INHIBITION OF *HELICOVERPA ARMIGERA* GUT ZYMOGEN ACTIVATION BY PLANT PROTEASE INHIBITORS

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INHIBITION OF *HELICOVERPA ARMIGERA* GUT ZYMOGEN ACTIVATION BY PLANT PROTEASE INHIBITORS

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BY

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MY BELOVED PARENTS

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BAMU-ICRISAT COLLABORATIVE RESEARCH EFFORT

RESEARCH WORK EMBODIED IN THIS THESIS WAS CARRIED OUT AT

Dr. Babasaheb Ambedkar Marathwada University

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CERTIFICATE

This is to certify that Shri. **VINOD DADARAO PARDE** has completed his research work entitled, "**Inhibition of *Helicoverpa armigera* gut zymogen activation by plant protease inhibitors**", under my guidance. It is further certified that the thesis submitted by him, was his original plan of work and carried out under my guidance.

Date:

Prof. M. S. Kachole
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CERTIFICATE

This is to certify that the work in this thesis entitled, "**Inhibition of *Helicoverpa armigera* gut zymogen activation by plant protease inhibitors**", submitted by **Mr. Vinod Dadarao Parde**, was carried out by the candidate under my supervision. No part of the thesis has been submitted by the student for any other degree or diploma. The author of the thesis has duly acknowledged all assistance and help received during the course of the investigation.

Date:

Dr. H. C. Sharma
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DECLARATION

I hereby declare that the thesis entitled, "**Inhibition of *Helicoverpa armigera* gut zymogen activation by plant protease inhibitors**", submitted for Ph. D. degree to the **Dr. B. A. M. University** has not been submitted by me to any other University for a degree or diploma. The material obtained from other sources has been duly acknowledged in this thesis. This work was carried out at the BAMU and ICRISAT, India.

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ABSTRACT

Protease inhibitors (PIs) play an in important role in host plant resistance to insect pests. Therefore, the present studies were carried out on host and non-host plant protease inhibitor for inhibition of zymogen activation, and their biological activity against the pod borer, *Helicoverpa armigera* (Hübner), the most important pest of agriculture and horticulture crops world wide.

The present studies indicated that *H. armigera* gut proteinases were synthesized in inactive isoforms, and get activated by trypsin-like proteinases. Six pro-proteinase bands were detected, and partially purified and characterized using substrate assays. Gelatin coating present on the X-ray film was used as a substrate to detect electrophoretically separated pro- and proteinases of *H. armigera* gut extract on native PAGE, SDS-PAGE, and two-dimensional gels. *In gel* activation method involved electrophoresis, followed by washing the gel with non-ionic detergent in case of SDS-PAGE, equilibration of the gel in trypsin (0.0001%) buffer, overlaying the gel on X-ray film, followed by washing the film with hot water to remove hydrolyzed gelatin, revealing activated pro-proteinase bands.

Helicoverpa armigera gut proteinases showed activity over a broad range of pH, maximum activity was observed between the pH 9 to 11, while pro-proteinase(s) showed

activity maxima at pH 8 and 10. Gut proteinases showed optimal activity upto 60°C, and the pro-proteinase at 40°C. Although gut pro-proteinase activation was affected by Ca^{2+} ions, the activity of its active isoforms was not affected.

The *H. armigera* gut pro-proteinase isoforms activation was observed in the fraction eluted on benzamidine sepharose 4B and on size exclusion column, and activation measured by substrate assays. Purification and substrate assay studies revealed that, 23,000 – 70,000 Da polypeptides were the likely trypsin/chymotrypsin-like pro-proteinases. Larvae of *H. armigera* fed on a cocktail of synthetic inhibitors (antipain, aprotinin, leupeptin, and pefabloc) showed maximum activation of pro-proteinases as compared to the larvae fed on individual inhibitors. The studies clearly indicated that the serine proteinase inhibitors were potent inhibitors of HaGPs and HaGPPs *in vitro* as well as *in vivo*. Activation of HaGPPs in different stadia of *H. armigera* increased with larval age, and maximum activity was observed in fifth-instars. The fecal matter of insects feeding on synthetic inhibitors exhibited profound pro-proteinase activity both with the substrate as well as *in gel* assay methods.

Seeds of 108 plant species were used for initial screening, of which 31 showed high inhibitory activity, while 18 and 22 samples showed moderate and low activity against *H. armigera* gut proteinases, respectively. All the non-host plants exhibited trypsin inhibitory activity, but most of them did not show chymotrypsin and HaGP inhibitory activity. *In vitro* activation of HaGPP in larvae fed on non-host plant PIs showed significant activation in solution as well as *in gel* assay method. The larvae fed on diet impregnated with *Datura alba ness* and *Psophocarpus tetragonolobus* PIs showed high activation of pro-proteinases as compared to the others. The present studies revealed that non-host plant PIs were good candidates as inhibitors of HaGPs as well as HaGPPs. Activation of pro-proteinases increased over instars, and maximum activation was observed in the fifth-instars. The larvae fed on non-host plant PIs exhibited activation of pro-proteinase when treated with partially purified HaGP. Out of 10 proteinases, HaGP 5 and 9 showed activation of pro-proteinases. HaGP 5 activated more HaGPPs than 9. The fecal matter of the larvae fed on non-host plant PIs showed high pro-proteinase activation, of which, *D. alba ness* showed highest pro-proteinase activation, followed by *C. annum*, and *P. tetragonolobus*.

The present studies demonstrated that non-host plant PIs were effective in inhibiting the isoproteinases and larval growth of *H. armigera*. Larval growth and development were reduced when fed on non-host plant PIs in artificial diet. Among the non-host plant PIs, *Datura alba ness* resulted in highest stunted growth. Non-host plant PIs have the potential to inhibit the growth of *H. armigera* larvae, which provides the basis for deploying these PI is in insect-resistant transgenic plants. Further research should focus on developing transgenic plants that express higher level of potent PIs to achieve protection against *H. armigera*.

CHAPTER 1

GENERAL INTRODUCTION

Helicoverpa armigera (Lepidoptera: Noctuidae) is major pest food, oilseed, fodder, and horticultural crops in Asia, Africa, Australia and the Mediterranean Europe. It causes an estimated loss of over US $2 billion annually in the semi-arid tropics, despite US $500 million worth of pesticides applied for controlling this pest (Sharma, 2001). The high pest status of *H. armigera* is due to its potential for polyphaghy, meaning that it is capable of feeding on a diverse array of plant species. It feeds on 181 plant species including chickpea (*Cicer arietinum*), pigeonpea (*Cajanus cajan*), tomato (*Lycopersicon esculentum*), okra (*Abelmoschus esculentus*), cotton (*Gossypium spp*.). It is also an important pest of other crops such as sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), maize (*Zea mays*), tobacco (*Nicotiana tabacum*), and groundnut (*Arachis hypogea*) (Manjunath et al., 1989).

Chemical control of *H. armigera* insects is often not effective, as it has developed high levels of resistance to oraganochlorides, organophosphates, and pyrethroids (Heckel et al., 1997). Natural defense of plants against insects, mediated by protease inhibitors can be used as one of the compound for controlling the pest (Ryan, 1990). Protease inhibitors (PIs) comprise one of the most abundant classes of proteins in plants. Most storage organs such as seeds and tubers contain 1 to 10% of their total proteins as PIs, which inhibit different types of enzymes. The function of the inhibitors is to control proteolysis within cells, organelles, or fluids while limited proteolysis is important for the biochemical of physiological process.

Plants synthesize various proteinaceous and non-proteinaceous compounds against insect attack, amongst these, proteinase inhibitors (PIs) are the most-studied class of plant-defense proteins. Protease inhibitors reduce the digestive capability of insects by inhibiting proteinases of the midgut, thereby arresting their growth and development (Broadway and Duffey, 1986a, b; Delano et al., 2008), and have been deployed in plant defense improvement against insects through transgenic technology (Jouanin et al., 1998; Lawrence and Koundal, 2002). Analysis of digestive proteinases of *H. armigera* has revealed the presence of serine proteinases, predominantly trypsin- and chymotrypsin-like enzymes (Johnston et al., 1991; Xu and Qin, 1994; Bown et al., 1997; 1998). In polyphagous insects such as *H. armigera*, diverse specificities and intricate changes in the expression of proteinases are responsible for the inactivation of host plant and newly

exposed PIs (Bown et al., 1997; Jongsma et al., 1995; Broadway, 1996; Llewellyn et al., 1997; Gruden et al., 2004; Koiwa et al., 1997). Most of PIs are small proteins that have been found in all plant species investigated thus far, and occur in both reproductive and vegetative tissues. In herbivorous insects, they act by inhibiting protein digestive enzymes in the guts of insect larvae or adults, resulting in amino acid deficiencies that lead to serious developmental delay, mortality, or reduced fecundity. Hence, the identification of PIs having specificities towards different insect gut proteinases with high binding efficiency is necessary for effective inhibition of midgut proteinases of *H. armigera*. Such PI(s) may have direct relevance and application in the development of transgenic plants with resistance to this insect.

In legumes, PIs accumulate in large amounts during seed maturation, and play an important role both in the deposition of storage protein and in plant defense (Koiwa et al., 1997). Protease inhibitors are induced under various stress-prone conditions such as insect chewing, mechanical wounding, pathogen attack, drought and UV exposure (Schaller and Ryan, 1995; Conconi et al., 1996).

Trypsin/chymotrypsin-like proteinases from the midgut of *H. armigera* have been purified and characterized by Johnston et al. (1991). Screening of the cDNA library prepared from the midguts of *H. armigera* reared on a high-protein and inhibitor free diet has revealed 18 genes encoding trypsin-like proteinases, 14 genes of chymotrypsin-like proteinases, and two genes of elastase-like proteinases (Gatehouse et al., 1997). Within the chymotrypsin family, there are several distinct subfamilies (Bown et al., 1997; Mazumdar-Leighton et al., 2000). Two cDNAs coding for elastase like proteinases from the midgut of *H. armigera* exhibit 44% and 46% identity with *Manduca sexta* elastase and chymotrypsin sequences, respectively (Gatehouse et al., 1997). Such a wide array of proteolytic enzymes is a distinct advantage to polyphagous insects such as *H. armigera* (Bown et al., 1997). It has also been demonstrated that *H. armigera* adjusts to new dietary PIs by producing a novel, inhibitor resistant suite of midgut serine proteinases (Wu et al., 1997; Bown et al., 1997). Wu et al. (1997) reported the induction of elastase-like proteinases activity in the midgut of *H. armigera* upon feeding the larvae on transgenic tobacco plants expressing giant taro PI.

Currently, the main emphasis of plant-PI studies is on identifying potential inhibitors of digestive proteinases of the target insects, and on understanding the dynamic nature of insect midgut proteinases at the molecular level (Bown et al., 1997; Lopes et al., 2004). Lepidopteron insects have serine proteinases as a major component of their digestive complement, and among them, trypsin-and/or chymotrypsin-like are the most commonly found proteinases (Purcell et al., 1992; Srinivasan et al., 2006). *Helicoverpa armigera* larvae have an alkaline gut, which can produce at least ten major, and several minor serine proteinases that are able to overcome the native PIs of its host plants (Johnston et al., 1991; Bown et al., 1997, Bown et al., 1998; Giri and Kachole, 1998). These enzymes play an important role in protein digestion by releasing amino acids from the peptides produced by endopeptidases, thus, completing the digestion process. The plants' natural defense mechanism against insect pest is to block digestive proteinases by proteinase inhibitors (PIs), thus starving them for amino acids and energy. The PIs enter in the insect digestive tract along with the food and block the protein digestion, and hence, starving the insect for amino acids and energy, resulting in retardation of growth and development (Ryan, 1990). Insects on the other hand exhibit mechanisms to produce inhibitor-insensitive or inhibitor-degrading proteinases in the midgut to overcome the effect of PIs (Jongsma et al., 1995; Michaud, 1997; De Leo et al., 1998; Volpicella et al., 2003). Insects capable of adapting to such plant defenses have a chance to survive and to emerge as potential pests of agricultural crops.

A useful strategy for enhancing plant defense systems in future is to identify PIs with high activity against the particular target insect (Koiwa et al., 1998). This will entail examination of plant species beyond the host group (Jongsma et al., 1996). An example of an insect that can adapt to a range of plant PIs is *H. armigera*, a polyphagous pest of many important crops, which often leads to heavy losses in the field (Manjunath et al., 1989).

While insect-resistant transgenic plant cultivars currently available commercially employ only *Bt* toxin genes, the development of transgenic plants expressing protease inhibitors (PIs) has emerged as an additional strategy for pest control (Hilder et al., 1987; Burgess et al., 1997). PIs act by binding with insect digestive proteases resulting in disruption of protein digestion, which leads to reduced growth and mortality. Although

the exact mode of action of PIs is complex, and not yet fully understood, it is fundamentally different from of the *Bt* toxins operate. PI-transgenic plants may therefore be a useful adjunct to the use of *Bt* as a biopesticide. Combinations of *Bt*- and PI- plants could be used to manage resistance development, either as seed mixes in adjacent plots, or through 'pyramiding', whereby the two genes are engineered into a single plant (McGauhey and Whalon, 1992; May, 1993; Christeller et al., 2004).

The studies of the protein digestion in the insect gut have concentrated mainly on the initial phases of protein and peptide degradation through endopeptidase (trypsin and chymotrypsin-like proteases) activities (Terra and Ferreira, 1994). However, the products of such endopeptidase cleavages are large peptides, which must be further degraded to be taken up by the insect gut cells (Billingley, 1990).

A large number of enzymes and physiologically active proteins are synthesized as inactive precursors that are subsequently converted to the active form by the selective cleavage (limited proteolysis) of peptide bonds. The ultimate agency of activating enzymatic function is limited proteolysis, either in single step activation or in a consecutive series (cascade). The specificity of each activation reaction is determined by the complementarities of the zymogen substrate and the active site of the attacking protease (Neurath and Walsh, 1976). A novel pro-carboxypeptidase (PCPAHa) from *H. armigera*, the first enzyme of this class from a lepidopteran insect, has been characterized by expressing its encoding cDNA in insect cells (Bown et al., 1998). The pre-proproteases sequence contains 426 amino acid residues and exhibits sequences homology with the metallo-carboxypeptidases from mammalian species, and with carboxypeptidases from other invertebrates. Removal of a predicted signal peptide and of the activation peptide results in a product signal peptide, and of the activation peptide results in a product of approximately 35.5 kDa, which is comparable in size to the pancreatic mammalian carboxypeptidases (Vendrell et al., 2000). PCPAHa belongs to the A form of carboxypeptidases and preferentially cleaves aliphatic and aromatic residues. This carboxypeptidase is active over a broad pH range (7.5 – 10), displaying maximal activity at pH 8.0, but trypsin/chymotrypsin shows optimum at 10.0 (Johnston et al., 1991). This is in agreement with the report of a maximal peptidase activity in the alkaline region determined in other phytophagous lepidopteran species (Lenz et. al., 1991; Ortego

et al., 1996; Ferreira et al., 1994). A crystal structure from the novel pro-carboxypeptidase (PCPAHa) has been observed in the gut extracts from *H. armigera* larvae (Estebanez et al., 2001). The metalloprotease is synthesized as a zymogen of 46.6 kDa, and upon *in vitro* activation, yields a pro-segment of 91 residues and an active carboxypeptidase moiety of 318 residues.

In present work, we focused on the purification and partial characterization of the zymogen(s) of *H. armigera* gut lumen protease(s), as well as preliminary studies on its activation *in vitro*. In many respects, the trypsinogen-trypsin/chymotrypsingoen-chymotrypsin isoforms of insect system resemble the trypsinogen-trypsin system of mammals. However, trypsins belong to endopeptidases, and are the most important proteases in insects because of their key role in food digestion and zymogen(s) activation.

The major objectives of the present studies are:

1. Identification and characterization of proteinase zymogen(s) present in *H. armigera* gut lumen.

2. Isolation and characterization of *H. armigera* gut proteinase(s) involved in zymogen(s) activation.

3. Preliminary screening of plant protease inhibitor isoforms of *H. armigera* gut proteinase(s).

4. Interactions of plant protease inhibitors with *H. armigera* gut zymogen proteinase(s): Identification of potent PIs from various plants for inhibition of zymogen activation.

5. Validation of *in vivo* inhibition of zymogen(s) activation by selected plant PIs.

CHAPTER 2

REVIEW OF LITERATURE

Plant Protease Inhibitors against Insect Digestive Enzymes

2.1 Introduction

During the last three decades, much effort has been put into the study of insect digestive enzymes, mainly of α-amylases and serine proteinases. These enzymes have amino acid sequences similar to mammalian enzymes, although both insect amylases and serine proteinases differ from mammalian enzymes in substrate specificity and behavior in the presence of protein inhibitors. These findings argue in favor of the necessity of studying properties of specific insect digestive enzymes to study their mechanisms, specificities, and behavior in the presence of inhibitors. However, information on enzymes of phylogenetically distant organisms should be used with caution when trying to infer properties of insect enzymes.

Digestive enzymes in insects occur in midgut luminal contents or might be restricted to midgut cells. In cells, they may be associated with the glycocalyx or bound to microvillar membranes. The compartmentalization of digestive enzymes and the occurrence of midgut fluid fluxes define the overall patterns of digestion, which are closely related to the phylogenetic position of the insects. These patterns provide a reasonable basis for evolutionary considerations and provide support for evolution of theory basic digestive processes of insects.

The discovery in plants of protein inhibitors affecting insect digestive enzymes called attention to the possibility of using these enzymes as targets in the development of new insect control techniques (Ryan, 1990). Proteinase inhibitors (PIs) are ubiquitous small proteins that are quite common in nature. They are natural, defense-related proteins often present in seeds and induced in certain plant tissues by herbivory or wounding (Koiwa et al., 1997; Browse and Howe, 2008). PIs are present in multiple forms in numerous tissues of animals and plants as well as in microorganisms. In plants they can be counted among the defensive mechanisms displayed against phytophagous insects and microorganisms. The defensive capacities of plant PIs rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for growth and development (De Leo et al., 2002). Protein and peptide inhibitors of various exogenous (from invertebrates, viruses, fungi, and mammals) and endogenous proteinases are widespread in seeds. Proteinase inhibitor II (PIN2), is a serine proteinase inhibitor with trypsin and chymotrypsin inhibitory

activities (Bryant et al., 1976; Lawrence and Koundal, 2002), and occurs in many solanaceaous plants, including tomato (Gustafson and Ryan, 1976), potato (Bryant et al., 1976), and tobacco (Pearce et al., 1993; Luo et al., 2009). A successful approach was to produce transgenic plants that express, trypsin inhibitors to provide resistance to insects (Hilder et al., 1987). This approach will benefit from knowledge gained on the properties (which differ among insect groups) and midgut distribution (which determine if target enzymes are or not accessible to inhibitors) of insect digestive enzymes.

2.2 Proteolytic enzymes

Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins. Proteases are degradative enzymes, which catalyze the total hydrolysis of proteins. Proteolytic enzymes, also called proteases, are the enzymes that catalyze the hydrolytic cleavage of specific peptide bonds in their target proteins. These enzymes are widely distributed in nearly all plants, animals, and microorganisms (Joanitti et al., 2006; Neurath, 1989; Valueva and Mosolov, 2004; Christeller, 2005; Mosolov and Valueva, 2005; Lawrence and Koundal, 2002; Ryan, 1990). Being essentially indispensable to the maintenance and survival of their host organism, proteases play key roles in many biological processes. The proteolytic events catalyzed by these enzymes serve as mediators of signal initiation, transmission and termination in many of the cellular events such as inflammation, apoptosis, blood clotting and hormone, processing (Ivanov et al., 2006).

Proteolytic enzymes are intricately involved in many aspects of plant physiology and development, and their action can be divided into two different categories: limited proteolysis and unlimited proteolysis. In limited proteolysis, a protease cleaves only one or a limited number of peptide bonds of a target protein leading to the activation or maturation of the formerly inactive protein, e.g., conversion of prohormones to hormones. In another example, trypsin can be considered as a prototype of the class of enzymes synthesized as inactive precursors. Synthesized as trypsinogen, it requires proteolytic processing to be activated. Once activated, trypsin acts specifically only on peptide bonds whose carboxyl functions are contributed by lysine or arginine residues.

Proteases are responsible for the post-translational modification of proteins by limited proteolysis at highly specific sites. Limited proteolysis results in maturation of enzymes, is necessary for protein, assembly and subcellular targeting, and controls the activity of enzymes, regulatory proteins and peptides. In unlimited proteolysis, proteins are degraded into their amino acid constituents. The proteins to be degraded are usually first conjugated to multiple molecules of the polypeptide ubiquitin. The processing of inactive precursors and the secretory mechanisms of trypsin in insects seem to include aspects that are not found in other animals. Studies of the unique processes may provide the basis for a detailed understanding of the physiology of digestion in insects.

2.3 Occurrence of proteases

Since proteases are physiologically necessary for living organisms, they are ubiquitous, and found in a wide diversity of sources such as plants, animals, and microorganisms (Kenny, 1999). Based on an analysis of complete sequences of several genomes, it is estimated that about 2% of all gene products are proteases (Barrett et al., 1998). Proteases play crucial roles in the physiology and pathology of living organisms by controlling the synthesis, turnover, and function of proteins (Turk, 1999).

2.3.1 Plant proteases

Proteolysis is essential for many aspects of plant physiology and development. It is responsible for removing abnormal/misfolded proteins, supplying amino acids needed to make new proteins, assisting the maturation of zymogens and peptide hormones by limited cleavage, controlling metabolism, and for programmed cell death of specific cells in plant organs. Production of proteases from plants is a time-consuming process. Papain, bromelain, keratinases, and ficin represent some of the well-known proteases of plant origin.

2.3.2 Animal proteases

The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennins (Boyer, 1971; Hoffman, 1974). These are prepared in pure form in

bulk quantities. However, their production depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies.

2.3.2.1 Trypsin

Trypsin (Mr 23,300) is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. It is a serine protease and hydrolyzes peptide bonds in which the carboxyl groups are contributed by the lysine and arginine residues. Based on the ability of protease inhibitors to inhibit the enzyme from the insect gut, this enzyme has received attention as a target for biocontrol of insect pests. The enzyme is specifically inhibited by *N*-α- tosyl lysine chloromethyl ketone that acts on histidine (Shaw et al., 1965; Omondi, 2005). Through the use of ester or amide derivatives of arginine, such as *N*- α- tosyl arginine methyl ester (TAME) or *N*- α- Benzoyl DL-arginine ethyl ester (BAEE) and *N*- α- Benzoyl-DL-arginine 4-nitroanilide (BA*p*NA), digestive trypsin-like activity has been reported in most insect species examined (Applebaum, 1985). Most trypsin Mr values are in the range 20,000 to 35,000 and p*I* values are variable (most of them in the range 4 - 5). The pH optima is always alkaline (most between 8 and 10), irrespective of the pH prevailing in midguts from which the trypsins are isolated. Nevertheless, trypsins isolated from Lepidoptera have higher pH optima that correspond to the higher pH values found in their midguts.

Isolation of inactive precursors (zymogens) of insect digestive proteinases has largely been unsuccessful (Applebaum, 1985). Graf et al. (1986) suggested the occurrence of an inactive form of trypsin (trypsinogen) in midgut cells of *Aedes aegypti*, (Stegomyia) based on the finding of trypsin immunoreactivity in midgut cells and on their failure to assay trypsin activity in homogenates of washed midgut cells. Nevertheless, trypsin is also immunolocalized in the glycocalyx of *A. aegypti* midgut cells (Graf et al., 1986), the site at which trypsin must be active and from where it cannot be removed by washing (Santos et al., 1986). The failure to assay trypsin in midgut homogenates indicates a low sensitivity of their assay procedure rather than in favor of the existence of a trypsinogen. Barillas-Mury et al. (1991) sequenced, what seemed to be a precursor of midgut trypsin in *A. aegypti*. Its sequence is similar to that of most trypsins, although it showed significant differences from the vertebrate trypsin precursors

in the region of the activation peptide. Similar results were found with a putative trypsinogen from *Drosophila melanogaster* (Meigen) (Davis et al., 1985) and from *Simulium vittatum* (Zetterstedt) (Diptera: Simuliidae) (Ramos et al., 1993). These differences suggest that the processing of precursors of insect trypsins may be different from that of vertebrates. There is evidence in *Tineola bisselliella* (Hummel) (Ward, 1975) and *Bombyx mori* (Eguchi and Iwamoto, 1976; Eguchi et al., 1982) that soluble trypsin is derived from membrane-bound forms. *Erinnyis ello* (Santos and Terra, 1984; Santos et al., 1986) and in *Musca domestica* (Linnaeus) (Espinoza-Fuentes et al., 1987; Terra et al., 1988; Lemos and Terra, 1991a, b), trypsin is synthesized in midgut cells in an active form, but is associated with membranes of small vesicles. These vesicles then migrate to the cell apex and trypsin precursors are processed to a soluble form before being secreted. It seems that insects may control the activity of their digestive proteinases, in the absence of inactive forms, by binding the proteinases to membranes until they are released into the midgut lumen. Secretory granules isolated from the opaque zone cells from *Stomoxys calcitrans* (L.) (Diptera: Muscidae) adults contain a trypsin-like activity, which increases during incubation according to an apparent autocatalytic reaction (Moffatt and Lehane, 1990). The finding by the authors that activation occurs to a different extent, if opaque zone cells are homogenized in the presence or absence of detergent, suggests that trypsin processing in this insect is also different from that found in vertebrates.

2.3.2.2 Chymotrypsin

Chymotrypsin (Mr 23,800) is found in animal pancreatic extract. Pure chymotrypsin is an expensive enzyme and is used only for diagnostic and analytical applications. It is specific for the hydrolysis of peptide bonds in which the carboxyl groups are provided by one of the three aromatic amino acids, i.e., phenylalanine, tyrosine, or tryptophan. It is used extensively in the de-allergenizing of milk protein hydrolysates. It is stored in the pancreas in the form of a precursor, chymotrypsinogen, and is activated by trypsin in a multistep process.

Chymotrypsin is specifically inhibited by TPCK that acts on histidine. It is usually assayed with ester or amide derivatives of tyrosine such as BTEE or BT*p*NA. Although there are fewer reports on chymotrypsin-like than on trypsin-like enzymes

among insects, it seems that its distribution in insect taxa is similar to that of trypsin (Applebaum, 1985; Borovsky and Schlein, 1998). Most insect chymotrypsin Mr values are in the range 20,000 - 30,000 and pH optima in the range 8 - 10, irrespective of the pH prevailing in the midguts from which the chymotrypsins were isolated. Lepidoptera chymotrypsins, as observed for trypsins, display higher pH optima, paralleling the lepidoptera midgut luminal pH values. However, some properties of insect chymotrypsins contrast to those of vertebrate chymotrypsins, such as their instability at acid pH (Ward, 1975; Jany et al., 1978) and their strong inhibition by soybean trypsin inhibitor (SBTI) (Jany and Pfleiderer, 1974; Baker, 1981; Sakal et al., 1988).

2.3.2.3 Pepsin

The Pepsin (Mr 34,500) is an acidic protease found in the stomach of almost all the vertebrates. The active enzyme is released from its zymogen, i.e., pepsinogen, by autocatalysis in the presence of hydrochloric acid. Pepsin is an aspartyl protease and resembles human immunodeficiency virus type 1 (HIV-1) protease, responsible for the maturation of HIV-1. It exhibits optimal activity between pH 1 and 2, while the optimal pH of the stomach is 2 to 4. Pepsin is inactivated above pH 6.0. The enzyme catalyzes the hydrolysis of peptide bonds between two hydrophobic amino acids.

2.3.2.4 Rennin

Rennet is a pepsin-like protease (rennin, chymosin; EC 3.4.23.4) that is produced as an inactive precursor, prorennin, in the stomach of all nursing mammals. It is converted to active rennin (Mr 30, 700) by the action of pepsin or by its autocatalysis. It is used extensively in the dairy industry to produce stable curd with good flavor. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in k-casein to generate insoluble para-k-casein and C-terminal glycopeptide.

2.3.3 Microbial proteases

Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their amenability to genetic manipulation. Proteases from

microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for biotechnological applications.

2.4 Classification of insect digestive proteases

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (IUB, 1992). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Digestive enzymes are hydrolases. Enzymes responsible for the complete hydrolysis of proteins down to amino acids are the proteases. Proteases (peptide hydrolases, EC 3.4) are enzymes acting on peptide bonds and include the proteases (endopeptidases, EC 3.4.21-24) and the exopeptidases (EC 3.4.11-19) (Figure 2.1). Proteinases are divided into sub-classes on the basis of catalytic mechanism, as shown with specific reagents or effect of pH. Specificity is only used to identify individual enzymes within sub-classes. Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure (Barett, 1994). Proteases are grossly subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960).

2.4.1 Exopeptidases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively.

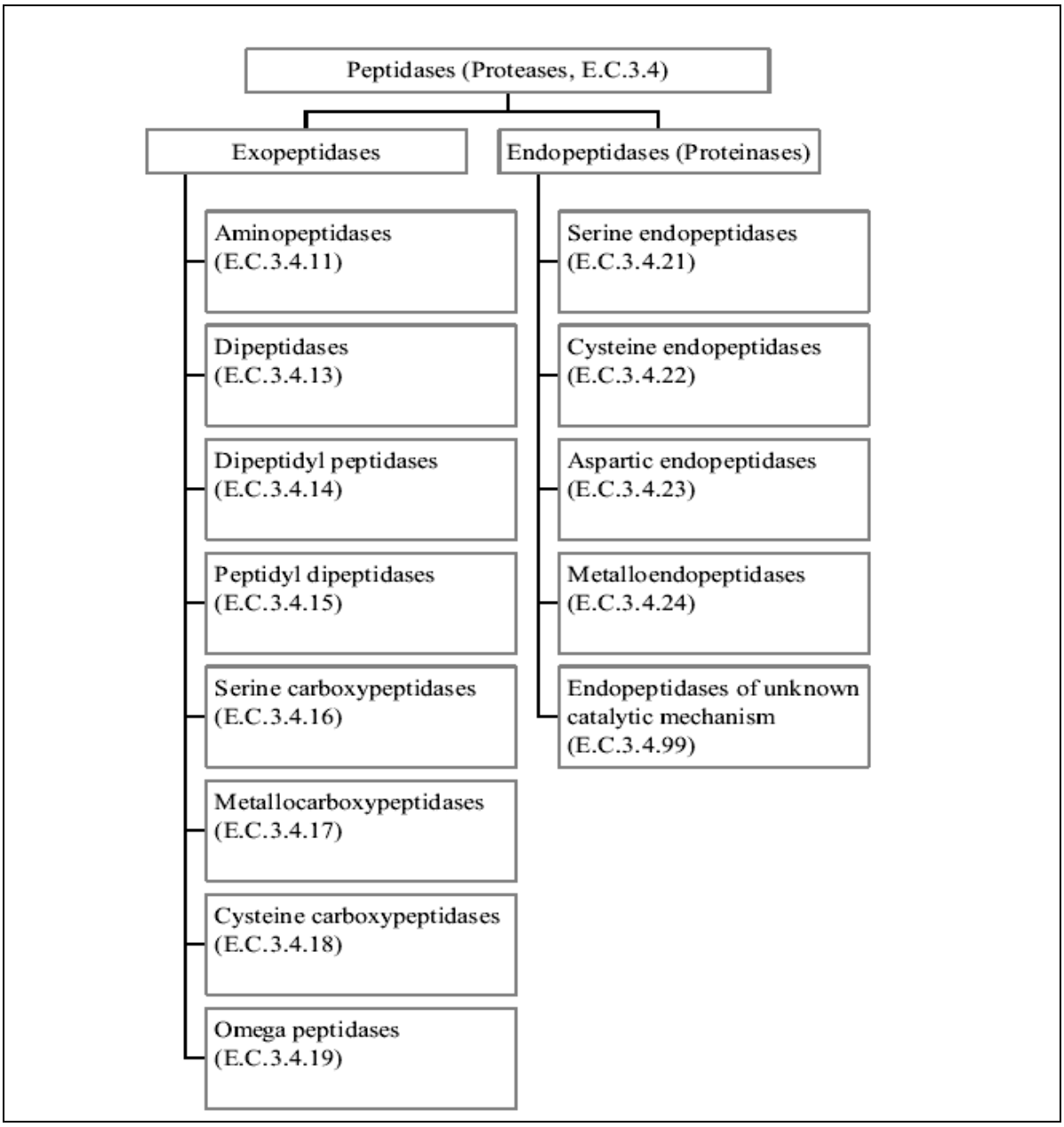


Figure 2.1 Classification of proteases (peptidases) according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB, 1992). NC-IUBMB recommended the term peptidase as the general term for all enzymes that hydrolyze peptide bonds. This is subdivided into exopeptidases cleaving one or a few amino acids from the N- or C-terminus, and endopeptidases cleaving internal peptide bonds of polypeptides. The classification of exopeptidases is based on their actions on substrates while the endopeptidases are divided by their active sites.

2.4.1.1 Aminopeptidases

Aminopeptidases act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. They are known to remove the N-terminal Met that may be found in heterologously expressed proteins, but not in many naturally occurring mature proteins. Aminopeptidases are usually metalloenzymes. Nevertheless, there have been a few attempts to study the role of metal ions in catalysis by insect midgut enzymes. The most complete study was performed with the partially purified major aminopeptidase from the microvillar membranes of *Rhynchosciara americana* (Houst. ex Mill.) midgut caeca cells (Ferreira and Terra, 1986). The pH optima of insect aminopeptidases are alkaline (range 7.2 - 9.0), irrespective of the pH of the midgut lumen from different species. Km values are similar (range 0.13 - 0.78 mM), except in *Anopheles stephensi* (Liston), where they are lower, and in *R. americana* (membrane-bound enzymes), where they are higher. The higher Km values observed in the membrane-bound aminopeptidases of *R. americana* are probably related to the specificity of these enzymes. Most aminopeptidases Mr values are in the range 90,000 - 130,000. Higher Mr values are usually found among detergent-solubilized membrane-bound enzymes, and may result from aggregation, as demonstrated with *R. americana* aminopeptidases (Ferreira and Terra, 1985).

Aminopeptidases play an important role in the intermediary digestion of proteins in insects, because they are usually more active than carboxypeptidases in these animals. Their role is best understood in larvae of *R. americana*, for which the following model for protein digestion was developed (Terra et al., 1979; Ferreira and Terra, 1980, 1984, 1985; Klinkowstrom et al., 1995). Oligopeptides produced by the action of proteinases (mainly trypsin) diffuse into the ectoperitrophic space, as soon as they become sufficiently small to pass through the peritrophic membrane (Terra, 1990). Once in the ectoperitrophic space, midgut fluxes direct oligopeptides to the midgut caeca. In the midgut caeca, oligopeptides are hydrolyzed mostly by the Mr 107,000 soluble aminopeptidase, which prefer larger peptides. The minor (20% of microvillar activity, Mr 107,000) microvillar aminopeptidase, which has substrate specificities similar to those of the soluble (Mr 115,700) aminopeptidase, complete the intermediary digestion of oligopeptides, and provide substrates for major (Mr 169,000) microvillar aminopeptidase.

Dipeptides resulting from the action of the major microvillar aminopeptidase on small peptides (usually tripeptides) are hydrolyzed by dipeptidases. The recently described soluble, glycocalyx-associated (Mr 117,000) aminopeptidase removes N-terminal aspartic or glutamic residues from peptides that are not efficiently attacked by the other aminopeptidases. This enzyme resembles vertebrate digestive aminopeptidase A (EC 3.4.11.7).

2.4.1.2 Carboxypeptidases

The carboxypeptidases (EC 3.4.16-18) act at the C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases (EC 3.4.16), metallo-carboxypeptidases (EC 3.4.17), and cysteine carboxypeptidases (EC 3.4.18), based on the nature of the amino acid residues at the active site of the enzymes. Serine carboxypeptidases are most active in the acidic range and have a serine in their active site, which is blocked by DFP or PMSF. Metallo-carboxypeptidases require bivalent cations, usually Zn, for activity and are inhibited by EDTA. Cysteine carboxypeptidases are inhibited by thiol-blocking reagents.

Insect digestive carboxypeptidases have been classified as carboxypeptidase A or B depending on activity against ZGlyPhe (or HPLA) or ZGlyArg (or HA), respectively. Most insect carboxypeptidase A-like enzymes have Mr values in the range 20,000 - 50,000. Those presenting higher Mr values are probably composed of subunits, as shown in *Erinnyis ello* (Linnaeus) (Santos and Terra, 1984) and *Spodoptera frugiperda* (Ferreira et al., 1994). Some may be overestimated due to associated detergent molecules, as occurs with the detergent-solubilized membrane-bound enzymes of *R. americana* and *M. domestica* (Jordao and Terra, 1989). Carboxypeptidases classified as B because of their alkaline pH optima and activity upon HA, and have been partially characterized in Diptera. Such information available for adults of *Stomoxys calcitrans* (pH optimum 7.5, Mr 28,500) and *Glossina morsitans* (pH optimum 7.8, Mr 22,000), and for larvae of *R. americana* (pH optimum 7.3, M, 104,000).

Recently, a novel procarboxypeptidase from *H. armigera* (PCPAHa) (Figure 2.2), the first enzyme of this class from a lepidopteran insect, has been characterized by

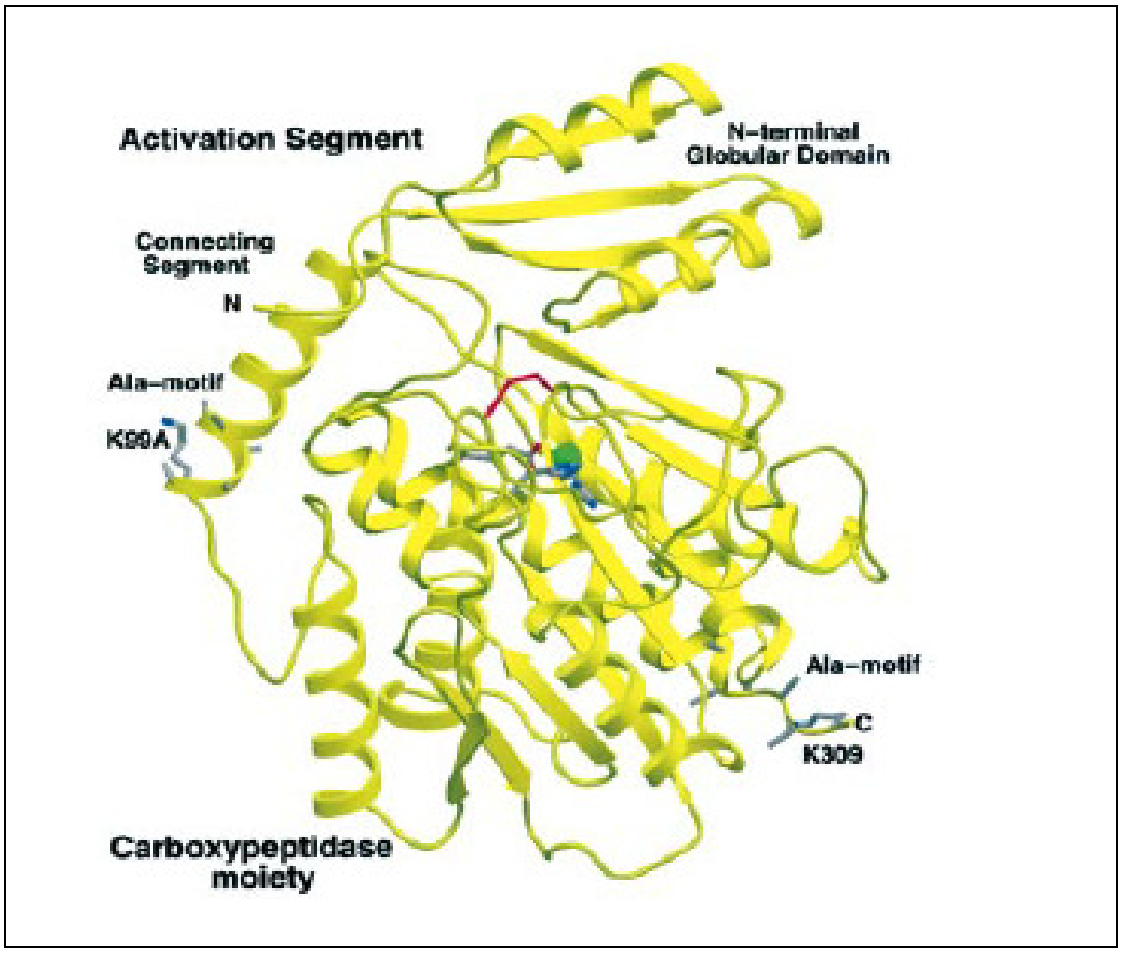


Figure 2.2 Three-dimensional structure of PCPAHa. Stereo ribbon plot representation of PCPAHa. Estebanez et al. (2001) reported the crystal structure of novel procarboxypeptidase (PCPAHa) found in the gut extracts of *H. armigera* larvae.

expressing its encoding cDNA in insect cells (Bown et al., 1998). The pre-proprotease sequence contains 426 amino acid residues and exhibits sequence homology with the metallo-carboxypeptidases from mammals, and with carboxypeptidases from other invertebrates. Removal of a predicted signal peptide and of the activation peptide results in a product of approximately 35.5 kDa, which is comparable in size to the pancreatic mammalian carboxypeptidases (Vendrell et al., 2000). PCPAHa belongs to the A form of carboxypeptidases and preferentially cleaves aliphatic and aromatic residues. This carboxypeptidase is active over a broad pH range (7.5 - 10), displaying maximal activity at pH 8.0. This is in agreement with the report of a maximal peptidase activity in the alkaline region determined in other phytophagous lepidopteran species (Lenz et al., 1991; Ortego et al., 1996; Ferriera et al., 1994; Estebanez et al., 2001).

2.4.2 Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. The presence of a free amino or carboxyl group has a negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanisms, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases, and (iv) metalloproteases.

2.4.2.1 Serine proteases

Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among the insects, mammals, viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. Based on their structural similarities, serine proteases have been grouped into 20 families, which have been further, subdivided into about six clans with common ancestors (Barett, 1994). Serine proteases are recognized by their irreversible inhibition by 3, 4-dichloroisocoumarin (3, 4-DCI), L-3-carboxytrans 2, 3-epoxypropyl-leucylamido (4-guanidine) butane (E 64), diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), and tosyl-L-lysine chloromethyl ketone (TLCK). Some of the serine

proteases are inhibited by thiol reagents such as *p*-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11. The isoelectric points of serine proteases are generally between pH 4 and 6. Serine alkaline proteases that are active at highly alkaline pH represent the largest subgroup of serine proteases have multiple specificities and include trypsins, chymotrypsins, elastases, cathepsin-B like proteases, aminopeptidases and carboxypeptidases, which are all responsible for protein digestion. Serine proteases are known to dominate the larval gut environment and contribute to about 95% of the total digestive activity (Srinivasan et al., 2005a). Beneath the complexity of multiple protease specificities, there usually exists an array of diverse protease isoforms; for example, the gut of *H. armigera* is known to contain about 20 different types of active serine protease isoforms at any given moment (Bown et al., 1997; Gatehouse, et al., 1997). Chougule et al. (2005) reported that *H. armigera* larvae fed on various host plants and in the larvae exposed to several types of plant serine PIs were expressed 7-trypsin, 4-chymotrpsin, 5-aminopepetidase, 3-carbosypepetidase and one elastase and cathepsin genes during the different larval instars.

Serine proteases usually follow a two-step reaction for hydrolysis in which a covalently linked enzyme-peptide intermediate is formed with the loss of the amino acid or peptide fragment (Fastrez and Fersht, 1973; Schechter and Plotnick, 2003). This acylation step is followed by a deacylation process, which occurs by a nucleophilic attack on the intermediate by water, resulting in hydrolysis of the peptide. Serine endopeptidases can be classified into three groups based mainly on their primary substrate preference: (i) trypsin-like, which cleave after positively charged residues; (ii) chymotrypsin-like, which cleave after large hydrophobic residues; and (iii) elastase-like, which cleave after small hydrophobic residues. The carboxypeptidases are unusual among the serine-dependent enzymes in that they are maximally active at acidic pH. These enzymes are known to possess a Glu- residue preceding the catalytic Ser, which is believed to be responsible for their acidic pH optimum. Although the majority of the serine proteases contain the catalytic triad Ser-His-Asp, a few use the Ser-base catalytic dyad. The Glu-specific proteases display a pronounced preference for Glu-Xaa bonds over Asp-Xaa bonds (Austew and Smith, 1976).

The serine proteinases exhibit different substrate specificities, which are related to amino acid substitutions in the various enzyme subsites interacting with the substrate residues. Some enzymes have an extended interaction site with the substrate. Others have a specificity restricted to the P1 substrate residue (Schaller, 2004). Three residues, which form the catalytic triad, are essential in the catalytic process, i.e. His 57, Asp 102, and Ser 195 (chymotrypsinogen numbering). The first step in the catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential Serine. Formation of this covalent intermediate proceeds through a negatively charged tetrahedral transition state intermediate, and then the peptide bond is cleaved. During the second step, or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme. The deacylation, which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water molecule is the attacking nucleophile instead of the Ser residue. The His residue provides a general base and accepts the OH group of the reactive Ser (Haq and Khan, 2003; Haq et al., 2004).

2.4.2.2 Aspartic proteases

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Aspartic proteinases are active at acid pH, hydrolyze internal peptide bonds in proteins, and some also attack synthetic substrates. The best-known animal aspartic proteinases are pepsin (EC 3.4.23.1) (Fruton, 1976) and cathepsin D (EC 3.4.23.5) (Barrett, 1977). Aspartic proteinase in insects was reported by Greenberg and Paretsky (1955), who found a strong proteolytic activity at pH 2.5 - 3 in homogenates of whole bodies of *M. domestica*. Most aspartic proteases show maximal activity at low pH (pH 3 to 4), and have isoelectric points in the range of pH 3 to 4.5. Their molecular masses are in the range of 30 to 45 kDa.

Aspartic endopeptidases depend on the aspartic acid residues for their catalytic activity. A marked conservation of cysteine residue is evident in aspartic proteases. The pepsins and the majority of other members of the family show specificity for the cleavage of bonds in peptides of at least six residues with hydrophobic amino acids in both the Pl

and Pl9 positions (Keil, 1992). The specificity of the catalysis has been explained on the basis of available crystal structures (Lindberg et al., 1981).

2.4.2.3 Cysteine/thiol proteases

Cysteine proteases occur in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized. Activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. The order of Cys and His (Cys-His or His-Cys) residues differs among the families (Barett, 1994.). Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups:
(i) papain-like, (ii) trypsin-like with preference for cleavage at the arginine residue,
(iii) specific to glutamic acid, and (iv) others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic pH.

Cysteine proteases catalyze the hydrolysis of carboxylic acid derivatives through a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate. The mechanism of action of cysteine proteases is very similar to that of serine proteases. The enzyme papain consists of a single protein chain folded to form two domains containing a cleft for the substrate to bind. The crystal structure of papain confirmed the Cys25-His159 pairing (Baker and Drenth, 1987). The presence of a conserved aspargine residue (Asn175) in the proximity of catalytic histidine (His159) creating a Cys-His-Asn triad in cysteine peptidases is considered analogous to the Ser-His-Asp arrangement found in serine proteases.

2.4.2.4 Metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases (Barett, 1995). They are characterized by the requirement for a divalent metal ion for their activity. They include enzymes from a variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Hibbs et al., 1985; Okada et al., 1986; Shannon et al., 1989; Weaver et al., 1977; Wilhelm et al., 1987). About 30 families of metalloproteases have been recognized, of which 17 contain

only endopeptidases, 12 contain only exopeptidases, and 1 (M3) contains both endo- and exopeptidases cells. Matrix metalloproteases play a prominent role in the degradation of the extracellular matrix during tissue morphogenesis, differentiation, and wound healing and may be useful in the treatment of diseases such as cancer and arthritis (Browner et al. 1995). In summary, proteases are broadly classified as endo- or exoenzymes on the basis of their site of action on protein substrates. They are further categorized as serine proteases, aspartic proteases, cysteine proteases, or metalloproteases depending on their catalytic mechanism. They are also classified into different families and clans depending on their amino acid sequences and evolutionary relationships. Based on the pH of their optimal activity, they are also referred to as acidic, neutral, or alkaline proteases.

2.5 Physiological functions of proteases

Proteases execute a large variety of complex physiological functions. Their importance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms. Proteases play a critical role in many physiological and pathological processes such as protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes. In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a critical role in the regulation of metabolism. In contrast to the multitude of the roles contemplated for proteases, our knowledge about the mechanisms by which they perform these functions is very limited. Extensive research is being carried out to unravel the metabolic pathways in which proteases play an integral role; and this research will continue to contribute significantly to our present state of information.

2.6 Mechanisms of digestive enzymes

The lepidopteran larval midgut lumen is a hostile environment for proteins. This follows from the primary function of this compartment as a site of digestion. The

ingested plant material encounters a host of secreted digestive enzymes, which cleave carbohydrates, lipids, and proteins into small molecules that are absorbed into the epithelial cells and passed via the circulatory system to the rest of the body. Most caterpillars (larvae of the insect order Lepidopteran larvae) are herbivorous and must digest large amounts of plant material to achieve high growth rates (Zalucki et al., 2002). The digestive system or gut is a simple tube designed for efficient extraction of nutrients in a constant flow-through system (Chapman, 1985). The midgut is the longest section of the gut, and it is here that most of the digestion and absorption of nutrients takes place, generally at a high pH. As the larva eats constantly between molts, a long continuous food bolus passes through the midgut to the hindgut. Here, water is removed, and waste filtered through the Malpighian tubules is added to the bolus, which is eventually excreted as frass or fecal pellets.

Proteins secreted into the lumen by the surrounding cells may be blocked or trapped within the peritrophic matrix (PM) if they are too large to pass through the meshwork of chitin fibrils or may pass through to contact the bolus of food if sufficiently small. Once inside the endoperitrophic space, proteins are carried posteriorly along with the mass movement of the food bolus and eventually excreted with the frass. However, it has been proposed that some proteins could be recycled by passing through the PM, and carried anteriorly by a countercurrent operating in the ectoperitrophic space (Terra, 2001). The main function carried out by lumen proteins is digestion, and we would expect to see a high abundance and activity of enzymes that cleave lipids, carbohydrates, and proteins into smaller molecules that can be efficiently absorbed by the transport systems in the microvillar membranes. In addition, many plants produce toxic compounds to deter herbivory, and the lumen represents a potential early site of enzymatic detoxification or sequestration by binding followed by excretion, to avoid absorbing toxins into the cells along with nutrients.

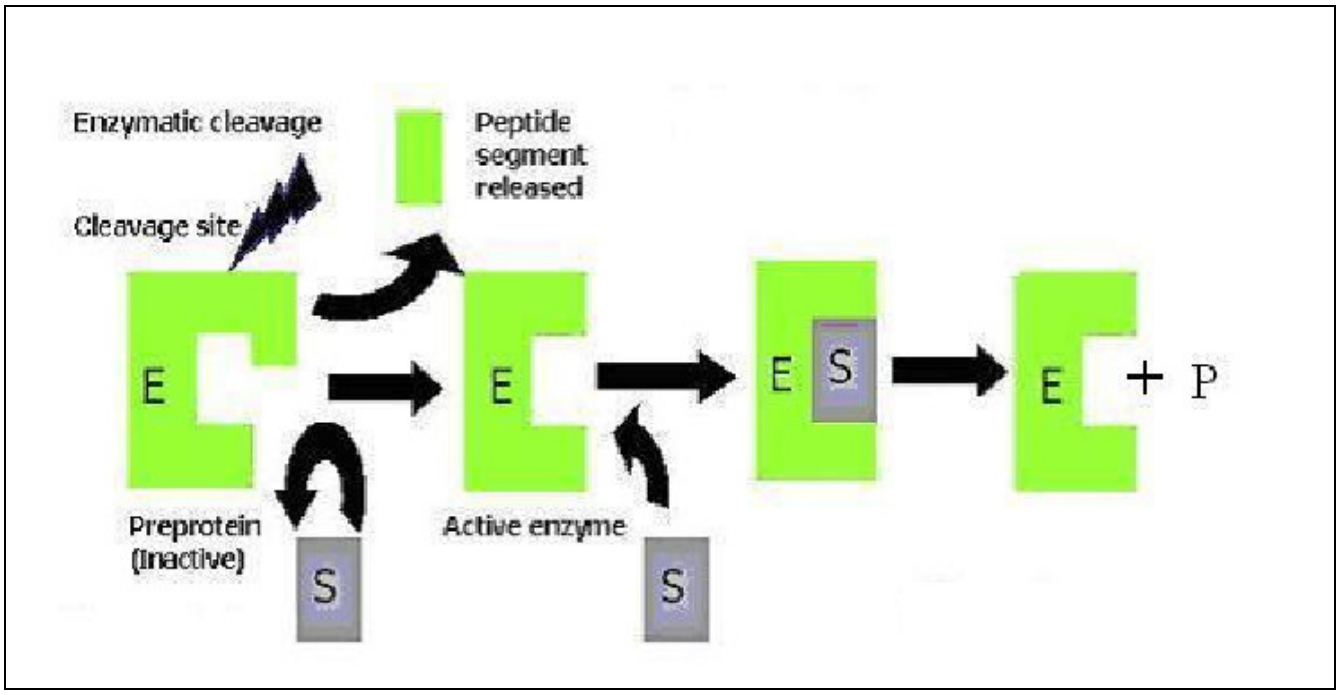
There are three main secretory mechanisms known for cells. In holocrine and apocrine secretion, secretory products are stored in the cytoplasm until they are released, at which time the whole (holocrine) or part (apocrine) of the secretory cell is lost to the extracellular space. In merocrine secretion (exocytosis), secretory products are contained in vesicles, which eventually fuse with the plasma membrane releasing their contents.

Exocytosis may occur through regulated or constitutive secretion (Willingham and Pastan, 1984; Kelly, 1985). Regulated secretion is the mechanism for secretion of proteins in a large number of cells, and is characterized by the concentration of the synthesized product and its retention in secretory vesicles (storage granules), whose contents are released only when the cell receives the appropriate signal (Palade, 1975). Constitutive secretion is observed in cells engaged in the continuous production of the secretory protein. No accumulation of storage granules occurs in the cytoplasm (Willingham and Pastan, 1984; Kelly, 1985). While most insect midgut cells appear to lack storage granules, exceptions are provided by the opaque zone cells of *S. calcitrans* (Lehane, 1976), the peritrophic-membrane secreting cells of *Calliphora erythrocephala* (Meigen) (Diptera: Calliphoridae) and *Forficula aurieularia* (L.) (Dermaptera: Forficulidae) (Peters et al., 1979), and the midgut cells of *Cubitermes severus* (Isoptera: Termitidae) (Bignell et al., 1982) and *A. stephensi* (Berner et al., 1983). Despite the lack of direct evidence, it is inferred with little doubt that the contents of storage granules are precursors of the peritrophic membrane in *C. erythrocephala* and *F. auricularia*, and digestive enzymes in *A. stephensi*.

2.7 Enzyme modification

Activation of the zymogenic precursor forms of enzymes and proteins by specific proteases represents an important step in the physiological regulation of many rate-controlling processes (Figure 2.3), such as generation of protein hormones, assembly of fibrils and viruses, blood coagulation, and fertilization of ova by sperm. Activation of zymogenic forms of chitin synthase by limited proteolysis has been observed in *Candida albicans* and *Aspergillus nidulans* (Bulawa, 1993). Kex-2 protease (kexin; EC 3.4.21.61), originally discovered in yeast, has emerged as a prototype of a family of eukaryotic precursor processing enzymes. It catalyzes the hydrolysis of prohormones and of integral membrane proteins of the secretory pathway by specific cleavage at the carboxyl side of pairs of basic residues such as Lys-Arg or Arg-Arg (Barett et al., 1998). Furin (EC 3.4.21.5) is a mammalian homolog of the Kex-2 protease that was discovered serendipitously and has been shown to catalyze the hydrolysis of a wide variety of precursor proteins at Arg-X-Lys or Arg-Arg sites within the constitutive secretory

A



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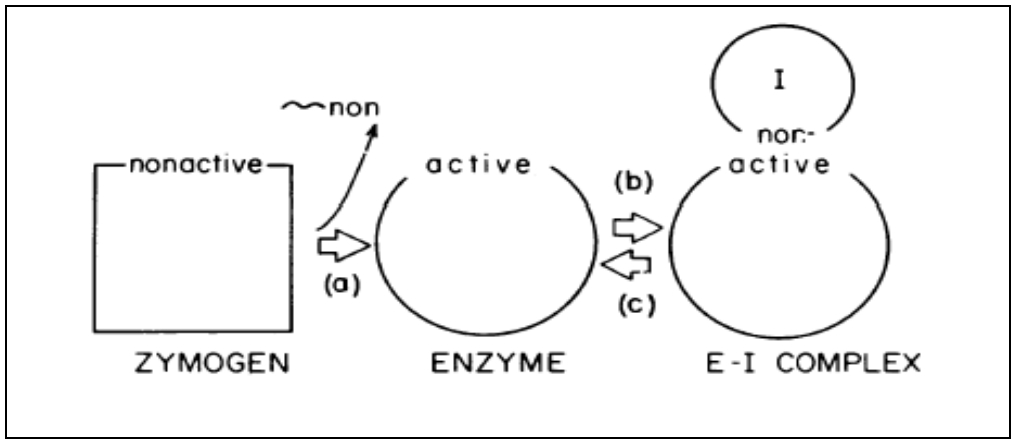


Figure 2.3 **A**. Schematic representation of the control of enzyme activity at the level of synthesis. Enzymes synthesized as inactive preproteins and are processed to be activated.
E - Enzyme, S - Substrate, P - Product.
B. Activation of zymogen and formation of E-I Complex
(a) Zymogen activation, (b) formation of an enzyme-inhibitor complex, and (c) dissociation of that complex. ~~non represents the activation peptide

pathway (Smeekens, 1993). Pepsin, trypsin, and chymotrypsin occur as their inactive zymogenic forms, which are activated by the action of proteases. Proteolytic inactivation of enzymes, leading to irreversible loss of *in vivo* catalytic activity, is also a physiologically significant event.

2.8 Applications of proteases

Proteases have a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations; whereas those that are used in medicine are produced in small amounts, but require extensive purification before they can be used.

Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures. The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes. The preparation of the first enzymatic detergent, "Burnus," dates back to 1913; it consisted of sodium carbonate and a crude pancreatic extract. The ideal detergent protease should possess broad substrate specificity to facilitate the removal of a large variety of stains due to food, blood, and other body secretions. Activity and stability at high pH and temperature and compatibility with other chelating and oxidizing agents added to the detergents are among the major prerequisites for the use of proteases in detergents. The key parameter for the best performance of a protease in a detergent is its p*I*.

2.9 Protease inhibitors

Protease inhibitors (PIs) are of very common occurrence. They have been isolated and characterized from a large number of organisms, including plants, animals, and microorganisms (Christeller, 2005; Haq et al., 2004; Supuran et al., 2002). Naturally

occurring PIs are essential for regulating the activity of their corresponding proteases and play key regulatory roles in many biological processes.

The natural antagonists of proteases are small proteins the PIs, which are quite common in nature and also present in all life forms (Fritz, 2000). The corresponding inhibitors of most proteases occur in nature. Most PIs interact with their target proteases by contact with the active (catalytic) site of the protease, resulting in the formation of a stable protease inhibitor complex that is incapable of enzymatic activity (Norton, 1991). Protease inhibitors have an enormous diversity of function by regulating the proteolytic activity of their target proteinases (Leung et al., 2000). Proteases can be either reversible or irreversible. Reversible proteases react in the absence or above critical concentrations of their inhibitors. Often, cocktails of inhibitors are used in varying combinations to inhibit a broad spectrum of proteases. For isolation and purification of protein (e. g. enzymes), specific mixtures of inhibitors are used, e.g., a cocktail of aprotinin, E-64 or leupeptin, EDTA, pefabloc, and pepstatin. A cocktail of PIs is a convenient and reliable solution for the inhibition of a broad spectrum of serine, cysteine, metalloproteases, as well as calpains. Optimized combination of synthetic PIs show excellent inhibition, and are well suited for protection of proteins isolated from animal tissue, plants, yeast, and bacteria. In the absence of information about which class of protease(s) may be degrading a particular sample, a protease cocktail can be obtained by using individual stocks (Table 2.1). Studies have been carried on protease inhibitors, which active against certain insect species, both in *in vitro* assays against insect gut proteases (Pannetier et al., 1997; Koiwa et al., 1998) and in *in vivo* artificial diet bioassays (Urwin et al., 1997; Vain et al., 1998).

2.10 Plant protease inhibitors

Protease inhibitors (PIs) are of common occurrence in the plant kingdom. Plant PIs (PPIs) are generally small proteins that have mainly been described as occurring in storage tissues, such as tubers and seeds, but they have also been found in the aerial parts of plants (De Leo et al., 2002). They are also induced in plants in response to injury or attack by insects or pathogens (Ryan, 1990). In plants, these PIs act as anti-metabolic proteins, which interfere with the digestive process of insects. One of the important defense strategies found in plants into combat the predators involves PIs which are in

Table 2.1 Protease inhibitors with their specificity towards enzymes and solubility and stability in water or organic solvents.

Synthetic inhibitor	Mr	Specificity of inhibitors	Solubility/ stability (Stock conc.)
Antipain-dihydrochloride	677.6	Papain, trypsin is inhibited to a small extent	Soluble in water (20 mg/ml), methanol or DMSO
Bestatin	308.4	Amino peptidase, including aminopeptidase B., leucine aminopeptidase, tripeptide aminopeptidase	soluble in 1 M HCl (20 mg/ml). Methanol (5 mg/ml) or 0.15 M NaCl (1 mg/ml).
Chymostatin	607.7	α-, β-, γ -, δ-chymotrypsin	Soluble in glacial acetic acid (20 mg/ml) or DMSO
E-64	357.4	Cysteine proteases	Soluble in 1:1 water/ethanol [v/v] (20 mg/ml)
Leupeptin	475.6	Serine and cysteine proteases such as plasmin, trypsin, papain, cathepsin B	Soluble in water (1 mg/ml)
Pepstatin	685 9	Aspartate proteases like pepsin, renin, cathepsin D, chymosin	Soluble in methanol (1.0 mg/ml)
Pefabloc SC	239.5	Serine proteases, e.g. trypsin, chymotrypsin, plasmin, thrombin	Soluble in water (100 mg/ml)
EDTA-Na2	372.2	Metalloproteases	Soluble in water to 0.5 M at pH 8-9.
Aprotinin	6512	Serine proteases	Soluble in water (10 mg/ml)

particular effective against phytophagous insects and microorganisms. The defensive capabilities of PPIs relies on inhibition of proteases present in insect gut or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development (Lawrence and Koundal, 2002; Chen et al., 2007). The plant protease inhibitors apparently may be involved in several processes, i.e., reveal multifunctional properties. At present, a multitude of biochemical pathways in which protein inhibitors may be involved is of particular interest with respect to the design of transgenic plant species containing proteinase inhibitor genes (Ryan, 1990; Michaud, 1997).

Protease inhibitors have been grouped into families and subfamilies, and into different clans on the basis of sequence relationship and the relationship of protein folds of the inhibitory domains or units. An inhibitor domain is defined as the segment of the amino acid sequence containing a single reactive site after removal of any parts that are not directly involved in the inhibitor activity. On the basis of sequence homologies of their inhibitor domains, PIs have been classified into 48 families (Rawlings et al., 2004). Proteins containing a single inhibitor unit are termed simple inhibitors, and those that contain multiple inhibitor units are termed complex inhibitors. A total of 11 families belong to the latter category and contain between 2 and 15 inhibitory domains. Most of these are homotypic, containing inhibitor units from a single family, some are, however, heterotypic, and contain inhibitor unit from different families (Richardson et al., 2001; Trexler et al., 2001, 2002).

Most of the studies have been carried out on crop plants viz., cereals, legumes, and solanaceous species. Soybean trypsin inhibitor was the first PI isolated and characterized. Since then many PIs have been found to be widely distributed throughout the plant kingdom. Most of plants PIs that have been characterized are from the Gramineae (Poaceae), Leguminosae (Fabaceae), and Solanaceae families (Brzin and Kidric, 1995). PLANT-PIs is a database developed to facilitate retrieval of information on the distribution and functional properties of protease inhibitors in higher plants (Table 2.2). Currently, PLANT-PIs, contains information for 495 inhibitors (plus several isoinhibitors) identified in 129 different plants (De Leo et al., 2002). PIs are usually found in storage organs, such as seeds and tubers, but their occurrence in the aerial part of

Table 2.2 Classification and distribution of some important families of plant protease inhibitors with their sources and target proteases.

Inhibitor family	**Type example**	**Source**	**Target Protease**	**References**
Bowman-Birk	Bowman–Birk plant trypsin inhibitor unit 1	*Glycine max*	Trypsin, Chymotrypsin	Odani and Ikenaka (1976)
	Bowman-Birk trypsin/chymotrypsin inhibitor	*Arachis hypogaea*	Trypsin, Chymotrypsin	Suzuki et al. (1987)
	sunflower cyclic trypsin inhibitor	*Helianthus annuus*	Trypsin, Cathepsin G, Elastase, Chymotrypsin and thrombin	Mulvenna et al. (2005)
Cystatin	onchocystatin	*Onchocerca volvulus*	Cysteine proteinase	Lustigman et al. (1992)
	ovocystatin	*Gallus gallus*	Thiol proteases	Laber et al. (1989)
	oryzacystatin II	*Oryza sativa*	Cysteine proteinases	Ohtsubo et al. (2005)
Kunitz	soybean Kunitz trypsin inhibitor	*Glycine max*	Trypsin, Chymotrypsin	Laskowski and Kato (1980)
	barley subtilisin inhibitor	*Hordeum vulgare*	Subtilisin,Alpha-amylase	Vallee et al. (1998)
	winged-bean chymotrypsin inhibitor	*Psophocarpus tetragonolobus*	Alpha-chymotrypsin	Habu et al. (1992)
	Kunitz cysteine peptidase inhibitor 1	*Solanum tuberosum*	Cysteine proteases	Gruden et al. (1997)
	proteinase inhibitor A inhibitor unit	*Sagittaria sagittifolia*	Trypsin, Chymotrypsin, Kallikerin	Laskowski and Kato (1980)
	Kunitz subtilisin inhibitor	*Canavalia lineata*	Subtilisin-type microbial serine proteases	Terada et al. (1994)
	cathepsin D inhibitor	*Solanum tuberosum*	Cathepsin D, Trypsin	Strukelj et al. (1992)
	trypsin inhibitor	*Acacia confusa*	Trypsin and alphachymotrypsin	Lin et al. (1991)
Potato type I	chymotrypsin inhibitor I	*Solanum tuberosum*	Chymotrypsin, Trypsin	Richardson (1974)
	glutamyl peptidase II inhibitor	*Momordica charantia*	Glu S.griseus protease Subtilisin	Ogata et al. (1991)
	subtilisin-chymotrypsin inhibitor CI-1A	*Hordeum vulgare*	Subtilisin , Chymotrypsin B.lichenoformis	Greagg et al. (1994)
	subtilisin/chymotrypsin inhibitor	*Triticum aestivum*	subtilisin, Alphachymotrypsin	Poerio et al. (2003)
Potato type II	proteinase inhibitor II	*Solanum tuberosum*	Trypsin, Chymotrypsin	Greenblatt et al. (1989)
	potato peptidase inhibitor II inhibitor unit 1	*Solanum tuberosum*	Trypsin, Chymotrypsin	Keil et al. (1986)
	tomato peptidase inhibitor II inhibitor unit 1	*Solanum lycopersicum*	Trypsin, Chymotrypsin	Graham et al. (1985)
	tomato peptidase inhibitor II inhibitor unit 2	*Solanum lycopersicum*	Trypsin, Chymotrypsin	Barrette-Ng et al. (2003)
Squash	trypsin inhibitor MCTI-1	*Momordica charantia*	Pancreatic elastase	Wiezorek et al. (1985)
	trypsin inhibitor MCTI-II	*Momordica charantia*	Trypsin	Huang et al. (1992)
	macrocyclic squash trypsin inhibitor	*Momordica cochinchinensis*	Trypsin	Hernandez et al. (2000)
	trypsin inhibitor CSTI-IV	*Cucumis sativus*	Trypsin	Wieczorek et al. (1985)

plants, as a consequence of several stimuli has also been widely documented (De Leo et al., 2002). PIs may accumulate to about 1 to 10% of the total proteins in these storage tissues. An increasing number of PIs is found in non-storage tissues, such as leaves, flowers, and roots (Brzin and Kidric, 1995; Xu et al., 2001; Sin and Chye, 2004). Some PIs also occur in yeast (Matern et al., 1979) and other fungi (Richardson, 1977). A trypsin inhibitor was found localized in the cytosol of mung bean cotyledonary cells (Chrispeels and Baumgartner, 1978). Soybean trypsin inhibitor (SBTI) is mainly present in the cell walls, with lesser amounts in protein bodies, the cytoplasm, and the nuclei of cotyledonary and embryonic cells. Soybean Bowman-Brik inhibitor (SBBI) occurs in protein bodies, the nuclei, and to a lesser extent the cytoplasm. In contrast to SBTI, some SBBI has been located in the intercellular space but not in the cell wall (Horisberger and Tacchini-Vonlanthen, 1983). The wound-induced inhibitors accumulate in vacuoles of tomato, wild tomato, and potato leaves. Xu et al. (2004) described the expression of a PIN2 protein from *Solanum americanum* Mill. in phloem of stems, roots, and leaves, suggesting a novel endogenous role for PIN2 in phloem. Further research showed that both SaPIN2a and SaPIN2b are expressed in floral tissues (Sin and Chye, 2004).

Protease inhibitors have been classified into serine, cysteine, aspartate and metallocarboxy PIs (De Leo et al., 2002; Laskowski et al., 2003; Koiwa et al., 1997). PIs that are active against all the mechanistic classes of proteases have also been described in plants.

2.10.1 Serine protease inhibitors (Serpins)

Serine protease inhibitors are universal throughout the plant kingdom and have been described in many plant species. Therefore, the number of known and partially characterized inhibitors of serine proteases is enormous (Haq et al., 2004). Serine protease inhibitors have been reported from a variety of plant sources and are the most studied class of protease inhibitors (Mello et al., 2002; Haq and Khan, 2003). Chiche et al. (2004) first introduced the squash inhibitor, a well-established family of highly potent canonical serine protease inhibitors isolated from Cucurbitaceae.

Plant serine PIs (serpins) have been shown to inhibit model trypsin like proteins (Roberts et al., 2003), but there are no obvious targets for these inhibitors in plants, which

may, apparently be involved in inhibiting proteases of plant pathogens (Hejgaard, 2005). Plant serpins have molecular mass of 39 - 43 kDa, with amino acid and nucleotide homology with other well-characterized serpins. The majority of serpins inhibit serine proteases, but serpins that inhibit caspases (Ray et al., 1992; Law et al., 2006) and papain like cysteine proteases (Schick et al., 1998; Irving et al., 2002) have also been reported. Plant serpins exhibit differing and mixed specificities towards proteases (Al-Khunaizi et al., 2002). Barley (*Hordeum vulgae*) serpin is a potent inhibitor of trypsin and chymotrypsin at overlapping reactive sites (Dahl et al., 1996). The inhibitors from at least four families belonging to serine PIs have been induced sequentially in various plants. These families include potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) inhibitors I and II in solanaceous plants (Melville and Ryan, 1972; Bryant et al., 1976; Plunkett et al., 1982; Valueva et al., 2001; Farran et al., 2002), Bowman-Birk inhibitors in alfa alfa (Brown and Ryan, 1984) and a Kunitz inhibitor in poplar trees (Bradshaw et al., 1989; Ledoigt et al., 2006).

The serpin family of PIs active against serine proteases also contains inhibitors of cysteine proteases (Heibges et al., 2003; Laskowski et al., 2003). Serine PIs belonging to various families have been reported either in storage organs or in the vegetative cells of a wide variety of plants (Garcia et al., 1987). Two oat (*Avena sativa*) serpins show specificity for chymotrypsin and / or elastase, and another one has specificity for trypsin and chymotrypsin at overlapping loop sites (Irving et al., 2002). Squash serpin Cmps-1 also inhibits elastase at two overlapping sites (Ligoxygakis et al., 2003). Serpins are irreversible inhibitors. The cleavage of an appropriate peptide bond in the reactive centre loop of the inhibitor triggers a rapid conformational change so that catalysis does not proceed beyond the formation of an acyl-enzyme complex (Huntington et al., 2000).

2.10.2 Bowman-Birk inhibitors (BBIs)

On the basis of sequence homology, this forms another family of serine PIs. The family is named after D.E. Bowman and Y. Birk, who were the first to identify and characterize a member of this family from soybean (*Glycine max*) (Bowman, 1946; Birk et al., 1963). The inhibitors have been found in legumes and cereals (Laing and McManus, 2002; Tanaka et al., 1997; Norioka and Ikenaka, 1983). The inhibitors of this

family are generally found in seeds, but are also wound-inducible in leaves (Eckelkamp et al., 1993; Moura and Ryan, 2001). The soybean inhibitor is now the most-well-studied member of this family and is often referred as the classic BBI.

Bowman Birk Inhibitors have been classified on the basis of their structural features and inhibitor characteristics. The inhibitors from dicotyledonous plants consist of a single polypeptide chain with the molecular mass of 8 kDa. These are double-headed, with two homologous domains each bearing a separate reactive site for the cognate proteases. These inhibitors interact independently, but simultaneously, with two proteases, which may be same or different (Raj et al., 2002; Birk, 1985). The first reactive site in these inhibitors is usually specific for trypsin, chymotrypsin, and elastase (Qi et al., 2005). The active site configuration in these inhibitors is stabilized by the presence of seven conserved disulfide bonds (Chen et al., 1992; Lin et al., 1993). In the case of double-headed BBIs, it has been found that the relative affinity of binding of proteases is altered when one site is already occupied. Peanut (*Arachis hypogoea* L.) inhibitor has been found to exhibit no activity against chymotrypsin when preoccupied with trypsin and vice versa (Tur et al., 1972). In the same way, the activity of soybean BBIs decreases 100-fold when trypsin is bound at the other site (Gladysheva et al., 1999).

2.10.3 Kunitz inhibitors

On the basis of sequence homologies, Kunitz-type inhibitors form a separate family. The members of this family are mostly active against serine proteases, but may also inhibit other proteases (Laing and McManus, 2002; Ritonja et al., 1990). The inhibitors in this family are widespread in plants and have been described in legumes, cereals and in solanaceous species (Chye et al., 2006; Ishikawa et al., 1994; Laskowski and Kato, 1980). A 20.5 kDa Kunitz-type trypsin inhibitor with antifungal activity has been reported from the roots of punce ginseng (*Pseudostellaria heterophylla*) (Wang and Ng, 2006).

The members of this family are mostly active against serine proteases and have been shown to inhibit trypsin, chymotrypsin and subtilisin (Laing and McManus, 2002; Park et al., 2005), but they also inhibit other proteases, including the aspartic protease cathepsin D and the cysteine proteinase papain. Kunitz-type PIs are also produced under

stress, as has been found in potato tubers (*S. tuberosum*) (Park et al., 2005; Ledoigt et al., 2006; Plunkett et al., 1982). The inhibitors usually have molecular mass of 18 - 22 kDa; have two disulfide bridges and one reactive site.

2.10.4 Squash inhibitors

Squash-family inhibitors have been described only in plants and form yet another family active against serine proteases. The members of this family have been described from many cucurbit families (Lee and Lin, 1995; Hamato et al., 1995; Felizmenio et al., 2001). Seven serine PIs belonging to this family have been isolated and characterized from the seeds of wild cucumber (*Cyclanthera pedata* L.) (Kuroda et al., 2001).

The members of this family consist of a small single peptide chain containing between 28 and 30 amino acids with a molecular mass of 3.0 - 3.5 kDa (Heitz et al., 2001). The structures of squash inhibitors, and inhibitor and proteinase complexes have been determined by X-ray crystallography and NMR spectroscopy (Holak et al., 1989; Nilges et al., 1991; Thaimattam et al., 2002). These inhibitors have been shown to follow the standard mechanism for inhibition.

2.10.5 Cysteine/Cystatin PIs

The cystatin is composed of several families and includes proteins that are related in structure and function to an inhibitor of cysteine proteinase, first described in egg white and referred to as 'chicken eggwhite cystatin' (Colella et al., 1989). The members these families inhibit the activity of cysteine proteases and are called cysteine PIs or cystatins. They are widely distributed in plants, animals and microorganisms (Oliveira et al., 2003). These inhibitors are grouped into four families based on sequence relationships, molecular mass, disulfide-bond numbers, and arrangements (Turk and Bode, 1991; Barrett, 1987). Cysteine proteinases isolated from insect larvae are inhibited by both synthetic and naturally occurring cysteine proteinase inhibitors (Wolfson and Murdock, 1987; Margis et al., 2008).

2.10.6 Aspartyl PIs

Aspartyl PIs have been described in sunflower, barley, and cardoon (*Cynara cardunculus*) flowers and in potato tubers (Park et al., 2000; Kervinen et al., 1999; Lawrence and Koundal, 2002; Marres et al., 1989; Wolfson and Murdock, 1987). The cathepsin D inhibitor, an aspartyl PI described in potato tubers shares considerable amino acid sequence homology with soybean trypsin inhibitor. It is a 27 kDa protein and inhibits serine proteases, trypsin and chymotrypsin, in addition to the aspartyl protease cathepsin D, but does not inhibit pepsin, cathepsin E, and rennin; which are all aspartyl proteases (Lawrence and Koundal, 2002). Pepstatin, a powerful and strong inhibitor of aspartyl proteases has been shown to inhibit proteolysis of the midgut enzymes of Colorado potato beetle (*Lptinotarsa decemlineata* Says) (Wolfson and Murdock, 1987).

2.10.7 Metallocarboxypeptidase PIs

The inhibitors that bind to metallocarboxypeptidases have been identified in solanaceaous plants, in the medicinal leech (*Hirudo medicinalis*), in the intestinal parasite roundworm *Ascaris suum*, in the blood tick *Rhipicephalus bursa* and in rat and human tissues (Arolas et al., 2005; Homandberg et al., 1989; Reverter et al., 1998; Normant et al., 1995; Liu et al., 2000). The plant inhibitors have been described in tomato and potato. These inhibitors are small peptide inhibitors consisting of 38 - 39 amino acid residues and have the molecular mass of about 4.2 kDa (Hass et al., 1975; Hass and Hermodson, 1981). These inhibitors inhibit strongly, but competitively, a broad spectrum of carboxypeptidases from both animals and microorganisms, but do not inhibit serine carboxypeptidases from yeast and plants (Havkioja and Neuvonen, 1985). A metallocarboxypeptidase inhibitor is found to accumulate in potato tuber tissues during development, along with the potato inhibitor I and II families of serine PIs. The inhibitor also accumulates in potato leaf tissues, along with the inhibitors of other families in response to wounding (Ryan, 1990).

2.11 Properties of plant PIs

Studies on the biosynthesis of several plant PIs has demonstrated the PIs are synthesized as either pre-proproteins (Graham et al., 1985a) or pre-proteins (Graham et

al., 1985b) that are processed *in vivo* either during or after synthesis to produce the native PIs (Nelson and Ryan, 1980). Some small PIs are derived *in vivo* from the post-translational processing of multidomain precursors (Sanchez-Serrano et al., 1986; McManus et al., 1994; Miller et al., 2000). Many PIs are produced in response to various stress conditions, e.g. pathogens, insect feeding, wounding, and environmental stresses (Koiwa et al., 1997). The plant PIs vary from 4 to 85 kDa, with the majority in the range of 8 to 20 kDa (Hung et al., 2003). Plant PIs usually have a high content of cysteine residues (Richardson, 1991) that form disulfide bridges (Greenblatt et al., 1989; Hung et al., 2003) and confer resistance to heat, extremes pH, and proteolysis (Richardson, 1991). For example, a trypsin inhibitor (BCTI) with molecular weight of 8 kDa was purified from seeds of *Brassica campestris* (L.). The BCTI was found to be a thermostable Bowman-Birk type TI that inhibits trypsin at the molar ratio 1:1. The stability of BCTI is apparently related to the presence of the disulfide bridge (Hung et al., 2003).

2.12 Functions of plant PIs

The main function of plant PIs is plant defense and the regulation of endogenous proteinases, but they may also function as storage proteins (Mosolov et al., 2001; Birk, 2003; Shewry, 2003; Chye et al., 2006). The possible role of PIs in plant protection was envisaged by Mickel and Standish (1947), who suggested observed that the larvae of certain insects were unable to develop normally on soybean products (Haq et al., 2004). PIs are of interest as potential sources of resistance against insect pests and pathogens in transgenic plants, and as drugs with antiviral and other properties as well as providing markers for studies on plant diversity and evolution (Konarev et al., 2002; Lawrence and Koundal, 2002; Korsinczky et al., 2004). The defensive role of PIs is based on their inhibitory activities towards the digestive enzymes of insects, and pathogen proteases involved in some vital processes, resulting either in a critical shortage of essential amino acids (Hilder et al., 1993; Jongsma and Bolter, 1997) or interfering with important biochemical or physiological processes of insects and other pathogens, such as the proteolytic activation of enzymes, molting of insects, or replication of viruses (Gutierrez-Campos et al., 1999). The activity of PIs is due to their capacity to form stable complexes with target proteases, blocking, altering, or preventing access to the enzyme active site.

PIs active toward serine proteases, the most widespread in nature, act as a potential substrate for proteases. Residues forming the scissile peptide bond are indicated as P1-P1' and are generally located on an external loop of the protein, interacting with proteases. The P1 residue determines the specific type of serine protease inhibited. Other residues around the reactive site also play a role in determining the strength of the PI-enzyme interaction (De Leo et al., 2002). Support for a defensive role of plant PIs initially came from studies of insects raised on artificial diets containing PIs and *in vitro* inhibition assays of insect gut proteases with purified PIs from various plant sources. The results of these studies strongly implicate plant PIs in interference with the growth and development of many phytophagous insects (Reeck et al., 1997). The first convincing evidence that PIs are part of the natural defensive chemicals of plants was the demonstration that wounding of tomato and potato leaves by Colorado potato beetles induced a rapid accumulation of proteinase inhibitor I (PIN1), not only in the damaged leaves, but also in distal, undamaged leaves (Green and Ryan, 1972). The correlation between the levels of PIs present in seeds of various cowpea varieties and the resistance to a major insect pest (*Callosobruchus maculates* (L.)) also indicated a protective role for PIs in crops (Gatehouse et al., 1979).

Proteinase inhibitors may play an important role in programmed cell death (PCD), which takes place in the course of development and aging of plant tissues. Cysteine and serine proteinases play a key role during the formation of conducting tissues in plants (Beers et al., 2000). Proteolysis control by the inhibitors may also take place in vegetative organs of plants. The activation of the proteinase in the course of germination is likely the consequence of dissociation of its complex with the inhibitor (Voskoboinikova et al., 1990; Belozersky et al., 1990).

Increased plant resistance to infestation with insects did not necessarily result from transfection with genes encoding proteinase inhibitors (Gatehouse et al., 2000; Girard et al., 1998). The main reason for such observations is the presence in phytophagous insects of a variety of mechanisms that counter adverse effects of plant proteinase inhibitors. The simplest mechanism involves increasing the secretion of the proteinase sensitive to the inhibitor (De Leo et al., 1998). Alternatively, the inhibitor may be cleaved by those intestinal proteinases that are resistant to it (Girard et al., 1998). In

yet other cases, new forms of proteinases are secreted which are also inhibitor-resistant (Jongsma et al., 1995; Michaud., 1997). All such responses are due to existence in insects of gene families encoding proteinases that differ in their sensitivity to inhibitors. For example, *H. armigera* has at least 28 serine proteinase genes (Bown et al., 1997). The enzymes resistant to the inhibitors are distinguished from their sensitive counterparts by the structure of those regions of their molecules that come into immediate contact with the inhibitor (Bown et al., 1997). The adaptation of the insect digestive system may be more intricate. This was the case of the weevil *Collosobruchus maculatus* when exposed to soybean cystatin scN (Zhu-Salzman et al., 2003), and Colorado potato beetle, (*L. decemlineata*) fed on the leaves of potato with an increased proteinase content (Gruden et al., 2003). A simultaneous increase in the expression of proteinases (both sensitive and resistant) enzymes initiating proteolytic degradation of the inhibitor took place in the weevil (Zhu-Salzman et al., 2003).

As shown in a number of studies, proteinase inhibitors of plant origin are capable of suppressing the activity of enzymes contained in the digestive tract of insects (Ryan, 1990; Gatehouse et al., 1999). It has also been demonstrated that maintaining insects on a diet rich in inhibitors of serine and cysteine proteinases suppresses growth, development, and reproduction (Broadway and Duffey, 1986a, b; Gatehouse and Boulter, 1983; Kuroda et al., 1996). Inhibitors of serine proteinases exerted more pronounced effects in lepidopterous insects (Lepidoptera), in which these enzymes play the major role in food protein cleavage (Ryan, 1990; Mosolov and Valueva, 2005). Cystatins more strongly affect coleopterans, which are known to have active cysteine proteinases in the intestine (Benchekroun et al., 1995; Lecardonnel et al., 1999). Based on the available evidence, it is concluded that adverse effects of plant proteinase inhibitors on insects is due to suppression of the normal assimilation of food proteins (Ryan, 1990; Gatehosue et al., 1999).

2.13 Proteolytic enzymes in plant protection

Plant PIs (PPIs) are small proteins generally present in high concentration in storage tissues, contributing upto 10% of the total protein content; they are also detectable in leaves in response to the attack of insects and pathogenic microorganisms

(Ryan, 1990). PPIs have been shown to play a potent defensive role against predators and pathogens. Many PIs have been shown to act as defensive compounds against insect pests by direct assay or by expression in transgenic crop plants, and a body of evidence for their role in plant defense has continued to accumulate (Krattiger, 1997; Lawrence and Koundal, 2002).

The first data suggesting that inhibitors of proteolytic enzymes are involved in plant defenses against insects were obtained in the mid-1960s: proteins acting as specific inhibitors of intestinal proteolytic enzymes of insects of the genus *Tribolium*, which cause severe loss of grain and grain products, were isolated from soybean and wheat seeds (*Triticum aestivum* L.) (Birk et al., 1963; Applebaum and Konijn, 1966). Another important line of evidence comes from experiments with tomato and potato leaves; their mechanical injury or damage done by Colorado potato beetle (*Leptinotarsa decemlineata*) caused systemic induction of serine protease inhibitors (Green and Ryan, 1972; Mosolov et al., 2001). Systemic induction of proteinase inhibitors by plant tissue injury was most extensively studied in experiments with representatives of the family Solanaceae, tomatoes, potato (*Solanum tuberosum* L.), and tobacco (*Nicotiana tabacum* L.) (Green and Ryan, 1972; Pena-Cortes et al., 1988; Ryan, 1992; Sanchez-Serrano et al., 1986). However, similar mechanisms induce the synthesis of proteinase inhibitors in other plants belonging to various families (Brown et al., 1985; Bradshaw et al., 1989; Eckelkamp et al., 1993; Mosolov et al., 2001). Many plant proteins primarily described as inhibitors of trypsin and chymotrypsin are effective in inhibiting proteinases contained in insect intestines (Birk and Applebaum, 1960; Broadway and Duffey, 1986b; Larocque and Houseman, 1990). This is due to the similarity of properties of intestinal serine proteinases from insects and mammals. Inhibition of proteinases in insects suppresses their growth and development and may eventually cause death. The first studies addressed only the effects of plant-derived inhibitors on insect serine proteinases. Further studies found other classes of digestive proteinases in insects. Phytophagous insects have acquired, in their evolution, some mechanisms attenuating the adverse effects of plant proteinase inhibitors.

The simplest mechanism is proteolytic cleavage of the enzyme by those proteinases of the insect intestine, which remain insensitive to the inhibitor. Because

intestines of most insects contain a set of proteolytic enzymes of various classes with different specificities, this mechanism is widespread (Michaud, 1997; Girard et al., 1998).

Phytophagous insects most probably acquired the ability to synthesize inhibitor-insensitive proteases during natural selection and this is a result of their constant contact with host plants whose characteristic feature is a high level of inhibitors of proteolytic enzymes (Jongsma et al., 1996). Because of this, the most effective inhibitors of insect proteases suitable for practical purposes must be searched for among plants that do not normally host the particular insect species. For example, the activity of intestinal proteinases from cotton noctuid was suppressed by inhibitors from the groundnut (*A. hypogea*), winged bean (*Psophocarpus tetragonolobus* (L.) Dc), and potato, but was not sensitive to proteinase inhibitors from cotton (*Gossypium arborentum* L.), or other natural hosts of this insect. It is important that the inhibitors belong to a different protein family, not the protein family of the host plant inhibitors (Broadway, 1996). So this is another possible search strategy to use protease inhibitors originating from non-host plant sources.

2.14 Applications of plant PIs

Plant PIs are involved in plant defense, regulation of endogenous proteinases, and protein storage. Whether plant PIs can be used in commerce has drawn great attention, and by 1991, plant PIs had already appeared in therapeutic use and laboratory applications (Richardson, 1991; Birk, 1993; Troll and Kennedy, 1993; Banerji and Fernandes, 1994; Abdel-Meguid et al., 2000). A great deal of early work on the therapeutic possibilities of plant PIs in the treatment of a wide range of disorders, such as pancreatitis, shock, allergy, and inflammation associated with enhanced proteolytic activities had resulted in several kallikrein trypsin inhibitor-based drugs (Richardson, 1977).

Plant PIs active towards proteases that regulate human physiological processess, e.g. cell signaling/migration, digestion, fertilization, growth, differentiation, immunological defense, wound healing and apoptosis, have great potential in therapeutic applications (Abdel-Meguid, 2000; Leung et al., 2000). Several plant PIs such as soybean trypsin inhibitor, which are readily available from commercial sources or conveniently

prepared in relatively large quantities at low cost, have been successfully used for the affinity purification of their inhibited proteases from a wide variety of sources (Richardson, 1977; 1991).

2.15 Transgenic plants: A biotechnological approach

Genetic engineering, a rapidly developing field of biotechnology is related to the use of transgenic plants as bioreactors for obtaining proteins of commercial or medical importance (enzymes, hormones, antibodies, blood plasma proteins, etc.) (Daniell et al., 2001). Plant expression systems used for obtaining recombinant proteins have certain advantages over their microbial or animal counterparts. The low net cost of the final product is the major advantage (Daniell et al., 2001; Goldstein and Thomas, 2004). Among the drawbacks in the low target protein (TP) yield, this was observed in a number of cases. One of the reasons for low yield is the rapid cleavage of foreign proteins by proteolytic enzymes (Doran, 2006). In this connection, extensive use of natural inhibitors of proteinases may become a potentially promising way to increase the yield of recombinant proteins in plant expression systems (Michaud, 2000; Outchkourov et al., 2003).

The use of methods of genetic engineering for obtaining plant forms that would be resistant to insects' pests is a major field of contemporary biotechnology (Schuler et al., 1998; Christou et al., 2006; Sharma et al., 2004). Representatives of the first generation of such plants harbor the genes of delta endotoxins (Cry proteins) of the Gram-positive soil bacterium *Bacillus thuringiensis* (Berliner) (*Bt* toxins) (de Maagd et al., 1999; Pigott and Ellar, 2007). The cultivation area of such plants amounted to 22 million hectares in 2004 (Ferry et al., 2006). In addition to developing approaches to increasing the efficiency of such plants, other proteins with pronounced insecticide and antimicrobial activities are currently being tested as candidates for expression in crop cultures. These proteins include, first of all, lectins and inhibitors of proteolytic enzymes, which constitute an important component of the natural plant defense system (Schuler et al., 1998; Ryan, 1990). Serine proteinases from different sources are involved in protoxin activation. In addition to serine proteinases in the bacterium, mammalian trypsin and chymotrypsin can degrade the protoxin (Chestukhina et al., 1982). Insect proteinases with

trypsin- or chymotrypsin like specificities can also hydrolyze *Bt* protoxins (Milne and Kaplan, 1993; Dai and Gill, 1993; Oppert et al., 1996; MartÌnez and Real, 1996; Carroll et al., 1997).

Several transgenic plants expressing PIs have been produced in the last two decades and tested for enhanced defensive capabilities, with particular efforts directed against insect pests (Valueva et al., 2001). Since the economically important orders of insect pests namely Lepidoptera, Diptera and Coleoptera use serine and cysteine proteinases in their digestive system to degrade proteins in the ingested food, efforts have generally been directed at genes encoding PIs active against these mechanistic classes of proteases for developing transgenic plants. The PI genes have been particularly useful in developing transgenic plants resistant to insect pests, as they require the transfer of a single defensive gene, and can be expressed from the wound-inducible or constitutive promoters of the host (Boulter, 1993). The first PI gene to be successfully transferred was that coding for CpTI and produced transgenic tobacco with significant resistance against tobacco hornworm (*Manduca sexta* L.) (Hilder et al., 1987). The efficiency of transgenic tobacco plants expressing CpTI was tested against armyworm (*Spodoptera litura* Fab.) in feeding trials under laboratory conditions. Reduction to the extent of 50% was observed in the biomass of armyworm larvae fed on transgenic leaves expressing 3 - 5 µg of CpTI/g of fresh leaves (Sane et al., 1997). Potato PI-II gene from potato was introduced into several japonica rice varieties to produce transgenic rice plants shown to be insect resistant in greenhouse trials. Wound-inducible PI-II promoter with the first intron of rice actin I gene was able to give high-level expression of PI-II gene in transgenic rice plants. These transgenic plants were resistant to pink stem borer (*Sesamia inferens*) (Duan et al., 1996).

PIs have potential for biotechnological use as natural resistance to insect pests. A number of transgenic plants expressing the genes of serine and cysteine proteinases have been obtained over the part two decades (Jongsma, et al., 1996; Michaud, 2000; Schuler et al., 1998). In a number of cases, the degree of plant protection (assessed by the level of damage or the effects on the insects) was as high as 50%. However, this value is still lower than those obtained for plants harboring the genes of *Bt* toxins (95% or higher) (Gatehouse, 2000). The main reason lies in rapid adaptation of the digestive tract of

phytophagous insects to the effects of the inhibitors, which is, in turn, due to genetic diversity of proteolytic enzymes (Christou et al., 2006; Ferry et al., 2006). Further refinement of the method requires that new, more efficient proteinase inhibitors, be identified (or those already known, modified, including by constructing hybrid proteins) (Urwin et al., 1995; Koiwa et al., 2001; Kiggundu et al., 2006). Yet another area has been delineated as a result of increasing use of plants for obtaining recombinant proteins (Daniell et al., 2001; Goldstein and Thomas, 2004). Processes of isolation and purification of recombinant proteins are frequently associated with considerable losses, due to degradation by proteinases (Doran, 2006; Outchkourov et al., 2003). An efficient means of protection of recombinant proteins may be explained by in coexpressing TPs and proteinase inhibitors (Komarnitsky et al., 2000). Because heterologous expression of proteinase inhibitors does not affect the plants significantly (Michaud et al., 2005; Van der Vyver et al., 2003), such an approach may also be used for solving other biotechnological problems (Michaud et al., 2005).

CHAPTER 3

MATERIALS AND METHODS

The materials utilized and methods used for identification and purification of *Helicoverpa armigera* gut pro-proteinases (HaGPPs), X-ray film processing and data recordings, interactions of HaGPs and HaGPPs with protease inhibitors (PIs), and the proteomics have been described. In this section Genstat10.1 was used for data analysis. Substrate assay methods used to measure total proteolytic activity and specific activity of serine proteases and their comparison with proteases belonging to the related families have been detailed. Methods used to separate and purify the *H. armigera* gut pro- and proteinases have also been described.

3.1 Analysis of *H. armigera* gut proteinases and pro-proteinases

3.1.1 Materials

Bovine trypsin, chymotrypsin, benzoyl-DL-arginyl-*p*-nitroanilide (BA*p*NA), *N*-glutaryl-L-phenylalinine *p*-nitroanalide (GLUPHEPA), *N*-α-benzoyl-L-arginine ethyl ether (BAEE), *N*-α-benzoyl-L-tyrosine ethyl ether (BTEE), succinyl-alanyl-alanyl-propyl-leucine-*p*-nitroanilide (SAAPL*p*NA), azocasein, casein, bovine serum albumin, sephadex G-75, sepharose 4B and 6B, blue dextran, and proenzymes trypsinogen and α-chymotrypsinogen A, and proteinase inhibitors kunitz soybean trypsin inhibitor (KSTI), soybean trypsin-chymotrypsin inhibitor, benzamidine hydrochloride, phenylmethylsulphonylflouride (PMSF), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and *N*-*p*-tosyl l-lysine chloromethyl ketone (TLCK) were procured from Sigma Chemicals, USA. Protease inhibitors containing synthetic inhibitors such as antipain-dihydrochloride, bestatin, chymostatin, E-64, leupeptin, pefabloc, EDTA-NA_2, and aprotinin were purchased from Roche Diagnostics Gmbh, Mannheim, Germany. Agfa X-ray films were obtained from SELVAS photographic Ltd., Silvassa. All other chemicals used were of analytical grade and purchased locally.

3.1.2 Rearing of *H. armigera* larvae in the laboratory

The *H. armigera* larvae were reared on an artificial diet (Armes et al., 1992) under controlled conditions in the laboratory at ICRISAT, Patancheru, India. The laboratory culture was regularly supplemented with field-collected larvae. The laboratory conditions were maintained at 26 ± 1°C temperature, 60 - 70% RH and 16 h daylight.

Adults emerged from the pupae after 6 - 10 days. Mating occurred 1 – 2 days after adult emergence. Adults can be sexed based on the colouration pattern of the wings. Females are chocolate brown coloured with variations in colour intensity, whereas males are pale brown. After a preoviposition period of 2 - 3 days, eggs were laid on nappy liners inside a 30 × 30 × 30 cm wire mesh covered cage. Adults survive for 8 - 10 days life. Moths were maintained in the cages until death. Honey (10%) was provided to the adults as a food on a cotton swab at the bottom of the cage. The adult food was changed on alternate days. The eggs laid on nappy liner were collected daily and treated with 0.1% sodium hypochloride solution and kept in a plastic box (10 × 5 cm) filled air. The eggs from each pair were kept in separate boxes. After hatching, neonate larvae were transferred into a plastic cup (diameter 20 cm) containing 2 – 3 mm layer of chickpea based diet on the bottom and on the rides. After 5 days, when the larvae become cannibastics, they were transferred individually into 6 – cell well plates.

Bioassays were conducted by feeding the *H. armigera* larvae on control (chickpea PIs removed artificial diet) or the diet having the host or non-host PIs. Composition of the artificial diet was given in Table 3.1. Thirty, early third-instar larvae were reared on the artificial diet with different treatments, and gain in weight was recorded on 5^{th} or 7^{th} day, at the time of termination of experiment, detailed in section 3.3.4. There were three replicates in a randomized complete block design.

3.1.3 Extraction of in- and active proteinases from *H. armigera* gut extract

Fresh, five hour starved *H. armigera* larvae or the larvae fed on artificial diet along with synthetic inhibitors were separately immobilized by keeping in freezer (-20 °C) for 30 min, and dissected out midventrally. The midguts were removed, collected separately, and stored at –20°C until needed. Midgut tissues were weighed and homogenized separately in 3 volumes of 0.2 M glycine-NaOH buffer (pH 10.0), and the larvae fed on SIs or extraction buffer containing a cocktail of synthetic inhibitors (aprotinin, E-64 or leupeptin, pefabloc sc and pepstatin), and the homogenates were centrifuged at 14000 rpm at 4°C for 20 min and the supernatants were used as crude preparations of *H. armigera* gut proteinases (HaGPs) and pro-proteinases (HaGPPs),

respectively. Proteins in crude gut extracts of HaGPs and HaGPPs were estimated by Lowry's method (1951), keeping bovine serum albumin as a standard.

Table 3.1 Composition of standard larval diet used for rearing *Helicoverpa armigera* under laboratory conditions.

Ingredients	Quantity
A. Chickpea flour	300.0 g
B. Ascorbic acid	4.7 g
C. Methyl-p-hydroxybenzoate	5.0 g
D. Sorbic acid	3.0 g
E. Auromycin powder	11.5 g
F. Vitamin stock solution	10.0 ml
G. Water	450.0 ml
H. Yeast	48.0 g
I. Agar	17.3 g
J. Water (Agar)	800.0 ml
Vitamin stock solution	
Nicotinic acid	1.528 g
Calcium pantothenate	1.528 g
Riboflavine	0.764 g
Aneurine hydrochloride	0.382 g
Pyridoxine hydrochloride	0.382 g
Folic acid	0.382 g
D-Biotin	0.305 g
Cyanocobal amine	0.003 g
Water	500.0 ml

3.1.4 Trypsinogen, chymotrypsinogen and proteinases assay

Trypsinogen, chymotrypsinogen and/or *H. armigera* gut trypsinogen, and chymotrypsinogen activation, total gut pro- and proteinases activity, trypsin, and chymotrypsin were measured by trypsinogen assay (Bermeyer et. al., 1974; Perlmann and Lorand, 1970), chymotrypsinogen assay (Rick 1974), caseinolytic (Belew and Porath, 1970), and /or azocaseinolytic (Brock et. al., 1982) assays, BA*p*NA assay (Erlanger et. al., 1964), and GLUPHEPA assay (Muller and Weder, 1989), respectively.

3.1.4.1 Trypsinogen assay

The *H. armigera* gut trypsinogen isoforms activity was measured by trypsinogen assay. For this, *H. armigera* gut extracts and trypsinogen solution standard were prepared. Activating reaction mixture was prepared in 2 ml of 1 M calcium chloride, 38 ml of 400 mM Tris-Cl buffer (pH 8.4) at 37°C, and 2 ml of trypsin enzyme solution, and mixed gently by swirling. Total trypsin activity of trypsinogen and/or HaGPP were measured at zero time, and then 0.1 ml of 5 mg/ml of trypsinogen and/or HaGPP extract was added to 1 ml of the activating mixture, and incubated at 5°C for 24 - 28 h, trypsin assay was carried out. Free trypsin activity was measured by keeping activating mixture (trypsinogen) for 24 - 28 h at 0 - 5°C. After 24 h, 0.1 ml of trypsinogen was added to 1 ml of the activating mixture, and trypsin activity measured immediately. For above tests, suitable blanks were kept and equilibrated at 37°C. Optical density was recorded at 253 nm until constant, using a suitable thermostat spectrophotometer. Activating mixture and enzyme solution were immediately mixed by inversion and recorded the increase in $A_{253\ nm}$ after 5 min. Observations were recorded at $A_{253\ nm/minute}$ using maximum linear rate for both the test and blank. One trypsinogen unit was defined as one BAEE unit that produces $A_{253\ nm}$ of 0.001 per minute, with BAEE as a substrate at 37°C (pH 7.6) in a reaction volume of 3.2 ml (1 cm light path).

3.1.4.2 Chymotrypsingoen assay

The *H. armigera* gut chymotrypsinogen isoforms activity was measured by the chromogenic substrate BTEE at 37°C, pH 7.8, using continuous spectrophotometer rate determination method. For this assay, 1 mM HCl solution, 80 mM Tris-Cl buffer (pH

7.8), 1.18 mM BTEE in methanol, 2 M calcium chloride solution ($CaCl_2$), 0.1% (w/v) trypsin enzyme solution, and α-chymotrypsinogen A (Chymo A, immediately before use) were prepared in 1 ml of trypsin solution incubated with chymotrypsinogen and/or HaGPPs at 37°C for 3 h. For recording free chymotrypsin activity, 10 mg of chymotrypsinogen and/or HGPPs were dissolved in 10 ml of 1 mM HCl solution, and immediately processed for chymotrypsin assay. In a 3 ml reaction mixture, the final assay concentrations were 38 mM Tris, 0.55 mM BTEE, 30% (v/v) methanol, 53 mM calcium chloride, 0.03 mM hydrochloric acid, 0.48 µg trypsin, and 4.3 µg of α-chymotrypsinogen A and/or HaGPPs. Tris buffer, substrate BTEE, and $CaCl_2$ were pipetted into two tubes separately. For the test, 0.1 ml activating mixture was added in one tube, while for the blank 0.1 ml of HCl solution was added in another tube. The increase in absorbance maxima at $A_{256\ nm/minute}$ was recorded for 5 min to obtain maximum linear rate for both, the test and the blank. One chymotrypsinogen activity unit was defined as one unit of enzyme that hydrolyzed 1.0 µmole of BTEE per minute at 37°C (pH 7.8).

3.1.4.3 Proteinase assay

Total gut proteinase activity was determined by caseinolytic assay. For this different concentration of *H. armigera* gut extracts were added to 2 ml of 0.5% casein (in 0.2 M glycine-NaOH buffer, pH 10.0) and incubated at 37°C for 20 min. The reaction was terminated after 30 min by the addition of 3 ml of 5% trichloroacetic acid (TCA). The optical density was measured at 280 nm. For azo-caseinolytic assay, 60 µl diluted enzyme was added to 200 µl of 1% azo-casein (in 0.2 M glycine-NaOH buffer, pH 10.0), and incubated at 37°C for 30 min. The reaction was terminated by the addition of 300 µl of 5% TCA. After incubation at room temperature for 30 min, the tubes were centrifuged at 10,000 rpm for 10 min and an equal volume of 1 N NaOH was added to the supernatant. The activity was estimated by measuring the OD at 450 nm. Trypsin and HaGPs activities were estimated using the chromogenic substrate *N* α-benzoyl-l-arginyl-*p*-nitroanilide (BA*p*NA). For trypsin assay, 150 µl of diluted *H. armigera* gut extract enzyme was added to 1 ml of 1 mM BA*p*NA (in 0.2 M glycine-NaOH, pH 10.0) and incubated at 37°C for 10 min. The reaction was terminated by the addition of 200 µl of 30 % acetic acid, the OD was measured at 410 nm. Bovine chymotrypsin (25 µg) or HaGP

extract were added to different tubes and the volume was made up to 700 µl with 0.2 M glycine-NaOH buffer (pH 10.0). Twenty-five microliters of GLUPHEPA (10 mg/ml in dimethyl formamide) was added to each tube and the reaction mixture was incubated at 37°C for 1 h. The reaction was terminated by the addition of 200 µl of 30% acetic acid, and the OD was measured at 405 nm. The *H. armigera* gut extracts were treated with trypsin (0.0001% in activation mixture), followed by proteinase assay for determining the activity of pro-proteinases.

3.1.5 Visualization of HaGP isoforms and activation of pro-proteinases on GXCP

Visualization of HaGP isoforms on native (Davis, 1964) and denatured (SDS) (Laemmli 1970) polyacrylamide gel electrophoresis was carried out using the gel X-ray film contact print technique. For the gel casting, vertical slab gel electrophoresis (160 × 140 × 1.5 mm) was used. Samples for activation and SDS-PAGE were used without heating, and without addition of reducing agents such as β-mercaptoethanol or dithiothreitol (DTT). Ten or 12% resolving gel was used for polymerization. The stacking gel (6% for native PAGE and 3% for SDS-PAGE) was poured on top and electrophoresis was carried out at 150 V at a constant current of 30 mA. After 6 - 8 h, when the tracking dye front reached at the bottom of the running gel, the gel was removed and shaken gently (100 rpm) for 15 min in 0.2 M glycine-NaOH buffer, pH 10.0. Proteinase activity bands in native gel were visualized. In the case of denatured gel, the SDS-PAGE was washed thrice for 5 min with 2.5% Triton X-100 dissolved in the 0.2 M glycine-NaOH buffer, pH 10.0, prior to incubation in assay buffer. After equilibration, the gel was overlaid on unprocessed X-ray film for 30 - 40 min at 37°C. For the second exposure, the gel was rinsed in 0.2 M glycine-NaOH buffer, pH 10.0, briefly for 5 min, and placed on another film with second side of the gel in contact with the film. These X-ray films were placed in tray containing hot water (60 - 70°C) and shaken to facilitate removal of hydrolyzed gelatin in the vicinity of proteinase activity. These X-ray films were developed and then contact printed. Two or three successively exposed films were overlapped carefully to match the profiles, to compare and confirm the mobility of proteinase bands on X-ray film.

For visualization of activation of pro-proteinase bands, all the above mentioned steps were carried out, but for activation of *H. armigera* gut pro-proteinases, trypsin (0.0001%) solution was used as a activator, which was prepared in 0.2 M glycine-NaOH buffer, pH 10.0. After activation, the gel was washed with the same buffer for twice for removal of excess of trypsin and the GXCP was carried out.

3.1.6 Factors affecting on activity of HaGP and activation of HaGPPs

The following discontinuous buffers were used for determination of activity of HaGPs and activation of HaGPPs at different pH values (pH 3, 200 mM glycine-HCl; pH 4 and 5, 200 mM sodium acetate; pH 6, 200 mM citrate-phosphate; pH 7, 200 mM phosphate buffer; pH 8, 100 mM Tris-Cl; pH 9, 10, and 11, 200 mM glycine-NaOH). Azocasein and BAEE were used as substrates for HaGPs and trypsinogen isoforms of HaGPPs, respectively.

For determination of temperature optima, for HaGPs, the insects were fed on chickpea (PIs removed), and HaGPPs the insects were fed on synthetic SIs. HaGPs and HaGPPs gut extracts were co-incubated with substrates azocasein and BAEE, respectively, for 30 min at 4, 10, 20, 30, 40, 50, 60, 70, and 80°C. Subsequently, the assays were carried out for determining temperature optima for the activities of HaGP and HaGPPs.

Effect of Ca^{2+} ion concentration on activation HaGPPs was measured by BAEE as a substrate at physiological (0 - 1 M) and non-physiological (1 - 10 M) concentration range in activation reaction buffer, respectively.

3.1.7 Pro-proteinase and proteinase purification and characterization by column chromatography and electrophoresis

The *H. armigera* gut pro-proteinases and proteinases were partially purified by column chromatography (affinity and size exclusive chromatography) and electrophoresis, and characterized using chromogenic substrates such as azocasein, BA*p*NA, GLUPHEPA, BAEE, and BTEE.

3.1.7.1 Affinity chromatography

Benzamidine sepharose 4B and/or 6B fast flow (high sub) were used for removal and/or purification of trypsin, trypsin-like serine proteinases, as well as, zymogens. These reversibly bound proteins can be eluted out by using either pH and/or salt gradients. Benzamidine 4B and 6B are excellent tools for removal and/or purification of trypsin and zymogens, and was prepared by attaching benzamidine (or *p*-aminobenzamidine, *p*ABA) to matrices of sepharose 4B and/or 6B (highly cross linked 4% agarose). A 1.6 × 13 cm of bed size, with 2 - 34 ml of bed volume was used in 1.6 × 30 cm size of the column. Slurry of sepharose 4B was prepared in pre-equilibrating the buffer, and poured in the column. The material was allowed to settle and the column was equilibrated with 0.25 mM Tris-Cl buffer, pH 7.2. 0.05 M Tris, 0.5 M NaCl, pH 7.4 buffer was used for binding and/or washing the matrices, while 0.1 M Tris-Cl, pH 8.0 buffer was used for elution. Acetone precipitated, concentrated protein was loaded onto a column and allowed to settle for 10 min on the upper surface of matrix bed. The column was then eluted (3 times the column volume) using the elution buffer as mentioned earlier, at a flow rate of 1 ml min^{-1}, and 2 ml fractions were collected. After that, a buffer salt (1 N NaCl in same elution buffer) gradient of five times the column volume was run. The 2 ml fractions were collected and their optical density (O.D.) was measured at 280 nm. Affinity chromatography was carried out under cold condition.

3.1.7.2 Size exclusion chromatography (Gel filtration column chromatography by Sephadex G75)

XK 16/30 (16 mm i.d.) column with bed volume of 2 - 40 ml and bed thickness of 1 - 20 cm was used for gel filtration. The column was packed with sephadex G-75 preparative grade matrix that has the capacity to separate 3,000 to 80,000 Da molecular weight compounds. The matrix was initially washed with 20% alcohol and degassed for 2 h under suction. It was prewashed with distilled water to remove the traces of alcohol and equilibrated with 0.1 M Tris-Cl buffer, pH 8.0, and loaded onto the column so as to fill the entire column (30 cm in length). It was then washed with 0.1 M Tris-Cl buffer, pH 8.0 (three times the column length).

Concentrated, acetone precipitated samples and/or affinity purified HaGPPs fractions were loaded on the column (1 ml), and eluted out by 0.1 M Tris-Cl buffer, pH 8.0. The fractions (2 ml) were collected manually at a fixed time interval, and protein concentration measured at 280 nm.

3.1.7.3 Electrophoresis

The *H. armigera* gut extract was loaded in single lane on 10% native-PAGE, and visualized by overlaying the gel on X-ray film as described in 3.1.5. These visualized bands on X-ray film were used for locating the position of proteinases in the gel, and were excised by cutting the gel. Immediately, the individual excised gel strips were stored in centrifuge tubes with activity buffer at -20°C. Proteinases were isolated by cutting the respective gel strips from the stored gel, and crushed in 0.2 M glycine-NaOH, pH 10.0, to elute out the proteinases. The clear supernatant containing the proteinases was obtained by centrifuging at 14,000 rpm at 4°C for 30 min, and its homogeneity checked. For pro-proteinases, the *H. armigera* gut was extracted in a cocktail of synthetic inhibitors. The gut proteinases were separated on 10% native-PAGE in one well, and lane was cut after electrophoresis. The gel strips with proteinases and pro-proteinases, and were used for activation. The activated proteinase bands were visualized on X-ray film, and the corresponding pro-proteinases were excised from gel, and stored in a centrifuge tube at -20°C. After detecting the position of the pro-proteinases, bands in gel strips from the stored gels were crushed and obtained in 0.2 M glycine-NaOH buffer, pH 10.0 to elute out the pro-proteinases.

Single lane preparative polyacrylamide gel was used for purification of pro-proteinases. For this small vertical gel strip was cut from the pro-proteinases separated on gel. The strip was processed for detection of pro-proteinases by the method described above. The rest of the gel was stored at 4°C. After detecting the position of the pro-proteinase bands in the gel, each band one was isolated by cutting it in 0.2 M glycine-NaOH buffer, pH 10.0, to elute out the pro-proteinases. After centrifugation at 14,000 rpm at 4°C for 15 min, the clear supernatant containing pro-proteinases was used for checking its homogeneity, and characterization.

3.1.8 SDS/PAGE analysis of pro-proteinases

Acetone precipitated *H. armigera* gut proteins from larvae fed on SIs and/or freeze dried proteins of pooled column fractions were dissolved in the sample buffer containing 0.5 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 50% (v/v) glycerol, and 0.01% (w/v) bromophenol blue, to obtain final concentrations of 25-30 mg of crude protein ml^{-1} and/or 1 mg purified protein ml^{-1}. Two units BA*p*NAase activity and/or four units BAEEase activity were loaded onto a denaturing 12 or 15% (w/v) polyacrylamide slab gel (Laemmli, 1970) without heating the samples, and/or without addition of reducing agents (β-mercaptoethanol/dithiothreitol). The gels were run at a constant current of 30 mA fixed, and stained for 30 min with a 0.5% Coomassie Brilliant Blue R (SRL, India) solution containing 40% methanol and 10% acetic acid or silver stained. Where indicated, densitometric quantitation of bands was also carried out. Gels were dried between two layers of cellophane as per gel drying procedure. Dried gels were scanned at 300 d.p.i. resolution in gray-scale mode, and the images were saved as JPEG files.

Visualization HaGP isoforms or activation of HaGPPs after12% SDS-PAGE were carried out using the gel X-ray film contact print technique. After electrophoresis, the gel was washed with 2.5% Triton X-100 in 0.2 M glycine-NaOH for removing SDS from the gel. Then, HaGPPs isoforms were activated by immersing the gel in 0.0001% trypsin solution (0.2 M glycine-NaOH, pH 10.0). After activation, excess trypsin was removed. The gel was equilibrated in the same buffer solution, and then overlaid on unprocessed X-ray film. After 30 min (the same exposer time period was maintained for visualization of HaGP and activation of HaGPPs), the gel was removed and the X-ray film was washed with tap water to reveal the proteinase activity and/or activated proteinase bands as hydrolyzed gelatin. The X-ray film was developed and then contact printed.

3.1.9 Analysis of fecal matter

Ten early fifth-instar or late fourth-instar of *H. armigera* were fed on the chickpea based (PIs removed) artificial diet having synthetic inhibitors and/or a cocktail of SIs for three days. Fecal matter was collected on the third day. The fecal matter was also collected from the fifth-instar larvae fed on control artificial chickpea (PIs removed) diet. The feces were freeze-dried and thus protein extracted in 0.2 M glycine-NaOH buffer, pH

10.0 and used for proteinase and pro-proteinase activation analysis, either on gel or in solution assays using specific substrates.

3.1.10 Two dimensional run of crude and purified *H. armigera* gut pro-proteinases

Acetone precipitated protein and freeze dried proteins pooled from the column were dissolved in sample buffer, and separated by non-denaturing polyacrylamide gel electrophoresis (native-PAGE), 10% gel in a vertical slab gel unit using a Davis buffer system. After polymerization of the resolving gel (10 or 12%), the stacking gel (6% for native PAGE) was poured on top, and electrophoresis was carried out at 25°C at constant current 20 mA. When the tracking due front reached at the bottom of the resolving gel (approximately 6 - 8 h), the gel was removed and incubated in activation solution (0.0001% trypsin prepared in 0.2 M glycine-NaOH buffer, pH 10.0) and shaken gently (100 rpm) at 37°C for 15 min. After activation of *H. armigera* gut pro-proteinases, the excess trypsin was removed by washing the gel strip twice with equilibration buffer, and placed on another 12% resolving gel (native-PAGE). The strip was inserted into glass plates and the strip was gently pushed in to that the lower edge of the strip came in contact with the top surface of the slab gel. No air boubbles were trapped between the gel strip, between the gel and the glass plate. The stacking gel stock was poured on top of resolving gel and allowed to polymerize. After polymerization, electrophoresis was carried out at a constant current 20 mA for 6 - 8 h. After electrophoresis, the gel was incubated in 0.2 M glycine-NaOH buffer, pH 10.0 for 10 min, followed by X-ray film contact printing. Two to three prints were taken for activation of HaGPPs and subsequently for inhibition of HaGPs in another experiment, and inhibition and activation profiles were compared. The same gel or different gels with the same sample were stained with silver nitrate using the standard protocol.

3.2 Analysis of host and non-host plant protease inhibitors

3.2.1 Materials

For assessing the PIs activity, seeds of medicinal plants were purchased from the local market, Aurangabad, India. Azocasein, BA*p*NA, BTEE, BAEE, and GLUPHEPA were obtained from Sigma chemicals, USA. Trypsin, chymotrypsin and

polyvinylpyrrolidon (PVP) were purchased from SRL. All chemicals were of analytical grade and purchased from Qualigens or SRL chemicals, India.

3.2.2 Preparation of host and non-host plant PIs extract

Seed powders of the host and non-host plants were prepared by grinding decorticated matured seeds in a mortal and pestle, and dehydrated, depigmented and defatted by washing at least six times with acetone, hexane, and Folch's mixture (chloroform: methanol, 2:1). The solvents were removed by filtration and the seed powders were air-dried. The defatted seed powders were mixed with 6 volumes of distilled water containing 1% PVP, and kept overnight at 4°C for extraction with intermittent shaking. After 12 h, the suspension was centrifuged at 10,000 rpm for 30 min at 4 °C, and the supernatant was stored in aliquots at -20°C. Protein concentration of the PIs extracts was quantified by Lowry's method (1951) using BSA as a standard.

3.2.3 Preliminary screening

3.2.3.1 Detection of protease inhibitors by dot-blot method

In our laboratory, we used a simple, rapid and sensitive technique, called X-ray film method, for the estimating host and non-host serine protease inhibitor activity, developed by Pichare and Kachole (1994).

Trypsin and chymotrypsin solutions were prepared in 0.1 M Tris-HCl buffer, pH 7.8 to obtain a final concentration of 0.1 mg ml^{-1}. Three varying concentrations of the enzyme and inhibitor 3:1, 1:1, and 1:3 (v/v), were prepared. The volume of the reaction mixture was adjusted with Tris-HCl buffer for trypsin and chymotrypsin and glycine-NaOH buffer for HaGP. The final volume was made upto 20 µl, and then spotted onto a strip of X-ray film. Spots were incubated for 20 min on X-ray film depending on the extent of gelatin hydrolysis. The film was washed with warm water. When the inhibitor is present, the trypsin/chymotrypsin did not degrade the gelatin on the X-ray film. When the inhibitor was absent, a clear zone formed at the site of sample application on the X-ray film. The reverse side of the film was cleared with trypsin/chymotrypsin, and the film scanned.

3.2.3.2 Electrophoretic visualization of proteinase inhibitor bands by GXCP

Seed extracts containing protease inhibitors were subjected to electrophoretic analysis by non-denaturing acidic and denaturing SDS-PAGE in a vertical slab gel electrophoresis. Native-PAGE and SDS-PAGE were carried out according to Davis (1964) and Laemmli (1970) methods, respectively. Basic gel electrophoresis was carried out in 10% acrylamide solution using the Davis buffer system with Tris-HCl buffer (pH 8.3) in the stacking gel. Denaturing gel electrophoresis i.e. SDS-PAGE (10 or 12%) was carried out according to the Laemmli method except that the sample was not containing β-mercaptoethanol/DTT, and/or, the samples were not heated in boiling water. The non-denaturing gel after electrophoresis was processed for activity profiling of PIs by the X-ray film contact print technique (Pichare and Kachole, 1994). The denaturing gels were washed with non-ionic detergent 2.5% Triton X-100 three times. Then, the gel was incubated in 0.1 M Tris-HCl buffer, pH 7.8, for 10 min, followed by incubation in 0.1% trypsin/chymotrypsin solution (0.1 mg ml^{-1}) for 15 min at room temperature. The gel was then briefly rinsed in Tris buffer in to remove the excess trypsin. The gel and X-ray film were placed in a tray and incubated at 37°C. The gel was removed after 5 - 10 min, and the extent of gelatin hydrolysis monitored visually. Depending on the extent of gelatin hydrolysis, the X-ray film was washed with either tap or warm water. For detection of bands with low PI activity, the same gel was overlaid three to four times with different pieces of X-ray films for 10, 15, and 20 min, respectively. Protease inhibitor bands appeared as non-hydrolyzed gelatin against the background of hydrolyzed gelatin. The reverse side of the film was cleared with trypsin, and the film scanned. After developing, the protease inhibitor bands on radiographic film became translucent against the dark opaque background, which produced black bands on a white background on the photographic paper after contact printing. For comparison of sensitivity of detection of inhibitor activity using X-ray film, gels containing triplicate samples were run on electrophoresis and processed under similar conditions.

3.2.4 *In vivo* inhibition of *H. armigera* gut proteinases

Host or non-host plant PIs or synthetic inhibitors incorporated in chickpea based (PIs removed) artificial diet were fed to *H. armigera* larvae, while the standard chickpea

(PIs removed) diet was kept as a control. The *H. armigera* gut proteinases were extracted in 0.2 M glycine-NaOH buffer, pH 10.0, and separated on 10 or 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) without the adding of β-mercaptoethanol/DTT, and/or without heating in boiling water. The entire gel was then equilibrated in 0.2 M glycine-NaOH buffer, pH 10.0 for 10 min. The gel was then overlaid on X-ray film, whilst taking care not to introduce any air boubble between the gel and the X-ray film (Pichare and Kachole 1994). After incubation at 37°C for 30, 40 and 50 min, the X-ray was washed with tap or warm water until the gelatin digested by the separated HaGPs.

3.2.5 Estimation of PIs (trypsin and chymotrypsin assay)

Inhibitory activity of PIs against trypsin, and chymotrypsin, and the HaGPs was measured by using synthetic substrates such as BA*p*NA, GLUPHEPA, and azocasein, respectively. Equal units of the seed extract and/or effective concentrations of SIs were mixed with 25 µg of trypsin, chymotrypsin, and/or equivalent amount of HaGP and allowed to stand for 30 min at 37°C. Total proteinase activity was determined by azocaseinolytic assay (0.2 M glycine-NaOH, pH 10.0 for HaGP) and incubated at 37°C for 30 min. The reaction was terminated by the addition of 300 µl of 5% TCA. After incubation at room temperature for 30 min, tubes were centrifuged at 10,000 rpm for 10 min, and an equal volume of 1 N NaOH was added to the supernatant. The activity was estimated by measuring the OD at 450 nm. The residual proteinase activity was measured by incubating the seed extract with 1 ml of 1 mM BA*p*NA (in 0.2 M glycine-NaOH, pH 10.0 for HaGP; and in 0.1 M Tris-HCl, pH 7.8 for trypsin), and with 25 µl of 1% GLUPHEPA (0.2 M glycine-NaOH buffer, pH 10.0 for HaGP; and for 0.1 M Tris-HCl, pH 7.8 for chymotrypsin) for 15 min at 37°C. The reaction was terminated by the addition of 200 µl of 30% acetic acid. After centrifugation at 10,000 rpm for 10 min, absorbance was measured at 410 nm. One unit of proteinase activity was defined as the amount of enzyme that caused a unit increase of optical density at 410 nm due to release of *p*-nitroaniline; while one PI unit was defined as the amount of inhibitor that inhibited 1 unit of proteinase activity.

3.2.6 Electrophoretic purification of host and non-host plant PIs

The protease inhibitors were partially purified in one step by electrophoretic elution, and separated in a single lane on 10% non-denaturing gel electrophoresis (native –PAGE). The PIs profile was visualized by putting the gel on X-ray film, as described in section, 3.3.3.2. Visualized bands on X-ray film were compared to the gel and used for locating and excising PI in the gel. Individual excised gel strips were stored in centrifuge tubes with 0.1 M Tris-HCl buffer, pH 7.8, at -20°C. The centrifuge tunes were thawed by keeping the tubes at a room temperature and used. Such preparations of individual PIs obtained from two or more preparative gels were pooled as and when necessary. Purity of protease inhibitors was checked by electrophoresis and subsequent printing by using GXCP. These partially purified protease inhibitors were used for analysis of inhibition study.

3.3 Interaction of *H. armigera* gut in- and active proteinases with proteinase inhibitors

3.3.1 Materials

Helicoverpa armigera larvae collected from the pigeonpea and chickpea fields near Hyderabad, which were reared on chickpea flour based artificial diet (Armes et al., 1992) at ICRISAT, Patancheru, India. Seed samples used for assay and inhibition studies were purchased from Davasaj, Aurangabad, India. Acrylamide, N, N'- methylene bisacrylamide, Tris-Cl, and glycine were of analytical grade, and obtained from Sisco Research Laboratories, Mumbai, India. All organic solvents of analytical grade were obtained from Qualigens, India. A chemical inhibitors set was purchased from Boehringer Mannheim, Germany. X-ray films were purchased from AGFA, Selvas photographics limited, Silvassa, India.

3.3.2 Extraction of gut enzyme

Fourth- and fifth-instars *H. armigera* larvae were reared on host and non-host plant PIs in the laboratory were used for the enzyme extraction. The larvae were starved for 5 h to prevent the proteolysis. Starved and freshly collected *H. armigera* larvae were killed by decapitation and the midguts dissected out over ice. The midgut contents

retained within the peritropic membrane were separated from the midgut tissue, and midguts washed with distilled water. The midgut contents were extracted in 0.2 M glycine-NaOH buffer, pH 10.0, containing 3 mg ml^{-1} soybean trypsin inhibitors and 10 mM benzamidine hydrochloride, and/or a cocktail of synthetic inhibitors to prevent activation of the pro-proteinases. The homogenate was allowed to stand for 2 h at 4°C. The suspension was then centrifuged at 14,000 rpm for 15 min at 4°C. The resulting supernatant was collected, frozen in aliquots, and used for purification of pro-proteinases. Protein contents of enzyme solutions were determined, using the Lowry's method (1951), with BSA Fraction V as standard.

3.3.3 Extraction of inhibitors from seeds

Dry mature seeds of host and non-host plants were ground in pestle-mortar, and/or mixer-blender to make a fine powder. The powder was dehydrated, depigmented, and defatted by several washes of acetone, followed by hexane, and Folch's mixture (chloroform: methanol, 2:1). The solvents were removed by filtration and the powders air-dried. The powders were mixed with six volumes of distilled water containing 1 % PVP and kept overnight at 4°C for extraction, with intermittent shaking. The suspension was centrifuged at 10,000 rpm for 20 min at 4°C and the supernatant was stored in aliquots at -20°C. Protein concentration of the host and non-host seed extracts was quantified by Lowry's method (1951).

3.3.4 Feeding assay

Studies on the effects of diet incorporated with synthetic inhibitors, and/or host and non-host plant PIs, on growth and development of *H. armigera* were carried on using third-instar larvae of known age. For this, newly moulted second-instar larvae were taken from the culture, and examined at 9:00 and 18:00 h daily. Any larvae moulting between the observation times were placed in labeled vials. The larvae were weighed, and divided into control and experimental groups. The same numbers of larvae from each chronological age were placed on the test diets, ensuring that variation between populations fed on each diet was minimized. Thirty larvae were placed on each diet, and each feeding assay was carried out in triplicates.

The larvae were initially placed in small, airtight plastic containers (3 cm dia X 4 cm high) with a cube of food (approximately 8 mm^3). By the time, the larvae moulted to the fourth-instar, small airholes were made in the lids, and the amount of food increased to 10 mm^3. Food was changed daily, and in cases where little diet was eaten, the food was changed every 2 days to reduce microbial contamination. Equal quantity of seed powder of host and non-host plant, was added to the chickpea flour in the artificial diet. The control diet (section 3.2.2), had no inhibitor.

Individual larval development was monitored at the same time each day. The feces were collected at the same time each day, freeze-dried, and used for analysis of pro-proteinase activation. The experiment was terminated at the same time on 5^{th} day, and the larval weights were recorded for computing weight gain and growth rate.

3.3.5 Electrophoretic determination of activation of gut pro-proteinases

Midgut serine pro-proteinases inhibition was determined on non-denaturing polyacrylamide (pH 8.8), using the method of Laemmli (1970). For inhibition study, newly moulted third-instar larvae were collected and divided into control-fed and inhibitor-fed (diet containing equal units of host and non-host PIs) groups. At known times, ten larvae from each group were killed by decapitation and their gut contents dissected out. The gut contents of each group of larvae were homogenized separately in 500 µl of 0.2 M glycine-NaOH buffer, pH 10.0. The proteins in these homogenates were precipitated with 1 ml cold acetone and stored overnight at -20°C. Ten insects from each of the two groups were sampled at known time throughout the third-instar stadium. When about 60 - 70% of the larvae had moulted to the fifth-instar, the assay was terminated.

The acetone-precipitated proteins were centrifuged at 10,000 rpm for 20 min at 4°C, and then redissolved in 100 µl of buffer and the protein solution used for electrophoretic separation and enzyme assay.

In inhibition tests, equal quantity of inhibitor fed *H. armigera* gut extracts were incubated with 0.0001% trypsin (activation solution in 0.2 M glycine-NaOH buffer, pH 10.0) at room temperature for 30 min before electrophoretic separation. Electrophoresis was performed at room temperature at constant current of 30 mA till the tracking dye front reached at the bottom the resolving gel (in approx. 4 - 6 h). The gel was removed

and gently shaken at 37°C for 10 min in 0.2 M glycine-NaOH buffer, pH 10.0. For proteinase activity visualization, the gel was overlaid on X-ray film for 30 min (Pichare and Kachole, 1994; Hursulkar et. al., 1998). In this way, three subsequent prints were taken and the results compared by visualization of proteinase activity.

3.3.6 Electrophoretic detection of HaGPPs activation on treatment of HaGP

The *H. armigera* larvae fed on host and non-host plant PIs incorporated in chickpea (PIs removed) diet were analyzed for the activation of HaGPPs by treating individual partially purified HaGPs. HaGPPs from insects fed on non-host plant PIs and electrophoretically purified 10 µg HaGP were co-incubated at 37°C for 30 min, and separated on 10% non-denaturing polyacrylamide gel. After electrophoresis, the gel was incubated in 0.2 M glycine-NaOH buffer, pH 10.0, and placed on an undeveloped X-ray film. The gel and the film were then placed in a tray and incubated at 37°C in a water bath. The appearance of activated proteinase band on X-ray was monitored visually. The film was then rinsed with tap water or placed in a water tray and shaken gently to remove the hydrolyzed gelatin. The gel was rinsed in 0.2 M glycine-NaOH, pH 10.0, and placed on another film with opposite side of the gel in contact with the film. For comparison of sensitivity of detection of activation of HaGPPs activation using X-ray film, a gel containing triplicate samples was cut into three pieces after electrophoresis and processed under similar conditions.

3.3.7 Proteinase and proteinase inhibitor assay for inhibition of HaGPPs

Pro-proteinase fractions were normalized for protein content. Partially purified pro-proteases were pre-incubated with electrophoretically purified trypsin inhibitors at 37°C for 30 min. Substrate in assay buffer was added along with activation buffer (0.0001% trypsin/HaGP in 0.2 M glycine-NaOH, pH 10.0; 2.5 mM $CaCl_2$). Azocasein, BA*p*NA, GLUPHEPA, BAEE, and BTEE, were used for the activation and inhibition assay, and the methods followed were as per section 3.2.4. Assays for inhibitor specificity were carried out in the presence of host and non-host plant PIs, and cocktail of synthetic inhibitors.

3.4.8 Fecal matter analysis

Thirty fifth-instar larvae were fed on chickpea (PIs removed) based diet incorporated with host and non-host plant PIs seed powder in vials for five days. The fecal matter was collected on 4th and 5th day. The fecal matter samples were extracted in 0.2 M glycine-NaOH buffer, pH 10.0, and used for pro- and proteinase analysis either on gel or in solution assay.

3.3.9 Separation and activation of HaGPPs on two-dimensional gel electrophoresis

The *H. armigera* larvae fed on a cocktail of protease inhibitors or non-host plants incorporated in to chickpea (PIs removed) based artificial diet were dissected, and gut lumen contents used for two-dimensional gel electrophoresis analysis. Larval gut contents were extracted in 0.2 M glycine-NaOH, pH 10.0, and the extract centrifuged (15,000 rpm, 30 min, and 4°C). The supernatant containing the gut lumen soluble proteins was kept, and protein concentration determined using the protein Dye reagent (Bio Rad) and bovine serum albumin (BSA) as a standard. The *H. armigera* gut proteins concentrated by chilled 100% acetone were centrifuged (15,000 rpm, 30 min, 4°C) and resultant protein pellet solubilized in IEF lysis buffer/thiourea rehydration buffer solution; 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% pharmalyte/IPG buffer, 1% bromophenol blue stock solution, without adding reducing agent DTT/β-mercaptoethanol in rehydration solution. Protein concentration was determined using the nanodrop method (Nanophore, GE healthcare). Twenty four centimeters Immobiline Dry strips, pH 3 - 10NL (GE healthcare) were used for isoelectric focusing on Ettan IPGphore system (GE healthcare). Total proteins (600 µg) in 450 µl of IEF lysis buffer was passively rehydrated for 16 h at 20°C. Electric conditions were as described by the supplier; IEF run was performed for a recommended program of 8 h at maximum current 50 µA perstrip, at 500 V for 1 h (step and hold); 500 – 1000 V for 1 h (gradient); 1000 - 10000V for 3 h (gradient); 10000 V for 2:45 – 4:15 h (step and hold total). After the first-dimensional run, the IPG gel strips were sealed in a plastic wrap and frozen at – 80°C or incubated at room temperature to equilibrate buffer (50 mM Tris pH 8.8, 30% v/v glycerol, 6 M urea, 2% (w/v) SDS) for 30 min to reduce and alkylate proteins. The IPG strips were then sealed with a solution containing 0.5% agarose in Laemmli SDS

electrophoresis buffer (Agarose sealing solution) on top of a 10% SDS-PAGE gel used for the second dimension. Electrophoresis was performed at a maximum power of 15 W per gel with a voltage limit of 300 V at 20°C using the Ettan DALT 6 apparatus (GE healthcare).

The *H. armigera* gut proteins from insects fed on with or without PIs and/or purified proteins were separated on two-dimensional IEFSDS-PAGE, and analyzed as described by O'Farrell (1975), with a pH range 3 - 10NL in the IEF gels without adding DTT/β-mercaptoethanol. Visualization of HaGPs and activated HaGPs after SDS-PAGE on 2D gel electrophoresis was carried out using the gel X-ray film contact print technique (Pichare and Kachole, 1994). After electrophoresis, the gel was washed with 2.5% Triton X-100 in 0.2 M glycine-NaOH (pH 10.0) for 20 min three times to remove SDS. Gel was overlaid on undeveloped X-ray film for 45 - 50 min time interval and subsequently three exposures were taken at three time intervals (1, 1:30, and 2 h). For *in-gel* activation, the same gel or the same protein loading sample gel was incubated at a low concentration of trypsin (0.0001%, prepared in 0.2 M glycine-NaOH buffer) activation solution for 20 min, and excess trypsin rinsed off, and the gel overlaid on an X-ray film for the same incubation time interval as mentioned earlier. For weakly active or low activation bands, the overlay time period was extended, but this may give rise to a loss of resolution of highly active bands. After exposure, the film was washed with tap water and proteinase activity and activation bands were visualized as hydrolyzed gelatin, while activated proteinases showed comparatively greater hydrolysis of gelatin than its active isoforms. After GXCP, the same gel was washed and stained for protein using Page blue according to the manufacturer's instruction or CBB R-250 in 40% methanol and 10% acetic acid, or by silver staining (Rabilloud et al., 1988).

CHAPTER 4

RESULTS

4.1 Gut proteinases and pro-proteinases of *Helicoverpa armigera*

Helicoverpa armigera (Lepidoptera: Noctuidae) is one of the most important pests of cereals, legumes, vegetables, fruit crops. Host plant resistance is one of the important components of integrated pest management. The levels of resistance to *H. armigera* in field crops are low to moderate, and therefore, there is need to identified species/cultivars with different mechanisms of resistance to this pest. Of the several morphological and biochemical traits, protease inhibitors play an important role in host plant defense against the herbivores. Therefore, the present studies were carried out to characterize proteases involved in digestion of food in the *H. armigera* midgut, and to identify potent protease inhibitors, which could be deployed in the commercial cultivars through genetic engineering to increase the levels, and diversify the basis of resistance to the pest. Pauchet et al. (2008) identified forty-gene proteases in the gut lumen of the generalist herbivore, *H. armigera*. These enzymes are predominantly responsible for digestion of carbohydrates, proteins, and lipids. The study focused on inactive isoforms of digestive enzymes secreted in the gut, and their activation by active forms of midgut proteinase(s). The activity of *H. armigera* gut proteinases is predominantly trypsin-like with a high pH optima (Johnston et al., 1991). Although gut pro-proteinase activation was affected by Ca^{2+} ions, but the proteinase activity was not. The present studies were undertaken to identify, partially purify, and characterize the serine pro- and proteinase(s) of *H. armigera* to provide a basis for assessment of plant serine protease inhibitors as protective agents insect damage. In addition, comparisons were also made between the insect's serine proteinase(s) and serine proteolytic activity of bovine trypsin. The specificity of the *H. armigera* digestive enzymes to synthetic inhibitors has been studied extensively. These digestive enzymes showed specificity mainly towards serine proteinase inhibitors, and if the serine PIs were fed to *H. armigera*, then predominantly pro-proteinase activity was obtained in the gut lumen as well as in the frass. Furthermore, these studies demonstrated the applicability of the gel X-ray film contact print technique for detection of pro- and proteinases after resolution on denaturing-gel, and two dimensional run of PAGE.

4.1.1 Gut proteinase profile of *H. armigera* and *in-gel* activation of pro-proteinases by trypsin treatment

The gut proteinases in the *H. armigera* larvae (HaGPs) fed on chickpea (PIs removed) based artificial diet were extracted in 0.2 M glycine-NaOH buffer, pH 10.0, and separated on a 10% native-PAGE, and further processed for proteinase activity visualization by gel X-ray film contact print technique (Figure 4.1.1). The total *H. armigera* gut proteinase activity was recorded in at least ten isoforms, of which four were the major proteinases (HaGP 2, 5, 7, and 9), four were relatively important, while the remaining two were minor. HaGP 5 and 7 showed the highest activity, while HaGP 4 and 8 exhibited moderate level of activity. HaGP 10 showed the lowest activity. Proteinase isoforms recorded in the resolving gel were categorized into three groups on the basis of mobility. HaGP 9 and 10 were closer and fast moving bands, while HaGP 1 and 2 exhibited very low mobility. By increasing the overlay time of gel on X-ray film (35 - 40 min, at 37°C), HaGP 8 and 10 could be detected. However, by then, other proteinases having high activity were diffused, and merged together. It was observed that when total *H. armigera* gut proteinase activity was resolved, no proteinase activity band was observed in stacking gel (Figure 4.1.1). On the basis of substrate specificity, inhibition by synthetic inhibitors and their molecular weight, and position of proteinase(s) in the gel, the HaGPs were classified into two major serine proteinase families. HaGP 1, 2, 3, 4 belonged to the chymotrypsin proteinase family, while remaining, having comparatively low mobility and molecular weight, (HaGP 5, 6, 7, 8, 9, and 10) showed trypsin-like activity. Activity of proteinase isoforms decreased as the incubation period increased. No new proteinase(s) or autoactivation of proteinase isoform(s) was observed on X-ray film.

There are several advantages of the gel X-ray film contact print method for detection of proteinase activity. We extended the use of this technique to visualize inactive isoforms of proteinases after activation on polyacrylamide gel. After resolving synthetic inhibitor(s) treated *H. armigera* gut extract on non-denaturing (10%) gel, the proteinase was activated by very low concentration of activator (0.0001% trypsin), and the activated proteinase profile visualized as shown in Figure 4.1.2. The *H. armigera* gut proteinase activity was distributed in 10 isoforms, but six inactive isoforms of proteinases were observed upon activation on X-ray film. HaGPP 1, 2, 3, 4, 5, and 6 were inactive

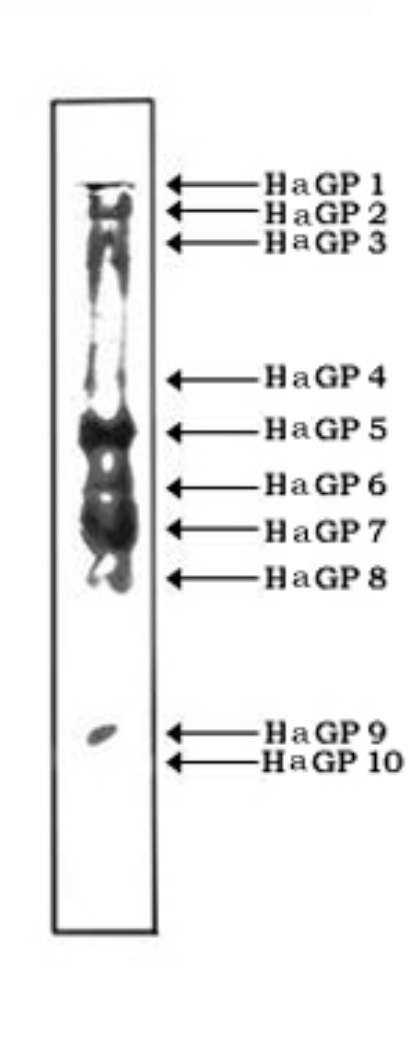


Figure 4.1.1 Gut proteinase profile of *H. armigera* fed on chickpea based artificial diet. PIs were removed from the chickpea powder by autoclaving and heat treatment. 0.02 U activity of HaGP was separated on 10% native-PAGE and the same gel was processed for X-ray film contact print technique for visualization of proteinase activity bands. The gel was exposed to X-ray film for 30 min.

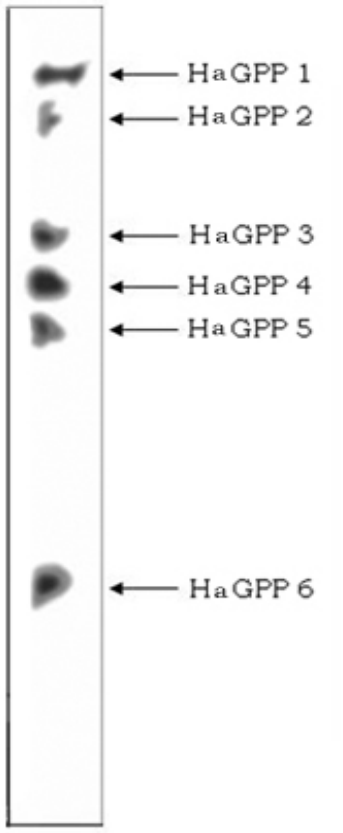


Figure 4.1.2 Electrophoretic analysis of the activation of *H. armigera* gut pro-proteinases (HaGPPs) visualized by gel X-ray film contact print technique by the treatment of activator bovine trypsin. The *H. armigera* larvae fed on a cocktail of synthetic inhibitors incorporated in chickpea (PIs removed) based artificial diet were separated on 10% native-PAGE. 2 U BAEE activity of HaGPPs gut extract was used for visualization of activation of pro-proteinases. The gel was exposed to X-ray film for 30 min.

isoforms of the HaGP 2, 3, 4, 5, 7, and 9, respectively. Pro-proteinase isoforms have less mobility as compared to their active isoforms as shown in Figure 4.1.3. In this case, the overlay time of the gel on X-ray film (30 min) was kept constant for activation and visualization.

The *H. armigera* larvae fed on a cocktail of synthetic inhibitors were starved for 5 h for obtaining high concentration of HaGPPs, and to prevent autoactivation. Mixture of synthetic inhibitors of serine proteinase inhibitors such as antipain, leupeptin, and aprotinin totally inhibited the activity of HaGPs.

Activity patterns of *H. armigera* gut proteinases and activated trypsin, chymotrypsin, and HaGPs after resolving on 12% SDS-PAGE in non-reducing conditions are shown in Figure 4.1.3. Activation of trypsinogen and chymotrypsingoen revealed active form of each proteinase band (24 kDa and 25.7 kDa, respectively). The apparent molecular masses of *H. armigera* gut proteinases were 61.8, 54.5, 46.9, 43.1, and 40.8 kDa, while HaGPPs had molecular weights of 47.6 and 42.1 kDa. The *H. armigera* gut proteases and pro-proteases showed optimum activity at pH 10.0.

4.1.2 Factors affecting on activation of *H. armigera* gut pro-proteinases

4.1.2.1 pH

To determine the pH optima of HaGPs and HaGPPs, various buffers with a pH range from 4 to 11 were used. Azocasein and BAEE substrates were used to measure the pH optima for HaGPs and trypsinogen isoforms of HaGPPs, respectively. It was observed that all proteinase isoforms were active over a board pH range, but the activity increased within the pH range of 4 to 9. Maximum activity of HaGP was recorded at pH 10, and thereafter, the activity declined. HaGPs were active at highly alkaline pH (between 9 and 11) [Figure 4.1.4 (A)].

Maximum pro-proteinase activation was obtained at pH 8 and 10 [Figure 4.1.4 (B)], pH 7 showed 50% activation, which further increased at pH 8. However, maximum proteinase activity as well as activation of pro-proteinases was observed at pH 10 (Figure 4.1.4). Subsequently, experiments with different substrates were carried out for 30 min. Proteinases are not stable at pH 12 as they degraded quickly (Ahmed et al., 1980).

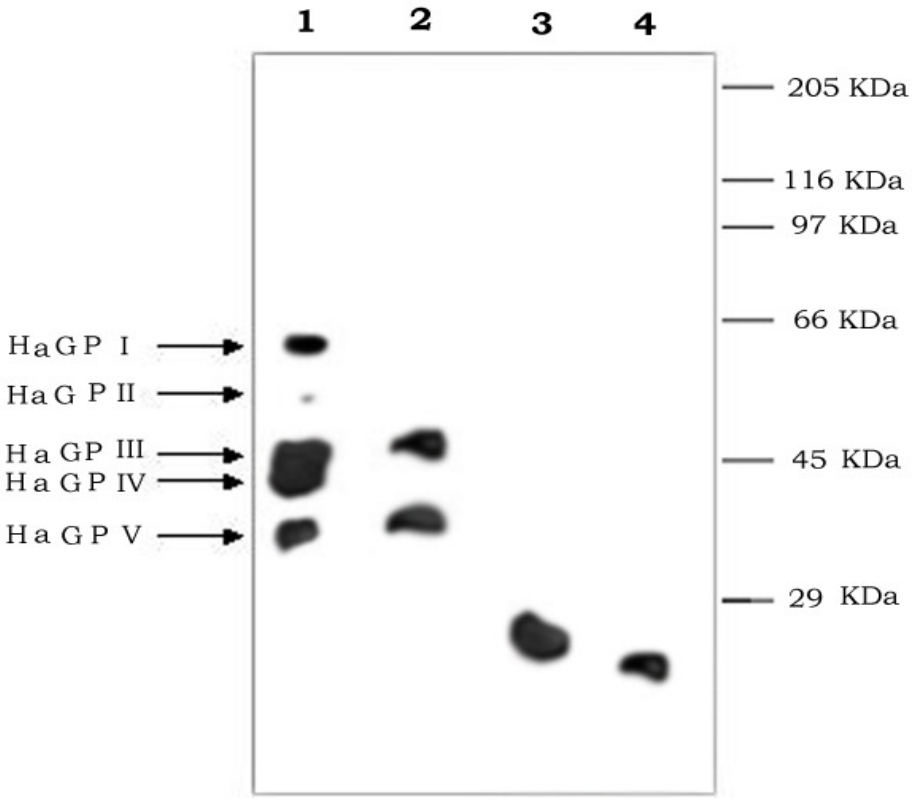
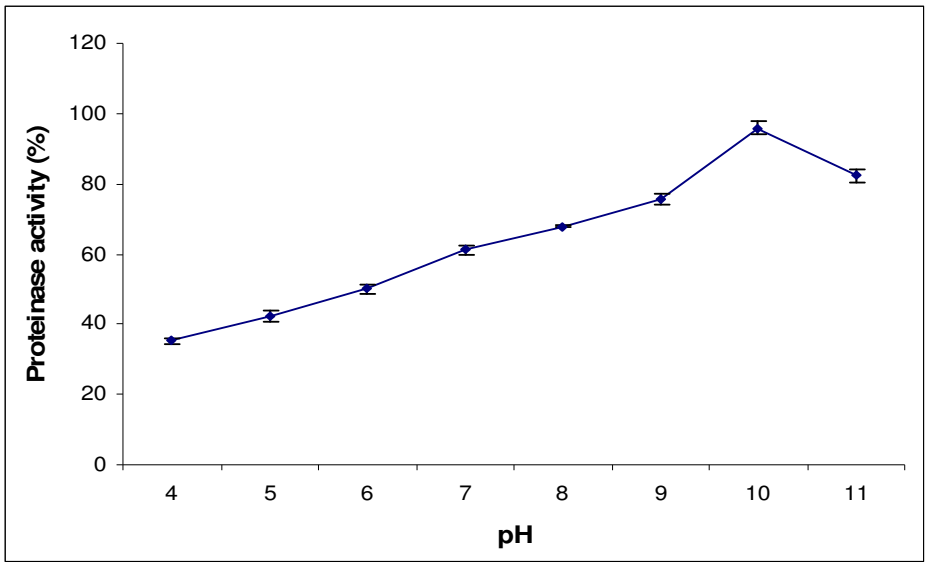


Figure 4.1.3 The *H. armigera* gut pro- and proteinases (HaGPs and HaGPPs), and proenzymes separated on denaturing gel 12% SDS-PAGE under non-reducing conditions. After electrophoresis, gel was washed with 2.5% Triton X-100 to remove SDS. Pro-proteinase and proenzyme activation were carried out in activation buffer, and the activity visualized by X-ray film. To visualize proteinase activity, the gel was processed separately. Lane 1, *H. armigera* gut extract; lane 2, *H. armigera* gut extract fed on synthetic inhibitors incorporated in chickpea (PIs removed) based artificial diet, followed by pro-proteinase activation was visualized by treatment of trypsin; lane 3, Chymotrypsinogen activated band; lane 4, Trypsinogen activated band.

A



B

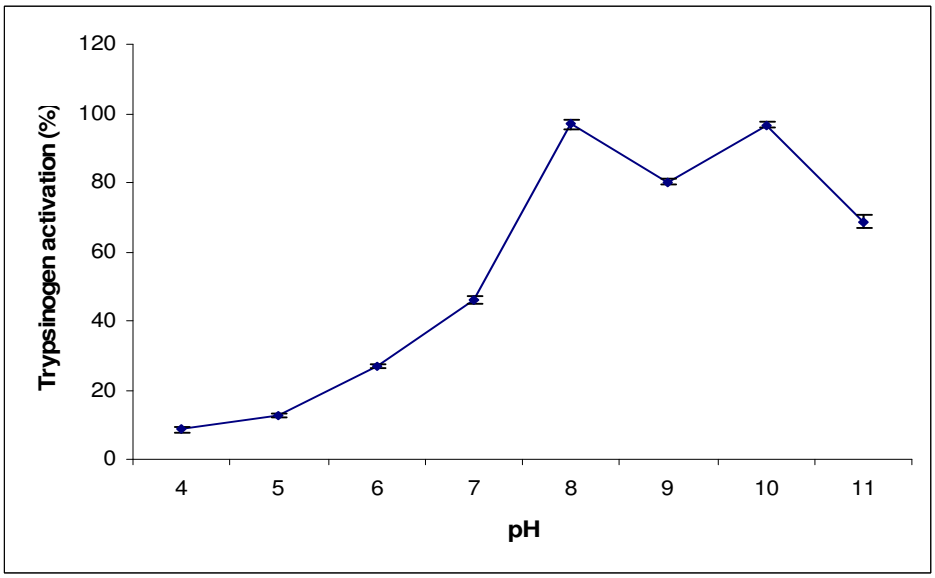


Figure 4.1.4 Determination of pH optima of HaGPs (A) and HaGPPs (B).
Total proteinase activity and activation of trypsinogen isoforms were estimated at various pH using synthetic substrates azocasein and BAEE, respectively. Maximum proteinase activity and activation were considered as 100%, and relative percent activity values were calculated.

Therefore, the experiments were carried out at pH 10 for proteinase activity, and for activation at pH 8 and pH 10.

4.1.2.2 Temperature

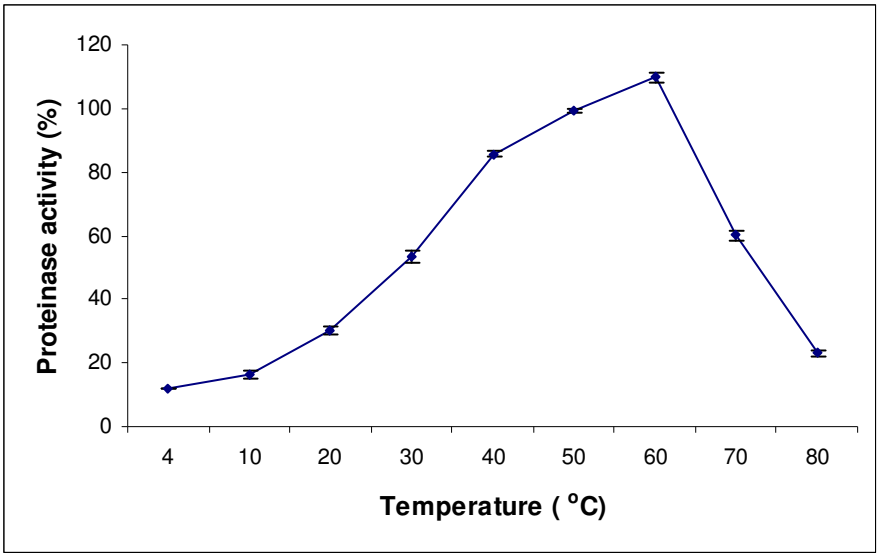
Activity of HaGP isoforms and subsequent activation of trypsinogen isoforms of *H. armigera* were analyzed by the substrate assay using azocasein and BAEE, respectively (Figure 4.1.5). In the substrate assay, proteinase activity of HaGPs of the larvae fed on chickpea based artificial diet (PIs removed) and activation of trypsinogen isoforms of *H. armigera* fed on a cocktail of SIs were carried out at 4, 10, 20, 30, 40, 50, 60, and 70°C. Total proteolytic activity increased with an increase in temperature, maximum activity was recorded at 60°C, which declined at temperatures > 60°C [Figure 4.1.5(A)].

When BAEE was used as a substrate for determination of activation of trypsinogen isoforms, 10% activation occurred at 4°C. Activation increased with increasing temperature, and maximum activation occurred at 40°C [Figure 4.1.5 (B)]. However, *H. armigera* gut pro-proteinases showed total activation of pro-proteinases at 4°C after 24 to 28 h incubation period (data not shown).

4.1.2.3 Ca^{+2} ion concentration

Activation was measured in 0.1 M Tris-Cl, pH 8.3 buffer at 37°C, both in the physiologically relevant Ca^{2+} concentration range [0 - 1 M in activation reaction mixture as described in Material and Methods; Figure 4.1.6 (A)], and at higher (unphysiological) Ca^{2+} concentration range [1 - 10 M in activation reaction mixture as described in Materials and Methods, Figure 4.1.6 (B)], which is usually tested in biochemical assays. The *H. armigera* gut pro-proteinases exhibited minimal activation in the absence of Ca^{2+} or at Ca^{2+} concentrations of 1 M and above. The rate of activation increased upto 5 M Ca^{2+}, while the activation rate declined slightly at higher concentrations (5 and 10 M), but still resulted in high levels of trypsin activity [Figure 4.1.6 (B)].

A



B

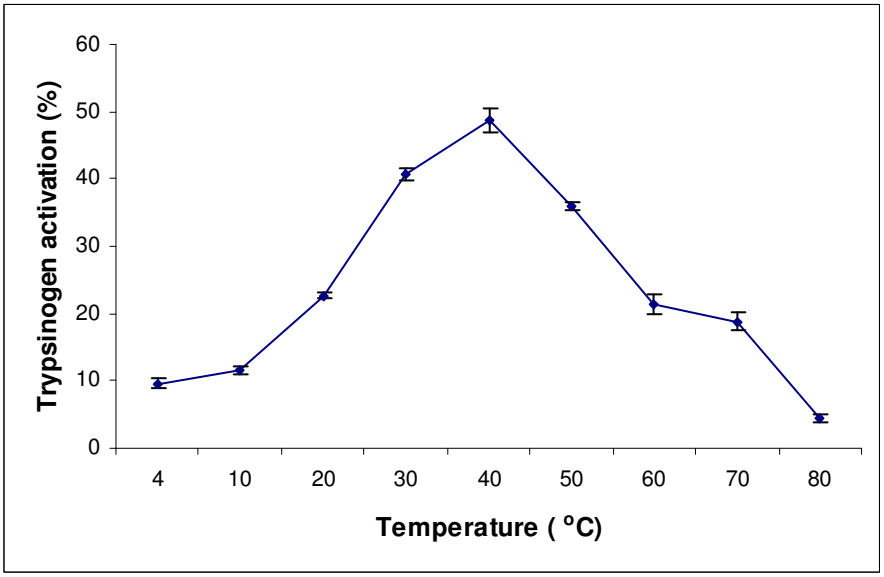
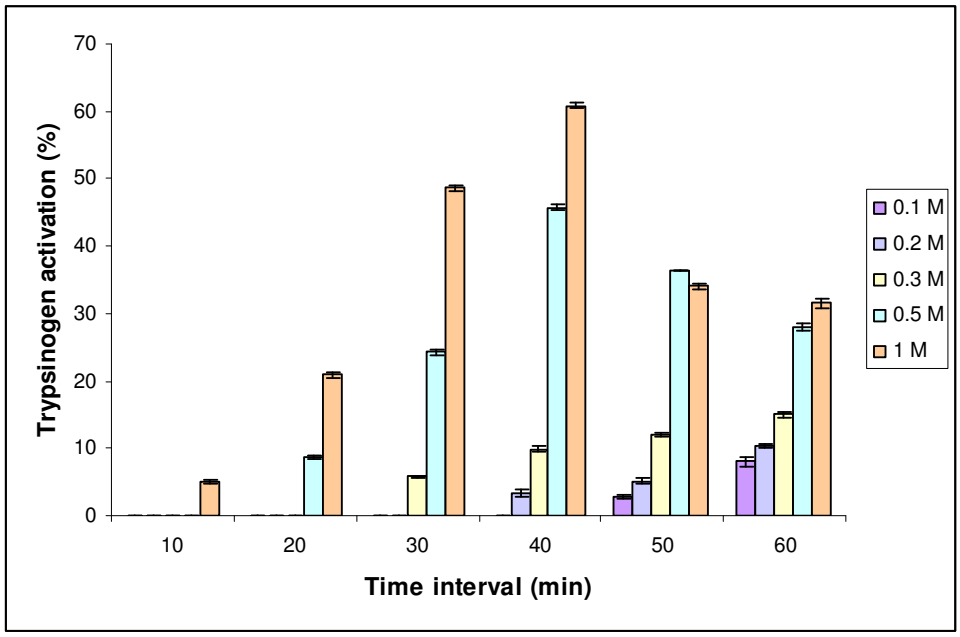


Figure 4.1.5 Determination of temperature optima of HaGPs (A) and HaGPPs (B). Total proteinase activity and trypsinogen activation of *H. armigera* gut trypsinogen isoforms were estimated at various temperatures using synthetic substrates azocasein and BAEE, respectively. Maximum proteinase activity of HaGPs in larvae fed on chickpea (PIs removed) based artificial diet and trypsinogen of HaGPPs in larvae fed on a cocktail of synthetic inhibitors were considered as 100%, and relative percent activity and activation values were calculated, accordingly.

A



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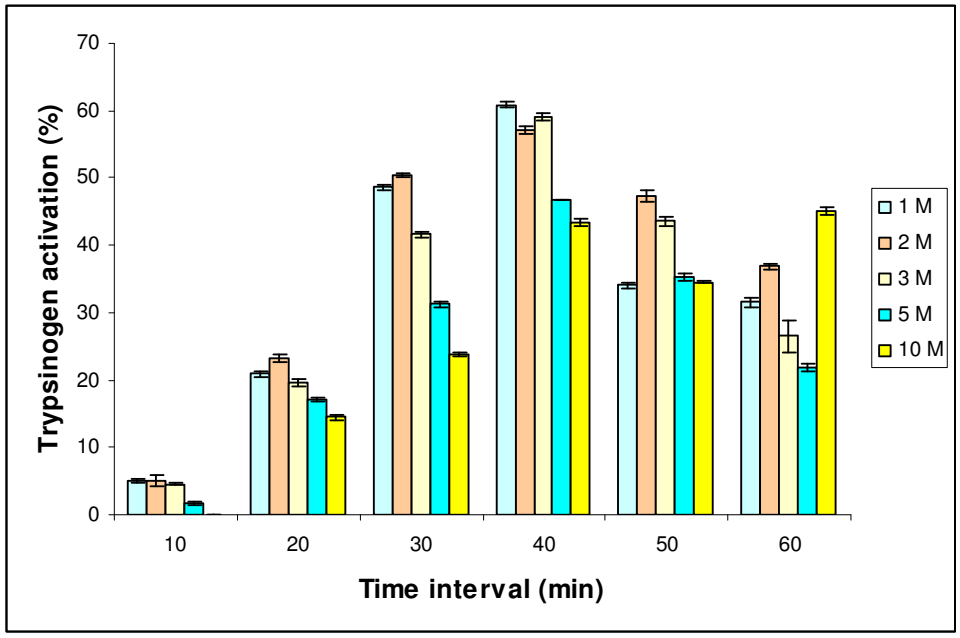

Figure 4.1.6 Effect of Ca^{2+} on the relative rate of autolysis. Approximately 50 µg of HaGPPs extract (in a final volume of 1 ml) was incubated at 37°C in 0.1 M Tris-Cl (pH 8.0) in activation reaction buffer. Trypsinogen activation was determined by BAEEase as a substrate at physiological and nonphysiological Ca^{2+} concentration ranges.

4.1.3 Purification and characterization of pro- and proteinases on affinity and size exclusion column chromatography

When an aqueous extract of gut of *H. armigera* larvae, fed on a cocktail of synthetic inhibitors was loaded onto a benzamidine sepharose 4B (fast flow) column affinity chromatography at pH 8.0, the separation and isolation of many gut proteins can be accomplished by elution with 0.1 M Tris-Cl buffer as shown in Figure 4.1.7. The major unbound in- and active proteinase isoforms were eluted with 0.1 M Tris-Cl buffer, pH 8.0 in first pool, while in the second pool, trypsin isoforms bound to column were eluted by 0 - 1 M NaCl gradient in the same buffer. The elution pattern is shown in Figure 4.1.7. There were two major peaks of unbound proteins between 6 – 10, and the second peak in 13 - 21 fractions. Protein contents and proteinase activity analysis at each purification step were measured by absorbance at 280 nm, and BA*p*NAase assay, respectively. Fraction 8 showed maximum activity in first peak, while fraction 17 showed highest proteinase activity (Figure 4.1.7). When, NaCl gradient was used for elution of bound trypsin isoforms of *H. armigera*, trypsin activity was first detected in fraction 41, and maximum activity was recorded in fraction 51, and declined thereafter.

The *H. armigera* gut pro-proteinase isoforms activation was observed in the same eluted fraction in activation buffer, and activation was measured by BA*p*NAase assay (Figure 4.1.8). First pool of fractions divided in two lots showed activation of pro-proteinases. Here also, fraction 8 showed maximum activation in first peak, while fraction 17 showed maximum activation of pro-proteinase isoforms. Fractions eluted by NaCl gradient did not show any activation (Figure 4.1.8). Finally, the fractions showing high pro-proteinase activation were pooled, concentrated, used for further purification on size exclusion column.

The purification steps employed in the present studies increased the specificity of HaGPs to activation of HaGPPs. In another partial purification of *H. armigera* gut pro- and proteinases fed on a cocktail of synthetic inhibitors, crude gut extract was subjected to gel filtration column chromatography using sephadex G 75 (Figure 4.1.9). Activity of proteinase(s) present in fractions and the relative abundance of pro-proteinase activation in the same fractions were estimated using general substrate, azocasein. Proteinase and activation of pro-proteinases were initially observed in fraction 6, and their activity

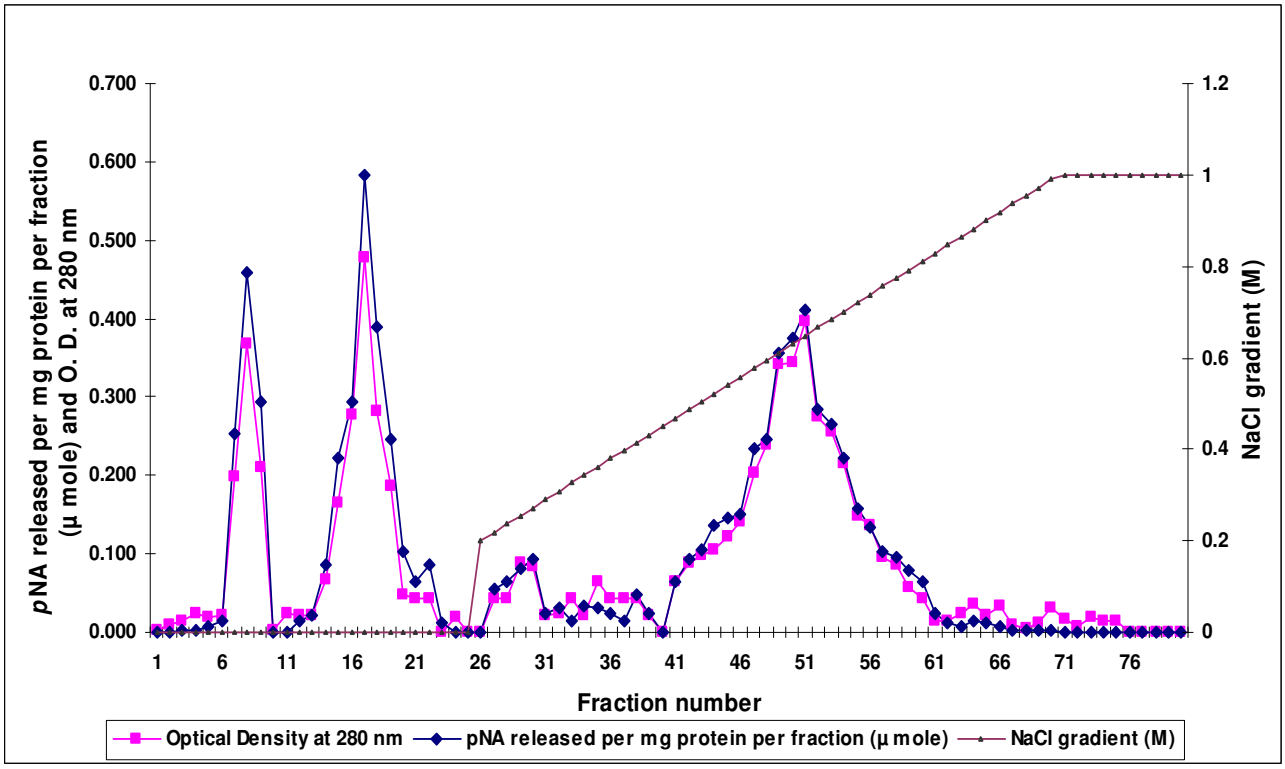

Figure 4.1.7 Benzamidine sepharose 4B column (fast flow), affinity chromatography of acetone precipitated proteins from *H. armigera* larval gut. The protein contents of fractions eluted by NaCl gradient was measured by absorbance at 280 nm, and enzyme activity by BA*p*NAase assay. 0.25 mM Tris-Cl, pH 7.2, and 0.05 M Tris-Cl, 0.5 M NaCl, pH 7.4 buffers were used for equilibration, and binding and/or washing the matrices, and 0.1 M Tris-Cl, pH 8.0 buffer was used for elution. Fractions (2 ml/tube) were collected at a flow rate of 1 ml/min.

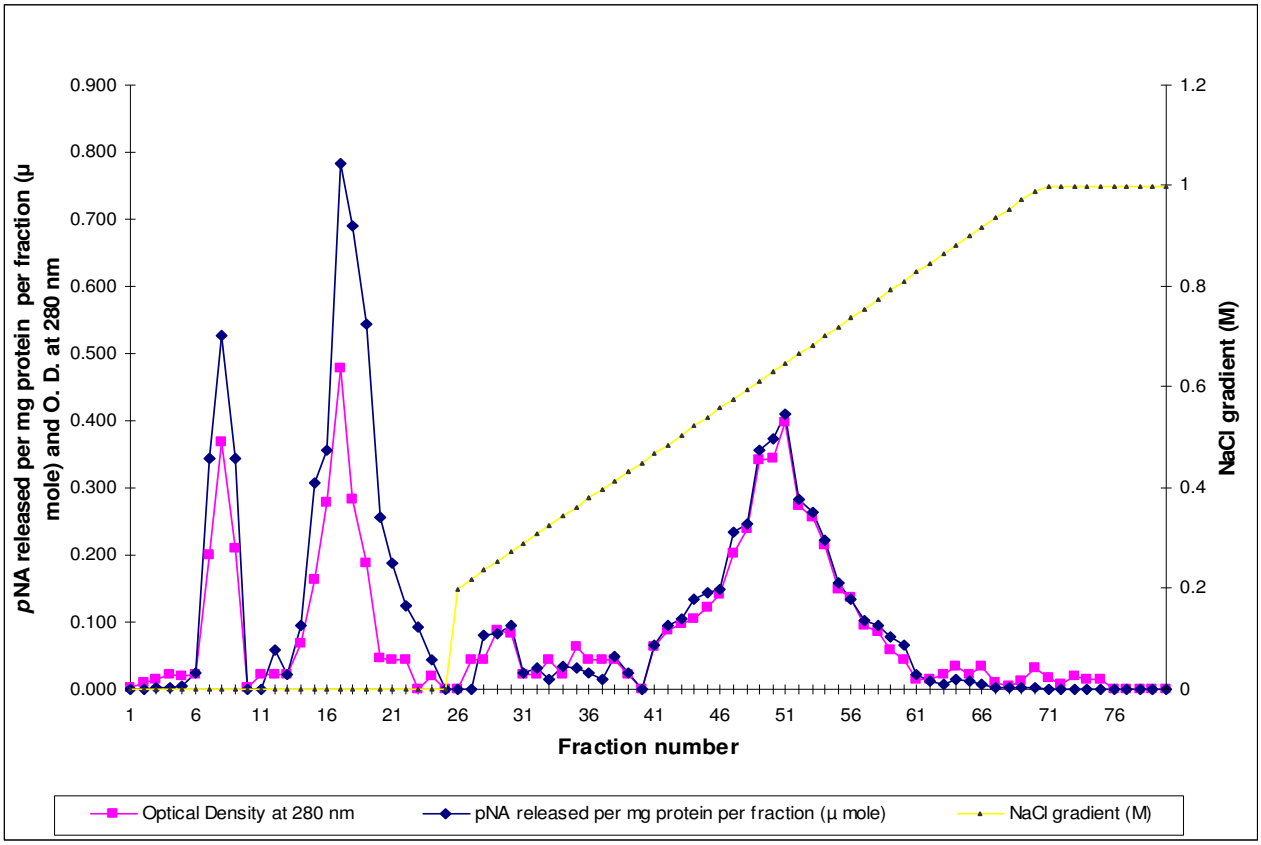

Figure 4.1.8 Activation of *H. armigera* gut pro-proteinases purified on benzamidine sepharose 4B column chromatography (fast flow). HaGPPs were purified on affinity chromatography, protein content of fractions eluted by a NaCl gradient was measured by absorbance at 280 nm, and the corresponding activation of pro-proteinases measured by BA*p*NAase assay in activation buffer as described in 'Materials and Methods'. 0.25 mM Tris-Cl, pH 7.2, and 0.05 M Tris-Cl, 0.5 M NaCl, pH 7.4 buffers were used for equilibration, and binding and/or washing the matrices, and 0.1 M Tris-Cl, pH 8.0 buffer was used for elution. Fractions (2 ml/tube) were collected at a flow rate of 1 ml/min.

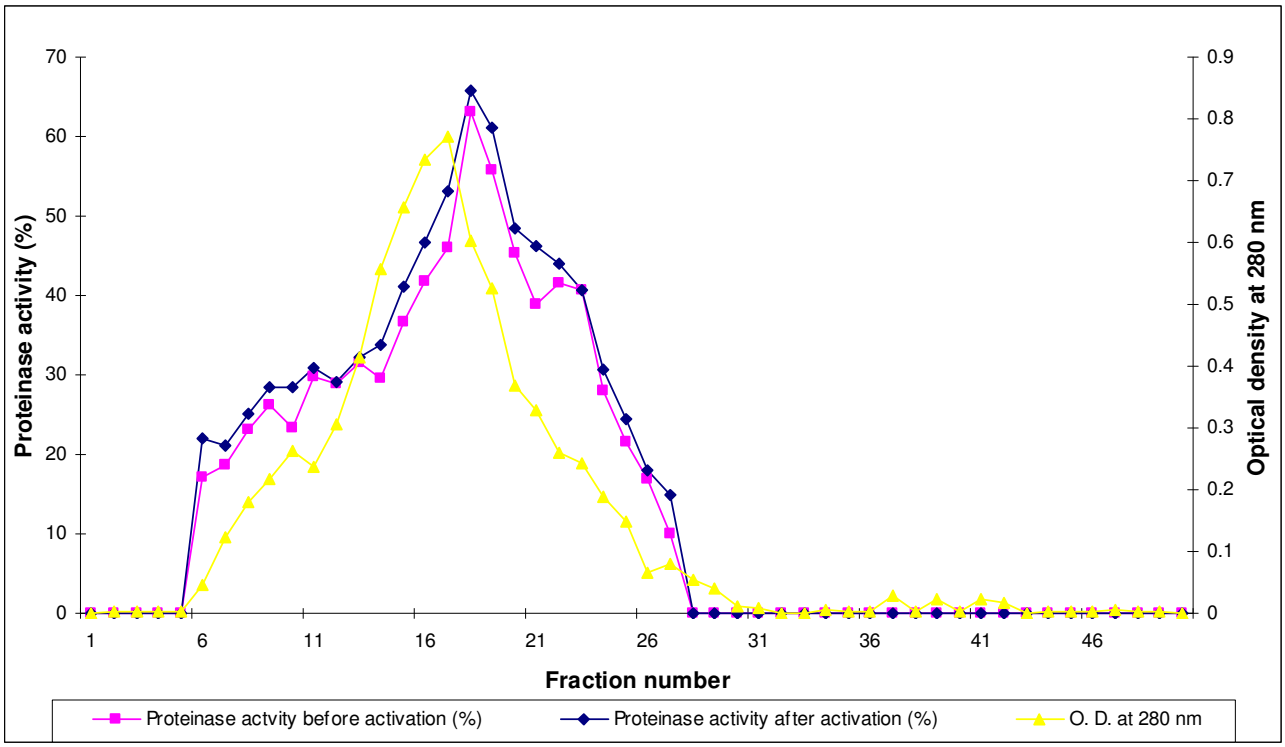

Figure 4.1.9 Chromatography on a sephadex G 75, gel filtration column of an aqueous extract of 30 mg of *H. armigera* gut extract fed on a cocktail of synthetic inhibitors. Detection of pro-proteinase was carried out by tryptic activation of fraction (10 µg bovine trypsin added to 200 µl of each fraction), and activation measured by synthetic substrate, azocasein. The column was equilibrated with 0.1 M Tris-Cl buffer, pH 8.0, and the fractions were eluted in the same buffer.

increased gradually. Fraction 18 showed maximum proteinase activity, while fraction 20 showed maximum activation of pro-proteinases. The activity and activation of partially purified HaGPs and HaGPPs were corroborated and analyzed for substrate specificities using GLUPHEPA and BTEE assay, respectively (Table 4.1.1). The substrates, which differ in the type of amino acid residue (leucine and tyrosine), must be hydrolyzed and are widely used to differentiate specificities for proteolytic activity. Since these fractions had a low amount of protein concentration, it was difficult to measure the activation of pro-proteinases.

A typical size exclusion separation, using sephadex G 75 gel filtration column was used for the separation of partially purified HaGPPs on affinity chromatography. About 90% of protein was eluted off the column between fractions 12 to 21, and these fractions also showed activation of partially purified HaGPPs, except the fraction 21, as shown in Figure 4.1.10. Fraction 17 and 18 showed nearly equal activation of the HaGPPs. Analysis of these protein fractions on SDS-PAGE under non-reducing conditions indicated that the eluted protein fractions contained a band with a Mr 23, 000-90, 000 Da (data not shown). In the *H. armigera* larvae fed on a cocktail of SIs (the same gut extract used for purification), the active isoforms of proteinase formed a complex with the inhibitors (E-I), and affected the mobility of proteinase isoforms.

4.1.4 Inhibition of *H. armigera* pro-proteinases activation by synthetic inhibitors

The *H. armigera* gut extracts were assayed for inhibition of pro-proteinases activation by synthetic inhibitors with respect to their specificity towards the active isoforms of proteinase(s) (Table 4.1.3). To examine the specificity of *H. armigera* gut proteinases (HaGP), the larvae were fed on chickpea based artificial diet (PIs removed by autoclaving and heat treatment). The *H. armigera* gut proteinases showed specificity towards synthetic inhibitors, and the proteinase activity was inhibited strongly as shown in Table 4.1.2. The HaGPs were inhibited strongly by serine proteinase inhibitors, such as PMSF (98%), pefabloc (78%), leupeptin (72%), aprotinin (69%), and antipain (63%), while chymostatin (26%) showed partial inhibition. Pepstatin (13%) and EDTA-Na_2 (6%), E-64 (4%) showed very low inhibition of *H. armigera* gut protease activity. Inhibition by the cocktail of synthetic inhibitors resulted in nearly total inhibition (91%)

Table 4.1.1 Activation of HaGPPs purified on sephadex G 75 column. The *H. armigera* larvae were fed on chickpea (PIs removed) based artificial diet having a cocktail of synthetic inhibitors, were purified by gel filtration column chromatography. Partially, purified gut protein fractions 6 - 28 showed azocaseinase activity, were analyzed for GLUPHEPAase and BTEEase activation assay as described in 'Materials and Methods'.

Fraction number	**Activity of purified pro-and proteinase (U/fraction) mean ± SE (n = 3)**	
	GLUPHEPAase	**BTEEase**
6	0.208 ± 0.041	3.275 ± 0.035
7	0.463 ± 0.202	5.144 ± 0.033
8	0.586 ± 0.264	4.964 ± 0.091
9	1.049 ± 0.071	4.832 ± 0.040
10	1.264 ± 0.048	4.673 ± 0.005
11	1.430 ± 0.030	4.522 ± 0.043
12	1.494 ± 0.010	4.472 ± 0.047
13	1.639 ± 0.028	4.501 ± 0.033
14	1.675 ± 0.032	4.290 ± 0.019
15	1.647 ± 0.023	4.041 ± 0.020
16	1.647 ± 0.020	4.530 ± 0.670
17	1.696 ± 0.026	5.823 ± 0.137
18	1.691 ± 0.012	6.711 ± 0.327
19	1.730 ± 0.036	7.216 ± 0.057
20	1.866 ± 0.015	7.994 ± 0.035
21	1.825 ± 0.011	7.314 ± 0.131
22	1.724 ± 0.025	6.688 ± 0.058
23	1.750 ± 0.019	6.812 ± 0.099
24	1.810 ± 0.028	5.427 ± 0.000
25	1.984 ± 0.073	4.221 ± 0.154
26	1.322 ± 0.083	3.738 ± 0.265
27	0.442 ± 0.023	1.272 ± 0.092
28	ND	ND

ND – Not Detectable.

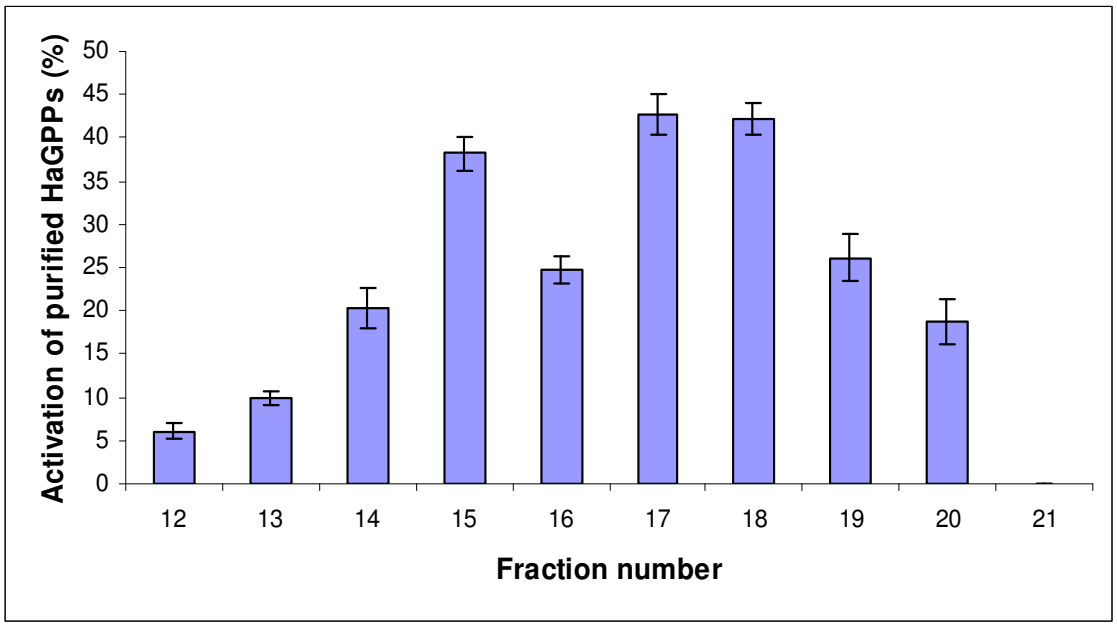

Figure 4.1.10 Activation of HaGPPs purified fractions on affinity followed by gel filtration column chromatography. *Helicoverpa armigera* gut pro-proteinase(s) were purified on benzamidine sepharose 4B (fast flow) column followed by sephadex G 75 column chromatography. Finally, GFC purified fractions 12-20 were showing pro-proteinase(s) activity and activation was measured by BAEE as a substrate. The bars represent standard error.

Table 4.1.2 Effect of synthetic inhibitors based artificial on gut proteinase activity of *H. armigera* larvae fed on chickpea (PIs removed) based artificial diet. Inhibition of 0.02 U proteinase activity was measured using azocasein as a substrate. Maximum inhibition obtained by individual inhibitor at respective concentrations is presented below.

Synthetic inhibitor	**Specificity of the inhibitor**	**Solubility/ stability**	**Effective concentration (per 0.02 U gut proteinase tissue)**	**Inhibition of gut proteinase activity (%) mean ± SE (n = 3)**
Antipain	Serine proteinases	Water/methanol/ DMSO	284 µM	63.69 ± 1.39
Aprotinin	Serine proteinases	Water	2.1 µM	69.49 ± 1.31
Chymostatin	Chymotrypsin	Glacial acetic acid/ DMSO	238 µM	26.95 ± 1.00
E-64	Cysteine proteinases	Water/methanol	128 µM	4.99 ± 1.12
EDTA-NA_2	Metallo-proteinases	Water	1 mM	6.20 ± 1.33
Leupeptin	Serine proteinases	Water	25 µM	72.07 ± 2.37
Pefabloc	Aspartic proteinases	Methanol	10 mM	78.06 ± 1.32
Pepstatin	Aspartic proteinases	Methanol	10 µM	13.45 ± 1.02
PMSF	Serine proteinases	Methanol	1 µM	98.29 ± 0.13
Cocktail of SIs	Serine proteinases	Water/methanol	---	91.55 ± 0.77

Table 4.1.3 *In vivo* effect of synthetic inhibitors on *H. armigera* gut proteinase activity, and *in vitro* activation of pro-proteinase(s) using trypsin as an activator. Inhibition of gut proteinase activity and activation of pro-proteinases were measured using azocasein as a substrate.

Synthetic inhibitor	**Effective concentration (gm^{-1} diet)**	**Inhibition of proteinase activity (%) mean ± SE (n = 3)**	**Activation of pro-proteinases (%) mean ± SE (n = 3)**
Antipain	284 mM	71.26 ± 2.99	11.91 ± 0.65
Aprotinin	2.1 mM	79.54 ± 1.44	21.20 ± 1.32
Chymostatin	238 mM	46.99 ± 3.91	16.47 ± 1.11
E-64	128 mM	14.46 ± 4.73	ND
EDTA-NA_2	1 M	26.8 ± 2.91	ND
Leupeptin	25 mM	81.82 ± 1.76	16.54 ± 2.11
Pefabloc	10 M	88.68 ± 1.09	23.76 ± 1.57
Pepstatin	10 mM	16.95 ± 4.94	ND
Cocktail of SIs	---	90.42 ± 0.43	33.27 ± 0.56

ND - Not Detectable.

of *H. armigera* gut proteinases. PMSF also showed nearly total inhibition to HGPs, but it is an irreversible inhibitor, and hence, was omitted for the *in vivo* assay.

The inhibition of major proteinases of *H. armigera* was observed in larvae fed on chickpea (PIs removed) based on artificial diet treated with specific synthetic inhibitors such as phenylmethylsulphonylflouride (PMSF), 4 (amidino) methane sulfonyl fluoride (APMSF), EDTA, E-64, leupeptin, pepstatin, chymostatin, antipain, and aprotinin. This E-I complex was resolved on 10% native polyacrylamide gel as shown in Figure 4.1.11. PMSF is an irreversible inhibitor of serine proteases, and inhibited all isoproteinases (Figure 4.1.11; lane 7). Antipain, pefabloc, and leupeptin inhibited serine proteases (Figure 4.1.11; lanes 2, 1, and 9, respectively). EDTA-Na_2 (Figure 4.1.11; lane 6) and pepstatin (Figure 4.1.11; lane 8) showed partial inhibition of the minor bands, while E-64 (Figure 4.1.11; lane 5) showed comparatively less inhibition.

In vivo studies revealed the specificity of HaGP in inhibiting the activation of pro-proteinases. Larvae fed on synthetic inhibitors when incorporated into chickpea (PIs removed) based artificial diet inhibited the activity of serine proteinases. When the larval guts were extracted in 0.2 M glycine-NaOH buffer, pH 10.0, inhibition of gut proteinase activity was measured using azocasein as a substrate. Cocktail of SIs resulted in 90% inhibition, while pefabloc, leupeptin, aprotinin, and antipain showed 88%, 81%, 79% and 71% inhibition to HaGPs, respectively. Chymostatin (49%) and EDTA-Na_2 (26%) showed partial inhibition, but pepstatin (16%) and E-64 (14%) resulted in very low inhibition of gut proteinase activity (Table 4.1.3). *In vitro* activation of pro-proteinases revealed apparently low levels of activation (activity of gut proteinases measured by azocaseinolytic assay). The cocktail of synthetic inhibitors showed 33% activation of pro-proteinases, followed by pefabloc (23%), and aprotinin (21%). Chymostatin and leupeptin showed equal activation of pro-proteinase (16%), while E-64, pepstatin, and EDTA-Na_2 fed larvae did not show the activation of pro-proteinases (Table 4.1.3).

To visualize the *in vivo* inhibition and *in vitro* activation of pro-proteinases, HaGPs of the larvae fed on synthetic inhibitors were treated with activation buffer. The results indicated that serine proteinase inhibitors were potent inhibitors of HaGP *in vitro* as well as *in vivo*. Pefabloc and leupeptin showed total inhibition of HaGPs (Figure 4.1.12; lane 1 and 6, respectively); while antipain, aprotinin, and chymostatin did not

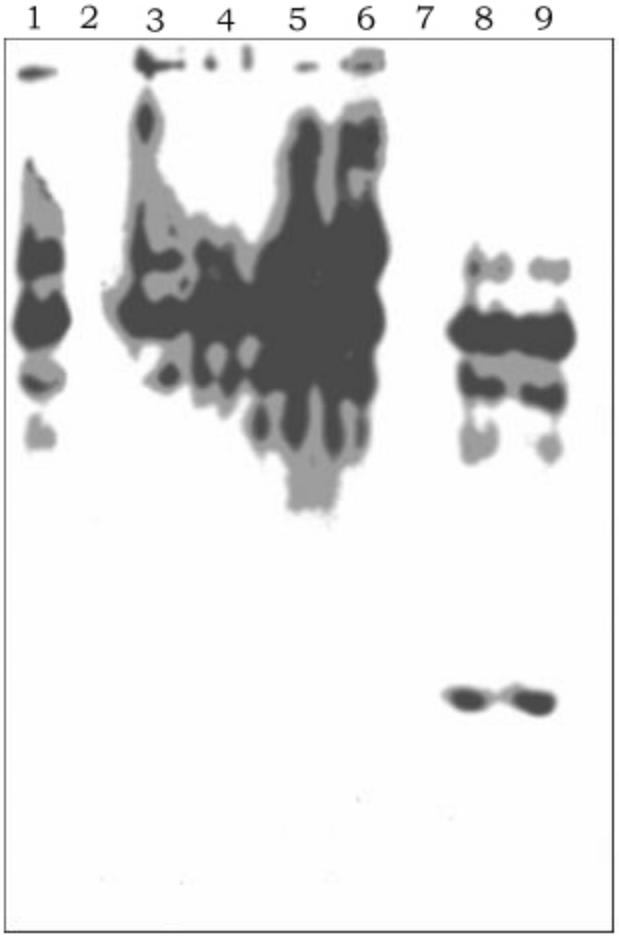

Figure 4.1.11 *In vitro* inhibition of HaGP isoforms by synthetic inhibitors. The *H. armigera* larvae fed on chickpea (PIs removed) based artificial diet were treated with synthetic inhibitors antipain (lane 1), APMSF/pefabloc (lane 2), chymostatin (lane 3), aprotinin (lane 4), E-64 (lane 5), EDTA-NA_2 (lane 6), PMSF (lane 7), pepstatin (lane 8) and leupeptin (lane 9) loaded on 10% native-PAGE. Inhibition of gut proteinase activity visualized by the gel X-ray film contact print technique.

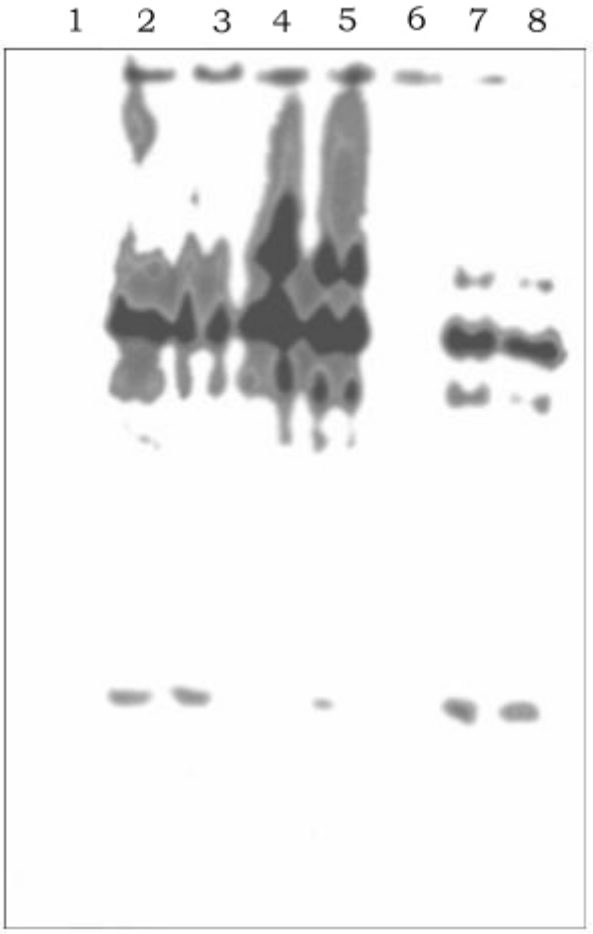

Figure 4.1.12 *In vivo* inhibition of HaGP isoforms by synthetic inhibitors. The *H. armigera* larvae fed on chickpea (PIs removed) based artificial diet with synthetic inhibitors APMSF/pefabloc (lane 1), antipain (lane 2), aprotinin (lane 3), E-64 (lane 4), EDTA-NA_2 (lane 5), leupeptin (lane 6), chymostatin (lane 7), and pepstatin (lane 8) loaded on 10% native-PAGE. Inhibition of gut proteinase activity visualized by the gel X-ray film contact print technique.

result in substantial inhibition of major proteinase activity (Figure 4.1.12; lanes 2, 3, 7, and 8, respectively). Cocktail of synthetic inhibitors (leupeptin, aprotinin, and pefabloc) totally inhibited the activity of HaGP (data not shown). *In gel* activation of HaGPPs showed major as well as minor proteinase activity bands, as visualized on X-ray film. Major proteinases isoforms formed a smear. Pefabloc resulted in activation of six HaGPPs, while another serine PI, leupeptin showed activation of two HaGPPs on X-ray film (Figure 4.1.13; lanes 8 and 3, respectively). Aprotinin and antipain showed the activated HaGPs 6, 7, 8, and 9, when observed on X-ray film (Figure 4.1.13; lanes 6 and 7, respectively).

The *H. armigera* larvae fed on synthetic inhibitors showed low to moderate levels of pro-proteinases after one-day, but two-days feeding exhibited maximum pro-proteinase levels (Data not shown). However, in larvae fed on diet incorporated with synthetic inhibitors more than two days, it was observed that the larval growth retarded, and pro- and proteinase levels were also reduced significantly.

4.1.5 Activation of HaGPPs in the different stadia of *H. armigera*

Different stadia of *H. armigera* larvae fed on a cocktail of SIs were assessed for their *in vitro* activation of total pro-proteinase, trypsinogen, and chymotrypsinogen on azocasein, BAEE, and BTEE, respectively (Table 4.1.4). Second-instar larvae showed 11.79 (azocaseinolytic) pro-proteinase activation, but there was no alteration of trypsinogen and chymotrypsinogen isoforms. HaGPPs activation increased in different instars of *H. armigera* larvae, and maximum activation was recorded in fifth instar larvae (total pro-proteinases, trypsinogen, and chymotrypsinogen isoforms). In the third-, fourth-, and fifth- instars, chymotrypsinogen activation was low as compared to trypsinogen.

To visualize *in vivo* inhibition and *in vitro* activation of pro-proteinases, different instars of *H. armigera* larvae were fed on chickpea (PIs removed) based artificial diet with effective concentration of cocktail SIs, and *in vitro* activation of pro-proteinase on treatment with trypsin (Figure 4.1.14). High amounts of proteinase activity in third-, fourth-, and fifth-instars of *H. armigera* fed on a cocktail of synthetic inhibitors incorporated in chickpea (PIs removed) artificial diet, and the gut extracts loaded on 10%

Figure 4.1.13 *In vitro* activation of HaGPP isoforms by the treatment of bovine trypsin. The *H. armigera* larvae fed on chickpea (PIs removed) based artificial diet synthetic inhibitors pepstatin (lane 1), chymostatin (lane 2), leupeptin (lane 3), EDTA-NA_2 (lane 4), E-64 (lane 5), aprotinin (lane 6), antipain (lane 7), and APMSF/pefabloc (lane 8) loaded on 10% native-PAGE. Activation of gut pro-proteinase isoforms visualized by the gel X-ray film contact print technique.

Table 4.1.4 *In vitro* activation of second-, third-, fourth-, and fifth-instars of *H. armigera* gut pro-proteinases fed on a cocktail of synthetic inhibitors incorporated in chickpea (PIs removed) based artificial diet. Activation of pro-proteinases, trypsinogen, and chymotrypsinogen isoforms was measured using azocasein, BAEE, and BTEE as substrates, respectively.

Larval Stage	**HaGPPs activation (U/gm gut tissue) mean ± SE (n = 3)**		
	Azocaseinase	**BAEEase**	**BTEEase**
Second-instar	11.79 ± 1.49	ND	ND
Third-instar	26.46 ± 1.33	77.27 ± 2.13	1.74 ± 0.37
Fourth-instar	42.71 ± 1.22	91.36 ± 4.99	6.58 ± 0.47
Fifth-instar	53.51 ± 0.07	186 ± 10.8	11.625 ± 0.35

ND - Not Detectable.

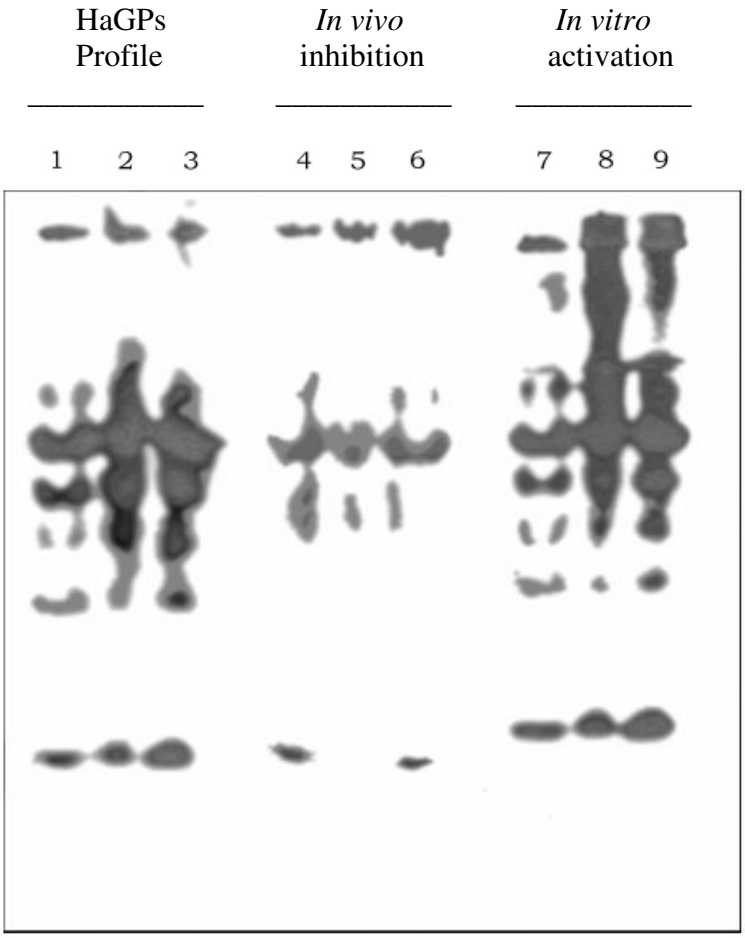

Figure 4.1.14 *In vivo* inhibition and *in vitro* activation of HaGPs and HaGPPs in the third-, fourth-, and fifth-instars of *H. armigera* larvae, respectively. 0.04 U proteinase activity of third- (lane 1), fourth- (lane 2), and fifth- instars (lane 3) were loaded on 10% native-PAGE and visualized HaGPs profile by gel X-ray film contact print method. The *H. armigera* fed on synthetic inhibitors incorporated in chickpea (PIs removed) based artificial diet was used for *in vivo* and *in vitro* analysis. High amount of activity units were loaded to visualize *in vivo* inhibition of third- (lane 4), fourth- (lane 5), and fifth-instars (lane 6) larvae of HaGPs; while *in vitro* activation of the gut extract with similar concentration was used for visualization of activation of instar of third- (lane 7), fourth- (lane 8), and fifth-instars (lane 9) HaGPPs on X-ray film as described in 'Materials and Methods'.

native-PAGE, and control proteinase profile was also studied (Figure 4.1.14; lane 1, 2, and 3). In *H. armigera* larvae fed on SIs, it was observed that HaGP 1, 7, 8 were totally inhibited, while HaGPs 4, 5, 6, and 9 showed partial inhibition (Figure 4.1.14; lanes 4, 5, and 6). After *in gel* activation, gut proteinase HaGPs 1, 3, 7, and 8 were activated, while HaGPs 2, 4, 5, 6, and 9 show-increased activity (Figure 4.1.14; lanes 7, 8, 9). Interestingly, HaGP 3 activation was observed in all the three instars of *H. armigera*. Second-instars of *H. armigera* did not show activation on X-ray film (data not shown).

4.1.6 Analysis of fecal matter

The fecal matter of *H. armigera* larvae fed on synthetic inhibitors incorporated in chickpea (PIs removed) based artificial diet was collected and assessed for pro-proteinase activation (Table 4.1.5). The fecal matter of larvae reared on artificial diet showed high proteinase activity, and this activity was totally inhibited by the synthetic inhibitors. Pro-proteinase and trypsinogen activation was measured by azocasein and BAEE as substrates, respectively (Table 4.1.5). Interestingly, fecal matter from the larvae fed on a cocktail of SIs showed the presence of trypsinogen like activities as observed by BAEE assay. Comparatively, the other pro-proteinase(s) such as chymotrypsingoen, showed low activation, as measured by BTEE assay (data not shown). The *H. armigera* larvae fed on chymostatin showed maximum pro-proteinase activation, followed by those fed pefabloc, leupeptin, antipain, and aprotinin. Pepstatin showed comparatively low activation of pro-proteinases, but E-64, and EDTA-Na_2 showed very low activation. Trypsinogen isoforms were measured by using BAEE as a substrate. Pefabloc and aprotinin showed maximum activation, followed by antipain, but E-64 and EDTA-Na_2 did not show the activation in BAEE assay (Table 4.1.5).

The study revealed that there was inhibition of *H. armigera* gut proteinase(s). Inactive proteinases were transported from the peritropic membrane to gut lumen, but were not activated due to inhibition of activator and remained in inactive form in the lumen and were excreted in frass.

Figures 4.1.15 and 4.1.16 depict electrophoretic patterns of proteinases and activation of pro-proteinases in fecal matter of larvae fed on chickpea (PIs removed)

Table 4.1.5 Pro-proteinase activation was obtained in fecal matter of *H. armigera* fed on different synthetic inhibitors incorporated into chickpea (PIs removed) based artificial diet. Activated pro-proteinases and trypsinogen isoforms were measured using azocasein and BAEE, respectively.

Synthetic inhibitor	**Pro-proteinase activation (U/g of gut tissue) mean ± SE (n = 3)**	
	Azocaseinase	**BAEEase**
Antipain	31.46 ± 1.52	100.89 ± 1.94
Aprotinin	28.96 ± 1.13	107.03 ± 2.29
Chymostatin	38.36 ± 1.80	58.75 ± 4.04
E-64	8.24 ± 0.87	ND
EDTA-NA_2	4.00 ± 0.19	ND
Leupeptin	32.24 ± 1.00	63.82 ± 1.54
Pefabloc	33.08 ± 2.30	107.37 ± 6.55
Pepstatin	14.67 ± 0.79	69.28 ± 4.20

ND - Not Detectable.

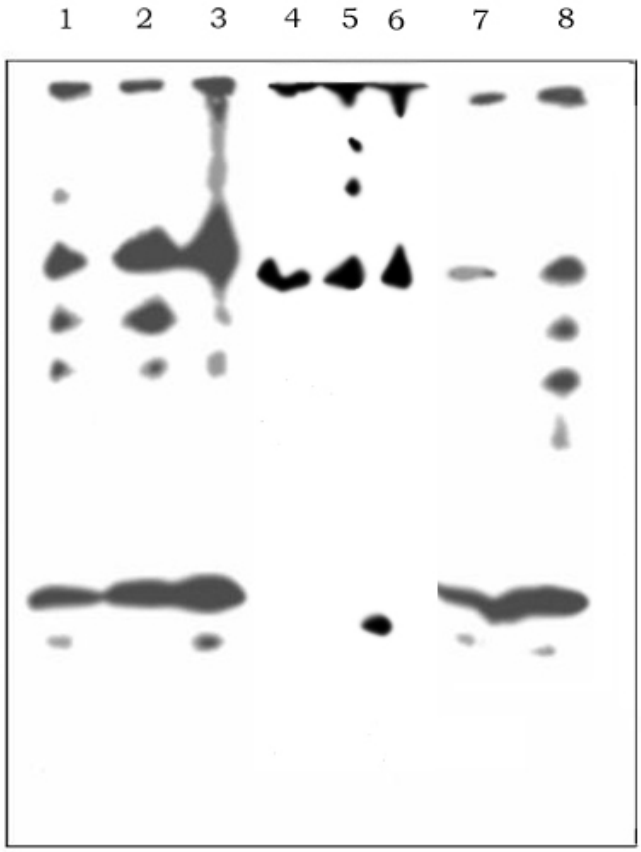

Figure 4.1.15 Proteinase profiles in the fecal matter of larvae fed on diet of synthetic inhibitors in the chickpea (PIs removed) based artificial diet. Proteinase profiles of the fecal matter of larvae fed on E-64 (lane 1), antipain (lane 2), aprotinin (lane 3), chymostatin (lane 4), leupeptin (lane 5), pefabloc (lane 6), pepstatin (lane 7), and EDTA-NA_2 (lane 8), loaded on 10% native-PAGE. Proteinase activity profile was visualized by gel X-ray film contact print method.

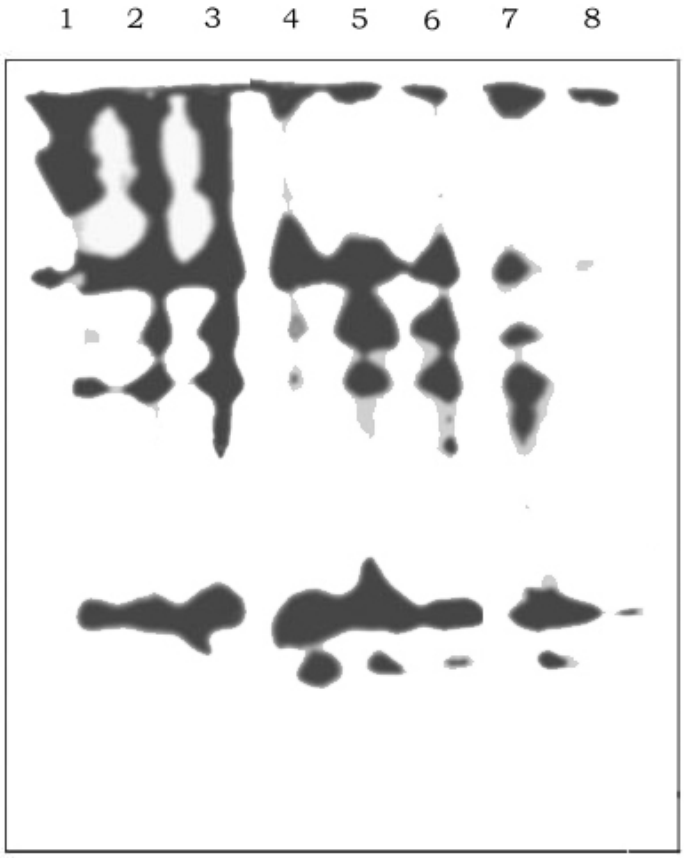

Figure 4.1.16 Activation of pro-proteinases in the fecal matter of larvae fed on synthetic inhibitors in artificial diet. For observing activation of pro-proteinases of the fecal matter of larvae reared on E-64 (lane 1), antipain (lane 2), aprotinin (lane 3), chymostatin (lane 4), leupeptin (lane 5), pefabloc (lane 6), pepstatin (lane 7), and EDTA-NA_2 (lane 8), loaded on 10% native-PAGE. Activation of pro-proteinases was carried out in activation buffer as described in 'Materials and Methods', and activated pro-proteinases visualized by gel X-ray film method.

based artificial diet with synthetic inhibitors, and separated on 10% native-PAGE by loading equal quantity of activity units and visualized on X-ray film.

Fecal matter of larvae fed on synthetic inhibitors showed similar proteinase profile of HaGP, because of hyper production of gut proteinases. HaGP 1 and 4 proteinase isoforms showed high activity in fecal matter of larvae fed on SI (Figure 4.1.15), while HaGP 9 did not show any activity in fecal matter of larvae fed on SIs such as chymostatin, leupeptin, and pefabloc as shown in Figure 4.1.15; lanes 4, 5, and 6, respectively. Interestingly, HaGP 10 isoform activity was observed in larvae fed on E-64, aprotinin, leupeptin, pepstatin, and EDTA-Na_2 (Figure 4.1.15; lanes 1, 3, 5, 7, and 8, respectively). Activation of pro-proteinases in fecal matter of larvae is (shown in Figure 4.1.16), suggested that there was high activation of all pro-proteinases, except HaGP 8, which did not show activation on X-ray film. Activated HaGPs 4, 5, and 6 were visualized in larvae fed on leupeptin, pefabloc, and pepstatin (Figure 4.1.16, lane 5, 6, and 7, respectively).

4.1.7 Activated proteinase profile on two dimensional run of electrophoresis

Diagonal HaGPs profile was obtained by two dimensional run of gut extract of larvae fed on chickpea (PIs removed) artificial diet (Figure 4.1.17). In the first dimension run, HaGPs were separated on the basis of their molecular weight and charge, but in the second dimension, the resolved proteinases were separated on the basis of their mobility. HaGPs separated on 10% native-PAGE in one dimension, again separated on second dimension, run with the same percentage of gel on the basis of mobility. Section 4.1.1, described the HaGP profile. In one dimension run, at least ten proteinase isoforms were detected, while the same activity loaded for two dimension run of HaGPs, eight HaGPs were visualized on X-ray film, but the minor bands were not visualized clearly on the X-ray film. HaGPs 2, 3; and HaGPs 4, 5, 6 formed a smear of band on X-ray film (Figure 4.1.17). HaGPs 5 and 9 showed high proteinase activity, while HaGPs 1 and 10 showed very low activity.

When the *H. armigera* larvae were fed on a cocktail of SIs, a complete inhibition of HaGP activity was observed, as described in section 4.1.3. Interestingly, three proteinases, HaGPs 5, 7, and 9 showed activation (Figure 4.1.18). After activation, gut

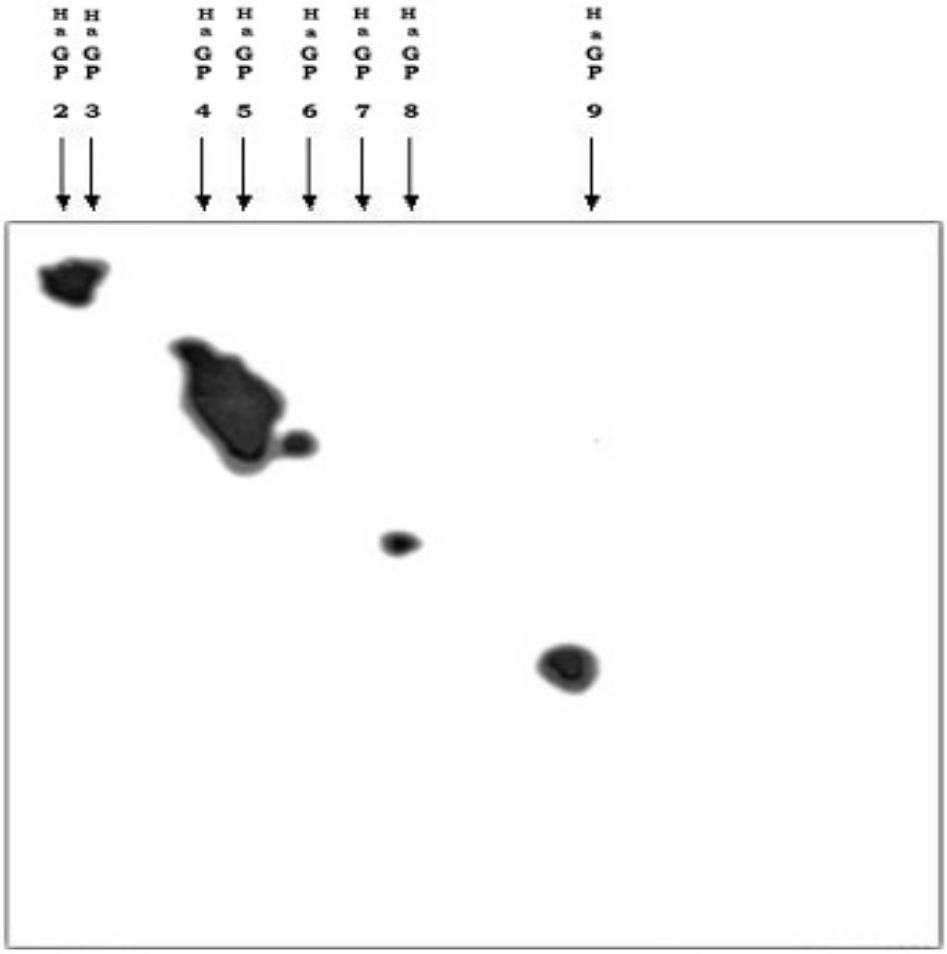

Figure 4.1.17 Two dimensional run of *H. armigera* gut proteinase(s) of larvae fed on artificial diet. Two unit BA*p*NAase activity of HaGPs was separated on 10% native-PAGE and the resolved HaGPs strip placed horizontally on second 10% resolving gel, and again the HaGPs were separated. Two dimensionally separated HaGPs profile was visualized by gel X-ray film contact print method.

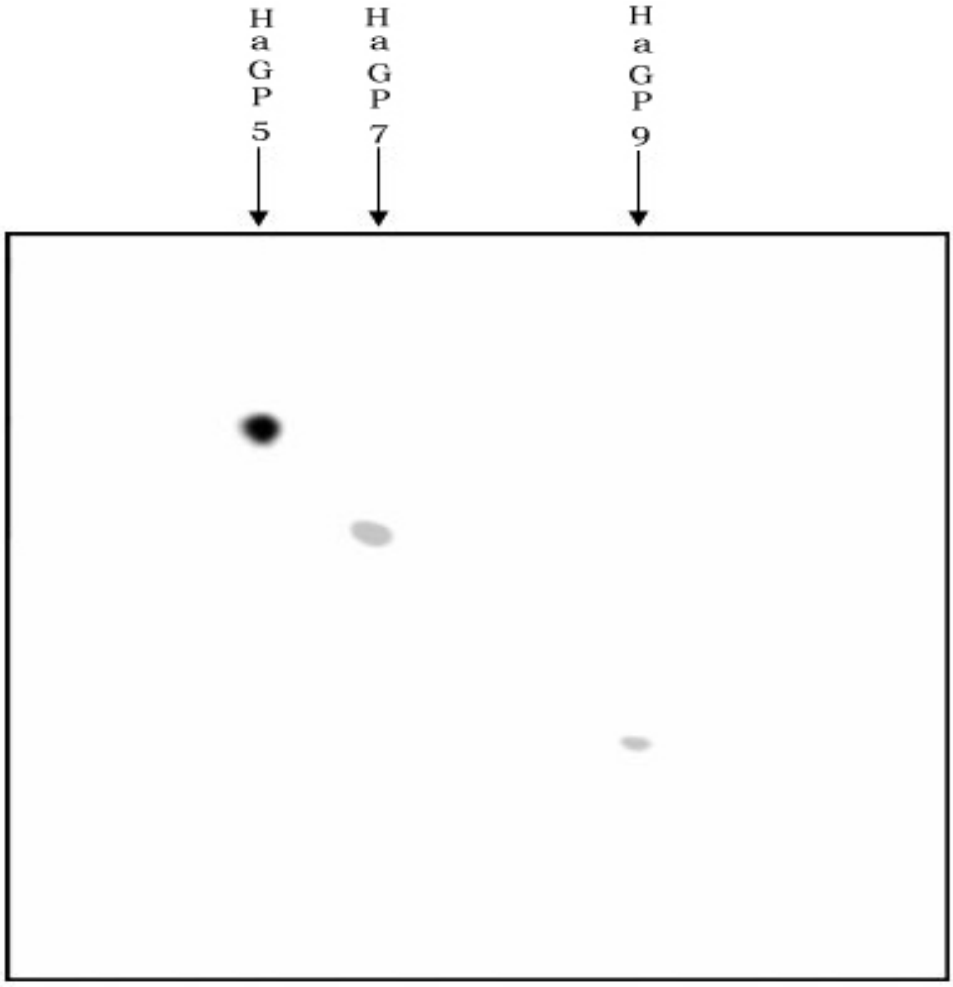

Figure 4.1.18 *In gel* activation of two dimensionally separated *H. armigera* gut pro-proteinases fed on a cocktail of SIs in artificial diet. High concentration of gut extract was loaded on 10% native-PAGE for one dimensional run, after separation the gel was incubated in activation buffer for 10 min, as described in 'Materials and Methods'. The same gel was placed horizontally on the second 10% resolving gel for 2nd run. The activation profile of HaGPPs was visualized by gel X-ray film contact print method.

proteinase mobility slightly shifted upward on X-ray film as compared to its active isoforms. The study implied that after activation of pro-proteinases, there was a cleavage of pro-region, and the pro-segment peptide separated and had a very low molecular weight, and hence, it was difficult to obtain its imprint *in gel* and on X-ray film and/or even after silver staining (data not shown).

4.2 Plant protease inhibitors of *H. armigera* gut pro- and proteinases

Plant protease inhibitors have been implicated in host plant defense against insect pests. Majority of protease inhibitors studied in plant kingdom originate from three main families; namely Leguminosae, Solanaceae, and Gramineae (Richardson, 1991). So far, serine, cysteine, aspartate, and metallo protease inhibitors have been studied for the inhibition of insect gut digestive enzymes. In earlier studies on plant protease inhibitors, the possible storage function of PIs had been postulated by Ryan (1973). Since then, data have shown that the relationship of protease inhibitors and storage proteins in plants is, in fact, more complex (Pusztai, 1972). Plant protease inhibitors are active against certain insect species, both *in vitro* assays against insect gut proteases (Pannetier et al., 1997; Koiwa et al., 1998), and *in vivo* artificial diet bioassays (Urwin et al., 1997; Vain et al., 1998). Protease inhibitors comprise a large and diverse group of plant proteins capable of forming reversible protein-protein complexes with enzymes, resulting in their inactivation (Richardson, 1977; Laskowski and Kato, 1980; Valueva and Mosolov, 2004). Recent studies have elucidated the properties, primary and spatial structures, and the mechanisms of interaction of PIs with the enzymes. However, the understanding of their physiological functions is still far from being complete (Laskowski and Kato, 1980; Valueva and Mosolov, 2004; Bode and Huber, 1992).

In the present studies, we assessed the role of host and non-host plant protease inhibitors in host plant defense against the herbivore insect pests, and the results suggested that these are potent candidates for inhibition of *H. armigera* gut proteinases, and activation of pro-proteinases.

4.2.1 *In vitro* screening of plant PIs by dot-blot assay

Dot-blot method developed by Pichare and Kachole (1994) was used to screen a large number of host and non-host plant PIs. Trypsin, chymotrypsin, and HaGPs activity or inhibition was detected by dot-blot assay method. Detection of PIs was observed by mixing various concentrations of protease and inhibitor, and spotted on the X-ray film. The results of detection of protease inhibitor activity by dot-blot are as shown in Figure 4.2.1. Results for screening of seed sample of 108 plant species for inhibition of proteinases are given in Table 4.2.1. At three concentrations of trypsin, chymotrypsin, and HaGP, and the inhibitor (1:3, 1:1, 3:1), 31 of species (*Acacia nilotica*, *Achyranthes aspera*, *Allium sativum*, *Blepharis edulis*, *Dalbargia latifolia*, *Dolichos lignosus*, *Emblica officinaris*, *Ferula asafetida*, *Foeniculum vulgare*, *Hordeum vulgare*, *Hygrophila schulli*, *Lowsonia inermis*, *Momordica charntia*, *Mucuna pruriens*, *Murraya koenigii*, *Nigela sativa*, *Phaseolus vulgaris*, *Pongamia pinnata*, *Psophocarpus tetragonolobus*, *Psoralea corywfolia*, *Raphanus sativus*, *Ricinus communis*, *Solanum nigrum*, *Terminalia chebula*, *Trigonella foenum-graecum*, *Urena lobata*, *Vigna unguiculata*, and *Zea mays*) resulted in complete inhibition. Similarly, 18 species (*Abelmoschus esculanta*, *Acacia arabica*, *Amonum aromaticum*, *Arachis hypogea*, *Cajanus cajan*, *Capsicum annum*, *Cheiranthis Cheiri*, *Cooculus suberosus*, *Dalbargia sisso*, *Dolichos uniflorus*, *Penganum harmala*, *Holarrhena pubescens*, *Ipomoea nil*, *Lablab purpurens*, *Linum utisacimum*, *Mesua ferrea*, *Punica grantum*, and *Schrebera sweitenoides*) showed nearly total or moderate level of inhibition of trypsin, chymotrypsin, and HaGPs. Out of 108 plant seeds, 31 showed high inhibitory activity, while 18 and 22 samples showed moderate and low activity against proteases, respectively (Table 4.2.1).

At equal concentrations of trypsin and the inhibitor, around 50 seed samples showed inhibition, while at higher inhibitor concentration, more number of plant seeds (72) showed inhibition. Seeds of 62 and 72 plant species showed inhibition of chymotrypsin at equal and high concentrations, respectively. Nearly 47 and 62 plant species showed inhibition at equal and higher inhibition concentrations, respectively.

Screening of a large number of plant species for inhibitory activity was ascertained by dot-blot method, which is more feasible and rapid than electrophoretic method. The results of detection of protease (trypsin, chymotrypsin, and HaGP) inhibitors

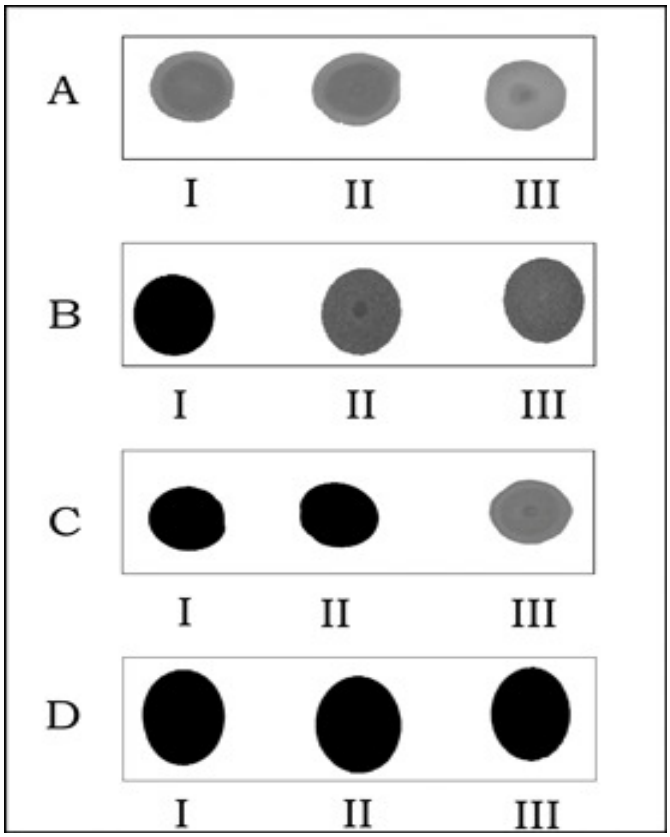

Fig 4.2.1 Detection of host and non-host plant trypsin, chymotrypsin, and *H. armigera* gut proteinase inhibitors by the dot-blot method. Three different concentrations of enzymes and inhibitors [I (3:1), II (1:1), III (1:3)] were used for screening of plant PIs. The faint gray spot indicate total inhibition of enzymes, while dark spot indicate no inhibition. TI profile of *Datura alba ness* (A), *Cajanus cajan* (B), *Cicer arietinum* (C), and *Avena sativa* (D). The CTI and HaGPI profiles for spot test are depicted in the same manner.

Table 4.2.1 Screening of trypsin, chymotrypsin, and *H. armigera* gut proteinase inhibitors from host and non-host plants by spot test. Enzyme and inhibitor concentrations were used for spot test.

Sr. No.	Botanical Name	Trypsin			Chymotrypsin			HaGP		
		3:1	1:1	1:3	3:1	1:1	1:3	3:1	1:1	1:3
1	*Abelmoschus esculanta*	P	Y	Y	P	Y	Y	Y	Y	Y
2	*Acacia arabica*	P	Y	Y	P	Y	Y	Y	Y	Y
3	*Acacia nilotica*	Y	Y	Y	Y	Y	Y	Y	Y	Y
4	*Achyranthes aspera*	Y	Y	Y	Y	Y	Y	Y	Y	Y
5	*Allium sativum*	Y	Y	Y	Y	Y	Y	Y	Y	Y
6	*Amonum aromaticum*	N	P	Y	P	Y	Y	Y	Y	Y
7	*Apium graveolens*	N	N	N	N	N	N	N	N	N
8	*Arachis hypogea*	N	Y	Y	Y	Y	Y	Y	Y	Y
9	*Avena sativa*	N	N	N	N	N	N	N	N	N
10	*Althaea officinalis*	N	N	N	N	N	N	N	N	N
11	*Ampecissus latifolia*	N	N	N	N	N	N	N	N	N
12	*Aethala officinalis*	N	N	N	N	N	N	N	N	N
13	*Azadricta indica*	N	N	P	N	P	Y	N	N	N
14	*Blepharis edulis*	Y	Y	Y	Y	Y	Y	Y	Y	Y
15	*Brassica campestrics*	N	P	Y	P	Y	Y	N	P	Y
16	*Butea monosperma*	N	P	Y	P	Y	Y	N	N	P
17	*Basela rubra*	N	P	Y	N	N	P	N	N	N
18	*Cajanus cajan*	N	Y	Y	N	Y	Y	N	Y	Y
19	*Capsicum annum*	Y	Y	Y	Y	Y	Y	P	Y	Y
20	*Celastrus paniculata*	N	N	N	N	N	N	N	N	N
21	*Cicer arietinum*	N	N	Y	N	P	Y	N	P	Y
22	*Cleistanthus collinus*	N	N	P	N	P	Y	N	N	N
23	*Cordia latifolia*	N	N	Y	P	Y	Y	N	P	Y
24	*Careya arboea*	N	N	N	N	N	N	N	N	N
25	*Celosia argentea*	P	Y	Y	Y	Y	Y	N	N	N
26	*Cheiranthis Cheiri*	Y	Y	Y	N	N	Y	N	N	N
27	*Cooculus suberosus*	Y	Y	Y	Y	Y	Y	N	P	Y
28	*Curculigo orchioides*	N	N	Y	N	N	P	N	P	Y
29	*Carum copticum*	N	N	Y	N	N	P	N	N	Y
30	*Carum carvi*	N	N	N	N	N	N	N	N	N
31	*Cinnamomum tamala*	N	N	N	N	N	N	N	N	N
32	*Carica papaya*	N	N	Y	N	N	Y	N	Y	Y
33	*Dalbargia sisso*	P	Y	Y	Y	Y	Y	Y	Y	Y
34	*Dalbargia latifolia*	Y	Y	Y	Y	Y	Y	Y	Y	Y
35	*Daucus carota*	N	N	N	N	N	N	N	N	N
36	*Dolichos uniflorus*	P	Y	Y	P	Y	Y	N	P	Y

Sr. No.	Botanical Name	Trypsin			Chymotrypsin			HaGP		
		3:1	1:1	1:3	3:1	1:1	1:3	3:1	1:1	1:3
37	*Diplocyclos oppasitifolia*	N	N	Y	P	Y	Y	N	P	Y
38	*Dolichos lignosus*	Y	Y	Y	Y	Y	Y	Y	Y	Y
39	*Delonix regia*	N	N	N	N	N	N	N	N	N
40	*Emblica officinaris*	Y	Y	Y	Y	Y	Y	Y	Y	Y
41	*Eugenia lambolina*	N	N	N	N	N	N	N	N	N
42	*Echinochloa frumentacea*	N	N	P	N	N	N	N	N	N
43	*Eugenia jambolina*	N	N	N	P	N	N	P	N	N
44	*Ferula asafetida*	Y	Y	Y	Y	Y	Y	Y	Y	Y
45	*Foeniculum vulgare*	Y	Y	Y	Y	Y	Y	Y	Y	Y
46	*Ficus benghalensis*	N	N	N	N	N	N	N	N	N
47	*Fimbristylis miliaceae*	N	N	N	N	N	N	N	N	N
48	*Fimbristylis schoenoids*	N	P	Y	N	N	P	N	N	N
49	*Glycine max*	Y	Y	Y	Y	Y	Y	Y	Y	Y
50	*Guizotia abyssinia*	N	N	N	N	N	N	N	N	N
51	*Gloriosa superba*	N	N	Y	N	Y	Y	N	N	N
52	*Gardina latifolia*	N	N	N	N	N	N	N	N	N
53	*Hordeum vulgare*	Y	Y	Y	Y	Y	Y	Y	Y	Y
54	*Penganum harmala*	Y	Y	Y	Y	Y	Y	P	Y	Y
55	*Hygrophila schulli*	Y	Y	Y	Y	Y	Y	Y	Y	Y
56	*Holarrhena pubescens*	P	Y	Y	P	Y	Y	N	Y	Y
57	*Hyoscyamus reticulatus*	N	N	N	N	N	N	N	N	N
58	*Ipomoea hederacea*	Y	Y	Y	Y	Y	Y	Y	Y	Y
59	*Indigophora tinctoria*	N	N	Y	N	Y	Y	N	N	N
60	*Ipomoea nil*	P	Y	Y	Y	Y	Y	P	Y	Y
61	*Lowsonia inermis*	Y	Y	Y	Y	Y	Y	Y	Y	Y
62	*Lepidium sativum*	N	N	P	N	P	Y	N	N	P
63	*Lablab purpurens*	N	P	Y	P	Y	Y	N	P	Y
64	*Lycopersicon esculentum*	P	Y	Y	P	Y	Y	P	Y	Y
65	*Linum utisacimum*	Y	Y	Y	Y	Y	Y	P	Y	Y
66	*Momordica charntia*	Y	Y	Y	Y	Y	Y	Y	Y	Y
67	*Mucuna pruriens*	Y	Y	Y	Y	Y	Y	Y	Y	Y
68	*Murraya koenigii*	Y	Y	Y	Y	Y	Y	Y	Y	Y
69	*Medicago sativa*	N	N	N	N	N	N	N	N	N
70	*Mitragyna parvifolia*	N	N	N	N	N	N	N	N	N
71	*Mentha piperata*	N	N	N	N	N	N	N	N	N
72	*Mesua ferrea*	P	Y	Y	P	Y	Y	N	N	P
73	*Mimosa pudica*	N	N	P	P	Y	Y	N	N	P
74	*Myristica fragrans*	P	Y	Y	Y	Y	Y	N	N	N
75	*Mathiola incana*	N	P	Y	N	P	Y	N	N	N
76	*Nigela sativa*	Y	Y	Y	Y	Y	Y	Y	Y	Y
77	*Phaseolus vulgaris*	Y	Y	Y	Y	Y	Y	Y	Y	Y
78	*Pongamia pinnata*	Y	Y	Y	Y	Y	Y	Y	Y	Y

Sr. No.	Botanical Name	Trypsin			Chymotrypsin			HaGP		
		3:1	1:1	1:3	3:1	1:1	1:3	3:1	1:1	1:3
79	*Psophocarpus tetragonolobus*	Y	Y	Y	Y	Y	Y	Y	Y	Y
80	*Psoralea corywfolia*	Y	Y	Y	Y	Y	Y	Y	Y	Y
81	*Punica grantum*	N	Y	Y	Y	Y	Y	N	P	Y
82	*Piper nigrum*	N	N	N	N	N	N	N	N	N
83	*Paspalum scrobiculantum*	N	P	Y	Y	Y	Y	N	P	Y
84	*Pennisetum americanum*	N	N	N	N	N	N	N	N	N
85	*Phyllanthus emblica*	N	P	Y	Y	Y	Y	N	P	Y
86	*Portulaea olercea*	N	P	Y	P	Y	Y	N	Y	Y
87	*Papaver somniferum*	N	P	Y	N	P	Y	N	N	N
88	*Pimpinella anisum*	N	N	Y	N	N	N	N	N	Y
89	*Piper longnum*	N	N	N	N	N	N	N	N	N
90	*Raphanus sativus*	Y	Y	Y	Y	Y	Y	Y	Y	Y
91	*Ricinus communis*	Y	Y	Y	Y	Y	Y	Y	Y	Y
92	*Sapindus laurifolius*	Y	Y	Y	Y	Y	Y	Y	Y	Y
93	*Schrebera sweitenoides*	P	Y	Y	P	Y	Y	P	Y	Y
94	*Solanum nigrum*	Y	Y	Y	Y	Y	Y	Y	Y	Y
95	*Spinacia oleracea*	N	N	N	N	N	N	N	N	N
96	*Salvia spinosa*	N	N	P	N	N	P	N	N	N
97	*Sida cardifolia*	N	P	P	N	N	P	N	P	Y
98	*Sphaeranthus indicus*	N	N	Y	N	N	Y	N	Y	Y
99	*Schleichera oleosa*	N	N	N	N	P	Y	N	N	P
100	*Setaria italica*	N	N	N	N	N	N	N	N	N
101	*Sesamum orientale*	N	N	Y	N	P	Y	N	N	N
102	*Terminalia chebula*	Y	Y	Y	Y	Y	Y	Y	Y	Y
103	*Trigonella foenum-graecum*	Y	Y	Y	Y	Y	Y	Y	Y	Y
104	*Terminalia belerica*	N	P	Y	N	Y	Y	N	P	Y
105	*Urena lobata*	Y	Y	Y	Y	Y	Y	Y	Y	Y
106	*Vigna unguiculata*	Y	Y	Y	Y	Y	Y	Y	Y	Y
107	*Vitis vinifera*	N	N	P	N	N	P	N	N	P
108	*Zea mays*	Y	Y	Y	Y	Y	Y	Y	Y	Y

N - No inhibition, P - Partial inhibition, Y - Total inhibition.

by dot-blot are shown in Figure 4.2.1. Host and non-host plant PIs were detected by mixing protease and inhibitors, and spotting on the X-ray film. For observations, clearance of film required mixing of individual protease and inhibitor samples before spotting on the X-ray film. However, for detection of low level of activity of inhibitors, protease and seed samples were mixed in different proportions as described earlier. The faint gray spots indicated total inhibition of trypsin activity by the non-host plant PIs, e.g., *D. alba ness*, while dark spot indicated that there was no inhibition of trypsin at all three concentrations, e.g., by *A. sativa* [Figure 4.2.1 (A) and (D), respectively]. *Cajanus cajan* and *C. arietinum* showed partial and low level of inhibition of trypsin [Figure 4.2.1 (B) and (C)].

4.2.2 Electrophoretic profile of TI, CTI, and HaGPI isoforms in host and non-host plant PIs

Trypsin, chymotrypsin, and HaGP inhibitory profiles in host and non-host plant PIs were detected in seed extracts of the sample run on denaturing gel, and visualized on X-ray film by immersing the gel in the same enzyme solution, separately, as shown in Figures 4.2.2 and 4.2.3, respectively. Thirty-one seed samples showed inhibitory activity in dot-blot assays and, were analyzed electrophoretically. Only 22 samples showed TIs, CTIs, or HaGPIs or all the three activity bands on X-ray film (Figures 4.2.2, 4.2.3, and 4.2.4, respectively). As in Table 4.2.2, several bands of TI and CTI activities were detected in both host and non-host plants. *Cajanus cajan* and *C. arietinum* are the common host plants of *H. armigera*, which exhibited nine and seven bands of TI (Figure 4.2.2; lanes 3 and 4), respectively. In *C. cajan*, out of nine, six fast-migrating TI bands were detected (Figure 4.2.2, lane 4). However, HaGPI activity bands were absent in C. *arietinum* and *C. cajan* (results not shown).

In seeds, of *G. max*, *P. tetragonolobus*, and *V. unguiculata*; 12, 8, and 10 TI and, 12, 12, and 10 CTI activity bands were detected, respectively (Figure 4.2.2, lanes 9, 18, and 22; Figure 4.2.3, lanes 6, 11, and 14), while two HaGPI bands were detected in all leguminous seed samples (Figure 4.2.2; lanes 5, 10, and 12).

From the seeds of *C. annum* and *M. koenigii*, four TI, and one HaGPI activity bands were resolved (Figure 4.2.2, lanes 5 and 15; Figure 4.2.4, lanes 3 and 8). While, *M.*

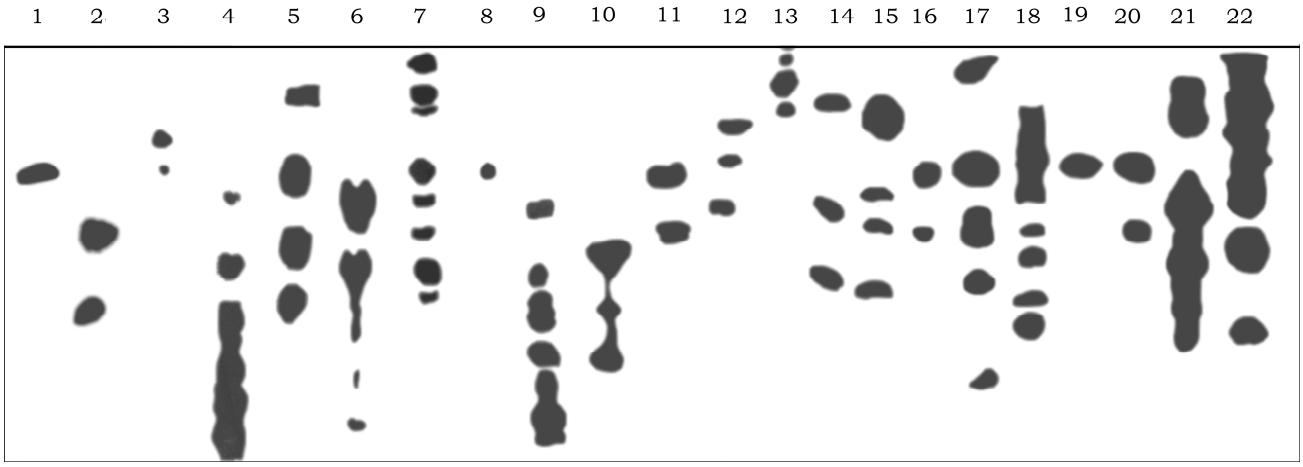

Figure 4.2.2 Visualization of TI profiles of host and non-host plants. Seed extract was separated on discontinuous polyacrylamide gel (10% resolving gel) using Davis buffer system (Davis 1964). In each lane, 30 µg of seed protein was loaded. Wherever low TI activity was detected, more amounts of protein were loaded.

Lane 1, *Acacia nilotica*; lane 2, *Achyranthes aspera*; lane 3, *Blepharis edulis*; lane 4, *Cajanus cajan*; lane 5, *Capsicum annum*; lane 6, *Cicer arietinum*; lane 7, *Datura alba ness*; lane 8, *Foeniculum vulgare*; lane 9, *Glycine max*; lane 10, *Hordeum vulgare*; lane 11, *Penganum harmala*; lane 12, *Hygrophila schulli*; lane 13, *Momordica charntia*; lane 14, *Mucuna pruriens*; lane 15, *Murraya koenigii*; lane 16, *Nigela sativa*; lane 17, *Pongamia pinnata*; lane 18, *Psophocarpus tetragonolobus*; lane 19, *Psoralea corywfolia*; lane 20, *Solanum nigrum*; lane 21, *Trigonella foenum-graecum*; and lane 22, *Vigna unguiculata*.

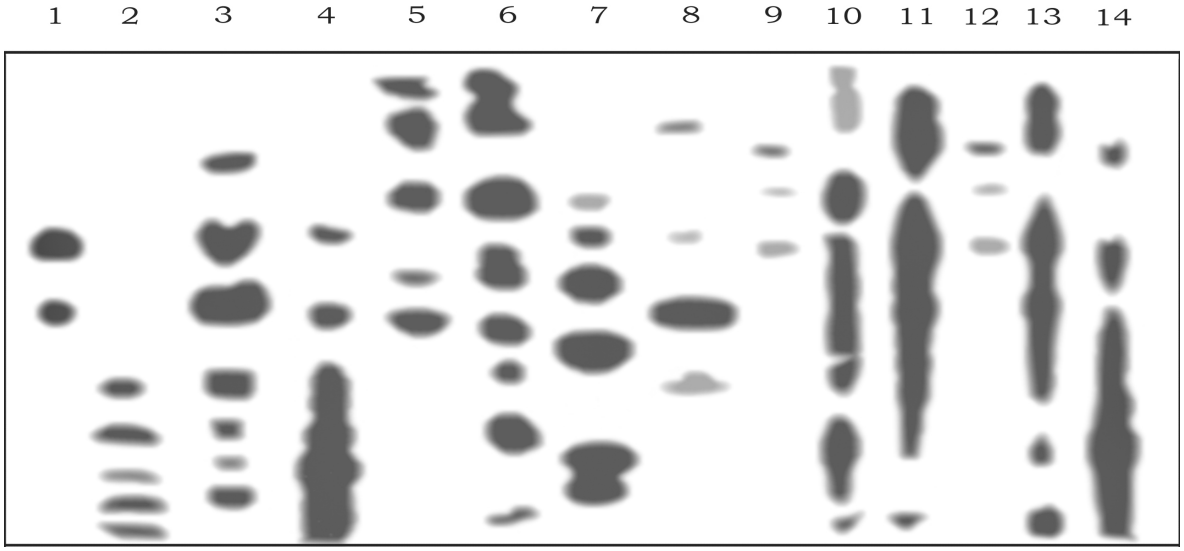

Figure 4.2.3 Electrophoretic separation of host and non-host plant CTIs on 10% native-PAGE. Chymotrypsin inhibitory activity visualized by gel x-ray film contact print method.

Lane 1, *Acacia nilotica*; lane 2, *Blepharis edulis*; lane 3, *Cajanus cajan*; lane 4 *Cicer arietinum*; lane 5, *Datura alba ness*; lane 6, *Glycine max*; lane 7, *Momordica charntia*; lane 8, *Mucuna pruriens*; lane 9, *Murraya koenigii*; lane 10, *Pongamia pinnata*; lane 11, *Psophocarpus tetragonolobus*; lane 12, *Psoralea corywfolia*; lane 13, *Trigonella foenum-graecum*; and lane 14, *Vigna unguiculata*.

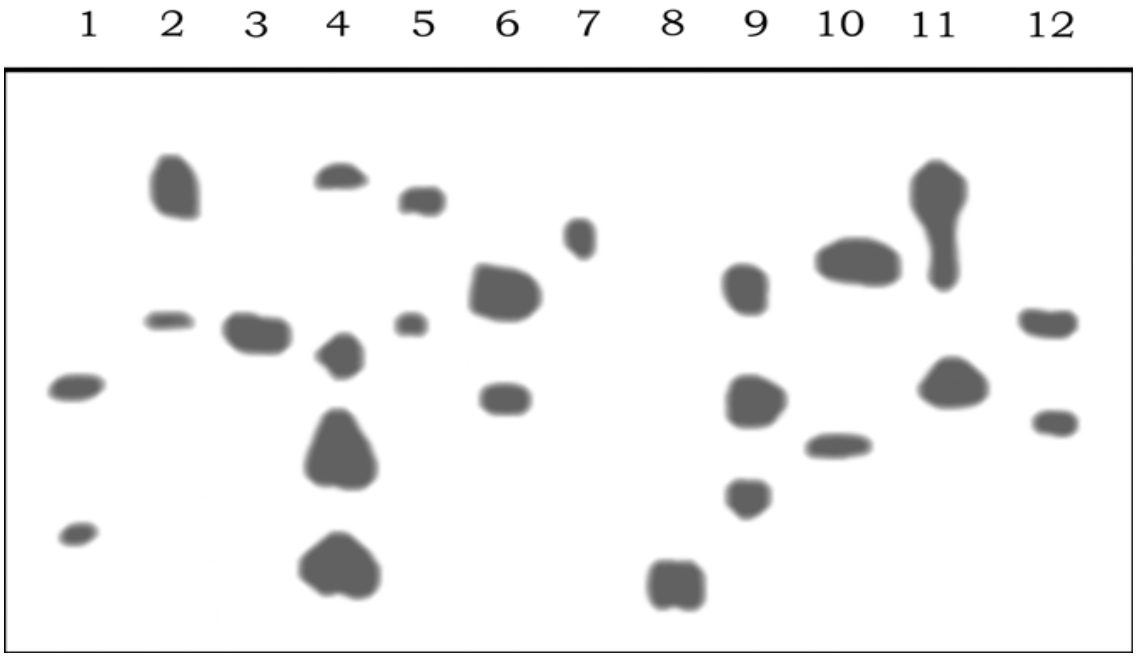

Figure 4.2.4 HaGPI profiles of host and non-host plant PIs. Seed extracts of host and non-host plants were separated on 10% native polyacrylamide gels. After electrophoresis, HaGPI bands were visualized as described in 'Materials and Methods'. Equal TI units were loaded in each lane.

Lane 1, *Acacia nilotica*; lane 2, *Blepharis edulis*; lane 3, *Capsicum annum*; lane 4, *Datura alba ness*; lane 5, *Glycine max*; lane 6, *Momordica charntia*; lane 7, *Mucuna pruriens*; lane 8, *Murraya koenigii*; lane 9, *Pongamia pinnata*; lane 10, *Psophocarpus tetragonolobus*; lane 11, *Trigonella foenum-graecum*; and lane 12, *Vigna unguiculata*.

Table 4.2.2 Electrophoretic detection of trypsin, chymotrypsin, and *H. armigera* gut proteinase inhibitor isoforms from host and non-host plants.

Host and non-host plant	**TIs**	**CTIs**	**HaGPIs**
Acacia nilotica	01	02	02
Achyranthes aspera	02	ND	ND
Blepharis edulis	02	05	02
Cajanus cajan	09	07	ND
Capsicum annum	04	ND	01
Cicer arietinum	07	08	ND
Datura alba ness	08	07	04
Foeniculum vulgare	01	ND	ND
Glycine max	12	12	02
Hordeum vulgare	03	ND	ND
Penganum harmala	02	ND	ND
Hygrophila schulli	03	ND	ND
Momordica charntia	04	06	02
Mucuna pruriens	03	04	01
Murraya Koenigii	04	03	01
Nigela sativa	02	ND	ND
Pongamia pinnata	06	10	03
Psophocarpus tetragonolobus	08	12	02
Psoralea corywfolia	01	03	ND
Solanum nigrum	02	ND	ND
Trigonella foenum-graecum	08	12	03
Vigna unguiculata	10	10	02

ND - Not Detectable.

koenigii showed three CTI bands on X-ray film (Figure 4.2.3, lane 9), *C. annum* did not exhibit CTI activity (results not shown).

Acacia nilotica, *F. vulgare*, and *P. corywfolia* had one trypsin inhibitor band in each sample, while *B. edulis*, *N. sativa*, and *S. nigrum* had two CTI bands as visualized on X-ray film in each sample. (Figure 4.2.2, lanes 1, 8, 19; and Figure 4.2.3, lanes 3, 16, and 20). *Datura alba ness*, which showed potent inhibitory activity against HaGPs, had eight TIs, seven CTIs, and four HaGPIs (Figure 4.2.2, lane 7; Figure 4.2.3, lane 5; Figure 4.2.4, lane 4). *Achyranthes aspera*, *F. vulgare*, *H. vulgare*, *P. hurmala*, *H. schulli*, *N. sativa*, and *S. nigrum* had two, one, three, two, three, two, and two TI bands, respectively (Figure 4.2.2; lanes 2, 8, 10, 11, 12, and 16, and 20).

Mucuna pruriens also exhibited potent inhibitory activity, and had three TIs, four CTIs, and one HaGPI band in seed extract (Table 4.2.2). *Momordica charntia*, *P. pinnata*, and *T. foenum-graecum* seed extracts had four, six, and eight TI activity bands (Figure 4.2.2, lane 13, 17, and 21); and six, ten, and twelve CTIs (Figure 4.2.3, lane 7, 10, and 13); and two, three, and three HaGPI bands (Figure 4.2.4, lane 6, 9, and 11), respectively.

All host and non-host plant PIs exhibited trypsin inhibitory activity, but most of them did not show chymotrypsin and HaGP inhibitory activity. *Capsicum annum* showed HaGPI activity, but CTI activity was absent. Host plants viz. *C. cajan* and *C. arietinum* did not show inhibitory activity towards *H. armigera* gut proteinases, but non-host plant PIs were potent inhibitors of HaGP.

4.2.3 *In vivo* inhibition of HGP isoforms by host and non-host plant PIs

The *H. armigera* larvae fed on host and non-host plant PIs were analyzed for *in vivo* inhibitions of HaGPs and trypsin isoforms activities by azocasein and BA*p*NA, respectively (Table 4.2.3). This is a simple approach to evaluate the specificity and potential of host and non-host plant PIs against *H. armigera* gut digestive enzymes. Hence, substrate assays were used to determine the percentage inhibition of total proteinase activity along with trypsin isoforms activity, and assessed the insensitive gut proteinase profile electrophoretically (Figure 4.2.5). To assess the *in vivo* inhibition of HaGPs, early third-instars of same size were fed on host and non-host plant PIs. The

4.2.3 *In vivo* inhibition of proteinase and further *in vitro* activation of pro-proteinases of *H. armigera* fed on host and non-host plant PIs. Various plant PIs from host and non-host were tested for their maximum inhibition potential against HaGPs and further activation of HaGPPs. Azocasein and BA*p*NA were used as substrates to measure the total proteinase and trypsin isoforms activities, respectively. Then, *in vitro* activation of pro-proteinases and trypsinogen isoforms were measured using azocasein and BAEE as substrates, respectively, in activation buffer as described in 'Materials and Methods'.

Host and non-host plant	**Inhibition of HaGPs (%) mean ± SE (n=3)**		**Activation of HaGPPs (%) mean ± SE (n=3)**	
	Proteinase	**Trypsin**	**Pro-proteinase**	**Trypsinogen**
Acacia nilotica	71.53 ± 1.87	53.78 ± 2.38	11.90 ± 0.65	27.81 ± 0.95
Achyranthes aspera	38.59 ± 2.48	34.83 ± 7.42	16.77 ± 1.61	23.96 ± 2.08
Blepharis edulis	50.96 ± 2.50	44.51 ± 1.47	11.68 ± 0.47	19.43 ± 0.31
Cajanus cajan	8.02 ± 4.35	10.62 ± 1.20	ND	ND
Capsicum annum	83.56 ± 1.43	65.68 ± 3.26	23.21 ± 1.16	33.29 ± 1.07
Cicer arietinum	16.02 ± 1.00	11.48 ± 2.61	ND	ND
Datura alba ness	91.33 ± 0.87	80.02 ± 0.31	26.04 ± 1.48	42.97 ± 1.31
Foeniculum vulgare	29.43 ± 5.29	40.08 ± 3.02	19.44 ± 0.59	18.59 ± 0.31
Glycine max	61.54 ± 2.38	70.60 ± 1.13	18.78 ± 3.71	30.83 ± 2.30
Hordeum vulgare	70.01 ± 1.70	67.02 ± 2.94	14.58 ± 0.61	12.37 ± 1.27
Penganum harmala	24.85 ± 0.42	33.43 ± 1.67	ND	ND
Hygrophila schulli	65.75 ± 1.56	69.90 ± 0.55	14.23 ± 0.51	11.50 ± 0.24
Momordica charntia	53.48 ± 1.90	58.98 ± 0.74	12.62 ± 1.29	12.37 ± 1.19
Mucuna pruriens	83.69 ± 0.58	84.23 ± 0.64	21.77 ± 1.28	21.34 ± 1.80
Murraya Koenigii	73.23 ± 2.80	73.89 ± 3.01	14.44 ± 0.42	15.01 ± 0.51
Nigela sativa	81.84 ± 2.17	86.28 ± 0.33	19.95 ± 1.14	22.55 ± 3.81
Pongamia pinnata	85.03 ± 1.35	83.94 ± 0.83	20.25 ± 1.99	25.16 ± 1.93
Psophocarpus tetragonolobus	87.98 ± 1.43	88.61 ± 1.85	24.17 ± 1.62	34.92 ± 1.58
Psoralea corywfolia	42.68 ± 2.67	43.61 ± 3.18	11.61 ± 0.57	12.94 ± 0.86
Solanum nigrum	50.65 ± 4.19	52.92 ± 2.47	ND	ND
Trigonella foenum-graecum	61.05 ± 1.74	62.96 ± 1.80	10.81 ± 0.20	12.47 ± 0.44
Vigna unguiculata	70.94 ± 1.62	74.97 ± 2.46	13.19 ± 0.34	13.57 ± 0.58

ND - Not Detectable.

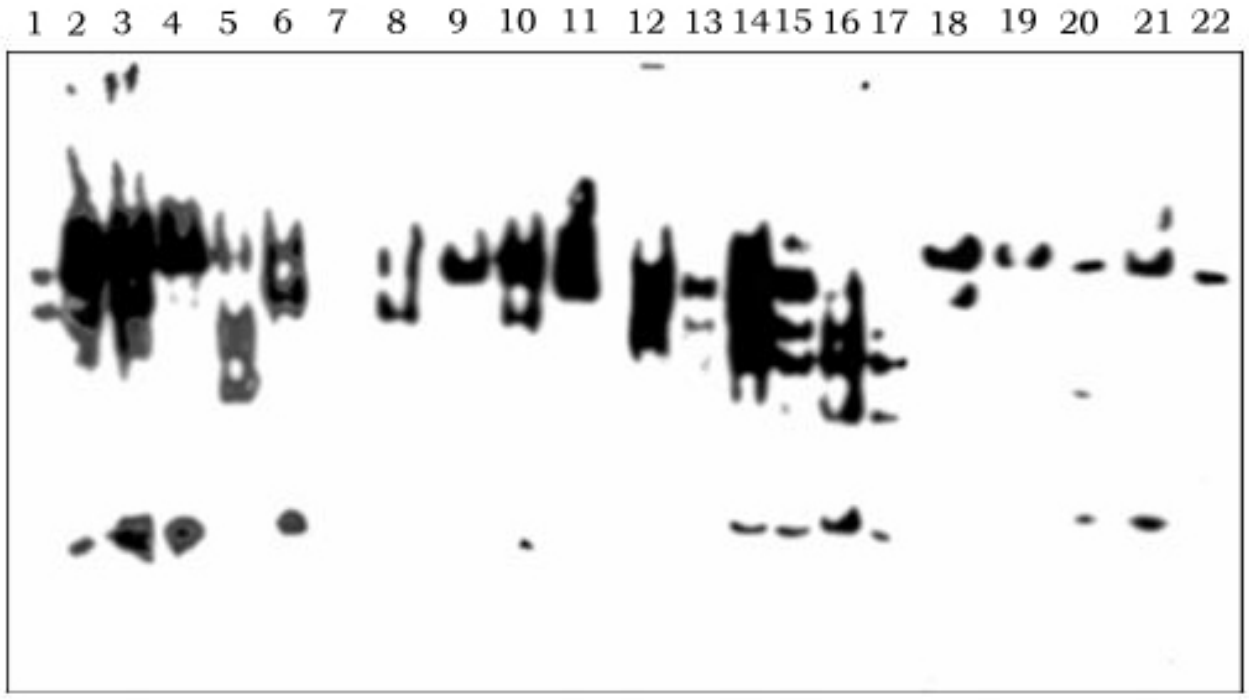

Figure 4.2.5 *In vivo* inhibition of HaGPs isoforms by inhibitors of host and non-host plant PIs. *H. armigera* fed on host and non-host plants incorporated in chickpea (PIs removed) diet with a proportion 1:1 and then gut proteinase activity was visualized by the gel X-ray film contact print technique.

Lane 1, *Acacia nilotica*; lane 2, *Cajanus cajan*; lane 3, *Cicer arietinum*; lane 4, *Achyranthes aspera*; lane 5, *Blepharis edulis*; lane 6, *Capsicum annum*; lane 7, *Datura alba ness*; lane 8, *Foeniculum vulgare*; lane 9, *Glycine max*; lane 10, *Hordeum vulgare*; lane 11, *Penganum harmala*; lane 12, *Hygrophila schulli*; lane 13, *Mucuna pruriens*; lane 14, *Momordica charntia*; lane 15, *Murraya koenigii*; lane 16, *Psoralea corywfolia*; lane 17, *Nigela sativa*; lane 18, *Pongamia pinnata*; lane 19, *Psophocarpus tetragonolobus*; lane 20, *Solanum nigrum*; lane 21, *Trigonella foenum-graecum*; lane 22, *Vigna unguiculata*.

resulting inhibition of total proteinase activity and/or trypsin activity was determined by azocasein and BA*p*NA assay, respectively. Non-host plants such as, *D. alba ness*, *P. tetragonolobus*, *P. pinnata*, *M. pruriens*, *C. annum*, and *N. sativa* showed 91.33, 87.98, 85.03, 83.69, 83.56, and 81.84% inhibition of HaGPs, while inhibition was observed in crude trypsin isoforms of 80.02, 88.61, 83.94, 84.23, 65.68, and 86.28%, respectively.

Murraya koenigii, *A. nilotica*, *V. unguiculata*, *H. vulgare*, *H. schulli*, *G. max*, *T. foenum-graecum*, *M. charntia*, *S. nigrum*, and *B. edulis* showed high to moderate levels of inhibition of gut proteinase activity; 73.23, 71.53, 70.94, 70.01, 65.75, 61.54, 61.05, 53.48, 50.65, and 50.96% as measured by azocasein assay, respectively. Inhibition of trypsin isoforms was 73.89, 53.78, 74.97, 67.02, 69.90, 70.60, 62.96, 58.98, 52.92, and 34.83% by BA*p*NA assay, respectively (Table 4.2.3). *Achyranthes aspera*, *F. vulgare*, and *P. corywfolia* resulted in 38.59, 24.85, and 42.68% inhibition of HaGP; and 34.83, 40.08, and 43.61% inhibition of trypsin isoforms, respectively. These plants showed low inhibition as compared to the other non-host plants. Host plants *C. arietinum* and *C. cajan* showed very low inhibition of HaGP (16.02, 8.02%) and (11.48, 10.62%) of trypsin isoforms of *H. armigera* (Table 4.2.3).

Electrophoretic visualization of *in vivo* inhibition of HaGP isoforms by host and non-host plant PIs is shown in Figure 4.2.5. Larvae reared on *C. arietinum* PIs showed overproduction of HaGP 9, and increased expression of HaGP 6 and 9, but decreased the expression of HaGP 3 and 7. HaGPs 1 and 8 were not detected on X-ray film (Figure 4.2.5). Larvae fed on another host plant, *C. cajan* PIs diet showed a profile of HaGPs similar to larvae fed on *C. arietinum* diet. HaGP 4 and 5 were highly expressed, but HaGPs 1, 2, and 8 were not detected, while HaGP 3 showed low expression.

Larvae fed on non-host plant PIs showed stunted growth, and the HaGPs activity was also inhibited significantly. Among the non-host plants, *D. alba ness* showed total inhibition to HaGPs (Figure 4.2.5, lane 7). *Glycine max*, *P. tetragonolobus*, and *V. unguiculata* PIs also inhibited the gut proteinase activity, except that of HaGP 5 (Figure 4.2.5, lanes 9, 19, and 22). Non-host plants such as *A. aspera* and *F. vulgare* showed less inhibition in azocasein assay, but HaGP activity was not detected on X-ray film (Figure 4.2.5, lanes 4 and 8). *Momordica charntia*, *M. koenigii*, and *P. corywfolia* resulted in increased expression of HaGP 4, 5, 6, 7, and 8 (Figure 4.2.5; lanes 14, 15, and 16).

Blepharis edulis showed partial inhibition, but low- and fast-moving proteinase bands were not observed on the X-ray film (Figure 4.2.5, lane 5). However, *P. harmala* did not show much activity and, low-moving bands (HaGPs 6, 7, 8 bands) were not visualized on the X-ray film. However, it resulted in high expression of HaGPs 4, 5, and 6. *Acacia nilotica*, *M. pruriens*, and *P. pinnata* showed maximum inhibition in azocaseinase and BA*p*NAase assay, and HaGP 5 and 6 were observed on X-ray film (Figure 4.2.5; lanes 1, 13, and 18). *Hordeum vulgare*, *S. nigrum*, and *T. foenum-graecum* inhibited low migrating bands, but did not affect the fast-moving bands (Figure 4.2.5; lanes 10, 20, and 21). Non-host PIs showed high inhibitory potential towards the HaGPs activity, while host plant PIs were weak inhibitors of HaGPs.

4.2.4 Activation and visualization of HaGPPs fed on host and non-host plant PIs

Host and non-host plant PIs were also incorporated into the artificial diet to test the *in vivo* inhibition potential against *H. armigera* gut pro- and proteinase(s). The chickpea flour possesses major amounts of bovine trypsin PIs of the kunitz type, which were inactivated by autoclaving and heat treatment. In the present study, larvae were fed on artificial diet of chickpea (PIs removed); with equal quantity of host and non-host plant PIs.

Effect of host and non-host plant PIs on *H. armigera* larvae was studied by activation of pro-proteinase by azocasein assay, and trypsinogen isoforms, respectively (Table 4.2.3). The larvae fed on diet with *D. alba ness* PIs showed highest inhibitory activity against HaGPs, and also resulted in *in vivo* inhibition of HaGPPs activation. Activation of HaGPPs by trypsin (activation of HaGPPs by trypsin described in Materials and Methods) resulted in 26.04% activation of HaGPPs and 42.97% activation of trypsinogen isoforms.

Psophocarpus tetragonolobus also exhibited high inhibitory potential towards HaGPs, and resulted in 24.17% activation of HaGPPs and 34.92% activation of trypsinogen isoforms of *H. armigera* by azocasein and BAEE assays, respectively (Table 4.2.3). *Capsicum annum*, *M. pruriens*, *P. pinnata*, and *N. sativa* showed high inhibitory potential towards HaGPs, and also resulted in *in vitro* 23.21, 21.77, 20.25, and 19.95%

activation of HaGPs; and 33.29, 21.34, 25.16, and 22.55% activation of trypsinogen isoforms, respectively (Table 4.2.3).

Larvae fed on non-host plant PIs from *P. harmala* and *S. nigrum* did not show the activation the HaGPPs and the trypsinogen isoforms. Interestingly, *F. vulgare* showed low inhibitory effect on HaGPs, but activated the HaGPPs (19.44%) and trypsinogen isoforms (18.59%) (Table 4.2.3).

Non-host plant PIs fed HaGPs from *A. nilotica*, *A. aspera*, *B. edulis*, *G. max*, *H. vulgare*, *H. schulli*, *M. charntia*, *M. koenigii*, *P. corywfolia*, *T. foenum-graecum*, and *V. unguiculata* showed 11.90, 16.77, 11.68, 18.78, 14.58, 14.23, 12.63, 14.44, 11.61, 10.81, and 13.81% activation of HaGPPs; and 27.81, 23.96, 19.43, 30.83, 12.37, 11.50, 12.37, 15.01, 12.94, 12.47, and 13.57% activation of trypsinogen isoforms, respectively (Table 4.2.5).

To visualize activation of *H. armigera* gut pro-proteinases after feeding on host and non-host plant PIs revealed that most of proteinases were inhibited and, further activation of pro-proteinases showed either increased activity or activated proteinase bands on X-ray film. In *H. armigera* larvae fed on plant PIs were resolved on 10% native-PAGE for visualization of proteinase activity bands, several major and minor proteinase bands were inhibited (Figure 4.2.5), and followed by *in-gel* activation, the inactive isoforms of proteinases were activated and visualized by X-ray film method (Figure 4.2.6). For visualization of activation of major and minor pro-proteinase, more activity units were loaded on the gel or overlay incubation time period increased, but resulted in high concentration of activated major proteinases or close mobility isoforms merged, and resulted in a smear of proteinase profile on X-ray film. To overcome this problem, variable incubation period of gel on X-ray film for inhibition and activation was followed, and the profiles compared. Gut proteinase(s) of larvae fed on *C. arietinum* PIs incorporated in chickpea (PIs removed) based artificial diet showed that minor bands with close mobility were inhibited, and after activation, there was a slight increase in activity of proteinase, but they merged and formed a smear. Similar profile was obtained in *C. cajan*, but larvae fed on both host plant PIs did not show any activated proteinase band (Figure 4.2.6). Non-host plants PIs are a good candidate for inhibition of HaGPs and HaGPPs. Many major and minor proteinases were inhibited after feeding (Figure

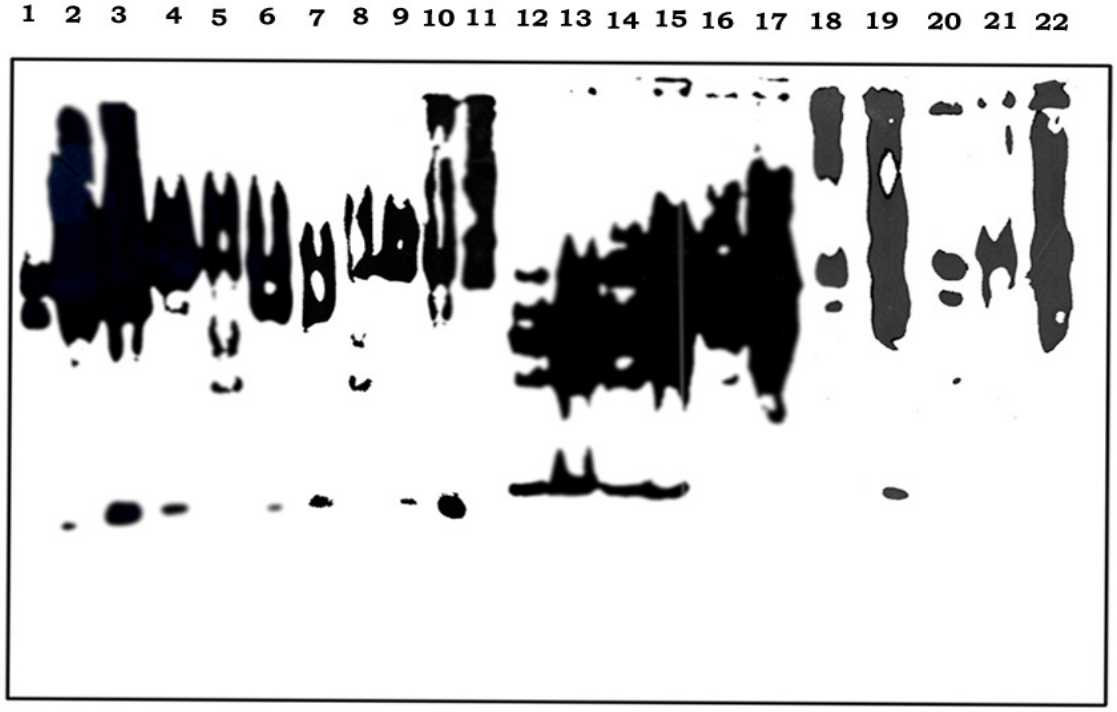

Figure 4.2.6 *In vitro* activation of HaGPP isoforms. The *H. armigera* larvae fed on host and non-host plant PIs with artificial diet in 1:1 proportion were used for *in-gel* activation. The *H. armigera* gut extract was loaded on 10% native-PAGE and the gel processed for *in-ge*l activation as described in 'Materials and Methods'. Activation was visualized by gel X-ray film contact print method.

Lane 1, *Acacia nilotica*; lane 2, *Cajanus cajan*; lane 3, *Cicer arietinum*; lane 4, *Achyranthes aspera*; lane 5, *Blepharis edulis*; lane 6, *Capsicum annum*; lane 7, *Datura alba ness*; lane, 8, *Foeniculum vulgare*; lane 9, *Glycine max*; lane 10, *Hordeum vulgare*; lane 11, *Penganum harmala*; lane 12, *Hygrophila schulli*; lane 13, *Mucuna pruriens*; lane 14, *Momordica charntia*; lane 15, *Murraya koenigii*; lane 16, *Psoralea corywfolia*; lane 17, *Nigela sativa*; lane 18, *Pongamia pinnata*; lane 19, *Psophocarpus tetragonolobus*; lane 20, *Solanum nigrum*; lane 21, *Trigonella foenum-graecum*; and lane 22, *Vigna unguiculata*.

4.2.5), followed by *in vitro* pro-proteinase activation visualized on X-ray film (Figure 4.2.6). *Datura alba ness* fed *H. armigera* larvae gut extract showed complete inhibition (Figure 4.2.5; lane 7). *In vitro* activation of HaGPPs showed that four proteinase were activated HaGP 4, 5, 6, and 9 visualized on X-ray film (Figure 4.2.6; lane 7). Larvae fed on *P. tetragonolobus* and *M. pruriens* showed high activation of HaGPPs, but the activated proteinases merged and formed a smear (Figure 4.2.6; lane 19 and 13). *Capsicum annum* showed activation of major proteinase bands, and HaGPs 4 and 5 were activated (Figure 4.2.6, lane 6), but no activation was observed in slow- or fast-migrating bands. *Acacia nilotica* showed increased activity of HaGPs 5 and 6 (Figure 4.2.6; lane 1), while *A. aspera* show activated isoforms HaGPs 4, 5, and 6 (Figure 4.2.6; lane 4). *Foeniculum vulgare* activated HaGPs 7 and 8, while *G. max* activated HaGPs 4 and 9 (Figure 4.2.5; lane 8 and 9). *Hordeum vulgare* showed activation of slow and fast-migrating HaGPP bands (Figure 4.2.5, lane 10). Interestingly, *P. harmala* and *S. nigrum* resulted in activation of slow-moving bands (Figure 4.2.6; lanes 11 and 20), but no activation was observed in substrate assays (Table 4.2.3). *Hygrophila schulli* showed activation of fast-moving bands, while *M. charntia*, *M. koenigii*, *P. corywfolia*, *N. sativa*, *P. pinnata*, *T. foenum-graecum*, and *V. unguiculata* showed activation of major pro-proteinase bands as well as slow-migrating HaGPPs (Figure 4.2.5; lanes 12, 14, 15, 16, 17, 18, 21, and 22). These results were corroborated by substrate assay. These studies indicated that non-host plant PIs are good candidates' as inhibitors of the HaGPs as well as HaGPPs, however, *H. armigera* larvae fed on host plant PIs did not show the activation of HaGPPs and trypsinogen isoforms, because they are weak inhibitors of HaGP.

4.2.5 Activation of gut pro-proteinases during the development of *H. armigera* larvae reared on non-host plant PIs

In order to examine the inhibition potential of host and non-host plant PIs against HaGPs in different instars, two host plants, *C. arietinum* and *C. cajan*, and two non-host plants, *D. alba ness* and *P. tetragonolobus*, and artificial diet fed larval HaGPs and HaGPPs were bioassayed.

Cicer arietinum and *C. cajan* PIs exhibited very low inhibition of HaGPs, and did not show activation in azocaseinolytic assay (data not shown). But larvae reared on non-host plant PIs (*D. alba ness* and *P. tetragonolobus*) showed significant inhibition of HaGPs (data not shown), and *in vitro* activation of HaGPPs (BAEEase and BTEEase) (Tables 4.2.4 and 4.2.5). However, *D. alba ness* fed larval azocaseinase, BAEEase, and BTEEase (HaGPPs) activities were higher than the larvae fed on PIs of *P. tetragonolobus*. Proteolytic activities were barely detected in first-instar larvae, and it was difficult to measure inhibition and activation of HaGPPs (data not shown). In second- instar larvae, low total pro-proteinase activation was detected in both non-host plant PIs fed larval gut extract, but BAEEase and BTEEase activation was not detected (Tables 4.2.4 and 4.2.5). Activation of HaGPPs significantly increased in the third-instar larvae, while maximum activation was observed on fifth-instar larvae (Table 4.2.4 and 4.2.5). Activity declined drastically in the sixth-instars (data not shown). Total pro-proteinase (azocaseinolytic) and trypsinogen isoforms (BAEEase) showed significantly higher activation in non-host plant PIs of *D. alba ness* and *P. tetragonolobus* fed HaGPPs. However, very low chymotrypsinogen isoforms (BTEEase) activation was detected in third-, fourth-, fifth-instar larval stages (Tables 4.2.4 and 4.2.5).

The gut extract of *H. armigera* larvae from various instar fed on non-host plant PIs from *D. alba ness* and *P. tetragonolobus*, and loaded on 10% native-PAGE showed differential *in vivo* inhibition of HaGPs, and followed by *in vitro* activation of HaGPPs (Figures 4.2.7 and 4.2.8). In first-instar larvae, proteinase activity bands were not detected on X-ray film due to very low activity, while second-instars exhibited detectable activity, but pro-proteinase activation was not detected on GXCP (data not shown). Third-, fourth-, and fifth-instar larval gut HaGPs profile and activation of HaGPPs (Figure 4.1.14) have been described in section 4.1.4. It is reveled from Figure 4.1.14 that third-instar larvae showed the presence of HaGPs 2, 4, 5, 6, 7, 8, and 9 inhibited by *D. alba ness* PIs, except HaGP 5 activity was visualized on X-ray film (Figure 4.2.7, lane 1; *in vivo* inhibition).

High proteinase activity units were loaded for visualization of major and minor bands of fourth- and fifth-instars larvae. The larval HaGPs 5 and 9 were not inhibited and HaGP 6 was observed on X-ray film in the fifth-instar larvae (Figure 4.2.7; lanes 2 and 3;

Table 4.2.4 *In vitro* activation from HaGPPs during the instars of larvae fed on non-host plant PIs *Datura alba ness*. Total gut pro-proteinase, trypsinogen, and chymotrypsinogen isoforms activation were measured using azocasein, BAEE, and BTEE as substrates, respectively.

Larval instar	**Activation of pro-proteinases (U/g of gut tissue) mean ± SE (n = 3)**		
	Azocaseinase	**BAEEase**	**BTEEase**
Second	5.66 ± 0.52	ND	ND
Third	12.51 ± 0.14	48.07 ± 1.29	0.371 ± 0.010
Fourth	16.85 ± 0.55	71.54 ± 1.29	0.355 ± 0.010
Fifth	20.52 ± 0.40	76.40 ± 2.50	0.394 ± 0.006

ND - Not Detectable.

Table 4.2.5 *In vitro* activation of HaGPPs during instars of *H. armigera* larvae fed on non-host plant PIs of *Psophocarpus tetragonolobus*. Pro-proteinases, trypsinogen, and chymotrypsinogen isoforms activation were measured using azocasein, BAEE, and BTEE as substrate, respectively.

Larval instars	**Activation of pro-proteinases (U/g of gut tissue) mean ± SE (n = 3)**		
	Azocaseinase	**BAEEase**	**BTEEase**
Second	4.71 ± 0.86	ND	ND
Third	8.09 ± 0.68	38.45 ± 1.08	0.306 ± 0.015
Fourth	11.80 ± 0.35	52.66 ± 0.17	0.277 ± 0.029
Fifth	15.18 ± 0.17	59.84 ± 2.35	0.343 ± 0.018

ND - Not Detectable.

Figure 4.2.7 *In vivo* inhibition of HaGPs and *in vitro* activation of HaGPPs of *H. armigera* larvae fed on host and non-host plant PIs from *Datura alba ness*. High amounts of equal proteinase activity units were loaded to visualize the maximum inhibition and activation bands on X-ray film during larval development.

Third-instar (lane 1), fourth-instar (lane 2), fifth-instar (lane 3).

Figure 4.2.8 *In vivo* inhibition of HaGPs and *in vitro* activation of HaGPPs of *H. armigera* larvae fed on host and non-host plant PIs from *P. tetragonolobus*. High amounts of equal proteinase activity units were loaded to visualize the maximum inhibition and activation bands on X-ray film during larval development.

Third-instar (lane 1), fourth-instar (lane 2), fifth-instar (lane 3).

in vivo inhibition). Significant increase in activation of HaGPs in third-, fourth-, and fifth-instar larvae was observed by gel X-ray film contact print technique (Figure 4.2.7; lane 1, 2, and 3; *in vitro* activation). In third-instar larvae, HaGPs 3, 4, 6, and 9 were activated; while in fourth-instar, HaGPs 3, 4, 6, and 7 were activated. Fifth-instar larvae showed maximum activation of all HaGPPs, HaGPs 8 and 10 bands were not detected. *Psophocarpus tetragonolobus* was also assayed for *in vivo* inhibition of HaGPs (Figure 4.2.7) and *in vitro* activation of HaGPPs (Figure 4.2.8). When high activity units were loaded on 10% native-PAGE, it observed that *P. tetragonolobus* showed considerable specificity for inhibition of HaGPs as compared to *D. alba ness*. *In vivo* inhibition results showed that third-instar larvae exhibited inhibition to HaGP 4, 6, 7, and 9, but not of HaGPs 2 and 5. In fourth-instars, HaGPs 2, 3, and 5 were inhibited, while in fifth-instars, HaGP 6 was not inhibited by plant PIs (Figure 4.2.8; lanes 2 and 3; *in vivo* inhibition). *In vitro* activation of HaGPPs on X-ray film visualized HaGPs 2, 4, and 5 in third-instar, while fourth- and fifth-instar larvae showed similar profile, but HaGPs 4 and 6 were activated (Figure 4.2.8; lanes 1, 2, and 3; *in vitro* activation).

4.2.6 Activation of HaGPPs on treatment of partially purified HaGPs

To assess the activation of pro-proteinase in crude *H. armigera* gut extract from larvae fed on non-host plant PIs, individual electrophoretically purified HaGPs from the fifth-instar were determined by azocaseinolytic and BAEE assay (Figure 4.2.9). Approximately 0.02 U activity of each proteinase was individually treated in different *H. armigera* gut extracts of larvae fed on *D. alba ness*. Total proteolytic activation and trypsinogen isoforms of HaGPPs were measured by azocasein and BTEE, respectively. Of all HaGP isoforms, HaGPs 5 and 9 were showed 19% and 32%; while HaGP 9 showed 17% and 27% activation of total pro-proteinase and trypsinogen isoforms, respectively (Figure 4.2.9). HaGP 5 and 9 are trypsin-like major proteinase isoforms, which showed specificity towards BA*p*NA (results not shown).

To visualize activation of pro-proteinase(s) of *H. armigera* fed on non-host plant PIs from *D. alba ness* fed larval gut extract was used. The experiment was carried out by the treating individual electrophoretically purified HaGP on HaGPPs, and activated gut proteinases visualized by gel X-ray film contact print technique (Figure 4.2.10). Equal

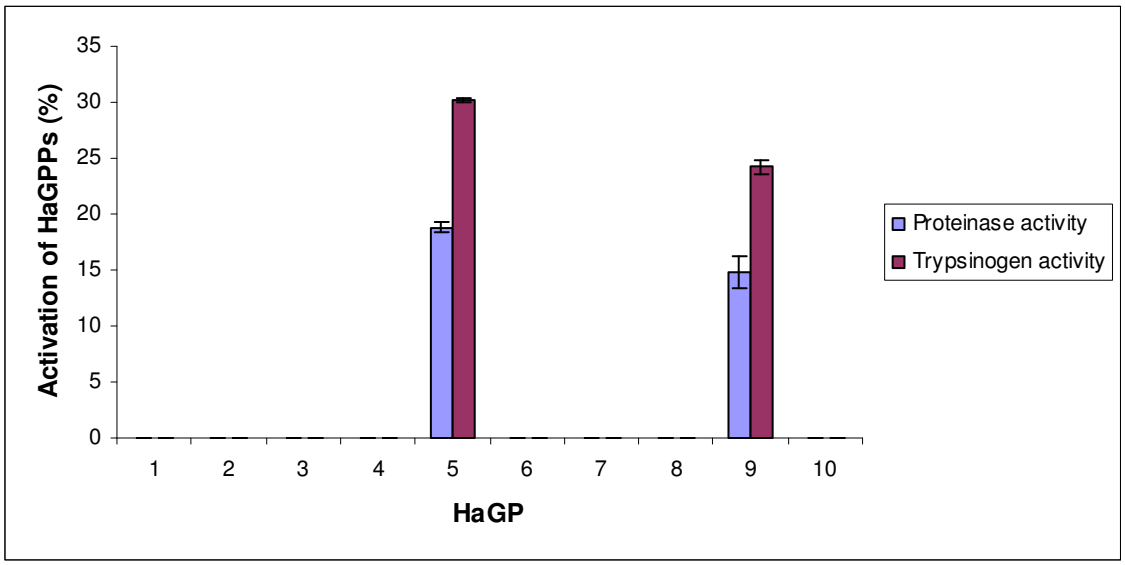

Figure 4.2.9 Effect of partially purified individual *H. armigera* gut proteinase(s) on activation of HaGPPs. Total proteolytic activation and trypsinogen isoforms activation of HaGPPs were determined by azocasein and BAEE, respectively.

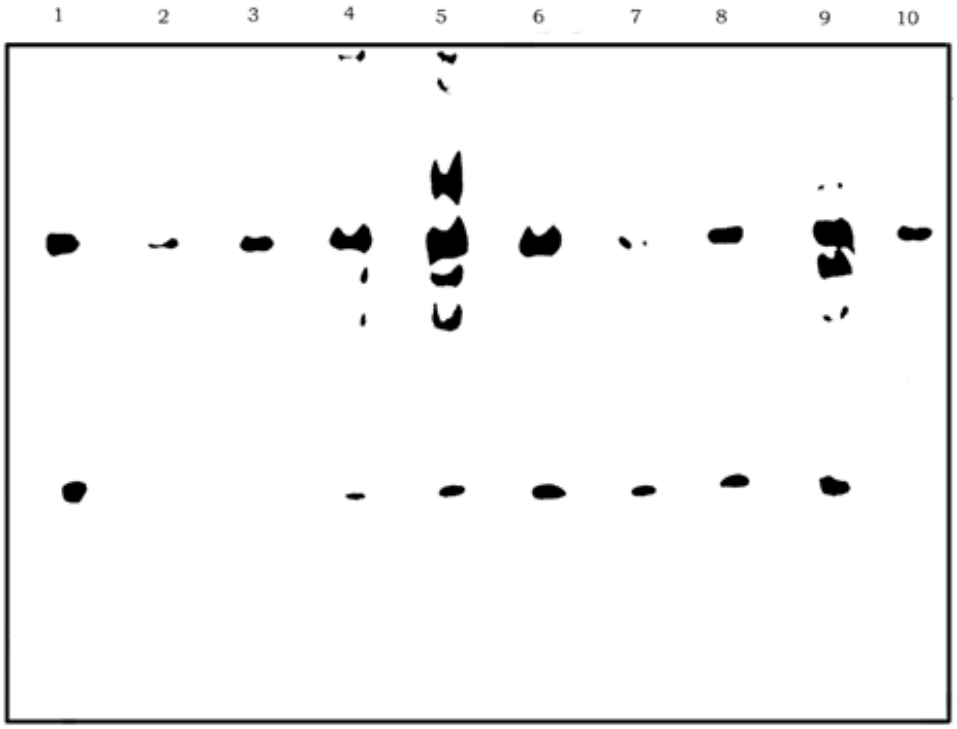

Figure 4.2.10 Effect of individual partially purified HaGP on extract of *H. armigera* larvae fed on non-host plant PIs from *Datura alba ness*. PIs fed gut extract was loaded on 10% native-PAGE and activation of HaGPPs was checked on the treatment of electrophoretically purified HaGP, separately, as described in 'Materials and Methods'. The activation profile of HaGPPs was visualized by gel X-ray film contact print technique.

BAEEase activity units were incubated with equal BA*p*NAase activity units partially purified HaGPs at 37°C for 30 min. After the incubation period, each HaGP treated HaGPPs gut extracts were loaded on 10% native-PAGE, and activation of HaGPPs was visualized on film (Figure 4.2.10). HaGPs 5 and 9 showed activation of pro-proteinases, however, HaGP5 activated the inactive isoforms of HaGPs 1, 2, 4, 6, and 7, which showed activation of pro-proteinases, while HaGP 9 activated inactive isoforms of HaGPs 4, 5, 6, and 7 (Figure 4.2.10; lanes 5 and 9). Both HaGPs 5 and 9 activated HaGP inactive isoforms. HaGP 4 did not activate HaGPP in azocaseinase or BAEEase assay, but activation was observed on X-ray film (activated HaGP 1, 6, and 7 isoforms). HaGPs 1, 6, 7, and 8 showed activity of major proteinases 5 and 9, but did not show activation of HaGPP. Similarly, HaGPs 2, 3, and 10 showed activity of HaGP 5, but no activation band was observed on X-ray film (Figure 4.2.10; lanes 1, 6, 7, 8, and 2, 3, 10, respectively).

4.2.7 Activation of pro-proteinases present in fecal matter

When *H. armigera* larvae were fed on host and non-host plant PIs, the larvae produced pro- and proteinases in response to dietary PIs. Fecal matter was examined by solution assay and electrophoresis. Fecal matter of larvae fed on artificial diet with host and non-host plant PIs were collected and estimated for their pro-proteinase activation (Table 4.2.6). The fecal matter of larvae fed on *C. arietinum* diet showed high proteinase activity, while *C. cajan* fed larvae showed low proteinase activity (data not shown). But fecal matter of *C. cajan* fed larvae showed pro-proteinase activation, whereas *C. arietinum* fed larvae did not show any activation (Table 4.2.6).

Non-host plant PIs fed larvae fecal matter showed high pro-proteinase activation (Table 4.2.6), while the proteinase activities were barely observed (results not shown). When the larvae were fed on plant or artificial diet incorporated with host or non-host plant, a huge food bolus passed through gut lumen, and the proteinase activity was inhibited by plant PIs. At the same time, pro-proteinase(s) were transported to gut lumen through peritropic membrane; however, enough amount of activator proteinase was not present in gut. As a result, HaGPPs passed in fecal matter and the inactive isoforms of proteinases were observed by substrate assay and gel X-ray film contact print technique.

Table 4.2.6 Pro-proteinase activation obtained in the fecal matter of *H. armigera* fed on host and non-host plant PIs. Total pro-proteinases and trypsinogen isoforms activations were measured by azocasein and BAEE as substrates, respectively.

Host and non-host plant	**Pro-proteinase activation (%) mean ± SE (n = 3)**	
	Total pro-proteinase	**Trypsinogen**
Acacia nilotica	24.05 ± 0.90	33.41 ± 2.14
Achyranthes aspera	19.24 ± 0.45	23.26 ± 0.55
Blepharis edulis	16.38 ± 0.56	21.45 ± 1.31
Cajanus cajan	6.68 ± 0.16	ND
Capsicum annum	30.45 ± 0.08	37.45 ± 0.61
Cicer arietinum	ND	ND
Datura alba ness	31.81 ± 0.49	35.82 ± 0.56
Foeniculum vulgare	21.75 ± 0.75	24.97 ± 0.55
Glycine max	21.66 ± 0.59	28.50 ± 0.71
Hordeum vulgare	21.51 ± 0.31	16.89 ± 1.03
Penganum harmala	ND	ND
Hygrophila schulli	19.23 ± 1.37	17.62 ± 1.60
Momordica charntia	18.71 ± 1.10	17.74 ± 1.23
Mucuna pruriens	23.14 ± 0.89	24.07 ± 0.40
Murraya Koenigii	17.51 ± 1.73	19.54 ± 1.34
Nigela sativa	22.84 ± 0.23	26.17 ± 1.95
Pongamia pinnata	22.62 ± 0.84	29.16 ± 0.87
Psophocarpus tetragonolobus	29.20 ± 0.64	36.51 ± 1.18
Psoralea corywfolia	15.42 ± 1.06	14.34 ± 1.70
Solanum nigrum	ND	ND
Trigonella foenum-graecum	16.57 ± 0.25	19.35 ± 0.88
Vigna unguiculata	14.44 ± 0.44	14.35 ± 0.67

ND - Not Detectable.

Among the non-host plant PIs fed group, *D. alba ness* showed highest pro-proteinase activation (31.81%), while *C. annum* showed comparatively low activation (30.45%) in the fecal matter of *H. armigera* (Table 4.2.6). *Psophocarpus tetragonolobus* showed 29.20% pro-proteinase activation and 36.51% trypsinogen isoforms activation. *Penganum harmala* and *S. nigrum* in non-host plant group did not result in activation of pro-proteinase and trypsinogen isoforms in the fecal matter. *Acacia nilotica*, *M. pruriens*, *N. sativa*, and *P. pinnata* also showed 24.05, 23.14, 22.84, and 22.62% activation of total pro-proteinases, and 33.41, 24.07, 26.17, and 29.16% activation of trypsinogen isoforms, respectively (Table 4.2.6). *Foeniculum vulgare*, *G. max*, and *H. vulgare* showed nearly 21.75, 21.66, and 21.51% total pro-proteinase activation, and 24.97, 28.50, and 16.89% trypsinogen isoforms activation, respectively. Other non-host plant fed larval fecal matter showed moderate levels of activation of pro-proteinase *A. aspera*, *B. edulis*, *H. schulli*, *M. charntia*, *M. koenigii*, *P. corywfolia*, *T. foenum-graecum*, and *V. unguiculata* showed 24.05, 19.24, 21.66, 18.71, 17.51, 15.42, 16.57, and 14.44% total pro-proteinase; activation; and 33.41, 24.97, 17.62, 17.74, 19.54, 14.34, 19.35, and 14.35% trypsinogen isoforms activation, respectively (Table 4.2.6).

The fecal matter extract of *H. armigera* larvae reared on host and non-host plant PIs were resolved on 10% native-PAGE for visualization of proteinase activity, and activation of pro-proteinases by loading with an equal unit activity (Figure 4.2.11). *Cicer arietinum* fed larval fecal matter showed total proteinase profile, which resembled the gut proteinase profile, but the fecal matter did not show slow-moving proteinase bands (Figure 4.2.11, lanes 8 and 9). No activation band was detected in the fecal matter of *C. arietinum* and *C. cajan* fed larvae, but high proteinase activity was observed. Closely moving proteinase bands merged and formed a smear (Figure 4.2.12; lanes 8 and 9).

In the non-host plant group, the fecal matter of larvae fed on PIs showed low proteinase activity, but detectable activation of its inactive isoforms (Figures 4.2.11 and 4.2.12). All non-host plant PIs fed larvae fecal matter showed fast-moving bands, except in *H. vulgare*. *Acacia nilotica*, *A. aspera*, *B. edulis*, *H. schulli*, *M. charntia*, which showed low-moving proteinase bands. *Penganum harmala* and *N. sativa* fed larvae showed a single fast-moving band in the fecal matter (Figure 4.2.11; lanes 1, 2, 3, 12, 13,

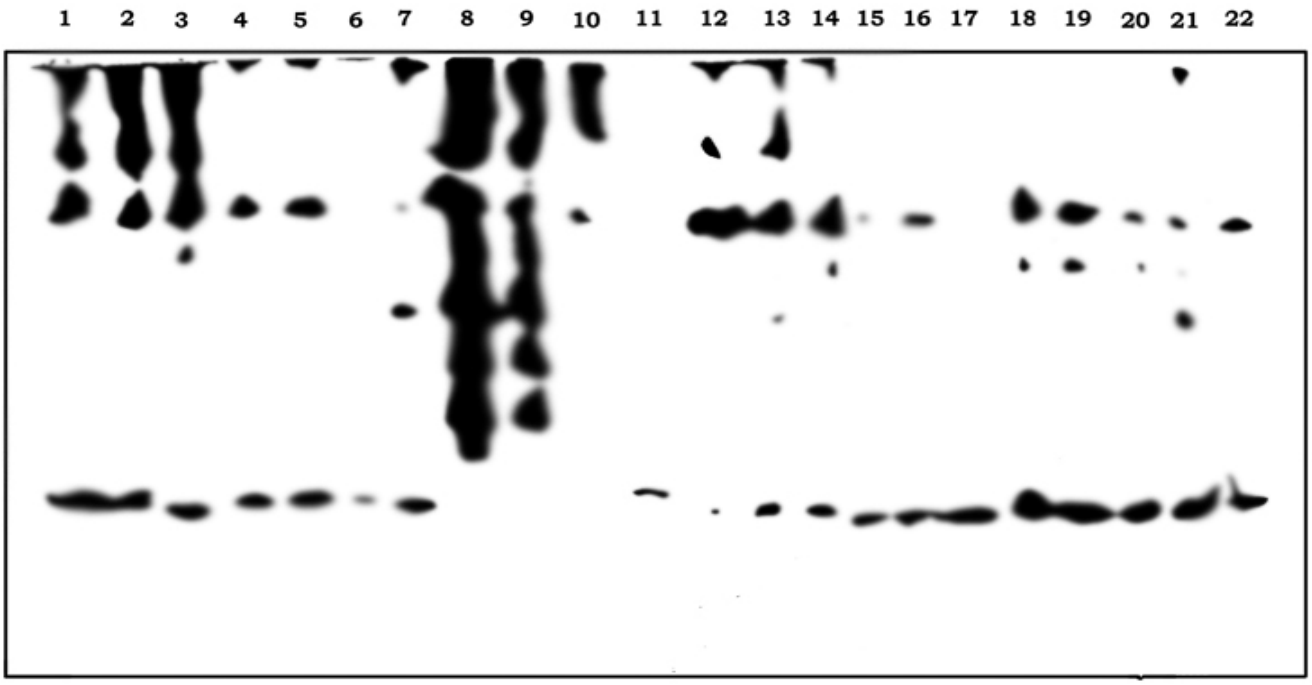

Figure 4.2.11 Proteinase profiles in the fecal matter of *H. armigera* fed on host and non-host plants PIs incorporated in chickpea (PIs removed) artificial diet with a proportion of 1:1. Proteinase profiles of the fecal matter of larvae fed on lane 1, *Acacia nilotica*; lane 2, *Achyranthes aspera*; lane 3, *Blepharis edulis*; lane 4, *Capsicum annum*; lane 5, *Foeniculum vulgare*; lane 6, *Glycine max*; lane 7, *Datura alba ness*; lane 8, *Cicer arietinum*; lane 9, *Cajanus cajan*; lane 10, *Hordeum vulgare*; lane 11, *Penganum harmala*; lane 12, *Hygrophila schulli*; lane 13 *Momordica charntia* ; lane 14, *Mucuna pruriens*; lane 15, *Murraya koenigii*; lane 16, *Psoralea corywfolia*; lane 17, *Nigela sativa*; lane 18, *Pongamia pinnata*; lane 19, *Psophocarpus tetragonolobus*; lane 20, *Solanum nigrum*; lane 21, *Trigonella foenum-graecum*; and lane 22, *Vigna unguiculata* loaded on 10% native-PAGE and proteinase activity was visualized by gel X-ray film contact print technique.

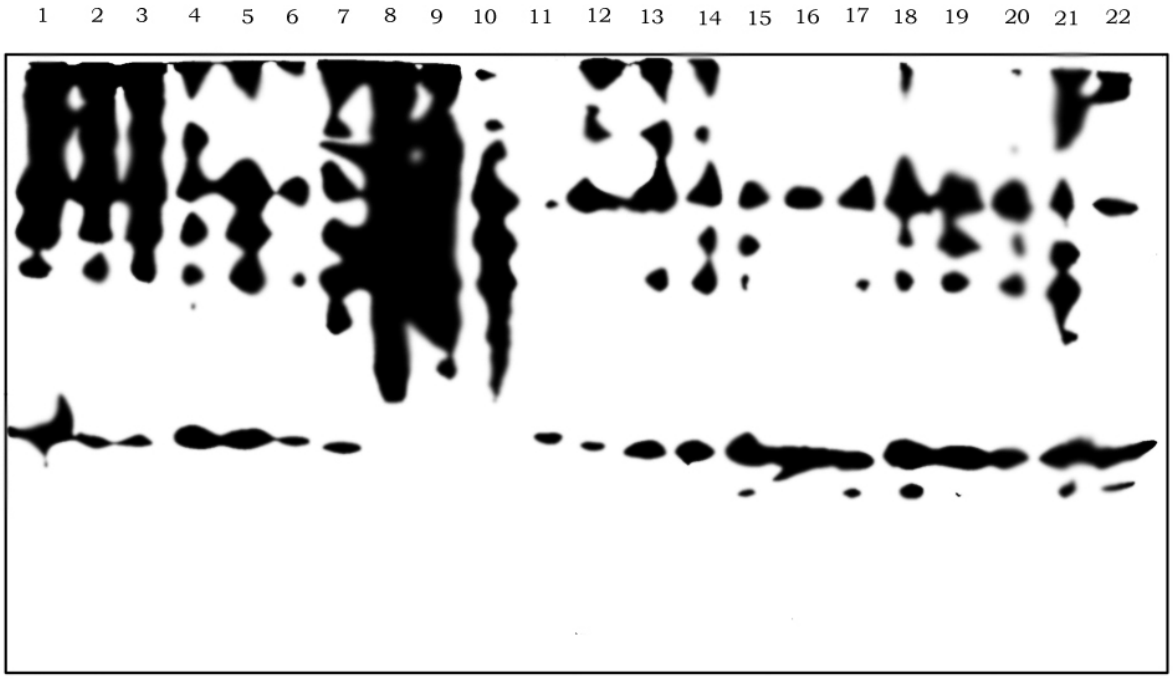

Fig 4.2.12 Activation of pro-proteinase in the fecal matter of larvae fed on host and non-host plant PIs incorporated in chickpea (PIs removed) artificial diet. For activation of pro-proteinases of the fecal matter of larvae reared on lane 1, *Acacia nilotica*; lane 2, *Achyranthes aspera*; lane 3, *Blepharis edulis*; lane 4, *Capsicum annum*; lane 5, *Foeniculum vulgare*; lane 6, *Glycine max*; lane 7, *Datura alba ness*; lane 8, *Cicer arietinum*; lane 9, *Cajanus cajan*; lane 10, *Hordeum vulgare*; lane 11, *Penganum harmala*; lane 12, *Hygrophila schulli*; lane 13 *Momordica charntia* ; lane 14, *Mucuna pruriens*; lane 15, *Murraya koenigii*; lane 16, *Psoralea corywfolia*; lane 17, *Nigela sativa*; lane 18, *Pongamia pinnata*; lane 19, *Psophocarpus tetragonolobus*; lane 20, *Solanum nigrum*; lane 21, *Trigonella foenum-graecum*; and lane 22, *Vigna unguiculata* were reared on 10% native-PAGE. Activation of pro-proteinase was carried out in activation buffer as described in 'Materials and Methods', and visualization by gel X-ray film contact print technique.

and 11, 17, respectively). Fecal matter of larvae fed on non-host plant PIs showed the presence of major proteinase activity bands.

Activation of pro-proteinases in the fecal matter of *H. armigera* fed on non-host plants showed the activation of major as well as fast- and slow-moving pro-proteinases. *Capsicum annum*, *F. vulgare*, and *G. max* showed activation of slow-moving bands, while *M. koenigii*, *N. sativa*, *P. pinnata*, and *P. tetragonolobus* showed activation of fast-moving pro-proteinases. Minor proteinase bands were detected on X-ray film (Figure 4.2.12; lanes 4, 5, 6, and 15, 17, 19, respectively). *Trigonella foenum-graecum* and *V. unguiculata* showed activation of both slow as well as fast-moving pro-proteinases, along with minor proteinase bands (Figure 4.2.12; lanes 21 and 22). *Datura alba ness* and *H. vulgare* showed high activation of all pro-proteinases in activation profile visualized by GXCP (Figure 4.2.12; lanes 7 and 10).

4.2.8 Inhibitory potential of non-host plant PIs against *H. armigera* gut proteinases and activation of pro-proteinases

To assess the inhibitory potential of non-host plant PIs against *H. armigera*, larval gut proteinases and activation of its inactive isoforms were analyzed by *in vivo* and *in vitro* experiments. The *H. armigera* larvae fed on artificial diet were used for *in vitro* inhibition. Proteinase inhibitors were evaluated for their potential to suppress the growth of herbivore insects. Spectrophotometeric assay methods were employed for analysis of inhibition of total proteinase and trypsin isoforms activity by azocasein and BA*p*NA, respectively (Table 4.2.7). The *H. armigera* larvae are known to exhibit differential gut proteinase activity. The fifth-instar larvae showed maximum proteinase activity, and hence, mid-fifth-instar larvae were used in these studies. Maximum inhibitor amount required to attain the maximum inhibition of HaGPs of chickpea based artificial diet (PIs removed) fed larvae was calculated for each inhibitor and used for inhibition assays. Twenty-two host and non-host plant PIs were analyzed for *in vitro* inhibition of HaGP and all of them showed inhibition of HaGPs and trypsin isoforms of *H. armigera* (Table 4.2.7). Among the host plants, *C. cajan* (47.68% and 51.23%) and *C. arietinum* (39.62% and 41.82%) showed partial/low inhibition of HaGP and trypsin isoforms, respectively.

Table 4.2.7 *In vitro* inhibition of proteinase of *H. armigera* fed on chickpea (PIs removed) based artificial diet. Various plant PIs from host and non-host were tested for their maximum inhibition potential against HaGPs. Azocasein and BA*p*NA were used as substrates to measure the total proteinase and trypsin isoforms activities, respectively.

Host and non-host plant	**Inhibition of HaGPs (%) mean ± SE (n = 3)**	
	Total proteinase	**Trypsin**
Acacia nilotica	69.18 ± 0.98	48.18 ± 2.17
Achyranthes aspera	45.22 ± 1.87	64.13 ± 3.76
Blepharis edulis	67.12 ± 1.83	63.98 ± 2.72
Cajanus cajan	47.68 ± 1.83	51.23 ± 0.91
Capsicum annum	78.60 ± 1.13	85.18 ± 1.79
Cicer arietinum	39.62 ± 1.55	41.82 ± 1.47
Datura alba ness	95.57 ± 1.37	93.28 ± 1.49
Foeniculum vulgare	36.72 ± 3.93	45.92 ± 2.72
Glycine max	69.64 ± 1.24	79.19 ± 2.35
Hordeum vulgare	58.62 ± 1.79	56.34 ± 0.89
Penganum harmala	37.66 ± 2.82	43.83 ± 1.89
Hygrophila schulli	75.85 ± 1.47	79.88 ± 1.85
Momordica charntia	68.39 ± 2.77	72.12 ± 0.94
Mucuna pruriens	81.25 ± 1.38	84.13 ± 1.87
Murraya Koenigii	77.43 ± 1.25	67.29 ± 2.87
Nigela sativa	84.48 ± 0.67	89.15 ± 1.59
Pongamia pinnata	86.23 ± 1.55	88.26 ± 1.48
Psophocarpus tetragonolobus	89.98 ± 2.33	95.65 ± 1.09
Psoralea corywfolia	52.26 ± 0.89	59.71 ± 1.17
Solanum nigrum	30.76 ± 2.28	43.34 ± 1.36
Trigonella foenum-graecum	69.29 ± 0.64	67.29 ± 1.09
Vigna unguiculata	78.64 ± 2.59	81.28 ± 1.39

Non-host plants PIs are potent inhibitors of HaGPs, and they showed high to moderate levels of inhibition of total proteinase and trypsin isoforms of HaGP. *Datura alba ness* PIs significantly inhibited the gut proteinase activity, and showed 95.57% inhibition of total proteinase, and 93.28% inhibition of trypsin isoforms of HaGP. *Psophocarpus tetragonolobus*, *P. pinnata*, and *N. sativa* also showed maximum inhibition of gut proteinase activity; and resulted in 89.68, 86.23, and 84.48% proteinase inhibition; and 95.65, 88.26, and 89.15% inhibition of trypsin isoforms, respectively (Table 4.2.7). Similarly, *C. annum*, *V. unguiculata*, *M. koenigii*, and *H. schulli* showed 78.60, 78.64, 77.43, and 75.85% inhibition of total proteinase; and 85.18, 81.28, 67.29, and 79.88% inhibition to trypsin isoforms, respectively. *Penganum harmala* and *S. nigrum* showed similar levels of *in vivo* inhibition. These resulted in low inhibition of proteinases 37.66% and 30.76%; and partial inhibition of trypsin isoforms 43.83% and 43.34%, respectively. *Acacia nilotica*, *G. max*, and *T. foenum-graecum* showed similar inhibition of total proteinases *(*69%), but differed in trypsin isoforms inhibition (48.18, 79.19, and 67.29%, respectively). *Momordica charntia*, *B. edulis*, *H. vulgare*, and *P. corywfolia* showed moderate levels of inhibition of proteinase 68.39, 67.12, 58.62 and 52.26%; and 72.12, 68.98, 56.34, and 59.71% inhibition of trypsin isoforms, respectively (Table 4.2.7).

Many host and non-host plant PIs showed similar inhibition of proteinase profile in *in vitro* and *in vivo* experiments, while the proteinases were inhibited partially *in vivo* assay appeared with increased activity in *in vitro* activation. The proteinases were inhibited totally due to potent PIs in *in vivo*, but its inactive isoforms appeared in activated forms in *in vitro* activation process (Table 4.2.8).

4.2.9 Effect of host and non-host plant PIs on *H. armigera* growth and development

Development of *H. armigera* larvae fed on host and non-host plant PIs incorporated into artificial diet was also evaluated for *in vivo* potential. The standard artificial diet (Table 3.1) used in the feeding assay was chickpea (PIs removed) flour based diet, supplemented with vitamins and salts. The chickpea flour possesses major amounts of bovine trypsin PI of the Kunitz type, which are inactivated by autoclaving and heat treatment. Equal quantity of host and non-host seed powder was added to

Table 4.2.8 Electrophoretic detection of *in vitro* and *in vivo* inhibition of HaGPs by host and non-host plant PIs

Host and non-host plant PI	***In vitro* inhibition of HaGP**										***In vivo* inhibition of HaGP**										
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	
A. nilotica	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-
A. aspera	+	+	+	-	-	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	-
B. edulis	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-
C. cajan	-	-	-	-	-	-	-	+	-	+	-	+	-	-	-	-	-	+	-	-	-
C. annum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	-
C. arietinum	-	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-	+	-	-	-
D. alba ness	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-
F. vulgare	+	-	+	-	-	-	-	+	-	+	+	+	+	-	-	-	+	+	+	+	-
G. max	+	+	+	-	-	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-
H. vulgare	+	-	+	-	-	-	+	+	+	+	+	+	+	-	-	-	+	+	-	+	-
P. harmala	+	+	-	-	-	-	-	+	-	+	+	+	+	-	-	+	+	+	-	+	-
H. schulli	+	+	+	+	-	+	-	+	+	+	-	+	+	-	-	-	+	+	+	+	-
M. charntia	+	-	+	-	-	+	-	+	-	+	+	+	+	-	-	-	-	-	-	+	-
M. pruriens	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-
M. Koenigii	+	-	+	+	-	+	+	+	-	+	+	+	+	-	-	-	-	+	-	+	-
N. sativa	+	+	+	+	-	-	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-
P. pinnata	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-
P. tetragonolobus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-
P. corywfolia	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	+	-
S. nigrum	+	-	+	-	-	-	-	+	-	+	+	+	+	-	+	+	+	-	-	+	-
T. foenum-graecum	+	+	+	+	-	+	+	+	-	+	+	+	+	-	-	+	+	+	-	+	-
V. unguiculata	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-

' + ' Inhibition of HaGP, ' - ' No inhibition of HaGP, ' -^{n} ' No activation of HaGPP, ' -* ' Increased activity of

chickpea powder, and the diet was used for feeding trials. Early third-instar larvae were released on diet ± inhibitors and the control (chickpea, PIs removed) diet. Larval weights were recordered at the time of releasing the larvae on the diet (Table 4.2.9). The *H. armigera* larvae reared on host plant; standard artificial diet, and non-host plants, e. g. *D. alba ness* showed the normal and stunted growth of larvae, respectively (Figure 4.2.13). Larval growth was significantly reduced by non-host plant PIs compared to the larvae fed on control diet (data set significant at $p < 0.001$, Genstat 10.1 analysis LSD at P 0.05 for initial and final weight) from the 7^{th} or 8^{th} day of larval feeding onwards (Table 4.2.9). As compared to control (*C. arietinum**), the host plant PIs from *C. cajan* and *C. arietinum* also showed reduction in weight gain.

Among the non-host plant PIs, *D. alba ness* resulted in highest stunted growth, followed by *C. annum*, *M. pruriens*, *H. schulli*, *P. pinnata*, *M. koenigii*, *N. sativa*, *H. vulgare*, *P. tetragonolobus*, *A. nilotica*, and *B. edulis* (Table 4.2.9). *Solanum nigrum*, *V. unguiculata*, *P. corywfolia*, *T. foenum-graecum* showed moderate reduction in larval weights. Larvae fed on diet with non-host plant PIs such as *A. aspera*, *G. max*, and *P. harmala* showed normal growth.

Non-host plants PIs have the potential to inhibit the growth of larvae, and this provides a basis for selection of potent PIs for developing transgenic crops with resistance to *H. armigera*.

4.2.10 Two-dimensional gel electrophoresis profile of *H. armigera* gut proteinases

At least nine proteinase bands between 65 and 20 kDa were detected in HaGP extracts on IEFSDS-PAGE using the gel X-ray film contact print technique (Pichare and Kachole, 1994) (Figure 4.2.14). The *H. armigera* larvae fed on artificial diet were used for gut lumen proteinase profile analysis on 2-D gels, using a pH gradient of 3-10NL for IEF run in the first dimension, and a 10% SDS-PAGE gel for the second dimension, followed by on X-ray film to be detected in gelatin hydrolyzing serine proteinase bands. As the mixture of luminal proteins contained active proteinases, precautions were taken to preserve proteinase activity by not heating the samples prior to electrophoresis. Reducing agents such as β-mercaptoethanol and dithiothreitol were excluded at the time of sample preparation as described in section 3.3.10 in 'Materials and Methods'. 2-D gel

Table 4.2.9 *H. armigera* fed on host and non-host plant PIs for inhibition of gut proteinase activity. The experiment was carried out in three replications and each replication contains thirty larvae.

Sample name	Initial Weight (mg)	Final Weight (mg)	Weight gain (mg)	Growth rate (%)
Acacia nilotica	30.72	143.10	112.50	381.90
Achyranthes aspera	19.93	319.30	299.30	1047.30
Blepharis edulis	49.00	280.70	231.70	469.00
Cajanus cajan	51.27	369.20	317.90	723.90
Capsicum annum	26.60	56.70	29.70	102.70
*Cicer arietinum**	22.24	426.80	404.60	1902.50
Cicer arietinum	25.50	377.40	351.90	1436.50
Datura alba ness	25.18	39.10	13.70	53.80
Foeniculum vulgare	28.98	316.60	287.40	976.00
Glycine max	60.95	330.90	269.40	1296.50
Hordeum vulgare	23.65	92.10	68.30	308.00
Penganum harmala	24.51	356.70	331.80	1352.40
Hygrophila schulli	24.74	85.80	61.10	274.30
Momordica charntia	96.11	389.00	292.90	326.80
Mucuna pruriens	26.26	70.60	44.30	181.00
Murraya koenigii	20.59	72.80	52.20	275.30
Nigela sativa	24.98	97.10	72.20	294.60
Pongamia pinnata	22.57	73.20	50.20	254.70
Psophocarpus tetragonolobus	23.32	94.00	71.40	334.30
Psoralea corywfolia	38.51	373.50	334.50	876.60
Solanum nigrum	20.27	126.30	106.00	513.90
Trigonella foenum- graecum	30.34	298.00	267.90	933.70
Vigna unguiculata	38.63	301.50	262.90	696.40
Fp	**<0.001**	**<0.001**	**<0.001**	**<0.001**
SE ±	**1.74**	**10.73**	**10.34**	**43.14**
LSD at P 0.05	**4.97**	**29.77**	**28.71**	**119.67**

* PIs removed from the sample.

Figure 4.2.13 Development of *H. armigera* fed on with and without plant PIs. Larvae fed on control diet-containing chickpea (PIs removed) showing normal growth (upper row), while larvae fed on test diet containing *Datura alba ness* along with chickpea (PIs removed) with a proportion of 1:1 showing retarded growth (lower row).

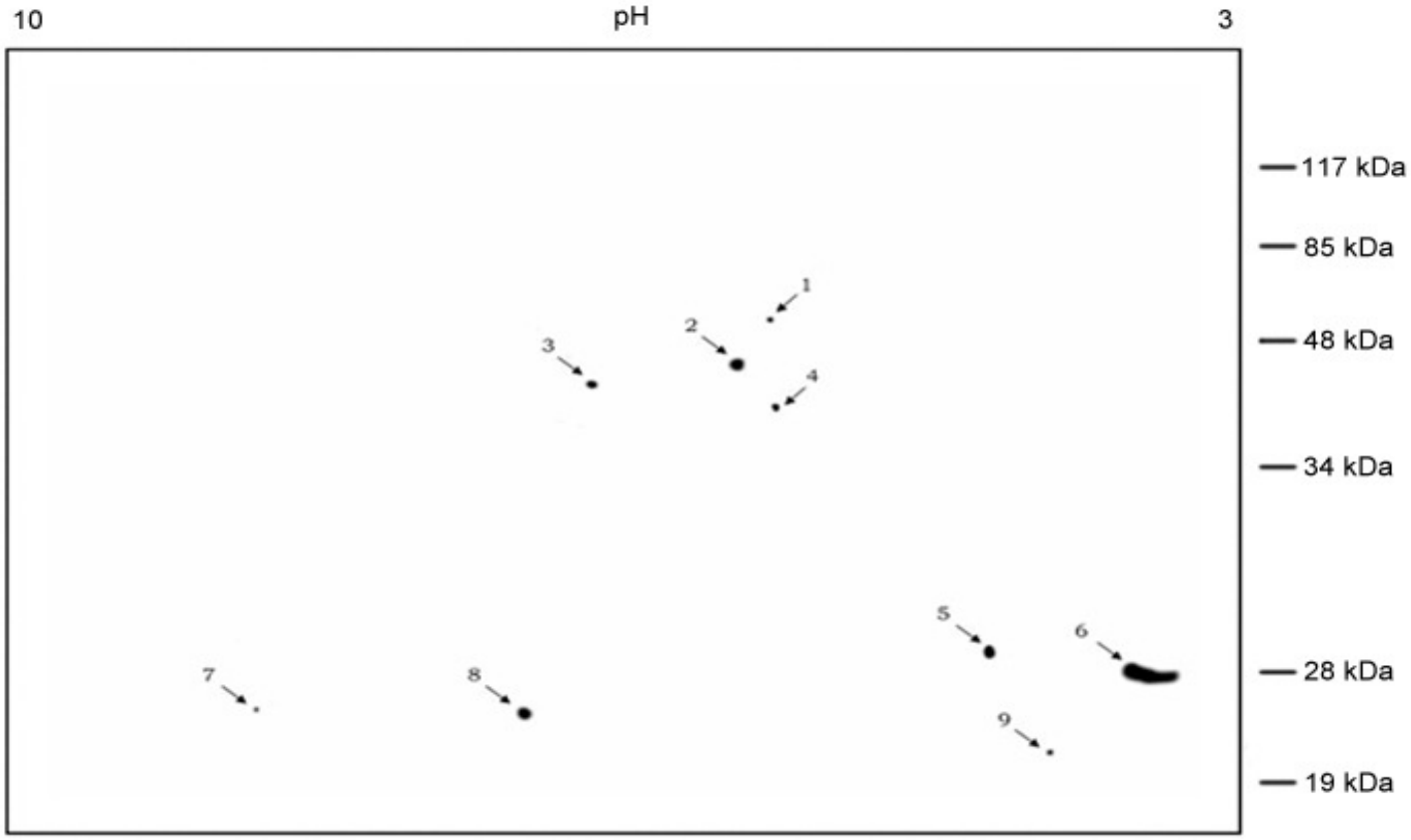

Figure 4.2.14 Separation of proteinases from *H. armigera* gut lumen by 2D-gel electrophoresis followed by gut proteinase activity visualized on gel X-ray film contact print technique. *H. armigera* fed on diet containing chickpea (PIs removed), and 2nd dimension run was carried out on 10% SDS-PAGE without adding reducing agent such as β-mercaptoethanol or dithiothreitol. The same or different gel with same loading concentration was used for silver staining.

required more time for exposure on X-ray film for visualization of proteinase activity bands as compared to one dimensional GXCP. With longer exposer 20, 21, and 64 kDa bands were detected, but 26 kDa proteinase band was diffused. Most of *H. armigera* gut proteinases have acidic pI, and four proteinase bands were detected in between 65 - 40 kDa range, while remaining five were in 29 - 20 kDa range. Other serine proteinase activity bands were also present in HaGP extract, but nine proteinase bands with apparent molecular weights of 53.2, 47.0, 43.8, 40.6, 28.4, 27.7, 25.7, 25.9, and 21.3 kDa were detected on X-ray film. The same loading concentration of HaGP activity was separated on other 2-D gels and used for Coomassie Brilliant Blue R 250 (CBBR) staining, as well as for silver staining, but identification of proteinase spot became difficult due to contamination of abundant proteins present in the food (results not shown). Gut proteinase profile was confirmed by two-three exposures of the same 2D-gel on X-ray film, while proteinase bands were not detected in shorter contact time; those appeared with longer time of exposure. In an attempt to visualize the *H. armigera* gut proteinase activity after 2-D gel electrophoresis, it was observed that proteinase remained active after exposure to several detergents and urea used in IEF run and SDS-PAGE.

4.2.11 *In-gel* activation of *H. armigera* gut pro-proteinases and visualization on two-dimensional gel electrophoresis

In the previous section 4.2.10, we described the proteinase profile of *H. armigera* using 2-D GXCP. This technique was extended to visualize activation of inactive isoforms of proteinase of *H. armigera* after treating with activator trypsin. When larvae were fed on a cocktail of synthetic inhibitors incorporated in artificial diet, and the gut protein contents extracted in 0.2 M glycine-NaOH buffer, pH 10.0, were separated on two-dimensional gel electrophoresis. HaGPPs separated on 2-D gel were activated by 0.0001% trypsin solution, and activated proteinase profile was visualized by gel X-ray film contact print technique (Figure 4.2.15). At least four pro-proteinases showed activation on X-ray film, and the apparent molecular weights of the activated proteinases were 47.8, 44.6, 32.7, and 26.3 kDa (Figure 4.2.15). All activated proteinases showed acidic p*I*.

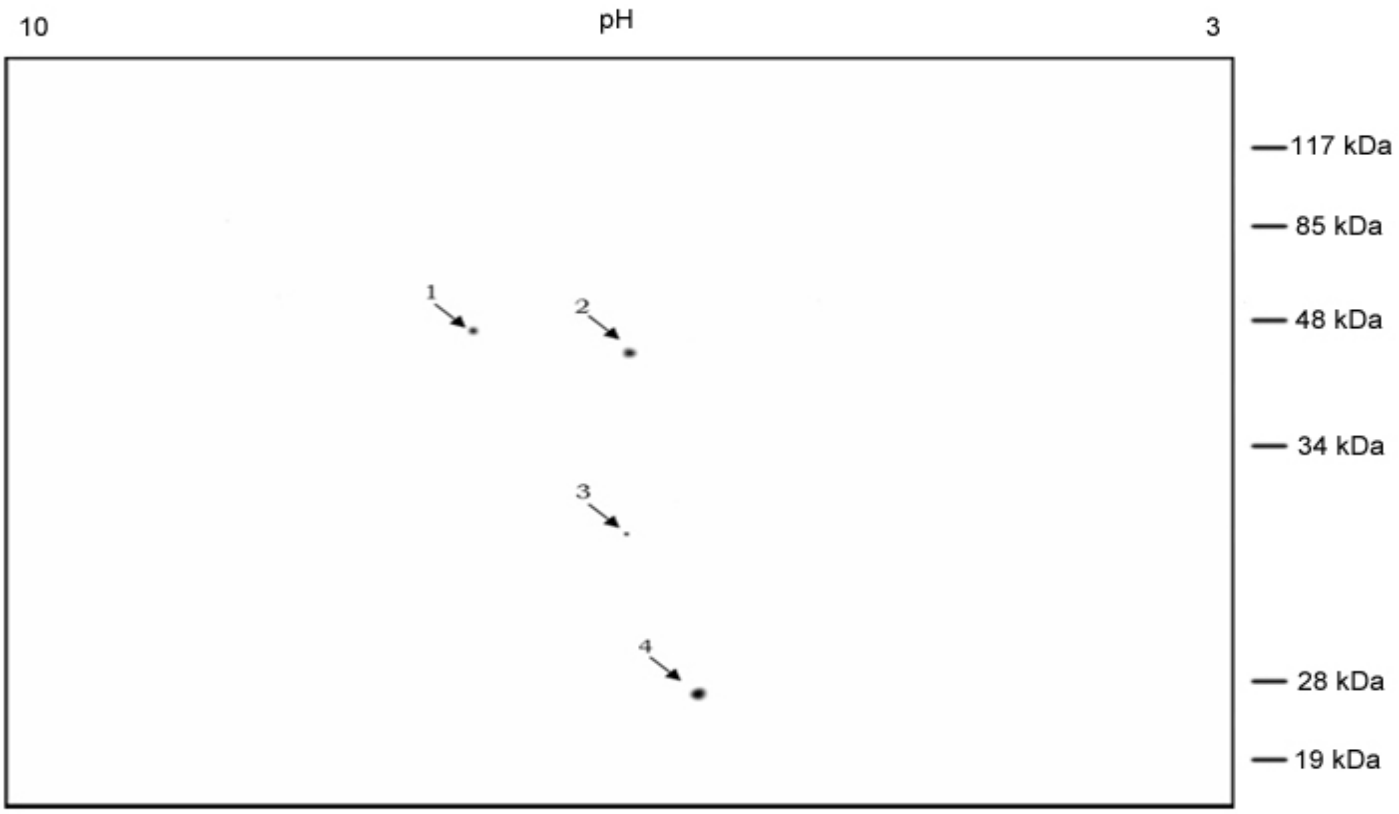

Figure 4.2.15 Activation of pro-proteinases from *H. armigera* gut lumen 2D-gel electrophoresis. Larvae fed on cocktail of synthetic inhibitors incorporated in chickpea (PIs removed) diet were used for two-dimensional gel analysis. HaGPPs separated on the basis of pI (pH 3-10NL) followed by 2nd dimension run on 10% SDS-PAGE without adding reducing agent and activation was carried out in activation buffer as described in 'Materials and Methods'. Activated gut pro-proteinases visualized by gel X-ray film contact print method and the same or different gel with same loading concentration was used for silver staining.

Another approach was used for the detection of HaGPPs activation in non-host plant PIs fed *H. armigera* larvae. *Datura alba ness* incorporated in artificial diet fed larvae were used for pro-proteinase activation analysis on two-dimensional gel electrophoresis, followed by GXCP. At least three activated proteinase bands were observed with apparent molecular weights 41.3, 35.4, and 30.2 kDa (Figure 4.2.16). Two high molecular weight activated proteinase bands showed acidic p*I*, but the third band showed alkaline.

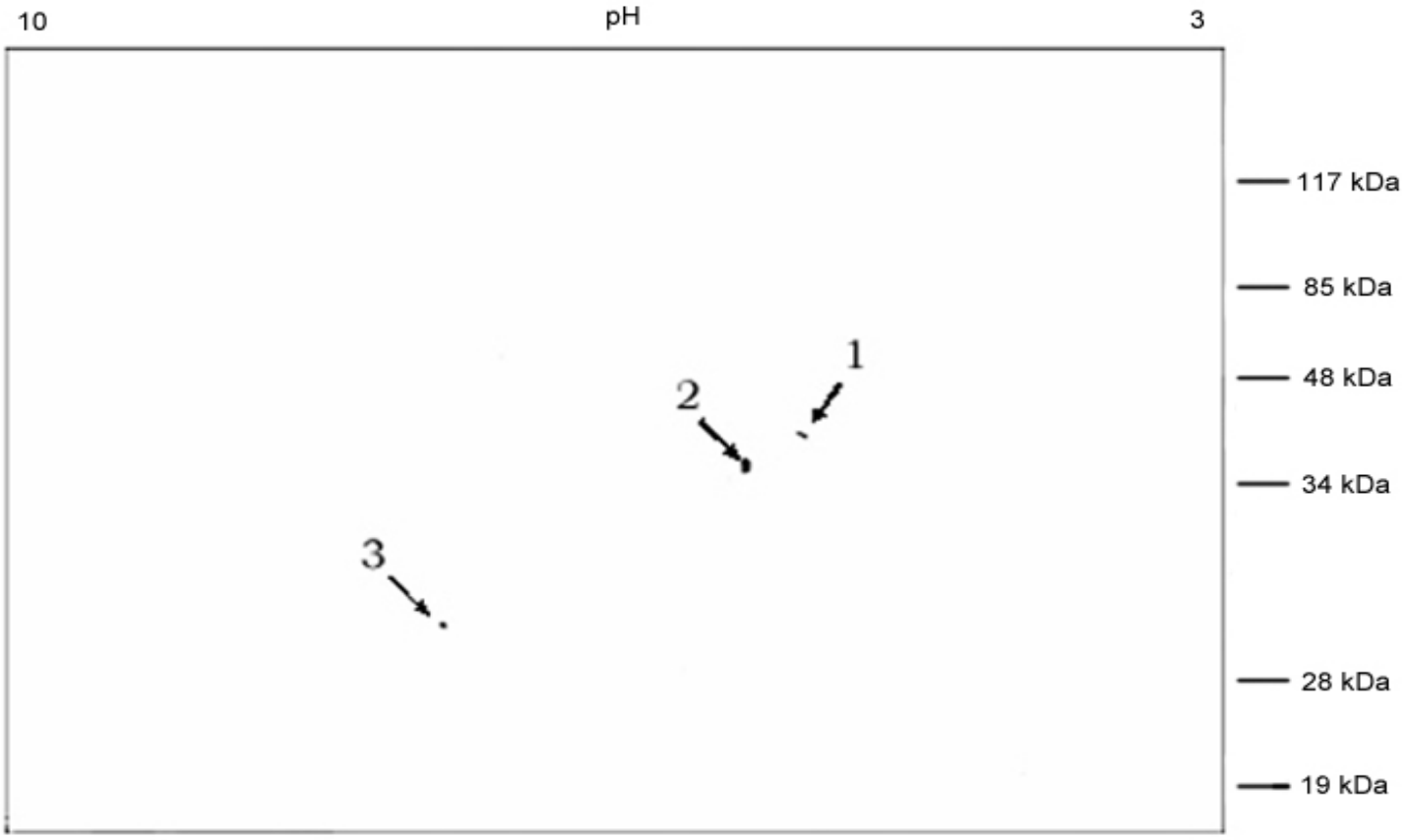

Figure 4.2.16 Activation of HaGPPs from gut lumen of larvae was analyzed by two-dimensional gel electrophoresis. Larvae fed on non-host plant PIs, *Datura alba ness* were incorporated in chickpea (PIs removed) diet with a proportion of 1:1 was used for analysis. The same gut extract was initially separated on pH strip 3-10NL followed by 2nd dimensionally on 10% SDS-PAGE in absence of reducing agent. Activation of pro-proteinase was carried out in activation buffer as described in 'Materials and Methods' and activated proteinases were visualized by gel X-ray film contact print technique. After GXCP process, the same or different gel with same loading concentration was used for silver staining.

CHAPTER 5
DISCUSSION

5.1 Introduction

Proteolysis is an essential metabolic process for protein processing and turnover. The activation of zymogens usually occurs by proteolytic cleavage of a peptide bond in a region that is amino terminal relative to the active site of the protein (Neurath and Walsh, 1976; Neurath, 1986). Upon proteolytic cleavage of zymogen, the interaction of the pro-segment with the enzyme moiety is weakened, allowing the release of the pro-segment. A novel procarboxypeptidase from *Helicoverpa armigera* (Hüb.) (PCPAHa), the first zymogen protease from a lepidopteran insect (*H. armigera*) has been characterized (Bown et al., 1998). Estebanez et al. (2001) studied the crystal structure of PCPAHa in the gut extract of *H. armigera* larvae.

The understanding of the digestive process in insect pests is a key step in the design of many insecticides, including insect-resistant transgenic plants (Terra and Ferreira, 1994; Ferry, 2004). Primary digestive enzymes of *H. armigera* larvae are predominantly serine proteinases of trypsin and chymotrypsin type, which are extracellular and active at alkaline pH (Johnston et al., 1991; Purcell et al., 1992; Chougule et al., 2005). Full-length of several aminopeptidases and carboxypeptidases has been reported from gut of *H. armigera* (Bown et al., 1998; Emmerling et al., 2001; Bayes et al., 2005). These enzymes play an important role in protein digestion by releasing amino acids from the peptides by endopeptidases, thus completing the digestion process (Terra and Ferreira, 1994; Terra et al., 1996). In the present study, we found that trypsin is the key enzyme, which can activate its inactive precursor as well as other zymogen protease. In this perspective, zymogen proteases(s) (trypsinogen- and chymotrypsinogen-like) were isolated from the larval gut contents, and characterized based on their substrate specificities.

Disruption of amino acid metabolism by inhibition of protein digestion has been a key target for use in insect control (Johnson et al., 1989; Hilder et al., 1993; Hilder and Boulter, 1999). Proteinase inhibitors have been evaluated as natural control agents against herbivorous insects, and have been shown to reduce the digestive proteolytic enzyme activity and/or larval development in different species of Coleoptera and Lepidoptera (Broadway et al., 1986; Christeller and Shaw, 1989; Hilder et al., 1989; Michaud et al., 1993; Johnson et al., 1989; Oppert et al., 1993; Chen et al., 2007).

However, insect pests are adapted to host plant PIs either by synthesis of insensitive proteinases (Broadway, 1995; Jongsma et al., 1995), their ability to possess them (Michaud, 1997; Girard et al., 1998), or by altering gut contents through up- or down-regulation of proteinases (Hilder et al., 1987). Insects have evolved and adapted to the effect of host plant PIs (Bolter and Jongsma, 1995; Broadway, 1995, 1996; Falco and Silva, 2003), and therefore, it is necessary to study the non-host plant PIs as a potential source of resistance genes against insect pests.

In the present studies, potency of non-host plant PIs was revealed in response to larval digestive enzymes after incorporating the PIs into the artificial diet. Seeds of a large number of species from different families (medicinal, legumes, and spices) were screened by dot-blot assay method. A large number of non-host plants possessed TI and CTI activity, but a few showed HaGPI activity. The gut contents and fecal matter of the larvae fed on synthetic inhibitors and plant PIs revealed zymogen protease activity. The non-host plant PIs showed the ability to inhibit the gut digestive enzymes, and activation of zymogens.

5.2 Applicability of gel X-ray film contact print technique for SDS-PAGE and two-dimensional gels

The X-ray film coated with gelatin has been used as a substrate for detection of proteolytic activity of enzymes (Manicourt and Lefebvre, 1993; Garcia-Carreno et al., 1993; Michaud et al., 1993; Kleiner et al., 1994) in dot-blot assay (Cheung et al., 1991). This method is suitable for detection of protease inhibitors (Pichare and Kachole, 1994; Mulimani et al., 2002) and an insect gut digestive enzymes (Hursulkar et al., 1998) was used for detection of *in gel* activation of *H. armigera* gut pro-proteinases. The method described herein requires neither ampholytes nor synthetic substrates, and therefore, is cheaper and convenient. Preparation of gels is simple as compared to the methods involving incorporation of substrates in gels (Seidl et al., 1978; Pellegrini et al., 1984; Hanspal et al., 1983). X-ray film can be stored as records and immediate photography is not essential. Time required for visualization of *H. armigera* protease bands was much shorter (50 min for native-PAGE, 50 - 60 min for SDS-PAGE, and 1 – 2 h for two-dimensional gel at 37°C) than other methods. This method allowed the interaction of a

large number of insect enzymes and inhibitors, circumventing the need of purification of individual enzymes by traditional methods.

In the present studies, we have described the use of gelatin coated X-ray film for detection of electrophoretically separated *H. armigera* gut pro-proteinases on native PAGE, SDS-PAGE, and two-dimensional gels. When the gels were prepared in SDS, washing thoroughly with a nonionic detergent and then activating the pro-proteinases with the treatment of activator, trypsin (0.0001%), followed by placing the gel on the unexposed X-ray film to visualize the activated pro-proteinase bands to hydrolyze the gelatin on the surface. Washing the X-ray film with hot water removed the hydrolyzed gelatin at the site of enzyme activation. It is easy to handle and stable in boiling water, and is compatible with most of laboratory reagents in a wide range of pH and molarity (Cheung et al., 1991; Peach et al., 1993; Pichare and Kachole, 1994; Mulimani et al., 2002).

The two-dimensional gel X-ray contact print method was used to identify proteinase activity in complex mixtures of gut proteins, and can be used to complement other techniques in proteomics. This approach permitted the comparison of various gut pro- and proteinase patterns in different instars. The method is also useful for comparing proteinase activities in different insect species, as well as other organisms, provided the enzymes are stable under the experimental conditions.

Substrate gel zymography was performed as described by Huessen and Dowdle (1980). Gels containing sodium dodecyl sulphate were prepared by co-polymerization of acrylamide and gelatin of the test substrate at the final concentration. However, substrate in the gel might interfere with the mobility of proteinases (Garcia-Carreno et al., 1993; Manicourt and Lefebvre, 1993; Choi et al., 2001). In the native PAGE, proteinases actively digest gelatin during the electrophoretic run. Insect gut proteinases were treated with synthetic inhibitors to detect the possible pro-proteinase profile, but the activity of some proteinases in the sample may interfere in electrophoretic run, resulting in tracking on the gel.

5.3 Complexity and specificity of *H. armigera* gut pro- and proteinases

Proteinases are the major digestive enzymes in the insect gut. They are responsible for continuous supply of essential amino acids and energy from the food source for development. Most of the midgut proteolytic enzymes in lepidopteran larvae have been shown to be extracellular serine proteases, exhibit high pH optima, and are well suited to the alkaline conditions of the midgut (Applebaum, 1985; Johnston et al., 1991). Five protease isoforms have been reported in *Plodia interpunctella* (Hub.) (Oppert et al., 1996) and two protease isoforms in *Ostrinia nubialis* (Hub.) (Houseman et al., 1989) gut extracts. Johnston et al. (1991) observed two major bands of BA*p*NAase activity in *H. armigera* gut extract, while Hursulkar et al. (1998) observed six HaGPs, and three bacterial proteinases in *H. armigera*. Using the GXCP method, we observed ten proteinases in *H. armigera* gut extract, of which four were chymotrypsin-like and six trypsin-like proteinases.

Six distinct pro-proteinase bands from the gut of *H. armigera* larvae were observed after native PAGE, of which two showed detergent stable activity with molecular masses of 47.6 and 42.1 kDa at pH 10.0. On the basis of substrate specificities, these pro-proteinases appeared to be serine pro-proteinases.

The existence of proteases in the form of inactive zymogens, activable by limited proteolysis, is widely documented in vertebrates (Cera, 2009; Neurath, 1999; Reeck et al., 1997). Proteolytic zymogens also occur in bacteria (Lin and Bustin, 1970). Among the invertebrates, indirect evidence for inactive protease(s) is available in a variety of systems, but direct demonstration of proteolytically activable zymogens (Camacho et al., 1970; Ariki et al., 2004), chemically or immunochemically distinct from the active enzymes, has not been documented. The inactive precursor (zymogen) of the insect protease cocoonase has been reported from galeae of the silkmoth, *Antheraea polyphemus* (Cramer) (Berger and Kafatos, 1970). Recently, Estebanez et al. (2001) reported a three dimensional crystal structure of procarboxypeptidase from the *H. armigera* gut extract. In the present study, we observed trypsinogen- and chymotrypsinogen-like pro-proteinase activity from the *H. armigera* gut extract in the solution assays as well as by the *in gel* activation method.

5.4 Biochemical properties of gut pro- and proteinase of *H. armigera*

5.4.1 pH dependence activity of HaGP and HaGPP

Majority of the insects belonging to the lepidopetran family exhibit alkaline pH in the midgut (Miller, 1974; Ahmad et al., 1980; Pritchett et al., 1981; Sasaki and Suzuki, 1982; Euguchi and Kuriyama, 1985; Broadway, 1989; Lam et al., 1999). Jongsma et al. (1996) reported that by using methemoglobin as a substrate, the pH maxima of crude *Spodoptera exigua* (Hub.) gut proteinase activity was found to be maximal at the highest pH range (11.5 – 12). *Helicoverpa armigera* also showed the alkaline gut pH maxima (Johnston et al., 1991).

The *H. armigera* gut proteinases showed stability and activity over a wide range of pH, having maximum activity at pH 9 to 11, the pro-proteinases pH maxima was observed at pH 8 to 10 (Figure 4.1.4). However, the same molarity of Tris-HCl (pH 8.0) and glycine-NaOH (pH 10.0) buffer indicated high activation of pro-proteinases in substrate assays.

5.4.2 Temperature and Ca^{2+} ions concentration influences the gut pro- and proteinase activity

Helicoverpa armigera gut proteinase showed remarkable heat stability even upto 60°C with azocasein as a substrate in solution assay. However, some of proteinase isoforms were also detected on X-ray film after placing the gel at this temperature. Earlier reports have indicated that proteinase isoforms of *Menduca domestica* (L.) and *H. armigera* are not stable beyond 53 and 50°C, respectively (Lemos and Terra, 1992). We found that pro-proteinase activation rate increased upto 40°C and then declined thereafter. Gut proteinase activity was detected at high temperatures, but activation of pro-proteinase declined due to degradation of activated proteinases.

Helicoverpa armigera gut proteinase activity is not affected by calcium ion or by the divalent chelating agents, EDTA (Johnston et al., 1991). However, in the present studies, we observed that gut pro-proteinase activity was significantly affected with an increase in concentration of Ca^{2+} ions (Figure 4.1.6).

Johnston et al. (1991) opined that presence of calcium is not necessary for insect enzymes to achieve maximum activity, as influenced in case of bovine trypsin. Ahmad et

al. (1980) also reported that calcium is not necessary for the catalytic action of the protease in *Spodoptera litura* (Fab.). However, Houseman et al. (1989) found that calcium activated the BA*p*NAase activity, but not BAEE hydrolysis in *O. nubilalis*. Our results suggested that *H. armigera* gut proteinase activity (BA*p*NA assay) did not show any differences across Ca^{2+} regions, but pro-proteinase activity (BAEE assay) increased significantly with an increase in Ca^{2+} ion concentration.

5.5 Purification and characterization of the serine pro- and proteinases from the midgut of pod borer, *H. armigera* larvae

Insects are known to make use of various enzymes such as cysteine proteinases, aminopeptidases, serine proteinases, carboxypeptidases, aspartyl-proteinases, and metallo-proteinases for digestion of food (Baker and Woo, 1981; Baker, 1982; Mcfarlane, 1985; Terra and Ferreira, 1994; Bown et al., 1997; Girard and Jouanin, 1999; Murdock and Shade, 2002; Ferry et al., 2004). However, presence of such a wide range of proteinase(s) in the insect gut implies multiple roles for these enzymes in insect development and their interaction with food ingredients (Broadway, 1996; Bown et al., 1997; Ferry et al., 2004). Well-documented digestive enzymes in the case of lepidopteran insects are serine proteinases. Although several trypsins, chymotrypsins, and carboxypeptidases have been characterized at the molecular level in polyphagous species such as *H. armigera* (Bown et al., 1997, 1998; Bown and Gatehouse, 2004), the biochemical properties of these enzymes have not been described in detail due to lack of appropriate expression systems.

Insect gut proteinases have been purified and characterized using chemical inhibitors, substrates of different specificities, and other activators or stabilizers (Christeller et al., 1989; Johnston et al., 1991; Jongsma et al., 1996; Denolf et al., 1997; Marchetti et al., 1998; Valaitis et al., 1999; Patankar et al., 2001). In the present studies, two major groups of pro-proteinases, trypsinogen- and chymotrypsinogen-like isoforms from the midgut extract of *H. armigera* were partially purified by affinity and size exclusion column chromatography, and characterized with substrate specificities. The aim of this study was to characterize trypsins involved in the activation of zymogens of insect digestive proteases. In the affinity column chromatography, benzamidine

sepharose 4B bound or inhibited the total activity of *H. armigera* gut trypsin-like proteinases in the column, and pro-proteinases eluted into two major pools (Figure 4.1.7). The *H. armigera* gut pro-proteinase isoforms activation was observed in the same eluted fraction in activation buffer. In an another approach, HaGPPs purified on size exclusion column chromatography, resulted in a group of fractions in which pro-proteinase activity was distributed. Partially purified HaGPs and HaGPPs activities were corroborated and analyzed with their substrate specificities. In *H. armigera*, the Mr 20,000 – 70,000 polypeptides were the likely serine pro-proteinases present in fractions with low BA*p*NAase activation.

5.6 Quantitive changes in gut pro-proteinases of *H. armigera* larvae fed on different synthetic PIs

It is essential to study the composition of insect mid-gut to plan the PI-based strategies to develop plants with insect resistance (Terra and Ferriera, 1994). The insect has the capacity to alter the midgut contents within the same generation for neutralizing the effect of PIs. For better understanding, the nature of gut proteinase activity together with the activity induced upon ingestion of PIs is important for selecting PIs or combination of PIs for developing plants with insect resistance.

Studies were undertaken to understand the nature of HaGP and HaGPP activity in *H. armigera* larvae fed on eight different synthetic inhibitors, viz., antipain, aprotinin, chymostatin, E-64, EDTA-Na_2, leupeptin, pefabloc, and pepstatin (with their effective concentration), during the larval development, and their response to different or cocktail of synthetic PIs.

The larvae fed on a cocktail of synthetic PIs showed more than 2- to 3-fold total as well as trypsinogen-like pro-proteinase activity than the larvae fed on the individual synthetic PIs (Table 4.1.3). High HaGPPs activity was observed in larvae fed on a cocktail of SIs because of total proteinase activity inhibited. Bown et al. (1997) reported that serine PIs antipain, leupeptin, and aprotinin inhibited maximum activity of HaGP. PMSF is a serine irreversible proteinase inhibitor, which inhibited 98% of HaGP activity in the larvae reared on chickpea (PIs removed) based artificial diet *in vitro* analysis (Table 4.1).

The relative effectiveness of synthetic inhibitors and/or their cocktail *in vitro* and *in vivo* indicated that these inhibitors were interfering with normal proteolysis, and effectively starving the larvae of protein or, more likely, of essential amino-acids. *In vivo* studies revealed that long-term exposure of larvae to synthetic PIs resulted in lower amounts of pro-proteinases in the gut. For obtaining adequate amount of pro-proteinases, *H. armigera* larvae were fed on synthetic PIs for 1 or 2 days. Pepstatin, E-64, and EDTA-Na_2 are aspartic-, cysteine-, and metallo-proteinases; but these did not inhibit pro-proteinase activation, because they are relatively non-specific for inhibition of target activator proteinases.

Hyperproduction of proteinases in response to ingested PIs has been shown to exert extra-load on the insect for energy and essential amino acids resulting in retardation of insect growth (Broadway and Duffey, 1986a, b). The present studies revealed that the fecal matter of larvae fed on synthetic PIs incorporated into the diet had lower proteinase activity than in larvae fed on artificial diet alone. Chemical protease inhibitors provided valuable information about the role played by the affected enzymes in the insects' digestive enzymes. We also evaluated gut pro- and proteinase activity of different larval instars fed on artificial diet, and the diet impregnated with synthetic PIs to understand qualitative and quantitive differences in gut composition during larval development. The results indicated a significant increase in activity of pro- and proteinases during larval development in *H. armigera* (Table 4.1.4 and Figure 4.1.14). The second-instar larvae showed low proteolytic activity, while the fifth-instar larvae exhibited predominantly serine pro-proteinase activity.

5.7 Protease inhibitors (PIs): Plant defense proteins

Plants have the capacity to synthesize certain biologically active substances, which play an important role in host plant defense against insect damage and microbial infection. Some of these include defense proteins such as proteinase inhibitors, amylase inhibitors, lectins, and pathogenesis-related proteins (Garcia-Olmedo et al., 1987; Ryan, 1990; Chrispeels and Luikhel, 1992; Tatyana et al., 1998; Chougule et al., 2003). Several studies have demonstrated that these protein PIs are specially produced in the plant upon biotic stress, and protect the plant tissue from damage (Ryan, 1990; Schaller and Ryan,

1995; Conconi et al., 1996; Tatyana et al., 1998). Protease inhibitors are the most exploited class of plant defense proteins for their use in developing plants with resistance to insects (Jouanin et al., 1998; Giri et al., 2003).

The main objective of these studies was to find out as to how protease inhibitors of the plant influence the gut pro- and proteinase activity in *H. armigera*. Among the known approaches of evaluating biological activity of PIs, the first and most common procedure is *in vitro* screening of PIs. For this, we used a simple, rapid, inexpensive and sensitive dot-blot assay and electrophoretic gel X-ray contact print method (Pichare and Kachole, 1994) for determining the ability of plant seed extracts to inhibit enzyme activity in the midgut of *H. armigera*. Evaluation of seeds of 108 species of host and non-host plants showed significant variation in their ability to inhibit trypsin, chymotrypsin, and *H. armigera* gut proteinase(s) at three different concentrations of the enzyme and the inhibitor (1:3, 1:1, 3:1). Of the 108 plant species tested, 31 showed high inhibitory activity, while 18 and 22 samples showed moderate and low activity against proteases. At equal concentration of trypsin and the inhibitor, 50 seed samples showed inhibition, but at higher concentration of inhibitor, the scenario changed, and more number of plant species (72) showed inhibition. Similar results were observed in chymotrypsin and HaGP inhibition. Screening of a large number of plant species for inhibitory activities was also ascertained by dot-blot assay, which is faster than the electrophoretic method (Pichare and Kachole, 1994). The present studies revealed that an appropriate concentration of inhibitor influences the activity of enzymes.

Electrophoretic gel X-ray film contact print method was used for the conformation of dot-blot assay results, and to visualize the separation pattern of protease inhibitor bands on X-ray film. Screening of non-host plants and/or wild relatives could be used for identification of strong insect gut PIs, which is a prerequisite for the application of PI-based strategy for developing insect-resistant transgenic plants (Chougule et al., 2003). *Helicoverpa armigera* is a polyphagous pest, feeding on more than 200 plant species belonging to 45 different families (Manjunath et al., 1989). It is exposed and acclimatized to PIs present in a wide range of host plant species. In this context, we need to find out the non-host potent PIs, which are not pre-exposed to *H. armigera*.

Protease inhibitors bind to the substrate site of proteolytic enzymes, e.g., trypsin-like enzymes recognize the amino acid residues arginyl and lysyl (Ryan, 1990; Terra and Ferriera, 1994). However, the reactive site of trypsin inhibitors includes either an arginyl or lysyl group of residue. In addition to this, there is a contact between amino acids that surrounds the reactive site of the inhibitor and the enzyme. The strength of the E-I interaction is determined by the compatibility of all amino acid residues in contact with the PI (Laskowski, 1985). Although serine proteinase inhibitors may contribute to the defense of plants against invading organisms, the efficacy of a specific inhibitor depends upon the structural compatibility of the reactive site of the plant proteinase inhibitor with the structure-binding site of the proteinases in the target organism. However, trypsin-like isoforms present in the midgut of *H. armigera* may have undergone minor substitution at the binding site, resulting in moderate inhibitor interactions by host and non-host plant PIs.

The present studies revealed the electrophoretic profiles of host and non-host plant PIs in a number of isoforms differing in intensity and mobility. However, some of PI bands exhibit less activity/low expression, and they appeared in two-three prints with long exposure of the X-ray print. Evaluation of 22 host and non-host plants showed significant variation in their TI, CTI, and HaGPI activity. The study implies that *in vitro* screening of PIs on the basis of dot-blot and electrophoretic inhibition showed that non-host plant PIs have a potential to inhibit HaGP isoforms.

5.8 Efficacy of non-host plant PIs in inhibition of *H. armigera* gut pro- and proteinases

Non-host plant PIs have been extensively studied and used as a model to obtain insight of plant defenses against herbivore attack. The present studies demonstrated the efficacy of non-host plant PIs against *H. armigera* larvae in feeding assay, which correspond to their effectiveness as inhibitors of gut pro- and proteinases, as estimated by *in vitro* inhibition assays. The results showed that *in vitro* assays are a useful means of screening for inhibitors with potential for increasing host plant resistance to insects. Both *in vitro* and *in vivo* results indicated that non-host plant PIs were good candidates for use in genetic transformation as a protective mechanism to impart resistance to attack by *H.*

armigera. Feeding bioassays with the non-host plant PIs provided valuable data about the role played by the activator enzymes in the digestive system. In the present study, non-host PIs from *D. alba ness*, *P. tetragonolobus*, *P. pinnata*, *M. pruriens*, *C. annum*, and *N. sativa* inhibited more than 80% of the total proteolytic (azocaseinolytic) activity of *H. armigera* larvae *in vivo*, while *in vitro* activation showed more than 20% pro-proteinase activity (Table 4.2.3).

Plant PIs have been extensively studied, and strategies for developing plants with resistance to insect pests have been suggested (Garcia-Olmedo et al., 1987; Hilder et al., 1987; Ryan, 1990; Boulter, 1993; Jongsma et al., 1996; Michaud, 1997; Ussaf et al., 2001). However, insect pests have adapted to host plant PIs by synthesizing proteinases that are either insensitive to PIs (Broadway, 1995; Jongsma et al., 1995; Lawrence and Koundal, 2002) or have the capacity to degrade them (Michaud, 1997; Girard et al., 1998). Therefore, it is necessary to study the non-host plant PIs as potential sources to overcome the problem of insect adaptation to the defense mechanism of the host plant. Non-host plant PIs showed high inhibitory potential against the HaGP activity, while host plant PIs were found to be weak inhibitors of HaGPs as well as HaGPPs (Table 4.2.3).

Chickpea seeds contain Bowmann-Birk (BBI) and Kunitz (CaKPI) type proteinase inhibitors, in which BBIs are ineffective against the digestive proteinases, but CaKTI causes antagonistic effects on developing *H. armigera* larvae (Srinivasan et al., 2005a; b). Chickpea PIs in flour used in the artificial diet were inactivated by autoclaving and heat treatment. Seed powder used for feeding assays was mixed with plant PIs in equal amounts.

Non-host plant, *D. alba ness* PIs showed highest inhibition of HaGPs as well as HaGPPs, followed by *P. tetragonolobus*. Giri et al. (2003) reported that *P. tetragonolobus* PIs especially, TIs have different binding potentials towards HaGP, although HaGP activity is trypsin-like. While earlier studies have indicated that *P. tetragonolobus* PIs may be good candidates for engineering resistance to *H. armigera* in host plants (Hursulkar et al., 1999). Mature seeds of *P. tetragonolobus* are known to contain several PIs, some of which are inhibitors of only trypsin and chymotrypsin, while others inhibit both types of proteinases (Shibata et al., 1986). *Penganum harmala* and *S. nigrum* failed to inhibit the activation of HaGPPs, while *F. vulgare* inhibited the activity

of HaGPs, but showed low level of inhibition of HaGPPs activation. Activation of pro-proteinases resulted in high concentration of proteinases, and isoforms moving closely merged and formed a smear. This is one of the disadvantages of GXCP.

In vitro studies on the fate of non-host plant PIs viz., *D. alba ness* and *P. tetragonolobus* in *H. armigera* guts indicated that these inhibitors reduced the larval growth efficiently. Quantitive analysis of *in vitro* activation of pro- and proteinases of *H. armigera* revealed that *D. alba ness* possessed greater capability to inhibit pro- and proteinase as compared to *P. tetragonolobus*. However, in second-instar larvae, pro- and proteinase activity was quite low, while fifth-instar larvae the showed highest activity.

Broadway and Duffey (1986a, b) proposed that hyperproduction of proteinases led to inhibition of growth by ingested PIs. In the present studies, larvae fed on artificial diet without non-host PIs showed normal growth in contrast to the inhibition of growth of larvae fed on non-host plant PIs impregnated in artificial diet. The larvae fed on diets with non-host PIs showed a decrease in gut proteinase activity. Interestingly, a high level of pro- and proteinase activity was recorded in the fecal matter of larvae fed on artificial diet impregnated with host and non-host plant PIs, suggesting hyperproduction of pro- and proteinases in response to ingested PIs.

5.9 Activation of HaGPPs by HaGPs

The columnar cells in the midgut epithelium are involved in absorption of digested food as well as secretion of enzymes. These enzymes are synthesized behind the peritropic membrane and then transported into the gut lumen by exocytotic process, and activated (Terra and Ferriera, 1994). Recently, Liu et al. (2008) reported that trypsins and serine endoproteases are the most important proteases in *H. armigera*, because of their key roles in food digestion and zymogen activation. A serine protease present in the gut lumen which activates zymogen phenol oxidase (PPO) of *S. litura* (Arora et al., 2009).

The *H. armigera* pro-proteinases were activated upon treatment of its active isoforms. Other biochemical parameters (Ca^{2+}, pH, and temperature) were also important for activation of zymogen proteinases of *H. armigera*.

Initially, we used bovine trypsin as an activator for activation of *H. armigera* gut pro-proteinase. We observed that partially purified trypsin isoforms of *H. armigera* activated its inactive isoforms as well as other pro-proteinases, and they were also responsible for autoactivation. The non-host plant, *D. alba ness* PIs resulted in greater activation of *H. armigera* gut pro-proteinase(s) as compared to *P. tetragonolobus* (Figure 4.2.9).

5.10 Adaptation of *H. armigera* larvae to plant PIs

Insects adapt to the PIs of their host plants through different mechanisms. They adapt to plant PIs by either *de novo* synthesis of inhibitor-insensitive (inhibitor-resistant) proteinase (Jongsma et al., 1995; Bown et al., 1997; Paulillo et al., 2000; Brito et al., 2001; Mazumdar-Leighton and Broadway, 2001a, b; Volpicella et al., 2003), by production of proteinases that here the ability to degrade PIs, i.e. inhibitor-degrading proteinases (Girard et al., 1998; Moon et al., 2004; Telang et al., 2005; Giri et al., 1998), or by rapidly altering gut contents in response to the ingested PIs through up- and down-regulation of proteinases (overproduction) (Hilder et al., 1987).

In the present investigation, we evaluated the physiological response of *H. armigera* larvae to the host and non-host plant PIs *in vivo*. Two host plants viz., *C. arietinum* and *C. annum* PIs were totally degraded by *H. armigera* gut proteinases. Giri et al. (1998) reported that *C. arietinum* defensive TIs are degraded by *H. armigera* gut proteinases. Ability to overcome the effect of host plant PIs is of great significance for adaptation and survival of polyphagous insects. The adaptation of insect pests to host plant PIs probably results from the selection pressure acting on the insect population when they encounter the PIs of their host plants. Jongsma et al. (1996) proposed that the non-host plants are the potential source of effective inhibitors for on insect pest in question, as the insect is not pre-exposed to the inhibitors. Trasgentically expressed PIs of non-host plants have been found to be effective against the target insect pests. The non-host plant PIs are of dual benefit, viz., they act against the proteinases of insect gut, and they also protect the host plant's defense proteins from proteolysis, thus giving the plants an edge over the insect pests. Hursulkar et al. (1999) reported several non-host plants of *H. armigera* as new sources of potent PIs. Continuous exposure of insects to different PIs

might result in insect adaptation to any or all of the available defense mechanisms. Earlier, studies have demonstrated that precursor proteins of Pin-II type inhibitors in various plants consist of 1 to 8 inhibitory repeat domains (IRD), which upon cleavage by endogenous proteinases release single inhibitor proteins that are active against one or multiple serine proteinases (Heath et al., 1995; Horn et al., 2005).

The insect proteinases might have undergone substitution of amino acids, resulting in weakened interactions with plant PIs (Broadway, 1995, 1996; Jongsma et al., 1996), e.g., *S. litura* and *Lephnotersa decemlineata* (Say) may adapt to plant PIs by producing inhibitor insensitive proteinases (Bolter and Jongsma, 1995; Jongsma et al., 1995). However, increase in secretion of additional or overproduction of proteinases in response to the inhibitors requires utilization of valuable amino acid pools, which may starve the insects (Broadway and Duffey, 1986b; Broadway, 1995). Incorporation of soybean TI and potato PI-II into the diet of *Helicoverpa zea* (Boddie) and *S. exigua* resulted in overproduction of trypsin, and a decrease in growth rate. In the present studies, we observed that *H. armigera* larvae responded by overproduction of gut proteinases to non-host plant PIs of *A. aspera*, *M. charntia*, *M. koenigii*, and *P. corywfolia* (Figure 4.2.5). Thus, the insect adapts to ingested PIs by synthesis of proteinases, which may be insensitive to and/or may degrade the PIs. These mechanisms of adaptation involving overproduction of proteolytic enzymes is a key to the toxicity of PIs to insects as it enforces insects to produce new proteinase(s) to compensate the effect of PIs. The demands for utilization of additional essential amino acids and energy, besides affecting digestion of proteins, results in poor growth of insects due to the presence of PIs.

A large proportion of HaGPs are insensitive to host plant PIs, while the PIs from the non-host plants such as *D. alba ness* and *P. tetragonolobus* inhibit the activity of HaGP (Figure 4.2.5) and HaGPPs (Figure 4.2.6). In view of the ability of the insects to alter the composition of midgut proteinases, no single defense gene can impart sustainable resistance. Therefore, optimization of the combination of PIs, and/or other genes is essential to develop insect-resistant plants for sustainable resistance.

5.11 Effect of host and non-host plant PIs on survival and development of *H. armigera* larvae

Edmonds et al. (1996) reported that strong inhibitors of gut proteinases *in vitro* do not necessarily retard larval growth and development. Insect feeding assays were therefore performed to assess the antibiosis exerted on *H. armigera* by the host and non-host plant PIs (Table 4.2.9). Development of *H. armigera* larvae fed on plant PIs incorporated into chickpea (PI removed) based artificial diet was evaluated *in vivo*.

The disruption of amino acid metabolism by the inhibition of protein digestion through PIs is the basis of PI-based defense in plants. The present studies revealed that larval growth and development were significantly reduced when the larvae were fed on non-host PI diet. Reduced feeding of larvae was observed in case of PI-incorporated diet as compared to those fed on control diet. The adverse effects were significant at higher/sublethal concentrations of non-host plant PIs. Significant mortality of larvae was also observed. The present studies demonstrated that larval stage is very crucial for accumulating nutrients and energy, which are used for pupal and adult development, and fecundity. Ashouri et al. (1998) reported that oryzacystatin-I affected fertility and fecundity of *Perillus bioculatus* (F.). Ingestion of potent PIs adversely affected the protein intake at the larval stage, which caused developmental abnormalities and also reduced fertility and fecundity of the adults. Apart from inhibition and disruption of molting, larval-pupal intermediates and malformed adults were also observed. The malformed adults were short lived and infertile. Adults emerging from PI-fed larvae had impaired fertility and fecundity. Low fecundity values symbolized less progeny production, which has a direct impact on the subsequent generation of *H. armigera*. However, starvation and added stress on gut proteinase expression system resulted in synthesis of new and/or higher amounts of proteinases and this could be the possible reason for arrested growth and mortality of *H. armigera*. Several researchers have observed growth retardation and mortality with high PI doses in various insects (Jongsma et al., 1996; Stotz et al., 1999; Murdock and Shade, 2002; Tamhane et al., 2005).

In the present studies it was observed that ingested PIs exerted physiological stress on the larvae and resulted in retarded the growth. Amongst the non-host plant PIs, *D. alba ness* resulted in highest stunted growth (Table 4.2.9; Figure 4.2.13). Thus, the

non-host plant PIs have the potential for deployment in transgenic plants with resistance to *H. armigera*.

CHAPTER 6

SUMMARY

Proteinaceous proteinase inhibitors (PIs) are ubiquitous in plant parts, which regulate proteolytic activity through protein-protein interaction, and are the plant's defense compounds produced in hyper amounts in response to insect and pathogen attack, and wounding. Once ingested, PIs impair protein digestion in insect gut, and thereby, limiting availability of essential amino acids resulting in growth retardation. However, it has been observed that insects adapt to the PIs of their host plants by various mechanisms. In some cases, it has been observed that *H. armigera* gut proteinases (HaGPs) are insensitive to inhibition by host plant PIs and can effectively degrade them, thereby, defeating the defense mechanism. On the other hand, the use of genetic engineering technology for transformation of crop plants for insect pest resistance has created access to genes from un-related organisms. *Bacillus thuringiensis* (*Bt*) toxin genes have been successfully expressed in several crops to impart resistance to herbivorous insects. However, recent studies shown that insects might develop resistance to *Bt* endotoxins by producing proteinases that inactivate the toxin or by lacking the proteinase allele required for activation of *Bt* protoxin. Development of insect-resistant transgenic plants expressing PI, amylase inhibitor, and lectin genes from plants is another approach to develop transgenic plants with resistance to insect pests. The use of non-host plant PIs in developing plants resistant to insects is of dual benefit, as they inhibit insect mid-gut proteinases, thereby, protecting other defense proteins from proteolytic degradation. As the PIs from non-host plants are not pre-exposed to insects, so they have the capability to block the digestive proteinases in insect gut and starve them of essential amino acids, and also affect a number of vital processes, including proteolytic activation of enzymes and moulting. The present studies were aimed at evaluating *in vitro* and *in vivo* effects of host and non-host plant PIs on *H. armigera*, and to identify promising potent PI for inhibition of zymogen activation in *H. armigera*.

In the present study, analysis of *H. armigera* gut extract revealed that it is a cocktail of at least ten proteinases having diverse specificities. The X-ray film coated with gelatin was used as a substrate to detect the electrophoretically separated proteinases and pro-proteinases of *H. armigera* gut extract on native-PAGE, SDS-PAGE, and two-dimensional gels. Using the *in-gel* activation method, at least six pro-proteinase isoforms of *H. armigera* gut extract were activated, and proteinase activity visualized on X-ray

film by degrading the gelatin. On the basis of substrate specificity and inhibition by synthetic inhibitors and their molecular weight and position, pro- and proteinases in the gel were classified into two major serine proteinase families i.e., trypsinogen-trypsin and chymotrypsinogen-chymotrypsin. Detectable pro-proteinase amounts were observed in *H. armigera* gut extract contents when the larvae were fed on a cocktail of synthetic inhibitors incorporated in chickpea (PIs removed) based artificial diet. The *H. armigera* larvae fed on synthetic inhibitors showed low to moderate levels of pro-proteinases after one-day, but two-days feeding resulted maximum activity, which declined thereafter.

The pH, temperature, and Ca^{2+} ion concentration affected the activity and activation of *H. armigera* gut pro- and proteinases. The trypsin- and chymotrypsin- like proteinase activity in *H. armigera* midgut had high pH maxima, being in the order of pH 9 – 11. But pro-proteinase activation was maximum at pH 8 and 10. *Helicoverpa armigera* gut pro- and proteinase activity was also affected by temperature. As the temperature increased, activity and activation rates also increased, and reached at the maximum at 40ºC and then declined thereafter. Gut proteinases showed maximum activity at 60ºC, and pro-proteinase activation at 40ºC. Although gut pro-proteinase activation was affected by Ca^{2+} ions, the proteinase activity was not.

The pro- and proteinases were extracted and partially purified by affinity and size exclusion column chromatography from the alimentary tract of larval *H. armigera* fed on a cocktail of synthetic inhibitors. The *H. armigera* gut pro-proteinase isoforms activation was observed in the elution fraction on benzamidine sepharose 4B column, and activation measured by BA*p*NA as a substrate. In an another approach, *H. armigera* pro- and proteinases were purified by gel filtration column chromatography using sephadex G 75. Size exclusively purified HaGPPs were corroborated and analyzed for substrate specificities using BTEE assay. In *H. armigera*, the Mr 23,000 – 70,000 Da polypeptides were the likely trypsin/chymotrypsin- like pro-proteinases, which were also present in fractions with low BA*p*NAase activity.

To understand the nature of HaGP activity in insects, larvae were fed on synthetic inhibitors with different specificities during larval development, and their response to a cocktail of synthetic PIs was studied. The *H. armigera* gut extracts were assayed for inhibition of pro-proteinases activation by synthetic inhibitors with respect to their

specificity towards the active isoforms of proteinase(s). It was observed that larvae fed on a cocktail of SIs (antipain, aprotinin, leupeptin, and pefabloc) showed maximum activation of pro-proteinases, as compared to the larvae fed on individual inhibitors. The results suggested that serine-proteinase inhibitors were potent inhibitors of HaGPs *in vitro* as well as *in vivo*. Activation of HaGPPs in different stadia of *H. armigera* increased with larval age, and maximum activity was observed in fifth-instars. The fecal matter of insects feeding on synthetic inhibitors exhibited profound pro-proteinase activity with the substrate as well as with *in gel* assay method. This might be due to the inhibition of insects' gut digestive enzymes by ingested synthetic PIs.

In the present study, we screened several non-host plant PIs for inhibition of insect digestive proteinase zymogen(s) activation. Protease inhibitors present in plant tissues were extracted in water and used for dot-blot and electrophoretic detection analysis. X-ray film method developed by Pichare and Kachole (1994) was used for screening the plant PIs. Seeds of 108 plant species were used for initial screening, of which 31 showed high inhibitory activity, while 18 and 22 showed moderate to low activity against *H. armigera* gut proteinases, respectively. Trypsin, chymotrypsin, and *H. armigera* gut proteinase inhibitor profiles were detected in host and non-host plant seed extracts by X-ray film method. All non-host plants exhibited trypsin-like inhibitory activity, but most of them did not show chymotrypsin and HaGP inhibitory activity. Host plants such as *Cajanus cajan* and *Cicer arietinum* exhibited trypsin and chymotrypsin inhibitory activity, but did not show HaGP inhibitory activity.

The *H. armigera* larvae fed on 22 different host and non-host plant PIs were analyzed for *in vivo* inhibition of HaGPs, and trypsin isoforms activity measured by substrate assays. This is a simple approach to evaluate the specificity and potential of host and non-host plant PIs against *H. armigera* gut digestive enzymes. Substrate assays were used to determine the percentage inhibition of total proteinase activity, and visualize the insensitive gut proteinase profile. Six non-host plants showed high inhibition, while 10 plants showed high to moderate activity, the remaining showed low activity. The host plants of *H. armigera* showed very low inhibition to HaGP and trypsin isoforms. Interestingly, in electrophoretic analysis, no band was observed in the lane where the larvae were fed on non-host plant PIs from *Datura alba ness*. However, non-host plant

PIs showed high inhibitory potential towards the HaGP activity, while host plants PIs are weak inhibitors of HaGP.

In vitro activation of HaGPPs in larvae fed on non-host plant PIs showed significant activation in solution as well as *in gel* assay method. The larvae fed on diet with *Datura alba ness* PIs showed highest activation followed by *Psophocarpus tetragonolobus*, which also exhibited higher activation as compared to other non-host plant species. Non-host plant PIs from *Capsicum annum*, *Mucuna pruriens*, *Pongamia pinnata*, and *Nigela sativa* showed high inhibitory potential towards HaGPs *in vivo*, and also exhibited moderate level of *in vitro* activation of pro-proteinases. However, some of non-host plant PIs such as those from *Penganum harmala* and *Solanum nigrum* did not show the activation of pro-proteinases. The present studies indicated that non-host plant PIs are good candidates as inhibitors of the HaGPs as well as HaGPPs.

Activation of gut pro-proteinases during the development of *H. armigera* larvae was examined by solution as well as *in gel* assay. Two non-host plants were selected, which possessed potent inhibitory activity against HaGPs and pro-proteinases. The second-instar larvae showed very low pro-proteinase activity, and no activation of trypsinogen/chymotrypsinogen isoforms was observed when the larvae were fed on both non-host plant PIs. Activation of pro-proteinases increased with the instars, and maximum activation was observed in the fifth-instar. *Datura alba ness* fed larvae showed greater activation as compared to the larvae fed on *P. tetragonolobus*.

The pro-protein could be activated by treatment with bovine trypsin; degradation of bound pro-region, rather than cleavage of pro-region form mature protein, and was the rate-limiting step in activation. Similarly, larvae fed on non-host plant PIs exhibited activation of pro-proteinases with the treatment of partially purified HaGP. Low concentrations of partially purified HaGPs were treated with gut extracts of larvae fed on *D. alba ness*, and activation measured by substrate assays. Out of 10 proteinases, HaGP 5 and 9 showed activation of pro-proteinases. Both appeared to be major trypsin-like proteinase isoforms, and showed specificity towards BA*p*NA. HaGP 5 showed more activation as compared to HaGP 9.

The fecal matter of larvae fed on different host and non-host plants in artificial diet was analyzed for pro- and proteinase activity. The larvae possessed putative hyper

production of proteinases in response to dietary PIs. The *H. armigera* larvae fed on host plant PIs exhibited high proteinase activity, but did not result in activation of pro-proteinase. It was observed that fecal matter of larvae fed on non-host plant PIs showed high pro-proteinase activation. Among the non-host plant PIs, *D. alba ness* showed highest pro-proteinase activation, followed by *C. annum*, and *P. tetragonolobus*.

The inhibitory potential of non-host plant PIs against *H. armigera* gut digestive enzymes was analyzed by *in vivo* and *in vitro* experiments. Spectrophotometric assay methods were employed for analysis of inhibition of pro- and proteinase activity by specific substrates. The fifth-instar larvae exhibited maximum pro- and proteinase activity, and hence, mid-fifth-instar larvae were used in these studies. Among the host plants, *C. cajan* and *C. arietinum* showed partial inhibition of gut proteinases *in vitro*. However, non-host plant PIs were found to be potent inhibitors of HaGPs, and showed high to moderate levels of inhibition of gut proteinase activity.

The present studies demonstrated that non-host plant PIs are effective in inhibiting the isoproteinases and growth of *H. armigera* larvae. Proteinases are the major digestive enzymes in the insect gut, and are responsible for a continuous supply of essential amino acids and energy from the food for development. The disruption of amino acid metabolism by the inhibition of protein digestion by the PIs is the basis of PI-based defense in plants; however, in nature, it might be coupled with other factors. To evaluate the *in vivo* effects of host and non-host plant PIs on *H. armigera*, feeding assays were conducted with added inhibitor protein in the artificial diet. Larval growth and development were reduced when the larvae were fed on non-host plant PIs in the artificial diet. Reduced feeding of larvae was observed in case of PI-incorporated diet as compared to control diet. The adverse effects were significant at higher concentrations of PIs. Significant mortality of larvae was also evident. Larval stage is crucial for accumulating nutrients and energy, which is used for pupal and adult development, fecundity, and fertility. Among the non-host plant PIs, *D. alba ness* resulted in highest stunted growth. Non-host plants PIs, which have the potential to inhibit the growth of larvae, can be exploited for developing insect resistant transgenic plants.

From the foregoing account, it is evident that the various non-host plant PIs have the capability to inhibit the gut digestive enzymes, and also activation of zymogen

protease(s). The results of the present studies demonstrated the usefulness and potential of the non-host plant PIs against *H. armigera*, which can be exploited, to develop genetically modified plants with resistance to this insect.

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