BASIC BIOLOGICAL CHARACTERS AND FUNCTIONS OF *PSEUDOPLUSIA INCLUDENS* PROTEIN PICYT1

by

SHU ZHANG

(Under the Direction of Michael Strand)

ABSTRACT

PiCyt1 is a protein which was identified from the hemocytes of *Pseudoplusia includens* (Lepidoptera: Noctuidae). This protein is the antigen of 43E9A10, a monoclonal antibody which recognizes specifically one type of hemocyte named plasmatocytes. In this project, I studied some basic biological characteristics and functions of this protein. The biological characters include the encoding cDNA and protein sequences, the expression tissue, transcriptional pattern in 5^{th} instar *P. includens* larvae, the influence of some immune challenges on the transcription and secretion of this protein. Based on the facts that PiCyt1 shares homology with a hemolytic protein from *Aspergillus fumigatus*, Asp-hemolysin, we hypothesize that it has a similar function. Here I transiently expressed PiCyt1 in selected insect cell lines and purified the recombinant protein through the attached 6xHis tag. The cytolytic activity assay of recombinant PiCyt1 was conducted against both prokaryotic and eukaryotic targets.

INDEX WORDS: Plasmatocyte, Plasmatocyte Spreading Peptide (PSP), *Pseudoplusia include*, pIZT/V5-His vector, cytolytic activity

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CHAPTER I

INTRODUCTION

The immune systems of multicellular animals can be divided into innate and acquired immunity. The innate immune system recognizes and kills foreign invaders by factors encoded by germline DNA, while the acquired immune system relies on rearrangement of somatic genes to produce receptors that recognize specific foreign antigens resulting in an immunological memory (Fearon, 1997). Insects lack an acquired immune system but have a well-developed innate response (Lavine and Strand, 2002). Innate immunity in insects is divided into two kinds of responses: humoral and cellular defense responses. Humoral defense refers to antimicrobial peptides and other soluble molecules in insect hemolymph that recognize and kill microorganisms (Lowenberger, 2001). Cellular defense involves the response of hemocytes to foreign targets. Hemocytes usually eliminate unicellular pathogens by phagocytosis while multicellular parasites are killed by encapsulation (Strand & Pech, 1995; Carton & Nappi, 2001). Normally, insect hemocytes circulate freely in the hemolymph. However, when hemocytes recognize an invader as foreign, they rapidly become adhesive to make a capsule around the target. Encapsulated organisms die in capsules but the mechanisms responsible for their death are poorly understood (Carton & Nappi, 2001; Lavine & Strand, 2002).

Previous studies with the moth *Pseudoplusia includens* (Lepidoptera: Noctuidae) resulted in production of antibody markers for identifying specific classes of hemocytes, methods for isolating these cells, and protocols for studying capsule formation in vivo and in vitro (Pech et al., 1994; Strand & Johnson, 1996; Pech & Strand 1996). With these techniques, it was found that encapsulation involves two kinds of hemocytes: plasmatocytes and granular cells.

Encapsulation begins when granular cells attach to the foreign target. This is followed by the attachment of a large number of plasmatocytes that form the bulk of the capsule. After the capsule has been made, granular cells bind to the outermost layer of plasmatocytes, which terminates the response (Lavine & Strand, 2002). A key factor regulating these events is the cytokine, plasmatocyte spreading peptide (PSP), which is produced by granular cells and fat body, and which activates plasmatocyte adhesion by inducing translocation of integrins to the cell surface. After the capsule is formed, the invader is always killed. Among the monoclonal antibodies generated against *P. includens* hemocytes, 43E9A10 is an antibody which specifically recognizes plasmatocytes. The antigen of 43E9A10, Picyt1, was identified recently. Based on sequence comparison, PiCyt1 shares significant amino acid identity with Asp-hemolysin, a cytolytic protein identified from *Aspergillus fumigatus*. Based on these data, I hypothesize that PiCyt1 is a cytolytic protein involved in killing encapsulated foreign organisms.

In the first part of this research project, I did experiments to study the basic characters of PiCyt1, including spatio-temperal patterns of expression in *P. includens* larvae, the effects of PSP and plasma on inducible expression, and the effects of PSP and microbial challenge on PiCyt1 secretion. These results are discussed in Chapter III. In the second part of this project, I expressed and purified recombinant PiCyt1 from *Drosophila* S2 cells. The cytolytic ability of this recombinant protein was tested against Gram negative bacteria, Gram positive bacteria and red blood cells from different animals. These studies are discussed in Chapter IV.

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CHAPTER II

LITERATURE REVIEW

Before discussing the results of my thesis project, I first briefly review important background information.

Insect immune system

Most multicellular organisms have an immune system which is very important in defense against invading pathogens. In mammals and other vertebrates, the immune system is divided into innate and acquired responses. Innate immunity depends on germline-encoded proteins, such as some antimicrobial peptides, to recognize and eliminate foreign invaders. Acquired immunity produces antibodies against invading organisms by the rearrangement of somatic genes in B and T lymphocytes. By this mechanism the acquired immune system can produce an unlimited array of antibodies and retain immunological memory to invaders. These two systems work together in vertebrates. Unlike vertebrates, insects are thought to lack an acquired immune system but possess a well-developed innate response (Fearon, 1997).

The innate immune systems of insects and vertebrates share several features. These include the expression of similar pattern recognition receptors (PRRs) that recognize pathogens via binding of pathogen-associated molecular pattern (PAMP) molecules. The innate immune system of insects and vertebrates is further divided into humoral and cellular immunity. In humoral immune responses, antimicrobial peptides and proteins are synthesized by fat body, midgut and hemocytes to kill the invading microorganisms. Cellular immunity refers to that aspect of the immune system in which certain types of hemocytes are responsible for recognizing

and eliminating invaders (Strand & Pech, 1995). In the process of an insect immune response, these two systems do not function separately. Some hemocytes secrete humoral factors and some humoral factors affect the behavior of hemocytes in cellular immunity (Lavine & Strand, 2002). These two immune responses also cooperate in the recognition of foreign invaders (Lavine & Strand, 2002). In the past few years, researchers have made important progress in characterizing the humoral immune system. Many immune molecules, particularly as it relates to humoral defenses, have been identified and many important pathways have also been clarified. Progress in studying the cellular immune response has been slower, however, primarily due to the small size of insects and limited number of hemocytes in circulation. Reseachers in this field have also encountered difficulties separating and identifying hemocytes. The improvements in the methods of collection and separation of different types of hemocytes have been made in some insect species. Antibodies and other markers have also been developed to identify different types of hemocytes, making it possible to study in greater detail the mechanisms of the cellular immune response (Strand & Johnson, 1996).

Organs involved in immunity

The first line of defense in insects against pathogens is their cuticle which functions as a physical barrier against invading pathogens. If pathogens succeed in entering the hemocoel, several humoral and cellular defenses protect the insect.

In those immune reactions, the fat body plays a very important role by synthesizing a number of immune molecules including antimicrobial peptides. Hemocytes (blood cells) are also an important component of the insect immune system. Hemocytes synthesize regulatory factors and

antimicrobial peptides and also are essential for phagocytosis, nodulation and encapsulation. Lepidopterans produce five types of hemocytes: plasmatocytes, granular cells, prehemocytes, oenocytoids and spherule cells. Plasmatocytes are large flat cells capable of spreading, movement, and aggregation. Granular cells contain numerous digitations and pinocytic vesicles in their cytoplasm. These two cell types comprise more than half of the total hemocytes and are responsible for most immune responses in Lepidoptera larva. Prohemocytes are spherical cells with a high nuclear/cytoplasmic ratio and are normally very rare in the circulatory blood. They are considered to be the stem cells of other types of hemocytes. Oenocytoids are normally round or oval cells with numerous ribosomes in their cytoplasm. Oenocytoids also contain high levels of phenoloxidase, which is involved in regulating melanization of hemolymph. Spherule cells contain large-cytoplasmic inclusions and are suggested to have transport functions. (Drif & Brehelin, 1993).

Innate immune responses in insects

Humoral immunity in insects relies on hemolymph components and eliminates invaders by hemolymph coagulation, melanization or antimicrobial peptides. Cellular immunity refers to immune responses mediated by hemocytes. Small targets such as microbes may either be phagocytosed or inactivated by a multicellular process referred to as nodulation. Large multicellular invaders, upon entering the insect hemocoel, induce a process referred to as encapsulation. Both humoral and cellular defenses proceed through similar steps that include non-self recognition, signal transduction and elimination of pathogens. Below, I discuss each of these steps.

1. Non-self recognition

Non-self recognition is an important step for insects to initiate the immune responses against foreign invaders. The process is complex and not well understood. In vertebrates, the immune system has developed pattern-recognition receptors (PRRs), which bind to pathogen-associated molecular pattern (PAMP) molecules produced by some microorganisms such as bacteria and fungi (Fearon, 1997).

In insects, some PRRs have also been identified that recognize molecules present on the surface of prokaryotes and fungi, such as LPS, peptidoglycan (PG), lipoproteins and CpG DNA (Medzhitov & Janeway, 1997). These candidate PRRs include lectins, hemolin, LPS-binding protein, Gram-negative bacteria binding protein (GNBP, also called β-glucan recognition protein, βGRP) and peptidoglycan recognition protein (PGRP). (Schmidt et al., 2001).

Insect PGRPs are divided into Short (such as PGRP-SA, -SB, -SC and -SD) and Long classes (such as PGRP-LA, -LB, -LC, -LD and -LE) on the basis of size (Tanji & Ip, 2004). Short-class PGRPs are usually extracellular peptides while long-class PGRPs are intracellular or transmembrane. These two classes of PGRPs are involved in recognition of Gram-positive or Gram-negative bacteria and activate downstream Toll or Immune deficiency (Imd) pathways respectively. Experiments showed that the third amino acid residue of the PG peptide bridge on the bacteria surface was important for this discrimination. For Gram-negative bacteria, the presence of meso-diaminopimelic (DAP) residues in this position activates PGRP-LC binding while for Gram-positive bacteria, the replacement of a lysine in the third amino acid position can selectively activate PGRP-SA binding (Stenbak et al., 2004). In the process of recognizing

Gram-positive bacteria, another PRR called Gram-negative binding protein-1 (GNBP-1) is also required. GNBP-1, perhaps named mistakenly, cooperates with PGRP-SA in recognition of Gram-positive bacteria and activating the Toll pathway (Gobert et al. 2003). Unlike Gram-positive bacteria, fungi activate the Toll pathway via a PGRP-SA/GNBP-1 independent pathway. This pathway involves a serine protease, Persephone, and a protease inhibitor, Necrotic (Ligoxygakis et al., 2002). Besides its important role in recognition of Gram-negative bacteria, PGRP-LC is also involved in some cellular defenses including phagocytosis of *E. coli* and development of *Plasmodium* inside the mosquito (Michel & Kafatos, 2005).

Another PRR candidate in insects is hemolin, a lepidopteran protein that can interact with Gram-negative bacteria LPS and lipoteichoic acid on the surface of Gram-positive bacteria (Yu & Kanost, 2002). Meanwhile, hemolin can also bind to hemocytes in a calcium-dependent manner, which suggests that it might increase phagocytosis efficiency (Kanost & Zhao, 1996).

In mammals, C-type lectins are a family of carbohydrate-binding proteins which can activate the complement pathway when binding to the ligands. It can also increase the phagocytosis of bacteria (Kawasaki et al., 1989). C-type lectins are also found in insects. In some lepidopteran and cockroaches, C-type lectins can increase phagocytosis, nodulation and activation of prophenoloxidase (Yu et al., 2002).

Integrins are another class of cellular PRRs that participate in recognition of microorganisms. These proteins are transmembrane proteins containing a (α, β)-heterodimer. Integrins are thought to be an important part of several signal transduction pathways. Binding to excellular factors induces conformation changes in integrins and thus induces changes in cells by affecting certain kinase cascades. The ligands of integrins include the Arg-Gly-Asp (RGD) tripeptide and related peptides (Gonzalez-Amaro & Sanchez-Madrid, 1999). There are five α–

and two β–subunits and three complete integrins in *Drosophila* whose functions have begun to be characterized in immunity (Hynes & Zhao, 2000).

Besides immune responses to microorganisms, which are genetically far different from arthropods, insects also possess an immune system which reacts with more phylogenetically similar organisms. These include other insect tissues and parasitoid eggs (Strand & Pech, 1995). The mechanism of non-self recognition toward these entities is not well understood. Working with Drosophila mutants about twenty years ago, researchers found that hemocytes encapsulated self tissues lacking an intact basement membrane. Transplanting of self-organs without an intact basement membrane into the insect hemocoel also caused encapsulation (Rizke & Rizke, 1984). From these results, we can conclude that there must be some characters of the basement membrane that are important in preventing activation of the immune response. Unfortunately, few molecules involved in recognition of self or related insect tissues have been identified. Among them, a candidate receptor called Croquemort (CRQ), which may play a role in non-self recognition, was identified from *Drosophila*. Experiments with a CRQ null mutant showed that this protein was essential for phagocytosis of apoptotic corpses but is not required for engulfment of bacteria (Franc et al., 1999). Some other evidence indicates that the structure of sugars on the basement membrane is important for non-self recognition (Schmidt et al., 2001).

2. Signal transduction

After the PRRs recognize a foreign invader, a series of immune reactions are activated. In the humoral immune response, the major final product is antimicrobial peptides whose

transcription is regulated by NF-kB transcriptional factors. There are two types of pathways which can lead to the activation of NF-kBs: the Toll pathway and the Imd pathway.

Toll-like receptors (TLRs) which are also found in mammals, can interact directly with pathogens. In *Drosophila*, there are a total of 9 Toll receptors. Unlike mammallian TLRs, insect Toll receptors have additional functions in development. They are expressed in many tissues throughout all developmental stages, and are very important in the dorsal-ventral axis determination (Gerttula et al., 1988). In the innate immune response, the Toll pathway is activated by Gram-positive bacteria and fungi. The infection of Gram-positive bacteria triggers a protease cascade and leads to a truncated form of Spatzle, which is the ligand of the Toll receptor. Infection by fungi can also induce this cascade through involvement of Persephone and Necrotic and finally truncated Spatzle.

Spatzle is a 41 kDa protein that when synthesized forms a homodimer (DeLotto & DeLotto, 1998). This factor is activated by proteolysis and is converted to a C-terminal 106 amino acid form. This proteolyzed form of Spatzle can trigger the dimerization of Toll receptors and therefore active the downstream pathway (Tanji & Ip, 2005).

Following activation of the Toll receptor, three cytoplasmic proteins, MyD88, Tube and Pelle are involved in activation of the downstream NF-kB factors. First, MyD88 binds to the active Toll receptor and then transforms into the active form. Subsequently, MyD88, Tube and Pelle form a complex which can lead to transphosphorylation of Pelle and thus activate the kinase activity of Pelle (Horng & Medzhitov, 2001; Shen & Manley, 2002). Following activation, Pelle kinase interacts with cytoplasmic Dorsal-Cactus and Dif-Cactus complexes. Dorsal and Dif are NF-kB transcriptional factors which are inactive when bonnd to the IkB Cactus (Haffmann, 2003). Phosphorylation by Pelle kinase, however, causes Cactus to

dissociate from Dorsal and Dif which in turn are free to translocate into the nucleus and induce expression of antimicrobial peptides, like drosomycin.

Unlike the Toll pathway, the Imd pathway is induced by Gram-negative bacteria infection. The PRRs in this pathway are PGRP-LC and –LE complex, which are regarded as transmembrane factors (Takehana et al., 2004). After the binding of peptidoglycan from Gram-negative bacteria with PRR complex, the cytoplasmic domain of PRRs interacts with Imd factor. There are several signal factors that are activated by Imd to regulate the transcription of the drownstream antimicrobial peptides. Transforming growth factor-β-activated kinase1 (TAK1) can be activated and interact with downstream IKK complex (Vidal et al., 2001). IKK complex subsequently cleaves an NF-kB factor Relish. Without immune challenge, Relish is present in the cytoplasm as the inactive form with C-terminal IkB-like ankyrin repeats. When activated by the Imd pathway, the IkB domain is cleaved and the remaining Relish domain translocates into the nucleus and activates transcription of antimicrobial peptides (Stoven et al., 2000). Another pathway involves association of the Fas-associated protein, FADD, and the caspase Dredd, which can also regulate the activity of Relish (Hu & Yang, 2000).

Compared with signal transduction pathways in humoral immunity, the mechanism of signal transduction in cellular immunity is not well understood. Recent studies indicate that the JAK/STAT pathway might play a role in cellular immunity. This pathway was first found in flies for its function in embryonic segmentation (Binari & Perrimon, 1994). By binding to the ligand, Upd, the receptor of this pathway, Dome, actives downstream JAK proteins. JAK proteins possess kinase domains and can active STAT which acts as a transcription factor when its C-terminal tyrosine is phosphorylated (Agaisse & Perrimon, 2004). One of the target genes of the JAK/STAT pathway is *dTEP1* which encodes a PRR, TEP1 (Lagueux et al., 2000). TEP1

can increase the phagocytosis of both Gram-positive and Gram-negative bacteria by binding to their surface (Michel & Kafatos, 2005). Besides this, the JAK/STAT pathway can also influence hemocyte differentiation and proliferation following immune challenge (Agaisse & Perrimon, 2004).

During the process of encapsulation, it is well known that epidermal tissue, fat body and hemocytes release factors that activate hemocytes and make them adhere to foreign targets (Chain & Anderson, 1982; Geng & Dunn, 1988). These factors include hemokinin, a protein that is released by epidermal cells and involved in wound healing (Cherbas, 1973), and encapsulation promoting factor (EPF), a factor that was found in the lepidopteran *Heliothis virescens* with the function of activating hemocytes to form capsules (Davies et al, 1988). A similar factor called plasmatocyte spreading peptide (PSP) was identified from another lepidopteran species, *Pseudoplusia includens* (Clark et al., 1997). PSP belongs to the ENF cytokine family and induces plasmatocytes spreading, movement and aggregation.

3. Killing of invaders

The final phase of an immune response is killing the invading pathogen. In humoral immunity, pathogens are cleared by secreted antimicrobial peptides while in cellular immunity the foreign invaders are eliminated by phagocytosis, nodulation or encapsulation.

Antimicrobial peptides (AMPs) are widely present not only in insects but also in plants and other animals, including mammals (Bulet & Stocklin, 2005). In holometabolous insects, AMPs are synthesized by fat body and released into hemolymph where they can be transported throughout the body (Bulet et al., 2003). In hemimetabolous insects, AMPs are synthesized

mainly in hemocytes (Lamberty et al., 2001). There are some common characters of insect AMPs, such as a low molecular weight (usually below 5 kDa), a positive charge at physiological pH, and the presence of amphiphilic α-helices or hairpin-like β–sheets (Bulet et al., 1999). Based on the spectrum of antimicrobial activity, the AMPs can be divided into three groups: Drosomycin and Metchnikowin are against fungi, Diptericin, Drosocin, Cecropins and Attacins are mainly used to kill Gram-negative bacteria and Defensin is the only antimicrobial peptide whose target is Gram-positive bacteria (Imler & Bulet, 2005). Based on the amino acid component and structural characters, AMPs can also be divided into three types: (1) linear peptides with α–helices and no cysteines; (2) Cyclic peptides with cysteines and (3) peptides with an overexpression of proline or glycine (Bulet et al., 1999).

Cecropins are a large group of antimicrobial peptides belonging to the α–helical linear AMP family. The first antimicrobial peptide identified from insects belongs to this group (Steiner et al., 1981). In a hydrophobic environment, Cecropins possess an N-terminal amphipathic α–helix and a more hydrophobic C-terminal α–helix (Holak et al., 1988). Structure-activity relationship studies indicate that charge, helicity, and hydrophobicity affect antimicrobial activity (Tossi et al., 2000). The mechanism of antimicrobial activity for cecropin is that it kills bacteria by disabling transmembrane electrochemical ion gradients (Silvestro et al., 1997).

The second class of AMPs is cysteine-rich peptides, such as defensins, drosomycin and thanatin. This class can form a compacted secondary structure by intramolecular disulfide bonds. All insect defensins have 6 cysteines that form three conserved disulfide bridges (Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6), which give insect defensins the secondary structure of αββ (Hetru et al., 1998; Bulet & stocklin, 2005). The mechanism of killing gram-positive bacteria

Micrococcus luteus has been studied. First, the defensin peptide destroys the permeability of the bacteria cytoplasmic membrane which induces the loss of potassium in the cell, followed by depolarization of the inner membrane and the loss of ATP. These events then result in inhibition of respiration (Cociancich et al., 1993).

Unlike defensins, drosomycin has 8 cysteines in the peptide and therefore has one more disulfide bond. The extra disulfide bond makes the molecule form a more compact βαββ structure which may be the reason for the protease resistance of this peptide (Michaut et al., 1996). The mechanism underlying the antifungal activity of drosomycin remains unknown but the mechanism of plant defensins, which have a consensus sequence similar to drosomycin has been studied. Plant defensins induce calcium influx and potassium efflux in fungi by changing membrane potential (Bulet et al., 1999).

Another cysteine-containing AMP is thanatin, which has only 2 cysteines and possesses a very simple secondary structure. The C-terminus is an antiparallel two stranded β-sheet structure which forms a hairpin-like structure while the N-terminus is a long chain. The disulfide bond connects the two β-sheets to maintain structure (Mandard et al., 1998). Thanatin is the only insect AMP which is found to have antimicrobial activity toward Gram-positive, Gram-negative and also fungi, but its mode of action is unknown.

The third class of AMPs is proline-rich peptides whose amino acid content consists of more than 25% proline residues. Proline residues often associate with arginine or histidine residues to form doublets or triplets which can be distributed evenly in the whole peptide sequence. Some of the proline-rich peptides are glycosylated, such as drosocin (Bulet & Stocklin, 2005).

Drosocin is an O-glycosylated AMP and was first identified from *Drosophila* (Bulet et al., 1993). Drosocin has antimicrobial activity against most Gram-negtive bacteria and also some

Gram-positive bacteria, and the glycan moiety seems important in its activity (Bulet et al., 1999). Although there were some experimental results indicating that the stereoselective element may play a role in the antimicrobial activity of drosocin, the detailed mechanism of its activity is unknown. Glycosylation also makes it difficult to solve the secondary structure of drosocin.

Besides all the antimicrobial peptides, insects can also eliminate invading targets by hemocyte-mediated phagocytosis, nodulation or encapsulation. Encapsulation is a very important process which is used to deal with large targets, such as parasitoid eggs. By using antibodies towards different hemocyte types, encapsulation in Lepidoptera has been studied in detail. In *P. includens*, the capsule is made mainly by plasmatocytes and granular cells. Encapsulation is initiated when granular cells recognize and attach to the target surface. Once the encapsulation starts, plasmatocytes will bind to foreign target and form the multilayer sheath around it. After the capsule is formed, granular cells will bind to the outside of it to terminate the whole process (Lavine & Strand, 2002).

Once capsule formation finishes, the target organism always dies. One important process involved in this target-killing event is melanization. In fact, melanization is not only used in encapsulation, it is also used in phagocytosis and humoral immunity. In addition to melanin, melanogenesis results in production of several cytotoxic molecules, including reactive intermediates of oxygen (ROI) and nitrogen (RNI).

Melanogenesis is the process by which monophenols and diphenols are oxidized and polymerized into melanin (Sugumaran, 2002). Melanogenesis is induced by several types of pathogens and invading parasites. The understanding of melanin synthesis in insects is based on the mammalian system. In mammals, the first step of melanogenesis is the hydroxylation of phenylalanine to tyrosine which is catalysed by phenylalanine hydroxylase. After that, tyrosine

is hydroxylated into dopaquinone by tyrosinase (TYR). Dopaquinone can react with thiol to form pheomelanins which is a yellow to reddish brown pigment (Napoliano et al., 2000). If there is no thiol, dopaquinone can undergo a series of reactions and finally produce DHI eumelanin or DHICA eumelanin.

In insects, one important enzyme in melanogenesis, Phenodoxidase (PO), has been identified. PO is the TYR-like molecule which can catalyze tyrosine hydroxylation into dopaquinone. PO is first synthesized as an inactive form prophenoloxidase, which can be activated by a serine protease. The activation of the serine protease requires additional upstream proteases which are activated by immune challenge (Cerenius & Soderhall, 2004). Besides the final products pheomelanin and DHI, some additional toxic molecules are generated during melanogenesis. The melanin synthesis process involves the one-electron redox reaction of quinones and semiquinones, and also produces reactive oxygen (ROI) and nitrogen (RNI) which are toxic (Nappi & Christensen, 2005). Since insects have an open circulatory system, it is very important for melanogenesis to be site specific. One hypothesis for this site specific mechanism is that during the melanogenesis reactions, the enzymes can bind to some substrate-like molecules which are expressed on the target surfaces and therefore the cytotoxic products of these enzymes can be located specifically to the target.

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CHAPTER III

BIOLOGICAL CHARACTERS OF *PSEUDOPLUSIA INCLUDENS* PROTEIN PICYT1[1]

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Abstract

Pseudoplusia includens (Lepidoptera: Noctuidae) produces five types of hemocyes that are distinguished by morphological, molecular and functional characters. Plasmatocytes are one of the most important hemocyte types and there are two subpopulations of plasmatocytes in circulation. One of these subpopulations is required for encapsulation and is recognized by monoclonal antibody 43E9A10. Here I report that the antigen of this antibody is a 22 kDa protein named PiCyt1. Northern blot and *in situ* hybridization experiments indicated that PiCyt1 is expressed specifically in plasmatocytes. The transcriptional abundance of *PiCyt1* mRNA in 5^{th} instar *P. includens* larva is high in the middle of the instar and low at two edges of the instar. The transcriptional abundance of *PiCyt1* decreases rapidly after hemocytes are plated and plasmatocyte spreading peptide (PSP) or plama does not affect this decrease. The secretion level of this protein is constant and neither the addition of PSP nor Gram-negative bacteria *E. coli* affects this level. Together, these data suggest that PiCyt1 is the antigen of 43E9A10 and is a plasmatocyte specific protein whose transcriptional abundance in 5^{th} instar larvae varies with time. The transcriptional abundance and secretion level is not affected by known immune inducing factors in vitro.

Key words

Plasmatocyte, Plasmatocyte Spreading Peptide (PSP), *Pseudoplusia includens.*

Introduction

Insects lack an acquired immune system but have a well-developed innate response which recognizes and kills foreign targets by factors encoded by germline DNA (Lavine and Strand, 2002). Innate immunity in insects is divided into humoral and cellular defense. Humoral defense refers to antimicrobial peptides and other soluble molecules in insect hemolymph that recognize and kill microorganisms (Lowenberger, 2001). Cellular defense involves the response of hemocytes to foreign targets. Hemocytes usually eliminate unicellular pathogens by phagocytosis while multicellular parasites are killed by encapsulation (Strand & Pech, 1995; Carton & Nappi, 2001). Normally, insect hemocytes circulate freely in the hemolymph. However, when hemocytes recognize an invader as foreign, they rapidly become adhesive to make a capsule around the invader. It is well known that the encapsulated organisms die in capsules but the mechanisms responsible for their death are largely unknown (Carton & Nappi, 2001; Lavine & Strand, 2002). Although the literature often discusses humoral and cellular immunity separately, in reality these systems are highly integrated. For example, hemocytes secrete humoral factors and some humoral factors affect the behavior of hemocytes in cellular immunity (Larvine & Strand, 2002).

Based on morphology and labeling patterns using monoclonal antibody (mAb) markers, the hemocytes of *P. includens* are divided into granular cells, plasmatocytes, spherule cells, oenocytoids, and prohemocytes (Pech et al, 1994; Strand & Johnson, 1996). Using these antibodies as markers, previous studies determined that two types of hemocytes are required for capsule formation. At the beginning of encapsulation, granular cells recognize and attach to the surface of the foreign target. Subsequently, granular cells recruit plasmatocytes recognized by

the mAb 43E9A10 attach by releasing a cytokine called plasmatocyte spreading peptide (PSP). Overlapping layers of 43E9A10 positive plasmatocytes then form a multilayer sheath around the target organism (Pech & Strand, 1996).

PSP is expressed in granular cells and fat body as a 142-residue propeptide that contains the PSP sequence in the final 23 amino acids of the C-terminus (Clark et al., 1998). The PSP propeptide is then cleaved by an unknown protease, thereby releasing the mature active PSP. The N-terminus of PSP contains a disordered chain (residues 1-6) and the remaining portion (residues 7-23) is highly structured. The structured region is stabilized by a disulfide bond between two cysteines and a B-hairpin structure (Volkman et al., 1999). Further studies also suggest PSP activates 43E9A10 positive plasmatocytes by mobilizing the translocation of integrins to the cell surface (Lavine & Strand, 2003). Addition of the ionophores Ionomycin and A23187 significantly inhibits plasmatocyte spreading, indicating that calcium ions are required for plasmatocyte activation. Reciprocally, forskolin, which activates adenylate cyclase, also induces plasmatocyte spreading, suggesting that cAMP also plays a role in the PSP signal transduction pathway (Zhang et al., unpublished).

The formation of a capsule ends when granular cells attach to the outermost layer of plasmatocytes and form a new basement membrane (Pech & Strand, 1996). The target inside the capsule dies after the encapsulation. As described in Chapter II, ROI and RNI are thought to play roles in killing encapsulated targets. However, other effector molecules such as antimicrobial peptides also play a role. Given that only plasmatocytes labeled by 43E9A10 participate in capsule formation, we sought in the current study to identify the antigen recognized by this antibody. Here I report that 43E9A10 recognizes a 23 kDa protein designated PiCyt1.

Materials and methods

Insect rearing and hemocyte collection *P. includens* larvae were reared on artificial diet (Strand, 1990) in 30 ml plastic cups with paper lids at 27±1ºC and a 16 hr light-8 hr dark photoperiod. Larvae were anesthetized with CO_2 and the surface was sterilized with 95% ethanol before bleeding. Blood was obtained through an incision in the cuticle and collected in 1 ml anticoagulant buffer (98 mM NaOH, 186 mM NaCl, 17 mM EDTA, 41 mM Citric acid, pH 4.5). After a 30 min incubation at 4^{o} C, hemocytes were collected by centrifugation and resuspended in the Excell medium. Plasmatocytes were purified on 46% /72.2% Percoll cushions (Pech et al., 1994).

DNA cloning and sequencing A cDNA expression library was constructed by synthesizing double-stranded cDNAs from polyadenylated mRNA isolated from *P. includens* hemocytes. cDNAs were then directionally cloned into ZAP II Lambda arms (Stratagene) and propagated in XL1-Blue strain cells. The library was screened using mAb 43E9A10, as previously outlined (Clark et al., 1998). Clones of interest were bidirectionally sequenced using the chain termination method and Abi Prism cycle sequencing kit (Perkin Elmer). Sequence analysis was performed using DNA Star (DNA Star Inc.) and the BLASTX program (NCBI).

Northern blotting cDNA probes were labeled to high specific activity by random priming in the presence of ^{32}PdCTP. Total RNA isolated from larval tissues or hemocytes was size fractionated on 6% formaldehyde-0.9% agarose gels (2.0 µg/lane) and transferred to nylon in 10x SSC. Northern blots were prehybridized for 2 h in the same hybridization buffer and probe

was added to filters at 1 x 10^6 cpm/ml and hybridized for 18 h at 60° C. Filters were washed at high stringency in 0.2x SSC, 1.0% SDS for 1 h at 60°C and autoradiographed.

***In situ* hybridization** Antisense RNA probes were synthesized by T7 RNA polymerase in 20 μl reaction with *PiCyt1* cDNA as template as previously described (Donnell et al., 2004). Sense probe was used as a control. Hemocytes were collected from 48 hour 5th instar *Pseudoplusia includens* larvae. The hemocytes were fixed in 4% paraformaldehyde in PBS for 20 minutes and permeablized in 5 μg/ml proteinase K in PBS for 90 seconds. The hemocytes were prehybridized at 55°C for 1 hour in 100 μg/ml salmon sperm and incubated with 50 μl RNA probe (50x) over night. After washing in PBST, the cells were incubated in PBST/α-Digoxigenin-alkaline phosphatase conjugated antibody (1: 2000) (The Jackson laboratory, Molecular Probes) for 1 hour and visualized using 1 ml RNA-RP buffer plus 4.5 μl NBT and 3.5 μl x-phosphate overnight. The stained hemocytes were examined using a Leica TCS microscope.

Real time-PCR To study the transcriptional pattern of PiCyt1 in 5th instar *P. includens* larvae, total RNA was isolated from hemocytes collected at 12, 36, 48 and 60 hour old 5th instar larvae using the High Pure RNA Isolation kit (Roche company). To study the influence of PSP or plasma on transcript abundance of PiCyt1, RNA samples were collected from *P. includens* hemocytes incubated with 10 μM PSP or 5 % plasma at 0 hr, 4 hrs, 8 hrs and 12 hrs. First strand cDNA was synthesized from total RNA using superscript III Reverse Transcriptase. The relative quantitative real-time PCR (rqRT-PCR) reactions were run using hemocyte cDNA as template and PiCyt1 specific primers (forward primer: 5'-CGATTCGAAAGGTAAAAGAGC-3'; reverse

primer: 5'-ATCCGCCAGCATCAA-ATC-3'). Each 10 μl reaction contained 4 μl template cDNA and 5 μl iQ^{TM}SYBR'Green Supermix (BioRAD). Final primer concentrations were 250 nM. Reactions were run on a Rotor Gene 2000 Real Time-PCR cycler (Corbett Research) for 45 cycles with denaturation at 95°C for 20 seconds, annealing at 50°C for 20 seconds and extension at 72°C for 20 seconds. All reactions were done in quadruplicate. The control template of the PCR reaction was 18s Ribosomal RNA. ΔΔCt was calculated and related transcription level of PiCyt1 was determined as described by Lavine & Strand (2003). The transcript abundance at the first selected time point was standardized to 1.0 and the transcript level at other time points were expressed as an increase or decrease relative to this standard.

Western blotting Plasmatocytes were collected and purified from 48 hours *P. includens* 5th instar larva and then incubated with PSP or challenged by *E. coli* in TC-100 medium. Final PSP concentration of PSP treatment was 10 μM and the final concentration of dead *E. coli* was 32,000 cells/μl. Time points were 0, 2 and 8 hours. The supernatant of the incubation solution was collected and added to sample loading buffer. The protein samples were then size fractionated on 8-16% gradient SDS gels and transferred to nitrocellulose membranes. After blocking using 5% dry milk PBST solution, membranes were incubated with 43E9A10 (1: 100) followed by an HRP-conjugated goat anti mouse secondary antibody (1: 2000) (Jackson labs). The membrane was visualized using a chemiluminescent substrate (Amersham).

Results

1. mAB 43E9A10 recognizes a protein designated as PiCyt1

Using the antibody 43E9A10 to screen a *P. includens* hemocyte cDNA library resulted in identification of multiple clones that each contained an 0.9 kb insert. Sequencing identified a 633 bp open reading frame that coded for a 209 amino acid protein with a predicted molecular mass of 22.7 kDa named PiCyt1 (Fig 3.1A). Sequence analysis indicated that PiCyt1 had a 25 amino acid hydrophobic domain at the N-terminus which was a typical eukaryotic signal peptide. Besides this domain, PiCyt1 had two other hydrophobic regions. One region was between Ile^{41} and Trp^{76} while the other was between Ile^{197} and Pro^{209}. There were also two putative N-glycosylation sites in PiCyt1 identified which start at Asn^{56} and Asn^{120} respectively. Sequence comparison revealed that PiCyt1 shared modest but significant homology with the cytolytic proteins Asp-hemolysin from the fungus *Aspergillus fumigatus*, and *cbm*17.1 and *cbm*17.2 from *Clostridium bifermentans* with BLAST e value 10^{-6} and 10^{-4}, respectively (Fig 3.1B). Although cbm17.1 and cbm17.2 have not been found to have cytotoxicity to sheep and horse red blood cells (Barloy et al., 1998), Asp-hemolysin has strong hemolytic activity against erythrocytes (Yokota et al., 1977).

2. *Picyt1* is specifically expressed by plasmatocytes

To assess where *Picyt1* was expressed, Northern blotting and *in situ* hybridization experiments were conducted. A 0.9 kb mRNA was detected in hemocytes but no signal was

detected in testes, salivary glands, Malpighian tubules, nervous tissue, gut, or fat body (Figure 3.2 A). Additional Northern blotting experiment against RNA from different hemocyte types further suggested that *Picyt1* was expressed specifically in plasmatocytes (Figure 3.2B). *In situ* hybridization experiment confirmed this result. The only type of hemocyte that hybridized with a *PiCyt1* antisence probe was plasmatocytes (Figure 3.2C). In contrast, *PiCyt1* sense probe did not hybridize to any hemocyte type. These results indicated that the *Picyt1* is specifically transcribed in plasmatocytes. These results also corroborate previous studies indicating that mAB 43E9A10 specifically labels plasmatocytes (Strand & Johnson, 1996).

3. Transcription level of PiCyt1 was higher at the middle of 5^{th} instar

To determine whether *PiCyt1* transcript abundance varies in plasmatocytes, I conducted relative quantitative real time PCR (rqRT-PCR) experiments using hemocyte RNA from 12, 36, 48 and 60 hours 5^{th} instar larvae as templates. Results indicated that *PiCyt1* transcript abundance increased to a maximum at 36 hours and declined to very low levels at 60 hours immediately prior to pupation (table 3.1A and figure 3.3A).

4. Neither PSP nor *E. coli* challenge affects transcription or translocation of PiCyt1 in plasmatocytes

To test whether PSP or some other factors in plasma affect PiCyt1 transcript abundance, hemocytes were placed into primary culture with 10 μM PSP, 5 % plasma, or TC100 medium alone. RNA was then isolated from cells 0-12 hours later and transcript abundance of *PiCyt1*

was measured by rqRT-PCR. The results of this experiment indicated that *PiCyt1* transcription levels decreased rapidly in all treatments (Table 3.1B and Figure 3.3B). Given that there was a signal peptide on the N-terminus of PiCyt1 and it was probably a secreted protein, we also tested the influence of PSP or *E. coli* challenge on secretion level of PiCyt1. My results indicated that there was a constant level of PiCyt1 detected outside the cells and there was no significant change when incubated with PSP or challenged by *E. coli* (Figure 3.4A, B).

Discussion

Plasmatocytes play a very important role in insect immune responses. They are the main cell type which make the capsule around the foreign invaders during encapsulation. The encapsulated target always die after encapsulation, so there must be some factors produced by plasmatocytes which are responsible for this target-killing process.

In this study, we characterized the antigen recognized by mAB 43E9A10. By screening a hemocyte cDNA expression library with 43E9A10, positive clones were identified and a 0.9 kb cDNA was characterized. This cDNA had an open reading frame of 633 bp and the deduced animo acid sequence had a molecular weight of 22.7 kDa. Using 43E9A10 to probe the total proteins from hemocytes, we found that one protein about 24 kDa, PiCyt1, was specifically labeled (data not shown). All the results confirmed that PiCyt1 was the antigen recognized by 43E9A10.

From the results of the sequence comparison, we found that PiCyt1 shares significant homology with Asp-hemolysin and cbm17.1. The function of cbm17.1 is unknown, while Asp-hemolysin has cytolytic activity to erythrocytes. Some studies showed that Asp-hemolysin binds

to low density lipoprotein (LDL) on the target cell surface and this binding ability is essential for its cytolytic activity. There was a negatively charged region from Asp^{34}-Asp^{45} which was similar to the cysteine-rich region in the ligand binding domain of the LDL receptor (Ebina et al., 1994). Compared with Asp-hemolysin, in the corresponding region of PiCyt1 (from Asp^{112} to Asn^{120}), this character was not so obvious. There were only 3 negatively charged residues while in Asp-hemolysin there were 6 (data not shown).

The Northern blotting and *in situ* hybridization experiments both indicated *Picyt1* mRNA was only detected in plasmatocytes. The levels of transcription and the secretion of PiCyt1, however, were not influenced by addition of PSP or *E. coli* challenge in vitro. Because hemolymph without PSP also induces plasmatocytes spreading, the effect of hemolymph on *PiCyt1* transcript abundance was also measured in vitro. The result was that PiCyt1 mRNA amount of hemolymph treatment was same as the control one. This indicates that while PiCyt1 is expressed specifically in plasmatocytes, the expression and release of this protein is unaffected by two factors previously implicated in plasmatocyte activation (Clark et al., 1997; Lavine et al., 2005). However, in these transcript or secretion level influence experiments, the hemocytes were cultured in primary culture medium (TC-100), so it is possible that this in vitro experiment condition affected the behavior of the hemocytes. It is also possible that the transcription or secretion level of PiCyt1 may be controlled by some other factors produced by other organs like fat body.

While the above transcript and secretion level experiments were conducted in vitro, the *PiCyt1* transcript abundance at different times in 5^{th} instar *P. includens* larvae was tested in vivo. The transcription level was higher in the middle of the instar and was lower at the biginnning and the end of the 5^{th} instar, and was extremely low at the end of instar just before the pupation. It

seemed that there was some relationship between the transcription level of PiCyt1 and the molting cycle. Recently, it was found that the sensitivity of plasmatocytes to PSP was affected by the molting cycle (Clark et al., 2005). The plasmatocytes from early instar *P. includens* larva were less sensitive compared with that from late instar. Although there is no significant connection between the results of these two experiments, it is possible that the PiCyt1 is involved in insect immunity.

Above all, although some results of this study do not support the hypothesis that PiCyt1 is involved in immune responses, given the limitation of the knowledge we have in insect cellular immunity and the potential effects of experiments conditions, it is still necessary for us to determine whether it has the cytolytic activity. The detail of these functional studies will be discussed in the next chapter.

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Tables and figures

Table 3.1

A. Relative transcript abundance of *PiCyt1* in 5th *P. includens* larvae (transcriptional level of 12 hours after molting was standardized as 1.0)

Time after molting into 5th instar lavae (hour)	Relative transcriptional level of PiCyt1
12	1.0± 0.1
36	4.46± 0.46
48	3.44± 0.17
60	(3.8± 0.5) x 10^{-5}

B. Relative transcript abundance of *PiCyt1* in different conditions (transcriptional level of Control at 0 hr was standardized as 1.0)

	0 hr	4 hr	8 hr	12 hr
Control	1.0 ± 0.1	0.15 ± 0.01	0.036 ±0.003	0.027 ± 0.002
PSP (10 μM)	1.52 ± 0.14	0.28 ± 0.03	0.091 ± 0.009	0.12 ± 0.02
Plasma (5%)	1.83 ± 0.08	0.15 ± 0.01	0.023 ± 0.002	0.015 ± 0.001

Figure 3.1 The sequence of PiCyt1 and its homology to Asp-homolysin and cbm17.1. A. The cDNA sequence and the deduced amino acid sequence of PiCyt1. The start codon is in bold and the putative N-glycosylation sites are double underlined. The predicted hydrophobic regions are underlined. The sequence in PiCyt1 that corresponds to the N-terminal sequence data obtained from protein purified from hemocytes is shaded. B. Sequence comparison of PiCyt1 and Asp-hemolysin and *cbm*17.1 from *Clostridium bifermentans*. Double dots indicate identical amino acid residues while single dots indicate conserve replacements of residues. PiCyt1 shares 63% and 61% similarity with Asp-hemolysin and cbm17.1 respectively.

Figure 3.2 The expression tissue of *PiCyt1*. A. Autoradiogram of a Northern blot to RNAs (2 μg/lane) from different tissues of *P. includens*. The tissues included testes (T), salivary gland (S), Malpighian tubules (M), nervous tissue (brain plus ventral nerve chord) (N), gut (G), fat body (F) and hemocytes (H). The blot was hybridized with a radiolabeled PiCyt1 cDNA probe, washed under conditions of high stringency and exposed to autogradiographic film using an intensifying screen at –80°C for 18 hours. B. Autoradiogram of a Northern blot to RNAs (2 μg/lane) from different types of hemocytes, including unseparated hemocytes (H) and Percoll gradient-purified granular cells (G), plasmatocytes (P) and spherule cells (S). The blot was processed as described in A and exposed to autoradiographic film using an intensifying screen at –80°C for 6 hours. C. *in situ* hybridization with PiCyt1 RNA probes to unseparated hemocytes. The spread cells are plasmatocytes and the non-spread round cells are granular cells and other cell types. The cells were hybridized with antisense RNA probe of *PiCyt1* and incubated with an α-Digoxigenin-alkaline phosphatase conjugated antibody (1: 2000) for 1 hour and visualized using 1 ml RNA-RP buffer plus 4.5 μl NBT and 3.5 μl x-phosphate) overnight.

Figure 3.3 Relative quantitative real-time PCR (rq-rtPCR) analysis of *PiCyt1* transcript levels in 5th instar *P. includens* larvae and in hemocytes under different conditions in vitro. A. Related transcript abundance of *PiCyt1* at 12, 36, 48 and 60 hours in 5th instar *P. includens* larvae. The transcript abundance at 12 hours after molting into 5th instar was standardized as 1.0 and transcript abundance at other time points were expressed as an increase or decrease compared to the 12 hours. B. Related transcript abundance of *PiCyt1* in hemocytes when incubated with 10 μM PSP or 5 % plasma. The time points were chosen as 0, 4, 8 and 12 hours and the transcript abundance of Control at 0 hour was standardized as 1.0. All other *PiCyt1* transcript levels were expressed as an increase or decrease compared with the control at 0 hour. The blue line presents the transcript level of Control, the green line presents transcript level in hemocytes incubated with PSP, and the red line presents the transcript level in hemocytes incubated with plasma.

Figure 3.4 Influence of PSP or *E. coli* challenge on secretion level of PiCyt1. The protein samples were from the supernatants of hemocytes incubated with PSP or *E. coli* at 0, 2 and 8 hours of incubation. The membranes were probed with 43E9A10 and an HRP-conjugated goat anti mouse secondary antibody (1: 2000) (Jackson labs). The membranes were visualized using a chemiluminescent substrate (Amersham). A. Influence of PSP (10 μM) on PiCyt1 secretion. Lane 1-3: Control medium at time points 0, 2 hours and 8 hours; Lane 4-5: PSP treatment medium at time points 0, 2 hours and 8 hours. B. Influence of *E. coli* challenge (32,000/μl) on secretion of PiCyt1 into medium. Lane 1-3: Control medium at time points 0, 2 hours and 8 hours; Lane 4-5: *E. coli* challenged cell medium at time points 0, 2 hours and 8 hours.

A

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GCACGA**ATG**AAGACCACATACGTAGTCAGGTTGGCCGCTTTGGCAGCGGTACTGCTCGCC   60
       M  K  T  T  Y  V  V  R  L  A  A  L  A  A  V  L  L  A     18

TCGGGGAAAGTCGACGCCGGCAACGCCCACTATGTTGAGCGCTTCGAACGTTTCTCGCCT  120
 S  G  K  V  D  A  G  N  A  H  Y  V  E  R  F  E  R  F  S  P     38

GAACAATCTATTGGAATAATAATGTGTCTTGCCAACTTAGTTTTTACTAACAACATTTCT  180
 E  Q  S  I  G  I  I  M  C  L  A  N  L  V  F  T  N  N  I  S     58

GTACTCTCGATAATGGCATGTGTGTCAGGCTTATCCGAACCGCAACCGTTCGCCTGGGGT  240
 V  L  S  I  M  A  C  V  S  G  L  S  E  P  Q  P  F  A  W  G     78

GAAGGTAATTACATAAATTTCAAAATTGTAAACGGTGAAAGTGTCAGCGAAAGCGGTATG  300
 E  G  N  Y  I  N  F  K  I  V  N  G  E  S  V  S  E  S  G  M     98

ATTATGAACGCCGAGCTGACCTGGGGCAAGTGGCAAAAGGATGGTAAAGATATCGACACA  360
 I  M  N  A  E  L  T  W  G  K  W  Q  K  D  G  K  D  I  D  T    118

GTCAATAGAACAGAGTTCGATTCGAAAGGTAAAAGAGCTGAATTCTCAGCGGTAGGCCGC  420
 V  N  R  T  E  F  D  S  K  G  K  R  A  E  F  S  A  V  G  R    138

ACTGGCGTTCCTAGTGGCGCCGAAGGATTTTTCGAAATCTTCGAAGATGATAAAGTGATC  480
 T  G  V  P  S  G  A  E  G  F  F  E  I  F  E  D  D  K  V  I    158

GCCAAGGTGATTTTCGATGTACCGTTTATGGGAGACAACAAGTTAAAAATCAAACAACTT  540
 A  K  V  I  F  D  V  P  F  M  G  D  N  K  L  K  I  K  Q  L    178

GACAAAGGCTTTTTATGTGACGATTCAGGATTTGATGCTGGCGGATCCCTGACCATCGAA  600
 D  K  G  F  L  C  D  D  S  G  F  D  A  G  G  S  L  T  I  E    198

ATCGTTTGCCATAAATTGGGCGCTGCTGTACCTTGAATCGAAATTATAAGAATCCAATTT  660
 I  V  C  H  K  L  G  A  A  V  P  *                            209

TCTTAACTAAATACCGAAATCGAACTCTTTAATGTTTAACTGTTGTCTTTTTAAATACAC  720
ATTTTGCATGAATAATGTATGTTTTTTTTACCCGACTGCCACCGATAGGAGGATAATGTT  780
TTTCATTCATGTGTATGTATGTAGATTGCTAAGTCCCCTGCTACAGCCTAAACGGCTGGA  840
AGGATTTTCAACGTATGTGGTGTCGTTAGATTCGTCTAGATAGCACAAGTGACACTAGGT  900
ACATAAGAAAAAAAAAAAAAA                                         922
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B

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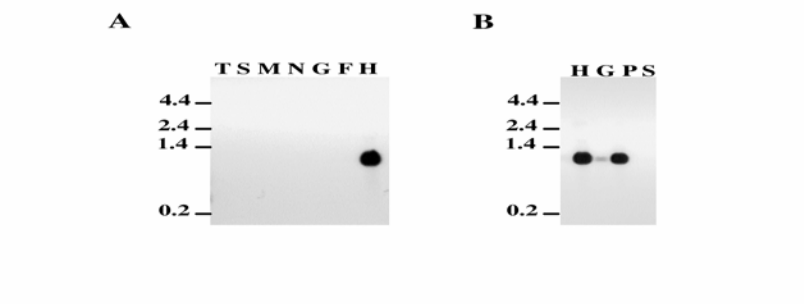
Asp-hem     AYAQWVTVHLINSMS-SETLSIKNASLSWGKWYK-DGDKDA----EITSEDVQQKTAPPGGSVNV   59
                ... ...:. : ::.  : :: :.:::: : :: ::     . ..   . .    :
PiCyt1      GEGNYINFKIVNGESVSESGMIMNAELTWGKWQK-DG-KDI----DTVN---RTEFDSKGKRAEF  133
                ...:.:   :.:.:.  : :: : :::..  .. :::    . ..   . .....
cbm17.1     AYRQWVKFHI---EAVNEGLKIRNASLKWGKFHDPNN-KDIPISPEDIS---KINIEKHDTAIIA   77


Asp-hem     NSCGRSDASSGTTGGFDLY-DGNTKIGRVHWDCPWGSKTNDFDVGERNKNYWVEIGTWNKYGGA   122
               ::. . ::. : :... . .  :..: .: :..   : . . . .:..  . . ..  ::.
PiCyt1      SAVGRTGVPSGAEGFFEIF-EDDKVIAKVIFDVPFMGD-NKLKIKQLDKGFLCDDSGFDA-GGS   194
            :. :. .. ::.:: : :  :..  :: . .: :. :. ::: : . .  :  ..:       :
cbm17.1     SS-GKENTASGTEGVFYICDENEDKIAAIYWDCPWSGS-NKLTIDKYNTKFAIEQSPTMI-SSS   139
```

Figure 3.1



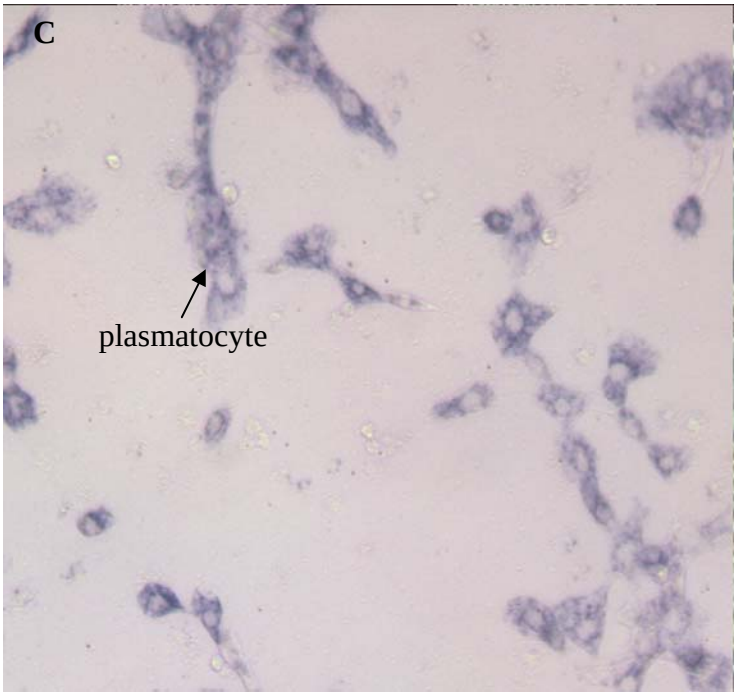


Figure 3.2

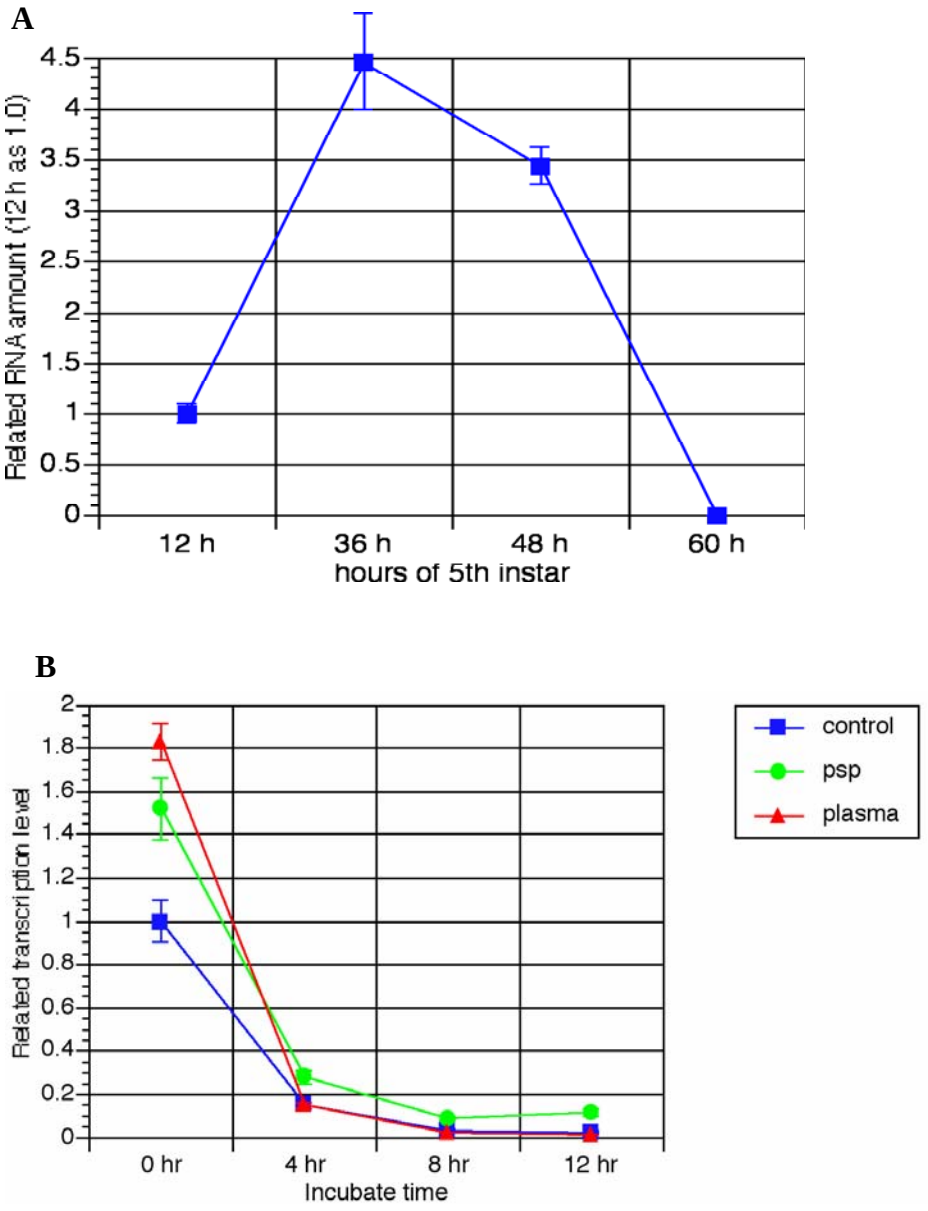

A
Related RNA amount (12 h as 1.0)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
12 h
36 h
48 h
60 h
hours of 5th instar
B
Related transcription level
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 hr
4 hr
8 hr
12 hr
Incubate time
control
psp
plasma

Figure 3.3

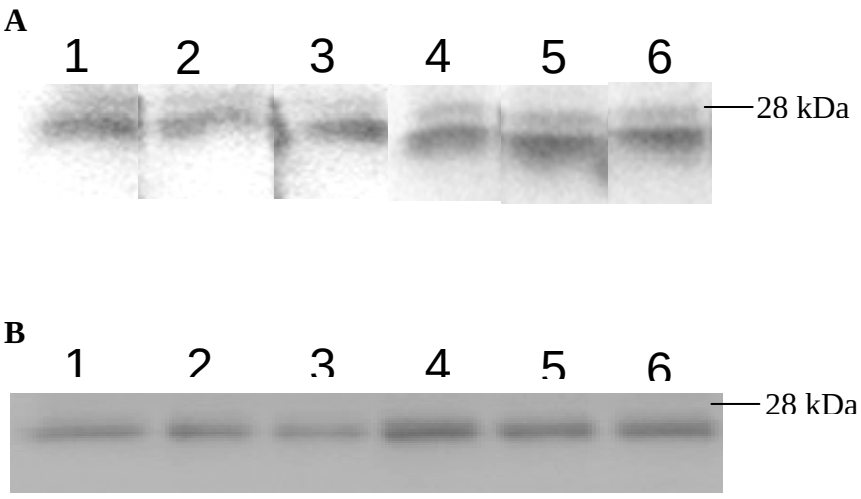


Figure 3.4

CHAPTER IV

TRANSIENT EXPRESSION AND FUNCTION STUDY ON *PSEUDOPLUSIA INCLUDENS* PROTEIN PICYT1[2]

[2] Zhang, S. and M. R. Strand. To be submitted to *Insect biochemistry and molecular biology*.

Abstract

PiCyt1 is a protein produced by *Pseudoplusia includens* (Lepidoptera: Noctuidae) that is expressed in a population of hemocytes called plasmatocytes. Because it shares significant homology with another protein, Asp-hemolysin, we hypothesize that PiCyt1 is also a cytolytic protein. Previous experimental results suggested that the efficiency of purifying PiCyt1 directly from *P. includens* by affinity column was quite low. Here we expressed both intact PiCyt1 and truncated PiCyt1 without signal peptide into a pIZT/V5-His vector and transfected into High Five and Drosophila S2 cells. The expression level of PiCyt1 in these cell lines was tested by Western blot and immunocytochemistry. The results indicated that the expression level in S2 cells is much higher than that in High Five cells. Through the attached 6xHis tag on the C-terminus of PiCyt1, we purified the recombinant protein by Ni^{2+}-beads. The cytolytic activity of the recombinant PiCyt1 was tested against bacteria and erythrocytes from chicken, sheep, mouse and human.

Key words

pIZT/V5-His vector, cytolytic activity

Introduction

Both prokaryotes and eukaryotes produce cytolytic peptides and proteins that are divided into three categories: peptides which change ion permeability of plasma membranes and then induce the death of the cells, peptides which cause enzymic degradation of the phospholipid bilayer membrane, and peptides which form channels through cell membranes (Bernheimer & Rudy, 1986). In the previous chapter, I reported that the monoclonal antibody marker 43E9A10 recognizes the 22 kDa protein PiCyt1. Sequence comparison further indicated that PiCyt1 shares some similarity with a fungal cytolytic protein Asp-hemolysin and cbm17.1 from *C. bifermentans*.

Hemolysins are a class of pore-forming proteins from invertebrates which share the ability to lyse erythrocytes from different vertebrates (Braun et al., 1993). Previous studies indicated that hemolytic activity of many hemolysins involves binding to sugars or lipids on the target cell membranes (Roch et al., 1981; Canicatti, 1988). Polymerization of hemolysins was also an important process during pore formation in some hemolysins (Canicatti, 1990). Asp-hemolysin is a hemolysin identified from *Aspergillus fumigatus* that exhibits cytolytic activity toward chicken and human erythrocytes (Yokota et al., 1977; Ebina et al., 1985). The mechanism of Asp-hemolysin pore formation activity is thought to involve binding to oxidized low density lipoprotein (LDL) on the membrane of red blood cells (Kudo et al., 1999). Asp-hemolysin has a negatively charged domain which is similar to the cystein-rich domain of the LDL receptor and thought to be essential for the LDL binding ability (Ebina et al., 1994). The corresponding sequence of PiCyt1, however, is less negatively charged than that of Asp-hemolysin.

Based on these data, the goal of this research was to assess whether PiCyt1 was involved in the killing of foreign targets during an immune response or whether the difference between the sequences of it and Asp-hemolysin reduces the cytolytic activity of PiCyt1. To meet this goal, I produced and purified different recombinant proteins of PiCyt1. Here I report that I successfully developed methods to purify PiCyt1. However, purified recombinant PiCyt1 was not cytolytic toward any targets tested.

Materials and methods

Insect cells growth High Five cells were grown in TC-100 medium with 15% serum and *Drosophila* S2 cells were grown in HyQ insect cell medium (Invitrogen) as previous described (Beck & Strand, 2005).

pIZT/V5-His-PiCyt1 plasmid construction I produced PiCyt1 expression constructs with and without a signal peptide by PCR cloning. For PiCyt1 with signal peptide, the forward primer was 5'-CATCTAGAGAATGGAGACCACATACG-3' and the reverse primer was 5'-GTCCGCGGT CTAGGTACAGCAG-3'; for PiCyt1 without signal peptide, the forward primer was 5'-CATCT AGAGCATGGCCCACTATGTTGAG-3' and the reverse primer was the same. PiCyt1 cDNA was amplified by Elongase (Invitrogen) using these primers and was blunt cloned into pCR2.1-TOPO vector. The plasmid DNA was digested with Xba I and Sac II and the insert was ligated into pIZT/V5-His vector and transformed into One-Shot competent *E. coli* cells. To produce constructs of PIZT/PiCyt1+ (with signal peptide) and PIZT/PiCyt1- (without signal peptide), positive clones were selected on low salt LB plates containing Zeocin (50 μg/ml).

Plasmid DNA was isolated from overnight cultures by Midi Prep Kit (Sigma) and the sequencing reaction was conducted using the Big Dye kit (Applied Biosystems) and sent to University of Wisconsin Biotechnology Center (Madison, WI).

Cell transfection High Five and S2 cells were transfected in 12-well culture plates as previously described (Beck & Strand, 2003; 2005). Briefly, $1x10^5$ cells were incubate with 3 μg/ml plasmid DNA and lipofectin (High Five cells) or Cellfectin (S2 cells) (Invitrogen) at room temperature for 6 hours. Cells were then resuspended in fresh medium and incubate at 27°C.

Immunocytochemistry Cells were fixed 48 hours after transfection in 4% paraformaldehyde and permeablized in PBS + 0.1% Triton x100. The primary antibody was 43E9A10 (1:5) and the secondary antibody was Texas Red conjugated goat anti mouse IgG (1:1000). Cells were examined using a Leica TCS fluorescence microscope.

Western blotting Protein samples were collected from both medium and cells 48 hours after transfection. Cells were lysed in lysis buffer (100 mM Tris, 150 mM NaCl, 0.1 % Nonidet P-40, pH 8.0), followed by centrifugation at 200x g for 2 minutes to eliminate insoluble debris. Protein samples were electrophoresed on 8-16% gradient SDS gels and transferred to Nitrocellular membranes. Membranes were then incubated with an anti-V5 epitope antibody (1:10000), an HRP-secondary antibody (goat anti mouse, 1:20000), and visualized by a chemiluminescent substrate (Amersham).

PiCyt1 purification Recombinant PiCyt1+ and PiCyt1- were partially purified by affinity chromatography using Ni-NTA superflow beads (QIAGEN). The Ni^{2+} beads were first equilibrated in cell lysis buffer. 4 ml cell extraction of 48 culture wells was incubated with 250 μl Ni^{2+} beads at 4°C for 3 hours. After washed 4x 1 ml of Wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), PiCyt1 was eluted by 4x 200 μl of Elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified protein was separated on 8-16% SDS Page gradient gels and visualized by both silver staining and Western blotting. The presence of recombinant PiCyt1 was determined by Western blotting described above and the purity was determined by silver stain. For silver staining, the gels were fixed in 10% acetic acid and 40% ethanol for 30 minutes and sensitized in 30% ethanol, 0.25% glutaraldehyde, 0.15 M $Na_2S_2O_3$ and 0.8 M CH_3COONa for 30 minutes. The silver reaction was conducted 15 mM $AgNO_3$ and 0.04% Formaldehyde and developed in 250 mM Na_2CO_3 and 0.02% Formaldehyde. The reaction was stopped by adding 40 mM EDTA disodium solution. The concentration of purified protein was determined with Protein Micro Assay (Bio-Rad).

Cytolytic activity bioassay The cytolytic activity of recombinant PiCyt1+ and PiCyt1- was tested against bacteria and erythrocytes. *Escherichia coli* and *Micrococus luteus* were used as the bacterial targets. *E.coli* and *M. luteus* were cultured in LB and Tryptic soy medium respectively. 5 μl bacteria culture (OD was about 0.5) was incubated in 45 μl purified PiCyt1 solution (final concentration was 1.0 μM) at room temperature for 2 hours. Bacteria after treatment were spread on agar petri dishes and cultured at 37°C overnight. Colony numbers were counted as the level of survival. The cytolytic activity of recombinant PiCyt1 against erythrocytes was determined as previous described (Yokata et al., 1977). Briefly, 30 μl of sheep,

chicken, mouse or human erythrocytes were washed by Alsever's solution (pH 6.1). The erythrocytes were incubated with 100 μl PiCyt1 solution (1.0 nM, 10 nM, 100 nM and 1.0 μM) at room temperature for 1 hour. The same amount of elution buffer dilution and Ni^{2+} beads purified extraction from non-transfected S2 cells were used as controls. The erythrocytes were eliminated by centrifugation at 2000 rpm for 5 minutes. The absorbance of the supernatant at 544 nm was measured.

Results

1. The sequence of recombinant PiCyt1 was same as that of native PiCyt1

To conduct further functional experiments, sufficient purified PiCyt1 was needed. I first tried to purify native PiCyt1 by affinity chromatography using 43E9A10. However, this purification method did not produce enough protein (data not shown), so I decided to overexpress PiCyt1 in insect cells. Expression of PiCyt1 in bacteria was not used because of potential concerns about appropriate processing and folding. PIZT/V5-His plasmid was used as the expression vector. Sequencing confirmed that PIZT/PiCyt1+ and PIZT/PiCyt1- corresponded to the *PiCyt1* cDNA.

2. The expression level of recombinant PiCyt1 was higher in S2 cells than that in High Five cells

To determine the expression level of recombinant PiCyt1 in S2 and High Five cells, Western blotting and immunocytochemistry experiments were conducted. The result of Western

blotting indicated that recombinant PiCyt1+ was approximately 30 kDa which matched the sequencing data and was detected both in the cell medium and cell lysates in both cell types. The amount of recombinant PiCyt1+ was much higher in the cell lysates of S2 cells than that of High Five cells (Figure 4.1A). Immunocytochemistry experiments also indicated that the amount of the recombinant Picyt1+ in S2 cells was higher than that in High Five cells (Figure 4.1B). Similar results were obtained for the recombinant PiCyt1- protein which was also retained primarily inside the cells and the expression level in S2 cells was very high while that in High Five cells was too low to be detected (data not shown). Due to the higher amount of recombinant protein in S2 cells, S2 cell lysates were used as the source of recombinant PiCyt1 for purification.

3. The recombinant PiCyt1 was purified by Ni^{2+} beads

Recombinant PiCyt1 was purified using Ni^{2+} beads (QIAGEN). Western blotting confirmed the presence of recombinant PiCyt1+ (Figure 4.2, Lane 1) and silver staining indicated that few other proteins were present (Figure 4.2, Lane 2). The concentration of recombinant PiCyt1+ was estimated to be 100 μg/ml. Given the molecular weight of recombinant protein is ~30 KD, the concentration of the recombinant PiCyt1+ was 3.5 μM. The purity of recombinant PiCyt1- was similar (data not shown) and the concentration was 44 μg/ml or 1.5 μM.

4. Cytolytic activity of recombinant PiCyt1

To determine whether PiCyt1 had cytolytic activity, cytolytic activity assays were conducted against bacteria and erythrocytes from different sources. Both gram-negative bacteria *E. coli* and gram-negative bacteria *M. luteus* were used as targets of the cytolysis bioassay. Colony assays in the control and recombinant PiCyt1+ treatments were not significantly different indicating that PiCyt1+ was not cytotoxic to bacteria at the concentrations I tested (Figure 4.3). The cytolysis bioassay against erythrocytes from different vertebrates was conducted by the methods described by Yokota et al. (1977). Light absorbance was used as a marker of cytolytic activity. The higher the absorbance, the more erythrocytes were lysed. The result of the cytolytic activity assays of recombinant PiCyt1+ indicated that there was little difference between the PiCyt1+ treatment and the control (Fig 4.4A). For the recombinant PiCyt1-, the cytolytic activity against erythrocytes of sheep and chichen were measured (Fig 4.4B). There was no difference between the PiCyt1 treatments and controls. The cytolytic activity of recombinant PiCyt1- was also tested against S2 cells and High Five cells and no cytolytic activity was detected (data not shown). From these results, I conclude that there is no significant cytolytic activity of the recombinant PiCyt1+ or PiCyt1- towards bacteria, insect cells or erythrocytes.

Discussion

PiCyt1 shares homology with Asp-hemolysin, which is cytolytic to some eukaryotic erythrocytes. In this project, I purified different forms of recombinant PiCyt1 and measured the

cytolytic activity of them against bacteria and different sources of erythrocytes. The results indicated that no significant cytolytic activity of PiCyt1 was detected.

Although these results indicated that recombinant PiCyt1 has no cytolytic activity, there are still two possibilities which can explain the results. One possibility is the difference between the recombinant protein and the native protein results in the loss of the cytolytic activity or the experimental conditions which are good for Asp-hemolysin to function are not suitable for the PiCyt1 protein. The other possibility is that the targets of PiCyt1 cytolytic activity are not the targets tested. As described above, Asp-hemolysin has a LDL binding domain which helps it bind to the surface of the target erythrocytes and then lyse the cells. The corresponding domain in PiCyt1 has fewer negatively charged amino acid residues which may reduce the LDL-binding ability and therefore reduce the cytolytic activity of PiCyt1 against erythrocytes. Because the detailed mechanism of Asp-hemolysin is still not well known, it is difficult to determine the important domains for cytolytic activity. The reduced number of negatively charged residues in the LDL-binding domain in PiCyt1 may result in a change of cytolytic activity targets, therefore requires further studies to verify these hypothesis.

Besides the two possibilities discussed above, it is also possible that the function of PiCyt1 is not cytolysis. Two proteins, *Pleurotus* and *Agrocybe* hemolysins, which also share homology with Asp-hemolysin, were identified from the edible mushrooms *Pleurotus ostreatus* and *Agrocybe aegerita* (Berne et al., 2002). These two proteins were found to be expressed specifically during fruiting initiation. The precise functions of them have not been identified. These results suggested that these two hemolysins may have some effect on development rather than cytolytic function. These facts make it possible that Asp-hemolysin may also have some other functions which have not been found and PiCyt1 shares those functions with Asp-

hemolysin. At the same time, because the homology between PiCyt1 and Asp-hemolysin is not high, the relationship between the functions of these two protein is not certain. Due to the limited information from the sequence comparison, it is very hard to determine the function of PiCyt1 at this time.

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Figures

Figure 4.1 The expression of recombinant PiCyt1+ in selected insect cell lines. A. The result of Western blot. The membrane was probed by an anti-V5 epitope antibody. Lane 1 The medium of High Five cells; Lane 2 The cell extraction of High Five cells; Lane 3 The medium of S2 cells; Lane 4 The cell extraction of S2 cells. B. The immunocytochemistry result. The cells were probed using 43E9A10 and a Texas Red-secondary antibody. The red color indicates the present of the protein PiCyt1.

Figure 4.2 The purified recombinant PiCyt1+. Lane 1. Western blot of purified protein. The membrane was probed by an anti-V5 epitope antibody. Lane 2. Silver staining of purified PiCyt1+ protein.

Figure 4.3 The cytolytic activity of PiCyt1+ to bacteria. *Micrococus luteus* and *Escherichia coli* were incubated in PiCyt1+ solution (final concentration was 1.0 μM) and spread on plates. The numbers of colonies were counted. The control was the bacteria incubated in elution buffer.

Figure 4.4 The cytolytic activity of PiCyt1 to erythrocytes from different sources. The absorbance of the supernatant at 544 nm was used as the marker of cytolytic activity. A. The cytolytic activity of recombinant PiCyt1+. a. The cytolytic activity of PiCyt1+ to chicken erythrocytes; b. The cytolytic activity of PiCyt1+ to human erythrocytes; c. The cytolytic activity of PiCyt1+ to mouse erythrocytes; d. The cytolytic activity of PiCyt1+ to sheep erythrocytes. The controls include the elution buffer and the purified products from non-transfected S2 cells

extract. The final PiCyt1+ concentrations were 1 nM, 10 nM, 100 nM and 1 μM. B. The cytolytic activity of recombinant PiCyt1-. a. cytolytic activity to chicken erythrocytes; b. cytolytic activity to sheep erythrocytes. The controls were elution buffer dilution in Alsever's solution and the final PiCyt1- concentration was 1 nM, 10 nM and 100 nM.

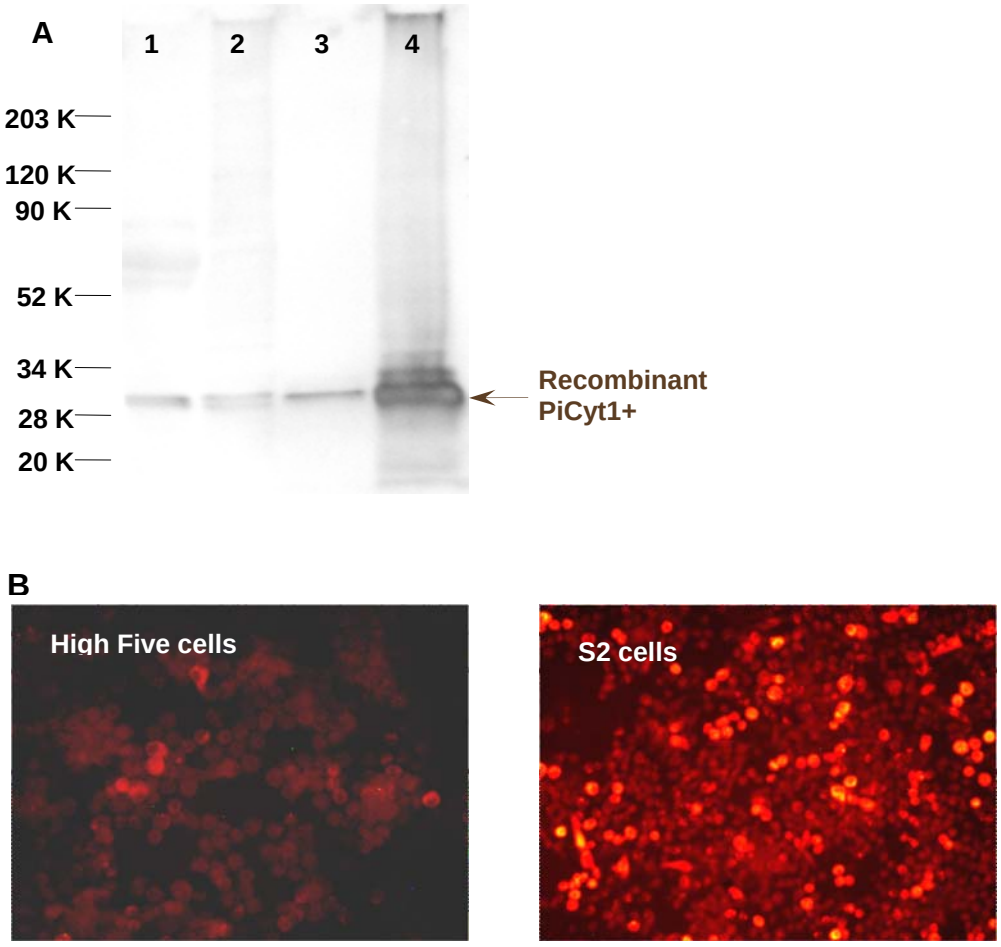

A
1
2
3
4
203 K
120 K
90 K
52 K
34 K
28 K
20 K
Recombinant
PiCyt1+
B
High Five cells
S2 cells

Figure 4.1

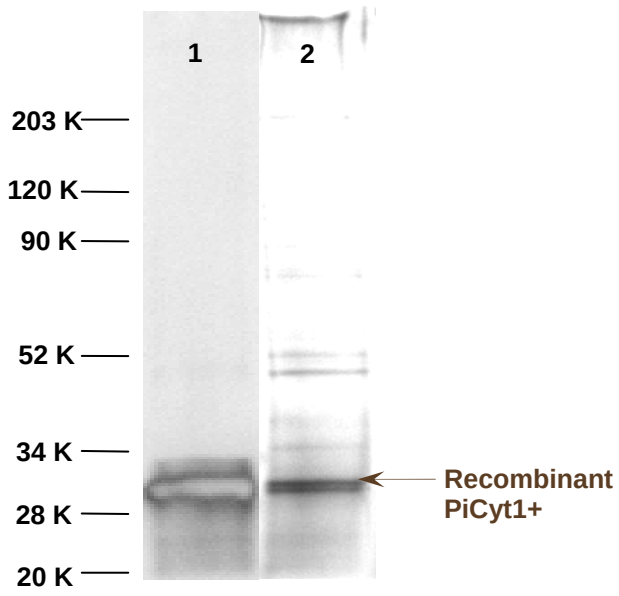


Figure 4.2

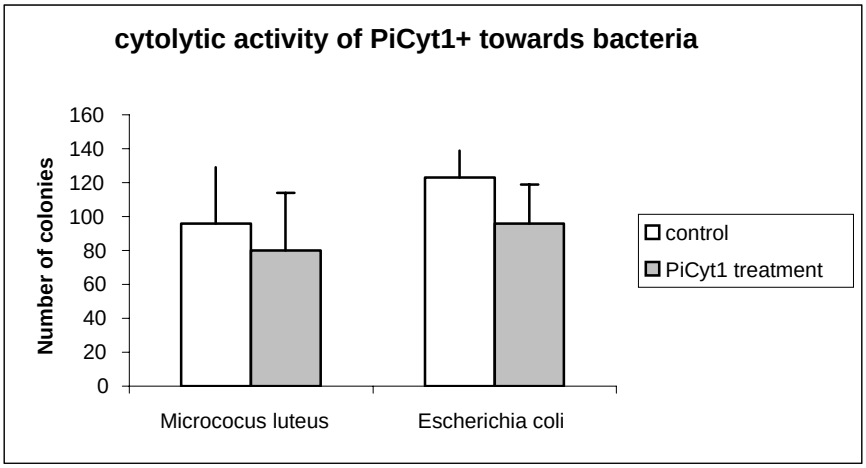
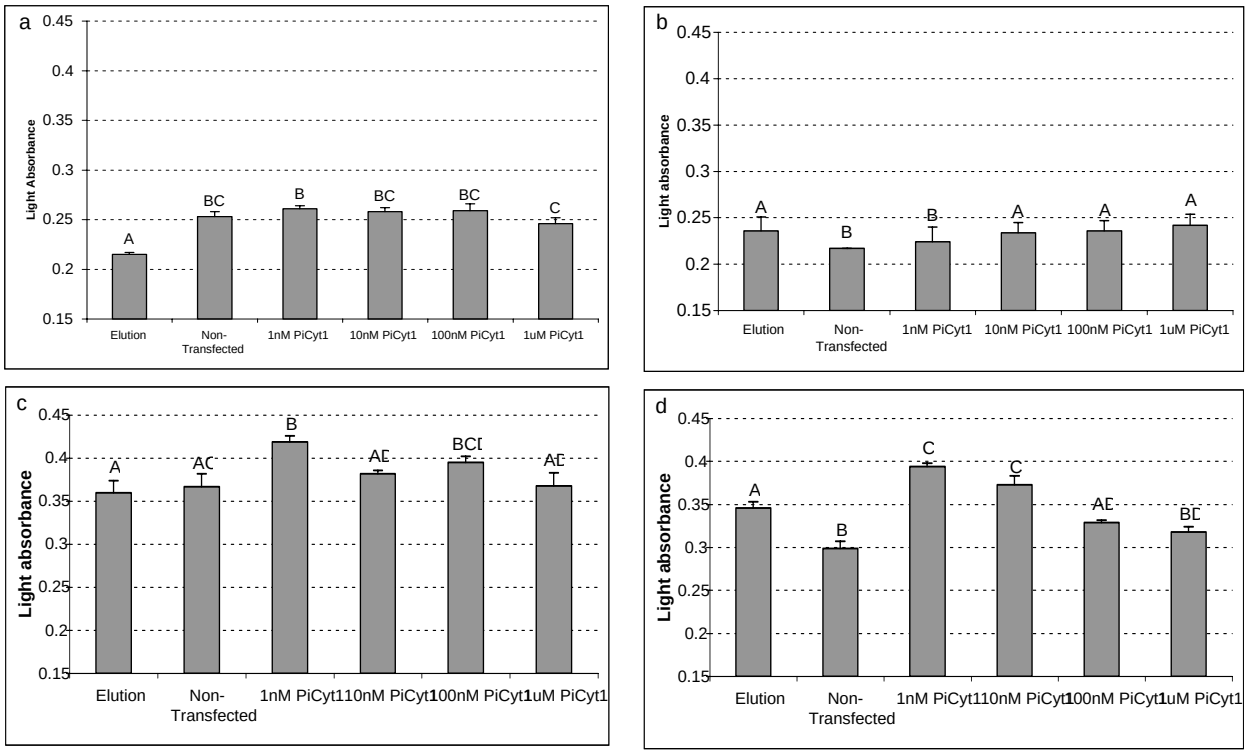

cytolytic activity of PiCyt1+ towards bacteria
Number of colonies
160
140
120
100
80
60
40
20
0
Micrococus luteus
Escherichia coli
control
PiCyt1 treatment

Figure 4.3

A


a
Light Absorbance
0.45
0.4
0.35
0.3
0.25
0.2
0.15
A
BC
B
BC
BC
C
Elution
Non-Transfected
1nM PiCyt1
10nM PiCyt1
100nM PiCyt1
1uM PiCyt1
b
Light absorbance
A
B
B
A
A
A
c
Light absorbance
A
AC
B
AE
BCE
AE
d
Light absorbance
A
B
C
C
AE
BE

B

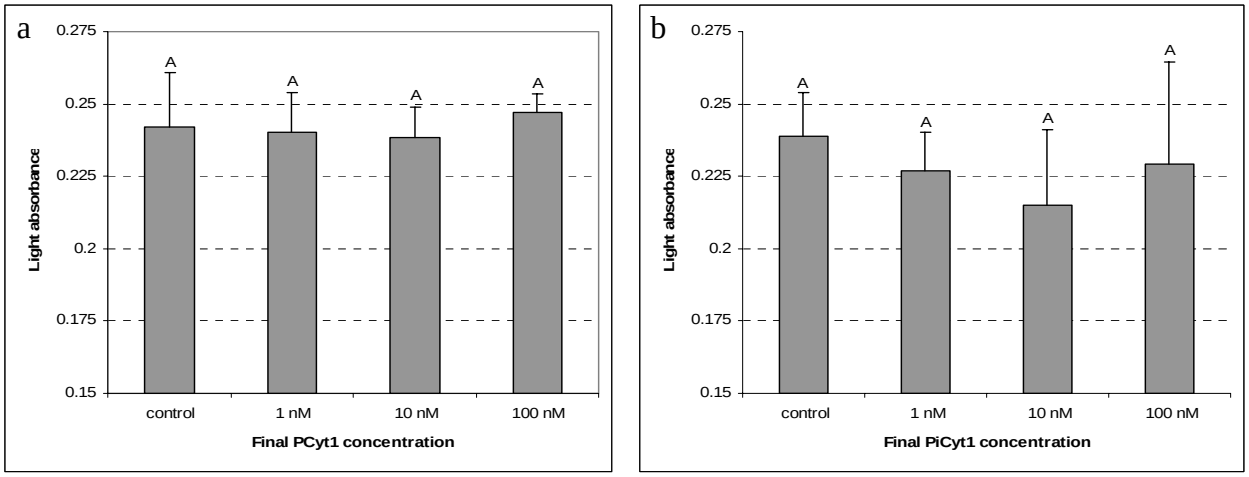

a
Light absorbance
0.275
0.25
0.225
0.2
0.175
0.15
A
A
A
A
control
1 nM
10 nM
100 nM
Final PCyt1 concentration
b
Light absorbance
Final PiCyt1 concentration

Figure 4.4

CHAPTER V

CONCLUSIONS

Insects produce several types of hemocytes which have essential functions in immunity. In lepidopterans, like *Pseudoplusia includens*, plasmatocytes are the primary hemocyte type involved in cellular immunity. Previous studies indicated that the monoclonal antibody 43E9A10 was a plasmatocyte-specific marker. The goal of this project was to identify and characterize the antigen recognized by this antibody.

In the first part of my project, I cloned the cDNA of *PiCyt1*. Sequence comparison indicated that PiCyt1 shared significant homology with a cytolytic protein called Asp-hemolysin. I thus hypothesized that PiCyt1 is a cytolytic protein involved in killing foreign organisms encapsulated by plasmatocytes. Northern Blotting and *in situ* hybridization studies indicated that *PiCyt1* was specifically expressed in plasmatocytes. Rq-RT PCR studies revealed that *PiCyt1* expression varied during the 5^{th} instar. Contrary to expectation, however, *PiCyt1* expression was not upregulated in plasmatocytes activated by plasma or the cytokine PSP.

To determine whether PiCyt1 was cytolytic , I conducted functional studies by transiently expressing recombinant PiCyt1 in High Five and S2 cells. Because of the higher expression level of PiCyt1, S2 cells were used as the expression system of the recombinant PiCyt1. Both full length PiCyt1 and the PiCyt1 without signal peptide were cloned into PIZT/V5-His plasmid, expressed, and purified by affinity chromatography. The cytolytic activity of the purified recombinant PiCyt1 towards bacteria and erythrocytes was then bioassayed. My results

indicated that PiCyt1 was not toxic to the targets tested. I conclude therefore that PiCyt1 is a plasmatocyte specific protein but that it is likely not involved in killing encapsulated prokaryotic and eukaryotic cells.