MATRIX METALLOPROTEINASE EXPRESSION VARIES THROUGHOUT DEVELOPMENT AND DURING THE INNATE IMMUNE RESPONSE OF TOBACCO HORNWORM, *MANDUCA SEXTA*

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Smitha Vishnuvardhan

In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Department:
Biological Sciences

April 2010

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Matrix metalloproteinase expression varies throughout development and during the innate immune response of tobacco hornworm, *Manduca sexta.*

By

Smitha Vishnuvardhan

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

ABSTRACT

Vishnuvardhan, Smitha, M.S., Department of Biological Sciences, College of Science and Mathematics, North Dakota State University, April 2010. Matrix Metalloproteinase Expression Varies Throughout Development and During the Innate Immune Response of Tobacco Hornworm, *Manduca sexta*. Major Adviser: Dr. Kendra J. Greenlee.

Matrix metalloproteinases (MMPs) are a family of conserved, proteolytic enzymes. MMPs can degrade most extracellular matrix proteins. As such, they are key enzymes in tissue remodeling processes, such as molting and metamorphosis in insects. The presence of MMP in tracheae of 4th and 5th instar *Manduca sexta* (*M. sexta*) larvae was investigated using zymograms and Western blots, and the expression was found to decrease at the end of the instar. Failure to completely inhibit gelatinolytic activity in a zymogram indicates the existence of various other proteases that are involved in the molting process. However, delay in the appearance of the dorsal blood vessel along with a reduction in the body mass upon inhibition of MMP supports our hypothesis that MMPs are required for molting.

MMPs are also known to be up-regulated in many disease states, where they may cause tissue damage. Their presence may be beneficial, as their inhibition may result in worsening of symptoms or increased mortality. We tested the hypothesis that MMPs play a role in innate immunity in the tobacco hornworm caterpillar, *M. sexta*. Larvae infected with *E. coli* DH5α had significantly more circulating hemocytes (insect blood cells) when compared to the larvae which received an MMP inhibitor, suggesting that MMPs may be important for hemocyte recruitment from the hematopoietic organs such as the lymph gland and hematopoietic cells present near the wing imaginal discs. The expression of MMP was increased by 50% in larvae that had been infected with *E. coli* DH5α. However, this response was abrogated upon inhibition of MMPs, reducing expression to

control levels. The majority of the circulating hemocytes were granulocytes, a cell type that phagocytoses foreign bodies, including pathogens. We used flow cytometry to assess phagocytic ability. Hemocytes in the presence of the MMP inhibitor were less able to phagocytose sephadex beads, supporting the hypothesis that this MMP contributes to the cellular innate immune response.

Our results suggest that MMP expression is high at the beginning of the instar while it reduces as the caterpillar reaches molting, during which other proteases like chitinases and molting fluid proteinases play a more prominent role. Our results also suggest that *M. sexta* larvae have a reduced immunity to bacterial infections when MMPs are inhibited. Collectively, inhibition of MMPs during the molting process indicates that the larvae, by down regulating the expression of MMPs, prevent the elicitation of immune responses that is generally seen during the larval pupal metamorphosis.

ACKNOWLEDGEMENTS

First, I would like to thank my advisor Dr. Kendra J. Greenlee for her continuous support in the masters program. Kendra has always been there to listen and to give advice. She showed me different ways to approach a research problem and the need to be persistent to accomplish any goal. My special thanks to her for helping me complete the writing of this thesis. Without her constant guidance and encouragement I would not have finished. I extend my thanks to all the professors who taught me throughout the masters program and also I would like to thank Drs. Mark Sheridan, Jane Schuh and Katie Reindl for serving on my advisory committee.

I would like to thank all my colleagues in Kendra's lab for their amazing support for the past few years. I am grateful to Drs. Peggy Biga, Mark Sheridan, Jodie Haring, Jane Schuh, and John McEvoy for allowing me to use their lab equipment for my experiments without which completing my experiments would have been a difficult task.

I especially thank all my friends here in the US and back in India who have always been there for me in need. Last, but not least, I thank my parents, sister, and brother for their unconditional support and encouragement to persue my interests, even when the interests were beyond the boundaries of geography.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: MATRIX METALLOPROTEINASES-AN INTRODUCTION

1.1. Introduction

Degradation of extracellular proteins is a phenomenon that is essential for the proper interaction of cells with their surrounding environments both in development and disease. There are a large number of enzymes and pathways involved in the degradation of the matrix proteins and connective tissue. The most common pathways with ECM (extracellular matrix) as substrates include plasmin-dependent pathways that are essential for extracellular degradation during tissue remodeling, cell migration, and tumor invasion. Plasmin-dependent pathways are mediated by plasmin, a serine proteinase, which is derived from plasminogen by the plasminogen-activating enzymes (Danø et al., 1985; Laiho and Keski-Oja, 1989). Other pathways with ECM as substrates are the polymorphonuclear leukocyte serine protease-dependent reactions. The third largest groups of enzymes with ECM as substrates are the matrix metalloproteinases (MMPs).

MMPs are a large family of proteolytic enzymes that require zinc as a cofactor. Generally, MMPs are found at very low concentrations of up to 10 to 100-fold lower than the plasmins (Birkedal-Hansen et al., 1993). MMPs are ancient enzymes that evolved even before the divergence of vertebrates from invertebrates and are highly conserved throughout both the groups (Massova et al., 1998). The first MMP that was discovered was the collagenase, found in 1962 in the tail of a metamorphosing tadpole larva of *Xenopus* (Gross and Lapiere, 1962). It was shown to cleave collagen into two smaller fragments at temperatures much lower than the melting point of collagen and at a neutral pH (Gross and Nagai, 1965). Since then, about 25 different MMPs have been identified in

vertebrates (Brinckerhoff and Matisian, 2002; Mannello et al., 2005a; Mott and Werb, 2004) with 24 MMPs just in humans (reviewed in Greenlee et al., 2007b; Overall and Lopez-Otin, 2002). In addition to vertebrates, MMPs have also been identified in plants, such as soybean, *Glycine max* (Graham et al., 1991; Ragster and Chrispeels, 1979); thale cress, *Arabidopsis thaliana* (Maidment et al., 1999); and green alga, *Clamydomonas reinhardtii* (Kinoshita et al., 1992). MMPs have also been identified in several invertebrates such as the nematode, *Caenorhabditis elegans* (Wada et al., 1998); echinoderm, *Holothuria glaberrima* (Quinones et al., 2002); and oyster, *Crassostrea gigas* (Takagi et al., 2004). Finally, MMPs have been identified in prokaryotes, like *Bacteroides fragilis* (Moncrief et al., 1995; Van Tassel et al., 1992). In *B. fragilis*, the MMP acts as an endotoxin, which is involved in human and animal intestinal infections (Obiso et al., 1995). Recently, MMPs also have been identified in insects, such as the fruit fly *Drosophila melanogaster* (Dm1-MMP and Dm2-MMP) (Llano et al., 2002; Llano et al., 2000), the wax moth *Galleria mellonella*, Gm1-MMP (Altincicek and Vilcinskas, 2008), and the flour beetle *Tribolium castaneum*, MMP-1, MMP-2, and MMP-3 (Knorr et al., 2009).

1.1.1. Overview of MMPs

Proteolytic enzymes can be broadly classified into two major groups based on the sites of cleavage of the target proteins: exopeptidases and endopeptidases. Exopeptidases cleave the terminal peptide bonds while the endopeptidases cleave internal peptide bonds. Matrix metalloproteinases are a class of endopeptidases belonging to the superfamily of metalloproteinases called the metzincins. The metzincins are sub-divided depending on the terminal amino acid in the conserved sequence of the catalytic site. Other metzincins

include ADAMs/adamalysins, astacins, and serralysins. MMPs have histidine as the terminal amino acid in the catalytic site. MMPs are further classified based on the primary sequences, the tertiary protein structure, the substrate specificity, and the inhibition of enzymatic activity by various MMP inhibitors (Table 1).

Table 1. Classification of mammalian MMPs based on their domain structures.

Domain	MMP	Common Name
Simple Hemopexin	MMP-1	Collagenase-1
	MMP-3	Stromelysin-1
	MMP-8	Collagenase-2
	MMP-10	Stromelysin-2
	MMP-12	Metalloelastase
	MMP-13	Collagenase-3
	MMP-18	Collagenase-4
	MMP-19	RASI-1
	MMP-20	Enamelysin
	MMP-22	CAMMP
Gelatin Binding	MMP-2	Gelatinase-A
	MMP-9	Gelatinase-B
Minimal	MMP-7	Matrilysin
Furin Activated	MMP-11	Stromelysin-3
	MMP-28	Epilysin
Trans Membrane	MMP-14	MT1-MMP
	MMP-15	MT2-MMP
	MMP-16	MT3-MMP
	MMP-24	MT5-MMP
Type II Transmembrane	MMP-23	Femalysin
GPI anchored	MMP-17	MT4-MMP
	MMP-25	Leukolysin
Vetronectin like	MMP-26	Endometase

1.1.2. Structure and function of MMPs

Although the first MMP was identified in 1962 (Gross and Lapiere, 1962), it took about a decade to recognize that virtually all the known MMPs identified by then, could be produced either as secreted or membrane-bound, inactive proenzymes or zymogens (Harper et al., 1971) which would be further activated as needed. Under *in vitro* conditions, MMPs can be activated using organomercurials, such as 4-aminophenylmercuricacetate (APMA), oxidants, heavy metals, and sulphydryl-alkylating agents.

Most of the MMPs identified to date have been fully sequenced and contain a common multi-domain structure with an auto-inhibitory pro-domain, a catalytic domain that has a conserved zinc-binding region (Nagase and Woessner, 1999), and a hemopexin-like C-terminal region (Fig. 1) (Llano et al., 2002; Llano et al., 2000). The MMP protein sequence starts with a hydrophobic signal peptide at the N-terminus. Upon cleavage of the signal peptide, the propeptide is exposed. This region with the amino acid sequence, PRCGVPD, is highly conserved and is called the "cysteine switch," because the cysteine residue of the propeptide is bound to the zinc atom in the catalytic domain. The removal of the cysteine residue is essential for the enzyme's activity (Springman et al., 1990). In addition to the zinc atom, the catalytic domain contains two to three calcium ions bound to histidine and glutamate residues (Lepage and Gache, 1990). The three histidine residues are part of a highly conserved sequence 'HEXXHXXGXXH,' where X is any amino acid. The histidines are essential for binding zinc to the catalytic site (Rowsell et al., 2002). There are a few fibronectin repeats spanning the catalytic domain in some, but not all, MMPs. These fibronectin repeats confer the gelatin-binding ability to MMPs (Goldberg et al., 1989). The catalytic region is connected to the hemopexin domain through a proline-

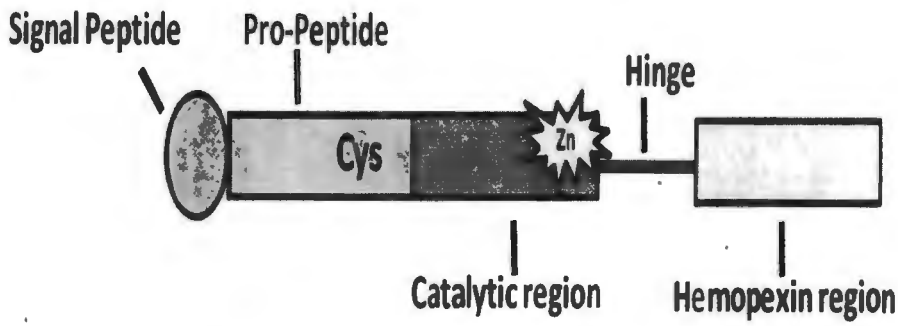

Figure 1. Generic structure of MMP. Basic structure contains a signal peptide, pro-peptide, catalytic domain, hinge region, and hemopexin domain. This basic structure is conserved in MMPs throughout vertebrates and invertebrates.

rich, hinge region of variable length. MMP-7 (matrilysin) is shorter in length, because it lacks the hinge region. The hemopexin domain is thought to be involved in substrate recognition and protein-protein interactions (Crabbe et al., 1994; Sternlicht and Werb, 2001).

Collectively, all of the known MMPs cleave most of the extracellular matrix proteins that have been tested (Page-McCaw et al., 2007; Sternlicht and Werb, 2001). MMP substrates also include cell adhesion molecules cytokines, chemokines, proteinases, and growth factors (Zhang et al., 2006). In addition to the ability to degrade many proteins, MMPs are also involved in the activation of latent growth factors from the matrix and adhesion molecules (Chakraborti et al., 2003). The presence of the large number of MMPs with overlapping and compensatory functions is helpful to ensure proper function in the case of dysregulation of other MMP family members (Lambert et al., 2003; Stickens et al., 2004), yet this ambiguity has made it difficult to identify the exact functions of MMPs.

1.1.3. Regulation of MMPs

Because of the capacity of MMPs to degrade the ECM, it is critical that they be tightly regulated, both at the gene and protein level to achieve their proper functions. Over-expression of MMPs may be due to the discrepancies in their regulation resulting in improper and/or unnecessary degradation of matrix proteins, while under expression may inhibit processes for normal development and cell migration. Hence, MMPs are highly regulated at the gene level, involving transcriptional, post-transcriptional and translational modifications and at the protein level they are regulated by various factors such as the activators, inhibitors, and the environment in which they are present.

Most of the MMPs except MMP-2, which is generally constitutively expressed (Strongin et al., 1995), are regulated by a large number of transcription activators and repressors such as the growth factors, hormones, cell adhesion molecules, and extracellular matrix proteins. Some signals may positively regulate the expression of some MMPs while negatively regulating others (reviewed in Greenlee et al., 2007b). For example, TGF-β up-regulates the transcription of MMP-13 but down-regulates the transcription of MMP-1 and -3 (Uria et al., 1998).

Regulation of MMPs can occur at the transcriptional level also. The key transcriptional regulators include transcription factors, such as tumor necrosis factor TNF-α and interferon IFN-γ (Kheradmand et al., 1998; Ning et al., 2004); growth factors, such as epidermal growth factor (EGF) (Kheradmand et al., 2002); and cytokines, such as interleukin IL-1 and IL-6 (Kusano et al., 1998). At the protein level, post-translational regulation is accomplished by activation, inhibition, secretion, and localization (Brinckerhoff and Matisian, 2002). For example, MMPs may be activated by cell surface proteins such as collagen I (Vogel et al., 1997), integrins (Brooks et al., 1996), and

transforming growth factors (TGF) (Yu and Stamenkovic, 2000). Inhibition of MMPs can be accomplished by the physiological MMP inhibitors called the tissue inhibitors of metalloproteinases (TIMPs) (Oslon et al., 1997). Localization and release of MMPs plays a vital role during inflammatory reactions. For example, pro-MMPs localized in neutrophil granules may be released to the surrounding environment upon the activation of leukocytes (Borregaard and Cowland, 1997).

1.1.4. Inhibitors of MMPs

Since all the metalloproteases require a metal ion for their proper function, they are highly susceptible to metal chelators. MMPs may be inhibited by metal chelators such as EDTA (Nagai et al., 1966), dithiothreitol (DTT), and 1, 10-phenanthroline (Banda and Werb, 1981), which bind and remove zinc from the enzyme (Springman et al., 1995). Antibiotics such as tetracyclines (Golub et al., 1983), erythromycin (Lubec and Ratzenhofer, 1979), and actinonin (Faucher et al., 1987) are also known to inhibit MMPs by metal chelation.

MMPs also can be inhibited by the natural inhibitors (Abe and Nagai, 1972). α_2-Macroglobulin (α_2M) is a plasma protein known to inactivate a variety of proteinases and irreversibly inhibit MMPs irrespective of their substrate specificities. Another type of inhibitor, specific to MMPs is the "reversion-inducing-cysteine-rich protein with kazal motifs" (RECK) (Brew et al., 2000; Knorr et al., 2009; Steetler-Stevenson, 2008) which is known to be a membrane-anchored, glycosylated protein. RECK negatively regulates MMP-9 and inhibits tumor invasion and metastasis (Takahashi et al., 1998).

The last group of specific, endogenous inhibitors of MMPs is the TIMPs. TIMPs are 20-29 kDa secreted proteins that reversibly inhibit MMPs (Brew et al., 2000; Gomez et

al., 1997). TIMPs bind to the active site of MMPs, thus inhibiting their activity (Gomis-Ruth et al., 1997). To date, four vertebrate TIMPs have been identified, although the reason for the presence of multiple kinds of inhibitors is not clearly known. All four TIMPs are very closely related structurally, with 12 invariant cysteine residues that form 6 intra-chain disulfide bonds which can bind to the active site of MMPs, thereby inactivating the MMPs (Murphy and Willenbrock, 1995). Even though most of the MMPs are inhibited by all 4 TIMPs, they exhibit specific inhibitory activity towards some MMPs. For example, TIMP-1 is known to completely bind to the catalytic site of MMP-3, inhibiting its exposure to its substrates (Gomis-Ruth et al., 1997). TIMP-1 and -2 bind to the hemopexin domain of MMP-2 and MMP-9 preventing the activation process (Woessner, 2002).

1.1.5. MMPs in disease

MMPs are important effectors in both innate and adaptive immunity. In many pathological conditions, MMPs are implicated in deterioration and degradation of matrix and tissue destruction (Woessner, 1998). MMPs are known to cleave the basement membrane, thus helping cancer cells to metastasize in cancer patients (Nelson et al., 2000). Attempts to use inhibitors of MMPs as therapeutics resulted in many side effects (Coussens et al., 2002), indicating their role even in the regulation of normal physiological processes. Genetic deletion of the singular and multiple MMPs in mice has often shown the importance of MMPs both in pathological conditions and in normal conditions such as tissue remodeling (Kuo et al., 2005), growth and metamorphosis (Page-McCaw et al., 2003), proliferation, differentiation, fertilization, embryogenesis, organogenesis, and tissue repair (reviewed inGreenlee et al., 2006). Some of the other diseases involving MMPs

include rheumatoid arthritis (Cawston, 1996, 1998), liver (Arthur, 1997), and renal fibrosis (Eddy, 1996), inflammatory lung diseases (Greenlee et al., 2007b), atherosclerosis (Amirbekian et al., 2009), heart failure (Satsuki et al., 2006), multiple sclerosis (Weaver et al., 2005), and hematological disorders (Guedez et al., 1996). MMPs are also involved in fighting bacterial infections, as evidenced by MMP-7 knockout mice with increased susceptibility to enteric pathogens like *Escherichia coli* and *Salmonella typhimurium* (Wilson et al., 1999).

1.1.6. Invertebrate MMPs

Even though the first vertebrate MMP was identified four decades ago, MMPs in insects have been identified only recently. *Drosophila melanogaster* MMPs (Dm1-MMP and Dm2-MMP) were the first insect MMPs to be identified and sequenced (Llano et al., 2002; Llano et al., 2000). Both the Dm-MMPs have a molecular weight of approximately 61 kDa, are closely related to human MMP-2 and MMP-9, and have the conserved, basic domain structure of MMPs (Fig. 1).

Interestingly, the Dm1-MMP gene has two splice variants suggesting that the *Drosophila* MMPs evolved to perform various functions. Both MMPs are involved in larval tracheal growth, metamorphosis, and tissue remodeling (Page-McCaw et al., 2003). Recombinant Dm1-MMP, which is capable of cleaving synthetic fluorescent peptides (Q-24, Q-35, Q-41) , is inhibited by *Drosophila* TIMP (Llano et al., 2000). Mutant phenotypes lacking one or both Dm-MMPs are less viable (Page-McCaw et al., 2003). The third insect MMP and the first from the order Lepidoptera to be identified and sequenced was Gm1-MMP, a 68 kDa collagenase found in the wax moth, *Galleria mellonella* (Altincicek and Vilcinskas, 2008). Gm1-MMP is closely related to Dm1-

MMP both in structure and function. It is essential for and up-regulated during tissue remodeling after pupation and during metamorphosis. Even though MMPs are essential for the remodeling process, knockout of the TIMP (Godenschwege et al., 2000) led to an inflated wing phenotype in *D. melanogaster* indicating that uncontrolled activity of MMPs is detrimental also in invertebrates (Godenschwege et al., 2000). In addition, Gm1-MMP was found to be important for innate immunity and was up-regulated in response to LPS and septic injury (Altincicek and Vilcinskas, 2008). Gm1-MMP is closely related to human MMP-19 and MMP-28, both of which are also involved in immunity (Altincicek and Vilcinskas, 2008).

Even more recently, three novel MMPs and their inhibitors TIMP and RECK, were identified in the red flour beetle *Tribolium castaneum* and characterized using RNA interference (Knorr et al., 2009). Phylogenetically, *Tribolium* MMP-1 is closely related to Dm1-MMP while *Tribolium* MMP-2 and MMP-3 are related to Dm2-MMP and *Anopheles gambiae* MMP-3, respectively. RNAi of the MMP-1 resulted in the irregular pupal metamorphosis and improper tracheal development during embryogenesis. *Tribolium* MMP, a collagenase, confers increased resistance to fungal infection by *Beauveria bassiana* (Knorr et al., 2009).

1.2. *Manduca sexta* as a model organism

Manduca sexta, also known as the tobacco hornworm, is a lepidopteran insect belonging to the family Sphingidae, present in most of the states in American continent. The tobacco hornworm is a pest that feeds on the leaves of plants belonging to the family Solanaceae (e.g., tomato, tobacco, and datura plants). The life cycle of *M. sexta* includes four morphologically distinct stages: egg, larva, pupa, and moth (Fig. 2).

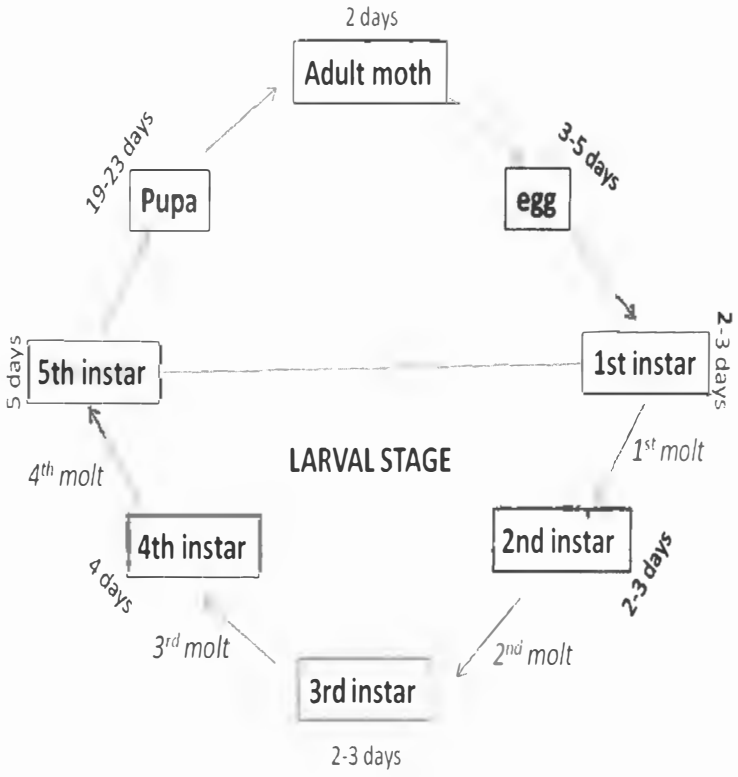

Figure 2. Life cycle of tobacco hornworm, *Manduca sexta*. The line connecting the 1st instar and 5th instar indicates the larval stage in the life cycle.

The translucent eggs are about a millimeter in diameter and typically hatch in four to five days. The larval stage is further divided into five stages called instars (Reinecke et al., 1980). All larval stages are marked by high food intake during which the larvae continuously feed and increase in size (Bell and Joachim, 1976; Ojeda-Avila et al., 2003; Yamamoto, 1969). At the end of each instar, larvae undergo, molting which is the removal of the old-exoskeleton. The shedding of the old exoskeleton reveals the underlying new exoskeleton (Truman, 1972). At the end of the fifth instar, the larva undergoes pupation and metamorphosis. During the pupal stage, most of the tissue degradation and remodeling occurs, making the insect highly susceptible to infections. After the pupal

stage, which typically lasts for 15-20 days in non-diapausing conditions, the adult moth emerges (Reinecke et al., 1980).

M. sexta is an excellent model organism for physiological studies, because during the fifth instar the larvae can weigh up to 10 or 11 g. In addition, nearly 1.5-2.0 ml of hemolymph, 4 million hemocytes, and large amounts of tissue can be obtained from a fully grown fifth instar larva, making it preferable for physiological research. Its large size also facilitates experimental manipulations such as injection of substances. In addition, these organisms can be easily maintained and propagated under laboratory conditions. Because of all these advantages, *M. sexta* has been gaining importance as a research model in neurobiology, as well as biomedical, and physiological research.

The overarching goal of this work is to establish a viable, alternative model system to study the role of MMPs in innate immunity. The central hypothesis explains that MMPs are critical for innate immune functions. *M. sexta* is predicted to have MMP, like other insects, yet it is a better model because of its large body size and the ease with which it can be manipulated. Before establishing the model of innate immunity, it was necessary to identify and characterize this enzyme during normal development. Therefore, the second chapter of this thesis describes the protein expression patterns and enzyme activity patterns throughout normal development. The third chapter describes the establishment of the model of innate immunity as well as the methods used to test the hypothesis that MMPs are important for phagocytosis and cell migration in response to bacterial infections.

1.3. References

Abe, S., Nagai, Y., 1972. Interaction between tadpole collagenase and human α2-macroglobulin. Biochimica et Biophysica Acta 278, 125-132.

Altincicek, B., Vilcinskas, A., 2008. Identification of lepidopteran matrix metalloproteinase with dual roles in metamorphosis and innate immunity. Develpomental Comparative Immunology 32, 400-409.

Amirbekian, V., Aguinaldo, J.G.S., Amirbekian, S., Hyafil, F., Vucic, E., Sirol, M., Weinreb, D.B., Greneur, S.L., Lancelot, E., Corot, C., Fisher, E.A., Galis, Z.S., Fayad, Z.A., 2009. Atherosclerosis and Matrix Metalloproteinases: Experimental Molecular MR Imaging *in vivo*. Radiology 251, 429-438.

Arthur, M.J., 1997. Matrix degradation in liver: a role in injury and repair. Hepatology 26, 1069-1071.

Banda, M.J., Werb, Z., 1981. Mouse macrophage elastase, purification and characterization as a metalloproteinase. Biochemical Journal 193, 589-605.

Bell, R.A., Joachim, F.G., 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. Annals of the Entomological Society of America 19, 365-373.

Birkedal-Hansen, H., Moore, W.G., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., Engler, J.A., 1993. Matrix metalloproteinases: A review. Critical Reviews in Oral Biology and Medicine 4, 197.

Borregaard, N., Cowland, J.B., 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 89, 3503-3521.

Brew, K., Dinakarpandian, D., Nagase, H., 2000. Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochimica Biophysica Acta 1477, 267-283.

Brinckerhoff, C., Matisian, L., 2002. Matrix metalloproteinase: a tail of a frog that became a prince. Nature Reviews in Molecular and Cellular Biology 3, 207-214.

Brooks, P.C., Stromblad, S., Sanders, L.C., von Schalscha, T.L., Aimes, R.T., Stetler-Stevenson, W.G., Quigley, J.P., Cheresh, D.A., 1996. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin avb3. Cell 85, 683-693.

Cawston, T.E., 1996. Metalloproteinase inhibitors and the prevention of connective tissue breakdown. Phramacology and Therapeutics 70, 163-182.

Cawston, T.E., 1998. Matrix metalloproteinases and TIMPs-properties and implications for the rheumatic diseases. Molecular Medicine Today 4, 130-137.

Chakraborti, S., Mandal, M., Das, S., Mandal, A., Chakraborti, T., 2003. Regulation of matrix metalloproteinases: An overview. Molecular and Cellular Biology 253, 269-285.

Coussens, L.M., Fingleton, B., Martisian, L.M., 2002. Matrix metalloproteinase inhibitors and cancer: Trails and tribulations. Science 295, 2387-2392.

Crabbe, T., Zucker, S., Cockett, M., Willenbrock, F., Tielke, S., O'Conell, J., 1994. Mutation of the active site glutamic acid of human gelatinase A: Effects on latency, catalysis, and the binding of tissue inhibitor of metalloproteinase-1. Biochemistry 33, 6684-6690.

Dano, K., Andreasen, P.A., Grøndahl-Hansen, J., Kristensen, P., Nielsen, L.S., Skriver, L., 1985. Plasminogen activators, tissue degredation and cancer. Advances in Cancer Research 44, 139-266.

Eddy, A.A., 1996. Molecular insights into renal interstitial fibrosis. Journal of the American Society of Nephrology 7, 2495-2508.

Faucher, D.C., Lelievre, Y., Cartwright, T., 1987. An inhibitor of mammalian collagenase active at micromolar concentrations from an actinomycete culture broth. Journal of Antibiotics (Tokyo) 40, 1757-1761.

Godenschwege, T.A., Pohar, N., Buchner, S., Buchner, E., 2000. Inflated wings, tissue autolysis and early death in tissue inhibitor of metalloproteinase mutants of *Drosophila*. European Journal of cell Biology 79, 495-501.

Goldberg, G.I., Manner, B.L., Grant, G.A., Eisen, A.Z., Wilhelm, S., He, C., 1989. Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteinases designated TIMP-2. Proceedings of National Academy of Science 86, 8207-8211.

Golub, L.M., Lee, H.M., Lehrer, G., Nemiroff, A., McNamara, T.F., Kaplan, R., Ramamurthy, N.S., 1983. Monocycline reduces gingival collagenolytic activity during diabetes. Preliminary observations and a proposed new mechanism of action. Journal of Periodontal Research 18, 516-526.

Gomez, D.E., Alonso, D.F., Yoshiji, H., Thorgeirson, U.P., 1997. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. European Journal of Cell Biology 74, 111-122.

Gomis-Ruth, F.X., Maskos, K., Betz, M., Bergner, A., Huber, R., Suzuki, K., Yoshida, N., Nagase, H., Brew, K., Bourenkov, G.P., Bartunik, H., Bode, W., 1997. Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. Nature 389, 77-81.

Graham, J.S., Xiong, J., Gillikin, J.W., 1991. Purification and developmental analysis of a metalloproteinase from the leaves of *Glycine max*. Plant Physiology 97, 786-792.

Greenlee, K.J., Corry, D.B., Engler, D.A., Matsunami, R.K., Tessier, P., Cook, R.G., Werb, Z., Kheradmend, F., 2006. Proteomic identification of *in vivo* substrates for matrix metalloproteinases 2 and 9 reveals a mechanism for resolution of inflammation. The Journal of Immunology 177, 7312-7321.

Greenlee, K.J., Werb, Z., Kheradmand, F., 2007b. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. Physiological Reviews 87, 68-98.

Gross, J., Lapiere, C.M., 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proceedings of National Academy of Science 48, 1014-1022.

Gross, J., Nagai, Y., 1965. Specific degeradation of the collagen molecule by tadpole collagenolytic enzyme. Proceedings of the National Academy of Sciences 54.

Guedez, L., Lim, M.S., Stetler-Stevenson, W.G., 1996. The role of metalloproteinases and their inhibitors in hematological disorders. Critical Reviews in Oncogenesis 7, 205-250.

Harper, E., Bloch, K.J., Gross, J., 1971. The zymogen of tadpole collagenase. Biochemistry 10, 3035-3041.

Kheradmand, F., Werner, E., Tremble, P., Symons, M., Werb, z., 1998. Role of Rac1 and oxygen radicals in collagenase-1 expression induced by cell shape change. Science 280, 898-902.

Kheradmand, P., Rishi, K., Werb, Z., 2002. Singaling through the EGF receptor controls lung morphogensis in part by regulating MT1-MMP-mediated activation of gelatinase A/MMP-2. Journal of Cell Science 839, 839-848.

Kinoshita, T., Fukuzawa, H., Shimada, T., Saito, T., Matsuda, Y., 1992. Primary structure and expression of gamete lytic enzyme in *Chlamydomonas reinhardtii*: similarity of functional domains to matrix metalloproteases., PNAS, 4693-4697.

Knorr, E., Schmidtberg, H., Vilcinskas, A., Altinicicek, B., 2009. MMPs regualte both develpoment and immunity in the *Tribolium* model insect. PLoS One 4, e4751.

Kuo, C.T., Jan, L.Y., Jan, Y.N., 2005. Dendrite-specific remodeling of *Drosophila* sensory neurons requires matrix metalloproteases, ubiquitin-proteome, and ecdysone signaling. Proceedings of the National Academy of Sciences 102, 15230-15235.

Kusano, K., Miyaura, C., Inada, M., Tamura, T., Ito, A., Nagase, H., Kamoi, K., Suda, T., 1998. Regulation of matrix metalloproteinases (MMP-2,-3,-9,and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. Endocrinology 139, 1338-1345.

Laiho, M., Keski-Oja, J., 1989. Growth factors in the regulation of pericellular proteolysis: A review. Cancer Research 49, 2533-2553.

Lambert, V., Wielockx, B., Munaut, C., Galopin, C., Jost, M., Itoh, T., Werb, Z., Baker, A., Libert, C., Hans-Willi, K., Foidart, J.M., Noel, A., Jean-Marie, R., 2003. MMP-2 and MMP-9 synergize in promoting choroidal neovascularization. FASEB Journal 17, 2290-2292.

Lepage, T., Gache, C., 1990. Early expression of collagenase-like hatching enzyme gene in the sea urchin embryo. EMBO Journal 9, 3993-3012.

Llano, E., Adam, G., Pendas, A.M., Quesada, V., Sanchez, L.M., Santamaria, I., Noselli, S., Lopez-Otin, C., 2002. Structural and enzymatic characterization of *Drosophila*

Dm2-MMP, a membrane-bound matrix metalloproteinase with tissue specific expression. Journal of Biological Chemistry 277, 23321-23329.

Llano, E., Pendas, A.M., Aza-Blanc, P., Korenberg, T.B., Lopez-Otin, C., 2000. Dm1-MMP, a matrix metalloproteinase from *Drosophila* with a potential role in extracellular matrix remodeling during neural development. Journal of Biological Chemistry 275, 35978-35985.

Lubec, G., Ratzenhofer, E., 1979. Collagenase activity of rat kidney with glomerulonephritis is inhibitted by erythromycin. Nephron 24, 93-95.

Maidment, J., Moore, D., Murphy, G., Clark, I., 1999. Matrix metalloproteinase homologues from *Arabidopsis thaliana*-expression and activity. Journal of Biological Chemistry 274, 706-734.

Mannello, F., Luchetti, F., Falcieri, E., Papa, S., 2005a. Multiple roles of matrix metalloproteinases during apoptosis. Apoptosis 12, 19-24.

Massova, I., Kotra, L.P., Fridman, R., Mobashery, S., 1998. Matrix metalloproteinases-structures, evolution oand diversification. FASEB Journal 12, 1075-1095.

Moncrief, J.S., Obiso, R.J., Barroso, L.A., 1995. The enterotoxin of *Bacteroides fragilis* is a metalloprotease. Infection and Immunity 63, 175-181.

Mott, J., Werb, Z., 2004. Regulation of matrix biology by matrix metalloproteinases. Current Opinion in Cell Biology 16, 558-564.

Murphy, G., Willenbrock, F., 1995. Tissue inhibitors of matrix metalloendopeptidases. Methods in Enzymology 248, 496-510.

Nagai, Y., Lapiere, C.M., Gross, J., 1966. Tadpole collagenase, preparation and purification. Biochemistry 5, 3123-3130.

Nagase, H., Woessner, J.F.J., 1999. Matrix metalloproteinases. Journal of Biological Chemistry 274, 21491-21494.

Nelson, A.R., Fingleton, B., Rothenberg, M.L., Matrisian, L.M., 2000. Matrix metalloproteinases: biological activity and clinical implications. Journal of Clinical Oncology 18, 1135-1149.

Ning, W., Li, C.J., Kaminski, N., Feghali-Bostwick, C.A., Alber, S.M., Di, Y.P., Otterbein, S.L., Song, R., Hayashi, S., Zhou, Z., Pinsky, D.J., Watkins, S.C., Pilewski, J.M., Sciurba, F.C., Peters, D.G., Hogg, J.C., Choi, A.M., 2004. Comprehensive gene expression profiles reveal pathways related to the pathogenesis of chronic obstructive pulmonary disease. Proceedings of National Academy of Science 101, 14895-14900.

Obiso, R.J., Lyerly, D.M., van Tassell, R.L., Wilkins, T.D., 1995. Proteolytic activity of the *Bacteroides fragilis* enterotoxin causes fluid secretion and intestinal damage *in vivo*. Infection and Immunity 63, 3820-3826.

Ojeda-Avila, T., Woods, T.A., Rausa, R.A., Raguso, R.A., 2003. Effects of dietary variation on growth, composition, and maturation of *Manduca sexta*. Journal of Insect Physiology 49, 293-306.

Oslon, M.W., Gervasi, D.C., Mobashery, S., Fridman, R., 1997. Kinetic analysis of the binding of human matrix metalloproteinase-2and -9 to tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. Journal of Biological Chemistry 272, 29975-29983.

Overall, C.M., Lopez-Otin, C., 2002. Strategies for MMP inhibition in cancer: Innovations for the post-trial era. Nature Reviews in Cancer 2, 657-672.

Page-McCaw, A., J., A., Ewald, A.J., Werb, Z., 2007. Matrix metalloproteinases and the regulation of tissue remodelling. Molecular Cell Biology 8, 221-233.

Page-McCaw, A., Serano, J., Sante, J.M., Rubin, G.M., 2003. *Drosophila* matrix metalloproteinases are required for tissue remodeling, but not embryonic development. Developmental Cell 4, 95-106.

Quinones, J.L., Rosa, R., Ruiz, D.L., Garcia-Arraras, J.E., 2002. Extracellular matrix remodeling and metalloproteinase involvement during intestine regeneration in the sea cucumber *Holothuria glaberrima*.l. Developmental Biology 250, 181-197.

Ragster, L., Chrispeels, M.J., 1979. Azocoll-digesting proteinases in soybean leaves: characteristics and changes during leaf maturation., Plant Physiology, 857-862.

Reinecke, J.P., Buckner, J.S., Grugel, S.R., 1980. Life cycle of laboratory-reared tobacco hornworms, *Manduca sexta*, a study of development and behavior, using time-lapse cinematography. The Biological Bulletin 158, 129-140.

Rowsell, S., Hawtin, P., Minshull, C.A., Brockbank, H.J.S.M.V., Barratt, D.G., McPheat, A.M.S.W., Waterson, D., Henney, A.M., 2002. Crystal structure of human MMP-9 in complex with a reverse hydroxamate inhibitor. Journal of Molecular Biology 319, 173-181.

Satsuki, M., Gregory, G., Charles, M.F., 2006. Differential expression of MMPs and TIMPs in moderate and severe heart failure in a transgenic model. Journal of Cardiac Failure 12, 314-325.

Springman, E.B., Angleton, E., Hansen, H.B., Van Wart, H.E., 1990. Multiple modules of activation of latent human fibroblat collagenase: Evidence for the role of Cys73

active-site zinc complex in latency and a cysteine switch mechanism for activation. Proceedings of the National Academy of Science, 364-368.

Springman, E.B., Nagase, H., Birkedal-Hansen, H., Van Wart, H.E., 1995. Zinc content and function in human fibroblast collagenase. Biochemistry 34, 15713-15720.

Steetler-Stevenson, W.G., 2008. Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. Science Signaling 1, re6.

Sternlicht, M.D., Werb, Z., 2001. How matrix metalloproteinases regulate cell behavior. Annual Reviews of Cell and Developmental Biology 17, 463-516.

Stickens, D., Behonick, D.J., Ortega, N., Heyer, B., Hartenstein, B., Yu, Y., Fosang, A.J., Schorpp-Kistner, M., Angel, P., Werd, Z., 2004. Altered endochrondal bone development in matrix metalloproteinase 13-deficient mice. Development 131, 5883-5895.

Strongin, A.Y., Collier, I., Bannikov, G., Marmer, B.L., Grant, G.A., 1995. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. Journal of Biological Chemistry 270, 5331-5338.

Takagi, M., Kikko, T., Hosoi, M., Hayashi, I., Toyohara, H., 2004. cDNA cloning of oyster matrix metalloproteinase and its possible involvement in hypoxic adaptation. Fisheries Science 70, 682-687.

Takahashi, C., Sheng, Z., Horan, T.P., Kitayama, H., Maki, M., Hitomi, K., Kiaura, Y., Takai, S., Sasahara, R.M., Horimoto, A., Ikawa, Y., Ratzkin, B.J., Arakawa, T., Noda, M., 1998. Regulation of matrix metalloproteinase-9 and inhibition of tumor

invasion by the membrane-anchored glycoprotein RECK. Proceedings of National Academy of Science 95, 13221-13226.

Truman, J.W., 1972. Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the molting cycle of larval tobacco hornworms. Journal of Experimental Biology 57, 805-820.

Uria, J.A., Jimenez, M.G., Balbin, M., Freije, J.M.P., Lopez-Otin, C., 1998. Differential effects of transforming growth factor-ß on the expression of collagenase-1 and collagenase-3 in human fibroblasts. Journal of Biological Chemistry 273, 9769-9777.

Van Tassel, R., Lyerly, D., Wilkins, T.D., 1992. Purification and characterization of an enterotoxin from *Bactroides fragilis*. Infection and Immunity 60, 1343-1350.

Vogel, W., Gish, G.D., Alves, F., Pawson, T., 1997. The discoidin domain receptor tyrosine kinases are activated by collagen. Molecular Cell 1, 13-23.

Wada, K., Sato, H., Kinoh, H., Kajita, M., Yamamoto, H., Seiki, M., 1998. Cloning of three *Caenorhabditis elegans* genes potentially encoding novel matrix metalloproteinases. Gene 211, 57-62.

Weaver, A., Goncalves da Silva, A., Nuttall, R.K., Edwards, D.R., Shapiro, S.D., Rivest, S., Yong, V.W., 2005. An elevated matrix metalloproteinase (MMP) in an animal model of multiple sclerosis is protective by affecting Th1/Th2 polarization. FASEB Journal 19, 1668-1670.

Wilson, C.L., Ouellette, A.J., Stachell, D.P., Ayabe, T., Lopez-Boado, Y.S., Strasman, J.L., Hultgren, S.J., Matrisian, L.M., Parks, W.C., 1999. Regulation of intestinal

alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science 286, 113-117.

Woessner, F., 2002. MMPs and TIMPs-An historical perspective. Molecular Biotechnology 22, 33.

Woessner, J.F., Jr., 1998. The matrix metalloproteinase family., in: W.C.P.a.R.P. Mecham (Ed.), Matrix metalloproteinases. Academic Press, San Diego, 1-14.

Yamamoto, R.T., 1969. Mass rearing of the tobacco hornworm. III. larval rearing and pupation. Journal of Economic Entomology 62, 1427-1431.

Yu, Q., Stamenkovic, I., 2000. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-ß and promotes tumor invasion and angiogenesis. Genes and Development 14, 163-176.

Zhang, S., Dailey, G., Kwan, E., Glasheen, B., Sroga, G., Page-McCaw, A., 2006. An MMP liberates the ninjurin A ectodomain to signal a loss of cell adhesion. Genes and Development 20, 1899-1910.

CHAPTER II: MMPs IN LARVAL MOLTING AND GROWTH

2.1. Introduction

To grow, larval insects shed their exoskeleton at the end of each instar in a process called molting. Since the cuticle is the non-growing part in the larval body which cannot be expanded, it must be removed. During this process, the cuticle lining the tracheal tubes also is pulled out along with the outer exoskeleton, thus allowing the tracheal tubes to dilate (Zhang et al., 2006). The complete removal of the old cuticle lining the tracheae is essential in order for proper gas exchange and survival of the larvae. The detachment of the old cuticle involves several different kinds of proteinases. For example, two trypsin-like proteases were purified from molting fluid of lepidpterans. One identified in the silkworm, *Bombyx mori* was a neutral protease, which was sensitive to metal chelators (Katzenellenbogen and Kafatos, 1971). The other was a serine protease identified in *M. sexta* (Bade and Shoukimas, 1974). Other enzymes involved in molting include chitinases (enzymes that cleave the glycosidic bonds in chitin) in *B. mori* (Jeuniaux and Amanieu, 1955) and molting fluid proteinases (MFP-1 and MFP-2) from *M. sexta* (Samuels et al., 1993a). In addition to these proteinases, the recently identified matrix metalloproteinases (MMPs) may also play a role in molting. In *Drosophila melanogaster*, MMPs and the extracellular, membrane-bound protein, 'Ninjurin A' has been hypothesized to play a major role in the tracheal remodeling during metamorphosis and during larval growth for tube expansion (Page-McCaw et al., 2003; Zhang et al., 2006).

MMPs have been shown to be involved in tissue remodeling, metamorphosis, and repair both in vertebrates and invertebrates. For example, in vertebrates, during bone development, remodeling of cartilage and bone matrices take place, involving MMP-2,

MMP-9, and MT1-MMP (membrane type-MMP) (Sternlicht and Werb, 2001). Natural mutations of MMP-2 can cause a heritable human disease, Nodulosis, Arthropathy, and Osteolysis Syndrome, which results in joint disease, short stature, small jaws and deformed hands (Martignetti et al. 2001). Yet in another example, MMP-2 and MMP-14 are co-expressed along with TIMP2 during the morphogenesis of lung bronchioles in mouse embryonic development (Greenlee et al., 2007b; Strongin et al., 1995). MMPs are also involved in the remodeling of apoptotic tissue during amphibian metamorphosis (Damjanovski et al., 2006; Gross and Lapiere, 1962; Gross and Nagai, 1965).

Invertebrates may also rely on MMPs for tissue remodeling during development. In the wax moth, *Galleria mellonella,* most of larval tissue remodeling and metamorphosis occurs in the early pupal stage during which there is a 16-fold increase in the expression of Gm-1 MMP (Altincicek and Vilcinskas, 2008). In the red flour beetle, *Tribolium castaneum,* inhibition of MMP-1 expression arrests the initial stages of pupation (Knorr et al., 2009). During the larval development, MMPs play a role in tracheal system development (Zhang et al., 2006), as shown by mutant forms of *D. melanogaster*, lacking both the *Mmp* genes (Dm1-MMP and Dm2-MMP), which had defective tracheal remodeling. However, MMP inhibition did not influence the development of tracheae in the embryonic stages of *D. melanogaster* (Page-McCaw et al., 2003). In *Mmp* knock-out larvae, the dorsal trunks of the tracheal system were shorter and did not completely extend from the anterior to the posterior spiracle, which caused a possible decrease in the supply of oxygen. The tracheal epithelial cells of the mutant forms could not properly attach to the extracellular matrix, which resulted in expanded and broken tracheal tubes, suggesting that MMPs are essential for release of old cuticle and also attachment of a new cuticle

during the enlargement of tracheal tubes through dilation process (Manning and Krasnow, 1993) and during molting (Page-McCaw et al., 2003). Although MMPs are important for tracheal development, their over-expression leads to increased stretching of the tracheal trunks resulting in breaks and internalized spiracles, again supporting the idea that there is an optimal concentration of these enzymes for proper development of the tracheae.

Similar to the effect of MMPs on the tracheal development in *D. melanogaster*, RNAi of MMP-2 in red flour beetle *T. castaneum* was shown to be detrimental for the development of gut during the embryogenesis leading to the death of larvae indicating MMPs role in embryogenesis. However, in case of the Mmp-1 knock out larvae despite these defects in tracheae, the larvae still developed into normal adult insects suggesting that MMP is not required for tracheal development (Knorr et al., 2009).

Clearly, MMPs are important for tracheal growth and larval development, however, it remains unclear as to how the MMPs are involved in molting process in the tobacco hornworm, *Manduca sexta*. Because, during the molting process, the exoskeleton is removed by dissolving the proteins in between the new and the old exoskeleton, proteolytic activity of MMP should increase in tracheae and cuticle as the larva reaches molting stage. To test the hypothesis that MMP is essential for the molting process in *M. sexta*, we identified the MMP using an antibody specific to *M. sexta* MMP and characterized the expression of MMP protein throughout the 4^{th} and 5^{th} instars. We also characterized MMP activity throughout these instars. Furthermore, we used *in vivo* inhibition of MMPs to determine their role in the molt cycle.

2.2. Materials and methods

2.2.1. Rearing of M. sexta larvae

Tobacco hornworm eggs were obtained from Carolina Biological Supply Company, USA. The eggs were kept for hatching on a block of wheat germ-based artificial diet. The larvae were maintained under a 16 L: 8 D photoperiod cycles at 25 °C and fed ad libitum (Ojeda-Avila et al., 2003). Larval age was determined visually by monitoring the presence of the head capsule which indicates the sign of molting.

2.2.2. Collection of tracheal tissue

Tracheal samples were collected daily from 4th instar and 5th instar larvae of known ages. Caterpillars were anesthetized on ice and surface sterilized with 75% ethanol. Using sterile scissors, an incision was made on the dorsal surface along the heart and the larva was opened. The gut was removed and the body was washed with ice cold manduca saline buffer (MSB; 4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2.6H_2O$ and 3 mM $CaCl_2$, pH 6.5) with few crystals of PTU (phenylthiourea) (Willot et al., 1994). Using a clean razor blade, the fat body was carefully removed to reveal intact tracheae. Tracheae were collected into sterile 1.5 ml tubes using sterile forceps. The tracheae were then macerated with sterile polypropylene micro pestles in 200 µl MSB without PTU. The samples were centrifuged for 5 min at 4 °C to settle the debris, and the supernatant was collected into fresh 1.5 ml tubes. The resulting supernatant was subjected to protein precipitation.

2.2.3. Protein precipitation and estimation of protein concentration

Proteins were precipitated from all samples using four volumes of methanol. After the addition of methanol, samples were vortexed, incubated at room temperature for 15 min, and centrifuged at 12,000 rcf for 10 min. The supernatant was discarded and the pellet was resuspended in 200 µl of MSB without PTU. Concentration of protein in the precipitated tracheal samples was estimated using a standard Bio-Rad protein assay as per

the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, 5 µl of sample was pipetted into a 96-well plate and 150 µl of Bradford reagent was added. Samples and standards (Bovine serum albumin) were run in duplicates. The wells were carefully mixed, kept at room temperature for 5 min, and the absorbance was read at 595 nm on a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.2.4. SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis)

Discontinuous polyacrylamide gel electrophoresis (PAGE)) (Laemmli, 1970) under denaturing and reducing conditions in the presence of SDS and β-mercaptoethanol (Bio-Rad, Hercules, CA, USA) was used to analyze the protein. After the estimation of proteins in the samples, 10 µg of protein was mixed with Laemmli buffer (Bio-Rad) and β-mercaptoethanol in the ratio of 2:1 and boiled at 95 °C for 5 min on a heating block. In samples with low protein concentrations, only 5 µg protein was used for SDS-PAGE analysis. Samples were subjected to SDS-PAGE on 12% acrylamide resolving and 5% acrylamide stacking gels at 100 V for 1.5 to 2 h at room temperature. After electrophoresis, the gels were removed from the electrophoresis apparatus, washed in ddH_2O for 5 min, stained with Bio-safe Coomassie-blue (G-250) staining solution (Bio-Rad) for 1-2 h, and then destained with ddH_2O to visualize bands. If bands were not visible with coomassie staining, gels were stained with silver stain (Bio-Rad), as per manufacturer's instructions.

2.2.5. Western Blotting

After submitting the proteins (10 µg/lane or 20 µg/lane were used for verifying our custom antibodies) to SDS-PAGE on a 12% acrylamide resolving, 5% acrylamide stacking gel, the proteins were electro-transferred (100 V) to a 0.45 µm Whatman nitrocellulose

membrane (Whatman GmbH, Germany) at 4 °C for 1 h. After transferring the proteins to the nitrocellulose membrane, the membranes were blocked in ~20 ml of 5% milk in Tris-buffered saline with Tween-20 (TBST; 0.1 M Tris, 1.5 M NaCl, and 0.5% Tween-20, pH 8) overnight at 4 °C. The membrane was incubated in 1:500 dilution of the custom-synthesized anti-*M. sexta* MMP primary antibody in 5% milk-TBST for 1 h at room temperature, after which the membrane was rinsed and washed with TBST three times for 10 min each. The membrane was then incubated in 1:100,000 dilution of the goat-anti-rabbit IgG-HRP secondary antibody in 5% milk for 1 h at room temperature. After washing the membrane for six times 5 min each, the proteins were subjected to the enhanced chemiluminescence (ECL) detection system (Thermo Scientific, Pierce, IL USA) containing the HRP-specific substrate and the signal was detected for 1 min and 3 min in an Alpha Innotech Chemi-imager (Alpha Innotech, CA, USA).

2.2.6. Antibodies

Polyclonal antibodies against the *M. sexta* MMP were generated against the partial MMP sequence using the peptide 'SKYWRYNGQKMDGD (ProSci Incorporated, Poway, CA), (Fig. 3). To validate the specificity of the antibody, the membrane containing tracheal proteins was incubated with anti-*M. sexta* antibody and secondary antibody. The blot was then stripped of the primary and secondary antibodies by incubating the blot with stripping buffer (Thermo Scientific, Illinois, USA) at 37 °C for 10 min, and re-probed with 1:500 dilution of anti-mouse/rat MMP-2 antibody (R&D systems, MN, USA) because of high sequence similarity between *M. sexta* MMP and mouse MMP-2 and 1:100,000 dilution of specific secondary antibody. In a second validation experiment, the membrane containing tracheal proteins was first probed with anti-*M. sexta* MMP antibody and

RRGTNLFQVAA**HEFGHSLGLSHS**DVRSALMAPFYRGFDPAF
QLDQDDIQGIQALYGHKTQTDIGGGSVGGGGLVPSVPRATT
QQPSAEDPALCADPRIDTIFNGADGSTFVFKGEHYWRLTED
GVAAGYPRLISRAWPNLPGNIDAAFTYKNGKTYFFKGSKYW
RYNGQKMDGDYPKEISEGFTGIPDNIDAALVWSGNGKIYFY
KGSKFWRFD

Figure 3. Deduced partial amino acid sequence of *M. sexta* MMP-1. The underlined sequence indicates the conserved, zinc-binding site of MMP. The sequence in red indicates the peptide that was used to generate the antibodies against *M. sexta* MMP-1 (ProSci Incorporated, Poway, CA).

secondary antibody. The blot was then stripped and reprobed with anti-*M. sexta* MMP pre-incubated with 2 µl of activated recombinant mouse MMP-2 and then probed with secondary antibody. Antibody detection was performed as described above.

2.2.7. Zymography

Gelatinolytic activity of MMPs in tracheal samples was detected by zymography. A 10% acrylamide resolving gel was embedded with 0.3% gelatin for zymography. Five micrograms of the tracheal sample protein was mixed with the zymogram sample buffer at a ratio of 1: 0.5. The samples were then subjected to electrophoresis at 4 °C at 120 V for 1.5-2 h. After electrophoresis, gels were washed in renaturation buffer (2.5% triton X-100) for 30 min at room temperature and incubated in developing buffer/incubation buffer (50 mM Tris, 5 mM $CaCl_2$, 0.02% w/v sodium azide pH 8) at 37 °C overnight. The gels were stained using 0.5% w/v coomassie brilliant blue (R-250) and destained (40%

methanol/10% acetic acid, v/v). To verify that the observed gelatinolytic activity was due to matrix metalloproteinases, the gels were incubated in incubation/developing buffers containing any of the various inhibitors enzyme inhibitors such as, 10 μM GM6001, 10 mM EDTA, 5 nM TIMP-4, 10 mM PMSF, 100 μM GM6001, or 100 μM 1, 10-Phenanthroline.

2.2.8. Preparation of GM6001

GM6001 (Millipore, Billerica, MA, USA) is a broad spectrum inhibitor of MMPs. The hydroxamic acid group of GM6001 binds to the zinc atom on the catalytic site of MMP thereby blocking the active site. In addition, the tryptophan side chain and isobutyl group of GM6001 prevent binding of MMP to the extracellular matrix proteins (Galardy, 1993; Strauss et al., 1996b). 100 μM stock of GM6001 was prepared by dissolving 5 g of GM6001 in 128.68 μl DMSO. A 10 μM working solution was prepared by diluting the stock with 1 X PBS to a final concentration of 0.001% DMSO.

2.2.9. Injection of GM6001

To test the affect of MMP inhibitor on the molting process, caterpillars from 4th instar larvae of similar body masses were injected with inhibitor and their behavior was compared to that of control (no injection) and PBS-DMSO-injected groups. The PBS and GM6001 larvae were injected with 10 μl PBS containing 0.001% DMSO and 10 μM GM6001 in PBS-DMSO (0.001% DMSO at the final concentration), respectively, from third day of the 4th instar until the dorsal heart was observed in the 5th instar. Before injection, caterpillar weights were noted daily and observed for any visual changes in the control versus treatment groups.

2.2.10. Data analysis

Statistical analyses were performed using both SPSS version 17 and MINITAB version 15. Differences among the treatment and control groups were detected by Analysis of Variance (ANOVA) and post hoc tests (Bonferroni-corrected post-hoc test). Effects of MMP inhibitor on growth were tested using a two-way, repeated measures ANOVA. A probability value 0.05 was used to indicate the significance unless otherwise indicated. Data are expressed as mean ± standard error of the mean throughout.

2.3. Results

2.3.1. Identification of MMPs in various tissues in M. sexta

Bands specific to *M. sexta* MMP-1 protein were found in hemolymph, hemocytes, fat body, and tracheae (Fig. 4). In addition to all of these tissues, the cuticle also expressed MMP (data not shown). Even though all of the tissues showed bands specific for MMP protein, the molecular weights varied with the tissue type.

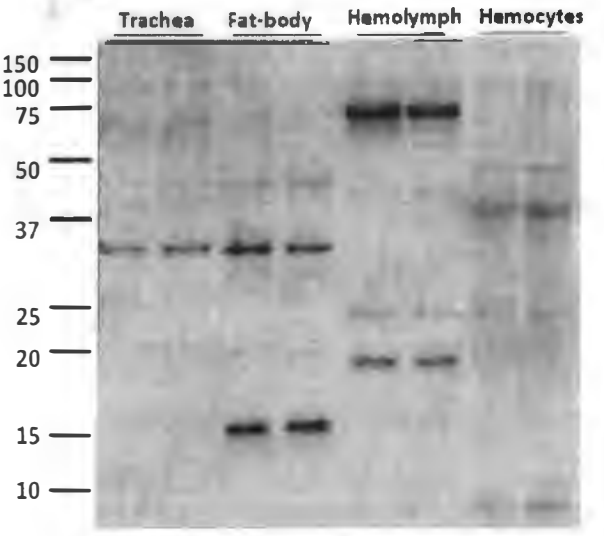

Figure 4. Western blots of various larval tissues showing MMP expression. Expression of MMP protein varies by tissues. Western blot of tracheae, fat body, hemolymph, and hemocytes detected MMP expression in all tissues.

2.3.2. Evidence that the bands are MMP specific

Since the molecular weights of the bands observed in different tissues varied, and the same tissue showed multiple bands, we validated the specificity of our primary *M. sexta* MMP antibodies using anti-mouse MMP-2.

2.3.2.1. Probing with anti-MMP-2

The bands observed when the blot was treated with our custom antibodies were similar in molecular weight to those detected after the blot was stripped and re-probed with anti-MMP-2 (Fig. 5a and b).

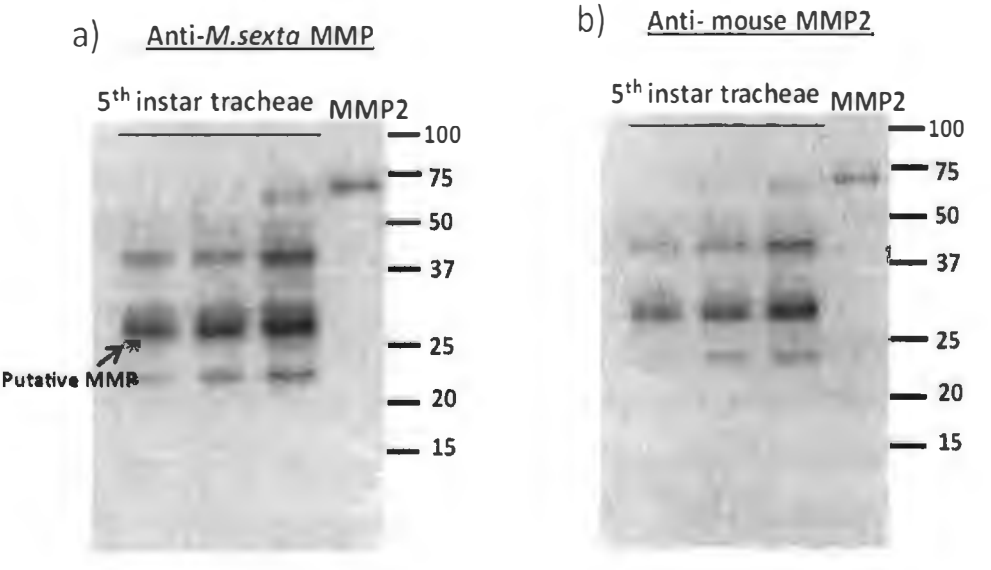

Figure 5. Identification of *M. sexta* MMP using anti-*M. sexta* MMP (a) and anti-mouse MMP (b). Both antibodies detected the same bands indicating that *M. sexta* MMP recognizes MMP protein.

2.3.2.2. Pre-incubation with mouse MMP

To further test whether the observed bands were MMP-specific, we pre-incubated activated recombinant mouse MMP-2 with the antibodies against *M. sexta* MMP and then

probed the blot containing tracheal samples and MMP-2 as a positive control. The intensity of the signal was much greater when the blot was treated with *M. sexta* MMP primary antibody alone, in contrast to that when the primary antibody was preincubated with activated mouse MMP-2, indicating that the *M. sexta* MMP antibody was pre-absorbed by MMP-2 protein (Fig. 6a and b).

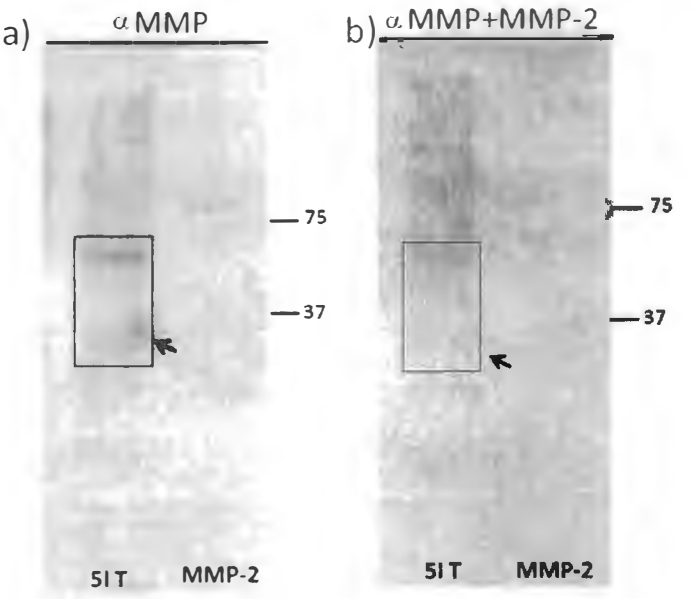

Figure 6. Anti-*M. sexta* MMP detects two bands in tracheae (a). Anti- *M. sexta* MMP is blocked by pre-incubation with recombinant mouse MMP-2 (b). Arrows indicate the putative MMP which disappeared after pre-incubation with recombinant MMP-2 antibody.

2.3.3. MMP expression profiles in tracheae

MMP protein expression was high during the beginning of an instar and gradually decreased towards the end of the 5th instar (Fig. 7a and b). Two bands, one between 25 kD-37 kD and another between 37 kD-50 kD, were observed in both 5th instar and 4th instar tracheal samples.

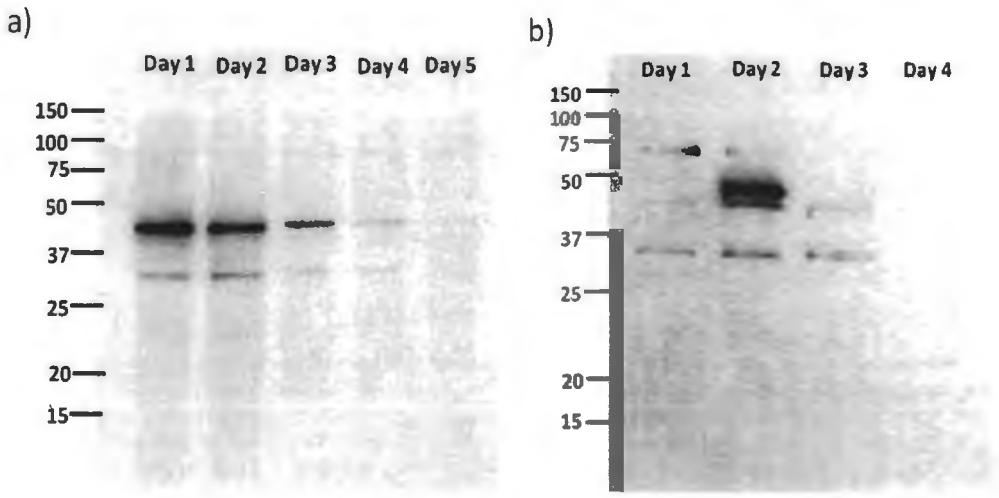

Figure 7. Western blots of 5th instar (a) and 4th instar (b) tracheae. MMP expression in the tracheae is high at the beginning of an instar and decreases through the instar.

2.3.4. Gelatinolytic activity in tracheae

Since some MMPs are known to be gelatinolytic (Gross and Lapiere, 1962), we attempted to identify MMPs using gelatin zymography. Similar to the results observed in the western blots, the zymograms of the tracheal proteins from the 4th and 5th instar larvae (Fig. 7a and b) showed high proteolytic activity during the beginning of each instar, which decreased as the instar progressed. A prominent proteolytic band corresponding to molecular weight ~30 kD was detected in both the instars. The low molecular weight band between 25 kD-37 kD observed in the western blots coincides (Fig. 7) with the MW of the proteolytic band observed in zymograms (Fig. 8).

2.3.5. Inhibition of gelatinolytic activity

To determine which proteases were responsible for the gelatinolytic activity (Figure. 9), zymography was performed and various inhibitors were added to the incubation buffer. Inhibitors included 10 μM and 100 μM GM6001, 10 mM EDTA, 10 mM PMSF, 5 nM TIMP and 100 μM 1, 10-Phenanthroline. Complete inhibition was

achieved by 10 mM EDTA and 100 μM GM6001; 10 mM PMSF inhibited most of the activity, while only slight inhibition was observed with 10 μM GM6001 and 5 nM TIMP (Figures. 9 and 10).

2.3.6. In vivo testing of the role of MMP on larval growth

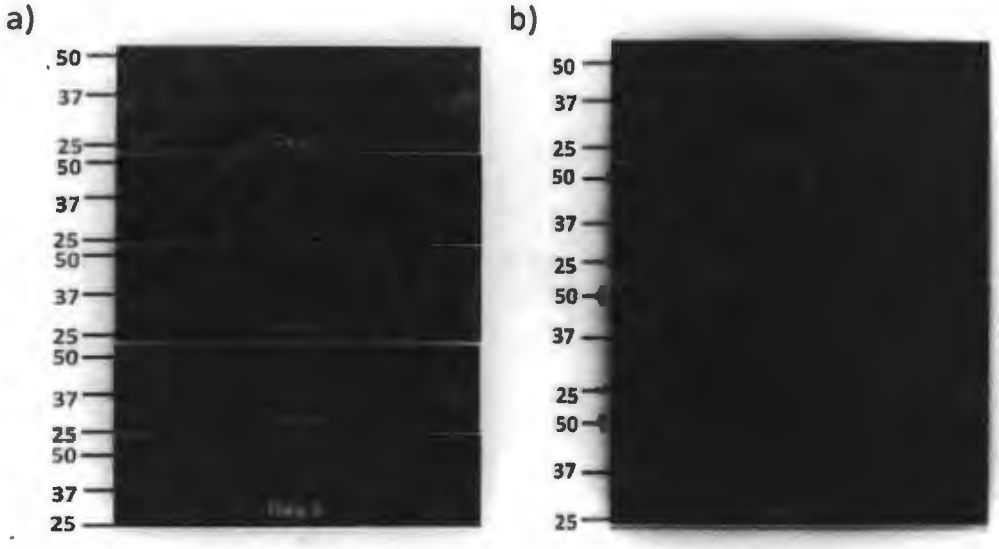

Figure 8. Zymograms of 4th and 5th instar tracheae. The gelatinolytic activity (band of clear zone below 37 kDa) is high at the beginning of the instar and decreases through the end of the instar. Each panel in both the gels shows tracheal samples from each day of the 4th (b) and 5th (a) instar (n = 6 for each day of the instar).

Since the gelatinolytic activity was difficult to inhibit in the zymograms using 10 μM GM6001, we attempted to see if it had any effect *in vivo*. Daily injections of the inhibitor significantly affected the growth of the caterpillars over time (Figure. 11; repeated measures ANOVA; significant interaction between time and treatment, $F_{(2, 11)} = 14.77$, $p = 0.001$). This result indicates that caterpillar growth varied differently depending on the treatment. All of the injected caterpillars survived the experiment and also did not

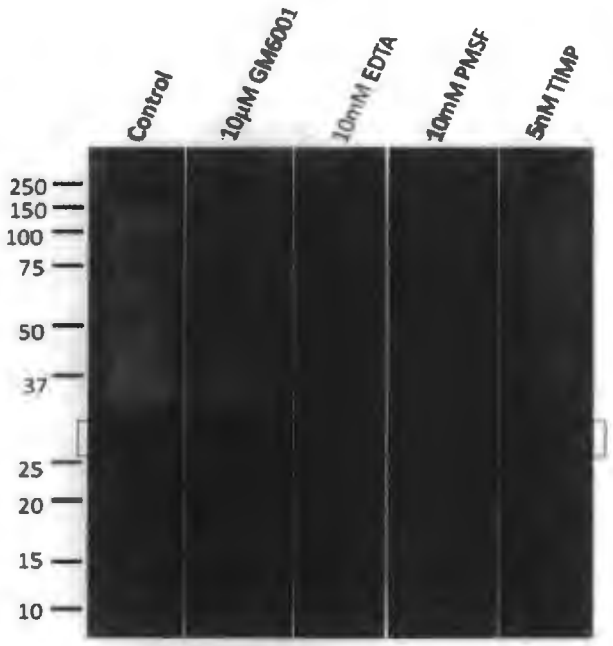

Figure 9. Inhibition of gelatinolytic activity using various proteinase inhibitors. Gelatinolytic activity was completely inhibited only 10 mM EDTA. 10 μM GM6001, 10 mM PMSF, and 5 nM TIMP showed partial inhibitory effect on the gelatinolytic activity.

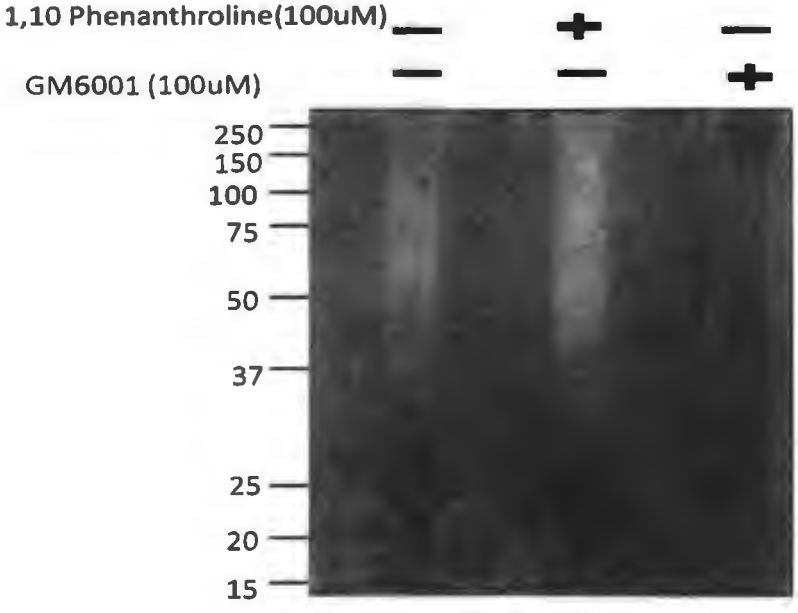

Figure 10. Inhibition of gelatinolytic activity by GM6001. High concentrations of GM6001 (100 μM) but not 100 μM 1, 10-Phenanthroline, inhibited gelatinolytic activity in the tracheae.

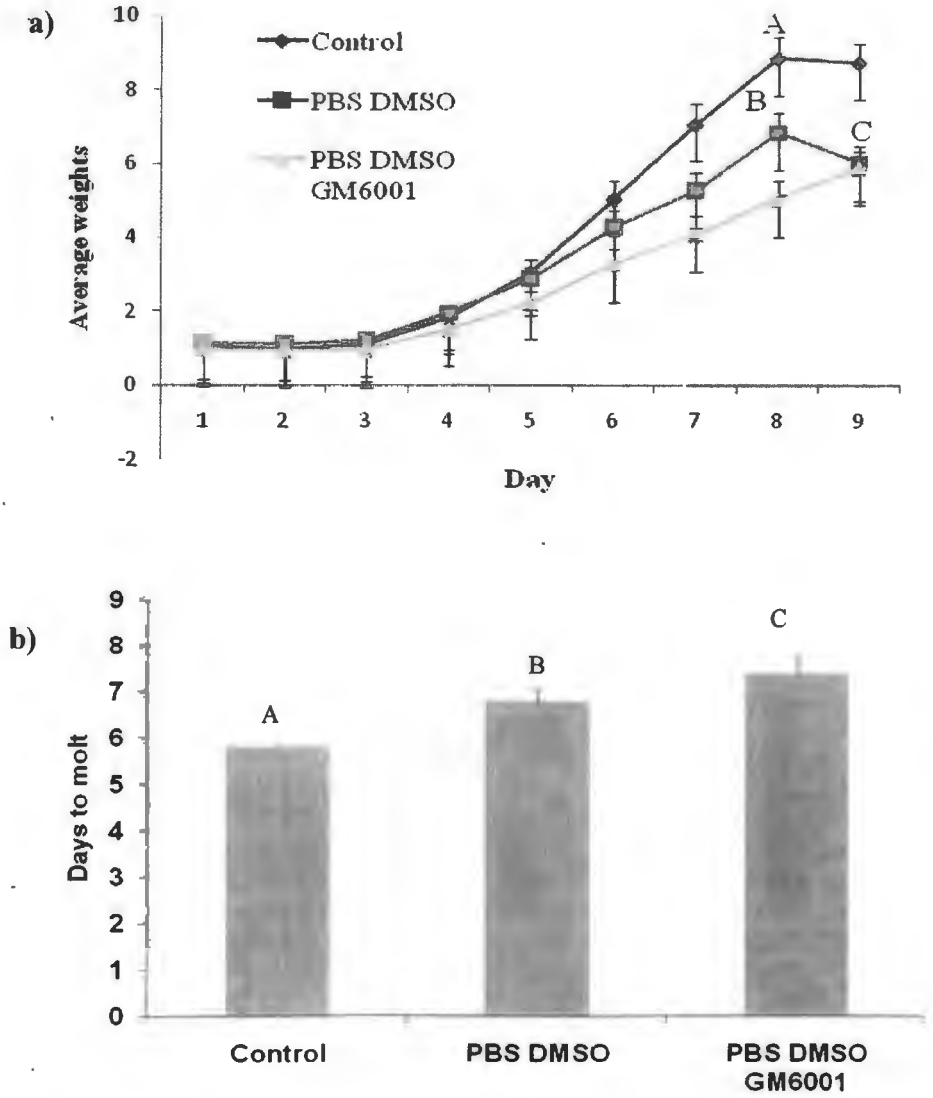

Figure 11. Effect of MMP inhibition of the growth of caterpillars. (a) Caterpillars injected with GM6001 weighed significantly less compared to the control or vehicle-injected caterpillars. (b) There was a significant delay in the appearance of dorsal heart in the MMP-inhibited larvae. Letters indicate that all the groups are significantly different.

show any signs of weakness throughout the experiment. Comparisons of 95% confidence intervals of the estimated marginal means showed that there was no difference between the masses of control caterpillars versus those that received injection of the vehicle. The caterpillars that received injections of GM6001were significantly smaller than control

caterpillars by day 7. Treatment with the inhibitor also affected the timing of development (ANOVA; $F_{(2, 12)} = 7.538$, $p < 0.008$). The control caterpillars showed normal growth as evidenced by the appearance of the dorsal vein at day 6 or 7 of the 5th instar. In addition, the dorsal vein for the vehicle-injected caterpillars was visible by day 7or day 8 of the 5th instar. Interestingly, the dorsal vein for GM6001 treated caterpillars did not appear until day 9 of the 5th instar.

2.4. Discussion

To date, the role of MMPs has been studied only in the larval-pupal transition and during metamorphosis. This is the first report in which MMP expression during larval to larval molting has been studied. MMPs play a role in larval development as evidenced by our *in vivo* inhibition assay. The presence of multiple bands with different molecular weights in the each tissue indicates that there may be more than one MMP or multiple isoforms of one MMP or the MMPs may be processed differently in each tissue. However, the exact role is unclear since the results obtained in the zymograms and western blots show that the activity of the MMPs diminishes as the larva reaches the end of an instar (Figs. 7 and 6). These findings are contrary to our prediction that MMP expression would be up-regulated during the molting process.

During the molting process, digestion of the old, and rigid surface cuticle takes place while, at the same time the new and more flexible exoskeleton below the old one is being replaced. After the synthesis of the new exoskeleton, the larva crawls out of the old exoskeleton entering a new larval instar (Wigglesworth, 1973b). During the dissolution of the old exoskeleton, detachment of epidermis from the old exocuticle, called apolysis takes place (Jenkin and Hinton, 1996). All of these processes require proteases which cleave the

extracellular matrix proteins such as fibronectin and type IV collagen (Fessler and Fessler, 1989) and adhesion molecules which connect the epidermis and exocuticle (Zhang et al., 2006). Since MMPs are the enzymes that cleave extracellular matrix proteins, it is anticipated that they would be up-regulated during the molting process. In this work, we analyzed tracheal samples for the presence and role of MMPs in the molting process since tracheae are lined with the invaginations of the outer exoskeleton/exocuticle, and is removed along with the exocuticle during the molting process.

Lack of MMPs expression and activity at the end of the instar (Fig. 7 and 8) suggests that other non-MMP proteases are active during this part of the molting process. Several proteases have been identified from molting insects including two trypsin-like proteases from the molting fluid of *Bombyx mori* (Katzenellenbogen and Kafatos, 1971); a neutral metal chelator-sensitive protease, and a serine protease in *M. sexta* (Bade and Shoukimas, 1974); chitinases in *B. mori* (Jeuniaux and Amanieu, 1955); and molting fluid proteinases (MFP-1 and MFP-2) from *M. sexta* (Samuels et al., 1993a). The presence of MMPs in early stages of the instar supports the hypothesis that MMPs are involved in the tracheal growth and differentiation (Page-McCaw et al., 2003; Zhang et al., 2006).

In contrast to the observations made by Page-McCaw et al. (2003), where northern blots of total RNA in *D. melanogaster* S2 cells showed constitutive expression of *mmp*1 throughout development, except during embryogenesis, our results show that MMP is expressed during the larval stages but not when the larvae are preparing to molt. Although gene expression may not reflect protein expression at a given time point. The down regulation of MMP protein during the molting process also may be explained in part by the fact that larval-larval molting does not require complete degradation of the larval tissue as

observed during the larval-pupal transition during which enhanced expression of MMP-1-like activity was detected in *Galleria mellonella* (Vilcinskas and Wedde, 2002).

The presence of multiple bands in the western blot (Figs. 4, 5, and 7) suggests that there may be significant post-translational modifications of the MMP, for example, pro-form, active-form, and inactive form. These types of modification are common among MMPs (Greenlee et al., 2007b). Furthermore, MMPs may form dimers even in denaturing and reducing conditions in a gel. We are confident that the polyclonal antibodies for *M. sexta* MMP detected MMP proteins because pre-absorption of our custom synthesized antibodies with recombinant mouse/rat MMP-2 resulted in the disappearance of a band at ~ 30 kD that was present in the tracheal samples (Fig. 6). Validation of anti-*M. sexta* MMP in other tissues such as hemolymph and hemocytes fat-body clearly needs to be performed because the protein expression patterns are so different. An alternative, but not mutually exclusive, explanation for the presence of multiple bands may be that this MMP has several isoforms. Obtaining the full MMP sequence will help our interpretation of the protein expression pattern.

A low concentration (10 μM) of GM6001 failed to inhibit the gelatinolytic activity of MMP in zymograms (Fig. 9), though 100 μM GM6001 successfully inhibited the gelatinolytic activity (Fig. 10), indicating that the proteolysis observed was MMP specific. Similar results were obtained in mouse lung bronchiole branching where only high concentrations of GM6001 could inhibit the branching morphogenesis (Gill et al., 2003). The fact that the gelatinolytic activity was inhibited completely by EDTA and to some extent by PMSF indicates the presence of trypsin-like enzymes (Bade and Stinson, 1978), neutral metal chelators, and/or serine proteases (Bade and Shoukimas, 1974) in the tracheal

tissue. It also could mean that the MMPs are expressed at that time in the instar, which is supported by our western data. However, because the MMP activity could not be inhibited by TIMP-4, an endogenous inhibitor known to inhibit Pro-MMP-2 (Bigg et al., 1997) and several other MMPs (-1,-2,-3,-7, and -9) (Douglas et al., 1997), the presence of MMP could not be unequivocally confirmed. Failure to completely inhibit the gelatinolytic activity using 5 nM TIMP-4 (Fig. 9) led us to inhibit MMPs *in vivo* (Fig. 11). We chose to begin injections from the third day of the 4th instar in the hopes of inhibiting molting to the 5th instar. However, since the 4th instar is short (the 4th instar larvae start to molt on the 4th day), injection from the first day of the 4th instar may help detection in the delay of molting.

Despite the inability to inhibit MMPs *in vitro*, *in vivo* inhibition with GM6001 resulted in a striking decrease in growth (Fig. 11). The smaller body mass of the inhibited caterpillars was not due to repeated injections, since there was no significant difference between control and vehicle-injected animals. However, repeated injuries must have some effect on growth, because of the increased energy required for the repeated clotting and tissue repair. We detected a delay in the development of 5th instar caterpillars in the presence of the MMP inhibitor, in which the appearance of the dorsal heart was nearly 2 days behind control caterpillars.

The appearance of the dorsal heart is a key point in the larval-pupal transition and is driven by changes in hormones throughout the instar. Even though both the larval-larval molting and larval-pupal metamorphosis involve degradation of the ECM proteins, they are dissimilar based on the hormonal requirements. Larval-larval molting occurs when the ecdsyone hormone produced by thoracic gland stimulates the digestion of surface epithelial

cells to digest the old cuticle and initiates synthesis of the new cuticle (Wigglesworth, 1973a). This process is supported by the high levels of juvenile hormone produced by the corpora allata. When juvenile hormone is reduced at the end of the last instar, differentiation of the imaginal discs begins, beginning the larval-pupal transition, a process which is led by prothoracicotropic hormone (PTTH) (Nijhout, 1981).

The delay in the appearance of the dorsal blood vessel suggests that there are possible functions for MMPs in larval-pupal transition: i) the regulation of PTTH which initiates the gut purging along with the appearance of dorsal heart, a process only after which the feeding larvae become wondering larvae (Nijhout and Williams, 1974a); ii) in the clearance of tissue surrounding the dorsal blood vessel initiating the degradation process (Nijhout and Williams, 1974a); or iii) in the clearing of juvenile hormone from the hemolymph (Nijhout, 1981) in the presence of which PTTH is inhibited. Similar results were obtained in the flour beetle *T. casteneum* where the larval-pupal transition was inhibited when MMP-1 was knocked out (Knorr et al., 2009). However, loss of MMP activity was not detrimental to the larvae since the larvae survived continuous injections of GM6001, supporting the fact that MMPs are not the only proteases that are involved in the molting process.

2.5. References

Altincicek, B., Vilcinskas, A., 2006. Metamorphosis and collagen-IV-fragments stimulate innate immune response in the greater wax moth, *Galleria mellonella*. *Developmental and Comparative Immunology*, 1108-1118.

Altincicek, B., Vilcinskas, A., 2008. Identification of lepidopteran matrix metalloproteinase with dual roles in metamorphosis and innate immunity. Develpomental Comparative Immunology 32, 400-409.

Bade, M.L., Shoukimas, J.J., 1974. Neutral metal chelator-sensitive protease in insect moulting fluid. *Journal of Insect Physiology* 20, 281-290.

Bade, M.L., Stinson, A., 1978. Digestion of cuticle chitin during the moult of *Manduca sexta* (Lepidoptera: Sphingide). *Insect Biochemistry* 9.

Bigg, H.F., Shi, Y.E., Liu, Y.L.E., Steffensen, B., Overall, C.M., 1997. Specific, high affinity binding of tissue inhibitor of metalloproteinases-4 (TIMP-4) to the COOH-terminal hemopexin-like domain of human gelatinase A-TIMP-4 binds procollagenase A and the COOH-terminal domain in a similar manner to TIMP-2. *Journal of Biological Chemistry* 272, 15,496-415,500.

Damjanovski, S., Amano, T., Li, Q., Ueda, S., Shi, Y.B., Ishizuya-Oka, A., 2006. Role ECM remodeling in thyroid hormone-dependent apoptosis during anuran metamorphosis. Annals of the New York Academy of Sciences 926, 180-191.

Douglas, D.A., Shi, Y.E., Sang, Q.X.A., 1997. Computational sequence analysis of the tissue inhibitor of metalloproteinase family. *Journal of Protein chemistry* 16, 237-255.

Fessler, J.H., Fessler, L.I., 1989. *Drosophila* extracellular matrix. *Annual Reviews in Cell Biology* 5, 309-339.

Galardy, R., 1993. Galardin. *Drugs Future* 18, 1109-1111.

Gill, S.E., Pape, M.C., Khokha, R., Watson, A.J., Leco, K.J., 2003. A null mutation for tissue inhibitor of metalloproteinase-3 (TIMP-3) impairs murine branchiole branching morphogenesis. *Developmental Biology* 261, 313-323.

Greenlee, K.J., Werb, Z., Kheradmand, F., 2007b. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. Physiological Reviews 87, 68-98.

Griesch, J., Wedde, M., Vilcinskas, A., 2000. Recognition and regulation of metalloproteinase activity in the haemolymph of *Galleria mellonella*: a new pathway mediating induction of humoral immune responses. *Insect Biochemistry and Molecular Biology* 30, 461-472.

Gross, J., Lapiere, C.M., 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proceedings of National Academy of Science 48, 1014-1022.

Gross, J., Nagai, Y., 1965. Specific degeradation of the collagen molecule by tadpole collagenolytic enzyme. Proceedings of the National Academy of Sciences 54.

Jenkin, P.M., Hinton, H.E., 1996. Apolysis in Arthropod moulting cycles. *Nature London Journal* 21, 871.

Jeuniaux, C., Amanieu, M., 1955. Mise en evidance d'une chitinase dans le liquide exuvial de *Bombyx mori* L. *Archives Internationales de Physiologie et de Biochimie* 63, 94-103.

Katzenellenbogen, B.S., Kafatos, F.C., 1971. Proteinase of silkmoth molting fluid: physical and catalytic properties. *Journal of Insect Physiology* 17, 775-800.

Knorr, E., Schmidtberg, H., Vilcinskas, A., Altinicicek, B., 2009. MMPs regualte both develpoment and immunity in the *Tribolium* model insect. PLoS One 4, e4751.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Manning, G., Krasnow, M.A., 1993. Development of *Drosophila* tracheal system., in: M. Bate, A. Martinez Arias (Eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 609-685.

Nijhout, H.F., 1981. Physiological control of molting in insects. *American Zoologist* 21, 631-640.

Nijhout, H.F., Williams, C.M., 1974a. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last instar larva and the decision to pupate. *Journal of Experimental Biology* 61, 481-491.

Ojeda-Avila, T., Woods, T.A., Rausa, R.A., Raguso, R.A., 2003. Effects of dietary variation on growth, composition, and maturation of *Manduca sexta*. Journal of Insect Physiology 49, 293-306.

Page-McCaw, A., Serano, J., Sante, J.M., Rubin, G.M., 2003. *Drosophila* matrix metalloproteinases are required for tissue remodeling, but not embryonic development. Developmental Cell 4, 95-106.

Samuels, R.I., Charnely, A.K., Reynolds, S.E., 1993a. A cuticle degrading proteinase from the molting fluid of tobacco hornworm, *Manduca sexta*. *Insect Biochemistry and Molecular Biology* 23, 607-614.

Sternlicht, M.D., Werb, Z., 2001. How matrix metalloproteinases regulate cell behavior. Annual Reviews of Cell and Developmental Biology 17, 463-516.

Strauss, B.H., Robinson, R., Batchelor, W.B., Chisholm, R.J., Ravi, G., Natarajan, M.K., Logan, R.A., Mehta, S.R., Levy, D.E., Ezrin, A.M., Keely, F.W., 1996. *In vivo*

vollegen turnover following experimental ballon angioplasty injury and the role of matrix metalloproteinases. *Circulation Research* 79, 541-550.

Vilcinskas, A., Wedde, M., 2002. Insect inhibitors of metallproteinases. *IUBMB Life* 54, 339-343.

Wigglesworth, V.B., 1973a. The significance of "apolysis" in the moulting fluid of insect. . *Journal of Entomology* 47, 141-149.

Wigglesworth, V.B., 1973b. The significance of "apolysis" in the moulting fluid of insect. . *Journal of Entomology* 47, 141-149.

Willot, E., Trenczek, T., Thrower, L.W., Kanost, M.R., 1994. Immunological identification of insect hemocyte populations: monoclonal antibodies distinguish four major hemocyte type in *Manduca sexta*. European Journal of Cell Biology 65, 417-423.

Zhang, S., Dailey, G., Kwan, E., Glasheen, B., Sroga, G., Page-McCaw, A., 2006. An MMP liberates the ninjurin A ectodomain to signal a loss of cell adhesion. *Genes and Development* 20, 1899-1910.

CHAPTER III: MMPs IN THE INNATE IMMUNITY OF *MANDUCA SEXTA*

3.1. Introduction

Like mammals, insects have the ability to recognize self from non-self (Leulier et al., 2003) and to defend themselves from pathogenic organisms by mounting a successful immune response (Horohov and Dunn, 1982). Interestingly, insects do this without the presence of antibody-mediated defenses. Thus, the insect immune system can be compared to the innate immune system of vertebrates (Hoffmann and Janeway, 1994; Janeway, 1994). Innate immune functions are highly conserved from vertebrates to invertebrates (Bulet et al., 1999b; Hoffmann et al., 1999; Janeway, 2001; Strand, 2008). The main barriers in the defense mechanism in insects are: a) the integument and gut, which serve as the preliminary defense by preventing the entry of microorganisms; b) the fat body, an organ in insects that is comparable to liver and the major site of anti-microbial protein synthesis during the immune response (Lemaitre and Hoffmann, 2007; Strand, 2008), and c) the hemocytes, the cellular components of the immune system which circulate in the hemolymph (Gillespie and Kanost, 1997). Together these immune defenses produce antimicrobial factors, encapsulate and phagocytose foreign objects, and kill pathogenic organisms (Ratcliffe and Rowley, 1981; Schmidt et al., 2001; Strand and Pech, 1995).

Hemocyte classification varies depending on taxa. For example, in the lepidopteran insects, (Horohov and Dunn, 1982; Lavine and Strand, 2002; Willot et al., 1994), *Hyalophora cecropia* (Lackie, 1988), and *Bombyx mori* (Nakahara et al., 2009), hemocytes are classified in four groups: plasmatocytes, granulocytes, spherulocytes, and

oenocytoids. However, in the fruitfly, *Drosophila melanogaster* (Lemaitre and Hoffmann, 2007), there are only three hemocyte classifications: crystal cells, plasmatocytes and lamellocytes. Granulocytes, Plasmatocytes, and lamellocytes (in the case of *D. melanogaster*) are recognized as important modulators of the immune system in insects (Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007). The hemocytes detect non-self via pathogen recognition receptors (PRR) present on their surfaces (Eleftherianos et al., 2007). These PRRs help in the identification and discrimination of various molecular patterns on the surface of invading pathogens, thereby assisting in the activation and proliferation of hemocytes. Upon activation, changes in hemocyte morphology and behavior occur (Lackie, 1988) in addition to increases in the total hemocyte count (THC) (Horohov and Dunn, 1982). This rise in the hemocyte numbers is due to the recruitment of hemocytes from the hematopoietic organs such as the lymph glands and cells in the wing imaginal discs (Horohov and Dunn, 1982; Lavine and Strand, 2002) and also by the proliferation and differentiation of the hemocytes that are already in circulation (Arnold and Hinks, 1975; Ratcliffe et al., 1985). Activated hemocytes bind to the pathogens with the help of their receptors and aggregate (nodule formation Lackie, 1988), phagocytose (Bayne, 1990), or encapsulate (Christensen and Severson, 1993) the pathogenic organisms.

Humoral responses during bacterial infection in invertebrates result in the production and release of several antimicrobial factors; including antibacterial proteins (Eleftherianos et al., 2007; Lowenberger, 2001; Meister et al., 2000), reactive oxygen or nitrogen species (Bogdan et al., 2000; Vass and Nappi, 2001) and phenoloxidase (Ashida and Bery, 1995). Among the antibacterial proteins, a class of antimicrobial peptides (AMPs), such as lysozyme, cecropins, and attacins function to kill bacteria. In addition to

these peptides, several pattern recognition proteins (PRPs), such as hemolin, peptidoglycan recognition proteins (PGRP), and c-type lectins (immulectin 1-4), and cell adhesion molecules are produced during the immune response (Bulet et al., 1999a; Hetru et al., 1998; Jiang, 2008; Trenczek, 1998). All the humoral and cellular responses along with the activation of complex enzymatic cascades that cause coagulation and melanization of hemolymph work in concert to fight the infection, thereby reducing or eliminating the pathogens (Cerenius and Soderhall, 2004; Gillespie and Kanost, 1997; Muta and Iwanaga, 1996).

In vertebrates, one family of enzymes that is particularly important to immune function is the matrix metalloproteinases (MMPs) (Madri and Graesser, 2000). These zinc-dependent proteases play an essential role in pathogenesis and immunity, however, their exact role remains unclear. MMPs are up-regulated during repair and remodeling, but they are also up-regulated in many diseases (Greenlee et al., 2007b; Parks et al., 2004; Vu and Werb, 2000). It is hypothesized that they may be detrimental during inflammatory responses and pathogenesis by contributing to tissue degradation. Up-regulation of several MMPs occurs in asthma, in which excessive inflammation and breakdown of tissue occurs (Demedts et al., 2005; Greenlee et al., 2007b). Contrary to this hypothesis, lack of MMP either by inhibition or gene deletion, lead to many deleterious side effects and can increased mortality (Corry et al., 2004; Coussens et al., 2002), indicating their contribution of MMPs to disease pathology. For example, mice lacking MMP-7 failed to survive when exposed to gram-negative bacteria (Wilson et al., 1999). Inflammation of the lung parenchymal cells was increased in MMP-2 and MMP-9 deficient animals(Corry et al., 2004). From these results in mammals, it is clear that the MMPs play a major role in the

immune system. But because of the large number of MMPs identified in humans and mice (24 and 25 respectivey, reviewed inGreenlee et al., 2007b) and their overlapping and compensatory roles, it is difficult to identify specific functions of MMPs in the immune system.

Thus, investigation of the roles of MMPs is facilitated by using a simplified model such as insects, which share conserved innate immune functions yet have been shown to express only 2 to 3 MMPs (Altincicek and Vilcinskas, 2008; Knorr et al., 2009). The first insect MMPs were identified in *D. melanogaster* (Page-McCaw et al., 2003), but the role of these MMPs, *Dm*1-MMP and *Dm*2-MMP in *Drosophila* immunity has not been investigated. *Dm*1-MMP localization was dramatically increased during tumor metastasis in the larval imaginal discs of *D. melanogaster* larvae, indicating the role of MMPs in cancer (Beaucher et al., 2007). Another MMP was identified in the wax moth, *Galleria mellonella,* where it was shown to be directly involved in innate immunity (Altincicek and Vilcinskas, 2008), as lipopolysaccharide (LPS) challenge to the insect up-regulated *Gm*1-MMP gene expression and collagenolytic activity in the hemocytes of *G. mellonella.* MMP-1 knock-out in the red flour beetle, *Tribolium castaneum,* rendered beetles more susceptible to entomopathogenic fungal infection (Knorr et al., 2009).

Manduca sexta is an ideal model organism for testing the role of MMPs in immunity because of its size, yielding a large volume of hemolymph and abundant hemocytes. *M. sexta,* like other insects, has an efficient innate immune system that initiates a complex set of humoral and cell-mediated responses involving hemolymph proteins and hemocytes in order to reduce the invasion by foreign and pathogenic organisms (Dickinson et al., 1988; Kanost et al., 1990; Mulnix and Dunn, 1994; Yu et al.,

2002). In this research, we test the hypothesis that MMPs are critical in innate immunity and phagocytosis. We predict that the hemocytes will lose the ability to phagocytose when MMPs are inhibited. To test this hypothesis, we used a broad spectrum MMP inhibitor, GM6001 to inhibit MMPs and challenged the 5th instar caterpillars with bacterial infection in presence and absence of GM6001 to study hemocyte behavior and to compare the MMP protein expression profiles of the hemolymph, hemocytes, and fat body of naïve and infected larvae.

3.2. Materials and methods

3.2.1. Culture and preparation of E. coli DH5α

E. coli DH5α was generously obtained from Dr. Brigit Pruess, Department of Veterinary and Microbiological Sciences, NDSU. For injection of bacteria into caterpillars, bacteria were cultured in LB broth in a shaking incubator at 37 ^{0}C overnight. After 24 h, the bacterial suspension was centrifuged at 4000 ref for 15 min at room temperature, and supernatant was discarded. The bacterial pellet was washed in sterile PBS and diluted to a final concentration of 1 x 10^{7}cfu/ml. Ten microliters of this suspension, which contained approximately 1 x 10^{5} cfu/ml, was injected into each larva.

3.2.2. Preparation of GM6001

GM6001 (Millipore, Billerica, MA, USA) is a broad spectrum inhibitor of MMPs. The hydroxamic acid group of GM6001 binds to the zinc atom on the catalytic site of MMP thereby blocking the active site. In addition, the tryptophan side chain and isobutyl group of GM6001 prevents binding of MMP to the extracellular matrix proteins (Galardy, 1993; Strauss et al., 1996a). One hundred μM stock of GM6001 was prepared by

dissolving 5 g of GM6001 in 128.68 µl DMSO. A 10 µM working solution was prepared by diluting the stock with 1 X PBS to a final concentration of 0.001% DMSO.

3.2.3. Injection of E. coli DH5α into larvae

Larvae in the second day of the fifth instar were used for the bacterial challenge experiments. Larvae were anesthetized on ice for 15-20 min and then surface-sterilized with 75% ethanol. Using a 250 µl glass syringe (Hamilton, Reno, NV) mounted on a micro-applicator, 10 µl of the bacterial suspension was injected laterally into the hemocoel. Larvae were divided into four treatment groups (n = 5 for each group): 1) naïve (no injection), 2) vehicle-injected (PBS- 0.001%DMSO), 3) bacteria-injected (*E. coli* DH5α), and 4) inhibited (PBS-DMSO-10 µM GM6001 and *E. coli* DH5α injected). The final concentration of DMSO was maintained at 0.001% of the injection volume. Inhibited insects received injection of inhibitor 1 h prior to injection of bacteria. Larvae were kept with ad lib access to food at room temperature for 24 h after injections. At the end of 24 h the larvae were observed for any visible external symptoms such as weakness, larvae not eating properly and then dissected for collection of fat body, hemolymph, and hemocytes.

3.2.4. Collection of fat body, hemolymph and isolation of hemocytes

Larvae were chilled on ice for 10 min and surface-sterilized with 75% ethanol. A small incision was made on the first proleg, and the hemolymph was collected into sterile 1.5 ml tubes containing 100 µl of chilled manduca saline buffer (MSB; 4 mM NaCl, 40 mM KCl, 18 mM $MgCl_2.6H_2O$ and 3 mM $CaCl_2$, pH 6.5) and a few crystals of phenylthiourea (PTU) (Sigma, St. Louis, MO, USA) as previously described (Willot et al., 1994). The fat body was collected by gently scraping the fat body into a sterile 1.5 ml tube

after removing the gut. Ten µl of the fresh hemolymph was diluted in 90 µl of MSB without PTU and 10 µl loaded onto a hemocytometer. THC was calculated as follows:

Total hemocytes /ml = Sum of hemocytes in 4 squares /4 X10^4 X dilution factor X total volume of suspension.

Twenty µl of the fresh hemolymph was subjected to cytospin (100 rcf) for differential counting of the hemocytes. The resulting slides were air-dried overnight and then stained with hematoxylin and eosin (HEMA stain kit, Fisher Scientific, MI, USA). The cells were counted on a light microscope (OLYMPUS DP 71) at 60 X magnification under visible light. At least 200 hemocytes were counted in 10 fields from each slide. The cells were classified based on their cell morphology. The remainder of the hemolymph sample was centrifuged at 400 rcf for 10 min at 4°C to pellet the hemocytes. The supernatant (cell-free hemolymph) was collected into fresh tubes and set aside, while the hemocytes were washed twice in 200 µl MSB without PTU at 400 rcf for 10 min at 4 °C. After the second wash, hemocytes were resuspended in 200 µl MSB, macerated using sterile polypropylene micro pestles for about 5 min to rupture the cells. The fat body sample was suspended in 200 µl of MSB, macerated, centrifuged at high speed, and the supernatant was collected into fresh tubes.

3.2.5. Protein precipitation and estimation of protein concentration

Proteins were precipitated from fat body and hemolymph samples by adding four volumes of methanol. Samples were then vortexed, incubated at room temperature for 15 min, and centrifuged at 12000 rcf for 10 min. The supernatant was discarded and the pellet resuspended in 200 µl of MSB without PTU. The concentration of protein in the precipitated fat body, hemolymph, and hemocyte samples was estimated using a standard

Bio-Rad protein assay as per the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, 5 µl of sample was pipetted into a 96-well plate and 150 µl of Bradford reagent was added. Samples and BSA standards were run in duplicate. The contents in the wells were carefully mixed, kept at room temperature for 5 min, and the absorbance was read at 595 nm on a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

3.2.6. SDS-PAGE

The standard protocol of discontinuous electrophoresis (Laemmli, 1970) under denaturing and reducing conditions in presence of SDS and β-mercaptoethanol (Bio-Rad, Hercules, CA, USA) was used. Ten µg of protein was mixed with Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA) and β-mercaptoethanol in the ratio of 2:1 and boiled at 95°C for 5 min. Samples were separated on 12% acrylamide resolving and 5% acrylamide stacking gels at 100 V for 1.5 to 2 h at room temperature. After electrophoresis, the gels were washed in ddH_2O for 5 min, stained with Bio-safe Coomassie-blue (G-250) staining solution (Bio-Rad Laboratories, Hercules, CA, USA) for 1-2 h, and then destained with ddH_2O to visualize protein bands. If bands were not clearly visible with bio-safe coomassie staining, gels were re-stained with silver stain (Bio-Rad Laboratories, Hercules, CA, USA), as per manufacturer's instructions.

3.2.7. Western Blotting

Proteins were separated as above except that either 10 µg/lane or 20 µg/lane was loaded onto gels. After electrophoresis, proteins were transferred to a 0.45 µm Whatman nitrocellulose membrane (Whatman GmbH, Germany) at 4 °C and 100 V for 1 h. Membranes were blocked in 20 ml of 5% dry milk in Tris-buffered saline with Tween-20

(TBST; 0.1M Tris, 1.5M NaCl, and 0.5% Tween-20, pH 8) overnight at 4 °C. The membrane was incubated with anti-*M. sexta* MMP primary antibody (1:500) in 5% milk-TBST for 1 h at room temperature. The membrane was rinsed and washed with TBST three times for 10 min each. The membrane was then incubated in goat-anti-rabbit IgG HRP (Horse Radish Peroxidase) secondary antibody (1:100,000) in 5% milk for 1 h at room temperature. After washing the membrane six times 5 min each, the proteins were visualized with an ECL detection system (Thermo Scientific, Pierce, IL USA) containing the HRP-specific substrate. Signal was detected for 1min and 3 min in an Alpha Innotech Chemi imager (Alpha Innotech, St San Leandro CA, USA). For densitometric analysis, four samples from each treatment group were electrophoresed in random order on 10%, 18-well Tris-HCl criterion gels (Bio-Rad Laboratories, Hercules, CA, USA). Bands of three samples with high intensity (Mol wt: 50 kDa in hemocytes and 20 kDa in hemolymph) in the western blots were used for densitometric analysis (Alpha Innotech, St San Leandro CA, USA).

3.2.8. Flow Cytometry

To test the hypothesis that MMPs function to aid phagocytic ability of hemocytes, flow cytometry was performed on hemocytes incubated with fluorescent beads. Collection and isolation of hemocytes from 5th instar larvae (n = 5) were performed as described above. After the second wash, the resultant hemocyte pellets from all the larvae were pooled and resuspended in one ml of MSB without PTU and THC of the pooled hemocytes was estimated as indicated above. THC of the pooled hemocytes was estimated to be $20.4*10^6$ hemocytes per ml and these cells were divided equally into four groups of approximately $1.7*10^6$ hemocytes in 300 µl (total of 12 tubes labeled 0 min, 10 min, and 30

min for each treatment). Except for the incubation periods, hemocytes were kept on ice throughout the experiment. Three treatment groups were treated with 100 μM, 50 μM, and 10 μM concentrations of the MMP inhibitor, GM6001, in 0.001% DMSO for 1 h at room temperature. After 1 h, fluorescent (yellow green fluorescence) labeled sephadex beads (No. F8823 FluoSpheres carboxylate-modified microspheres, 1.0 μM in size, yellow green fluorescent, Molecular Probes, Invitrogen, Eugene, Oregan, USA) were added to all the groups in the ratio of 1:2 (cells:beads) and incubated at room temperature for 0 min, 10 min, and 30 min respectively. Excess or unbound beads were removed by centrifugation at 400 rcf after the end of incubation periods and the cells were analyzed using a FACS caliber flow cytometer equipped with an argon-ion laser (488 nm). When the cells are passed through an argon laser beam, light is scattered in all directions. The light scattered through narrow angles to the axis (forward scatter) indicates cell size while the light scattered at right angles (side scatter) indicates the granularity of the cells. The fluorescence of the bound beads was plotted in histograms, and Flow-Jo software version 8.7.1 was used to analyze the data. Cells that were close to or on the y-axis were considered to be dead cells. Live cells in the side-scatter versus forward-scatter plots were gated (22.8 % of the total hemocytes) and this population was used to estimate the number of hemocytes that had engulfed fluorescent beads, using mean fluorescence intensity. Because n = 1 for each group and samples were not run in triplicate, we did not perform statistical analyses for this experiment.

3.2.9. In vitro bacterial killing assay

Hemolymph was collected from the second or third day 5th instar larvae into tubes containing 100 μl MSB with PTU. Hemocytes from individual caterpillars were isolated,

suspended in 600 ml of sterile MSB and counted as described above. Hemocytes from each caterpillar (n = 8) were equally divided into four tubes labeled-control, vehicle, bacteria (*E. coli* DH5α), and inhibited (PBS-DMSO-10 μM GM6001-*E. coli* DH5α). Simultaneously, a 24 h culture of *E. coli* DH5α was washed and resuspended in sterile PBS. Hemocytes labeled vehicle and inhibited were added with 10 μl of PBS-DMSO and 10 μl of 10 μM GM6001 in PBS-DMSO respectively and incubated for 1 h at room temperature. While the tubes labeled vehicle and inhibited were kept in incubation period, the tubes labeled bacteria were mixed with washed *E. coli* DH5α at a ratio of 2:1 and incubated at room temperature for 1 h. At 0 min, 30 min, and 1 h time points, a volume of hemocyte-bacterial suspension that would yield ~100-200 cfu upon culture was taken into fresh 1.5 ml tubes. Hemocytes were lysed with a solution of 2% NP-40 in sterile LB broth for 5 min at room temperature and the remaining bacteria were plated in duplicate on sterile LB agar (Sigma, St Louis, MO) plates. The control tubes did not receive bacteria. The control hemocytes were lysed with a solution of 2% NP-40 in sterile LB broth for 5 min at room temperature and plated on LB agar plates. Plate counts were compared to the controls of only bacteria. The above process was repeated for the tubes labeled vehicle and inhibited at the end of 1 h incubation period with DMSO and 10μM GM6001. Inoculated plates were incubated at 37°C for 24 h, and number of cfu were counted for each plate.

3.2.10. Data analysis

Statistical analyses were performed using both SPSS version 17 and MINITAB version 15. Differences among the treatment and control groups were detected by Analysis of Variance (ANOVA) and Bonferroni-corrected post-hoc test. A probability value less

than 0.05 was considered significant. Data are expressed as mean ± standard error of the mean throughout.

3.3. Results

3.3.1. Bacterial injection experiment

3.3.1.1. Total hemocyte count (THC)

The mean THC of the naïve larvae was 4.8±0.68 million hemocytes per ml. Total hemocyte count varied depending on treatment (Fig. 12), (ANOVA, $F_{(3, 16)} = 17.093$, $p < 0.001$). The vehicle-injected larvae which received 10 µl PBS-0.001% DMSO showed a

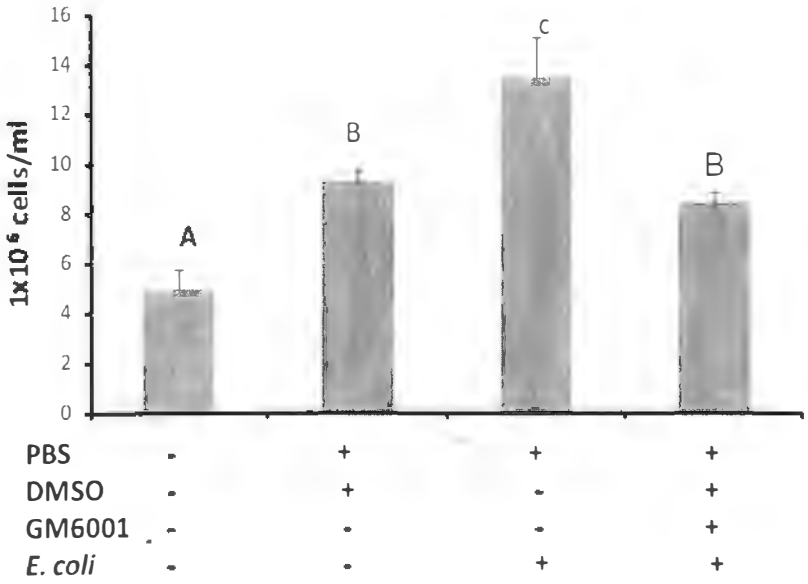

Figure 12. Total hemocyte count (THC) of naïve, vehicle-injected, bacteria-injected, and inhibited larvae. The letters on the bars indicate a significant difference between the groups. Same letters on the vehicle-injected and inhibited bars indicate that they are not significantly different.

slight increase in the cell count compared to naïve controls (Bonferroni-corrected post-hoc test, $p = 0.02$). This increase was not comparable to that of bacteria-injected larvae, which had a 50% increase in the number of hemocytes 24 h after of bacterial injection when

compared to naïve controls (Bonferroni-corrected post-hoc test, $p < 0.001$). In a separate experiment, PBS injections resulted in THC that were similar to those of vehicle-injected caterpillars (data not shown). In contrast to bacteria-injected larvae, the THC of the inhibited larvae that received 10 μM GM6001 was 60% lower (Bonferroni-corrected post-hoc test, $p < 0.004$) and was not significantly different from the vehicle-injected larvae (Bonferroni-corrected post-hoc test, $p < 1.0$).

3.3.1.2. Differential hemocyte count (DHC)

Similar to the results obtained in the total hemocyte counts, numbers of granulocytes (ANOVA, $F_{(3, 16)} = 5.50$, $p < 0.001$) and plasmatocytes (ANOVA, $F_{(3, 16)} = 7.84$, $p = .002$) varied with treatment (Fig. 13). Larvae that were injected with bacteria

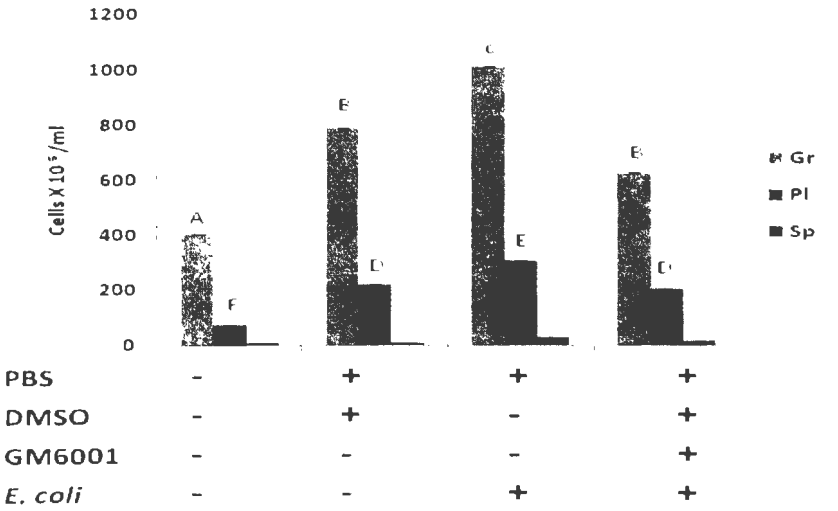

Figure 13. Differential hemocyte count (DHC) of naïve, vehicle-injected, bacteria-injected, and inhibited larvae. Significant differences were detected between bacteria-injected and inhibited granulocyte counts and plasmatocyte counts. Letters on the bars indicate the differences between groups. Same letters indicate that they are not different.

showed more than double the number of granulocytes and about 5 times more plasmatocytes than those of controls. No significant change was obsereved in the spherulocyte counts in the bacteria-injected or inhibited groups (Fig. 13). The larvae injected with *E. coli* DH5α showed degranulation of hemocytes 24 h after injection (Fig. 14. a-d). Hemocytes from larvae that were injected with both bacteria and the MMP inhibitor 10 μM GM6001, did not show degranulation (Fig. 14 d). Also, signs of activation, such as excessive granulation of granulocytes, were not observed in the inhibited (GM6001-injected) larvae (Fig. 14 d).

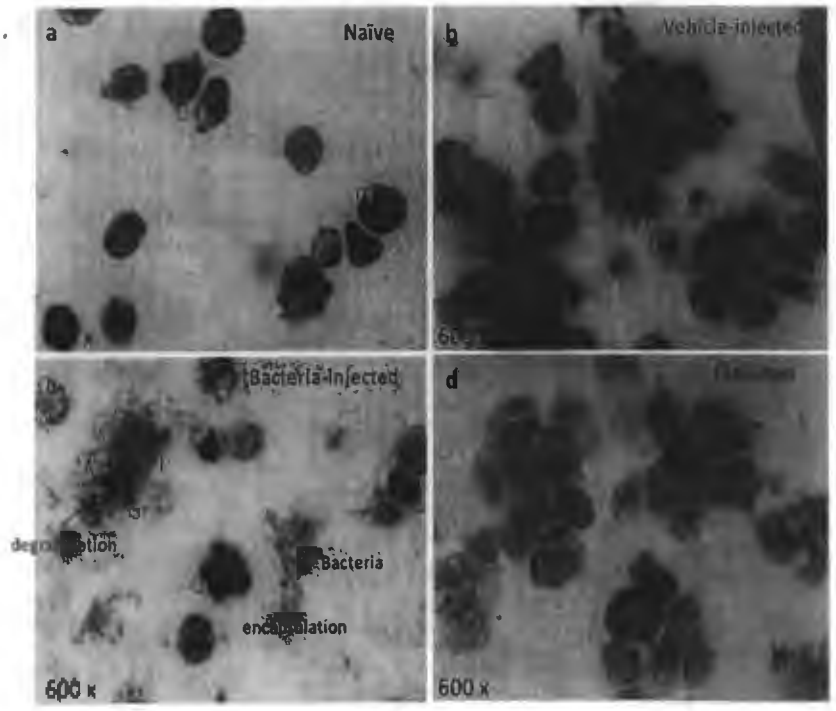

Figure 14. Morphology of granulocytes (Gr), plasmatocytes (Pl), and spherulocytes (Sp) from (a) control, (b) vehicle-injected, (c) bacteria-injected, and (d) inhibited larvae. Degranulation and bacterial encapsulation were only observed in bacteria-injected larvae (c) cell-clustering was observed in vehicle-injected, and inhibited larvae (d).

3.3.1.3. SDS-PAGE and western blot

MMP expression appeared to increase with bacterial injections, but because of the low sample size and large variation between individuals, MMP protein expression did not vary in the hemolymph (ANOVA $F_{(3, 8)} = .945$, $p > 0.05$) or hemocytes (ANOVA $F_{(3, 8)} = 1.246$, $p > 0.05$) of bacteria-injected larvae (Fig. 15 and 16).

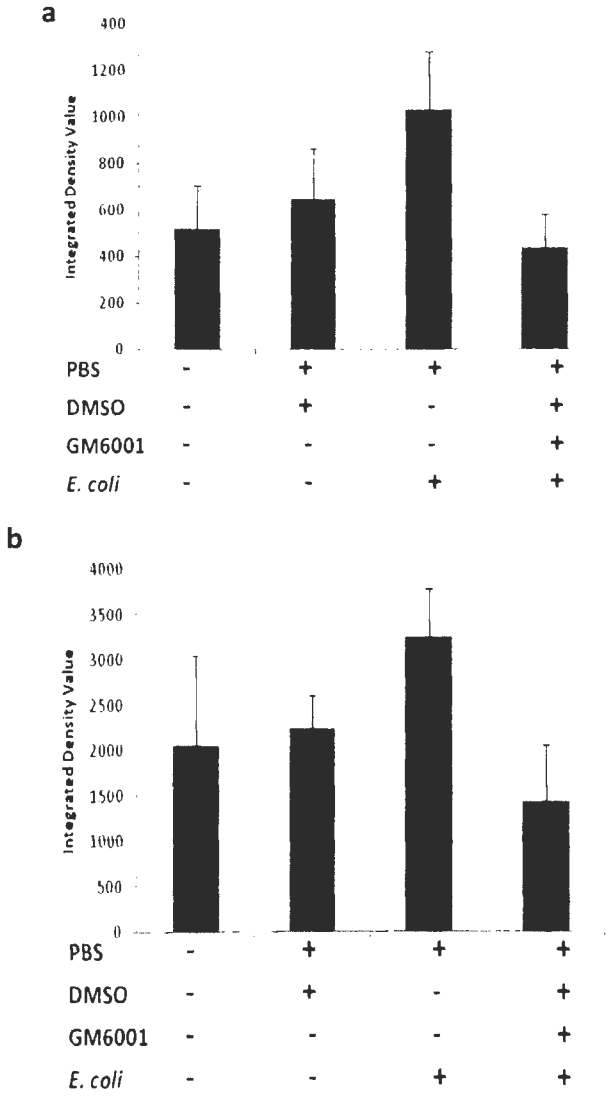

Figure 15. Densitometry analysis of MMP expression from western blots of a) hemocytes and b) hemolymph.

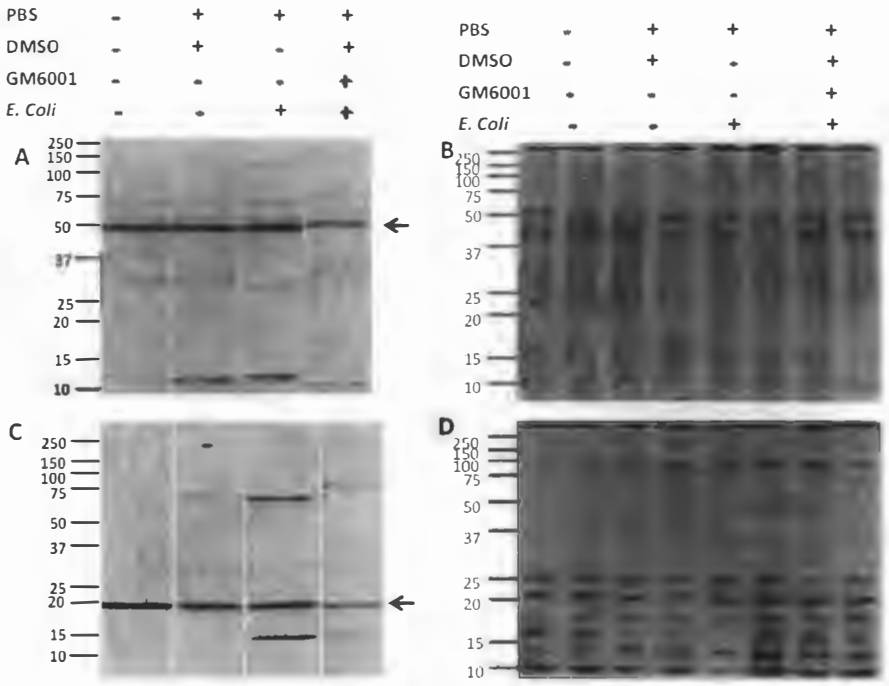

Figure 16. Representative western blots showing MMP expression profiles (a, c) and SDS-PAGE showing whole protein expression profiles (b, d) in hemocytes (a, b) and hemolymph (c, d) from naive, vehicle-injected, bacteria-injected, and inhibited larvae. Arrows indicate the bands from western blots that were used for densitometry (Mol wt: 50-kDa in hemocytes and 20- kDa in hemolymph).

3.3.2. Flow cytometry

Forward-scatter and side-scatter parameters (FSC and SSC) showed a distinct population of hemocytes (Fig. 17 a). This population was gated and the resulting fluorescence was plotted as a histogram (Fig. 17 b). The fluorescence intensity of the gated populations showed five distinct peaks. One of the peaks had little or no fluorescence indicating cells that did not take up beads. The other four populations had fluorescence which varied in intensity depending on the number of beads the cells had phagocytosed. For example, the cells that had only one bead on/in them had less fluorescence when compared to the cells that had four beads on/in them (Fig. 17 b, c and d). In the control hemocytes, almost 60% of the gated population had beads on them

within 10 min of incubation (Fig. 17 a and b). However, when the hemocytes were treated with 100 μM GM6001, the number of hemocytes that phagocytosed beads decreased substantially (Fig. 17 c and d). While the number of cells that phagocytosed beads at10 μM GM6001 was about 50%, at 100 μM concentration the phagocytosis was reduced to less than 30% within 10 min of incubation with beads (Fig 17 d).

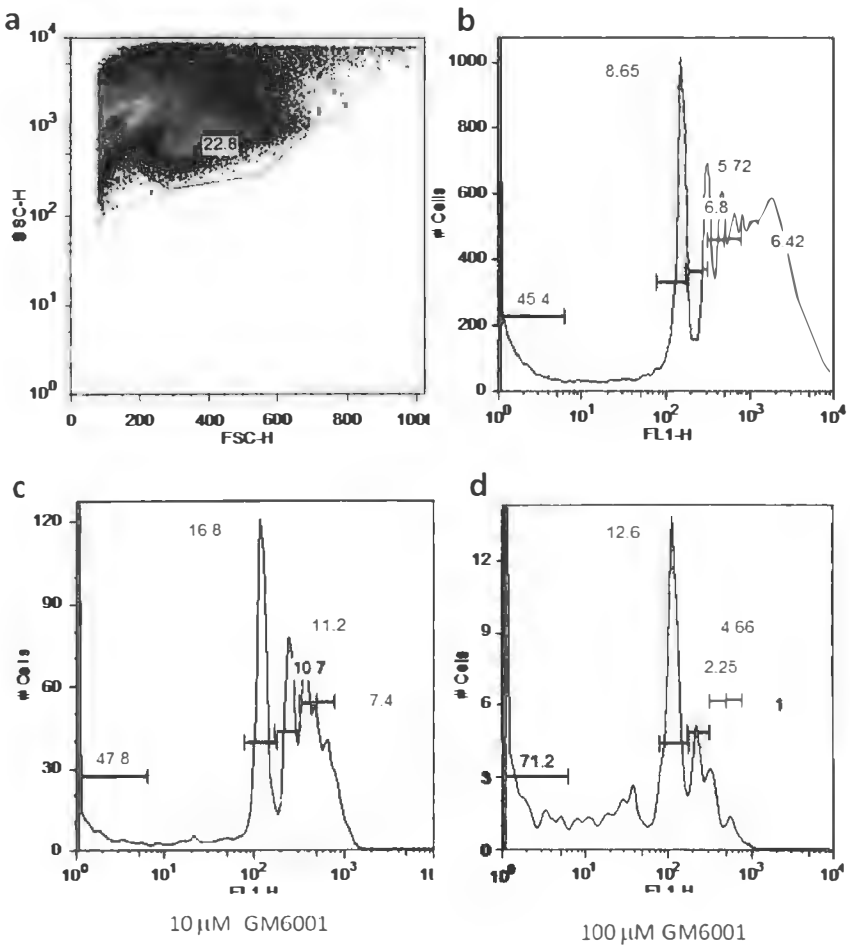

Figure 17. Flow cytometric analysis of the ability of hemocytes to phagocytose fluorescent sephadex beads, a) forward scatter and side scatter of hemocytes. The gate indicates live cells which were used for analysis of phagocytosis, b) Fluorescence of cells with or without beads. Subsequent peaks indicate cells with 1, 2, 3 or ≥ 4 beads, c) in the presence of 10 μM GM6001 or d) in the presence of 100 μM GM6001. Percent of the cell population is noted above each peak.

3.3.3. Bacterial killing assay

The results for the bacterial killing assay are not shown since all groups had a large number of cfu after 24 h incubation. As a result, cfu could not be counted and therefore the effect of inhibitor on the hemocytes could not be estimated.

3.4. Discussion

From the results obtained, it is clear that MMPs play an important role in the immune response of the tobacco hornworm. MMP expression was upregulated during infection in *M. sexta*, indicating that it does indeed play a role in the immune response. To elucidate the role that MMP may play in this process, we used the broad-spectrum MMP inhibitor, GM6001. Our results provide strong evidence that MMPs are critical for proper hemocyte function. First, circulating hemocyte numbers increased in the presence of bacteria and this response was abolished with the inhibitor (Fig 12). Inhibition of MMPs also affected the composition of the circulating hemocyte population (Fig 13). Together these results suggest that MMP may also play a role in cell differentiation and/or proliferation. The second major role of MMPs lies in the hemocytes' ability to phagocytose foreign agents. Phagocytosis of bacteria and beads was clearly inhibited in the presence of the MMP inhibitor (Fig 17). The loss of phagocytic ability due to inhibition of MMPs may have been caused either by prevention of activation of hemocytes or due to the inhibition of their proliferation and recruitment to the site of infection along with the maturation of the hemocytes.

3.4.1. MMPs in immune response

Insect hemocytes are known to promptly remove or reduce the circulating foreign particles by phagocytosis, nodulation, and encapsulation (Salt, 1970) along with the

synthesis and activation of antimicrobial peptides similar to that observed in mammalian infections (Greenlee et al., 2007b; Nagase and Woessner, 1999; Sternlicht and Werb, 2001). MMP-3-dependent T cell migration was abrogated in MMP-3$^{-/-}$ mice in the colon infected with *Citrobacter rodentium* (Chris et al., 2004); however, the mechanism by which MMP-3 affects T-cell migration is under investigation. Other studies of cellular MMP expression support our findings. For example, in *Galleria mellonella*, a septic injury with LPS resulted in enhanced MMP activity in hemocytes (Altincicek and Vilcinskas, 2008). The increase in the expression of MMPs was diminished when larvae were injected with GM6001 prior to the injection of bacteria and the collagen degrading property of MMPs observed in *G. mellonella* was eliminated when GM6001 was co-injected with LPS (Altincicek and Vilcinskas, 2008). Expression of MMP-8 and -9 present in the human neutrophil granules were upregulated during the inflammatory response for basement membrane degradation and infiltration neutrophils (Borregaard and Cowland, 1997). Mice lacking MMP-12 that is expressed in tissue macrophages had reduced bacterial clearance and increased mortality (Houghton et al., 2009), indicating that MMP expression may facilitate phagocytosis or cell adhesion.

While protein expression data may not accurately reflect the functional state of a proteolytic enzyme, the zymography revealed extremely high levels of gelatinolytic activity (data not shown) which could be inhibited only by high levels of GM6001 suggesting that other proteases with gelatinolytic activity, such as the serine proteases, may also be involved in the immune response. It has already been shown that serine proteases play a role in cell migration (Yoshida et al., 1998) and phagocytosis (Raptis et al., 2005) in vertebrate immunity.

3.4.2. MMPs in hemocyte function

Twenty four h after bacterial infection, the larvae showed no visible changes and no mortality was observed. However, the numbers of circulating hemocytes nearly tripled after infection. This response was abrogated in the presence of the MMP inhibitor, GM6001 (Fig. 12), suggesting that MMPs play a role in the immune responses. Underlying the increase in the total hemocyte count (Fig. 12) was the alteration of the hemocyte population (Fig. 13). This is a typical immune response where a considerable increase in the granulocyte and plasmatocyte population occurs during the immune response (Horohov and Dunn, 1982). The change in the hemocyte population was prevented in the presence of the MMP inhibitor (Fig. 12). This result suggests three possible, but not mutually exclusive, roles for MMP-1) recruitment of new hemocytes from hematopoietic organs, 2) proliferation of prohemocytes or other cell populations, and/or 3) maturation of existing prohemocytes.

The increase in the number of hemocytes during infection occurs either due to the mitotic division of circulating hemocytes (Arnold and Hinks, 1982; Arnold and Hinks, 1975) or due to the production of hemocytes from other hemopoietic organs such as lymph gland (Lanot et al., 2001) to the site of infection. Circulating prohemocytes, granulocytes, and spherule cells have been shown to undergo mitotic divisions (Arnold and Hinks, 1982; Arnold and Hinks, 1975). Prohemocytes are terminally differentiated to plasmatocytes (Arnold and Hinks, 1975). However, it is hypothesized that plasmatocytes have an additional source such as the hematopoietic organs associated with the wing discs and lymph glands (Hinks and Arnold, 1977). Granulocytes and spherule cells are known to undergo mitotic or sometimes amitotic divisions producing granulocytes and spherulocytes

respectively (Hinks and Arnold, 1977). However in this study, when larvae received MMP inhibitor before bacterial infection, the hemocyte population differed, suggesting that MMPs play a role in immune cell differentiation and maturation. That MMPs could have a role in immune cell development is not unheard of in the literature. MMP-9 inhibition reduced the maturation and migration of langerhans cells (dendritic cells) in female BALB/c mice (Kobayashi et al., 1999).

3.4.3. MMPs in phagocytosis and killing

Plasmatocytes and granulocytes of insects are similar to the vertebrate macrophages since they are involved in the encapsulation and phagocytosis of foreign agents (Gillespie and Kanost, 1997; Lemaitre and Hoffmann, 2007). Similar to the results observed during *in vivo* bacterial infection experiments, the naturally highly phagocytic hemocytes lost their ability to phagocytose fluorescently-labeled sephadex beads when administered with medium (50 μM) and high concentrations (100 μM) of GM6001 (Fig. 17). These results are parallel to the results observed in *T. castaneum*, where knock-down of MMP-1 impaired their ability to fight infection with *Beauveria bassiana* thus rendering them more susceptible to these infections (Knorr et al., 2009). Inhibition of MMPs may impair the hemocytes' ability to identify the foreign agents as a result of which they fail to combat infections. We hypothesize that MMPs may be involved in the activation of cytokines or chemokines which are essential for mediating the immune response.

Alternatively, MMP may activate adhesion molecules (Zhang et al., 2006) to initiate a signaling cascade. The incomplete inhibition of phagocytosis observed in the samples incubated with GM6001 indicates the presence of other proteases such as serine

proteases which have been documented to be involved in the phagocytosis in vertebrates (Raptis et al., 2005).

To determine whether MMPs are involved in bacterial killing we used an *in vitro* model by incubating hemocytes with live bacteria. Cfu obtained were too high to count and higher than the bacteria alone. This result may have occurred because the hemocytes were suspended in MSB rather than cell culture media or because we used hemocytes from naïve caterpillars. The loss of nutrition might have resulted in the death of hemocytes. In addition, the bacteria may have utilized the dead hemocytes as source of energy and multiplied and hence the cfu formed were much higher than the original volume plated (~100-200 cfu). Under ideal conditions, the hemocytes should phagocytose the bacteria and lyse them. Hence, when the hemocytes are lysed after the incubation period and remaining bacteria plated, the number of cfu obtained in the bacteria treated samples will be higher than those obtained from samples incubated with MMP inhibitor, GM6001.

3.5. References

Abe, S., Nagai, Y., 1972. Interaction between tadpole collagenase and human α2-macroglobulin. Biochimica et Biophysica Acta 278, 125-132.

Altincicek, B., Vilcinskas, A., 2008. Identification of lepidopteran matrix metalloproteinase with dual roles in metamorphosis and innate immunity. Develpomental Comparative Immunology 32, 400-409.

Amirbekian, V., Aguinaldo, J.G.S., Amirbekian, S., Hyafil, F., Vucic, E., Sirol, M., Weinreb, D.B., Greneur, S.L., Lancelot, E., Corot, C., Fisher, E.A., Galis, Z.S., Fayad, Z.A., 2009. Atherosclerosis and Matrix Metalloproteinases: Experimental Molecular MR Imaging *in vivo*. Radiology 251, 429-438.

Arnold, J.W., Hinks, c.F., 1975. Heamopoiesis in Lepidoptera. I. The multiplcation of circulating hemocytes. *Canadian Journal of Zoology* 54, 1003-1012.

Arnold, J.W., Hinks, C.F., 1982. Heamopoiesis in Lepidoptera. III. A note on the multiplication of spherule cells and granular hemocytes. Canadian Journal of Zoology 61, 275-277.

Arthur, M.J., 1997. Matrix degradation in liver: a role in injury and repair. Hepatology 26, 1069-1071.

Ashida, M., Bery, P.T., 1995. Role of integument in insect defense: pro-phenoloxidase cascade in the cuticular matrix. Proceedings of National Academy of Science 92, 10698-10702.

Bade, M.L., Shoukimas, J.J., 1974. Neutral metal chelator-sensitive protease in insect moulting fluid. *Journal of Insect Physiology* 20, 281-290.

Bade, M.L., Stinson, A., 1978. Digestion of cuticle chitin during the moult of *Manduca sexta* (Lepidoptera: Sphingide). *Insect Biochemistry* 9.

Banda, M.J., Werb, Z., 1981. Mouse macrophage elastase, purification and characterization as a metalloproteinase. Biochemical Journal 193, 589-605.

Bayne, C.J., 1990. Phagocytosis and non-self recognition in invertebrates. *Bio-Science* 40, 723-731.

Beaucher, M., Hersperger, E., Page-McCaw, A., Shearn, A., 2007. Metastatic ability of *Drosophila* tumors depends on MMP activity. Developmental Biology 303, 625-634.

Bell, R.A., Joachim, F.G., 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. Annals of the Entomological Society of America 19, 365-373.

Bigg, H.F., Shi, Y.E., Liu, Y.L.E., Steffensen, B., Overall, C.M., 1997. Specific, high affinity binding of tissue inhibitor of metalloproteinases-4 (TIMP-4) to the COOH-terminal hemopexin-like domain of human gelatinase A-TIMP-4 binds procollagenase A and the COOH-terminal domain in a similar manner to TIMP-2. *Journal of Biological Chemistry* 272, 15,496-415,500.

Birkedal-Hansen, H., Moore, W.G., Bodden, M.K., Windsor, L.J., Birkedal-Hansen, B., DeCarlo, A., Engler, J.A., 1993. Matrix metalloproteinases: A review. Critical Reviews in Oral Biology and Medicine 4, 197.

Bogdan, C., Rollinghoff, M., Diefenbach, A., 2000. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Current Opinion in Immunology 12, 244-248.

Borregaard, N., Cowland, J.B., 1997. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 89, 3503-3521.

Brew, K., Dinakarpandian, D., Nagase, H., 2000. Tissue inhibitors of metalloproteinases: evolution, structure and function. Biochimica Biophysica Acta 1477, 267-283.

Brinckerhoff, C., Matisian, L., 2002. Matrix metalloproteinase: a tail of a frog that became a prince. Nature Reviews in Molecular and Cellular Biology 3, 207-214.

Brooks, P.C., Stromblad, S., Sanders, L.C., von Schalscha, T.L., Aimes, R.T., Stetler-Stevenson, W.G., Quigley, J.P., Cheresh, D.A., 1996. Localization of matrix

metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin avb3. Cell 85, 683-693.

Bulet, P., Hetru, C., Dimarcq, J.L., Hoffmann, D., 1999a. Antimicrobial peptides in insecta: structure and function. Developmental and Comparative Immunology 23, 329-344.

Bulet, P., Hetru, C., Dimarcq, J.L., Hoffmann, D., 1999b. Antimicrobial peptides in insects: structure and function. *Developmental and Comparative Immunology* 23, 329-344.

Cawston, T.E., 1996. Metalloproteinase inhibitors and the prevention of connective tissue breakdown. Phramacology and Therapeutics 70, 163-182.

Cawston, T.E., 1998. Matrix metalloproteinases and TIMPs-properties and implications for the rheumatic diseases. Molecular Medicine Today 4, 130-137.

Cerenius, L., Soderhall, K., 2004. The prophenoloxidase-activating system in invertebrates. Immunological Reviews 198, 116-126.

Chakraborti, S., Mandal, M., Das, S., Mandal, A., Chakraborti, T., 2003. Regulation of matrix metalloproteinases: An overview. Molecular and Cellular Biology 253, 269-285.

Chris, K.F., Pender, S.L.F., Pickard, K.M., Chance, V., Holloway, J.A., Huett, A., Goncalves, N.S., Mudgett, J.S., Dougan, G., Frankel, G., MacDonald, T.T., 2004. Impaired immunity to interstitial bacterial infection in stromelysin-1 (matrix metalloproteinase-3)-deficient mice. Journal of Immunology 173, 5171-5179.

Christensen, B.M., Severson, D.W., 1993. Biochemical and molecular basis of mosquito susceptibility to *plasmodium* and filaroid nematodes., in: S.N.T. N.E. Beckage,

B.A. Federici (Ed.), *Parasites and Pathogens of Insects*. Academic, San Deigo, 245-266.

Corry, D.B., Kiss, A., Song, L.Z., Xu, J., Lee, S.H., Werb, Z., Kheradmand, F., 2004. Overlapping and independent contributions of MMP-2 and MMP-9 to lung allergic inflammatory cell regression through decreased CC chemokines. FASEB Journal 18, 995-997.

Coussens, L.M., Fingleton, B., Martisian, L.M., 2002. Matrix metalloproteinase inhibitors and cancer: Trails and tribulations. Science 295, 2387-2392.

Crabbe, T., Zucker, S., Cockett, M., Willenbrock, F., Ticlke, S., O'Conell, J., 1994. Mutation of the active site glutamic acid of human gelatinase A: Effects on latency, catalysis, and the binding of tissue inhibitor of metalloproteinase-1. Biochemistry 33, 6684-6690.

Damjanovski, S., Amano, T., Li, Q., Ueda, S., Shi, Y.B., Ishizuya-Oka, A., 2006. Role ECM remodeling in thyroid hormone-dependent apoptosis during anuran metamorphosis. Annals of the New York Academy of Sciences 926, 180-191.

Danø, K., Andreasen, P.A., Grøndahl-Hansen, J., Kristensen, P., Nielsen, L.S., Skriver, L., 1985. Plasminogen activators, tissue degredation and cancer. Advances in Cancer Research 44, 139-266.

Demedts, I.K., Brusselle, G.G., Bracke, K.R., Vermaelen, K.Y., Pauwels, R.A., 2005. Matrix metalloproteinases in asthma and COPD. Current Opinions in Pharmacology 5, 257-263.

Dickinson, I., Russell, V.W., Dunn, P.V., 1988. A family of bacterial-regulated cecropin D-like peptides from *Manduca sexta*. Journal of Biological chemistry 263, 19424-19429.

Douglas, D.A., Shi, Y.E., Sang, Q.X.A., 1997. Computational sequence analysis of the tissue inhibitor of metalloproteinase family. *Journal of Protein chemistry* 16, 237-255.

Eddy, A.A., 1996. Molecular insights into renal interstitial fibrosis. Journal of the American Society of Nephrology 7, 2495-2508.

Eleftherianos, I., Gokcen, F., Felfoldi, G., Millichap, P.J., Trenczek, T.E., ffrench-Constant, R.H., Reynolds, S.E., 2007. The immunoglobulin family protein hemolin mediates cellular immune responses to bacteria in the insect *Manduca sexta*. Cell Microbiology 9, 1137-1147.

Faucher, D.C., Lelievre, Y., Cartwright, T., 1987. An inhibitor of mammalian collagenase active at micromolar concentrations from an actinomycete culture broth. Journal of Antibiotics (Tokyo) 40, 1757-1761.

Fessler, J.H., Fessler, L.I., 1989. *Drosophila* extracellular matrix. *Annual Reviews in Cell Biology* 5, 309-339.

Galardy, R., 1993. Galardin. Drugs Future 18, 1109-1111.

Gill, S.E., Pape, M.C., Khokha, R., Watson, A.J., Leco, K.J., 2003. A null mutation for tissue inhibitor of metalloproteinase-3 (TIMP-3) impairs murine branchiole branching morphogenesis. *Developmental Biology* 261, 313-323.

Gillespie, J.P., Kanost, M.R., 1997. Biological mediatiors of insect immunity. Annual Reviews in Entomology 42, 611-643.

Godenschwege, T.A., Pohar, N., Buchner, S., Buchner, E., 2000. Inflated wings, tissue autolysis and early death in tissue inhibitor of metalloproteinase mutants of *Drosophila*. European Journal of cell Biology 79, 495-501.

Goldberg, G.I., Manner, B.L., Grant, G.A., Eisen, A.Z., Wilhelm, S., He, C., 1989. Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteinases designated TIMP-2. Proceedings of National Academy of Science 86, 8207-8211.

Golub, L.M., Lee, H.M., Lehrer, G., Nemiroff, A., McNamara, T.F., Kaplan, R., Ramamurthy, N.S., 1983. Monocycline reduces gingival collagenolytic activity during diabetes. Preliminary observations and a proposed new mechanism of action. Journal of Periodontal Research 18, 516-526.

Gomez, D.E., Alonso, D.F., Yoshiji, H., Thorgeirson, U.P., 1997. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. European Journal of Cell Biology 74, 111-122.

Gomis-Ruth, F.X., Maskos, K., Betz, M., Bergner, A., Huber, R., Suzuki, K., Yoshida, N., Nagase, H., Brew, K., Bourenkov, G.P., Bartunik, H., Bode, W., 1997. Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. Nature 389, 77-81.

Graham, J.S., Xiong, J., Gillikin, J.W., 1991. Purification and developmental analysis of a metalloproteinase from the leaves of *Glycine max*. Plant Physiology 97, 786-792.

Greenlee, K.J., Corry, D.B., Engler, D.A., Matsunami, R.K., Tessier, P., Cook, R.G., Werb, Z., Kheradmend, F., 2006. Proteomic identification of *in vivo* substrates for

matrix metalloproteinases 2 and 9 reveals a mechanism for resolution of inflammation. The Journal of Immunology 177, 7312-7321.

Greenlee, K.J., Werb, Z., Kheradmand, F., 2007b. Matrix metalloproteinases in lung: multiple, multifarious, and multifaceted. Physiological Reviews 87, 68-98.

Gross, J., Lapiere, C.M., 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proceedings of National Academy of Science 48, 1014-1022.

Gross, J., Nagai, Y., 1965. Specific degeradation of the collagen molecule by tadpole collagenolytic enzyme. Proceedings of the National Academy of Sciences 54.

Guedez, L., Lim, M.S., Stetler-Stevenson, W.G., 1996. The role of metalloproteinases and their inhibitors in hematological disorders. Critical Reviews in Oncogenesis 7, 205-250.

Harper, E., Bloch, K.J., Gross, J., 1971. The zymogen of tadpole collagenase. Biochemistry 10, 3035-3041.

Hetru, C., Hoffmann, D., Bulet, B., 1998. Antimicrobial peptides from insects., in: P.T.a.H. Brey, D (Ed.), Molecular mechanisms of immune responses in insects. Chapman & Hall, London, 40-66.

Hinks, C.F., Arnold, J.W., 1977. Haemopoiesis in Lepidoptera. II. The role of the haemopoietic organs. Canadian Journal of Zoology 55, 1740-1755.

Hoffmann, J.A., Janeway, C.A.J., 1994. Phyogenetic perspectives in immunity: The insect host defense. CRC Press, Boca Raton.

Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., Ezekowitz, R.A.B., 1999. Phylogenetic perspectives in innate immunity. Science 284, 1313-1318.

Horohov, D.W., Dunn, P.E., 1982. Changes in the circulating hemocyte population of *Manduca sexta* larvae following injection of bacteria. Journal of Invertebrate Pathology 40, 327-339.

Houghton, A.M., Hartzell, W.O., Robbins, C.S., Gomis-Ruth, F.X., Shapiro, S.D., 2009. Macrophage elastase kills bacteria within murine macrophages. Nature 460, 637-641.

Janeway, C.A., 2001. How the immune system works to protect the host from infection: A personal view., Preceedings of the National Academy of Sciences, USA, 7461-7468.

Janeway, C.A.J., 1994. The role of microbial pattern recognition in self: non-self discrimination in innate and adaptive immunity., in: J.A. Hoffmann, C.A.J. Janeway, S. Natori (Eds.), Phylogenetic Perspectives in Immunity: The Insect Host Defense. CRC Press, Boca Raton, 115-122.

Jenkin, P.M., Hinton, H.E., 1996. Apolysis in Arthropod moulting cycles. *Nature London Journal* 21, 871.

Jeuniaux, C., Amanieu, M., 1955. Mise en evidance d'une chitinase dans le liquide exuvial de *Bombyx mori* L. *Archives Internationales de Physiologie et de Biochimie* 63, 94-103.

Jiang, H., 2008. The biochemical basis of antimicrobial responses in *Manduca sexta*. Insect Science 15, 53-66.

Kanost, M.R., Kawooya, J.K., Law, J.H., Ryann, R.O., Van Heusden, M.C., Ziegler, R., 1990. Insect hemolymph proteins. Advances in Insect Physiology 22, 299-396.

Katzenellenbogen, B.S., Kafatos, F.C., 1971. Proteinase of silkmoth molting fluid: physical and catalytic properties. Journal of Insect Physiology 17, 775-800.

Kheradmand, F., Werner, E., Tremble, P., Symons, M., Werb, z., 1998. Role of Rac1 and oxygen radicals in collagenase-1 expression induced by cell shape change. Science 280, 898-902.

Kheradmand, P., Rishi, K., Werb, Z., 2002. Singaling through the EGF receptor controls lung morphogensis in part by regulating MT1-MMP-mediated activation of gelatinase A/MMP-2. Journal of Cell Science 839, 839-848.

Kinoshita, T., Fukuzawa, H., Shimada, T., Saito, T., Matsuda, Y., 1992. Primary structure and expression of gamete lytic enzyme in *Chlamydomonas reinhardtii*: similarity of functional domains to matrix metalloproteases., PNAS, 4693-4697.

Knorr, E., Schmidtberg, H., Vilcinskas, A., Altinicicek, B., 2009. MMPs regualte both develpoment and immunity in the *Tribolium* model insect. PLoS One 4, e4751.

Kobayashi, Y., Matsumoto, M., Kotani, M., Makino, T., 1999. Possible involvement of matrix metalloproteinase-9 in langerhans cells migration and maturation. The Journal of Immunology 163, 5989-5993.

Kuo, C.T., Jan, L.Y., Jan, Y.N., 2005. Dendrite-specific remodeling of *Drosophila* sensory neurons requires matrix metalloproteases, ubiquitin-proteome, and ecdysone signaling. Proceedings of the National Academy of Sciences 102, 15230-15235.

Kusano, K., Miyaura, C., Inada, M., Tamura, T., Ito, A., Nagase, H., Kamoi, K., Suda, T., 1998. Regulation of matrix metalloproteinases (MMP-2,-3,-9,and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. Endocrinology 139, 1338-1345.

Lackie, A.M., 1988. Hemocyte behavior. *Advances in Insect Physiology*. 21, 85-178.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Laiho, M., Keski-Oja, J., 1989. Growth factors in the regulation of pericellular proteolysis: A review. Cancer Research 49, 2533-2553.

Lambert, V., Wielockx, B., Munaut, C., Galopin, C., Jost, M., Itoh, T., Werb, Z., Baker, A., Libert, C., Hans-Willi, K., Foidart, J.M., Noel, A., Jean-Marie, R., 2003. MMP-2 and MMP-9 synergize in promoting choroidal neovascularization. FASEB Journal 17, 2290-2292.

Lanot, R., Zachary, D., Holder, F., Meister, M., 2001. Post embryonic hematopoiesis in *Drosophila*. Developmental Biology 230, 243-257.

Lavine, M.D., Strand, M.R., 2002. Insect haemocytes and their role in immunity. Nature Immunology 32, 1295-1309.

Lemaitre, B., Hoffmann, J., 2007. The host defense of *Drosophila melanogaster*. *Annual Reviews in Immunology* 25, 697-743.

Lepage, T., Gache, C., 1990. Early expression of collagenase-like hatching enzyme gene in the sea urchin embryo. EMBO Journal 9, 3993-3012.

Leulier, F., Parquet, C., Pili-Floury, S., Ryu, J.H., Caroff, M., Lee, W.J., Mengin-Lecreulx, D., Lemaitre, B., 2003. The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition. Nature Immunology 4, 478-484.

Llano, E., Adam, G., Pendas, A.M., Quesada, V., Sanchez, L.M., Santamaria, I., Noselli, S., Lopez-Otin, C., 2002. Structural and enzymatic characterization of *Drosophila*

Dm2-MMP, a membrane-bound matrix metalloproteinase with tissue specific expression. Journal of Biological Chemistry 277, 23321-23329.

Llano, E., Pendas, A.M., Aza-Blanc, P., Korenberg, T.B., Lopez-Otin, C., 2000. Dm1-MMP, a matrix metalloproteinase from *Drosophila* with a potential role in extracellular matrix remodeling during neural development. Journal of Biological Chemistry 275, 35978-35985.

Lowenberger, C., 2001. Innate immune responses of *Aedes aegyptii*. Insect Biochemistry and Molecular Biology 31, 219-229.

Lubec, G., Ratzenhofer, E., 1979. Collagenase activity of rat kidney with glomerulonephritis is inhibitted by erythromycin. Nephron 24, 93-95.

Madri, J.A., Graesser, D., 2000. Cell migration in the immune system: the evolving inter-related roles of adhesion molecules and proteinases. Developmental Immunology 7, 103-116.

Maidment, J., Moore, D., Murphy, G., Clark, I., 1999. Matrix metalloproteinase homologues from *Arabidopsis thaliana*-expression and activity. Journal of Biological Chemistry 274, 706-734.

Mannello, F., Luchetti, F., Falcieri, E., Papa, S., 2005a. Multiple roles of matrix metalloproteinases during apoptosis. Apoptosis 12, 19-24.

Manning, G., Krasnow, M.A., 1993. Development of *Drosophila* tracheal system., in: M. Bate, A. Martinez Arias (Eds.), The Development of *Drosophila melanogaster*. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 609-685.

Massova, I., Kotra, L.P., Fridman, R., Mobashery, S., 1998. Matrix metalloproteinases-structures, evolution oand diversification. FASEB Journal 12, 1075-1095.

Meister, M., Hetru, C., Hoffmann, J., 2000. The antimicrobial host defense of *Drosophila*., in: L.D.P.a.G. Litman (Ed.), Current Topics in Microbiology and Immunology. Springer-Verlag, Berlin.

Moncrief, J.S., Obiso, R.J., Barroso, L.A., 1995. The enterotoxin of *Bacteroides fragilis* is a metalloprotease. Infection and Immunity 63, 175-181.

Mott, J., Werb, Z., 2004. Regulation of matrix biology by matrix metalloproteinases. Current Opinion in Cell Biology 16, 558-564.

Mulnix, A.B., Dunn, P.E., 1994. Structure and induction of lysozyme gene from tobacco hornworm *Manduca sexta*. Insect Biochemistry and Molecular Biology 24, 271-281.

Murphy, G., Willenbrock, F., 1995. Tissue inhibitors of matrix metalloendopeptidases. Methods in Enzymology 248, 496-510.

Muta, T., Iwanaga, S., 1996. The role of hemolymph coagulation in innate immunity. Current Opinion in Immunology 8, 41-47.

Nagai, Y., Lapiere, C.M., Gross, J., 1966. Tadpole collagenase, preparation and purification. Biochemistry 5, 3123-3130.

Nagase, H., Woessner, J.F.J., 1999. Matrix metalloproteinases. Journal of Biological Chemistry 274, 21491-21494.

Nakahara, Y., Shimura, S., Ueno, C., Kanamori, Y., Mita, K., Kiuchi, M., Kamimura, M., 2009. Purification and characterization of silkworm hemocytes by flow cytometry. *Developmental and Comparative Immunology* 33, 439-448.

Nelson, A.R., Fingleton, B., Rothenberg, M.L., Matrisian, L.M., 2000. Matrix metalloproteinases: biological activity and clinical implications. Journal of Clinical Oncology 18, 1135-1149.

Nijhout, H.F., 1981. Physiological control of molting in insects. *American Zoologist* 21, 631-640.

Nijhout, H.F., Williams, C.M., 1974a. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last instar larva and the decision to pupate. *Journal of Experimental Biology* 61, 481-491.

Ning, W., Li, C.J., Kaminski, N., Feghali-Bostwick, C.A., Alber, S.M., Di, Y.P., Otterbein, S.L., Song, R., Hayashi, S., Zhou, Z., Pinsky, D.J., Watkins, S.C., Pilewski, J.M., Sciurba, F.C., Peters, D.G., Hogg, J.C., Choi, A.M., 2004. Comprehensive gene expression profiles reveal pathways related to the pathogenesis of chronic obstructive pulmonary disease. Proceedings of National Academy of Science 101, 14895-14900.

Obiso, R.J., Lyerly, D.M., van Tassell, R.L., Wilkins, T.D., 1995. Proteolytic activity of the *Bacteroides fragilis* enterotoxin causes fluid secretion and intestinal damage *in vivo*. Infection and Immunity 63, 3820-3826.

Ojeda-Avila, T., Woods, T.A., Rausa, R.A., Raguso, R.A., 2003. Effects of dietary variation on growth, composition, and maturation of *Manduca sexta*. Journal of Insect Physiology 49, 293-306.

Oslon, M.W., Gervasi, D.C., Mobashery, S., Fridman, R., 1997. Kinetic analysis of the binding of human matrix metalloproteinase-2and -9 to tissue inhibitor of

metalloproteinase (TIMP)-1 and TIMP-2. Journal of Biological Chemistry 272, 29975-29983.

Overall, C.M., Lopez-Otin, C., 2002. Strategies for MMP inhibition in cancer: Innovations for the post-trial era. Nature Reviews in Cancer 2, 657-672.

Page-McCaw, A., J., A., Ewald, A.J., Werb, Z., 2007. Matrix metalloproteinases and the regulation of tissue remodelling. Molecular Cell Biology 8, 221-233.

Page-McCaw, A., Serano, J., Sante, J.M., Rubin, G.M., 2003. *Drosophila* matrix metalloproteinases are required for tissue remodeling, but not embryonic development. Developmental Cell 4, 95-106.

Parks, W.C., Wilson, C.L., Lopez-Boado, Y.S., 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nature Reviews in Immunology 4, 617.

Quinones, J.L., Rosa, R., Ruiz, D.L., Garcia-Arraras, J.E., 2002. Extracellular matrix remodeling and metalloproteinase involvement during intestine regeneration in the sea cucumber *Holothuria glaberrima*.l. Developmental Biology 250, 181-197.

Ragster, L., Chrispeels, M.J., 1979. Azocoll-digesting proteinases in soybean leaves: characteristics and changes during leaf maturation., Plant Physiology, 857-862.

Raptis, S.Z., Shapiro, S.D., Simmons, P.M., Cheng, A.M., Pham, C.T.N., 2005. Serine protease cathepsin G regulation adhesion-dependent neutrophil effector function by modulating integring clustering. Immunity 22, 679-691.

Ratcliffe, N., Rowley, A., 1981. Invertebrate Blood cells. Academic Press, London.

Ratcliffe , N.A., Rowley, A.F., Fitzgerald, S.W., Rhodes, C.P., 1985. Invertebrate immunity: basic concepts and recent advances. *International Review of Cytology* 97, 186-350.

Reinecke, J.P., Buckner, J.S., Grugel, S.R., 1980. Life cycle of laboratory-reared tobacco hornworms, *Manduca sexta*, a study of development and behavior, using time-lapse cinematography. The Biological Bulletin 158, 129-140.

Rowsell, S., Hawtin, P., Minshull, C.A., Brockbank, H.J.S.M.V., Barratt, D.G., McPheat, A.M.S.W., Waterson, D., Henney, A.M., 2002. Crystal structure of human MMP-9 in complex with a reverse hydroxamate inhibitor. Journal of Molecular Biology 319, 173-181.

Salt, G., 1970. The cellular defense reactions of insects. Cambridge University Press, London.

Samuels, R.I., Charnely, A.K., Reynolds, S.E., 1993a. A cuticle degrading proteinase from the molting fluid of tobacco hornworm, *Manduca sexta*. *Insect Biochemistry and Molecular Biology* 23, 607-614.

Satsuki, M., Gregory, G., Charles, M.F., 2006. Differential expression of MMPs and TIMPs in moderate and severe heart failure in a transgenic model. Journal of Cardiac Failure 12, 314-325.

Schmidt, O., Theopold, U., Strand, M.R., 2001. Innate immunity and evasion by insect parasitoids. Bioessays 23, 344-351.

Springman, E.B., Angleton, E., Hansen, H.B., Van Wart, H.E., 1990. Multiple modules of activation of latent human fibroblat collagenase: Evidence for the role of Cys73

active-site zinc complex in latency and a cysteine switch mechanism for activation. Proceedings of the National Academy of Science, 364-368.

Springman, E.B., Nagase, H., Birkedal-Hansen, H., Van Wart, H.E., 1995. Zinc content and function in human fibroblast collagenase. Biochemistry 34, 15713-15720.

Steetler-Stevenson, W.G., 2008. Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. Science Signaling 1, re6.

Sternlicht, M.D., Werb, Z., 2001. How matrix metalloproteinases regulate cell behavior. Annual Reviews of Cell and Developmental Biology 17, 463-516.

Stickens, D., Behonick, D.J., Ortega, N., Heyer, B., Hartenstein, B., Yu, Y., Fosang, A.J., Schorpp-Kistner, M., Angel, P., Werd, Z., 2004. Altered endochrondal bone development in matrix metalloproteinase 13-deficient mice. Development 131, 5883-5895.

Strand, M.R., 2008. The insect cellular immune response. Insect Science 15, 1-14.

Strand, M.R., Pech, L., 1995. Immunological basis for compatibility in parasitoid-host relationships. Annual Review of Entomology 40, 31-56.

Strauss, B.H., Robinson, R., Batchelor, W.B., Chisholm, R.J., Ravi, G., Natarajan, M.K., Logan, R.A., Mehta, S.R., Levy, D.E., Ezrin, A.M., Keely, F.W., 1996a. *In vivo* collagen turnover following experimental ballon angioplasty injury and the role of matrix metalloproteinases. Circulation Research 79, 541-550.

Strauss, B.H., Robinson, R., Batchelor, W.B., Chisholm, R.J., Ravi, G., Natarajan, M.K., Logan, R.A., Mehta, S.R., Levy, D.E., Ezrin, A.M., Keely, F.W., 1996b. *In vivo* collagen turnover following experimental ballon angioplasty injury and the role of matrix metalloproteinases. *Circulation Research* 79, 541-550.

Strongin, A.Y., Collier, I., Bannikov, G., Marmer, B.L., Grant, G.A., 1995. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloprotease. Journal of Biological Chemistry 270, 5331-5338.

Takagi, M., Kikko, T., Hosoi, M., Hayashi, I., Toyohara, H., 2004. cDNA cloning of oyster matrix metalloproteinase and its possible involvement in hypoxic adaptation. Fisheries Science 70, 682-687.

Takahashi, C., Sheng, Z., Horan, T.P., Kitayama, H., Maki, M., Hitomi, K., Kiaura, Y., Takai, S., Sasahara, R.M., Horimoto, A., Ikawa, Y., Ratzkin, B.J., Arakawa, T., Noda, M., 1998. Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. Proceedings of National Academy of Science 95, 13221-13226.

Trenczek, T., 1998. Endogenous defense mechanisms of insects. Zoology 101, 298-315.

Truman, J.W., 1972. Physiology of insect rhythms. I. Circadian organization of the endocrine events underlying the molting cycle of larval tobacco hornworms. Journal of Experimental Biology 57, 805-820.

Uria, J.A., Jimenez, M.G., Balbin, M., Freije, J.M.P., Lopez-Otin, C., 1998. Differential effects of transforming growth factor-ß on the expression of collagenase-1 and collagenase-3 in human fibroblasts. Journal of Biological Chemistry 273, 9769-9777.

Van Tassel, R., Lyerly, D., Wilkins, T.D., 1992. Purification and characterization of an enterotoxin from *Bactroides fragilis*. Infection and Immunity 60, 1343-1350.

Vass, E., Nappi, A.J., 2001. Fruit fly immunity. Bioessays 51, 529-535.

Vilcinskas, A., Wedde, M., 2002. Insect inhibitors of metallproteinases. *IUBMB Life* 54, 339-343.

Vogel, W., Gish, G.D., Alves, F., Pawson, T., 1997. The discoidin domain receptor tyrosine kinases are activated by collagen. Molecular Cell 1, 13-23.

Vu, T.H., Werb, Z., 2000. Matrix metalloproteinases: Effect of development and normal physiology. Genes and Development 14, 2123-2133.

Wada, K., Sato, H., Kinoh, H., Kajita, M., Yamamoto, H., Seiki, M., 1998. Cloning of three *Caenorhabditis elegans* genes potentially encoding novel matrix metalloproteinases. Gene 211, 57-62.

Weaver, A., Goncalves da Silva, A., Nuttall, R.K., Edwards, D.R., Shapiro, S.D., Rivest, S., Yong, V.W., 2005. An elevated matrix metalloproteinase (MMP) in an animal model of multiple sclerosis is protective by affecting Th1/Th2 polarization. FASEB Journal 19, 1668-1670.

Wigglesworth, V.B., 1973a. The significance of "apolysis" in the moulting fluid of insect. . *Journal of Entomology* 47, 141-149.

Wigglesworth, V.B., 1973b. The significance of "apolysis" in the moulting fluid of insect. . *Journal of Entomology* 47, 141-149.

Willot, E., Trenczek, T., Thrower, L.W., Kanost, M.R., 1994. Immunological identification of insect hemocyte populations: monoclonal antibodies distinguish four major hemocyte type in *Manduca sexta*. European Journal of Cell Biology 65, 417-423.

Wilson, C.L., Ouellette, A.J., Stachell, D.P., Ayabe, T., Lopez-Boado, Y.S., Strasman, J.L., Hultgren, S.J., Matrisian, L.M., Parks, W.C., 1999. Regulation of intestinal

alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. Science 286, 113-117.

Woessner, F., 2002. MMPs and TIMPs-An historical perspective. Molecular Biotechnology 22, 33.

Woessner, J.F., Jr., 1998. The matrix metalloproteinase family., in: W.C.P.a.R.P. Mecham (Ed.), Matrix metalloproteinases. Academic Press, San Diego, 1-14.

Yamamoto, R.T., 1969. Mass rearing of the tobacco hornworm. III. larval rearing and pupation. Journal of Economic Entomology 62, 1427-1431.

Yoshida, S., Taniguchi, M., Suemoto, T., Oka, T., He, J.P., Shoska, S., 1998. cDNA cloning and expression of a novel serine protease, TLSP[1]. Biochimica Biophysica Acta:Gene structure and Development 1399, 225-228.

Yu, Q., Stamenkovic, I., 2000. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-ß and promotes tumor invasion and angiogenesis. Genes and Development 14, 163-176.

Yu, X.Q., Zhu, Y., Ma, C., Fabrick, J.A., Kanost, M.R., 2002. Pattern recognition proteins in *Manduca sexta* plasma. Insect Biochemistry and Molecular Biology 32, 1827-1834.

Zhang, S., Dailey, G., Kwan, E., Glasheen, B., Sroga, G., Page-McCaw, A., 2006. An MMP liberates the ninjurin A ectodomain to signal a loss of cell adhesion. *Genes and Development* 20, 1899-1910.

CHAPTER IV: GENERAL CONCLUSION

Our results suggest that MMP expression is high at the beginning of the instar while it reduces as the caterpillar reaches molting indicating that other proteases like chitinases and molting fluid proteinases play a more prominent role. However, the delay in the appearance of dorsal blood vessel along with the reduction of the body weight when MMPs are inhibited remains to be elucidated. Reduction of body mass below the required critical body weight will inhibit the larval to pupal metamorphosis and the larva will undergo molting leading to a bigger larval instar (Nijhout and Williams, 1974a). It remains to be studied whether the inhibition of MMPs will result in the reduction of body weight to less than the critical mass thus preventing the larval to pupal metamorphosis. Our results are in contrast to the upregulation of MMPs that was observed during the larval to pupal metamorphosis, a tissue remodeling during which excessive tissue degredation takes place (Altincicek and Vilcinskas, 2008) and RNAi of MMP resulted in the larval pupal intermediates (Knorr et al., 2009). Our results also suggest that *M. sexta* larvae have a reduced immunity to bacterial infections when MMPs are inhibited. This is due to the decline in the production of hemocytes from the hemopoietic organs and also due to inability of the hemocytes to phagocyte bacteria. It is shown that migration of the $CD4^+$ T-lympohcytes is reduced in MMP-3 knock-out mice (Li et al., 2004).

Collectively, inhibition of MMPs during the molting process indicates that the larvae by down-regulating the expression of MMPs prevent the elicitation of immune responses that is generally seen during the larval pupal metamorphosis. However the precise role of MMP/MMPs both in larval-larval molting and innate immunity remains to be elucidated for which identifying the complete sequence of MMP (/MMPs) and any

potential isoforms is essential. Once the complete sequence of MMP/MMPs in identified, MMP can be knocked-out and its role on larval-larval molting studied.

Even though it is clear that MMP expression increases during infection and that its inhibition results in the loss of insects' ability to mount an efficient immune response against infections, the exact molecular mechanism by which MMPs affect the immune modulators still need to be investigated. In order to identify the molecular mechanisms, it is essential that the substrates for the MMPs be identified. Once the substrates of these enzymes are identified, blocking of these substrates along with knocking out the MMP genes can give insights into how the MMPs function in immune cells of insects. Since unlimited up-regulation of MMPs is also detrimental to the insect as it will cause destruction of the surrounding tissue, it is also important to explore how they are down-regulated. Hence, identifying the endogeneous inhibitor of MMPs is necessary which can in turn be used to inhibit the MMPs for studying the molecular mechanisms of MMPs in innate immunity. Alternatively, MMPs can also be inhibited by RNA interference (RNAi) and the role of MMPs studied in response to highly pathogenic bacteria and fungi such as *Photorhabdus luminiscens* and *Aspergillus fumigatus* and effect of inhibition of MMPs on the production of antimicrobial peptides during their immune response can be studied.

4.1. References

Altincicek, B., Vilcinskas, A., 2008. Identification of lepidopteran matrix metalloproteinase with dual roles in metamorphosis and innate immunity. Develpomental Comparative Immunology 32, 400-409.

Knorr, E., Schmidtberg, H., Vilcinskas, A., Altinicicek, B., 2009. MMPs regualte both develpoment and immunity in the *Tribolium* model insect. PLoS One 4, e4751.

Li, C.K.F., Pender, S.L.F., Pickard, K.M., Chance, V., Holloway, J.A., Huett, A., Goncalves, N.S., Mudgett, J.S., Dougan, G., Frankel, G., MacDonald, T.T., 2004. Impaired immunity to intestinal bacterial infection in stromelysin-1 (matrix metalloproteinase-3)-deficient mice. Journal of Immunology. 173, 5171-5179.

Nijhout, H.F., Williams, C.M., 1974a. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): growth of the last instar larva and the decision to pupate. Journal of Experimental Biology 61, 481-491.