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Functional Role Of Recombinant Cysteine Protease On Spodoptera Frugiperda Peritrophic Matrix

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FUNCTIONAL ROLE OF RECOMBINANT CYSTEINE PROTEASE ON *Spodoptera frugiperda* PERITROPHIC MATRIX

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Fall armyworm larvae (FAW), which are serious pests in the southern United States, show retarded growth when they feed on insect-resistant maize inbreds Mp704 and Mp708. These maize genotypes are not only resistant to FAW, but to a number of other lepidopteran pests. In these genotypes, a unique, extracellular, 33-KDa cysteine protease (Mir1-CP) rapidly accumulates in the whorl in response to insect feeding. Initial morphological studies on larvae feeding on resistant maize plants over-expressing the cysteine protease showed severe damage in insect's first line of defense, the peritrophic matrix (PM). But it is not known whether the cysteine protease has unprecedented effect on insect defense mechanisms.

This study focuses on understanding the functional involvement of the cysteine protease (Mir1-CP) in a plant-herbivore defense mechanism. I used purified, recombinant 33-KDa cysteine protease (Mir1-CP) and its two mutated forms (Mut1 and Mut2) to determine their effects on the permeability of PMs from fall armyworm and other lepidopteran larvae. The purified Mir1-CP was also used to determine its minimal effective dosage on lepidopteran larval growth as well as to qualitatively determine their direct morphological effects on PM and gut regions of fall armyworm larvae.

In vitro permeability studies demonstrated that the recombinant Mir1-CP directly permeabilized the PM and requires both cysteine at the active site and the terminal 25 amino acids to achieve complete permeabilization. Dose response study suggested that physiologically relevant concentrations of Mir1-CP in the maize whorl would be effective in controlling a broad range of lepidopteran pests. The study also suggested that stacking Mir1-CP and Bt-toxin (Bt-CryIIA) genes in transgenic plants could broaden the normal range of both Mir1-CP and Bt-toxin. Morphological studies using three different microscopic techniques showed damaged PM in larvae fed on Mir1-CP diet. These results suggest that by directly permeabilizing and damaging the PM, the Mir1-CP provides critical defense in host plants against lepidopteran pests.

DEDICATION

I would like to dedicate this research to my parents, Mr. Mohan Srinivasaraghavan and Ms. Jayashee Mohan.

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CHAPTER I

INTRODUCTION

Higher plants have been attacked for millions of years by chewing, sucking and piercing insects. Plants respond to insect herbivory by inducing defensive secretion of various proteins. The defensive response can vary from minutes to years, depending on the plant and the reaction time (Turning et al., 1998). As a direct defense response, plants release either protease inhibitors, cell wall proteins, lectins, oxidative enzymes and enzymes catalyzing the production of secondary products to slow down the herbivore growth by decreasing nutrient availability (Halitschke et al., 2000; Bergey et al., 1996; Constabel et al., 1999) or secondary metabolites, which are toxic to the attacking herbivores. Plants also respond indirectly by releasing volatiles to attract predators or parasitoids, which will have detrimental effects on attacking herbivores (Halitschke et al., 2000; Bergrey et al., 1996). Major studies of plant-herbivore defense mechanisms have been conducted on dicotyledonous plants (Ryan and Pearce, 1998; Ryan 2000; Schaller and Ryan 1996; Scheer and Ryan 1999; Stout et al., 1994; Stramann and Ryan 1997). However, interactions between insect herbivore and monocot plants have received less attention, although maize plants' ability to correlate the amount of volatile released, with lepidopteran larval attack has been studied (De Mores et al., 1998; Turnings et al., 1995;

Turlings and Tumlinson, 1992; Engelberth et al., 2004; Lait et al., 2003; Stettner et al., 2000; Pare et al., 1998; Rose et al., 1996). This is unfortunate because the world's most important food crops are the monocots.

This present study focuses on understanding a novel maize–herbivore defense system. A unique 33-KDa cysteine protease (Mir1-CP) that rapidly accumulates in the whorls of maize (*Zea mays* L.) lines that have genetic resistance to leaf feeding by *Spodoptera frugiperda* and a number of other Lepidoptera (Davis et al., 1988) has been identified. Unlike in many other plant species (Ryan, 1990), fall armyworm larval feeding causes the accumulation of the unique 33-KDa cysteine protease (Mir1-CP) at the feeding site, rather than a cysteine protease inhibitor. Maximum accumulation of the Mir1-CP occurs in the yellow-green mid-whorl region and increases in abundance for up to 7 days after larval infestation (Pechan et al., 2000). Furthermore, the accumulation occurs more rapidly than other insect-induced plant defense proteins (Ryan, 2000). Physiological studies show lepidopteran larvae to be less effective in converting their ingested and digested food into body mass when feeding on yellow-green tissues of resistant maize inbreds (Chang et al., 2000), indicating that the yellow-green tissues have the greatest inhibitory effect on larval growth. Field and laboratory experiments indicate that insects reared on these lines have reduced growth and impaired nutrient utilization (Chang et al., 2000). Larval growth was also reduced approximately 70% when larvae were reared on transgenic maize callus over-expressing Mir1-CP. All these results suggested that we examine the insect to determine the potential targets of Mir1-CP. For

initial studies, we selected a component of the insect midgut, the peritrophic matrix (PM) for investigation.

The PM is a membranous, extracellular, semipermeable midgut epithelial secretion that surrounds the food bolus. Basically it is composed of glycoproteins, proteoglycans and chitin. It protects the larval midgut from chemical and physical damage and is involved in digestive processes (Lehane, 1997). A number of studies demonstrated that disruption of the chitin network with lectins or Calcofluor increased PM permeability and insect mortality (Cohen, 1987). The metalloprotease, enhancin, which is produced by the *T. ni* granulosis virus has been shown to specifically degrade insect intestinal mucin (IIM), a structural protein in the *Tricoplusia ni* PM. Using a dual chamber apparatus (Spence and Kawata, 1993) designed to determine PM permeability *in vitro*, Wang and Granados (2000) determined that *in vitro* treatment with enhancin increased the permeability of the *Tricoplusia. ni* PM to Blue Dextran 2000 and the baculovirus *Autographa california* (Peng et al., 1999).

Scanning electron microscopy (SEM) studies indicated that the peritrophic matrix (PM) of larvae reared on yellow-green tissues of either transgenic maize leaves or its callus was severely damaged (Pechan et al., 2002). Cracks, holes and fissures were evident in the PM and were more abundant on the luminal side of the matrix that was in contact with the food bolus. The PM of larvae reared on BMS (Black Mexican Sweetcorn) callus expressing Mir1-CP also showed considerable damage (Pechan et al., 2002). These results suggest that Mir1-CP inhibits larval growth *in vivo* by attacking the PM and disrupting the digestive process. This study focuses on understanding the

functional involvement of the cysteine protease (Mir1-CP) in a plant-herbivore defense mechanism. We used purified recombinant 33-KDa cysteine protease (Mir1-CP) and its two mutated forms (Mut1 and Mut2) for determining their effects on the permeability of PMs from fall armyworm larvae. The purified Mir1-CP was also used to determine their minimal effective dosage on lepidopteran larval growth as well as to qualitatively determine their direct morphological effects on PM and gut regions of fall armyworm larvae.

BACKGROUND INFORMATION

Differential plant response towards wounding and herbivore damage

Plants can distinguish damage done mechanically from those done by insect feeding by producing differential responses towards different wound stimuli. This discrimination is due to plants ability to recognize insect derived elicitors (Alborn et al., 1997; Korth and Dixon, 1997; Halitschke et al., 2001). Studies on *Arabidopsis* and potato leaf genes have shown that lepidopteran larvae-elicited transcriptional changes differed from those caused by mechanical wounding by forceps (Reymond et al., 2000, Korth and Dixon, 1997). Furthermore, plants initiate synthesis of specific blends of volatiles to attract natural enemies of the herbivore (Alborn et al., 1997; Pare and Tumlinson, 1997; Schittko et al., 2001; De Mores et al., 1998) and form neoplasmic tissues to impede larval entry (Doss et al., 2000). Oral secretions of chewing and sucking insect pests not only stimulate wound-inducing genes in plants (Korth and Dixon 1997)

but also have caused release of parasitoid-attacking volatiles (Alborn et al., 1997; Halitschke et al., 2001).

Plants defense response to phloem-feeding insects differs from insects with chewing and sucking mouthparts (Reymond et al., 2000). Phloem-feeding insects penetrate plant epidermal and parenchymal cells, causing limited intracellular or intradermal plant damage (Miles, 1999). For defense against phloem-feeders, plants activate genes that generally provide resistance against bacterial and fungal pathogens (Fidantsef et al., 1999; Moran et al., 2002; Zhu-Salzman et al., 2004). This elevates cellular concentration of salicylic acid (SA) and increases pathogenesis-related (PR) gene expression to provide disease resistance (Wu et al., 1997; Chamnongpol et al., 1998). Salicylic acid has been found to be an effective signaling hormone in inactivating both local and systemic defense against pathogens in many plant species (Cao et al., 1998; Dempsey et al., 1999; Zhang et al., 1999). Plants response to pathogen attack is similar to those of phloem-feeders, because insect stylets and fungal hyphae may have similar effects on the host plant (Fidantsef et al., 1999).

The hydrolytic enzymes present in the insect saliva serve as elicitors for host plants (Miles, 1999; Felton et al., 2001). The addition of gut regurgitant to artificially damaged leaves has released a volatile blend similar to those of herbivore-damaged plants. Insect saliva also induces systemic acquired resistance (SAR) in host plants (Felton et al., 1999; Sticher et al., 1997). Interestingly, plant derived fatty acid portion in insects oral secretions like volicitin, may interact with the octadecanoid (OD) pathway in plants to release volatile compounds. The OD pathway stimulates plants to trigger

endogenous defense responses and signals to parasitic organisms (Thaler, 1999). Thus plants have developed diverse mechanisms for defense against various insect attacks.

Plants also produce a variety of endogenous signaling molecules to regulate signal transduction cascades for activating and modulating defense-related genes. Among the various plant defense responses, wound induction plays a central role. It can occur either at the injury site or in distal systemic tissue (Ryan and Pearce, 1998), through the action of jasmonic acid (JA). In addition to commonly known signaling molecules, considerable attention has been received by oligogalacturonic acid (OGA) in up-regulating gene encoding defensive proteins. OGA is produced by the hydrolysis of polygalacturonides by polygalacturonase (John et al., 1997) and is considered downstream of JA signal transduction pathway (Orozco-Cardenas and Ryan, 1999). OGA is responsible for production of reactive oxygen species (ROS) like H_2O_2 (Orozco-Cardenas et al., 2001), which leads to defensive gene induction (Orozco-Cardenas and Ryan, 1999). H_2O_2 production has been observed in several plant species in response to both mechanical wounding and herbivore attack. Thus, JA and OGA production link the wound response and H_2O_2 production.

Insects adaptation to plant defense mechanism

In the evolutionary race between insect and plant, insects' salivary secretions have defeated plant defense. Insect salivary glands produce ROS (reactive oxygen species) to suppress the induced resistance by increasing SA production and disrupting the octadecanoid pathway for JA in tobacco (Niki et al., 1998; Doarse et al., 1995; Bi et al., 1997b, c). The enzyme GOX (glucose oxidase), present in larval salivary glands and

spinnerets, oxidizes systemin at the feeding site (Felton and Eischenseer, 1999) and inhibits lipoxygenase activity (Bi and Felton, unpublished data). Plant lipoxygenase is an indicator of induced resistance and a key enzyme in JA biosynthesis (Bi et al., 1994, Felton et al., 1994; Bi and Felton, 1995; Bi et al., 1997a). Thus, GOX suppresses the defensive secretion by indirectly inhibiting JA biosynthesis (Musser et al., 2002).

Although protease inhibitors have been shown to control insect pests including phloem-feeders (Rahbe and Febvay, 1993; Tran et al., 1997), their effectiveness was significantly lowered by insect digestive proteases. Insects suppress protease inhibitor effects by either overproduction of existing digestive proteases (De Leo et al., 1998) or synthesizing inhibitor-insensitive proteases (Bolter and Jongsma, 1995; Jongsma et al., 1995; Bown et al., 1997; Cloutier et al., 2000; Mazumdar-Leighton and Broadway, 2001; Zhu-Salman et al., 2003). Insects may also develop insensitivity by developing numerous protease isoforms with varying sensitivity to a specific plant protease inhibitor (Orr et al., 1994) or inactivate the plant protease inhibitors by direct proteolytic fragmentation (Michaud et al., 1995; Giri et al., 1998; Zhu-Salman et al., 2003). Thus, the insects' counter-defense mechanism involves broad-transcriptional regulation of large numbers of genes in its digestive tract cell lining to overcome effects of plant protease inhibitors (Moon et al., 2004).

Plant cysteine proteases

Cysteine proteases are found in bacteria, eukaryotic microorganisms, animals and plants (Barrett, 1986). They are endopeptidyl-hydrolases with a cysteine residue at its active site. They are usually identified based on their active site inhibitors (iodoacetate,

iodoacetamide and E64) and are activated by thiol compounds at acidic pH optima. They play an essential role in storage protein mobilization during seed germination (Becker et al., 1994), environmental stress such as drought salinity, chilling and changes in N-levels (Harrak et al., 2001; Jones and Mullet, 1995; Koizumi et al., 1993; Schaffer and Fisher, 1988, Ho et al., 2000), hypersensitive response leading to programmed cell death (PCD) and senescence (Pechan et al., 2000; Funk et al., 2002; Yamada et al., 2001; Ceros and Carbonell, 1993; Zhao et al., 2000; Kruger et al., 2002; Avrova et al., 1999; D'Silva et al., 1998; Solomon et al., 1999). The cysteine proteases are also involved in defensive roles in response to wounding (Linthorst et al., 1993; Ueda et al., 2000; Kinoshita et al., 1999; Gorlach et al., 1996). Based on their structural and evolutionary relationship, cysteine proteases are classified into at least six superfamilies or clans by Rawlings and Barrett (1993). Plant cysteine proteases usually belong to the papain (C1) and legumain (C13) families (Barrett et al., 1984). Other members of the cysteine protease family include caspases (C14), calpins (C2), ubiquitin C-terminal hydrolases (C12) and ubiquitin-specific proteinases (C19) (Vierstra, 2003).

The cysteine proteases are synthesized as preproproteins on membrane bound polysomes in the cytoplasm (Wiederanders, 2003). The 'pre' sequence is required for intracellular localization and 'pro' sequence is removed during enzyme activation (Mitsuhashi and Minamikawa, 1989; Koehler and Ho, 1990; De Barros and Larkins, 1994; Mitsuhasti and Oaks, 1994; Domoto et al., 1995; Drake et al., 1996). The large precursor consists of a short N-terminal and long C-terminal propeptide. The inactive proenzymes enter the endoplasmic reticulum lumen and are transported to the vacuole or

cell wall. Most soluble proteases would have a tetrapeptide sequence (KDAEL/HDEL) at their C-terminal region, which are recognized by receptors on the Golgi apparatus for transport into the trans-Golgi network (Okamoto and Minamikawa, 1995). However, papain like cysteine proteases having endoplasmic reticulum (ER) retention signal are transported into large vesicles that buds off from the ER. These types of proteases fuse directly with protein storage vacuoles or cell wall (Toyoka et al., 2000; Okamoto and Minamikawa, 1995). The retention signal in the endoplasmic reticulum also regulates delivery of proteins to other compartments (Yamada et al., 2001). The N- and C-terminal propeptides in the vacuoles or cell wall are sequentially removed to produce the mature form (Yamada et al., 2001). The papain family of proteases has the ERFNIN ($EX_3RX_3FX_2NX_3I/VX_3N$) motif in their N-terminal propeptides. The ERFNIN motif is involved in the post-translational modifications required for the formation of the mature N-terminus of cysteine protease (Karrer et al., 1993). This also function as an auto-inhibitory domain (Beers et al., 2000). The catalytic site of these cysteine protease are involved in thiolate-imidazolium ion pair between Cys^{25} and His^{159} as well as the Asn^{175} residue (papain numbering) for proper histidine side chain orientation (Beers et al., 2004).

Legumains are also synthesized in the rough endoplasmic reticulum. The nascent polypeptide is segregated into the lumen of the endoplasmic reticulum and is packed for transport in an inactive state in dense vesicles that move to the vacuoles or cell wall (Fischer et al., 2000; Schlereth et al., 2001). They are then processed at their final destination site by two proteolytic cleavage reactions (Kuroyanagi et al., 2002). The first

cleavage involves removal of C-terminal pro-peptide by autocatalysis. This helps to auto-inhibit the enzymatic activity during transit. The N-terminal peptide is removed in the second cleavage reaction, which might contribute to appropriate folding and targeting of the enzyme.

Plants self-regulate cysteine protease activity by synthesizing protease inhibitors (Turk and Bode, 1991). The protease inhibitors are subdivided into four families: stefin, cystatin, staphostatins and kininogen-based on their sequence homology, intrastrand disulfide position and their molecular mass. However, little is known about the gene families (Fernandes et al., 1993; Waldron et al., 1993) encoding these plant cysteine protease inhibitors. Protease inhibitor gene expression is usually limited to specific plant growth phase or particular organs. They are expressed during germination (Botella et al., 1996), early leaf senescence (Huang et al., 2001), drought (Waldron et al., 1993) or cold and salt stress (Pernas et al., 2000; Van der Vyver et al., 2003). Wounding or methyl jasmonate induction also evokes similar pattern of gene expression (Botella et al., 1996; Zhao et al., 1996).

Characteristics of *mir1* and Mir1-CP.

A single copy gene *mir1,* encodes for Mir1-CP (Pechan et al., 1999). It has amino acid sequence similar to other cysteine proteases in the papain super family (Pechan et al., 1999). These proteases are synthesized as a preproproteins that are post translationally processed to become enzymatically active. Cysteine proteases from several baculoviruses that infect Lepidoptera including *A. californica* and *Spodoptera exigua* nucleopolyhedrovirus (Rawlings et al., 1992; Slack et al., 1995; Wi et al., 1999)

have significant levels of similarity to Mir1-CP. The predicted molecular mass for mature Mir1-CP is 25.4 KDA (Pechan et al., 1999), which does not agree with the 33-KDa mass obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectroscopy, and suggests that Mir1-CP may be glycosylated. Unpublished data (Shivaji and Luthe) indicated that Mir1-CP is glycosylated. Additional amino acid sequence comparisons indicated a region of Mir1-CP from position 188-236 has approximately 70% similarity to the chitin-binding domains found in a *Volvox* cysteine protease (Amon et al., 1998), wheat germ agglutinin (Raikhel et al., 1993; Shen 1999) and hevein (Garcia-Olmedo et al., 1998; Koo et al., 1998). This similarity suggests that Mir1-CP has chitin binding activity and is confirmed by its capability of binding to ovamucoid beads (Shivaji and Luthe, unpublished data).

Amino acid sequence comparison also showed no match in the database for the 281-bp sequence of *mir1* at its 3' end. Of these, 75-bp are found in the coding sequence and the remaining 206 are in the 3' untranslated region. A database search for short nearly identical matches using the completely translated 25 carboxyl-terminal amino acid sequence revealed some small regions of similarity with other proteins. These included regions from an *Aspergillus nidulans* chitinase (Bostwick et al., 1992), a mouse apoptosis activating factor (Apaf1) (Takaya et al., 1998) and insect intestinal mucin (IIM) (Wang and Granados, 1997a, b; Cecconi et al., 1998). Also, the 30-amino acid C-terminal sequence of Mir1-CP has considerable similarity to the citrus tatter virus movement protein (Ohira et al., 1994) (Figure 1.1).

Figure 1.1 Sequence comparison of last 25 amino acids.

Insect digestive system

Insect digestive system is basically a continuous tube running from the mouth to the anus. But, the structure and function of the alimentary canal may be modified based upon whether the insect feeds on solid versus liquid food and/or animal versus plant food. Despite evolutionary diversity, the three major divisions of the alimentary canal-foregut (stomodeum), midgut (mesenteron) and hindgut (proctodeum) – can be identified at embryological, morphological, and physiological level in all insects (House, 1974; McFarlane, 1985; Nation, 1983). The cells of the foregut and hindgut are ectodermal in their origin and possess protective cuticular lining called intima, which is continuous with

that covering the outside of the body. The foregut and hindgut cuticular lining are usually unsclerotized, consisting only of endocuticle and epicuticle. Midgut cells lack the cuticular lining, as they are endodermal in origin.

The foregut usually consists of flattened and undifferentiated cells. They are not involved in absorption or secretions, but assumed to be concerned in moving the food back towards the midgut. Starting from the mouth, foregut is commonly differentiated into the buccal cavity (mouth), pharynx, esophagus, crop and proventriculus. The buccal cavity or the mouth acts as an enlarged cavity receiving either slightly chewed food from mandibulate insects or fluids from insects with piercing and sucking mouthparts. The salivary glands are positioned diverticular to their anterior part and secrete enzymes (mostly amylase) and fluids for extra-oral digestion and lubrication of the food (Cohen, 1995). The salivary glands are also modified in some insects for producing silk, toxin and antimicrobes. The backward movement of the ingested food is aided by pharynx, with the help of its well-developed musculature. The food passes though the esophagus into the crop for storage. Depending upon the type of insect species or the developmental stage, the crop may either appear dilated or may be a diverticulum from the main part of the foregut (eg: Diptera). The inner walls of the crop are usually folded longitudinally and transversely. The folds are flattened as the crop is filled, allowing a very large increase in volume. The crop periodically releases some of its content though the proventriculus and into the midgut. The proventriculus is very variable in form and is often composed of stomodeal valve, which prevents the backward movement of solid food but permits liquid movement in both directions. The stomodeal valves have been

modified into finger-like projections (eg: honey bee), flap-like extensions (eg: mole crickets) or sclerotized for mechanical grinding (eg: beetles).

The midgut is the principal site for digestive enzyme secretion and for digestion and absorption in many insects (Dow, 1986). It acts as an important site of detoxification as it is the only site in an insect body where external substances can have direct contact with the insect cells. The three major cell types of the midgut are principal cells, regenerate cells and globlet cell. These endodermal cells are protected by peritrophic matrix. The principal cells are basically tall columnar epithelial cells that are involved in digestive enzyme synthesis and secretion. Enzyme synthesis occurs only at the time of secretion so that stores of enzymes do not accumulate in the cells. The microvilli extend from their apical side and are covered by glycocalyx, which in most insects, is a layer of filamentous glycoproteins. The microvilli greatly increase the surface area of the cell membrane, enabling maximal nutrient absorption.

Since the principal cells are secretory in function, they possess rough endoplasmic reticulum, golgi bodies and membrane bound vesicles. They secrete enzymes by apocrine or microapocrine method in the anterior midgut and by eccrine method in the posterior region. Holocrine secretion has also been reported, but is considered rare. The enzyme secretion could be constitutive or may be under prandial, endocrine or paracrine control. Most insects utilize constitutive rather than regulated secretion. Some insects contain specialized cells called mycetomes in their midgut to secrete enzymes for polymeric food digestion (termites) or for extra-oral digestion (blow-flies).

The principal cells have a limited life and, in most insects are continuously replaced from regenerative cells at the base of the midgut epithelium. These often occur in groups, known as nidi, and are positioned at the base of mature cell in larval Diptera and Lepidoptera, or as clusters in Orthoptera and Odonata, or at the apex of gastric caeca in Coleoptera. Scattered among the principal cells of the midgut are the flask-shaped globlet cells. They help in nutrient absorption and maintain gut pH though their V-ATPase pump (Chao et al., 1991). The folds in the midgut, gastric caeca and microvilli help in increasing the surface area. The absorbed nutrients are stored in fat body cells, found either free floating (peripheral) or around the gut (peri-viseral/trophocytes).

The malphigian tublule at the end of the midgut marks the beginning of the hindgut. The junction is connected by pylorus and a valvular structure occurs here. The region posterior to the malphigian tubule is known as the ileum, which runs into the rectum. The ileum plays an active role in acid-base balance and is also a major site of isosmotic fluid reabsorption. However some insects have a distinct region called colon. The terminal rectal region is an enlarged sac consisting of groups of cells called rectal papilla/rectal pads. They play an essential role in reabsorption of water, ions and nutrients against strong osmotic gradients and produces highly concentrated hypertonic excreta in many insects. Aquatic insects have specialized cells called chloride cells for inorganic ion reabsorption. In comparison with the foregut, the hindgut has a thinner cuticular lining and are two times more permeable. However, the hindgut cells are impermeable to high molecular compounds like polysaccharides, inulin, etc.

Peritrophic matrix (PM)

The extra-cellular midgut epithelial secretion that surrounds the food bolus in most arthopods was first named as peritrophic membrane (Balbiani, 1890). Later, it was renamed as peritrophic envelope as it is composed of several layers (Terra, 1990). The membranous, extracellular and non-lipid bilayer nature has led to the gradual replacement of its name to peritrophic matrix (PM). Basically, the PM is composed of glycoproteins, proteoglycan and chitin. Chitin (poly ß-1,4-N-acetyl-D-glucosamine) forms between 3 and 13% of the PM (Becker, 1980; Zimmermann et al., 1975, Peters, 1992) and are usually present in their crystallized form as chitin fibrils. PMs from different insect species have shown to contain all three forms (α, β and γ) of chitin crystallites (Peters, 1992). The chitin fibrils are normally arranged as a random felt work or in a more ordered configuration (Peters, 1969; Peters et al., 1979). The microvilli are generally believed to force the ordered hexagonal or orthogonal arrangement of microfibril by acting as a physical mold or template.

Proteins accounts for 35-55% of PM by weight (De Mets and Jeuniaux, 1962; Ono and Kato, 1968; Zimmermann et al., 1975; Adang and Spence, 1983) and their number varies from species to species (Stamm et al., 1978; Adang and Spence, 1983; Dorners and Peters, 1988; Derksen and Granados, 1988; Lehane et al., 1996; Moskalyk et al., 1995). It is believed that the chitin-protein matrix may be responsible for the strength and elasticity of the PM. The reminder of the PM is formed by proteoglycans, that differ from glycoproteins in having long and unbranched carbohydrate chains covalently attached to the protein (Lehane, 1976; Lehane et al., 1996; Nisizawa et al., 1963; Peters,

1976). The chains are usually repeating disaccharide units of hexosamine and uronic acid residues of either D-glucuronic or iduronic acid. The carboxylic and/or sulfate side chain on their sugar residues make proteoglycans highly charged (Kjellen and Lindahl, 1991) and hydrophilic, thereby creating turgor pressure within the PM by attracting active cations, which in turn attracts large amounts of water (Hardingham et al., 1990; Hardingham and Fosang, 1992). These properties enable proteoglycans to be the major space filling molecule in PM (Kjellen and Lindahl, 1991) and to provide strength against compressive forces (Jackson et al., 1991) They also play essential role in determining permeability characteristics of the peritrophic matrix.

The PM can be categorized as type I and type II based on their method of formation (Wigglesworth, 1933). However, the functional and molecular distinction of having two types of PM in different insect species or at different life stages of the same insect is unclear. Type I PMs are produced by the midgut epithelial cells by delamination and is found in cockroaches (Dictyoptera), grasshoppers (Orthoptera), beetles (Coleoptera), bees, wasps and ants (Hymenoptera), moths and butterflies (Lepidoptera), and in hematophagous adult mosquitoes (Diptera) (Waterhouse, 1953a, b; Richards and Richards, 1977; Wigglesworth, 1972; Jimenez and Gilliam, 1990; Ferreira et al., 1990; Peters, 1992). The formation of these PMs is induced only after food ingestion (Bolognesi et al., 2001). Some insects produce type I PMs only in their middle third (*Ptinus)* or posterior end (*Cionus*) of the midgut (Richards and Richards, 1977). When the type I PM is not continuously produced, a viscous material called peritrophic gel protects the midgut epithelium (Ferreira and Terra, 1989). Peritrophic gel differs from

PM in having no mechanical resistance, high permeability and protects midgut cells from food only when the enzymes and fluids are in their forward movement (Ferreira et al., 1999).

Type II PMs are produced by specialized cells called cardia, present either in the anterior midgut or between crop and proventriculus of the foregut. They are produced as concentric or unbroken sleeves lining the midgut (Peters, 1992; Waterhouse, 1953a, b). Larval and adult (non-hematophagous) mosquitoes and flies (Diptera) and few adult Lepidoptera contain type II PMs. Hemiptera and Thysanoptera midgut cells are not lined by PMs but by protective perimicrovillar membrane (Silva et al., 1995). However, insects such as some adult ants (Hymenoptera), most adult moths and butterflies (Lepidoptera), lice (Phthiraptera), book lice (Psocoptera), adult fleas (Siphonaptera), bruchid beetles, Zoraptera, Strepsiptera, Raphidioptera and Megaloptera, lack PM or other protective lining in their midgut (Peters, 1992).

The chemical properties of the PM such as strength, elasticity and porosity, plays an essential role in the intestinal biology of the insect. The most common function of the PM in many insects is the mechanical protection of the midgut epithelium and acting as lubricants for food passage though the gut (Sutha and Muthu, 1988). PMs ability to withstand high pressure (Zhuzhikov, 1970; Zimmermann and Mehlan, 1976) prevents ingested food of high molecular weight from swelling and rupturing the midgut (Zhuzhikov, 1964). The PM plays an essential role in digestion by compartmentalizing the midgut into ecto and endo-peritrophic space as well as regulating passage of luminal digestive enzymes based on its permeability. The compartmentalization helps recirculate

digestive enzymes and increases the efficiency of polymeric food. It also helps in the efficient hydrolysis of oligomeric food by allowing its transfer to the ectoperitrophic space. Their semi-permeability characteristics, helps in enzyme immobilization as well. The PMs ability to separate the epithelium from the food shows its similarity as a protective layer to the mucus in vertebrate gut (Tse and Chadee, 1991). The PMs antioxidant function protects the insect from toxic plant allochemicals, pathogens and other damaging chemicals. These characteristics indicate the insect's adaptation to decrease the detrimental effects that may be caused by ingested food.

PM proteins

The traditional view of PM is under revision based on recent knowledge of the molecular characteristics of the peritrophic matrix proteins. PM protein structure and biochemical function have been investigated in different insect species (Elvin et al., 1996, Wang and Granados, 1997, a b; Casu et al., 1997; Schorderet et al., 1998), and based on their association with the PM (Tellam, 1996a, b; Tellam et al., 1999) have been grouped into four classes. Class I proteins are weakly attached to PM and are removed by physiological buffers (Tellam, 1996a, b). They lack binding domains for specific interactions with chitin and other PM components. Presumably some of these proteins were trapped during their transit though the PM, although no experimental evidence of their immobilization is known (Tellam et al., 1999). Class II, are pheripheral proteins that are removed by mild detergents that disrupt weak protein-protein, protein-oligosaccharide or protein-chitin interactions, as PM contains little or no lipids (Peters, 1992). The third class of PM proteins requires strong denaturants such as 6 M urea or 6

M guanidine HCl for their extraction. They are also referred to as peritrophins (Elvin et al., 1996; Tellam, 1996a) and have a strong interaction with the PM. They are the major fraction of the PM proteins and have been characterized at the molecular level from *L. cuprina* (Elvin et al., 1996; Schorderet et al., 1998; Casu et al., 1997), *C. bezziana* (Wijffels, unpublished data), *A. gambiae* (Shen and Jacobs-Lorena, 1998) and *T. ni* (Wang and Granados, 1997b). The fourth classes of PM proteins are non-extractable by strong denaturants or detergent treatments. They are thought to be covalently cross-linked either to themselves or to other PM constituents.

To date, five peritrophins have been identified from three insect species (Elvin et al., 1996; Wang and Granados, 1997a, b; Casu et al., 1997; Schorderet et al., 1998; Shen and Jacobs-Lorena, 1998). The first PM protein to be identified and studied at its biochemical and molecular level was peritrophin-44, from type II PM of *L. cuprina* larvae. Peritrophin-48, peritrophin-30 and peritrophin-95 were the other PM proteins that were later identified from the same species. The only type I PM protein to be identified, characterized (Wang and Granados, 1997a) and sequence determined (Wang and Granados, 1997b), was insect intestinal mucin, from *T. ni* larvae. Although the Ag-Aper1 from *A. gambiae,* was initially considered as type I PM protein, it requires further conformation as its expression occurs independently of PM formation (Shen and Jacon-Lorena, 1998). All identified PM proteins contain cysteine rich domains, which contribute towards PM structural strength, elasticity and porosity by forming intra-domain disulfide bonds. They also contain glycosylated chitin-binding domains (Wang and Granados, 2000) and with the exception of peritrophin-30, have acidic isoelectric

points (Tellam et al., 1999). The high PM binding affinity of the peritrophins enables them to survive the digestive enzyme-rich environment in the gut (Wang and Granados, 2000) and protects the PM from pathogens and toxins. However, the peritrophin binding affinity can be affected by competitive inhibitors like calcofluor (Wang and Granados, 2000) and wheat germ agglutinin (Czapla and Lang, 1990), commercial insecticides like Dimilin (diflubenzuron) and enzymes like chitinase, which inhibit chitin synthesis, or by using peritrophin-degrading baculovirus enzymes like enhancin (Peng et al., 1999). At the moment, an increasing number PM proteins are been sequenced from various insects and their partial sequences have been deposited in Genbank. (http://www.ncbi.nlm.nih.gov/genbank/index.html). This could provide sufficient resource for designing recombinant proteins with potentials to interfere with PM formation.

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CHAPTER II

DEGRADATION OF THE *Spodoptera frugiperda* PERITROPHIC MATRIX BY AN INDUCIBLE MAIZE CYSTEINE PROTEASE

ABSTRACT

A unique 33-KDa cysteine protease (Mir1-CP) rapidly accumulates at the feeding site in the whorls of maize (*Zea mays* L.) lines that are resistant to herbivory by *Spodoptera frugiperda* and other lepidopteran species. When larvae were reared on resistant plants, larval growth was reduced due to impaired nutrient utilization. Scanning electron microscopy (SEM) indicated that the peritrophic matrix (PM) was damaged when larvae fed on resistant plants or transgenic maize callus expressing Mir1-CP. To directly determine the effects of Mir1-CP on the PM *in vitro*, dissected PMs were treated with purified, recombinant Mir1-CP and the movement of Blue Dextran 2000 across the PM was measured. Mir1-CP completely permeabilized the PM and the time required to reach full permeability was inversely proportional to the concentration of Mir1-CP. Inclusion of E64, a specific cysteine protease inhibitor prevented the damage. The lumen side of the PM was more vulnerable to Mir1-CP attack than the epithelial side. Mir1-CP damaged the PM at pH values as high as 8.5 and more actively permeabilized the PM than equivalent concentrations of the cysteine proteases papain, bromelain and ficin. The effect of Mir1-CP on the PMs of *Helicoverpa zea*, *Danaus plexippus*, *Ostrinia nubilalis,*

Periplaneta americana and *Tenebrio molitor* also was tested, but the greatest effect was on the *S. frugiperda* PM. These results demonstrate that the insect-inducible Mir1-CP directly damages the PM *in vitro* and is critical to insect defense in maize.

INTRODUCTION

Plants often defend themselves against attack from insect herbivores by accumulating defensive proteins. These include protease inhibitors, lectins, chitinases, oxidative enzymes and enzymes that catalyze the formation of secondary defense compounds (Constabel et al., 1999). These defensive proteins have different types of deleterious effects on insects. One of the potential targets for plant defensive proteins is the insects' peritrophic matrix (PM). The PM consists of a thin, extracellular lamina that lines the midgut of most insects (Lehane, 1997; Barbehann, 2001; Wang and Granados, 2001; Terra, 2001). In addition to being a semi-permeable structure that is essential for nutrient passage and absorption (Wang and Granados, 2001; Terra, 2001), the PM is often the insects' first line of defense. It protects the midgut against pathogens and toxins (Barbehenn, 2001); improves digestion and protects epithelial microvilli from abrasion by food particles (Richards and Richards, 1977; Miller and Lehane, 1993; Santos and Terra, 1986; Derksen and Granados, 1988; Wang and Granados, 2001; Terra, 2001). It also provides an antioxidant defense for the midgut epithelium (Summers and Felton, 1996; Barbehennn and Stannard, 2004). Because of its importance in defense and digestion, disruption of the PM is often deleterious, if not lethal to the insect (Wang and Granados, 2001).

A model for the PM of lepidopteran larvae consists of chitin fibrils held together with chitin-binding proteins (CBP) (Wang and Granados, 2001; Wang et al., 2004). Disulfide bonding stabilizes the interaction of the CBPs with the chitin fibrils. Insect Intestinal Mucin (IIM), a highly glycosylated protein, associates with the CBP and protects them from degradation by digestive proteases. Agents that impair any of these interactions can disrupt PM formation and structure (Wang and Granados, 2001). For example, disruption of the chitin network with lectins or calcofluor increased PM permeability and insect mortality (Cohen, 1987; Harper et al., 1998; Wang and Granados, 2000). The metalloprotease, enhancin, which is produced by the *Tricoplusia ni* granulosis virus, has been shown to specifically degrade IIM. Using a dual chamber apparatus (Spence and Kawata, 1993) designed to determine PM permeability *in vitro*, Peng et al. (1999) determined that *in vitro* treatment with enhancin increased the permeability of the *T. ni* PM to Blue Dextran 2000 and subsequent susceptibility to infection by the baculovirus *Autographa California*.

We have identified another novel insect defense protein that appears to attack the PM (Pechan et al., 2002). A unique 33-KDa cysteine protease (Mir1-CP) rapidly accumulates in the whorls of maize (*Zea mays* L.) lines that have genetic resistance to leaf feeding by *Spodoptera frugiperda* and a number of other Lepidoptera (Davis et al., 1988). Field and laboratory experiments indicate that insects reared on these lines have reduced growth and impaired nutrient utilization (Chang et al., 2000). Larval growth also was reduced approximately 70% when larvae were reared on transgenic maize callus, over-expressing the gene encoding Mir1-CP (Pechan et al., 2000). This suggested that

Mir1-CP has a direct detrimental effect on larval growth. Scanning electron microscopy (SEM) was conducted to determine if there was physical damage to the insect midgut in response to feeding on tissues that expressed Mir1-CP. SEM indicated that the peritrophic matrix (PM) of larvae reared on resistant maize leaves or transgenic callus was severely damaged (Pechan et al., 2002). Cracks, holes and fissures were evident in the PM and were more abundant on the luminal side of the matrix that was in contact with the food bolus. These results suggest that Mir1-CP inhibits larval growth *in vivo* by attacking the PM and disrupting the digestive process.

Although studies using SEM indicated that the PMs of larvae that fed on maize tissue over-expressing Mir1-CP were damaged (Pechan et al., 2002), this study was done to extend this prior work and determine if purified Mir1-CP could attack the PM *in vitro.* We also hoped that it would provide a quantitative estimate of the amount of permeabilization. The results reported here indicate that Mir1-CP completely permeabilizes the PM and that the time required for complete permeabilization depends on the concentration of Mir1-CP. Although it has been shown that baculovirus metalloprotease, enhancin, attacks the PM of *T. ni* (Peng et al., 1999), we believe this is the first report of a plant protease damaging the insect PM.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Fisher Biotech (Fair Lawn, NJ). HPLC columns (size exclusion and reverse phase chromatography) were purchased from

Phenomenex (Torrance, CA). The MALDI-TOF calibration kit was purchased from Perceptive Biosystems (Foster City, CA). Commercial proteases with the following specific activities were obtained from Sigma (St. Louis, MO): papain (33 U/mg), bromelain (8.7 U/mg), ficin (19 U/mg), thermolysin (91 U/mg) and chymotrypsin (52 U/mg). Deionized water (18 MΩ) was used in all experiments.

Insect rearing

All fourth instar larval colonies of *Spodoptera frugiperda, Helicoverpa zea, Periplaneta americana, Danaus plexippus, Ostrinia nubilalis, and Tenebrio molitor*, reared on wheat germ artificial diet (Davis, 1976) with a photoperiod of 16:8 were obtained from a laboratory colony maintained by the USDA-ARS Corn Host Plant Resistance Research Unit at Mississippi State University.

Purification of Mir1-CP

Mir1-CP was expressed in the hemolymph of *S. frugiperda* instead of *Trichoplusia ni* larvae as previously described (Pechan et al., 2004). Mir1-CP was purified from the hemolymph using size exclusion and reverse phase HPLC. For size exclusion HPLC, 10µl hemolymph was applied to a TSK–Gel column (7.8 mm ID X 30 cm dimension and 6 µm particle size, Phenomenex) and was eluted in 0.1 M phosphate buffer (pH 7$\pm$0.2) at flow rate of 0.75 ml/min and a pressure of 33 bars using an Hewlett Packard 1050 high pressure liquid chromatography. Column was pre-calibrated with known standards in the 11-60 KDa range. Protein elution was monitored at 214 nm using a Hitachi UV detector. Fractions (0.5 ml) corresponding to 33-KDa mass of Mir1-CP

were collected between seventh and eight minute based on the calibration. Eluted fractions with protease activity were subjected to further purification using a pre-calibrated reverse phase chromatographic column (250 x 2 mm dimension, 5 μm particle size), with gradient elution from 90% H_2O/ 10% acetonitrile to 70% H_2O/ 30% acetonitrile, 30 min; to 90% H_2O/10% acetonitrile, 5 min; 10% H_2O /90% acetonitrile, 4 min; to 90% H_2O/ 10% acetonitrile, 1 min, at a flow rate of 0.5ml/min and pressure of 3129psi. Fractions (0.5ml) collected at 12 min with protease activity were subjected to a second round of reverse phase HPLC using a modified gradient elution of 90% H_2O/ 10% acetonitrile to 60% H_2O/ 40% acetonitrile, 30 min; to 90% H_2O/10% acetonitrile, 5 min; to 10% H_2O/ 90% acetonitrile, 4 min; to 90% H_2O/ 10% acetonitrile, 1 min; while maintaining all other parameters as the previous HPLC runs. Enzymatically active fractions were concentrated by vacuum drying and stored at –80°C.

Enzyme activity determination and immunoblotting

Protease activity in the HPLC fractions was determined using an in-gel activity assay (Michaud et al., 1993) or a rapid protease microassay (Mohan, et al., 2005). To determine the specific activity of the purified enzyme, protease activity was determined in a reaction mixture containing 0.4 M sodium phosphate (pH 6.0), 8 mM cysteine, and 4 mM EDTA (ethylenediaminetetraaceticacid), and 1.25 μM-17.5 μM of Z-Phe-Arg-AMC (Bachem, Switzerland) as substrate (Kirschke and Shaw, 1981, Tchoupe, 1991). Each reaction was incubated for 2 min at 37°C and the reaction was terminated by adding 50 mM iodoacetic acid in acetate buffer (pH 4.3). The amount of AMC liberated from

substrate was monitored spectrofluorometrically (HOEFER scientific, CA) at 460 nm. One unit of protease activity was defined as the release of 1 μmol of AMC per minute. Protein concentration was determined using the Bradford method (Bradford, 1976) with BSA as a standard. Immunoblotting of HPLC fractions was conducted as previously described (Pechan et al, 2004).

Mass Spectrometry (MALDI-TOF MS)

The molecular mass of the purified Mir1-CP was determined using an ABI Voyager Elite MALDI-TOF MS (Matrix Activated Light Desorption Ionization- Time Of Flight Mass Spectroscopy) Voyager™ Experimental station (Perspective Biosystems) (Mikes and Man, 2003). Fractions were mixed with sinapinic acid matrix in the ratio of 1:24, and excited using a nitrogen laser intensity of 3000 at 20 KV, 94% grid voltage, and 100 ns extraction delay time. Each spectrum was an average of 50 laser shots. Data Explorer™ software (Perceptive Biosystems) was used for further analysis of spectra.

Permeability apparatus and measurement

The permeability apparatus was modeled after that used by Spence and Kawata (1993) and Peng et al. (1999). The main body of the permeability apparatus was machined from a solid Teflon rod (25.4 mm diameter). The apparatus consists of two cells with a mating surface of 25 mm diameter at one end of the cell. A well of 12.5 mm diameter X 21 mm depth was made in each cell 33 mm from the mating surface. The bottom of the well was connected to the mating surface via a 9.6 mm diameter channel, fitted with a hole (2 mm diameter) drilled to within 1 mm of the other end of the cylinder.

The two ends of the cylinder were connected via a channel of 0.7112 mm diameter. To maintain proper alignment during assembly, the cylinder of one cell was machined such that the cylinder was recessed 1 mm into the cell, whereas the cylinder of the other cell was machined such that the cylinder protruded 1 mm out of the mating surface. These two surfaces insured proper alignment of the two cells when assembled. To further enhance the assembly of the apparatus, the two cells were keyed using a 2-56 X 25 mm screw on the mating surface of each cell. The solutions in the wells were stirred with a 1.5 mm (diameter) X 8 mm (length) micro-stir bar (Fisher Scientific, NJ). Proper mixing of the solutions was further enhanced by circulating the solution from the mating end of the cylinder back into the well using a dual channel peristaltic pump (Watson-Malow 400 F/DM2) running at 1 ml/min.

The PM was dissected from fourth instar *S. frugiperda* larvae, reared on artificial diet (Davis, 1976). The PMs of other fourth instar larval species were similarly obtained. The midgut of the intestinal tract was removed and transferred to a Petri dish containing modified Weever's saline (Weevers, 1966). The midgut was cut longitudinally and the PM containing the food bolus was removed. The PM was then cut open longitudinally with fine scissors, rinsed in Weever's saline and aligned flat over the pore of the permeability apparatus. The orientation of PM was established in such a way that the lumen side of the PM faced the chamber that contained Mir1-CP. To insure that the PM was not damaged during dissection, a 30 min pre-run was performed by adding 2 ml of Blue Dextran 2000 (diameter: 99 nm, Pharmacia Biotech, Inc., Piscataway, NJ) solution (10 mg/ml in Weever's saline) to the lumen-side chamber. The same volume of modified

Weever's saline was simultaneously added to the epithelial-side chamber to avoid hydrostatic stress on the PM. After the pre-run, both chambers were carefully emptied. A mixture containing 10 mg of Blue Dextran 2000 in 1 ml of Weever's saline containing 0.64 mg/ml of Mir1-CP, at pH 8.5 was added to the lumen-side chamber. An equal volume of Weever's saline was added to the epithelial-side chamber simultaneously. The test was conducted for 30 min at room temperature (24°C). Aliquots (0.1 ml) were removed from both chambers of the apparatus at 5 min intervals and refilled simultaneously. The concentration of Blue Dextran 2000 in each aliquot was determined by measuring the optical density at 610 nm. The experiment was repeated six times using a new, undamaged PM for each replicate.

RESULTS

Purification of recombinant Mir1-CP from hemolymph

Recombinant Mir1-CP was purified from hemolymph using size exclusion and reverse phase HPLC. The fraction corresponding to 33-KDa, the size of Mir1-CP (Fig. 2.1a), was collected by size exclusion HPLC and further purified by two reverse phase chromatography steps (Fig. 2.1b). The single peak from the last reverse phase HPLC showed a band of protease activity of approximately 33-KDa when it was analyzed by an in-gel activity assay (Fig. 2.2a). It cross-reacted with antibody to Mir1-CP isolated from maize plants (Fig. 2.2b), and had a molecular mass of 33.219-KDa when analyzed by MALDI-TOF MS (Fig. 2.2c). Proteolytic activity was blocked when the enzyme was preincubated with the cysteine protease inhibitor E64 (data not shown). A fluorometric

assay using Arg-Phe-AMC as substrate was used to determine the activity of the purified Mir1-CP (0.6 mg protein/ml) and it was 9 U/mg protein.

In vitro **analysis of PM permeability**

PM permeability

To determine if Mir1-CP could damage the PM *in vitro*, freshly dissected PMs from *S. frugiperda* were placed in the permeability apparatus and the flow of Blue Dextran between the chambers was measured. Following an initial lag phase of 5 min, PM permeability increased sharply when the lumen side was treated with the highest concentration (0.6 mg/ml) of Mir1-CP. Blue Dextran leakage across the PM ceased after 30 min and reached a steady state absorbance of 0.6 (Fig. 2.3). The initial Blue Dextran absorbance prior to addition of Mir1-CP was approximately 1.3, thus it appeared that the PM became completely permeable to Blue Dextran after 30 min. When the PM was treated without Mir1-CP or with Mir1-CP pre-treated with the specific cysteine protease inhibitor E-64, there was no permeability to Blue Dextran (Fig. 2.3). When the epithelial side of the PM was treated with Mir1-CP, additional time was required to reach steady state permeability and complete permeability was not attained (Fig. 2.4).

When larvae were fed on resistant maize plants, it is unlikely that they encountered concentrations of Mir1-CP as high as 0.6 mg/ml. Therefore, PMs were treated with serial dilutions of the enzyme to determine the lowest effective concentration. As the concentration decreased, the length of time required to reach steady state permeability increased. Permeability was complete after 15 h incubation at

the lowest effective concentration (12 ng/ml; 1/50000 dilution). Regardless of the Mir1-CP concentration that was tested, complete permeability was attained. A 1/60000 dilution of Mir1-CP did not permeabilize the PM.

pH and temperature

To determine if Mir1-CP could attack the PM at alkaline pH values that are typically found in the lepidopteran midgut, PM permeability was measured at pH 8.5, 9 and 10. At pH 8.5 and 9.0, it took approximately 13 and 17 min to reach one-half maximal permeability, respectively, and complete permeability was attained after 25 min (Fig. 2.5). At pH 10.0, 22 min were needed to reach one-half maximal permeability and complete permeability was not attained.

The effect of temperature on the ability of Mir1-CP to damage the PM also was tested. The recombinant enzyme was preincubated at 30°C for 30 min, overnight (approximately 12 h), or 24 h prior to testing in the permeability apparatus. There was little difference in the permeability attained when Mir1-CP was preincubated for 30 min or overnight, (Fig. 2.6), but pre-incubation at 30˚C for 24 h increased the time required to reach complete permeability. When Mir1-CP was preincubated at 40°C for 1 min, it was still active, but complete permeability was not attained. Preincubation at 40˚C for longer periods of time prevented PM damage by Mir1-CP.

Enzyme specific permeability

To determine if the ability to permeabilize the PM was specific to Mir1-CP, we tested the three additional plant-derived cysteine proteases at concentration of 0.6 mg/ml,

which was equivalent to the following number of units per treatment: bromelain, 5.2 U; papain, 19.8 U; ficin, 11.4 U. In comparison with Mir1-CP, treatment of the PM with each of the cysteine proteases increased the time required to reach steady state permeability and they did not completely permeabilize the PM (Fig. 2.7). The steady state permeability resulting from papain, bromelain and ficin treatments was approximately 70%, 33% and 17%, respectively, of that caused by Mir1-CP. At higher pH values (pH 9 and 10) these proteases had no effect on PM permeability. The two serine proteases, chymotrypsin (31.2 U/treatment) and thermolysin (54.6 U/treatment) did not permeabilize the PM at pH 8.0 (Fig. 2.7), at the other pH values, or temperatures tested (data not shown.)

Mir1-CP effect on the PMs from other insect species

To determine if Mir1-CP effect was specific to the *S. frugiperda* PM, we tested its effect on three other Lepidopterans, *Helicoverpa zea*, *Danaus plexippus* and *Ostrinia nubilalis* (Fig. 2.8). Although Mir1-CP was most effective against *S. frugiperda*, it also permeabilized the PMs of *H. zea* and *D. plexippus* when they were incubated with 0.6 mg/ml of Mir1-CP at pH 8.0. More time was required to reach one-half permeability and complete permeability was not attained for either insect. However, Mir1-CP appeared to be more effective in permeabilizing the PM of *H. zea* than *D. plexippus*. Mir1-CP did not affect the permeability of the *O. nubilialis* PM. We also tested the effect of Mir1-CP on PMs isolated from the Orthopteran *Periplaneta americana* and the Coleopteran, *Tenebrio molitor*. Mir1-CP did not increase the permeability of these PMs under any of the tested conditions (Fig. 2.8).

DISCUSSION

In previous work (Pechan et al., 2002), we used SEM to show that PMs of *S. frugiperda* larvae were severely damaged when they fed on transgenic plant material over-expressing Mir1-CP. Although there was little PM damage when larvae fed on controls, there still was the possibility that the damage could be due to the interaction of Mir1-CP with other plant components. This study was conducted to determine if purified Mir1-CP could permeabilize the PM *in vitro*. This should provide unequivocal evidence that Mir1-CP caused PM damage. The *in vitro* system also allowed us to test the effects of pH and temperature on Mir1-CP activity.

The study was facilitated by our ability to express a large quantity of Mir1-CP using a modified baculovirus system (Pechan et al., 2004). Mir1-CP was expressed in *S. frugiperda* larvae and the recombinant enzyme was purified from the hemolymph. Typically, we obtained approximately between 0.6 and 1 μg of purified Mir1-CP for each 10 μl of hemolymph. Considering that one larva contains between 100 and 200 μl of hemolymph, it is possible to obtain a significant amount of Mir1-CP from a few larvae. Characterization of recombinant Mir1-CP indicated that it had the same properties as the plant-derived enzyme.

When PMs isolated from *S. frugiperda* larvae were incubated with enzymatically active recombinant Mir1-CP, they became completely permeable to Blue Dextran 2000. Complete permeability to Blue Dextran was obtained at every Mir1-CP concentration tested, but the time required to reach complete permeability increased as the concentration decreased. The lowest effective concentration was a 1/50000 dilution of

stock Mir1-CP or 1.08 x 10^{-4} U. This was equivalent to 12 ng/ml, which is likely to be a physiologically relevant concentration in the maize whorl. To determine if proteolytic activity was required to permeabilize the PM, we blocked cysteine protease activity by pre-treating Mir1-CP with the specific inhibitor E64. In the presence of the inhibitor, Blue Dextran movement though the PM was abolished, which suggests that cysteine protease activity is required to permeabilize the PM.

According to Dow (1992) the gut pH of *S. frugiperda* is alkaline with pH range between 8.5 and 9. However, the pH optimum for Mir1-CP is acidic (Jiang et al., 1995). For Mir1-CP to function *in vivo*, it must retain some enzymatic activity under alkaline conditions corresponding to those found in the gut. The *in vitro* experiments conducted in this study indicated that Mir1-CP could permeabilize the PM *in vitro* under alkaline conditions up to pH 10. Consequently, it is likely that Mir1-CP has similar activity *in vivo*.

Heat treatment of Mir1-CP was conducted to determine the effect of temperature on activity. The temperature of 30 and 40˚C were selected because they are at the lower and upper range of temperature that might be encountered by the host plant and insects in the field. Mir1-CP was still fully active after incubation at 30˚C for 12 h and retained some activity after 24 h incubation. Preincubation at 40˚C for longer than 1 min probably inactivated the enzyme. Because preincubation at 40˚C abolished activity, higher temperatures were not tested.

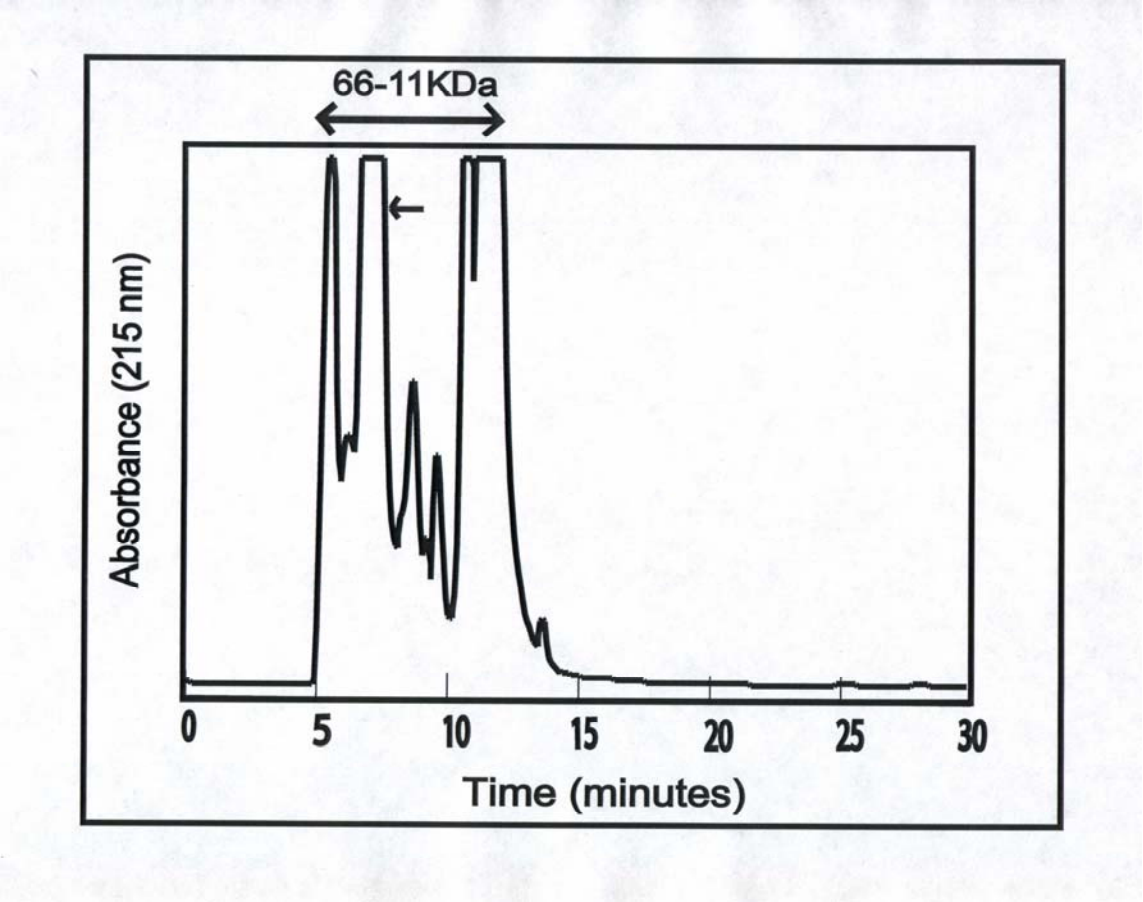
Recent studies by Konno et al. (2004) indicated that cysteine protease in the latex of papaya (*Carica papaya)* and fig (*Ficus virgata)* are crucial in defending plants from

feeding by *Samia ricini, Mamestra brasssicae and Spodoptera litura.* Therefore, the efficacy of three additional plant derived cysteine protease was tested. Papain, bromelain and ficin only partially permeabilized the *S. frugiperda* PM, even when they were used at higher concentration than Mir1-CP. The two serine proteases, chymotrypsin and thermolysin, had no effect on PM permeability. The effect of the Mir1-CP on the PM permeability was more pronounced when the luminal side was exposed to the enzyme. When the epithelial side was exposed to Mir1-CP, it took longer time to reach steady state permeability and was approximately 20% less that completely permeable. These data suggested that the protein or proteins attacked by Mir1-CP are more accessible on the luminal side of the PM than on the epithelial side.

Mir1-CP is found in maize germplasm that is resistant to a number of stem boring Lepidoptera (Davis et al., 1988). Consequently we wanted to determine if it could attack the PM of other insects. Mir1-CP effectively permeabilized the PM of the two noctuids tested, *H. zea* and *S. frugiperda*. It affected the PM of the danaid, *D. plexippus*, but not that of the crambid, *O. nubilalis*. This implies that there may be evolutionary differences in PM structure and sensitivity to Mir1-CP among the Lepidoptera. It is interesting that the insect resistant maize germplasm inhibits the growth of a number of Lepidoptera including noctuids, crambids and pyralids in the field and in bioassays. Because insect resistance in these lines is a multigene trait, it is likely that additional factors are needed to confer resistance to a wide range of lepidopterans. The PMs of *P. americana* and *T. molitor* also were resistant to Mir1-CP induced permeability. Evolutionary differences

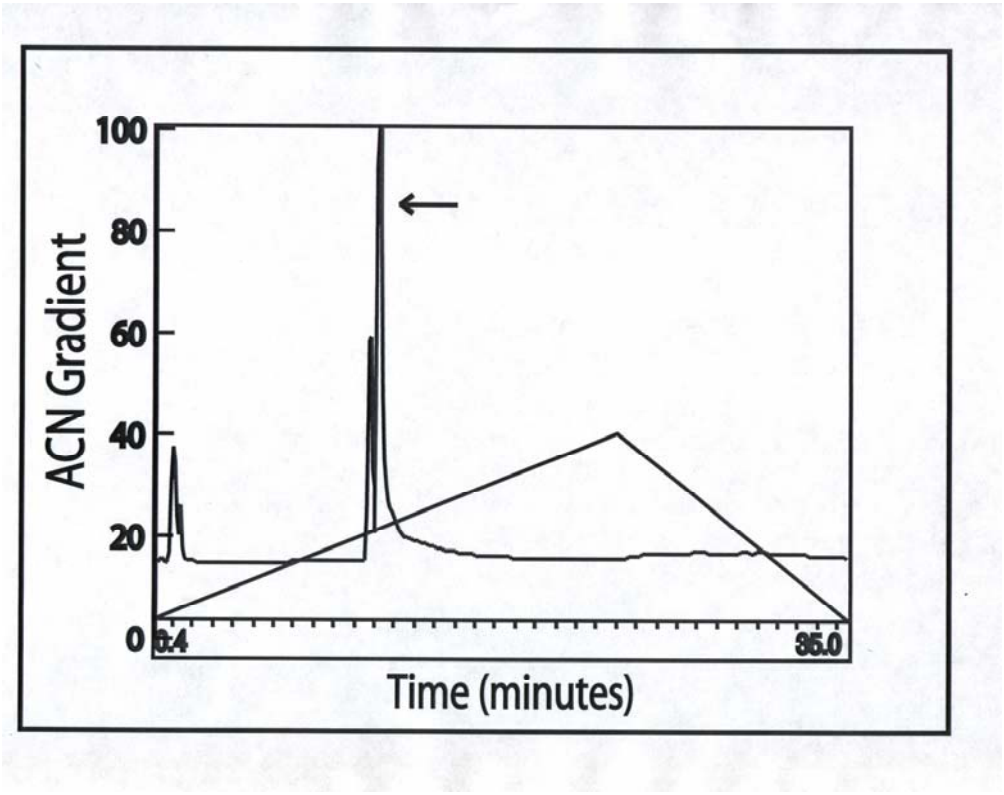
among species in PM formation or structure (Barbehenn and Martin, 1995) could greatly affect the interaction of Mir1-CP with the PM chitin and proteinaceous components.

We have demonstrated that a unique maize cysteine protease Mir1-CP is capable of directly permeabilizing the PM of several Lepidoptera larvae. Inhibition of Mir1-CP activity with E64 prevented permeabilization indicating that proteolytic activity is required to damage the PM. It is likely that Mir1-CP attacks and degrades the PM proteins, but we currently do not know if it attacks specific proteins like IIM, or if it non-specifically degrades all PM proteins. Nevertheless, it appears that Mir1-CP plays a critical role in maize defense against herbivory by lepidopteran larvae.



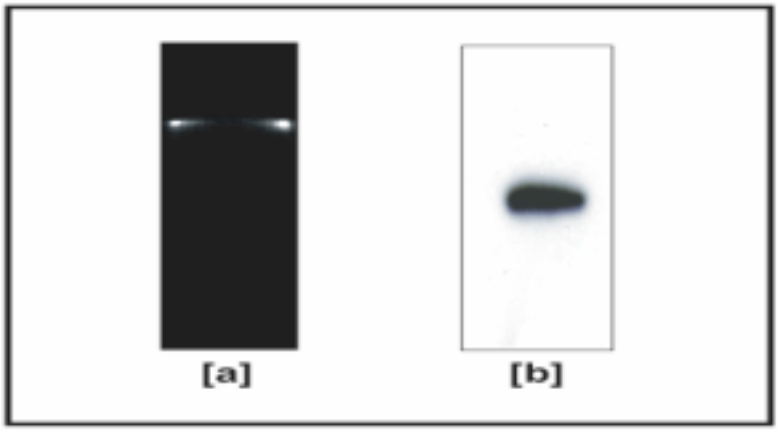
Note: (a) Size exclusion HPLC. Arrow marks the position of the 33-KDa peak corresponding to Mir1-CP.

Figure 2.1 Cysteine protease isolation and purification using size exclusion and reverse phase HPLC.

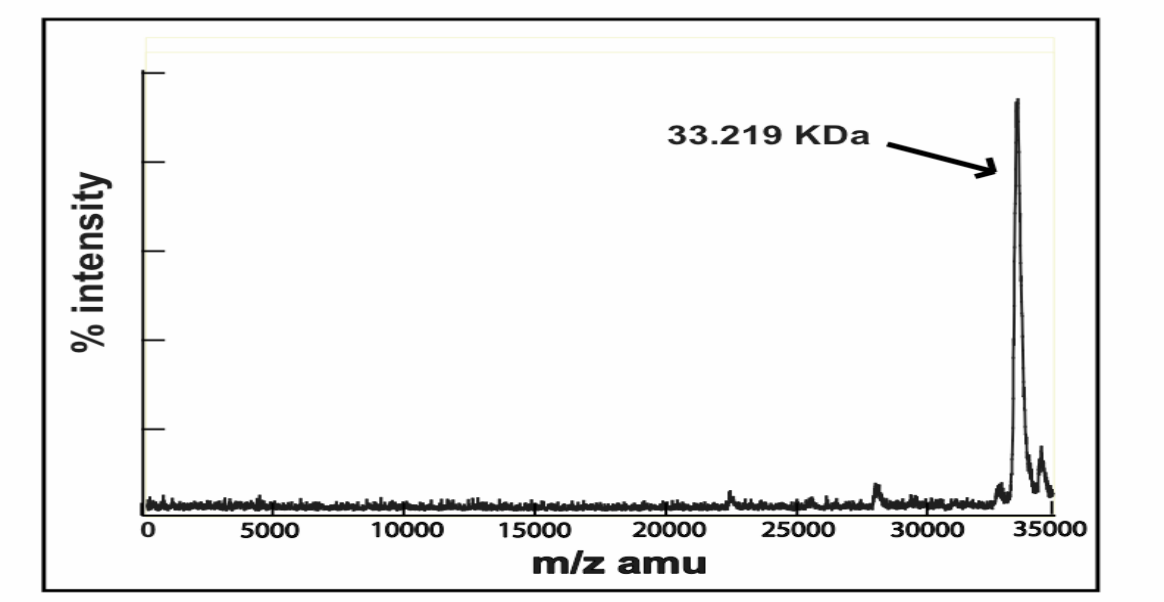


Note: (b) Revere phase HPLC. Arrow marks the position of the 33-KDa peak corresponding to Mir1-CP.

Figure 2.1 (continued).

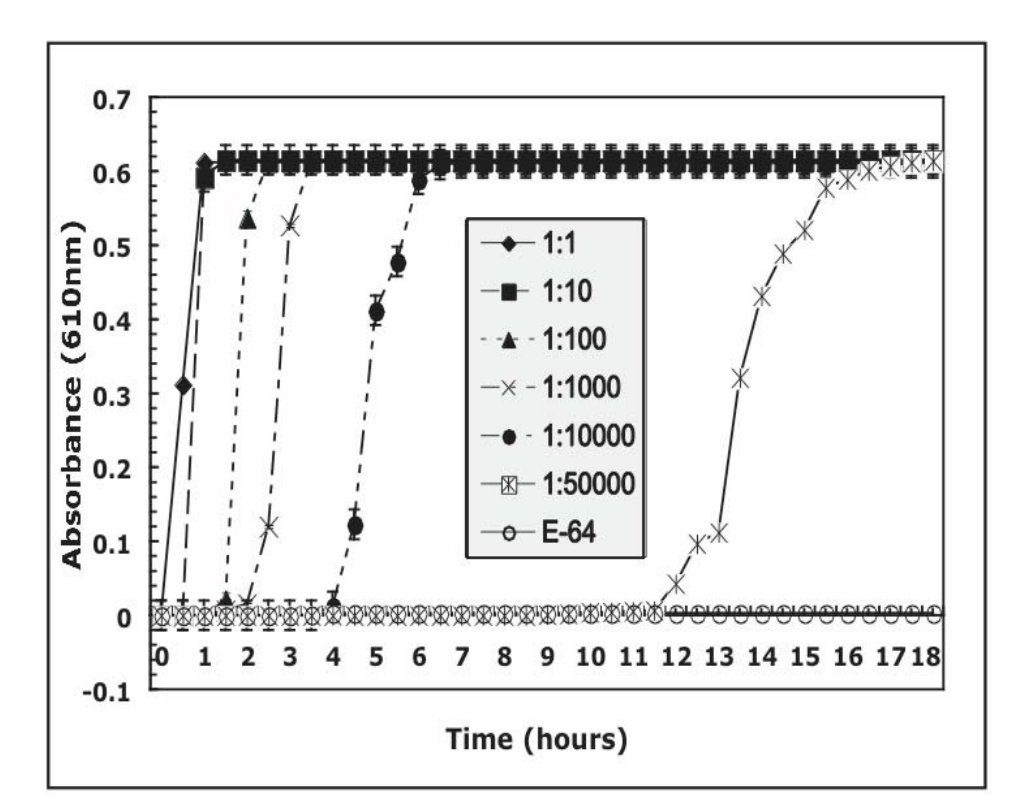


Note: (a) Purified HPLC fraction showing a white active band of protease activity at 33-KDa in gelatin-acrylamide gel assay. (b) Immunoblot analysis exhibiting cross-reactivity of Mir1-CP specific antisera with 33-KDa band of HPLC fraction.



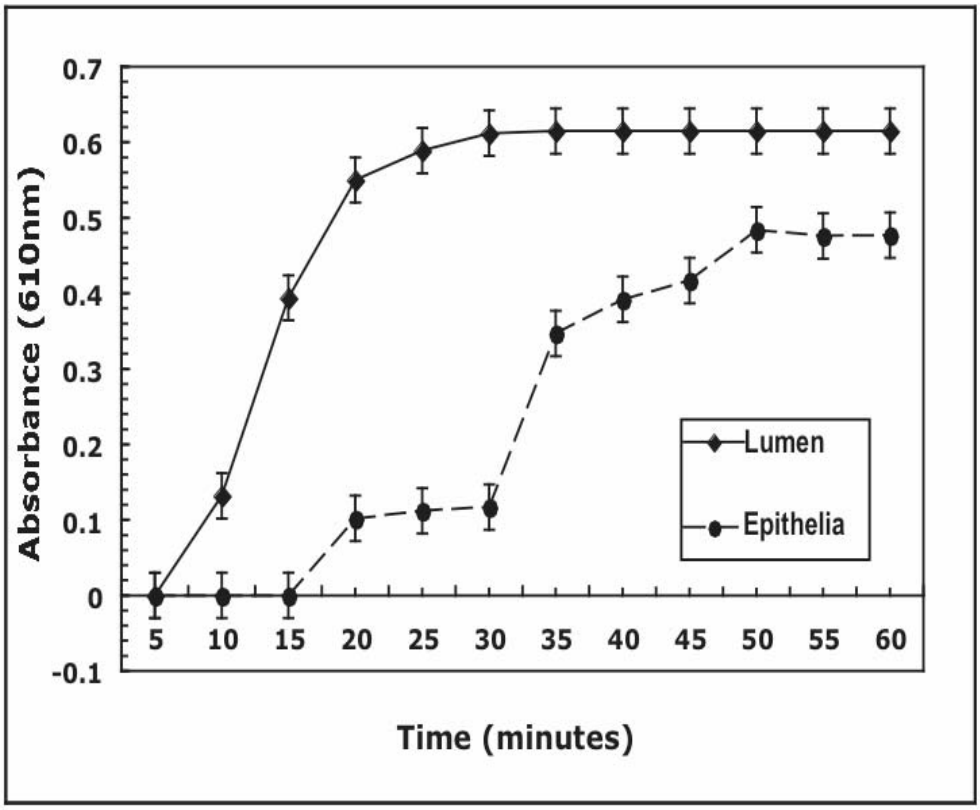
Note: (c) MALDI-TOF MS analysis indicated that the purified fraction is a homogenous peak of 33-KDa.

Figure 2.2 Analysis of Mir1-CP purity and activity.



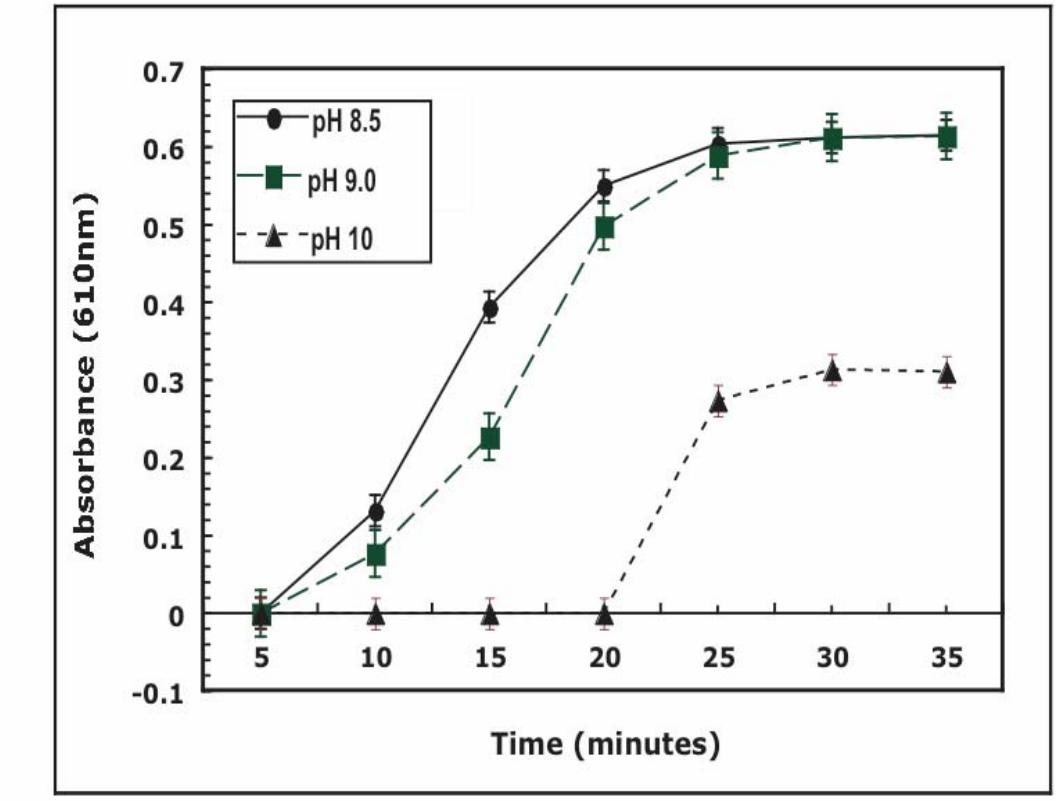
Note: (◆) Pure Mir1-CP at 0.6 mg/ml, (■) 60 µg/ml (1:10), (▲) 6 µg/ml (1:100), (✕) 600 ng/ml (1:1000), (•) 60 ng/ml (1: 10000), (✶) 12 ng/ml (1: 50000), (❍) No CP or CP (0.6 mg/ml) + E-64 (1 mg/ml) treatment or CP of 10ng/ml (1: 60000) were used for PM treatment at pH 8.0 and 24°C. Standard error bars are based on six different PM preparations tested.

Figure 2.3 *S. frugiperda* PM permeability in response to various concentrations of recombinant Mir1-CP as measured by Blue Dextran 2000 flow across the PM.



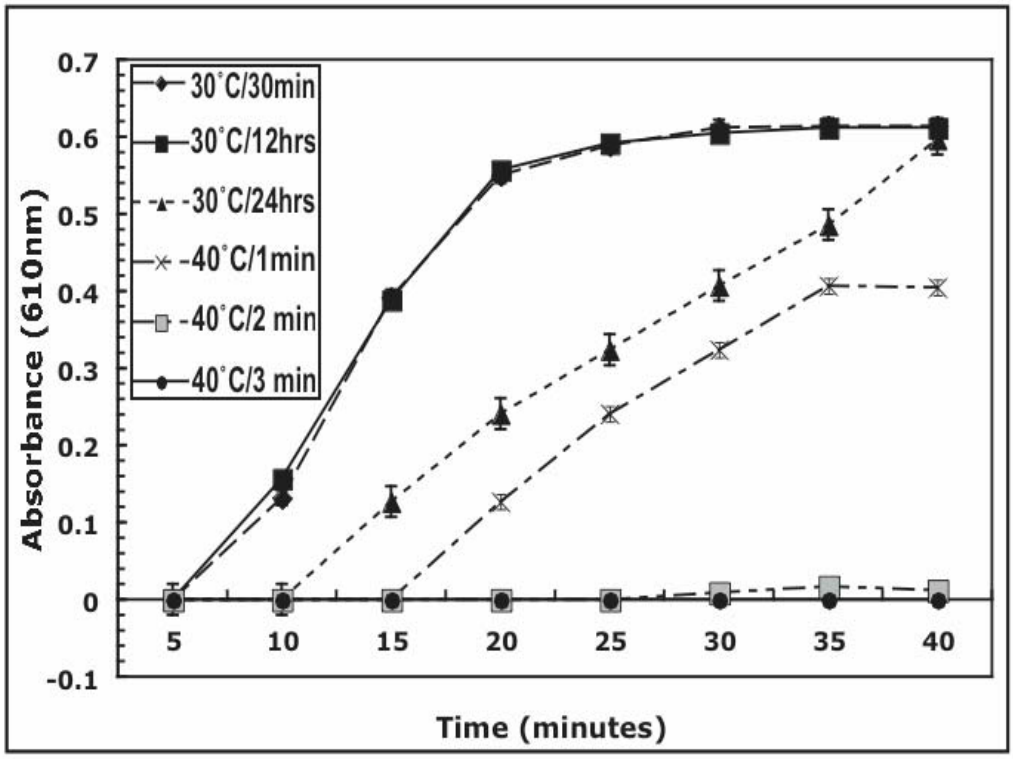
Note: The epithelial (●) and luminal (◆) sides of the PM were treated with Mir1-CP at 0.64 mg/ml at pH 8.0 and 24°C. Standard error bars are based on six different PM preparations tested.

Figure 2.4 *S. frugiperda* PM permeability in response to Mir1-CP and PM orientation as measured by Blue Dextran 2000 flow across the PM.



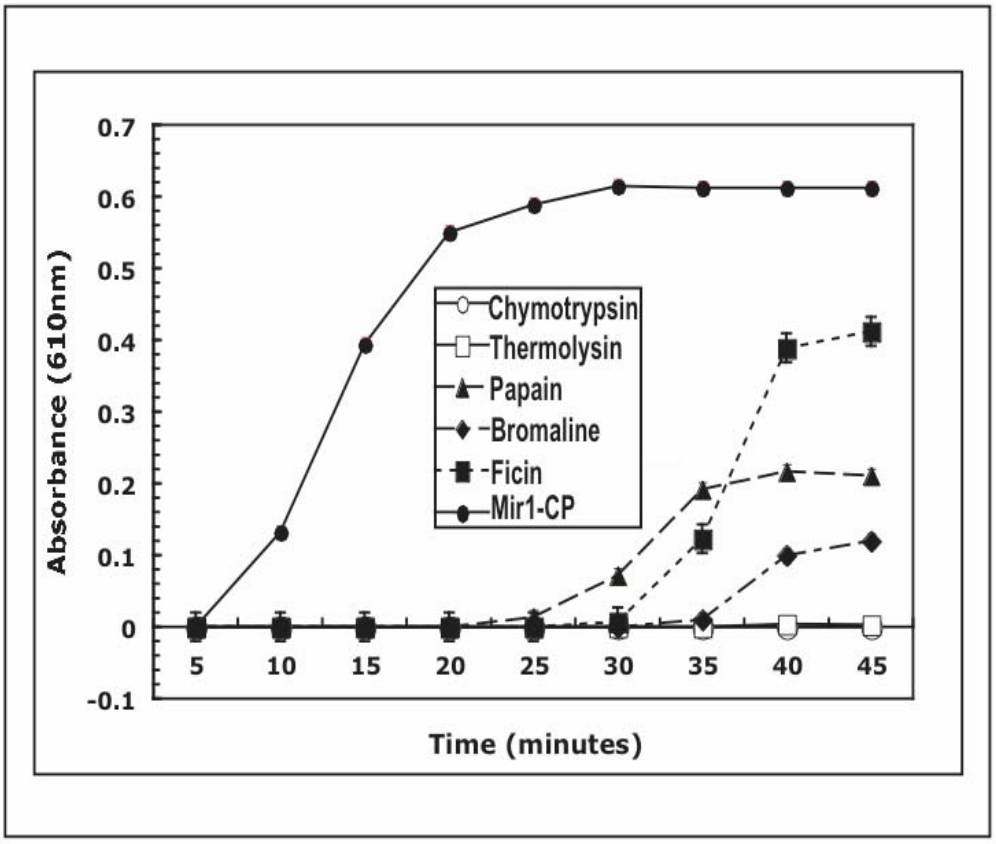
Note: PMs were incubated at 24°C with Mir1-CP (0.64 mg/ml) at pH 8.5 (●), pH 9.0 (■) and pH 10 (▲). Standard error bars are based on six different PM preparations tested.

Figure 2.5 *S. frugiperda* PM permeability in response to Mir1-CP and pH as measured by Blue Dextran 2000 flow across the PM.



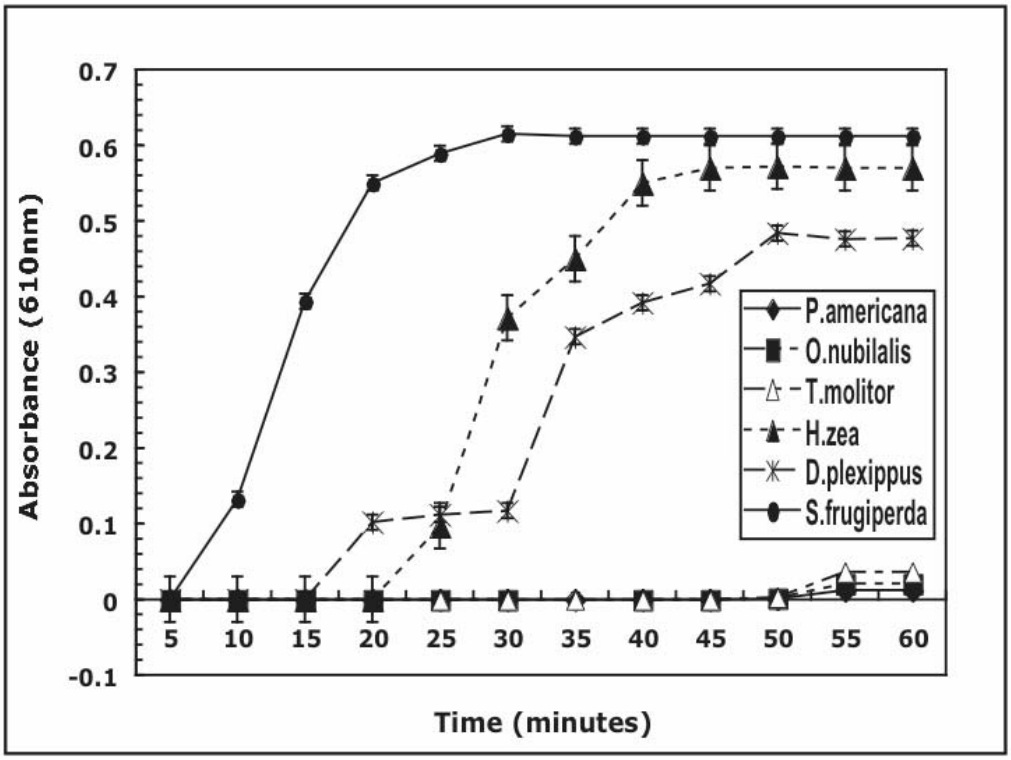
Note: Prior to PM treatment at pH 8.0 and 24°C, Mir1-CP (0.64 mg/ml) was incubated at 30°C for 30 min (◆), 30°C overnight (12 h) (■), 30°C for 24 h (▲), 40°C for1 min (✕), 40°C for 2 min (□) and 40°C for 3 min (●). Standard error bars are based on six different PM preparations tested.

Figure 2.6 *S. frugiperda* PM permeability in response to Mir1-CP preincubation temperature as measured by Blue Dextran 2000 flow across the PM.



Note: PMs were treated with 0.64 mg/ml of the cysteine proteases Mir1-CP (●), bromelain (◆), papain (▲), ficin (■) and the serine proteases, chymotrypsin (O) and thermolysin ( ) at pH 8.0 at 24°C. The specific activities of the enzymes are given in Materials and Methods. Standard error bars are based on six different PM preparations tested.

Figure 2.7 *S. frugiperda* PM permeability in response to various proteolytic enzymes as measured by Blue Dextran 2000 flow across the PM.



Note: The PMs of *S. frugiperda* (●), *H. zea* (▲), *D. plexippus* (✶), *O. nubialis* (■), *P. americana* (◆) and *T. molitor* (△) were treated with 0.64 mg/ml of Mir1-CP at pH 8.0 and 24°C Standard error bars are based on six different PM preparations tested.

Figure 2.8 Effect of Mir1-CP on the permeability of PMs isolated from several different insect species as measured by Blue Dextran 2000 flow across the PM.

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CHAPTER III

IMPORTANCE OF Cys^{181} AND C-TERMINAL REGION OF A PLANT CYSTEINE PROTEASE (Mir1-CP) IN *Spodoptera frugiperda* LARVAL PM PERMEABILITY

ABSTRACT

A unique 33-KDa cysteine protease (Mir1-CP) rapidly accumulates at the yellow-green region of the resistance maize (*Zea mays* L.) inbreds. *In vitro* analysis indicated that recombinant Mir1-CP increases PM permeability to Blue Dextran 2000 by degrading integral PM proteins. To determine the importance of active site cysteine and unique C-terminal tail of Mir1-CP in PM permeability, two mutated genes, *mut1* and *mut2*, were expressed. The absence of any detectable activity for *mut1* gene product (Mut1) and only low activity for *mut2* gene product (Mut2) indicated that complete processing did not occur. PMs incubated with unprocessed Mut1 showed no increase in permeability, but unprocessed Mut2 was able to partially permeabilize the PM. By processing both Mut1 and Mut2 to its mature form with minute quantities of Mir1-CP, there was an increase in permeability, but the level of permeabilization was only one-half of the Mir1-CP level. Processed Mut1 and Mut2 also showed no pronounced PM permeability effect when the epithelial side was exposed to these enzymes or at pH values greater than 9 and temperatures higher than 35˚C. This indicated that processing is essential for maximal

Mir1-CP activity and that both cysteine at the active site and terminal 25 amino acids are required for complete permeabilizaton.

INTRODUCTION

Plant-herbivore defense mechanism studies have shown that maize plants with genetic resistance to herbivory by fall armyworm (*Spodoptera frugiperda*) and other lepidopteran larvae defend themselves by accumulating a cysteine protease (Mir1-CP) at the feeding site in the whorl (Pechan et al., 2000). Mir1-CP inhibited larval growth by 70% when it was ectopically expressed in maize tissue culture cells (Pechan et al., 2000). When larvae fed on the transgenic material, scanning electron microscopy (SEM) indicated that insects' peritrophic matrix (PM) was damaged and contained many cracks and fissures (Pechan et al., 2002). In dose-response bioassays using purified recombinant Mir1-CP (Luthe and Mohan, unpublished data), 600 ppb (6 ng/ml) reduced the larval relative growth rate by approximately 50% and mortality increased from 8% in the control to 45% in the Mir1-CP-fed larvae. Mir1-CP completely permeabilized the PM in a concentration-dependent manner, when it was used for *in vitro* permeability studies (Mohan et al., 2006). Mir1-CP permeabilized the PM more completely than the other plant cysteine proteases, papain and ficin (Mohan et al., 2006; Konno et al., 2004). When Mir1-CP was preincuated with E64, a cysteine protease inhibitor, the PM was not permeabilized. This indicated that proteolytic activity was necessary for PM degradation.

Mir1-CP has been purified from maize callus (Jiang et al., 1995) and the cDNA *mir1* encoding the enzyme has been sequenced (Pechan et al., 1999). These analyses confirmed that Mir1-CP is a papain-like cysteine protease (Pechan et al., 1999). It is

designated as Clan A, family C1 protease (Barrett and Rawlings, 1996). Like other cysteine proteases, it is synthesized as preproprotein or zymogen that must be post-translationally processed to be fully active. The 'pre' sequence is required for intracellular localization and 'pro' sequence is removed during enzyme activation by limited proteolysis at acidic pH optima (Mitsuhashi and Minamikawa, 1989; Koehler and Ho, 1990; De Barros and Larkins, 1999; Mitsuhasti and oaks, 1994; Domoto et al., 1995; Drake et al., 1996) by a process called maturation, activation or proteolytic processing (Vernet et al., 1995). The predicted molecular mass of the Mir1-CP preproprotein is 42.6 KDa. It contains a 23 amino acid pre-sequence and a 134 amino acid prosequence. The predicted molecular mass of the processed or mature Mir1-CP is 25.4 KDa. However, both SDS-PAGE and mass spectroscopy indicated that it is 33-KDa (Jiang et al., 1995; Pechan et al., 2004). The discrepancy in molecular mass might be the result of post-translational modifications such as glycosylation. Unpublished data (Shivaji and Luthe) indicated that Mir1-CP is glycosylated. Sequence analysis of *mir1* indicated that 281 bp sequence on the 3' end of the gene had no matches in the databases. Of these 281bp, 75 encoded the last 25 amino acids on the C-terminus of the protein. This 25 amino sequence also has no matches in the databases. This amino acid sequence has limited sequence similarity to chitin-binding proteins and it contributes to the unique properties of Mir1-CP. This sequence is not similar to the granulin-like sequence found on other plant cysteine proteases (Tolkatchev et al., 2001).

The catalytic site of mature cysteine proteases is the thiolate-imidazolium ion pair between Cys^{25} and His^{159} (papain numbering) that forms the reactive nucleophile (Barrett

1986). The Asn^{175} residue is used for proper histidine side chain orientation (Beers et al., 2004), but is not absolutely essential for activity (Vernet et al., 1991). In Mir1-CP, Cys^{25} and His^{160} align with those of papain and other plant cysteine protease (Pechan et al., 1999). In this study, mutagenesis was used to convert cysteine at the Mir1-CP active site to an alanine and to remove 25 C-terminal amino acids to determine if they are critical for Mir1-CP's enzymatic activity and ability to permeabilize the fall armyworm PM.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Fisher Biotech (Fair lawn, NJ). HPLC columns (size exclusion and reverse phase chromatography) were purchased from Phenomenex (Torrance, CA). The MALDI-TOF calibration kit was purchased from Perceptive Biosystems (Foster city, CA). Deionized water (18MΩ) was used in all experiments.

Insect rearing

Fourth instar larval colonies of *S.frugiperda* reared on artificial diet (Davis, 1976) under a photoperiod of 16:8 at 28˚C were obtained from a laboratory colony maintained by the USDA-ARS Corn Host Plant Resistance Research Unit at Mississippi State University.

Purification, processing and activity determination

Site directed mutagenesis of *mir1* was used to generate *mut1* and *mut2* gene constructs (Pechan et al., 2004). The accession number in the Genbank Database is AF019145. For *mut1*, the active site cysteine (Cys^{181}) when numbered from the N-terminal of the preproprotein or Cys^{25} when numbered from the N-terminal of the mature, processed protein) was converted to alanine and in *mut2* insertion of a premature stop codon eliminated the last 25 C- terminal amino acids (Pechan et al. 2004). These constructs were expressed in the hemolymph of fourth instar *S. frugiperda* larvae as previously described (Pechan et al., 2004). Expressed recombinant Mut1 and Mut2 were purified using HPLC methods (Mohan et al., 2006) and the qualitative activity of the column fractions was determined by a rapid protease microassay (Mohan et al., 2005). Because we expected Mut1 and Mut2 to have less enzymatic activity than Mir1-CP, we assumed that they would not be able to perform the autocatalysis to form the mature active forms. Therefore, purified Mut1 (0.54mg/ml) and Mut2 (0.7 mg/ml) were processed by treatment with 1 ng/ml Mir1-CP at room temperature at 24˚C for 12 h, which is approximately a 10^6/1 ratio. Specific activity determination and immuno-blotting analysis of purified Mut1, Mut2, as well as their processed forms, were performed as previously described (Mohan et al., 2005). Protein concentration was determined using the Bradford method (Bradford, 1976) using bovine serum albumin (1 mg/ml) as standard. Active unprocessed Mut1, Mut2 as well as their processed forms were aliquoted and stored at –80˚C.

Mass Spectrometry (MALDI-TOF MS)

The molecular mass of the purified recombinant Mut1, Mut2 and their processed forms were determined by an ABI Voyager Elite MALDI-TOF MS (Matrix Activated Light Desorption Ionization-Time Of Flight Mass Spectrometry) Voyager™ Experimental station, as previously described (Mohan et al., 2006). However, the fractions were excited at a lower nitrogen laser intensity of 2300, 20KV and 91% grid voltage. Data explorer™ software (Perspective Biosystems) was used for further analysis of spectra.

Permeability apparatus and measurement

The modified dual chamber permeability apparatus (Mohan et al., 2006) was used for determining the biological activity of either processed or unprocessed Mut1 and Mut2 on PM permeability. Procedure for PM dissection and experimental measurement conditions were followed as mentioned by Mohan et al. (2006). The experimental measurements were performed at room temperature (24˚C) and repeated for a minimum of four times using freshly dissected PM for each replicate.

RESULTS

Purification of recombinant Mut1 and Mut2 from hemolymph

Recombinant *mut1* and *mut2* genes were expressed in fourth instar *S. frugiperda* larval hemolymph and purified using one size-exclusion and two reverse phase chromatographic steps (data not shown). The single peak fraction from the last reverse phase chromatographic step had a molecular mass of 42.8 and 36 KDa for unprocessed

Mut1 and Mut2 respectively, as determined by MALDI-TOF MS (Figure 3.1a, c). These correspond to the molecular mass of the preproprotein forms of these two enzymes. These precursor forms were incubated with 1 ng of Mir1-CP overnight to convert them to the mature processed forms. Similar mass spectrometric analysis showed molecular masses of 33.2 and 30.5 KDa for Mir1-CP (1 ng/ml) processed Mut1 and Mut2 fractions (Figure 3.1b, d). The mass of Mut1 is similar to what would be expected if the cysteine in Mir1-CP was converted to an alanine and that of Mut2 is what would be predicted if 25 amino acids were removed. In addition, unprocessed and processed Mut1 and Mut2 cross-reacted with Mir1-CP antibody and showed bands corresponding to molecular masses similar to those determined in MALDI-TOF MS (Figure 3.2). A fluorometric activity assay of recombinant HPLC fractions showed no significant activity for unprocessed Mut1 and a very low activity of 0.699 U for unprocessed Mut2. Processed Mut1 and Mut2 showed activities of 0.967 and 1.027 U, compared to a specific activity of 1.986 U for Mir1-CP.

PM permeability

To determine the effect of Mir1-CP and unprocessed and processed Mut1and Mut2 on PM permeability *in vitro*, the flow of Blue Dextran 2000 between the chambers of the permeability apparatus was measured. Mir1-CP (0.06 mg/ml) completely permeabilized the PM after 1 hour (Figure 3.3). In comparison, when the PM was treated with 0.54 mg/ml of processed Mut1 there was an increase in permeability after an initial 2 h lag. However, permeability was not complete and was only one-half of that attained with Mir1-CP. Treatment with 10-fold lower concentration of Mut1 (0.05 mg/ml)

increased the time required to reach one-half of the complete permeability. Unprocessed Mut1 did not effect the permeability. Preincubation of processed Mut1 (0.54 or 0.05 mg/ml) with E64, a cysteine protease inhibitor, also prevented permeabilization (Figure 3.3).

The effect of unprocessed and various dilutions of processed Mut2 (0.7mg/ml) on PM permeability were tested (Figure 3.4). When compared to Mir1-CP, only one-half complete permeability was obtained with processed Mut2. The length of time needed to reach steady state increased as the concentration of Mut2 decreased. Unprocessed Mut2 permeabilized the PM less than processed Mut2 and it took 9 hours to reach the steady state level. When Mut2 was treated with E64, there was no increase in PM permeability (Figure 3.4).

A prior study indicated that the luminal side of the PM was more susceptible to attack by Mir1-CP than the epithelial side (Mohan et al., 2006). Figure 3.5 shows that both processed Mut1 and Mut2 preferentially permeabilized the luminal surface of the PM. However, processed Mut2 was more effective than Mut1 and neither enzyme caused the complete permeability attained by Mir1-CP (steady state absorbance approximately 0.6). When the epithelia surface was treated with processed Mut2, it took 9 h to reach steady state permeability and the permeability was less. Processed Mut1 was unable to permeabilize the epithelial surface of the PM.

pH and Temperature

To determine the effect of pH on the activity of processed Mut1 and Mut2, PM permeability was measured at pH 8.5 and 9.0. Both processed Mut1 and Mut2 took 6 and

4 h, respectively to achieve saturation at pH 8.5, but failed to permeabilize the PM at pH 9 or greater (Figure 3.6). Again, the level of permeability was only one-half of the complete permeability achieved within 1 h with Mir1-CP.

Preincubation of Mir1-CP at 30° for 4 h did not affect its ability to completely permeabilize the PM. When processed Mut1 was pre-incubated at 30˚C for 4 h prior to PM treatment, saturation was achieved in less than 1 h, but permeabilization was not complete (Figure 3.7). Incubation of processed Mut1 at 35°C for 4 h increased the time required to reach the same steady state level. Pre-incubation of processed Mut1 at temperatures greater than 35˚C for a short period of time resulted in enzyme that was unable to permeabilize the PM. When processed Mut2 was pre-incubated either at 30˚C or higher temperatures for 1 min it did not increase PM permeability.

DISCUSSION

Earlier studies (Mohan et al., 2006) showed complete permeability of fourth instar *S. frugiperda* PM when it was incubated with enzymatically active recombinant Mir1-CP. Although the time needed to reach complete permeability increased with decreasing Mir1-CP concentrations, complete permeability was obtained at every Mir1-CP concentration greater that 12 ng/ml. The present study was conducted to understand the importance of cysteine at the active site and the unique 25-amino acid C-terminal tail region of Mir1-CP on FAW PM permeability. To pursue this study, we used two previously constructed mutant forms of the cysteine protease namely, *mut1*, in which alanine replaced the Cys^{25} at the active site and *mut2*, in which the codon for Asp^{232} was replaced with stop codon to prevent the translation of the last 25 amino acids. When

mut1 and *mut2* were expressed in fourth instar *S. frugiperda* larval hemolymph, protein bands similar in size to the Mir1 preproprotein were detected by immuno-blot assays. The molecular masses of 42.8 and 36 KDa for Mut1 and Mut2 by MALDI-TOF mass spectrometry confirmed the immuno-blot assay results. These molecular masses and the absence of any detectable activity for the unprocessed Mut1 protein encoded by *mut1* and only a low activity for *mut2* gene product (Mut2) indicated that complete processing did not occur. Processing of cysteine proteases to their mature form is believed to be autocatalytic (Vernet et al., 1991; Bromme et al., 1993; Mach et al., 1994). Since the active site cysteine was changed in Mut1, its autocatalytic activity was probably abolished. We do not know why Mut2 was unable to conduct autocatalysis. To obtain the mature processed forms of these two proteins, they were incubated with 1 ng/ml Mir1-CP. To minimize the effect of Mir-CP on subsequent PM permeability assays, the ratio of the mutant enzyme to Mir1-CP was approximately 10^6:1. This resulted in Mut1 and Mut2 with molecular masses of 33.2 and 30.5 KDa, respectively. The specific activity of Mut1 and Mut2 was approximately one-half that of Mir1-CP.

We expected the processed form of Mut1 to be inactive, and we do not know why it retained activity. Table 3.1 shows an alignment of the amino acids near the active site of Mir1-CP, Mir3-CP, papain and bromelain. Mir1-CP is the only protease in the group that has three glutamine residues (Q) preceding the Cys^{22} that is involved in disulfide-bonding. Perhaps the presence of three contiguous glutamines affects protein folding in some way that alters the active site. Also, in Mir1-CP (Pechan et al., 1999), Cys^{22} is separated from the active site Cys^{25} by two glycines (G). In the other proteases, the

separating amino acids are either serine (S) or theonine (T). We speculate that the presence of the two small glycine residues between $Cys^{22}$and Cys^{25} may reduce steric hindrance and allow Cys^{22} to partially substitute for Cys^{25} at the active site.

When the PM was incubated with unprocessed Mut1, there was no increase in PM permeability. However, when Mut1 was processed, there was an increase in permeability, but it was not as great as that achieved by Mir1-CP. This indicates that processing is essential for activity and that changing the cysteine at the active site, reduces, but does not abolish its activity. Unprocessed Mut2, lacking the terminal 25 amino acids, was able to partially permeabilize the PM, but it took longer time than Mir1-CP. Processed Mut1 and Mut2 were able to increase PM permeability, but only partial saturation was reached. The level of permeabilization was only one-half of the Mir1-CP level.

Previous studies indicated that Mir1-CP preferentially attacked the luminal surface of the PM (Mohan et al., 2006). Although the epithelial side was attacked, the level of permeability was approximately 25% less and it took longer to reach the steady state level. In this study processed Mut1 and Mut2 partially permeabilized the PM when the luminal surface was exposed. Mut2 permeabilized the epithelia surface less than the luminal one and Mut1 was completely unable to permeabilize the epithelial surface. We do not know if this is due to alteration of the cysteine at the active site, or to generally lower levels of enzymatic activity. To determine whether processed Mut1 and Mut2 proteolyic activity was required to permeabilize the PM, cysteine protease activity was blocked by pre-treating the enzymes with the specific inhibitor E64. The inability of the

Blue Dextran 2000 to move across the PM in the presence of the inhibitor, suggested that both Mut1 and Mut2 permeabilized the PM by proteolytically attacking PM proteins.

The optimal pH for Mir1-CP activity is between pH 5 and 7 (Jiang et al., 1995, Luthe and Bassford, unpublished data), but the gut pH of *S. frugiperda* larvae is alkaline with a pH range between 8.5 and 9 (Dow, 1992). Mir1-CP was able to completely permeabilize the FAW PM at pH 8.5 and 9 and partially permeabilize it at pH 10 (Mohan et al., 2006). To understand whether Mir1-CP requires the active site Cys^{25} as well as its unique 25 amino acid terminal sequence to retain enzymatic activity under alkaline conditions, the *in vitro* experiments were conducted with processed Mut1 and Mut2 at pH 8.5 and 9.0. The *in vitro* experiments showed that both processed Mut1 and Mut2 could partially permeabilize the PM at pH 8.5, but neither had activity at pH 9.0. Since processed Mut1 took longer time to achieve partial PM permeability than processed Mut2, it is likely that the mutation in the active site results in an enzyme that is more sensitive to changes in pH than the enzyme lacking the 25 C-terminal amino acids.

We also conducted the *in vitro* assay at varying temperature conditions to determine the degree of enzymatic stability and activity of processed Mut1 and Mut2. Temperature range between 30 and 40˚C were selected because they are at the lower and upper range of temperatures that might be encountered by the host plant and insect in the field. When processed Mut1 was preincubated at 30˚C and then assayed, the lag time was reduced to 30 min when compared to Mut1 used at room temperature of 24˚C (Figure 3.3). Preincubation at 35˚C increased the lag time to 2 hours. It is possible that the 30˚C incubation activates the enzyme. The exact reason for the relationship between

pre-incubation temperature and time required to achieve partial saturation level by processed Mut1 is still unclear. The ability of processed fractions of Mut1 to show PM permeability when incubated up to 35˚C/4 h but loss of enzymatic activity and PM permeability effect by processed Mut2 after incubating at 30˚C/1 min further shows the importance of the terminal 25 amino acids in maintaining stable cysteine protease enzymatic activity at higher temperatures. Because pre-incubation at temperature slightly above 35˚C abolished activity, higher temperatures were not tested.

These results indicated that processing is essential for maximal Mir1-CP activity and that both cysteine at the active site and terminal 25 amino acids are required for complete permeabilizaton.

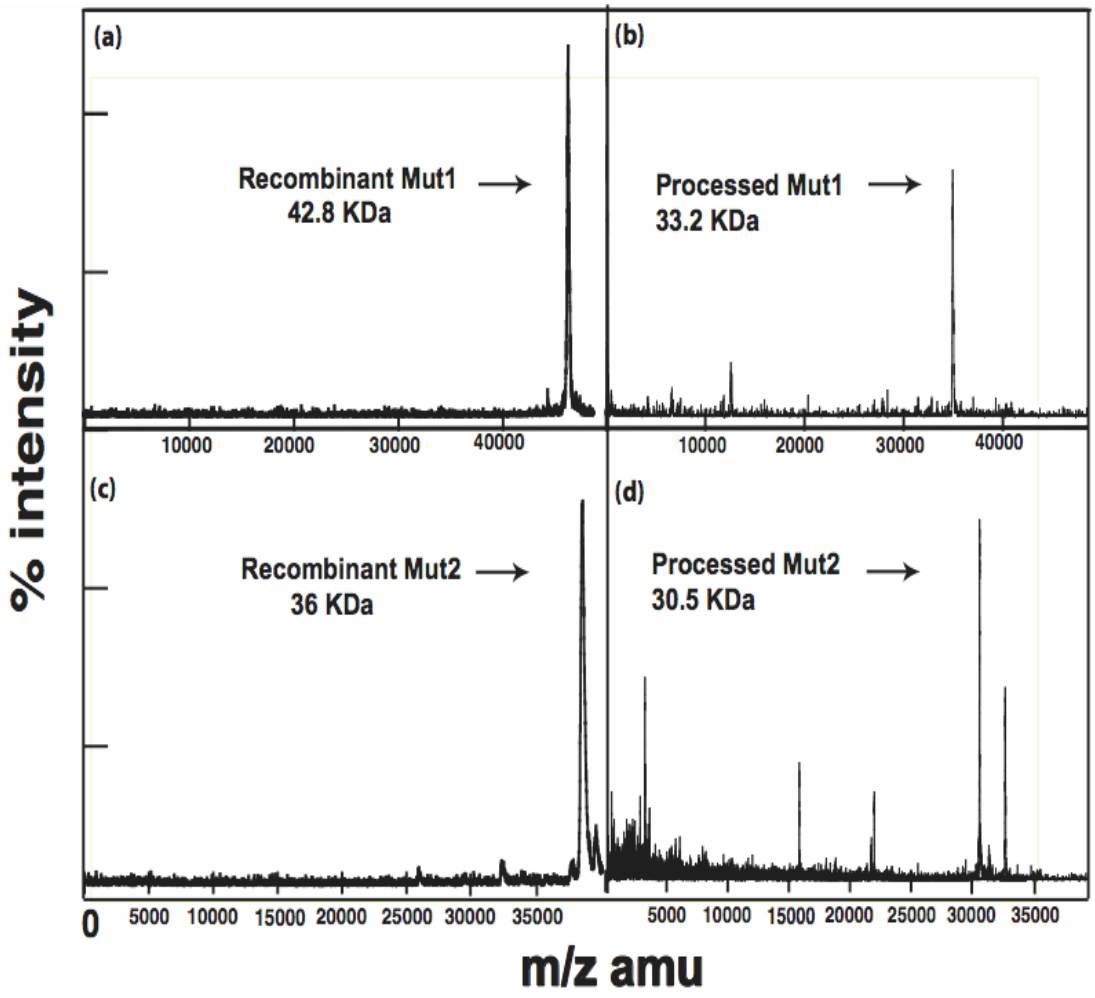
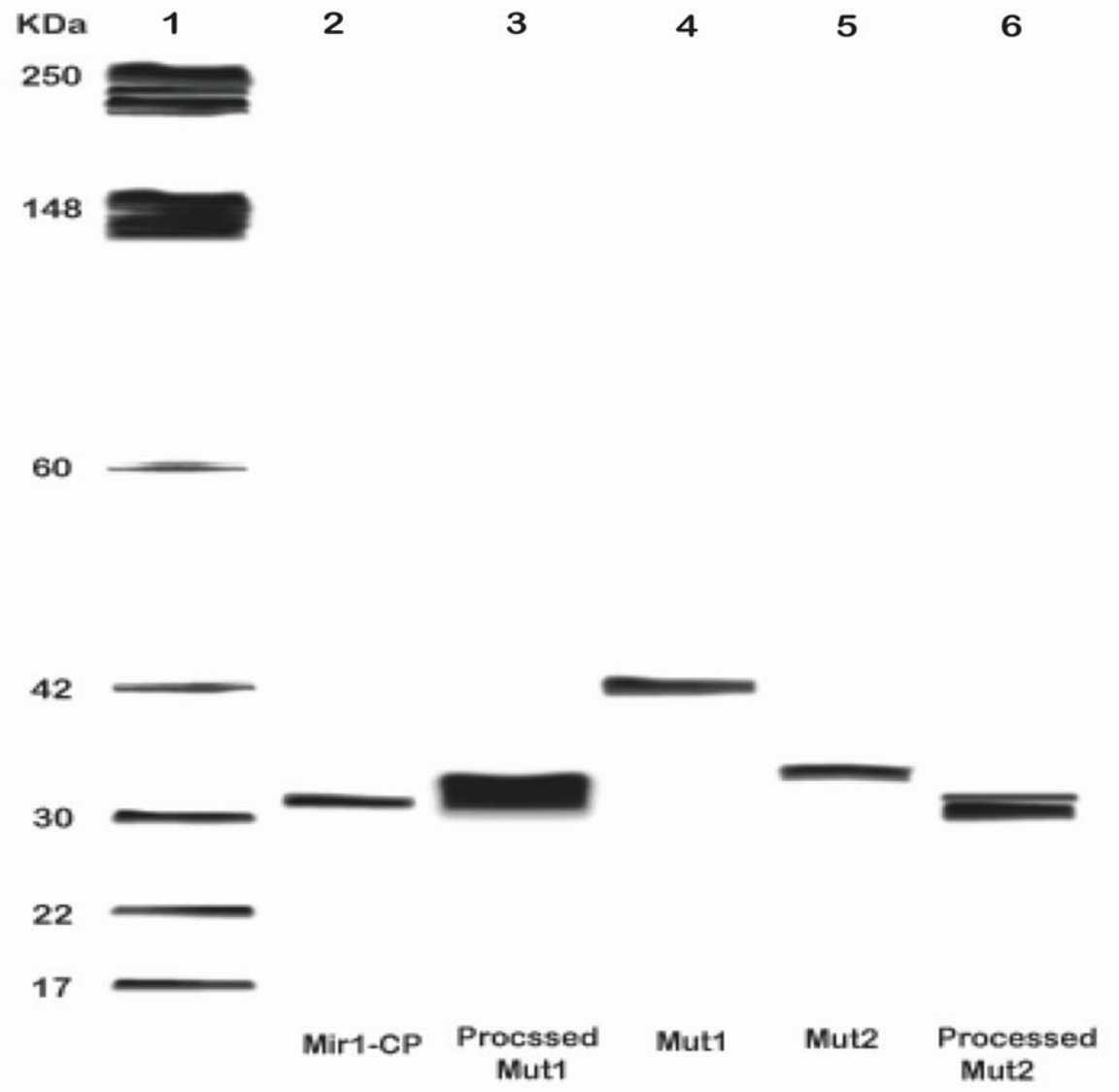
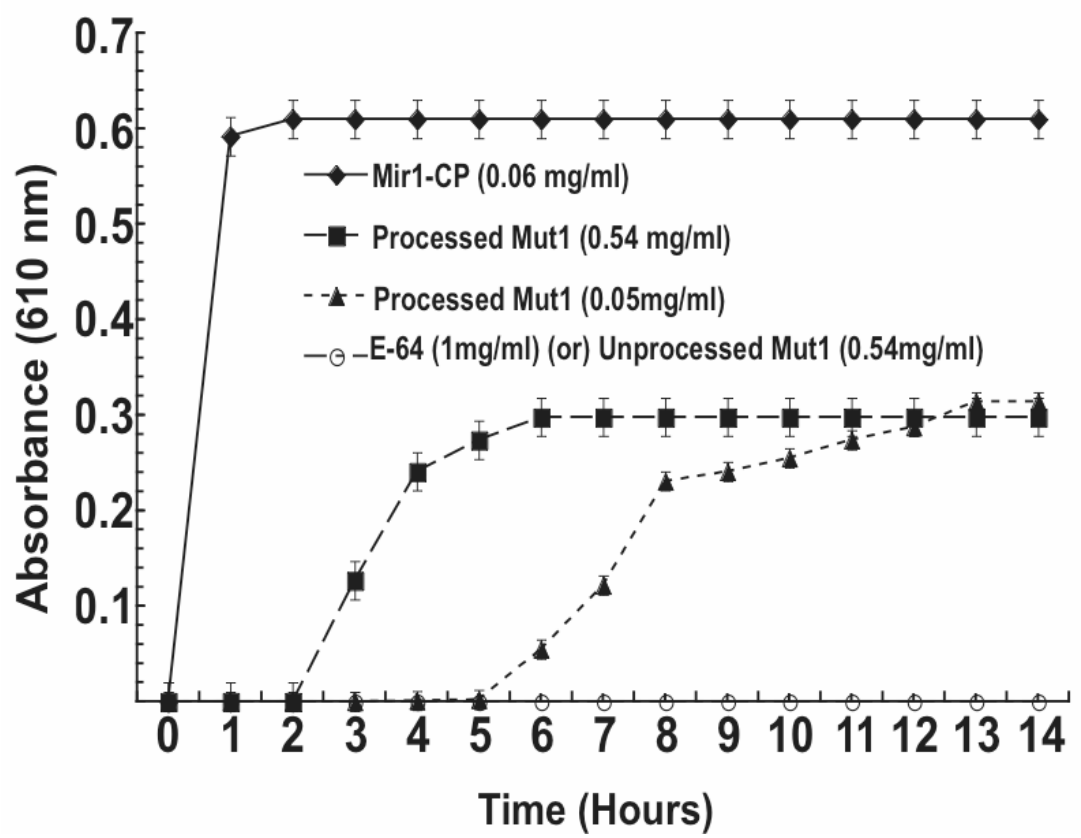


Figure 3.1. MALDI-TOF MS analysis of unprocessed Mut1 (a) and Mut2 (c), and processed Mut1(b) and Mut2 (d).



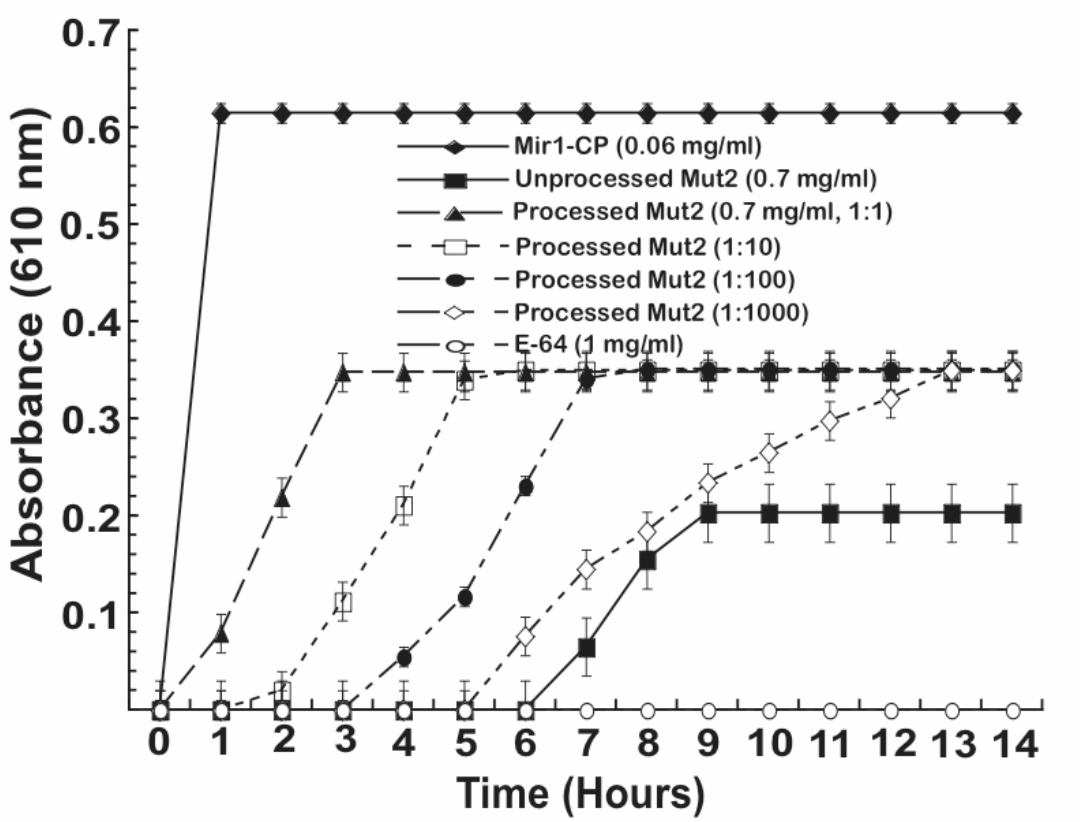
Note: Lane 1, molecular weight marker; lane2, Mir1-CP at 0.06 mg/ml; lane3, Mut1at 0.54 mg/ml processed with 1 ng/ml of Mir1-CP; lane 4, unprocessed Mut1 at 0.54 mg/ml; lane 5, unprocessed Mut2 at 0.7 mg/ml; lane 6, Mut2 at 0.7 mg/ml processed with 1 ng/ml of Mir1-CP.

Figure 3.2 Immunoblot analysis of unprocessed and processed Mut1 and Mut2 fractions using antibody specific to Mir1-CP.



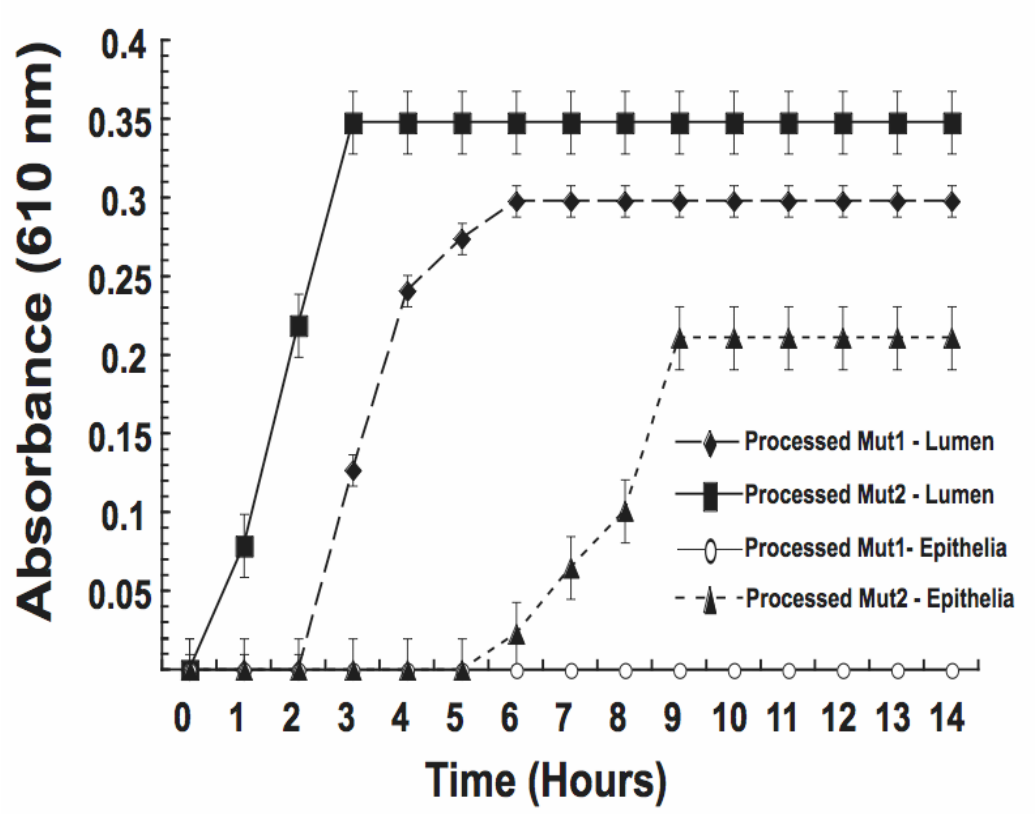
Note: PM permeability in response to Mir1-CP at 0.06 mg/ml (◆), processed Mut1 at 0.54mg/ml (■), processed Mut1 at 0.05 mg/ml (▲). Open circles (O) represent two different samples: (1) unprocessed Mut1 at 0.54 mg/ml, and (2) processed Mut1 (0.54 mg/ml) preincubated with 1 mg/ml E-64. PM treatments were conducted at pH 8.0 and 24˚C. Standard error bars are based on four different PM preparations tested.

Figure 3.3 *S. frugiperda* PM permeability in response to processed and unprocessed Mut1.



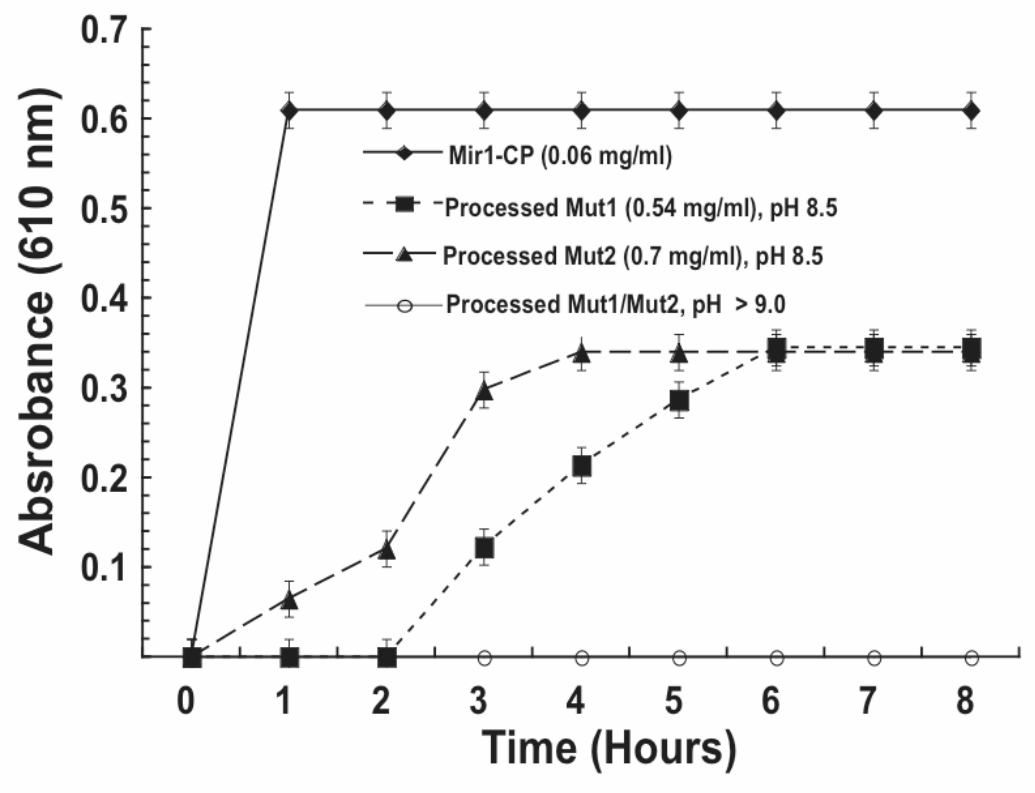
Note: PM permeability in response to Mir1-CP at 0.06 mg/ml(◆), unprocessed Mut2 at 0.7 mg/ml (■), processed Mut2 at 0.7 mg/ml (▲), 70 µg/ml (1:10) (□), 7 µg/ml (1:100) (●) and 700 ng/ml (1:1000) (✧), or processed Mut2 (0.7 mg/ml) preincubated with 1 mg/ml E-64 treatment (O). PM treatments were conducted at pH 8.0 and at room temperature of 24˚C. Standard error bars are based on four different PM preparations tested.

Figure 3.4 *S. frugiperda* PM permeability in response to processed and unprocessed Mut2.



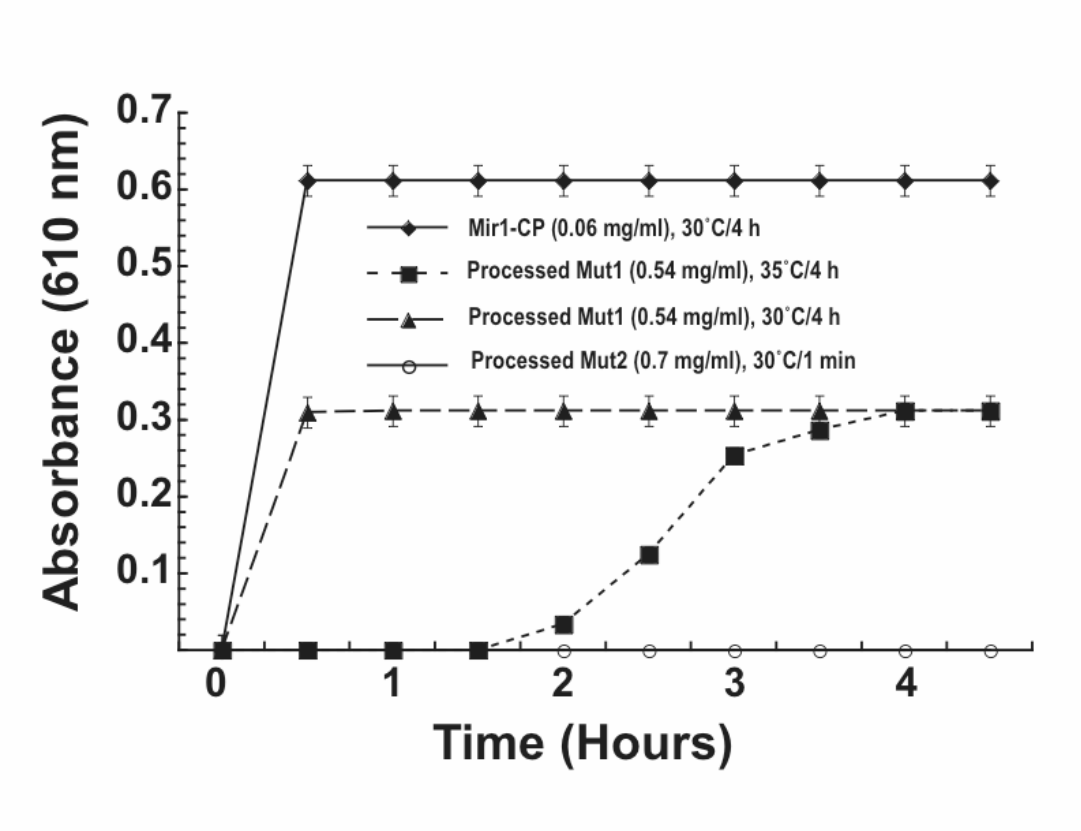
Note: Luminal (◆) and (O) epithelial sides of the PM were treated with processed Mut1 (0.54 mg/ml). Luminal (■) and (▲) epithelial sides of PM were treated with processed Mut2 (0.7 mg/ml). PM treatments were conducted at pH 8.0 and at room temperature of 24˚C. Standard error bars are based on four different PM preparations tested.

Figure 3.5 *S.frugiperda* PM permeability response to PM orientation.



Note: PM incubated at pH 8.5 with Mir1-CP (0.06 mg/ml) (◆), processed Mut1 (0.54 mg/ml) (■), processed Mut2 (0.7 mg/ml) (▲). PM preincubated with either processed Mut1 or Mut2 at pH at 9.0 or greater (O). PM treatments were conducted at room temperature of 24˚C. Standard error bars are based on four different PM preparations tested.

Figure 3.6 *S. frugiperda* PM permeability in response to Mut1 and Mut2 at alkaline pH conditions.



Note: Mir1-CP (0.06 mg/ml) was preincubated at 30˚C for 4h (◆) , processed Mut1 of 0.54 mg/ml was preincubated at either (▲) or 30˚C for 4 h (■) 35˚C for 4 h, and processed Mut2 of 0.7 mg/ml was preincubated at (O) 30˚C/1 min. Standard error bars are based on four different PM preparations tested.

Figure 3.7 *S. frugiperda* PM permeability in response to Mut1 and Mut2 preincubated at 30° and 35°C at pH 8.0

Table 3.1 Comparison of amino acid sequence of the 33-KDa Mir1-CP with those of several cysteine proteases.

Source	Amino Acid Sequence
	† *
Mir1	[11] GAVTEVKDAQQQ**C**GG**C**WAFSA[30]
Mir3	GAVAEVKDAQGS**C**GT**C**WAFST
Papain	GAVTPVKNQGS**C**GS**C**WAFSA
Bromelain	GAVNEVKNQNP**C**GS**C**WSFA
	*
Mir1	PCGTSLD**H**GVTAVGY
Papain	PCGNKVD**H**AVAAVGY
Bromelain	PCGTSLN**H**AITIIGY

Note: (†) indicates Cys^{22} position. (*) indicates either Cys^{25} or His^{159}.

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CHAPTER IV

POTENTIAL SYNERGISM BETWEEN Bt-CryIIA AND MAIZE CYSTEINE PROTEASE (Mir1-CP) ON LEPIDOPTERN LARVAL GROWTH

ABSTRACT

In previous studies we have shown that the peritrophic matrix (PM) of larvae that feed on plant material expressing Mir1-CP was damaged and treatment of isolated PMs with purified recombinant Mir1-CP increased PM permeability in a concentration dependent manner. This study was conducted to determine the minimal effective dosage of purified Mir1-CP for larval growth reduction and to determine if it could enhance the effect of *Bacillus thuringensis* (Bt-CryIIA) toxin *in vitro*. The relative growth rates (RGR) of fall armyworm decreased at concentrations greater than 600 ng/ml Mir1-CP and 0.75 µg/ml of Bt-CryIIA. Southwestern corn borer (SWCB), tobacco budworm (TBW) and corn earworm (CEW) required higher Mir1-CP and Bt-CryIIA concentrations to reach similar larval growth reduction. However, a single sub-lethal Mir1-CP concentration of 60 ng/ml was effective in synergizing Bt-CryIIA concentrations of 0.4 µg/ml for FAW, 0.5 µg/ml for either CEW or TBW and 1 µg/ml for SWCB. Statistical analysis showed significantly greater decrease in all four larval mean RGR values and at least 12-fold higher mortality percentage at combined Bt-CryIIA and Mir1-CP dosage

than at their individual treatments. These results suggest that there is a synergistic decrease in growth rate when larvae are reared on diet that contains sublethal concentrations of Bt-CryIIA and Mir1-CP.

INTRODUCTION

Lepidopteran pests constitute a major constraint in crop production. Although the use of "hard" chemical insecticides has provide some control, their high toxicity poses serious negative effects on non-target animals (and humans) (Hung et al., 2005). In addition, there is increasing risk that the Lepidopteran population will develop resistance to these pesticides. Transgenic crops, including cotton, potato, maize and other field crops, producing parasporal crystalline inclusions (Cry-toxins) from the gram-positive, endo spore-forming *Bacillus thuringiensis* have been developed (Fujimoto et al., 1993; Qaim and Zilberman, 2003; Huang et al., 2002; Eiaguirre et al., 2006; Vaughn et al., 2005; Koziel et al., 1993; Shelton et al., 2002). These plants contain polypeptides (Δ-endotoxins) that are toxic to a variety of insect species (Hofmann et al., 1988). The toxins, called Cry-toxins have significant advantages over chemical pesticides for controlling Lepidopteran pests in the field (Betz et al., 2000), as their pathogenicity is restricted to insects and crustaceans, and they can be used concurrently with chemically synthesized insecticides. They represent an attractive alternate class of biopesticides to the broad-spectrum of chemical insecticides.

Presently, 150 insecticidal crystalline Bt proteins (Cry-toxins) have been discovered in *Bacillus thuringiensis* and *Bacillus cereus* (Schnepf et al., 1998). All Bt-toxins share a high degree of structural and functional similarities in their conserved

domains and effects on the larval midgut (Gill et al., 1992, Knowles and Dow, 1993). Bt toxicity depends on a series of complex steps involving proteolytic processing of the protoxin to its active form by proteases in the alkaline midgut environment (Lecadet et al., 1967; Tojo and Aizawa, 1983). The toxin subsequently binds specific midgut receptor(s) on the midgut brush border membrane with very high affinity (Hofmann et al., 1988; Van Rie et al., 1990). This is followed by irreversible insertion into the membrane (Jenkins et al., 2000), which then forms lytic pores resulting in cell lysis, cessation of feeding and larval death (Luthy and Webersold, 1981).

The strain most widely used for controlling lepidopteran larval infestation is *Bacillus thuringiensis* subsp. *Kurstaki* HD-1. This strain usually contains genes encoding for at least five insecticidal crystal proteins: cryIA(a), CryIA(b), CryIA(c), cryIIA, and CryIIB (Hofte and Whiteley, 1989), which are expressed at high concentrations in the green portion of leaf tissues (Cooper et al., 1998, English et al., 1991, Ferre and Van Rie, 2002). The relative toxicities of individual CryIA proteins from HD-1varies widely among species (Hofte and Whiteley, 1989; MacIntosh et al., 1990; Milne et al., 1990; Moar et al., 1990; Van Frankenhuyzen et al., 1991) and CryIIA are essentially non-toxic at low concentrations (Tabashnik et al., 1993; Tang et al., 1995). The inverse correlation of Bt-toxin expression level on insect control has also increased the survival rate of lepidopteran pests (Adamczyk et al., 2001).

But, field evolved resistance to Bt-toxins are relatively low and restricted to larvae of the diamondback moth (*Plutella xylostella),* a global pest of cruciferous vegetables (Hama et al., 1992; Kirsch and Schmutterer, 1988; Shelton et al., 1993;

Tabashnik et al., 1990). Studies show such larval resistance to Bt-toxins to be an unstable (Hama et al., 1992;Tabashnik et al., 1994), inherited autosomal or incompletely recessive trait (Hama et al., 1992, Martinez-Ramirez et al., 1995; Tabashnik et al., 1992), which are usually controlled by one or few loci (Hama et al., 1992; Tabashnik et al., 1992).

Although resistance against transgenic Bt-toxin expressing plants among other lepidopteran larvae has yet to be seen in the fields, laboratory results have shown three types of resistance mechanisms. The most predominant resistance mechanism is the alteration of Bt-toxin binding site on the midgut receptors, which reduces the toxin's ability to bind to the cells (Ferre and Van Rie, 2002). This is usually considered either as decrease in binding affinity or as reduction in their binding sites. Alteration in the proteolytic processing of the Bt-protoxin as well as rapid degeneration of the damaged midgut epithelium, also contribute to Lepidopteran resistance to Bt-toxin (Ferre and Van Rie, 2002). So additional methods of sustainable insect pest control are being sought.

Maize lines that are genetically resistant to feeding by fall armyworm (*Spodoptera frugiperda*) and other Lepidopteran larvae (Davis et al., 1988; Williams et al., 1990) accumulate another toxic protein, Mir1-CP. Mir1-CP is a 33-KDa cysteine protease that rapidly accumulates in maize leaves in response to larval feeding (Pechan et al., 2000). Maize callus tissue was transformed with *mir1*, the gene encoding Mir1-CP. When larvae fed on the transgenic lines expressing Mir1-CP, their growth was inhibited approximately 70% (Pechan et al., 2000). Subsequent scanning electron microscopy (SEM) studies indicated that the peritrophic matrix (PM) of larvae feeding on the

transgenic callus was severely damaged and contained numerous holes and fissures (Pechan et al., 2002). *In vitro* studies using purified recombinant Mir1-CP indicated that it completely permeabilizes the PM, probably by attacking PM proteins (Mohan et al., 2006). These studies also indicated that Mir1-CP was most effective on lepidopteran larvae belonging to the noctuidae family (Mohan et al., 2006).

Mir1-CP has amino acid sequence similarity to a number of cysteine proteases including those from several baculovirus that infect Lepidopteran larvae such as *A. californica* and *Spodoptera exigua* nucleopolyhedrovirus (Rawlings et al., 1992; Slack et al., 1995; Wi et al., 1999). Another baculovirus metalloprotease, enhancin, attacks the PM of *T. ni* and specifically degrades the integral PM protein Insect Intestinal Mucin (IIM) (Peng et al., 1999, Wang and Granados, 1997a, b; Gendler and Spicer, 1995). However, Mir1-CP is the only plant defensive protease that has been shown to directly damage the lepidopteran PM.

Because Mir1-CP and Bt-toxins have different toxicity mechanisms, we used bioassays with the purified proteins to determine if there was synergism between them in reducing larval growth rate and increasing mortality. The proteins were tested on fall armyworm (*Noctuidae*), southwestern corn borer (*Diatraea grandiosella, Crambidae*), corn earworm (*Helicoverpa zea, Noctuidae*) and tobacco budworm (*Heliothis virescens, Noctuidae*). These insects were selected because they are economically important pests of many crops that are being controlled using Bt-containing transgenic crops.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Fisher Biotech (Fair lawn, NJ). Purified 33-KDa Mir1-CP of 0.6 mg/ml was prepared as previously described by Mohan et al. (2006). Processed 68-KDa Bt-CryIIA toxin (2 mg/ml) and its unprocessed 143-KDa form (2.3 mg/ml) in 50 mM potassium phosphate, 50 mM sodium chloride, 1 mM EDTA, with a final pH of 10.8, was obtained from Monsanto Plant Science/Regulatory Sciences (St. Louis, MO). Deionized water (18MΩ) was used in all experimental dilutions.

Insect rearing

All second instar larval colonies of fall armyworm (*Spodoptera frugiperda*), southwestern corn borer (*Diatraea grandiosella*), corn earworm (*Helicoverpa zea*) and tobacco budworm (*Heliothis virescens*) reared on artificial diet (Davis, 1976) under a photoperiod of 16:8 were obtained from a laboratory colony maintained by the USDA-ARS Corn Host Plant Resistance Research Unit at Mississippi State University.

Insect bioassay

Dose response bioassays with second instar larval colonies of fall armyworm (FAW), southwestern corn borer (SWCB), tobacco budworm (TBW) and corn earworm (CEW) were conducted in 96 well plates containing approximately 250 µl of artificial diet. The diet surface was covered with 50 µl solutions containing dilutions of either

recombinant Mir1-CP (0.6 mg/ml), processed Bt-cryIIA (2 mg/ml) or combined concentrations of both. Untreated wells served as control. The plates were air dried for 3-4 h and covered with Saran Wrap®. Previous experiments indicated that purified Mir1-CP lost only 20% of its activity after incubating at 30˚C for seven days (unpublished data). This indicates that the purified cysteine protease can retain a major portion of its activity during the bioassays.

Prior to the bioassay, larvae were weighed and placed in individual wells and the plates were covered with Breath Easy™ (USA Scientific Inc, FL, USA). Each bioassay treatment involved 24 larvae and was performed with three replicates. Larvae were incubated at 30˚C/48 h in 16:8 photoperiod before final larval weight was measured. Each bioassay treatment condition was repeated independently for 3-5 times. Larval growth reduction was measured as Δmg mg_{Avg}^{-1} $days^{-1}$, based on classical relative larval growth rate (RGR) (Hoffmann and Poorter, 2002) as

RGR = (final larval weight)-(initial larval weight) / (average larval weight) * days

The presence of a synergistic response between RGR of larvae fed Bt-CryIIA and Mir1-CP as well as percentage mortality was determined using SAS® software (Cary, NC).

PM protein extraction

Five larval PMs from fourth instar FAW and SWCB larval PM were dissected, cleaned in weever's saline and homogenized after 2 h incubation in 1% Calcofluor containing 4 µg/ml aprotinin, 1 µg/ml leupeptin, 1mM EDTA and 2mM

phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 10000 g/5 min and the resulting PM pellet was re-extracted. The supernatant collected from the two extractions were combined and further clarified by centrifugation at 15000 g/ 5 min. The calcofluor in the protein preparation was removed by using 10 DG setpac column (Bio-Rad, CA). The PM proteins in the resulting fractions were separated by pre-calibrated TSK-Gel size-exclusion column (7.8 mm ID x 30 cm dimension and 6 µm particle size, Phenomenex) and eluted in 0.1 M phosphate buffer (pH 7 $\pm$ 0.2) at a flow rate of 0.75 ml/min and a pressure of 36 bars using Hewlett Packard 1050 HPLC unit. The SEC-HPLC chromatograms were compared using Chromic™ software, for identifying any difference in proteins content between the two lepidopteran larval PMs. Eluted PM protein fractions were further purified using a pre-calibrated reverse-phase chromatographic column (250 x 2 mm diameter, 5 µm particle size) in a linear gradient, at a flow rate of 0.5 ml/min and 3124 phi. The purified fractions were concentrated by vacuum drying and stored at –80˚C.

One portion of the purified protein(s) were dissolved in 10% acetonitrile and loaded on to a pre-conditioned filter for Edman degradation sequence analysis at Iowa State University Protein Facility. The N-terminal sequences were compared with other nearly exact protein sequences in the database. The differential response of Mir1-CP on PM protein(s) was determined by incubating the other portion of individually purified proteins in 6000 ppb of Mir1-CP at 30˚C/24 h and separating them again by pre-calibrated reverse phase chromatographic column, in a linear gradient at a flow rate of 0.5 ml/min and 3000 psi. The study was repeated a minimum of at least four times.

RESULTS

Dose-response bioassays

The four lepidopteran larval species, fall armyworm (FAW), southwestern corn borer (SWCB), tobacco budworm (TBW) and corn earworm (CEW), showed varying dose response effects with diluted solutions of Mir1-CP, Bt-CryIIA or at combined sub-lethal concentrations. When larvae of the four lepidopteran species were reared on artificial diet alone, they all had similar RGR values of 0.59 $\pm$ 0.06. FAW larval growth rate decreased to 0.31 $\pm$ 0.04 at concentrations of Mir1-CP greater than 600 ppb and was 0.197 $\pm$ 0.02 with 0.75 ppm Bt-CryIIA (Figure 4.1a, b). The percentage mortality was 46% and 33% at the highest concentrations of Mir1-CP and Bt-CryIIA tested, respectively. When larvae were treated singly with 60 ppm of Mir1-CP, the RGR was 0.320 $\pm$ 0.09 and the mortality was 42% (Figure 4.1a). Larvae reared on 0.25 ppm Bt-CryIIA had a RGR of 0.639 $\pm$ 0.04, which was not significantly different from the control and there was no decrease in mortality (Figure 4.1b). At a concentration of 0.5 ppm Bt-CryIIA, the RGR decreased to 0.586 $\pm$ 0.059 and the percentage mortality was 13%. The concentrations of 60 ppb Mir1-CP and 0.4 ppm Bt-CryIIA were selected to test the effect of the components in combination (Figure 4.1c). When these two doses of Mir1-CP and Bt-CryIIA were combined the RGR value was reduced to 0.189 $\pm$ 0.054 (Figure 4.1c) and the percentage mortality increased to 50%, which was higher than those of the individual treatments (Table 4.1a, b).

Higher concentrations of Mir1-CP and Bt-CryIIA were required to reduce the RGR of other lepidopterans to the FAW level. For SWCB, 6000 ppb of Mir1-CP were required to reduce the RGR to 0.2 $\pm$ 0.01 and increase the mortality to 45% (Figure 4.2a). A concentration of 1.5 ppm of Bt-CryIIA was needed to reduce the RGR to 0.19 $\pm$ 0.023 and increase the mortality to 50% (Figure 4.2b). When used singly, the concentrations 60 ppb of Mir1-CP and 1 ppm Bt-CryIIA did not significantly change the RGR or the percentage mortality (4% for each) (Figure 4.2c) relative to the control. In combination, however, the RGR was reduced to 0.13 ± 0.03 and the mortality increased to 63% (Figure 4.2c and Table 4.1).

TBW larvae required 3600 ppb of Mir1-CP to decrease the RGR to 0.212 $\pm$ 0.017 (Fig. 4.3a) and increase the mortality to 42%. One ppm of Bt-CryIIA reduced their RGR to 0.201 $\pm$ 0.02 (Figure 4.3b) and increased the mortality to 43%. The lower doses of 600 ppb Mir1-CP and 0.5 ppm of Bt-CryIIA did not significantly decrease the RGR or percentage mortality (Figure 4.3a, b) relative to the control. When 60 ppb Mir1-CP and 0.5 ppm of Bt-CryIIA were used together, the RGR was 0.19 $\pm$ 0.03 and the mortality increased to 57% (Figure 4.3c, Table 4.1).

For CEW, 1800 ppb of Mir1-CP were needed to reduce the RGR to 0.18 $\pm$ 0.03 (Figure 4.4a). This resulted in 39% mortality. Bt-CryIIA (0.75 ppm) reduced the RGR to 0.191 $\pm$ 0.02 and the mortality was 47% (Figure 4.3b). Doses of Mir1-CP (600 ppb) and Bt-CryIIA (0.25 ppm) did not significantly decrease the RGR for CEW relative to the control. The percentage mortality (4 and 5%) was slightly higher than the control (Figure 4a, b). When CEW larvae were fed a combination of Mir1-CP (60 ppb) and Bt-CryIIA

(0.5 ppm), the RGR dropped to 0.17 ± 0.01, and the percentage mortality increased to 63% (Figure 4.4c, Table 4.1) .

Mir1-CP differential response

Comparative studies using size-exclusion chromatograms showed two proteins, PM protein 1 and 2, to be commonly found in both FAW and SWCB PMs with an additional unique PM protein in SWCB (Figure 4.5). The PM proteins 1 and 2 have molecular masses of 35012 and 34143 Da and the unique SWCB PM protein had a molecular mass of 24034 Da (Figure 4.6), in MALDI-TOF MS. Reverse phase chromatographic study showed proteolytic degradation of the two common PM proteins by Mir1-CP after 24 h treatment. However, the purified unique SWCB PM protein was resistant under similar conditions (Figure 4.7). Database analysis with first 10 N-terminal amino acid sequence of PM proteins 1 and 2 showed 100% homology with human carboxypeptidase A and B. The unique SWCB protein had 90% homology with dogfish trypsin precursor (Table 4.2).

DISCUSSION

This study was conducted to determine the minimum physiological concentration of Mir1-CP required for Lepidopteran larval growth reduction and to test its possible synergistic response with Bt-toxin (Bt-CryIIA). The study was facilitated by our ability to express, isolate and purify recombinant Mir1-CP in large amounts from fall armyworm hemolymph (Pechan et al., 2004; Mohan et al 2006). This also allowed us to quantitatively determine sensitivity of Mir1-CP towards a non-noctuid Lepidopteran

species, SWCB. There was a 10-fold difference in sensitivity to Mir1-CP among the Lepidopteran species tested. To lower the RGR from the control level to approximately 0.2 required 600, 1800, 3600 and 6000 ppb for FAW, TBW, CEW and SWCB, respectively (Figure 4.8). The larval growth reduction of these Lepidopteran species shows that physiologically relevant concentrations of Mir1-CP in the maize whorl would be effective in controlling broad range of Lepidopteran pests. These bioassays also indicate variability in Mir1-CP sensitivity towards noctuidae family. Interestingly, the crambid, SWCB was the most resistant to Mir1-CP. We isolated proteins from SWCB and FAW PMs for understanding whether morphological or biochemical differences among PM proteins of Lepidopteran larval families may account for the different sensitivities to Mir1-CP. Our studies showed a unique 24-KDa protein in SWCB PM having high level of resistance to proteolytic degradation by Mir1-CP of 6000 ppb for 24 h, among other PM proteins. We speculate that this unique protein in SWCB PM may be offering resistance against Mir1-CP in unknown ways.

The concentrations of Bt-CryIIA required to lower the RGR to approximately 0.2 were 0.75, 1.0, 0.75 and 1.5 ppm for FAW, TBW, CEW and SWCB, respectively. However when only 60 ppb of Mir1-CP and concentrations of Bt-CryIIA from 0.4 to 1 ppm were combined, both the RGR and percentage mortality was lower than the controls (Table 4.1). Thus it appears that 60 ppb Mir1-CP can synergize sublethal amounts of Bt-CryIIA in bioassays with the lepidopterans tested. It is possible that increasing the Mir1-CP concentration used in combination with Bt-CryIIA could result in complete mortality, but that was not tested in this study.

Both SEM and *in vitro* permeability experiments indicated that the PM is the target of Mir1-CP (Pechan et al., 2002; Mohan et al., 2006). SEM indicated that there were many cracks and fissures in the PM of FAW larvae that fed on plant material containing Mir1-CP (Pechan et al, 2002). The *in vitro* studies indicated that the PM could be completely permeabilized by Mir1-CP in a concentration dependent manner (Mohan et al, 2006). Therefore, it appears that Mir1-CP exerts its effect on these lepidopterans by making holes in the PM. Damage to the PM results in the loss of the midgut protective barrier, disrupts nutrient cycling in the midgut and slows larval growth (Wang and Granados, 2001). The Bt-endotoxins have a different toxicity mechanism than Mir1-CP. They act by binding to receptors in the midgut cells and forming lytic pores (Jenkins et al., 2000, Knowles and Ellar, 1987). Consequently, there are two possible ways Mir1-CP might synergize Bt-endotoxin. First, Mir1-CP, which permeabilizes the PM, might facilitate Bt-endotoxin movement though the PM to bind to midgut cells. Alternatively, Mir1-CP might process the pro-form of the endotoxin to the more toxic form in the bioassay or the midgut. Since the processed form of the toxin was used in the bioassays, the second possibility is unlikely. SDS-PAGE analysis indicated that Mir1-CP could cleave the protoxin, but not the processed form (Figure 4.9).

We used classical growth analysis to present our bioassay results, since it provides the formula for calculating the RGR for single individual larvae (Hoffmann and Poorter, 2002). It also decreased the chances of bias by decreasing the variance in RGR or in sample size. Other interpretations, such as the pairing method or the functional

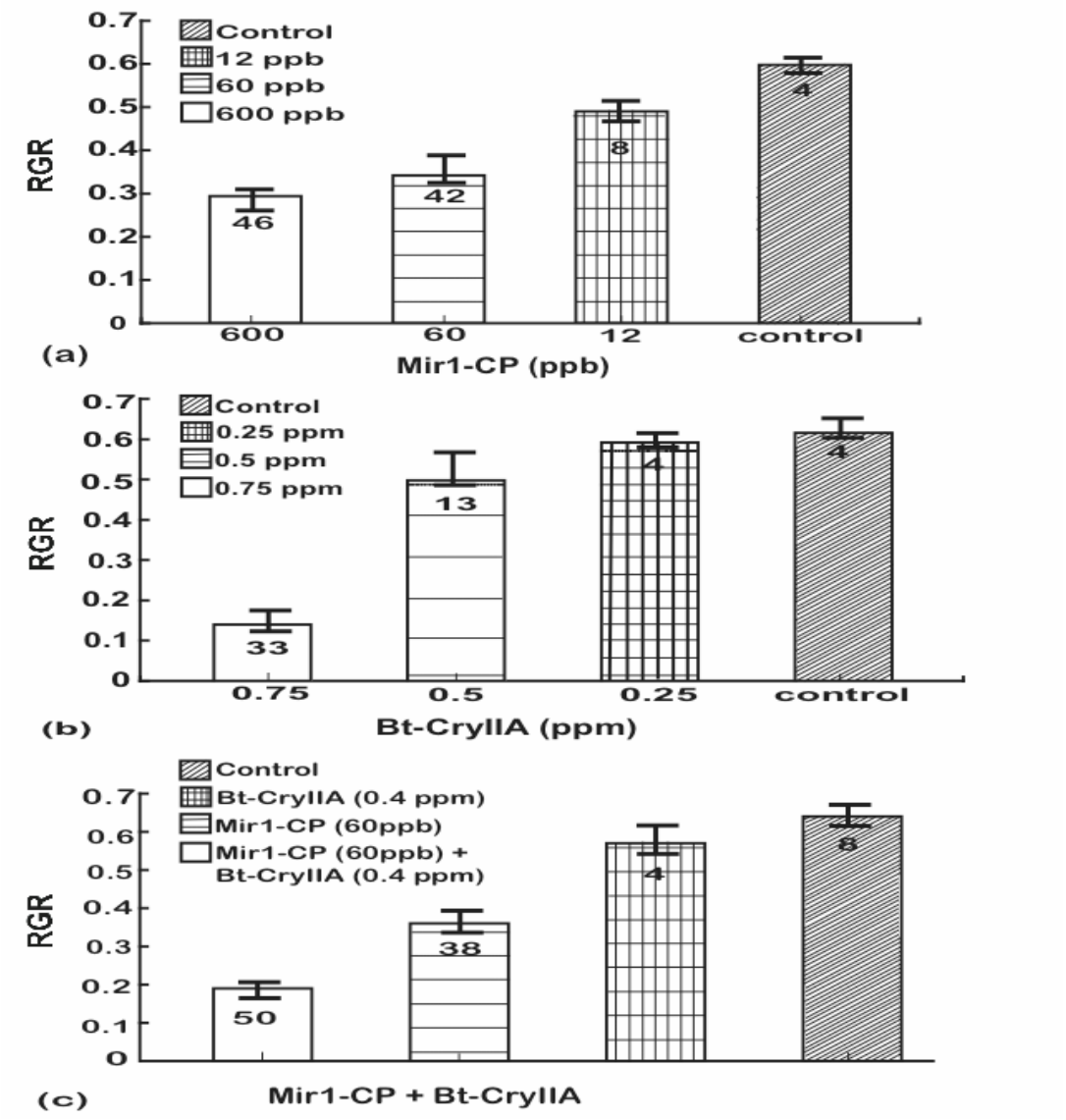
approach (Evans, 1972) to the classical growth analysis do exist and might yield different estimates of RGR, but they have not been recommended by others (McGraw and Garbutt, 1990; Venus and Causton, 1979; Radford, 1967; Evans, 1972; Hunt, 1982, 1990; Chiariello et al., 1991).

Tabashnik et al. (1993) and Tang et al. (1995) found low concentrations of Bt-CryIIA to be essentially non-toxic to both resistant and susceptible larvae (ca. up to 100 ppb), but our present bioassay results have shows Lepidopteran larval species to be susceptible to high Bt-CryIIA concentrations (ca. between 0.75 to 1.5 ppm). Resistance to other Bt endotoxins has already been observed in laboratory colonies and field poplulations of some insects (Tabashnik, 1994, 2000; Chaufaux et al., 1997; Perez and Shelton, 1997; Gould, 1998; Huang, et al., 1999; Burd et al., 2000) with high levels of resistance to HD-1 spore and all CryIA toxins isolated from *B.thuringiensis* subsp. *Kurstaki* (Tabashnik et al., 1993). Although CryIB, CryIC, CryID and CryIF has no field-evolved resistance to both resistant and susceptible larvae, Luttrell et al (1999) have reported that lepidopteran larvae have the ability to develop greater than 100-fold resistance to Bt-toxins after several generations of selection. Previous monitoring studies and historical models have also shown the propensity of multi-fold Bt resistance in noctuidae family. The responsible mechanism for resistance is unknown, but factors like altered protoxin activation by midgut proteinases (Oppert et al., 1994) and/or increased non-specific binding in midgut tissues (Moar et al., 1995) may be more likely in providing enough protection for larvae to survive on transgenic plants.

To suppress the prevailing threat of developing Bt-resistance in these target pest species, we combined the larval growth retarding effect of Mir1-CP with Bt-CryIIA. It has also been suggested that mixture of two or more of functionally diverse toxins might be more effective and also delays evolution of resistance in target insects (Gill et al., 1992; Tabashnik et al., 1991; Van Rie et al., 1990). This study showed the remarkable ability of a single sub-lethal Mir1-CP concentration of 60 ppb to effectively synergize Bt-CryIIA concentrations of 0.4 ppm for FAW, 0.5 ppm for either CEW or TBW and 1 ppm for SWCB. Statistical analysis showing greater decrease in the mean RGR values and high mortality percentages for larvae treated with combined Mir1-CP and Bt-CryIIA than with their individual concentrations, further confirms the synergistic response between them.

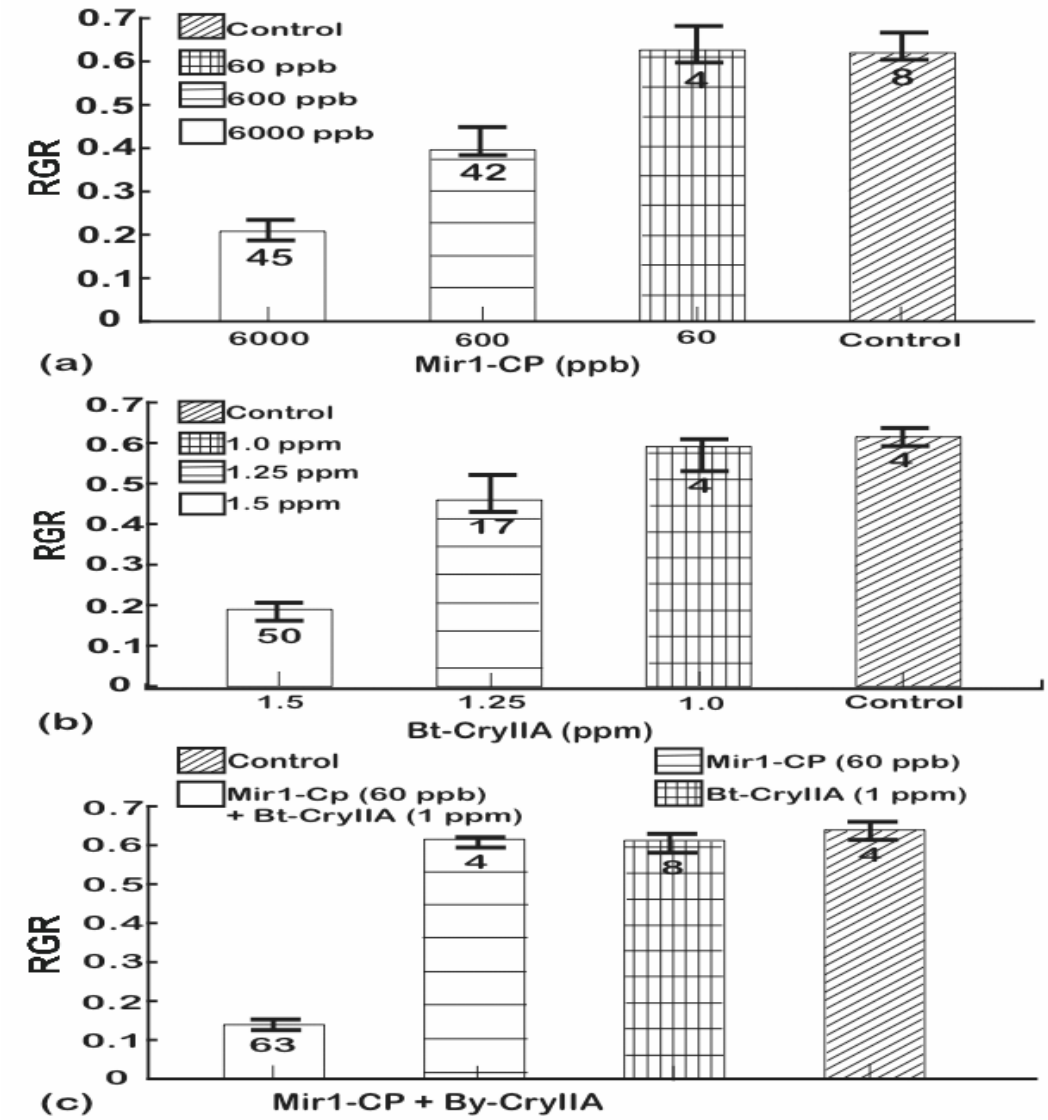
However, all tested Lepidopteran species required different combined sub-lethal dosage of Mir1-CP or Bt-CryIIA to achieve similar reduction in their growth. This suggests the degree of selectivity between Mir1-CP and Bt-CryIIA in their combined response towards the different larval species and deploys the high-dose strategy for suppressing the pest population (Via 1986; Shaw 1989; Caretto et al., 1994). The synergistic treatment also demonstrates the possibility of broadening the normal range of both Mir1-CP and Bt-toxins.

The synergism between Mir1-CP and Bt-CryIIA suggests that stacking these two genes in transgenic plants could enhance the effectiveness of Bt in the plant, potentially reduce the development of insect resistance to B-toxins, and ultimately result in more sustainable control of Lepidopteran pests.



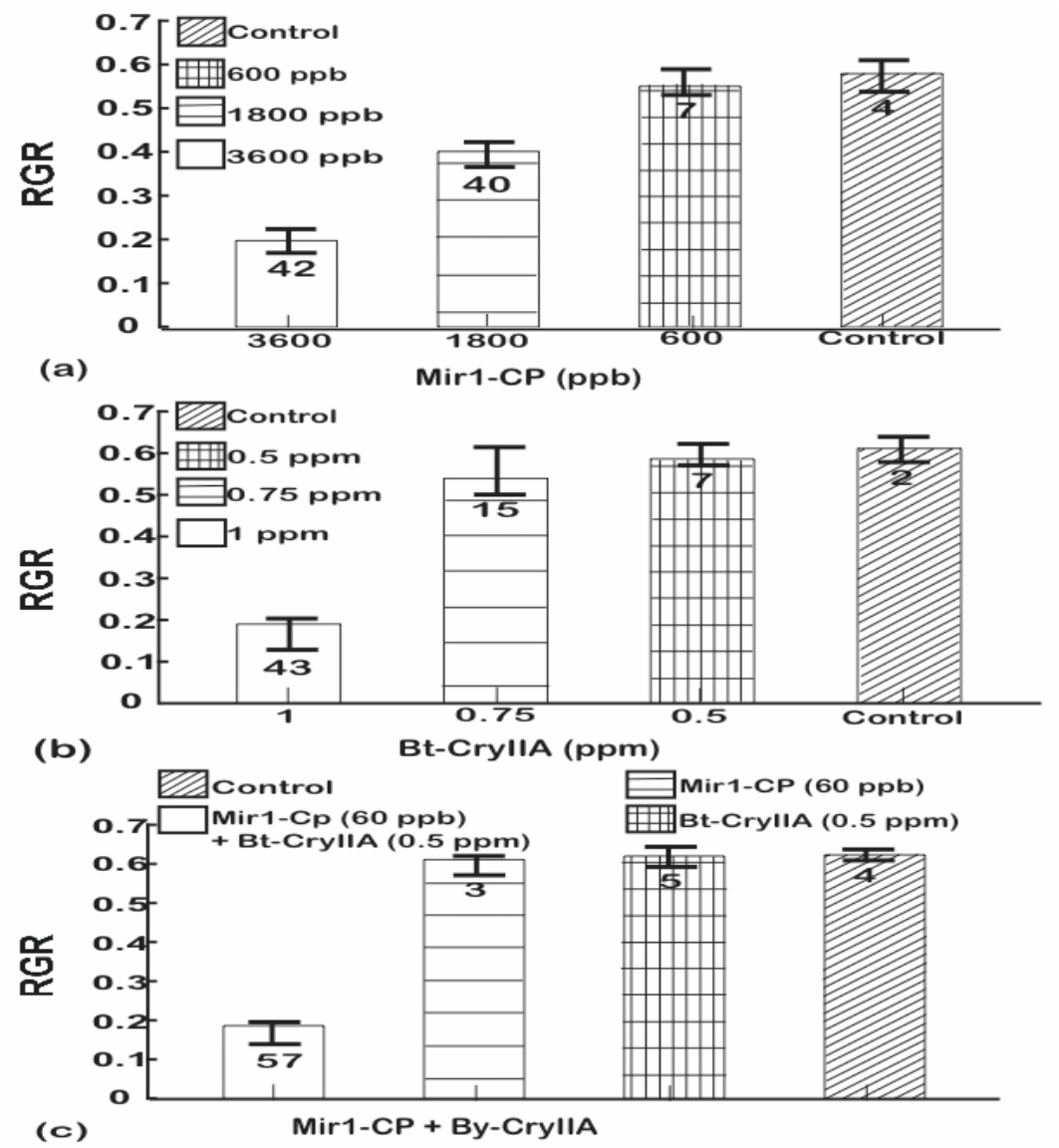
Note: Larval growth and mortality of (a) Mir1-CP, (b) Bt-CryIIA, and (c) sub-lethal doses of Bt-CryIIA and Mir1-CP. The number on the histogram bars represent average percentage mortality and has $P \leq 0.05$. Vertical bars indicate standard deviation. Results are based on four independent bioassays. ppm = parts per million. ppb = parts per billion. Larval RGR was measured as $\Delta mg\ mg_{Avg}^{-1}\ days^{-1}$.

Figure 4.1 Dose response bioassays for fall armyworm larvae.



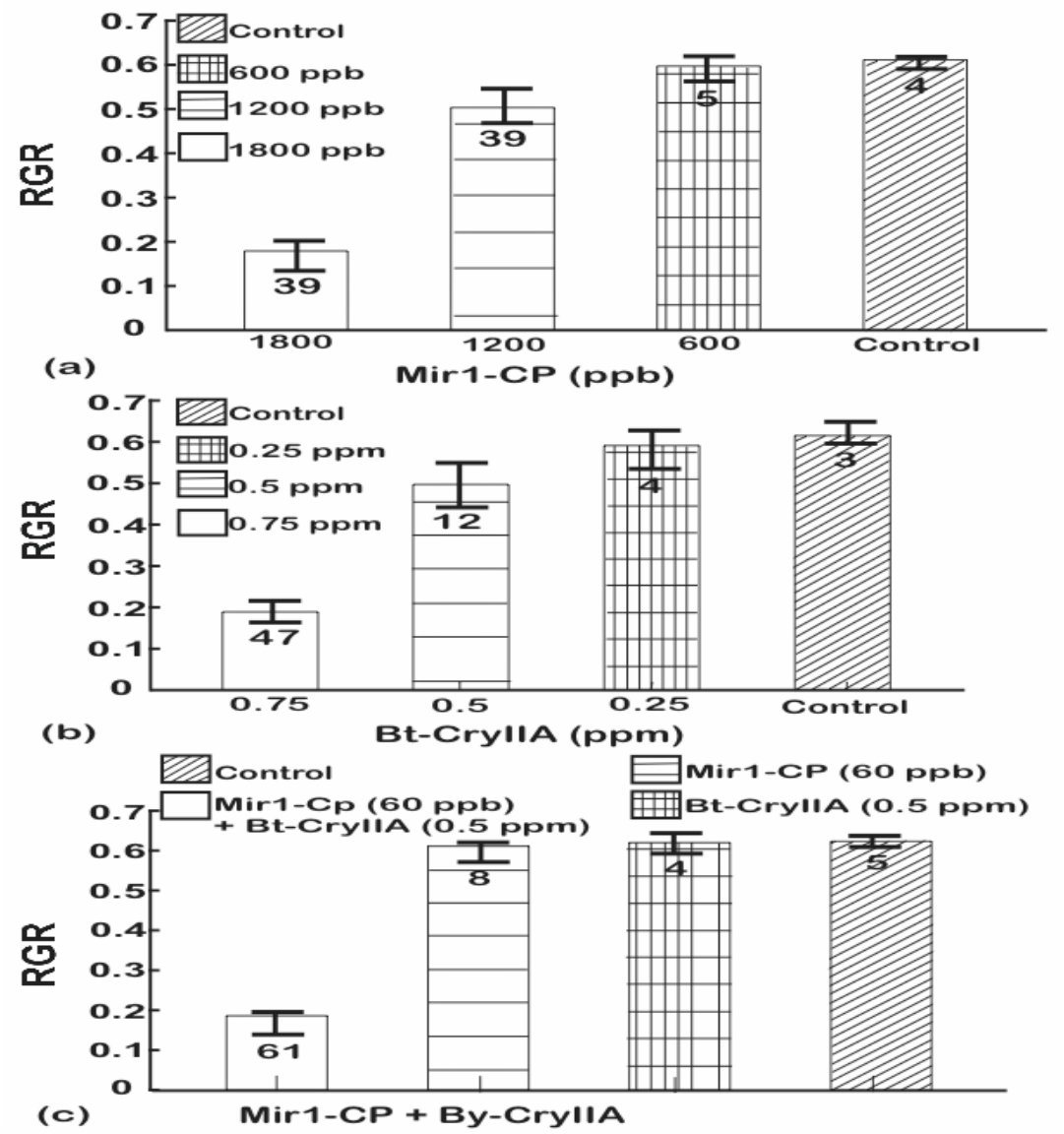
Note: Larval growth and mortality of (a) Mir1-CP, (b) Bt-CryIIA, and (c) sub-lethal dose of Bt-CryIIA and Mir1-CP. The number on the histogram bars represent average percentage mortality and has $P \leq 0.05$. Vertical bars indicate standard deviation. Results are based on four independent bioassays. ppb = parts per billion. Larval RGR was measured as Δmg mg_{Avg}^{-1} $days^{-1}$.

Figure 4.2 Dose response bioassays for southwestern corn borer larvae.



Note: Larval growth and mortality of (a) Mir1-CP, (b) Bt-CryIIA, and (c) sub-lethal dose of Bt-CryIIA and Mir1-CP. The number on the histogram bars represent average percentage mortality and has $P \leq 0.05$. Vertical bars indicate standard deviation. Results are based on four independent bioassays. ppm = parts per million. ppb = parts per billion. Larval RGR was measured as $\Delta mg\ mg_{Avg}^{-1}\ days^{-1}$.

Figure 4.3 Dose response bioassays for tobacco budworm larvae.



Note: Larval growth and mortality (a) Mir1-CP, (b) Bt-CryIIA, and (c) sub-lethal dose of Bt-CryIIA and Mir1-CP. The number on the histogram bars represent average percentage mortality and has $P \leq 0.05$. Vertical bars indicate standard deviation. Results are based on four independent bioassays.ppm = parts per million. ppb = parts per billion. Larval RGR was measured as Δmg mg_{Avg}^{-1} $days^{-1}$

Figure 4.4 Dose response bioassays for corn earworm larvae.

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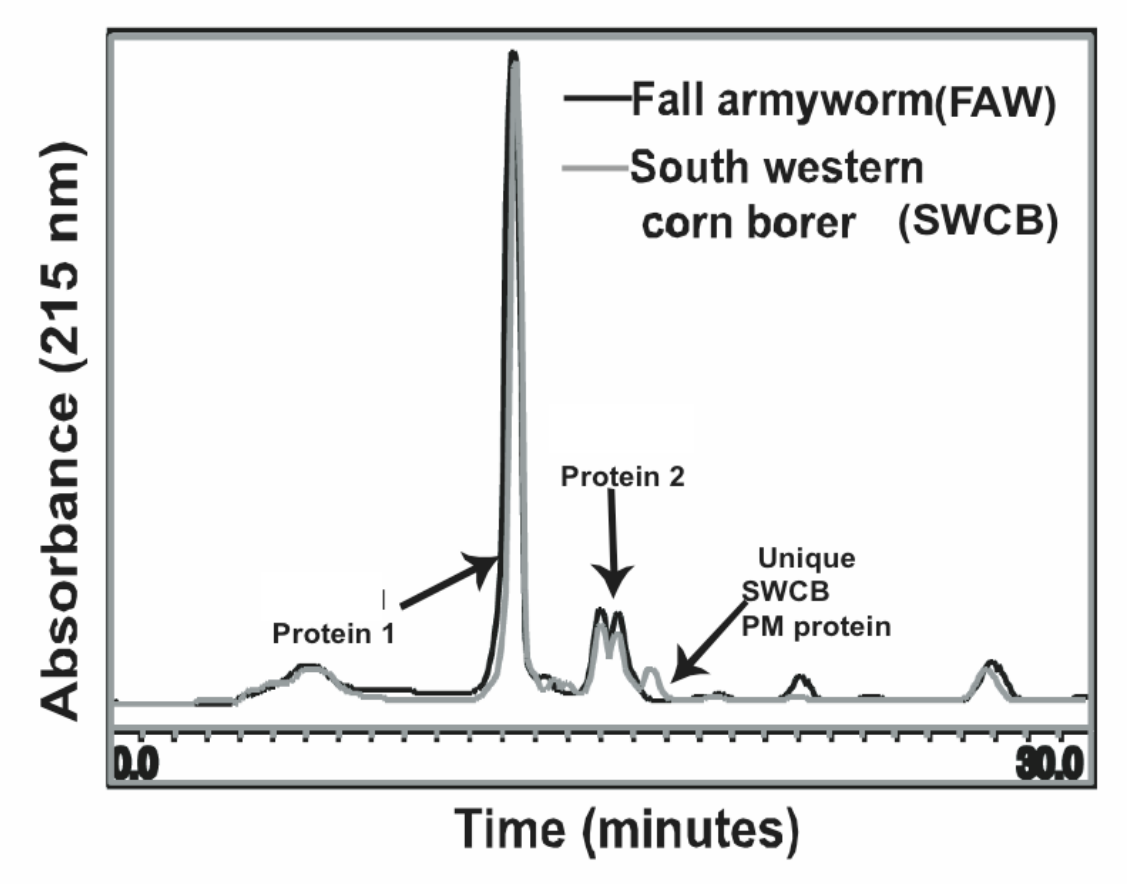


Figure 4.5 Comparative analysis of proteins from SWCB and FAW PM using size exclusion HPLC.

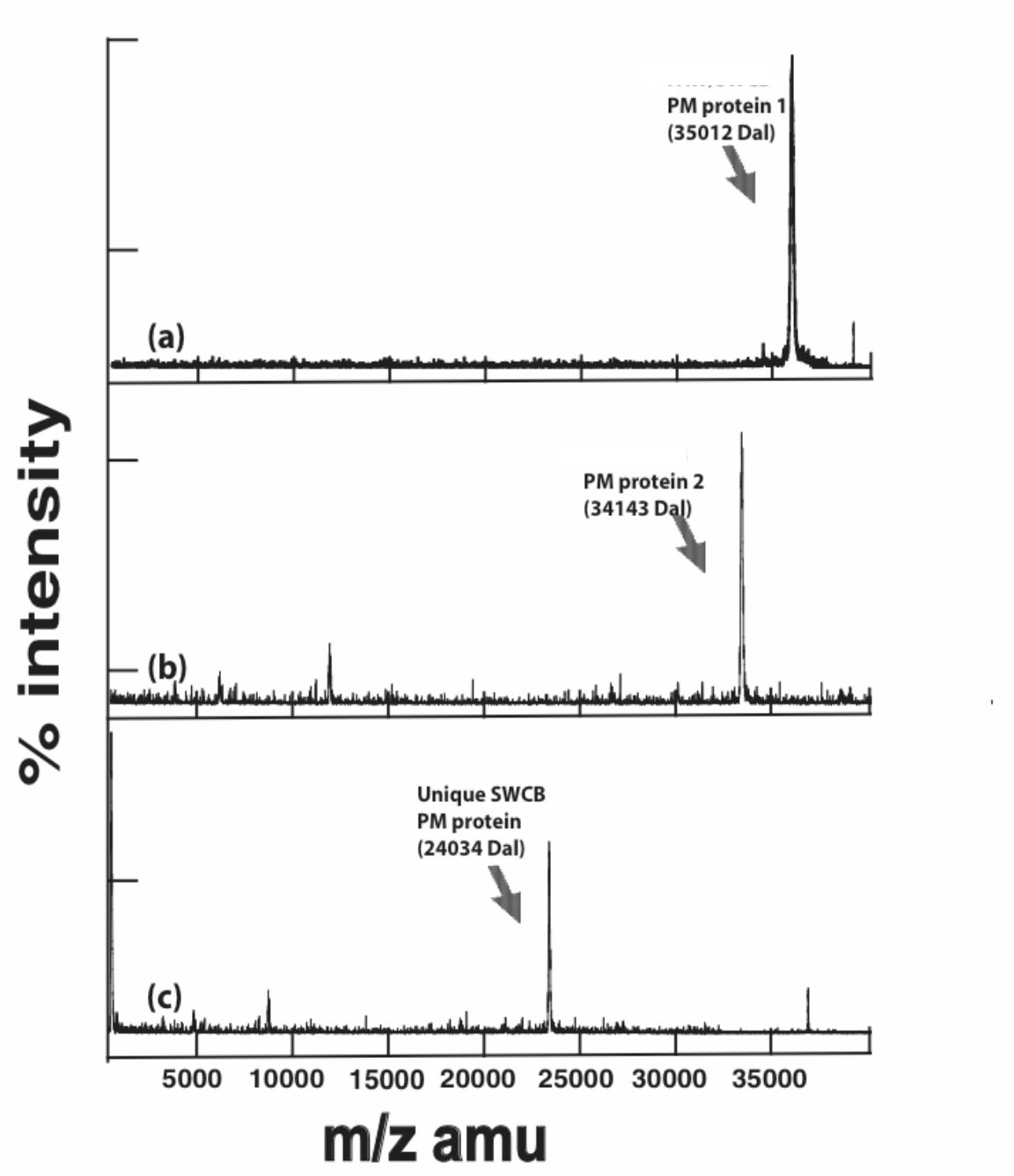


Figure 4.6 MALDI-TOF MS analysis on individual FAW and SWCB PM proteins.

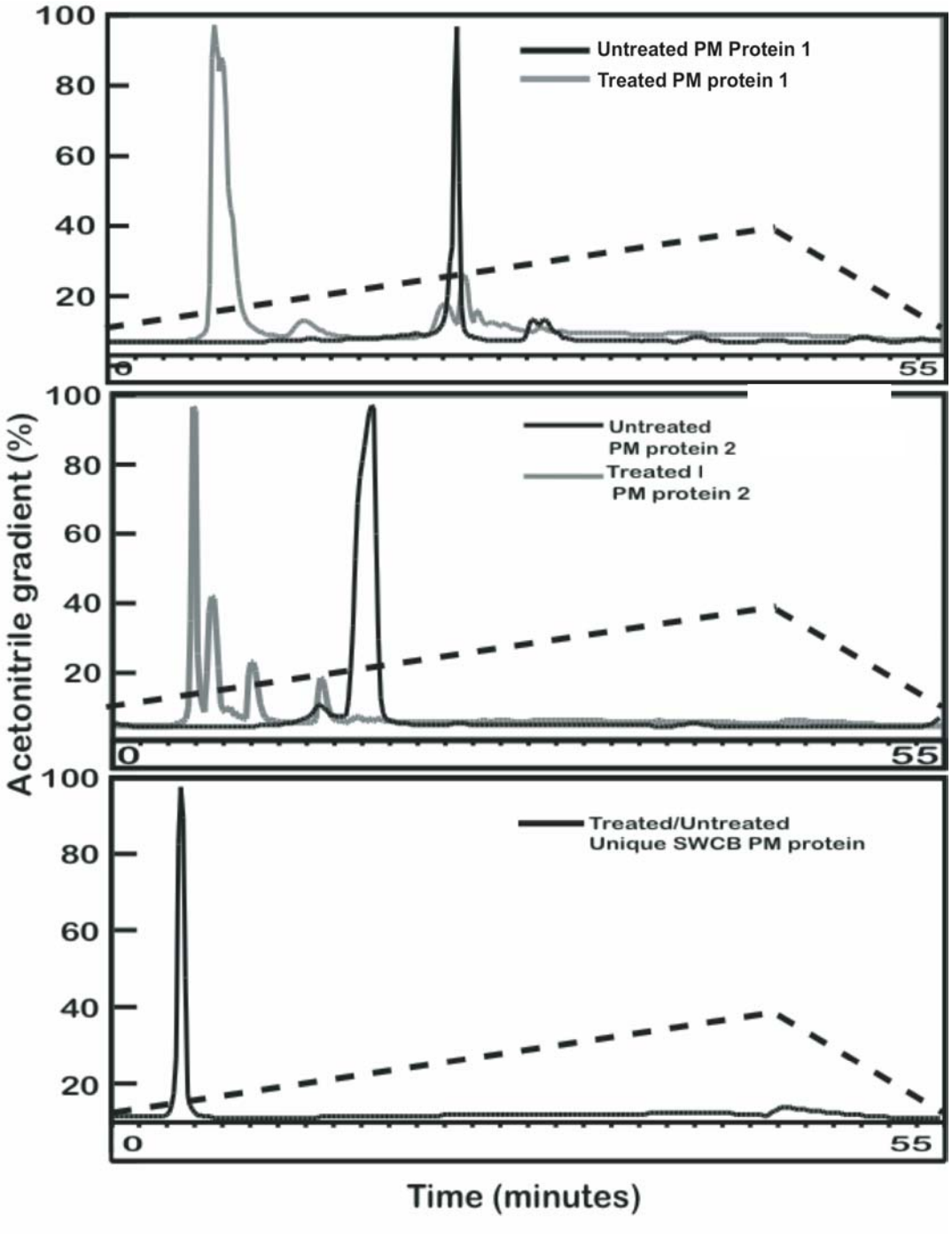


Figure 4.7 Differential Mir1-CP response on individual PM proteins from SWCB and FAW larvae.

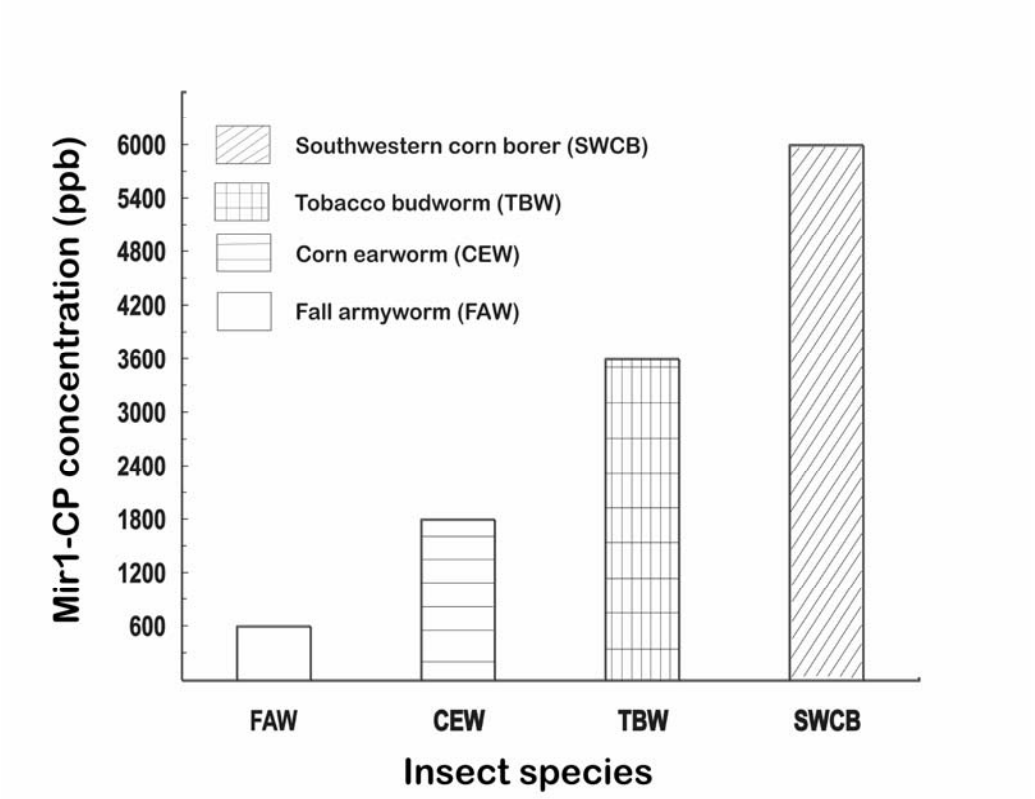
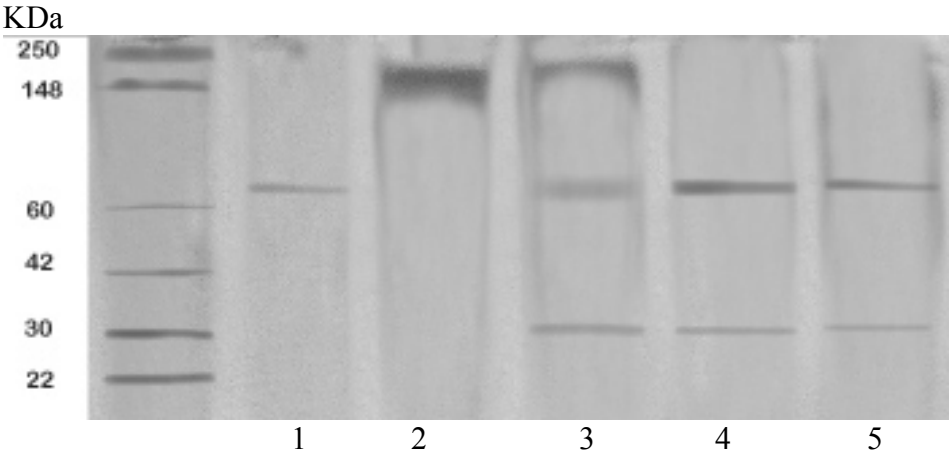


Figure 4.8 Mir1-CP concentration requirement for reduced lepidopteran larval growth.



Note: Lanes, Processed Bt-CryIIA (1), Unprocessed Bt-CryIIA (2), Unprocessed Bt-CryIIA incubated for 8 h with 60 ppb of Mir1-CP (3), Processed Bt-CryIIA incubated with 60 ppb of Mir1-Cp for 8 h (4) and 24 h (5).

Figure 4.9 SDS-PAGE analysis of Mir1-CP effect on Bt-CryIIA protoxin and its processed form.

Table 4.1 Larval mean relative growth rate (RGR) and percentage mortality.

(a)	Fall armyworm		Southwestern corn borer		Corn earworm		Tobacco budworm	
	Mir1-CP (ppb)	Bt-CryIIA (ppm)	Mir1-CP (ppb)	Bt-CryIIA (ppm)	Mir1-CP (ppb)	Bt-CryIIA (ppm)	Mir1-CP (ppb)	Bt-CryIIA (ppm)
Dosage	60	0.4	60	1	60	0.5	60	0.5
Mortality (%)	38	4	4	8	8	4	3	5
RGR Mean (Δmg mg_{Avg}^{-1} d^{-1})	0.3330	0.5683	0.609	0.596	0.574	0.514	0.5620	0.5740

(b)	Fall armyworm	Southwestern corn borer	Corn earworm	Tobacco budworm
Dosage	60 ppb Mir1-Cp + 0.4 ppm Bt-CryIIA	60 ppb Mir1-CP + 1 ppm Bt-CryIIA	60 ppb Mir1-CP + 0.5 ppm Bt-CryIIA	60 ppb Mir1-CP + 0.5 ppm Bt-CryIIA
Mortality (%)	50	63	61	57
RGR Mean (Δmg mg_{Avg}^{-1} d^{-1})	0.223	0.132	0.197	0.201
P value	0.033	0.042	0.037	0.039

Table 4.2 N-terminal sequence comparison.

Source	Amino Acid Sequence	Homology
SWCB/ FAW PM protein 1	S R S T F T F N Y A	100 %
Human Carboxypeptidase A precursor	- R S T F T F N Y A	
SWCB/FAW PM protein 2	R R G H S Y E K Y N	100 %
Human Carboxypeptidase B precursor	- - G H S Y E K Y N	
Unique SWCB PM protein	A P D D P D K I V G	90 %
Dogfish trypsin precursor	A P D D D D K I V G	

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CHAPTER V

MORPHOLOGICAL STUDY OF A NOVEL CYSTEINE PROTEASE EFFECT ON LEPIDOPTERAN LARVAL PERITROPHIC MATRIX

ABSTRACT

We have shown that the peritrophic matrix (PM) of FAW larvae that feed on plant material expressing Mir1-CP was damaged and treatment of isolated PMs with purified recombinant Mir1-CP increased PM permeability in a concentration dependent manner. Dose-response bioassay showed a decreased relative growth rate for FAW larvae fed on artificial diet containing 600 ppb Mir1-CP. Previous studies using scanning electron microscopy (SEM) indicated that the PMs of fall armyworm larvae were damaged when they fed on maize tissue expressing Mir1-CP. This study extends the prior work by qualitatively determining the morphological effects of purified Mir1-CP's on larval PM and gut regions, using three different microscopic techniques. Light microscopy studies showed morphological PM damage for larvae fed with 600 ppb Mir1-CP or higher. Damage to fall armyworm larval PM under similar Mir1-CP treatment conditions was also observed by scanning and transmission electron microscopy techniques. These results suggests that by damaging PM, which is insects first line of defense, Mir1-CP impairs the normal highly organized digestive system when used at relatively

physiological concentrations of 600 ppb or higher. The unusual host-plant resistance mechanism of the 33-KDa Mir1-CP may have applications in agricultural biotechnology.

INTRODUCTION

Fall armyworm, *Spodoptera frugiperda*, damages plants by feeding on leaves within the whorls and is a serious pest of wheat, rice, corn and other monocotyledonous food crops (*Z. mays* L.) in southern United States. But little is known about the insect-defense response mechanisms in these species. However, over the past 25 years, maize inbreds with genetic resistance to several lepidopteran species have been developed from Antiguan germplasm (Williams and Davis, 1982; Williams et al., 1990). These lines showed resistance to fall armyworm and other lepidopteran larvae through a novel defense mechanism involving both antibiosis and non-preference (Wiseman et al., 1981,1983). These resistant plants accumulates a unique 33-KDa cysteine protease at the wound site within one hour of fall armyworm feeding and its abundance increases for up to seven days after infestation (Pechan et al., 2000). The 33-KDa cysteine protease seems to have properties that are distinct from other insect-induced plant defense proteins, such as protease inhibitors, lectins, cell wall proteins, oxidative enzymes and enzymes catalyzing the production of secondary metabolites (Constabel et al., 1999).

Fall armyworm larvae reared on these resistant maize lines had lower initial growth rate, delayed pupation and approximately 50% reduction in body weight than those reared on susceptible genotypes (Chang et al., 2000; Williams et al., 1992). To understand this detrimental effect on larval growth, scanning electron microscopy (SEM) was conducted to morphologically determine if there was physical damage to the insect

midgut in response to feeding on resistant maize tissues expressing Mir1-CP. SEM studies indicated that the peritrophic matrix (PM) of the larvae, which surrounds the food bolus, assists in digestive processes and protects the midgut region, was severely damaged in larvae reared on resistant maize leaves or transgenic callus (Pechan et al., 2002). A number of studies have shown that damage to PM occurs due to disruption of its chitin network, which usually affects normal nutrition and enzyme recycling between endo and ectoperitrophic spaces (Terra, 2001; Bolognesi et al., 2001; Harper et al., 1998). In dose-response bioassays using purified recombinant Mir1-CP (Luthe and Mohan, unpublished data), 600 ppb reduced the larval relative growth rate by approximately 50% and mortality increased from 8% in the control to 45% in the Mir1-CP-fed larvae. Purified recombinant Mir1-CP completely permeabilized the PM in a concentration-dependent manner, when it was used for *in vitro* permeability studies (Mohan et al., 2006). Mir1-CP permeabilized the PM more completely than the other plant cysteine proteases, papain and ficin (Mohan et al., 2006; Konno et al., 2004). When Mir1-CP was preincuated with E64, a cysteine protease inhibitor, the PM was not permeabilized. This indicated that proteolytic activity was necessary for PM degradation. These results suggest that Mir1-CP inhibits larval growth *in vivo* by attacking the PM and disrupting the digestive process.

Although previous studies using SEM indicated that the PMs of FAW larvae were damaged when they fed on maize tissue expressing Mir1-CP, this study extends the prior work and qualitatively determines the morphological effects of purified Mir1-CP's on larval PM and gut regions using three different microscopic techniques.

MATERIALS AND METHODS

Insect rearing and treatment conditions

Fourth instar larval colonies of *S. frugiperda* reared on artificial diet (Davis, 1976) under a photoperiod of 16:8 at 28˚C were obtained from the corn insect laboratory at Mississippi State University. Larvae were allowed to feed on normal artificial medium as well as on diet containing 60, 600 or 6000 ppb Mir1-CP and midguts were dissected after 24 hours.

Microscopic studies

Midguts dissected from larvae feeding on normal and Mir1-CP-treated artificial diet were fixed overnight at 4˚C in half-strength Karnovsky's fixative. Midguts of larvae feeding on normal medium were used as control. For light microscopy, the midguts were dehydrated in ethanol, embedded in paraffin blocks and sectioned tangentially as 6 µm ribbons. The sections were mounted on to slides, air dried for 30 min and then baked in 50˚C oven overnight. The slides were de-paraffinized and stained with hematoxylin and eosin stain.

For scanning electron microscopy (SEM), the midguts fixed in half strength Karnovsky's fixative were post-fixed in 2% osmium tetraoxide (OsO_4) for 2 h at room temperature (24˚C). The midgut tissues were then dehydrated in ethanol, cryo-fractured in liquid nitrogen and dried using hexamethyldislazane (HMDS). The specimens were later air-dried overnight at room temperature and mounted on aluminum stubs. Mounted stubs were coated with gold/palladium at 2.5 KV and examined using JOEL JSM-6500F

field emission scanning electron microscopy.

For transmission electron microscopy (TEM), PMs dissected from larvae fed on normal or Mir1-CP-treated diets were fixed in Karnovsky's fixative overnight at 4˚C. The PMs were later post-fixed in 2% OsO_4, dehydrated in ethanol and incubated for 1 hour at room temperature in transitional solution of propylene oxide with two changes. The PMs were then infiltrated and embedded in Spurr's resin. The resin blocks were trimmed and thin sectioned of 50-60 nm were cut. The sections were mounted onto copper grids, post-stained with uranyl acetate and lead citrate, and examined using JOEL-JEM-100CXII transmission electron microscope.

At least three individual larvae per treatment were used to prepare specimens for each of the three microscopic studies. Samples were thoroughly examined and micrographs were taken to document the morphological study.

RESULTS

Light microscopy studies showed intact midgut region for larvae feeding on normal artificial diet. The PM was also intact and appeared to be tightly appressed to food bolus (Figure 5.1a). When midgut regions of larvae feeding on diet containing 60 or 600 ppb Mir1-CP was examined, mechanical damage in the PM and dissociation of its layers was evident (Figure 5.1b, c). The food bolus in these specimens did not appear to be completely filled. Larvae fed on diet treated with 6000 ppb of Mir1-CP, had no PM barrier and showed highly disintegrated midgut epithelial regions (Figure 5.1d).

Scanning electron microscopic studies on midgut specimens of larvae fed on 60 ppb Mir1-CP revealed no major differences in PM structure at lower magnifications

(500-1000X) other than cracks and tears (Figure 5.2b, c). Careful examination at higher magnifications (3000-5000X) showed damaged PM having net-like appearance in the endoperitrophic layer that was in direct contact with the food bolus (Figure 5.2d, e). At Mir1-CP dosage of 600 ppb, the PM lost its integrity and enclosed the micro-fibrilar layer of the gut as a feathery sheath (Figure 5.3b, c). Complete loss of PM barrier and severe damage to epithelial layer, which are in closer contact with the food bolus, were observed in larvae fed on diet containing 6000 ppb Mir1-CP (Figure 5.3d, e).

Next, PMs dissected from larvae fed on control and Mir1-CP treated diet were examined using transmission electron microscopy. In this study, PMs derived from larvae feeding on artificial diets containing Mir1-CP dosages of 60 ppb showed significant degree of dissociation of its layers (Figure 5.4b). As predicted, more complete dissociation of PM layers occurred at higher Mir1-CP dosages (Figure 5.4c, d).

DISCUSSION

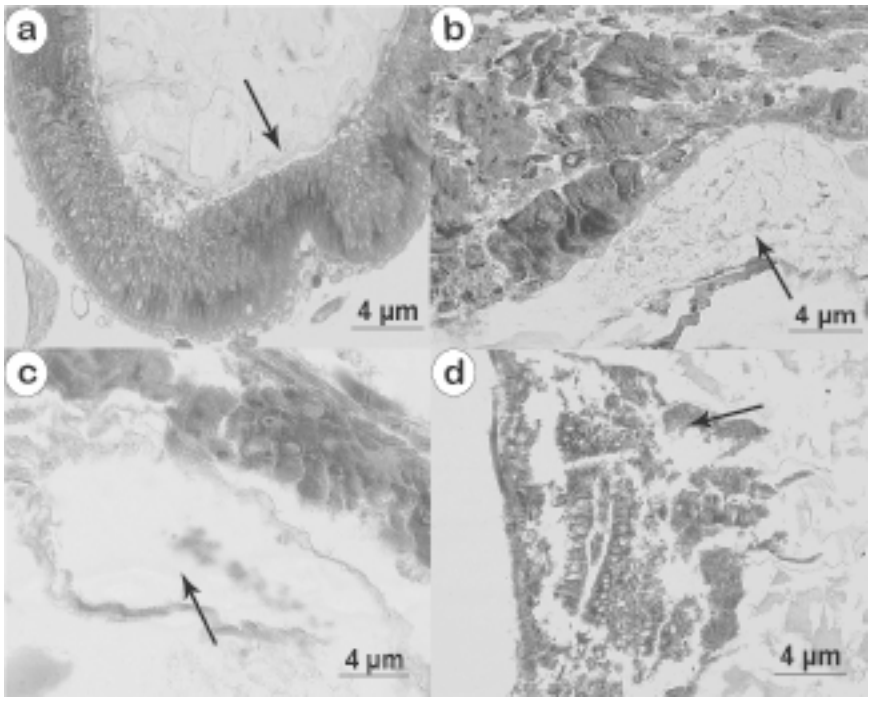
In previous work (Pechan et al., 2002), SEM was used to show that the PMs of fall armyworm larvae were severely damaged when they fed on resistant maize plants or Black Mexican Sweetcorn (BMS) callus over-expressing Mir1-CP. In both cases, damage was observed on the endoperitrophic layer, which was in direct contact with the food bolus. This current study extended those by examining the direct effect of purified recombinant Mir1-CP on the fall armyworm PM, hence there was no interference from the substances that might be present in the maize leaves or BSM callus. Three different microscopic techniques were used that provided qualitative observations on how Mir1-CP affects PM morphology. We treated larval diet medium with Mir1-CP in the range of

60-6000 ppb, based on dose response bioassay studies, which showed 600 ppb to be effective in reducing fall armyworm larval growth (Luthe and Mohan, unpublished data)

The light microscopic study showed disintegration, degradation or absence of the PM barrier in midgut specimens that were obtained from larvae feeding on different concentrations of Mir1-CP in their diet. But better understanding of level of morphological damage caused by Mir1-CP became more evident when similar specimens were analyzed using scanning and transmission electron microscopy. SEM studies showing damaged PM having net-like appearance from specimens derived from larvae fed on diet containing 600 ppb of Mir1-CP, indicating disruption of chitin network in the PM. Harper et al. (1998) have shown that disruption of chitin network would increase both PM permeability and insect mortality. The Mir1-CP might have influenced these effects by binding to chitin or to either glycoconjugates present on midgut epithelia, or glycosylated digestive enzymes. Although Mir1-CP has an acidic pH optimum (Jiang et al., 1995; Luthe and Bassford, unpublished data) it has shown severe damaging effect in alkaline environment of the larval gut in specimens derived from larvae feeding on higher Mir1-CP treated diets. This suggests that the digestive enzymes that are released from the anterior midgut cells by microapocrine process (Bolognesi et al., 2001) may have created microenvironment with a favorable pH condition for keeping Mir1-CP active. TEM studies at all tested Mir1-CP treatment conditions shows disintegration of the different PM layers, which could have occurred due to proteolytic degradation of structural proteins (Luthe and Ozkan, unpublished data). One of the major structural proteins in the PM, the Insect Intestinal Mucin (IIM) (Bolognesi et al., 2001; Wang and

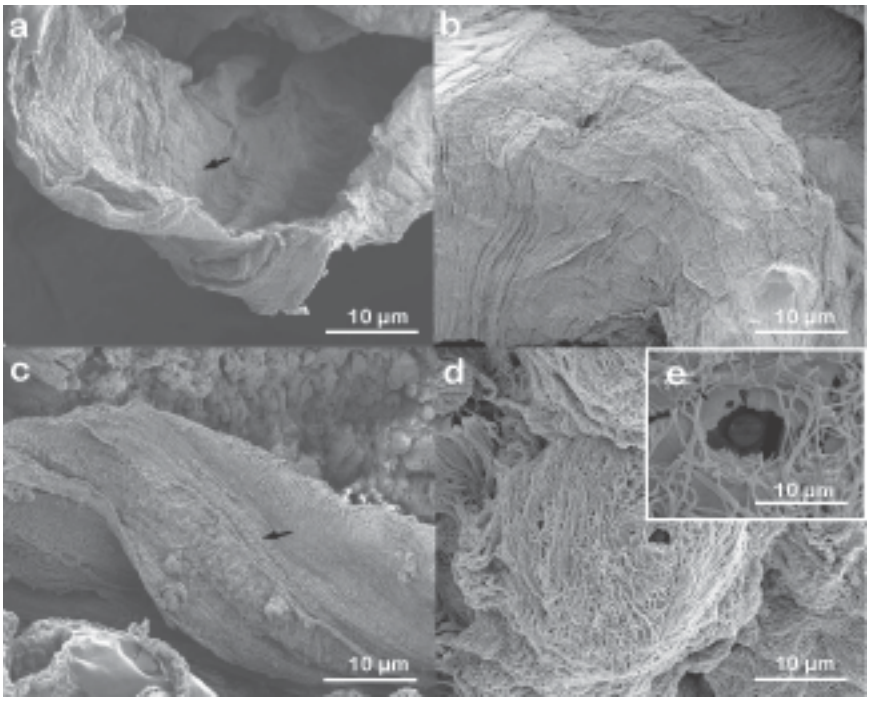
Granados, 2001) was degraded by the recombinant Mir1-CP (Luthe and Ozkan, unpublished data). Degradation of such structural proteins could also increase the PM permeability level and also increases susceptibility of larvae to baculoviral infections (Peng et al., 1999).

These results suggests that by damaging PM, which is the insect's first line of defense, Mir1-CP impairs the normal highly organized digestive system when used at concentrations of 600 ppb or higher. The unusual host-plant resistance mechanism of the 33-KDa Mir1-CP may have applications in agricultural biotechnology.



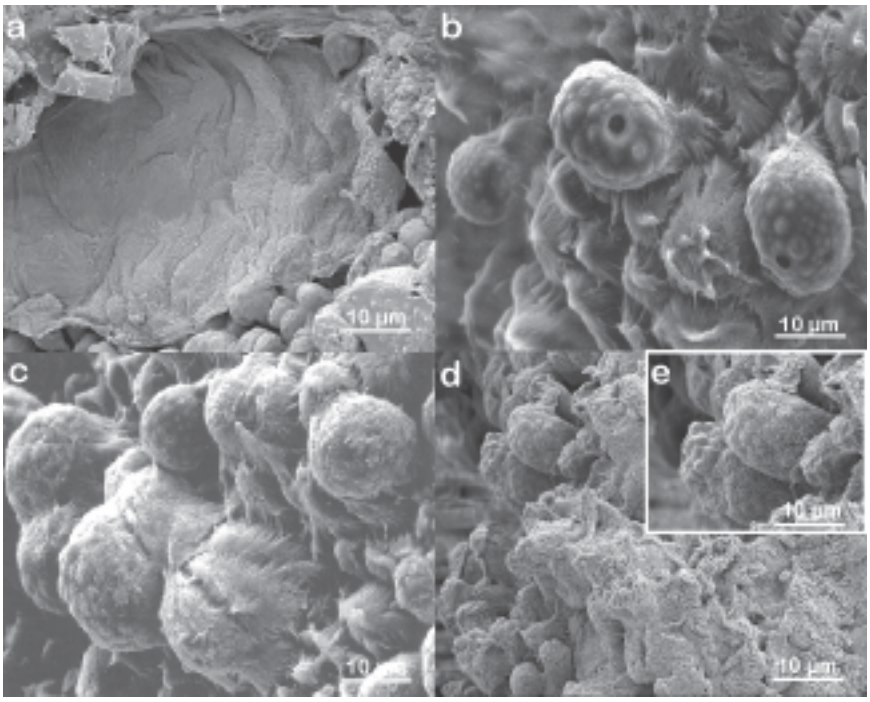
Note: Micrographs show midgut section from the control (a), or larvae fed 60 (b), 600 (c) or 6000(d) ppm recombinant Mir1-CP. PM shearing and dissociation was evident when larvae fed on diet containing 60 and 600 ppb Mir1-CP (a, b). Disintegration of midgut epithelial layers was evident when they fed on 6000 ppb (d). Results were based on three independent trials. Magnification was at 10 X for control specimens and 20X magnification for midgut specimens of larvae fed on Mir1-CP containing diets.

Figure 5.1 Light microscopic study of fourth instar FAW midgut cross-sections.



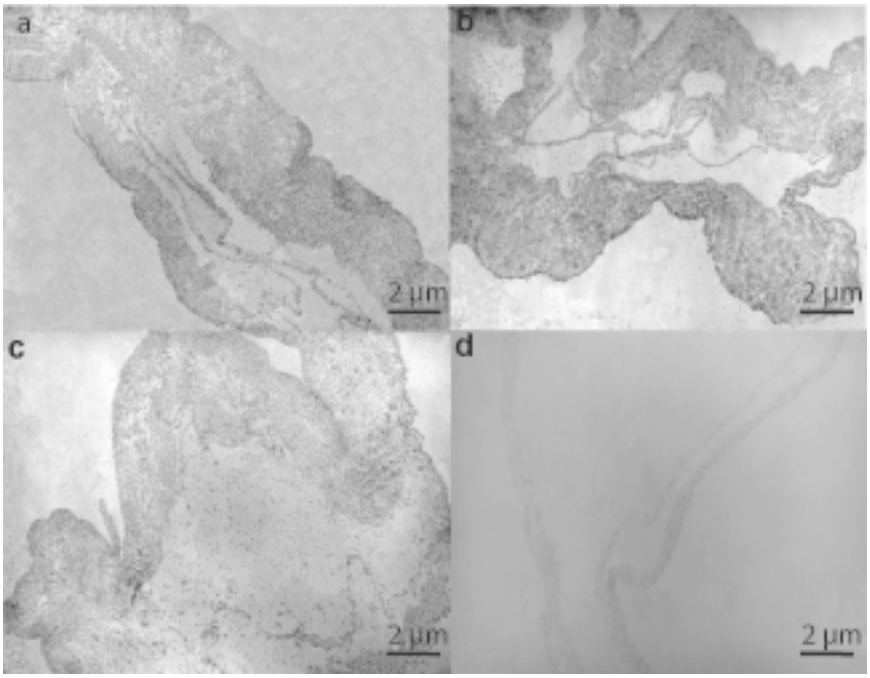
Note: SEM show the control (a) or PMs from larvae fed 60 ppb Mir1-CP (b and c) at 1000X magnification, while (d) and (e) show PM damage at 3000 and 5000X magnification, respectively. Results were based on three independent trials.

Figure 5.2 Scanning electron microscopy of 60 ppb Mir1-CP effect on FAW PM.



Note: Micrographs are the control (a), larvae feed 600 ppb (b and c) or 6000 ppb (d and e) of Mir1-CP at 1000X magnification. The PM of larvae fed 6000 ppb are also shown at 3000X magnification (e). Results were based on three independent trials.

Figure 5.3 Scanning electron microscopy FAW PM from larvae that fed on diet containing 600 and 6000 ppb Mir1-CP.



Note: Micrographs show the control (a) and the dissociation of PM layers from larvae fed 60, 600 and 6000 ppb Mir1-CP (b, c and d, respectively). Micrographs were documented at 5000X magnification. Results were based on two independent trials.

Figure 5.4. Transmission electron microscopy study of Mir1-CP effect on FAW PMs.

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CHAPTER VI

SUMMARY

Fall armyworm larvae (FAW), which are serious pests in the southern United States, show retarded growth when they feed on insect-resistant maize inbreds Mp704 and Mp708 (Williams and Davis, 1982; Williams et al., 1990). These maize genotypes are not only resistant to FAW, but to a number of other lepidopteran pest (Davis et al., 1988, 1999). In these genotypes, a unique, extracellular, 33-KDa cysteine protease (Mir1-CP) rapidly accumulates in the whorl in response to insect feeding. Initial morphological studies on larvae feeding on resistant maize plants transgenically over-expressing Mir1-CP showed severe damage in insect's first line of defense, the peritrophic matrix (PM). To separate the effects of the plant tissue containing Mir1-CP from those of the purified enzyme, our goal was to test the effects of the purified Mir1-CP on various lepidopteran pests, including the FAW. A modified baculovirus system was used to express the recombinant form of the plant-derived cysteine protease in fall armyworm hemolymph. Characterization of the HPLC purified recombinant Mir1-CP indicated that it had the same properties as the plant-derived enzyme.

To directly determine the effects of Mir1-CP on the PM *in vitro*, dissected PMs from fall armyworm larvae were treated with purified recombinant Mir1-CP and the movement of Blue Dextran 2000 across the PM was measured. Mir1-CP completely

permeabilized the PM and the time required to reach full permeability was inversely proportional to the concentration of Mir1-CP (Mohan et al., 2006). The lowest concentration of Mir1-CP affecting PM permeability under *in vitro* conditions was 12 ng/ml, which is likely to be a physiologically relevant concentration in the maize whorl. Inclusion of E64, a specific cysteine protease inhibitor prevented the damage, indicating that proteolytic activity is required to permeabilize the PM. The luminal side of the PM was more vulnerable to Mir1-CP attack than epithelial side. Although Mir1-CP has acidic pH optima, it permeabilized the PM more actively at alkaline pH values than equivalent concentrations of other cysteine proteases like papain and ficin. The effect of Mir1-CP on PMs from different insect species showed greatest effect on noctuid larval PMs. These results demonstrated that the insect-inducible Mir1-CP directly damages the PM *in vitro* and is critical to insect defense in maize.

To understand the importance of having an active site cysteine and unique 25 amino acid-tail in Mir1-CP for achieving complete PM permeability, we expressed and two previously constructed mutant forms of the cysteine protease namely, *mut1,* in which alanine replaced the cysteine at the active site, and *mut2*, in which the translation of the last 25 amino acids was prevented by replacing the codon for Asp^{232} with a stop codon. The absence of any detectable activity for Mut1 and only low activity for Mut2 indicated that complete processing did not occur. PMs incubated with unprocessed Mut1 showed no increase in permeability, but unprocessed Mut2 was able to partially permeabilize the PM. By processing both Mut1 and Mut2 to its mature form with minute quantities of Mir1-CP, there was an increase in permeability, but the level of permeabilization was

only one-half of the Mir1-CP level. Processed Mut1 and Mut2 also showed no pronounced PM permeability effect when the epithelial side was exposed to these enzymes or at pH values greater than 9 and temperatures higher than 35˚C. This indicated that processing is essential for maximal Mir1-CP activity and that both cysteine at the active site and terminal 25 amino acids are required for complete permeabilizaton.

We also conducted dose response bioassay studies to determine the minimal effective dosage of purified recombinant Mir1-CP for reducing lepidopteran larval growth and also to determine if it could enhance the effect of Bt-CryIIA *in vitro*. The relative growth rates (RGR) of fall armyworm decreased at concentrations greater than 600 ppb Mir1-CP or 0.75 ppm of Bt-CryIIA. Southwestern corn borer (SWCB), tobacco budworm (TBW) and corn earworm (CEW) required higher Mir1-CP and Bt-CryIIA concentrations to reach similar larval growth reduction. However, a single sub-lethal Mir1-CP concentration of 60 ppb was effective in synergizing Bt-CryIIA concentrations of 0.4 ppm for FAW, 0.5 ppm for either CEW or TBW and 1 ppm for SWCB. Statistical analysis showed significantly greater decrease in all four larval mean RGR values and at least 12-fold higher mortality percentage at combined Bt-CryIIA and Mir1-CP dosage than at their individual treatments. The synergistic study suggests that stacking Mir1-CP and Bt-CryIIA genes in transgenic plants could broaden the normal range of both Mir1-CP and Bt-toxins, and ultimately result in more sustainable control of lepidopteran pests.

To understand how the recombinant cysteine protease affects the larval PM and gut regions at various concentrations, we conducted morphological study using three different microscopic techniques. Light microscopy studies showed morphological PM

damage for larvae fed with 600 ppb Mir1-CP or higher. Damaged fall armyworm larval PM and disintegration of its layers under similar Mir1-CP treatment conditions was observed in both scanning and transmission electron microscopy studies.

These results suggest that by damaging PM, which is insects first line of defense, Mir1-CP could probably impair the normal highly organized digestive system when used at relatively physiological concentrations of 600 ppb or higher. The unusual host-plant resistance mechanism of Mir1-CP may have applications in agricultural biotechnology for sustaining lepidopteran pest populations.

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