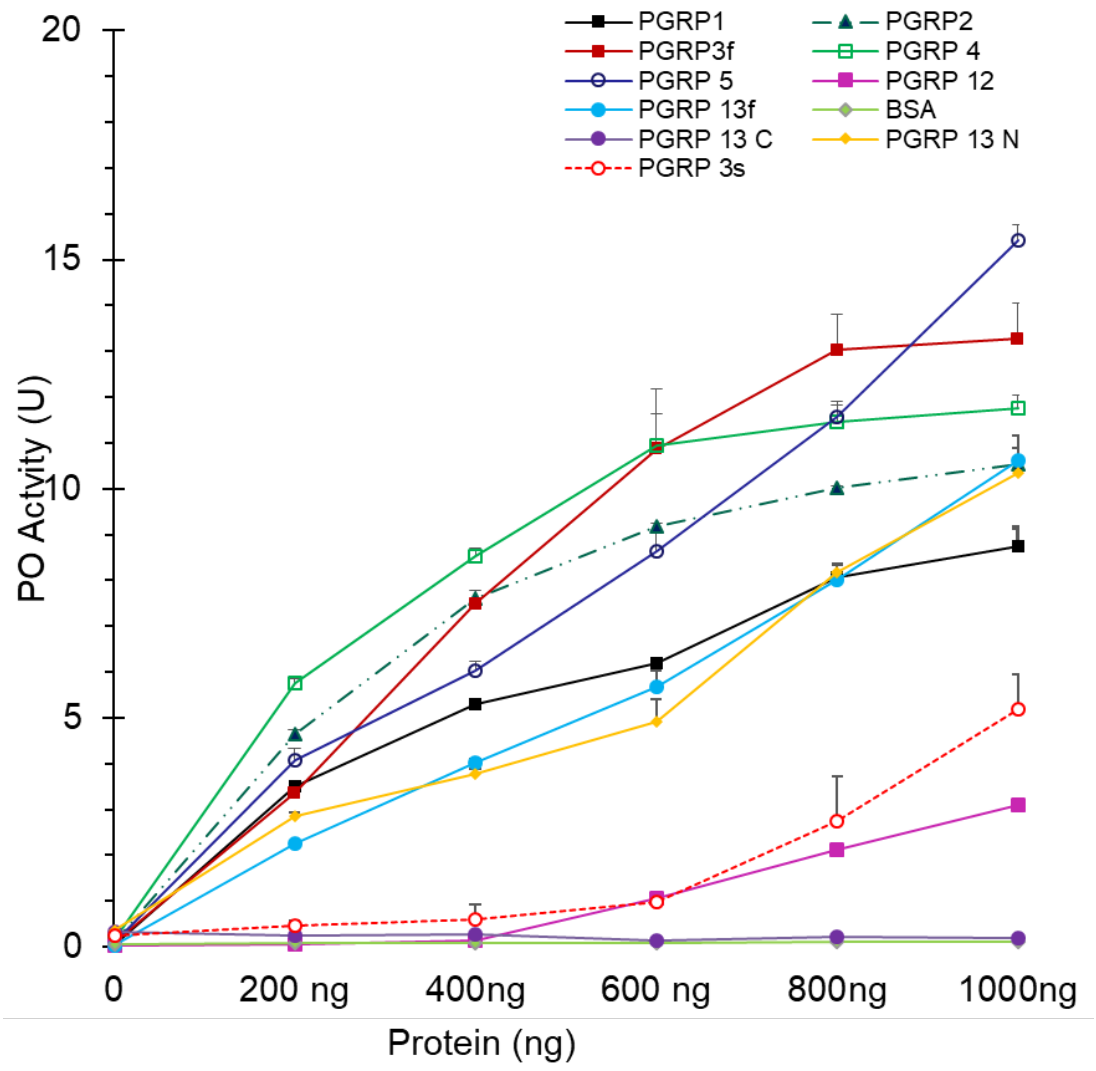
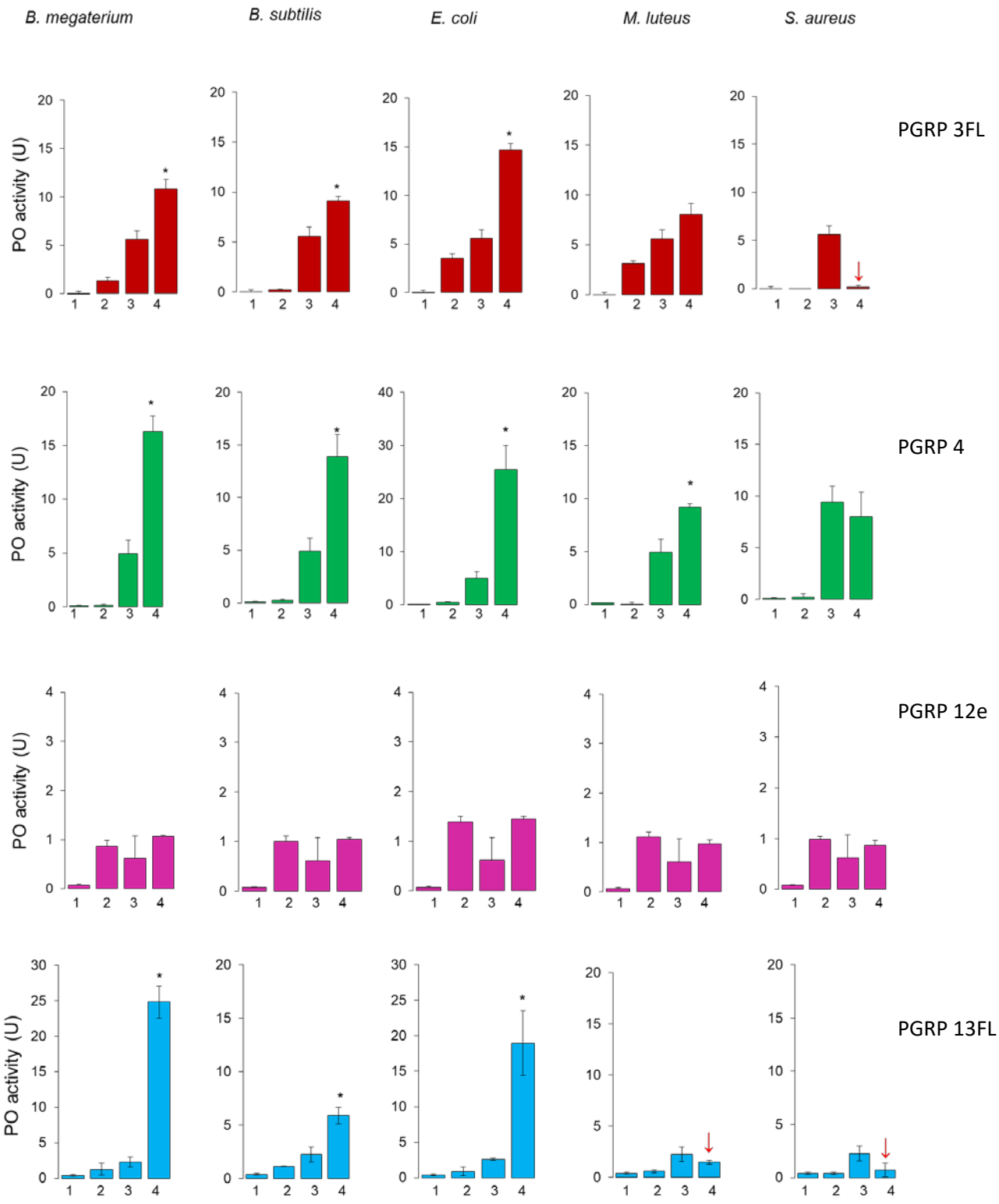


Fig. 6: Concentration-dependent increase in proPO activation caused by the purified *M. sexta* PGRPs in the absence of microbial elicitor. As described in Section 3.6, aliquots of cell-free hemolymph from day 2, fifth instar naïve larvae were incubated at room temperature with 0.2 to 1.0 µg of MsPGRP or BSA in a final volume of 24 µl. PO activities were determined and plotted as mean ± SEM (n=3).


B. megaterium
B. subtilis
E. coli
M. luteus
S. aureus
PO activity (U)
PGRP 3FL
PGRP 4
PGRP 12e
PGRP 13FL

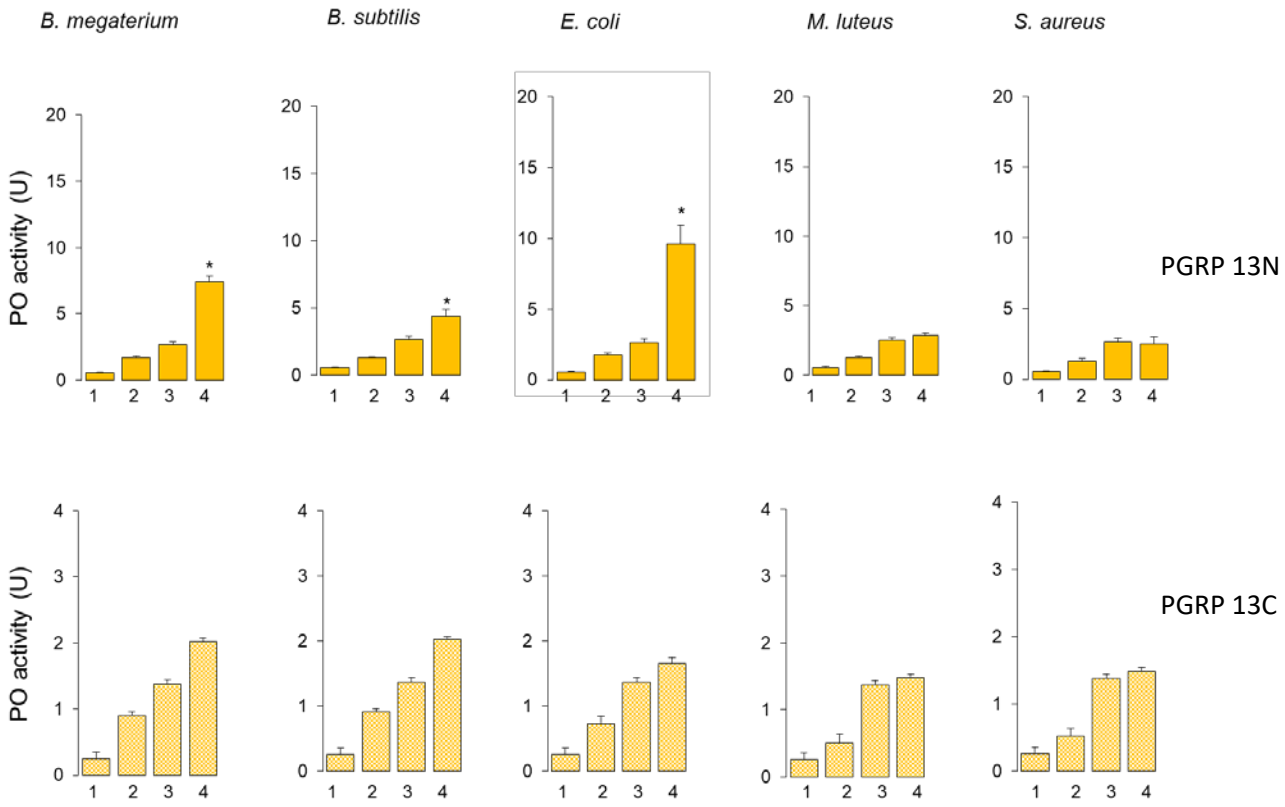
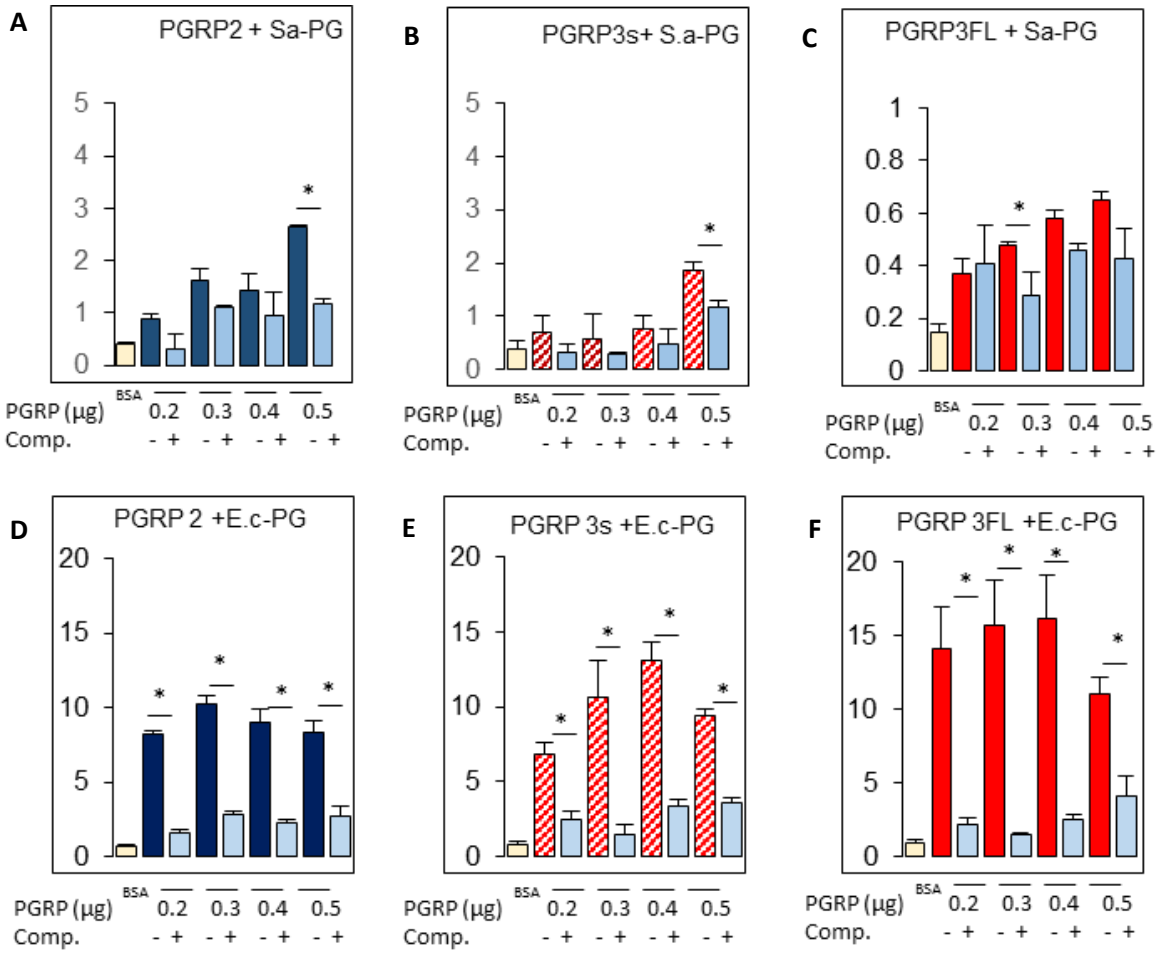
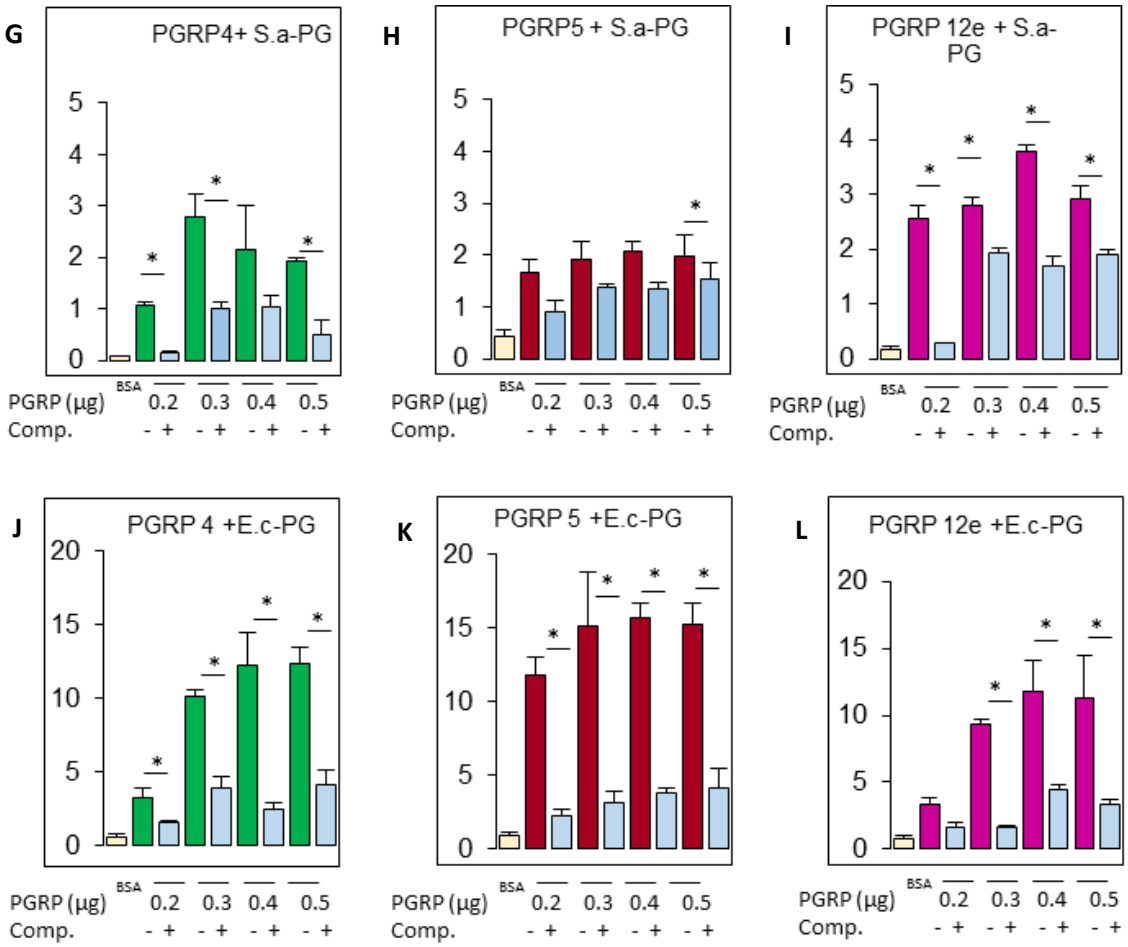


Fig. 7: Enhancement of proPO activation in plasma from naïve larvae by PGNs in the absence or presence of *M. sexta* PGRPs. As described in Section 3.7, PGNs of *B. megaterium* (**A**), *B. subtilis* (**B**), *E. coli* (**C**, soluble), *M. luteus* (**D**) and *S. aureus* (**E**) were separately incubated with 1:10 diluted plasma and purified recombinant MsPGRPs 3, 4, 12ecto, 13FL, 13N and 13C separately for 60 min at room temperature. PO activity (#4) was measured and plotted as mean ± SEM (n = 3), along with those of the controls (#1, plasma only; #2, plasma and PG; #3, plasma and PGRP1). Since interaction of plasma factors with elicitor (#2 - #1) and co-presence of plasma and exogenous MsPGRP (#3 - #1) both lead to proPO activation, an interaction of elicitor and exogenous MsPGRP in plasma (#4 - #1) is expected to increase proPO activation to a level significantly higher than the sum of the two components [*i.e.* (#2 - #1) + (#3 - #1)]. To detect a possible synergistic effect of PGN-MsPGRP interaction, the PO activity changes represented by (#4 - #1) and (#2 + #3 – 2×#1) are compared using unpaired t-test. An asterisk (*) on #4 indicates that (#4 - #1) is significantly higher ($p < 0.05$).


A
PGRP2 + Sa-PG
B
PGRP3s+ S.a-PG
C
PGRP3FL + Sa-PG
D
PGRP 2 +E.c-PG
E
PGRP 3s +E.c-PG
F
PGRP 3FL +E.c-PG
PGRP (µg) BSA 0.2 0.3 0.4 0.5
Comp. - + - + - + - +


G
PGRP4+ S.a-PG
H
PGRP5 + S.a-PG
I
PGRP 12e + S.a-PG
J
PGRP 4 +E.c-PG
K
PGRP 5 +E.c-PG
L
PGRP 12e +E.c-PG
PGRP (µg) BSA 0.2 0.3 0.4 0.5
Comp. - + - + - + - +

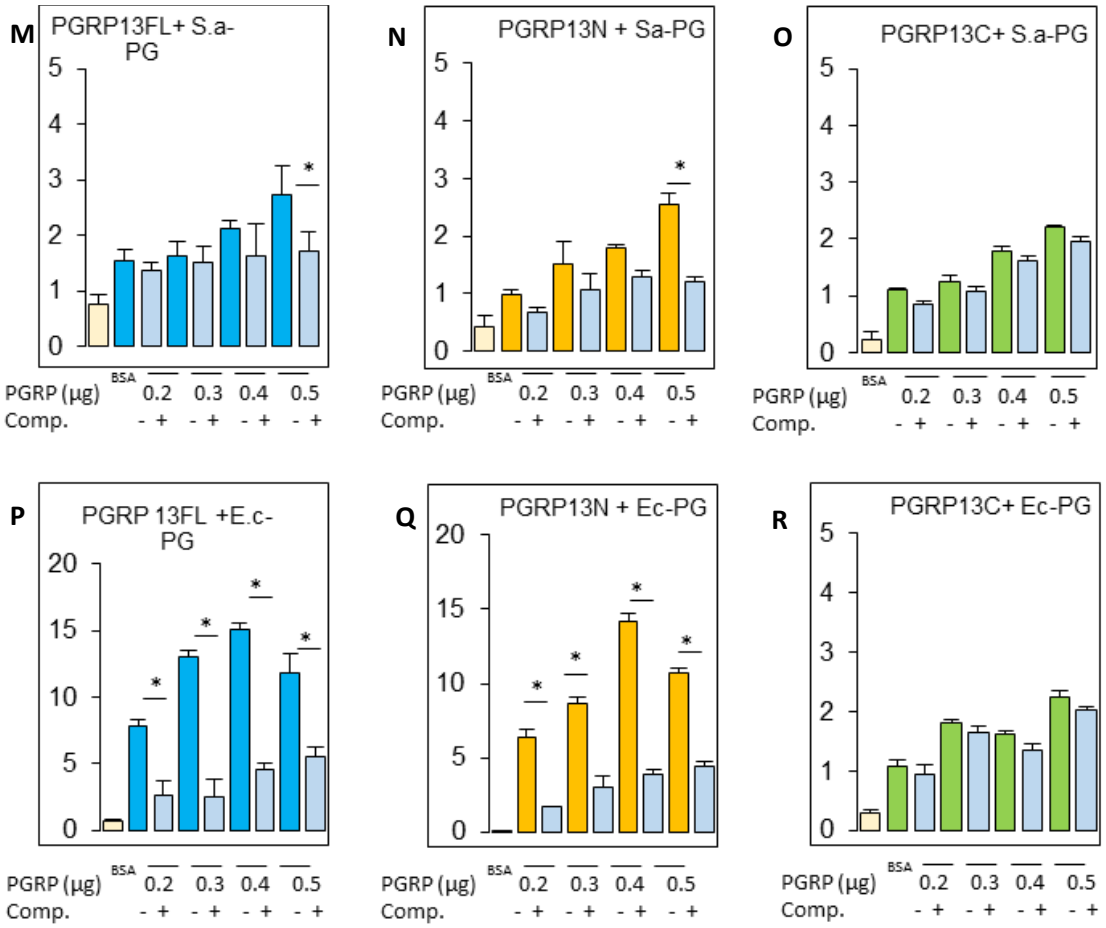


Fig. 8: ELISA of the *M. sexta* PGRPs interacting with soluble peptidoglycans from *S. aureus* (A, B, C, G, H, I, M, N, O) and *E. coli* (D, E, F, J, K, L, P Q R). MsPGRPs 2, 3s, 4, 5, 12ecto and 13 were tested for binding with PGNs from both *E. coli* and *S. aureus* separately. As described in Section 3.8, the purified MsPGRPs were incubated with soluble PG immobilized on a 96-well microplate. The binding was detected via ELISA and alkaline phosphatase activity is shown as mean ± SEM (*n*=3). Binding without a competitor, with excess soluble PG as competitor (c), and the negative control of BSA (BSA).

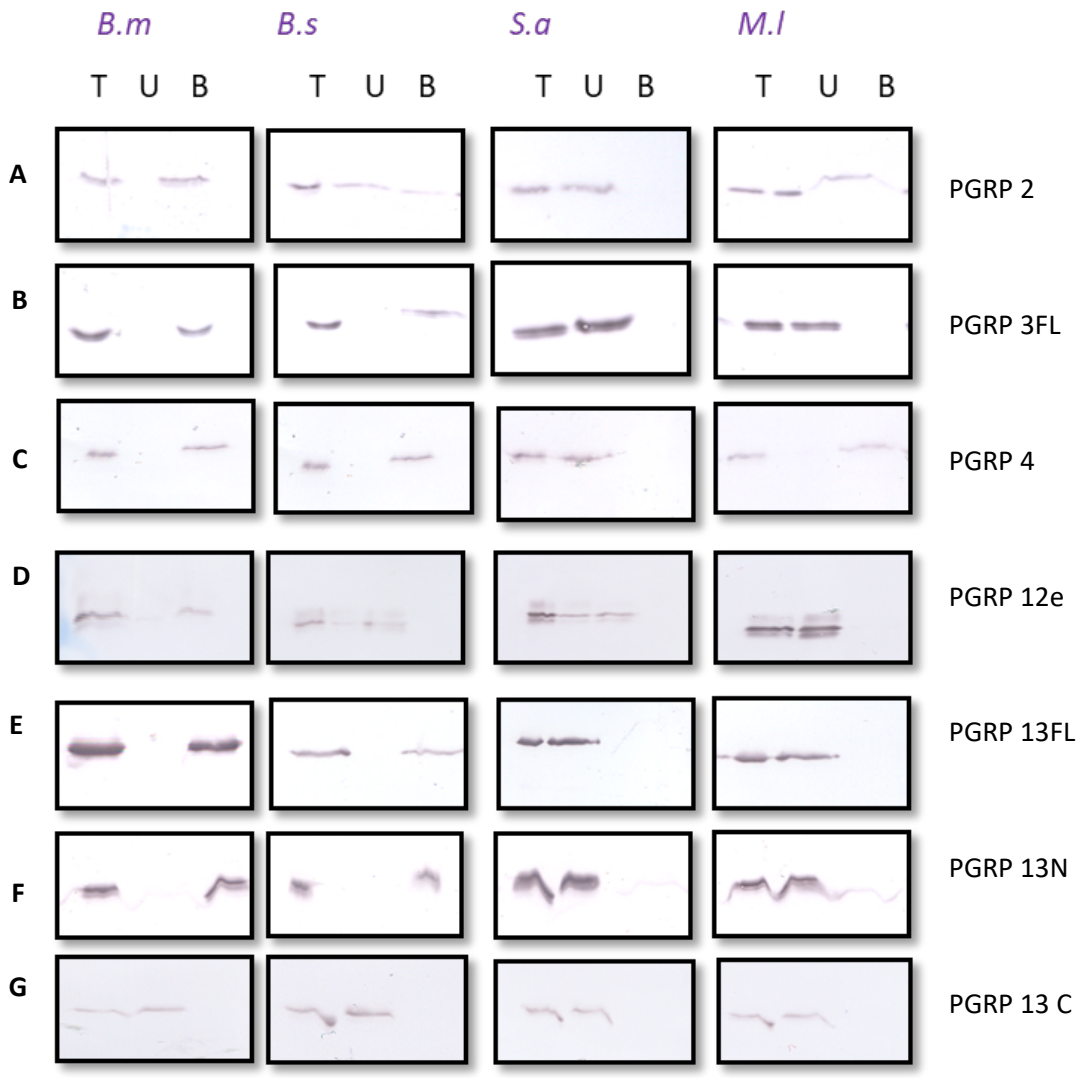
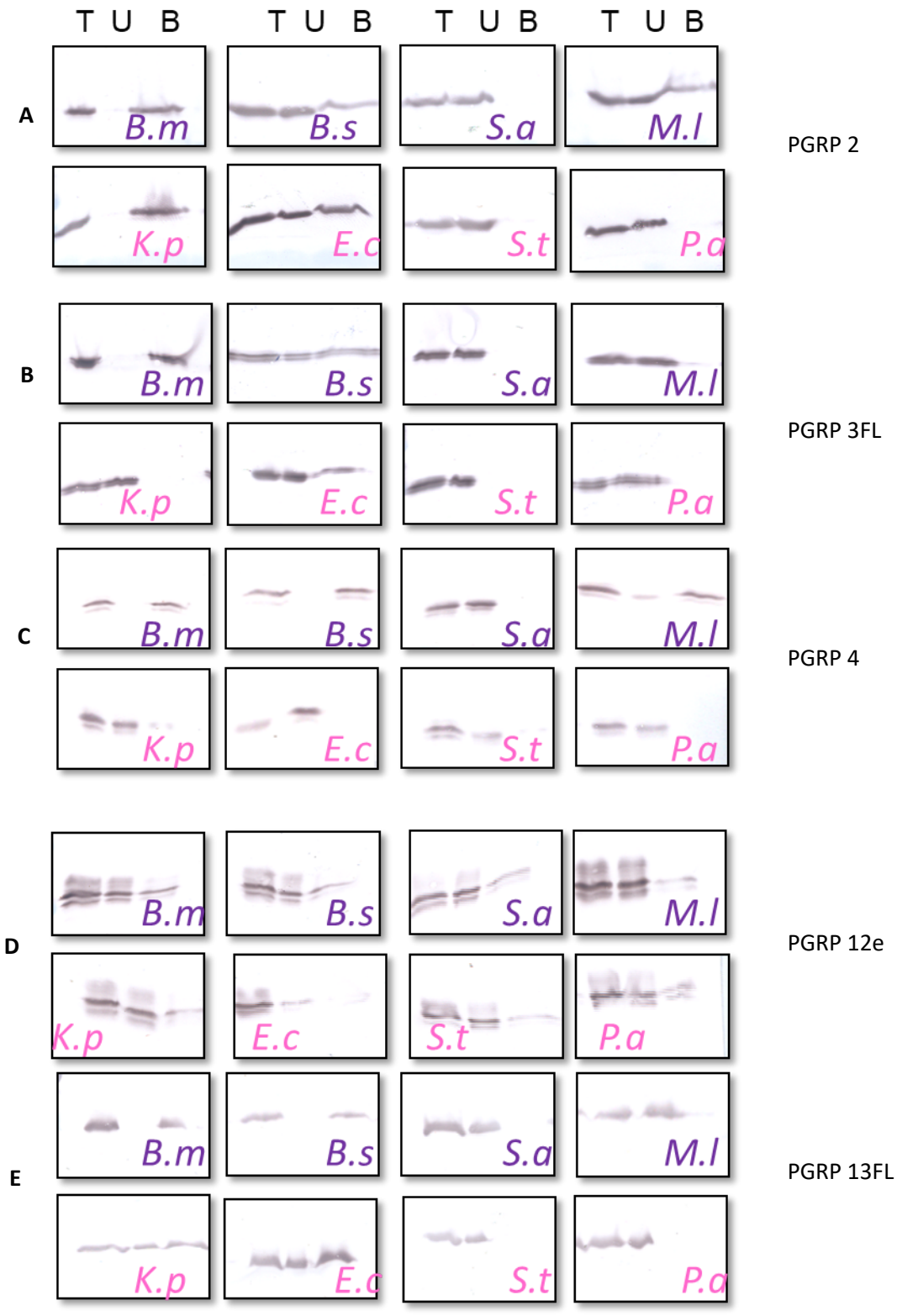


Figure 9: Binding of *M. sexta* PGRPs 2-4 12ecto, 13FL, 13N and 13C to peptidoglycans. Binding assays were performed using the purified PGRP2 (A), PGRP3 (B), PGRP 4 (C), PGRP12e (D), PGRP13FL (E) PGRP13N (F) and PGRP13C (G) and insoluble PGNs. T, U, B represents total, unbound, and bound fractions which were separated by SDS-PAGE followed by immunoblot analysis using anti-$(His)_5$ as the first antibody.


T U B
T U B
T U B
T U B
A
B.m
B.s
S.a
M.l
K.p
E.c
S.t
P.a
PGRP 2
B
B.m
B.s
S.a
M.l
K.p
E.c
S.t
P.a
PGRP 3FL
C
B.m
B.s
S.a
M.l
K.p
E.c
S.t
P.a
PGRP 4
D
B.m
B.s
S.a
M.l
K.p
E.c
S.t
P.a
PGRP 12e
E
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B.s
S.a
M.l
K.p
E.c
S.t
P.a
PGRP 13FL

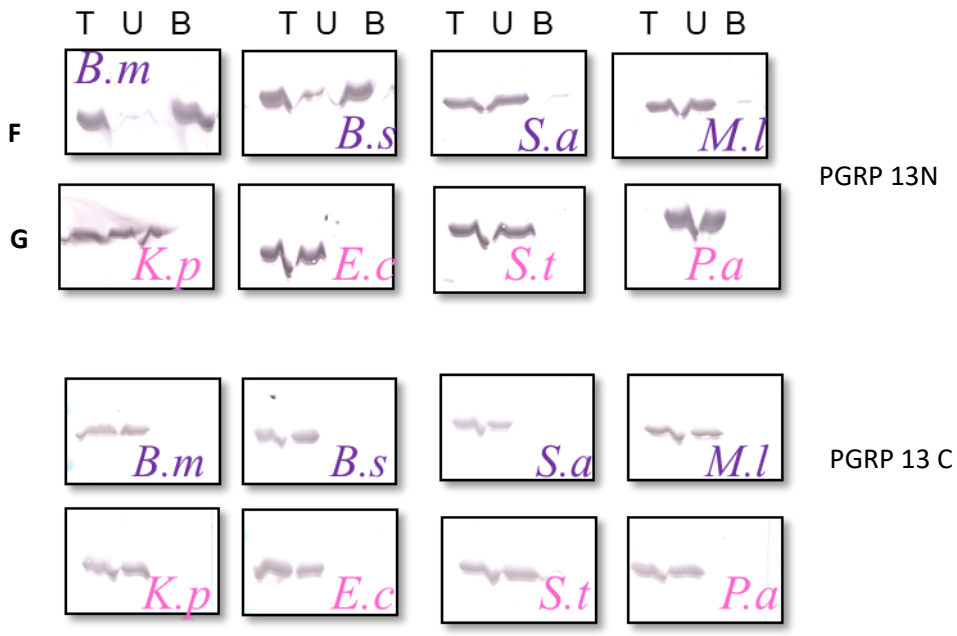

Fig. 10, **Binding of *M. sexta* PGRP 2-4, 12ecto, 13FL, 13N and 13C to different bacteria**. Binding assays were performed using the purified PGRP2 (A), PGRP3 (B), PGRP 4 (C), PGRP12e (D), PGRP13FL (E) PGRP13N (F) and PGRP13C (G) and whole bacteria. T, U, B represents total, unbound, and bound fractions which were separated by SDS-PAGE followed by immunoblot analysis using anti-$(His)_5$ as the first antibody.

TABLES

Table 1: Relationship between binding of exogenous PGRPs and increase in proPO activation

		Lys-type PG		DAP-type PG		
		M. luteus	*S. aureus*	*B. megaterium*	*B. subtilis*	*E. coli* (s)
PGRP2	PG binding	PB	No	CB	PB	N.a
	proPO activation	+	-	+	-	+
PGRP3	PG binding	No	No	CB	CB	N.a
	proPO activation	-	↓	+	+	+
PGRP4	PG binding	CB	No	CB	CB	N.a
	proPO activation	+	-	+	+	+
PGRP12	PG binding	No	PB	PB	PB	N.a
	proPO activation	-	-	-	-	-
PGRP13	PG binding	No	No	CB	CB	N.a
	proPO activation	↓	↓	+	+	+
PGRP13 N	binding	No	No	CB	CB	N.a
	proPO activation	-	-	+	+	+
PGRP13 C	binding	No	No	No	No	N.a
	proPO activation	-	-	-	-	-

PB : partial binding; CB complete binding; No : No binding + : synergistic enhancement of proPO activation.

↓: synergistic down regulation of proPO activation N.a : Not applicable

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APPENDICES

List of Abbreviations

AMP	antimicrobial peptide
BSA	bovine serum albumin
CLIP	clip-domain serine protease
DAP	meso-diaminopimelic acid
DHI	5,6-dihydroxyindole
DREDD	death-related ced-3/Nedd2 like protein
DS	dextran sulfate-Sepharose CL-6B
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas-associated protein with Death Domain
GlcNAc	N-acetylglucosamine
GNBP	Gram-negative bacteria-binding protein
HP	Hemolymph proteinase
His	Histidine
IgG	Immunoglobulin-G
Imd	immune deficiency
IPTG	isopropyl-β-D-thiogalactopyranoside
LDLa	Low Density Lipoprotein receptor class A
L-DOPA	L-dihydroxyphenylalanine
LPS	lipopolysaccharide
LTA	Lipoteichoic acid
Lys	Lysine
MAMP	microbe-associated molecular pattern
MurNAc	N-acetylmuramic acid
NADA	N-acetyldopamine
NBAD	N-β-alanyldopamine
NF-κB	nuclear factor-Kb
Ni-NTA	Ni-nitrilotriacetic acid
PAMP	pathogen-associated molecular pattern
PAP	proPhenoloxidase activating proteinase
PEG	polyethylene glycol
PCR	Polymerase chain reaction

PGN	peptidoglycan
PGRP	peptidoglycan recognition protein
PGRP-L	peptidoglycan recognition protein long form
PGRP-S	peptidoglycan recognition protein short form
PO	phenoloxidase
proPO	prophenoloxidase
PRR	pattern recognition receptor
SDS-PAGE	sodium dodecyl sulfate- Polyacrylamide gel electrophoresis
Serpin	serine proteinase inhibitor
Sf9	*Spodoptera frugiperda*
SP	serine proteinase
SPH	serine proteinase homolog
SPE	Spätzle-processing enzyme
TBS	Tris buffered saline
UPGMA	unweighted pair group method with arithmetic mean

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