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IDENTIFICATION AND CHARACTERIZATION OF HYDROLYTIC ENZYMES OF SUNN PEST (EURYGASTER INTEGRICEPS) AND COTTON BOLLWORM (HELICOVERPA ARMIGERA)

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IDENTIFICATION AND CHARACTERIZATION OF HYDROLYTIC ENZYMES OF SUNN PEST (EURYGASTER INTEGRICEPS) AND COTTON BOLLWORM (HELICOVERPA ARMIGERA)

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Approval for the Graduate School of Natural and Applied Sciences

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ABSTRACT

Identification and Characterization of Hydrolytic Enzymes of Sunn Pest (*Eurygaster integriceps*) and Cotton Bollworm (*Helicoverpa armigera*)

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In this study, hydrolytic enzymes from sunn pest (*Eurygaster integriceps*) and cotton bollworm (*Helicoverpa armigera*) midguts were identified and characterized in terms of their optimum pH, K_m and V_{max} values. Hydrolytic activities were also tested for inhibition by several protease and α-amylase inhibitors which can be used for the development of insect resistant plants through transgenic technologies.

For sunn pest midgut, a low proteolytic activity, belonging mostly to trypsin-like and leucine aminopeptidase-like proteases, and a very high α-amylase activity was found in sunn pest midgut, reflecting its high carbohydrate diet. Proteolytic activities could not be inhibited by natural protease inhibitors (SBTI and aprotinin) but inhibited significantly by a general serine protease inhibitor PMSF and metalloprotease inhibitors

$CdCl_2$ and $CuCl_2$. α-Amylase activity of sunn pest midgut is resistant to inhibition by bean α-amylase inhibitor, but inhibited by chickpea, wheat and maize α-amylase inhibitors by 26 %, 37 % and 40 %, respectively.

For cotton bollworm midgut, a very high proteolytic activity, belonging to serine and metalloprotease type, was detected. α-Amylase activity was lower compared to sunn pest midgut, but there were higher and diverse type of proteases, might be reflecting its wide range of host preference. Proteolytic activity was significantly inhibited by both natural protease inhibitors (SBTI and aprotinin). It was also inhibited by several synthetic protease inhibitors (PMSF, E-64, TPCK, $CdCl_2$, $CuCl_2$, Chymostatin). α-Amylase activity was inhibited by 60 % by wheat α-amylase inhibitor, while maize, chickpea and bean α-amylase inhibitors had no effect on cotton bollworm midgut α-amylase activity.

Keywords: *Eurygaster integriceps*, *Helicoverpa armigera*, proteases, α-amylase, protease inhibitors, α-amylase inhibitors.

ÖZ

Süne (*Erygaster integriceps*) ve Pamukta Yeşilkurt (*Helicoverpa armigera*) Zararlılarının Hidrolitic Enzimlerinin Belirlenmesi ve Karakterizasyonu

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Bu çalışmada, süne (*Eurygaster integriceps*) ve pamukta yeşil kurt (*Helicoverpa armigera*) zararlılarının sindirim sistemlerinde bulunan hidrolitik enzimlerin belirlenmesi ve optimum pH, K_m ve V_{max} değerleri bakımından karakterizasyonu amaçlanmıştır. Ayrıca, hidrolitik enzimlerin çeşitli proteaz ve α-amilaz inhibitörleri ile inhibisyonları test edilmiştir. Bu inhibitörler böceklere dayanıklı bitkilerin transgenik teknolojiler ile geliştirilmesi için kullanılabilir.

Süne sindirim sisteminde tripsin ve lösin aminopeptidaz benzeri aktivitelere bağlı düşük bir proteaz aktivitesi, diğer taraftan, oldukça yüksek bir α-amilaz aktivitesi tespit edilmiştir. Bu durum, sünenin yüksek karbonhidrat içeren diyeti ile uyum göstermektedir. Süne

proteolitik aktivitesi doğal proteaz inhibitörlerine (SBTI ve aprotinin) karşı direnç gösterirken, sentetik serin proteaz inhibitörü PMSF, ve metaloproteaz inhibitörleri $CuCl_2$ ve $CdCl_2$ ile önemli ölçüde inhibe olmuştur. Süne sindirim sisteminde bulunan α-amylase aktivitesi fasülye α-amilaz inhibitörüne karşı direnç gösterirken, nohut, buğday ve mısır α-amilaz inhibitörleri ile sırasıyla %26, %37 ve %40 oranlarında inhibe olmuştur.

Pamukta yeşil kurt zararlısının sindirim sisteminde çoğunluğu serin ve metaloproteaz benzeri yüksek bir proteolitik aktivite tayin edilmiştir. α-Amilaz aktivitesi ise süne ile kıyasladığında düşüktür, ancak proteaz aktivitesinin daha yüksek olduğu ve çeşitlilik gösterdiği gözlenmiştir. Bu durum, zararlının çok çeşitli bitki türlerinden beslenmesinin sonucu olabilir. Proteaz aktivitesi doğal proteaz inhibitörleri olan SBTI ve aprotinin ile önemli ölçüde inhibe edilmiştir. Ayrıca sentetik proteaz inhibitörleri (PMSF, E-64, TPCK, $CdCl_2$, $CuCl_2$, Chymostatin) ile de inhibisyon gözlenmiştir. α-Amilaz aktivitesi mısır, nohut ve fasülye α-amilaz inhibitörlerine karşı direnç gösterirken, buğday α-amilaz inhibitörü ile % 60 oranında inhibe olmuştur.

Anahtar Kelimeler: *Eurygaster integriceps*, *Helicoverpa armigera*, proteazlar, α-amilaz, proteaz inhibitörleri, α-amilaz inhibitörleri.

To my Family

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LIST OF ABBREVIATIONS

BAAI	Bean α-amylase inhibitor
BApNa	Nα-benzoyl-DL-arginine p-nitroanilide
CpAAI	Chickpea α-amylase inhibitor
E-64	L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane
HA	Hippuryl-L-arginine
HPA	Hippuryl-phenylalanine
LpNa	L-leucine p-nitroanilide
MAAI	Maize α-amylase inhibitor
PAGE	Polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonyl floride
SA_2PPpNa	N- succinyl – alanine – alanine – proline - phenylalanine p-nitroanilide
SA_3pNa	N-succinyl-alanine-alanine-alanine p-nitroanilide
SBTI	Soybean trypsin inhibitor
SDS	Sodium dedocyl sulfate
TCA	Trichloroacetic acid
TPCK	1-Chloro-3-tosylamido-4-phenyl-2-butanone
WAAI	Wheat α-amylase inhibitor

CHAPTER 1

INTRODUCTION

1.1 Sunn Pest (*Eurygaster integriceps*)

1.1.1 General Information

Sunn pests, also known as sunne pests, suni pests or wheat shield bugs are a complex of species belonging to two closely related families, namely Pentatomidae and Scutelleridae. Two important genera occur within these families: *Aelia* F. (Pentatomidae) and *Eurygaster* Lap. (Scutelleridae). Within these two genera some species are strongly migratory while others are sedentary or only subjected to minor displacements. The migratory species tend to be of greater economic importance because of their ability to survive or hibernate in "overwintering" areas, usually under fairly harsh and remote mountainous conditions at high altitudes. The species *Eurygaster integriceps* falls within this category as it breeds and feeds in wheat fields at relatively low altitudes in the spring and early summer and in late summer migrates first to aestivation areas and then to well established hibernating areas where it spends the autumn and winter.

The taxonomic position of *Eurygaster integriceps* Puton. in the class Insecta is within the order Hemiptera, sub-order Heteroptera (true bugs), family Scutelleridae. Adults of *E. integriceps* are 11.5-13 mm

long, rounded, with a long scutellum which covers the entire abdomen. They have a broad body with a length-to-width ratio of 1.56. Body coloration can be very variable, it ranges from yellow-brown to dark brown, red or black with darker markings on the lighter shades.

Figure 1.1 Sunn pest (*Eurygaster integriceps* Put.).

E. integriceps is predominantly distributed in the region of Near and Middle East to eastern Europe. It is found in south east of Turkey, most of Iran, Iraq, Syria, Lebanon and Israel. In Eastern Europe it is found in Croatia, Serbia, Yugoslavia, Greece, Bulgaria, Romania, and Ukraine. In Western Asia it is found in Russian Federation of CIS, Kazakhstan, Uzbekistan, Kyrgyzstan, Tajikistan, Afghanistan and Pakistan. Figure 1.2 shows the distribution of *Eurygaster* species in Turkey.

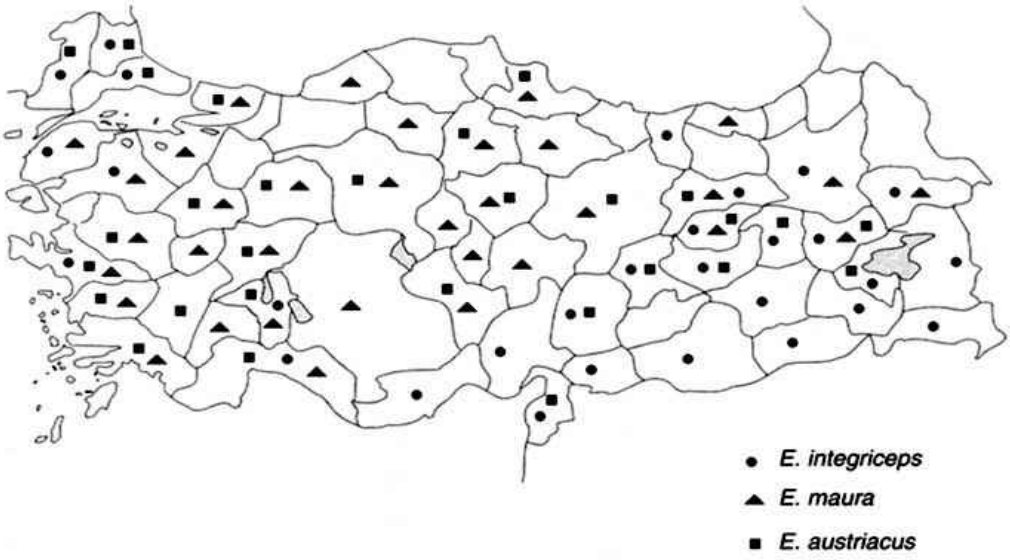


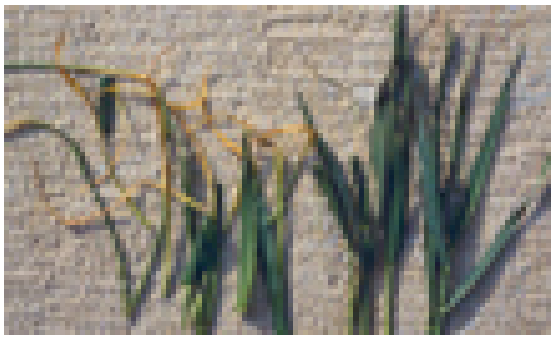
Figure 1.2 Distribution of *Eurygaster* species in Turkey.

E. integriceps feeds predominantly on Gramineae, particularly wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), with rye (*Secale cereale*), maize (*Zea mays*), grasses and other wild species of graminaceous plants. All parts of the cereal plant can be attacked from the leaves and stems to the ears, depending on the stage of growth of the insect as well as the plants. Nymphs, from the second instar onwards, will generally commence feeding on the leaves in the early developmental stages of the plant growth prior to heading, but once they reach adulthood will then feed almost exclusively on the ears and kernels in order to build up adequate food reserves as quickly as possible. In severe attacks particularly by overwintering adults on young wheat plants, feeding on the leaves and stems can cause the plants to wither through the destruction of the growing point of the central leaf known as "deadheart" (kurtboğazı). Later in the season, the ripening plant is damaged and this is often the most serious aspect of the injury. If the ear is attacked in the bud stage, it is usually aborted causing the

so-called “white ears” (akbaşak). Attacked wheats and damage caused by sunn pest was shown in Figure 1.3.

A

B



C

Figure 1.3 Sunn pest infestation on wheat. A) A wheat kernel infested by sunn pest. B) A sunn pest infested wheat field with white ears syndrome. C) Sunn pest infested wheat seedlings with deadheart syndrome.

The damage can range from complete destruction if the kernel is attacked in the milky stage, to slightly shriveled if attacked in late maturity. The presence of a black central point on the grain surrounded by a pale or discolored halo is the sign that it has been attacked by these bugs. The toxic saliva that the insect injects destroys the gluten in

the grain so that the bread made from the flour does not rise properly. Attacks on grain are manifested in three ways:

(a) Reduction in the actual yield: In grain attacked by sunn pest, 1000-grain weight can be reduced to 78-92 % of that of undamaged grain.
(b) Reduction in seed germination: If 14% of kernels are attacked, only 88% germination can be obtained.
(c) Reduction in the gluten index: In grain damaged by sunn pests, the gluten in the grain is degraded by enzymes while other characteristics, such as elasticity, are lost. Dough made from damaged grain has an abnormal consistency (due to gluten becoming soft and sticky) and does not knead properly. Baking is unsatisfactory and the bread is of poor quality. Only as little as 2-3% of the grain needs to be damaged for it to be rendered unacceptable for baking purposes.

1.1.2 Life Cycle

The schematic representation of *E.* integriceps life cycle is given in Appendix A. *Eurygaster integriceps* lives for one year and produce a single generation per year. During the life span only 2.5-3 months are spent as an active insect on graminaceous plants. The rest of the year is spent resting in vegetation and litter on hillsides and includes an obligatory diapause. This resting period or diapause includes two phases: "aestivation" during the hot dry months of the late summer and autumn and "hibernation" during the cold and often severe winter months. The whole of resting period is termed as "overwintering period". The mountain hibernating sites are usually 10-20 km from the wheat fields they infest although they can be as far away as 150 km. In south eastern Turkey *E. integriceps* overwinters at altitudes between 1000 and 2000 m above sea level. During the overwintering period, about a

quarter of the insects stored fat reserves is consumed and almost a quarter of a population may die if these fat reserves are inadequate. As soon as temperature attains 12-13 ^{o}C in spring, the surviving adults migrate down to these fields in one non-stop flight. The fields to which they fly in spring is termed as "invasion area" (Lodos, 1998).

After feeding, a reproductive phase in the annual cycle occurs and a new generation appears in the crops about June. Eggs are laid in small clusters, typically in even multiples of 14, on the leaf blades of the crop in the invasion area. The eggs take 9-10 days to develop under field conditions of about 20 ^{o}C. After hatching, the nymphs pass through five instars before developing into the adult. Each of the first four instars take about 4-7 days to complete, depending mainly on temperature but also food availability, with the final instars taking the longest time at 10-11 days. The development process takes a minimum of 35-37 days but could be as long as 50-60 days depending on the food availability and field conditions (Critchley, 1998).

The nymphal stages one and two are rarely seen on the stem of wheat as they spend all their time deep in the crop canopy to escape predation. During the third instar they begin to disperse up the plants and then as fourth and fifth nymphal instars they spend most of their time on the stem and ears of the cereal crop (Critchley, 1998).

1.1.3 Population Regulation

Sunn pest population is regulated by biotic factors (predators and parasits) and abiotic factors (availability of food, climate, farming, pesticides). Sunn pests are attacked by a number of arthropod predators such as spiders, carabid beetles and tachinid flies. They are also attacked by more specialized parasites, the most important of which being hymenopteran egg parasitoids. Among the abiotic factors affecting the population dynamics of sunn pest lack of food is the major

cause of mortality. When large number of overwintered adults destroy wheat fields and prevent the production of any grain, the developing nymphs die before maturity owing to lack of food or not able to build up sufficient fat reserves to survive. Climatic conditions also affect their survival. Sunn pest is a heat and sun-loving insect. All its vital processes including spring migration, sexual maturation, feeding, egg-laying, hatching, nymphal growth and development, *etc*., occur most successfully if the weather is warm, reasonably moist and free of high winds. Damp, cold, cloudy weather and high winds have adverse affects on population development and hence on survival. Apart from these, pesticides are the major interfering substances of sunn pest population (Critchley, 1998).

1.1.4 Prevention and Control

Losses due to sunn pests are highly variable depending on the density of the insect, water availability, features of wheat cultivar and duration of the crop-growing period. Some 10-15 million hectares under cereal cultivation are threatened by these bugs. If not controlled, it may cause up to 90% yield loss (Paulian and Popov, 1980). Integrated pest management (IPG) approach, which includes cultural, biological and chemical control mechanisms are being used for the control of sunn pest damage to wheat.

Cultural control practices include:

(a) Manipulation of crop growing factors to achieve as short a growth season as possible;
(b) Early and rapid harvesting;
(c) Planting early or resistant varieties;
(d) Thorough weeding;
(e) Adequate rotation with non-cereal crops.

Planting of cereals should be done in a way that optimizes growth and development, ensures early and rapid seeding and results in early and rapid harvesting. Other measures that can be taken are to grow winter wheat varieties that are resistant to *E. integriceps* on the basis of the energy that needs to be expended by the pest to utilize their available protein. Rotation of crops is a very important component in the cultural control of pests. Another cultural control measure is weeding of alternative wild host plants, such as camel-thorn, both within crops and in surrounding fields. This may appreciably reduce the chances of sunn pest nymphs surviving to adulthood or of adults surviving through the winter particularly if weather conditions turn harsh. For the cultural control measures to be useful, a comprehensive package of these measures has to be conducted on a regular basis (Critchley, 1998; Lodos, 1998).

Biological control strategies include the release of general predators and egg parasitoids. There are also attempts to use microorganisms or fungal pathogens such as *Bacillus thuringiensis* and *Beauveria bassiana* respectively. In some cases Bt toxins is applied together with some doses of insecticide but this may not be good strategy as it could lead to the insects developing insecticide resistance much more rapidly owing to the selective removal of only small, weak susceptible insects. Entomopathogenic fungi of *E. integriceps* named *Beauveria bassiana* have been reported to be effective in hibernating sites (Parker *et al*., 2003). It may significantly affect the survival of overwintering populations if uninfected sites are infected with the spread of fungus from infected sites (Critchley, 1998). In Turkey several natural enemies of sunn pest have been identified including some egg parasitoids (from the order Hymenoptera), endoparasitoids and adult and nymph predators (Diptera:Tachinadae) and egg predators (Acarina) and adult parasitic fungi (Ascomycetes) (Lodos, 1998). However, studies for the use of predators for pest management are limited because of the

difficulty of simultaneously managing three different biological populations – predator, prey (pest) and crop. They are particularly difficult to use in annual field crops and so find their greatest application in glasshouses and forestry where population dynamics are more easily regulated (Hilder and Boulter, 1999).

Chemical control is carried out through the use of conventional broad-spectrum insecticides (chlorinated hydrocarbons, organophosphates and synthetic pyrethroids) or selective insecticides. Conventional pesticides are highly toxic to sunn pests. However, timing of pesticide spray and stage of development of sunn pest are critical for mortality rate. The pesticide of low mammalian toxicity and low persistence should be chosen. While chemical control methods kill sunn pests, they are not always as effective as one would expect nor they are economic. In 2003 Turkey has spent about $7 million to prevent the sunn pest damage over 15 million decare areas (www.tarim.gov.tr). The side effects include the destruction of natural enemies, the problem of undesirable residues on wheat grain, contamination of food chain and water resources, development of resistance to the insecticides applied, and reduction of endurance of plants to damage by sunn pest and other organisms through their intensive use. Selective insecticides include such compounds as insect growth regulators (IGRs) and pheromones. However no practical application is available neither for IGRs nor pheromones (Critchley, 1998).

1.2 Cotton Bollworm (*Helicoverpa armigera)*

1.2.1 General Information

Cotton bollworm (*Helicoverpa armigera* Hüber.), also known as Old World bollworm and corn earworm, is highly distributed all over the world. It is a major pest on a wide range of crops in Europe, Africa, Asia

and Australia. Cotton bollworm is one of the most destructive polyphagous pest species of world-wide economical importance. At different developmental stages, *H. armigera* feeds on most plant structures including stems, leaves heads and fruits. They may even bore into the plants, for example at the base of flower buds, fruits, bolls of maize-cobs. This habit of boring can promote secondary infection of the damaged tissues, and can lead to rotting and fruit or leaf drop.

Crops affected by cotton bollworms include barley, beans, citrus, cucurbit, flax, garlic, groundnuts, leek, maize, oat, onion, pea, pine, potato, soyabean, stone fruits, sunflower, tobacco, tomato, wheat and many others.

The taxonomic position of *Helicoverpa armigera* Hübner. in the class of Insecta is within the order Lepidoptera, family Noctuidae and subfamily Heliothinae. Females lay several hundred eggs on all parts of the plant, flowers and fruits included. The eggs are approximately 0.5 mm in diameter and are white to brown in color. Eggs may hatch in less then 3 days at an optimum temperature of 27 – 28°C. Caterpillars pass through four developmental stages (instars) and ultimately reach 30 – 40 mm in length.

Figure 1.4 Cotton bollworm (*Helicoverpa armigera* Hübner.)

The caterpillar's body varies in color from green, yellow, pink and red-brown, to almost black, and the heads are usually pale brown. The caterpillars pupate in soil in silk-lined chamber. Pupae are 14 – 20 mm long, pale brown in color, turning darker brown as the moth develops within.

Adults can migrate over long distances, borne by wind, for example from southern Europe to UK. Movement in international trade is mainly on ornamental plants and on cut flowers; also in cotton bolls and in tomato fruits.

1.2.2 Life Cycle

Cotton bollworm give 2,5 generation per year. Adults emerge in the first three weeks of May and, 2-6 days later, oviposition begins. This period lasts 5-24 days and, within this time, a female may lay up to 3180 eggs. The first generation larvae (*i.e*. the larval progeny of the overwintering generation) appear in May and feed for 24-36 days; those of the second generation develop in 19-26 days. When fully fed, the larvae descend to the soil and after 1-7 days, pupate in the earthen cell, 2-8 cm below the surface. The overwintering pupae remain in the soil for 176-221 days.

1.2.3 Population Regulation

The population density of cotton bollworm is regulated by several biotic (parasitoids, predators and pathogens) and abiotic factors (availability of food, climate, farming and pesticides). Parasitoids of *Helicoverpa armigera* include egg parasitoids (*Trichogramma* & *Telenomus* wasps), larval parasitoids (*Micrropilitis demolitor, Netelia producta* -Orange caterpillar parasite, & Tachinid flies), larval-pupal parasitoids (*Heteropelma scaposum* - Two-toned parasite, & *Lissopimpla excelsa* - orchid dupe) and pupal parasitoids (*Ichneumon promissorius* - banded

caterpillar parasite). The most common *Helicoverpa* predators in field crops are predatory bugs, predatory beetles, spiders, lacewings and ants. Some predators are relatively permanent residents in fields (*e.g.* ants); others migrate from nearby fields, other vegetation or even further away. Pathogens are viruses, fungi or bacteria that infect insects. The commonest pathogens infect larvae, including the nucleopolyhedrovirus (NPV) and fungal pathogens (*Metarhizium, Nomurea* and *Beauvaria*). Another disease, ascovirus, stunts larval development, and is spread by wasp parasitoids.

1.2.4 Prevention and Control

Control of cotton boll worm population is carried out by chemical and biological means. Upon development of resistance against pyrethroid insecticides, the chemical control is replaced by endosulfan, an organochloride that has proved effective on both susceptible and resistant bollworms (CIRAD, 2003). Biological control includes use of parasitoids and pathogens. Two *Helicoverpa* pathogens are available commercially to control larvae: Nucleopolyhedrovirus (NPV) and the bacterial toxin from *Bacillus thuringiensis* (commonly called Bt). NPV occurs naturally and frequently causes natural outbreaks (epizootics) in *Helicoverpa* populations. The commercial *Helicoverpa* NPV is a highly selective biopesticide that infects only *H. armigera* and *H. punctigera* larvae. NPV is harmless to humans, wildlife and beneficial insects.The Bt toxin is available as a selective spray that only kills moth larvae. Genes from the Bt organism have been used to genetically modify cotton plants so that the toxin is expressed in the plant's tissues. When young *Helicoverpa* larvae feed on a *Bt* cotton plant, the *Bt* toxin kills susceptible individuals.

Integrated pest management approach is employed with the aim of reducing insecticide use while maintaining profitability, yield, quality and crop maturity.

Cultural control measures include:

- Deep ploughing and clean cultivation to expose resting pupae
- Crop rotation and avoidance of rooting
- Use of tolerant varieties
- Use of trap crops (like tomato) or intercrops (maize, cowpea) and mechanical removal of adults and larvae from the trap crop
- Use of baited sex pheromone traps

1.3 Hydrolytic Enzymes

1.3.1 Proteases

Proteases, also called proteinases or peptidases, hydrolyze peptide bonds of proteins. They frequently exist as zymogens in contact with potential substrates which are hydrolyzed only when the zymogen is activated by another protease. Proteases may function outside the cells (extracellular proteases), within the cell or may be present at cell surface which function in hydrolysis of relatively simple peptides rather than proteins (Kenny, 1999).

Proteases that require presence of unsubstituted N- or C-terminus in the substrate are exopeptidases, those that do not are endopeptidases. Exopeptidases remove a single amino acid, a dipeptide or a tripeptide from one or other terminus, action which are basis of classification of the exopeptidases. Similar considerations of specificity cannot be applied to endopeptidases, which are grouped according to the chemical nature of the catalytic sites yielding the four main classes: serine, cysteine, aspartic and metallo-endopeptidases. Most recently a group of threonine endopeptidases have been discovered. Same catalytic mechanisms operate among the exopeptidases, so that some of them can be further subdivided on the same basis (Fig 1.6).

All the hydrolases are designated by the International Union of Biochemistry and Molecular Biology as E.C.3.- and peptidases as E.C.3.4. The main classes of peptidases are defined by a third numeral (11 to 24) as listed in Table 1.1.

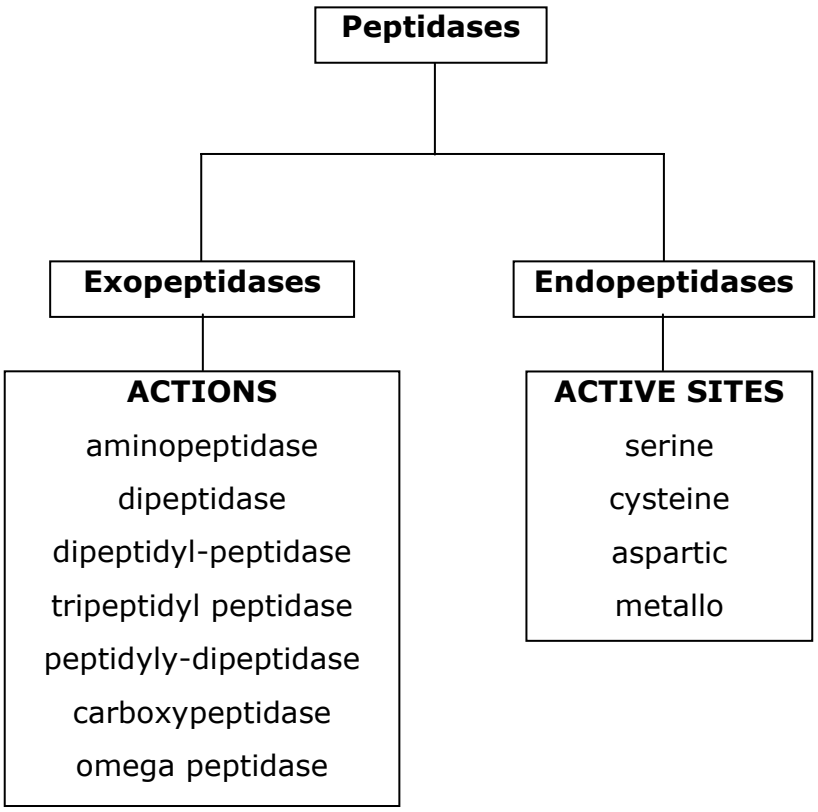


Figure 1.5 Main classes of petidases (Kenny, 1999)

Table 1.1 Types of peptidase defined in the Enzyme Nomenclature list of the International Union of Biochemistry and Molecular Biology (Kenny, 1999).

E.C. Number	Peptidase Type	Action
EXOPEPTIDASES		
3.4.11.-	Aminopeptidase	N-terminal residue released
3.4.13.-	Dipeptidase	Acts only on dipeptides
3.4.14.-	Dipeptidyl peptidase	N-terminal dipeptide released
	Tripeptidyl peptidase	N-terminal tripeptide released
3.4.15.-	Peptidyl dipeptidase	C-terminal dipeptide released
3.4.16.-	Carboxypeptidase (serine)	C-terminal residue released
3.4.17.-	Carboxypeptidase (metallo)	C-terminal residue released
3.4.18.-	Carboxypeptidase (cysteine)	C-terminal residue released
3.4.19.-	Omega peptidase	Release modified residue from N- or C-termini
ENDOPEPTIDASES		
3.4.21.-	Serine endopeptidase	
3.4.22.-	Cysteine endopeptidase	
3.4.23.-	Aspartic endopeptidase	
3.4.24.-	Metallo-endopeptidase	
3.4.99.-	Endopeptidase of unknown catalytic mechanism	

1.3.1.1 Serine Proteases

Peptidases in which catalytic mechanism depends on the hydroxyl group of a serine residue acting as the nucleophile that attacks the peptide bond are termed as serine peptidases.

There are in total of 50 families of serine peptidases that are distinguished on the basis of amino acid sequences. Most of these families are grouped into 9 clans by comparing the tertiary structures and the order of the catalytic residues in the sequences. The catalytic machinery usually involves, in addition to the serine that carries the nuclephile, a proton donor (or general base). In several clans of serine family, the proton donor is a histidine residue, and there is a catalytic triad because a third residue is required, probably for the orientation of imidazolium ring of the histidine, this is usually an aspartate or another histidine. In other clans a lysine residue has the role of proton donor, and a third catalytic residue is not required (Rawlings and Barrett, 2005a).

The best known of all serine peptidases are those such as trypsin, chymotrypsin and elastase.

Trypsin (E.C. 3.4.21.4) is one of the several digestive enzymes secreted into the intestine of animals. It is found in all animals, including insect, fish and mammals. It is synthesized and stored as proenzyme trypsinogen in secretory granules. Upon release into the gut, trypsinogen is activated by enteropeptidase or by trypsin itself. Once activated, the enzyme is responsible for activation of the proenzymes of all the other digestive enzymes such as chymotrypsin and elastase. Trypsin strongly prefers to cleave amide substrates following arginine or lysine residue at position 1. The preference for these basic amino acids

is reflected by its catalytic efficiency, which is 10^5 greater compared to other amino acids. Preference for arginine over lysine is 2- to 10-fold. Active trypsin consists of a single polypeptide chain, in which catalytic residue bridge two β-barrel domains**.** The most important catalytic residues are Asp102, His57 and Ser195 forming catalytic triad. Ser195 acts as a nucleophile in the cleavage reaction, producing an acyl enzyme intermediate. His57 is thought to act as a general base and Asp102 is believed to stabilize the correct tautomer of His57, and to provide compensation for the developing positive charge during the catalytic reaction (Halfon *et al*., 2005).

Chymotrypsin (E.C. 3.4.21.1) is another serine type endoprotease which has the same catalytic triad, Asp102/His57/Ser195, as trypsin. Catalytic acitivity involves the formation of acyl-enzyme intermediate, as in the case of trypsin. It is also secreted and stored as proenzyme, chymotrypsinogen, and then activated by an enteropeptidase or trypsin. Chymotrypsin structure is different from trypsin in that its 15 amino acid propeptide remains linked to the enzyme through a disulfide bridge between Cys1 and Cys122, which functions in keeping the proenzyme stable against non-specific activation. Chymotrypsin specifically acts on the peptide bonds formed by the carboxyl group of aromatic amino acids (tyrosine, phenylalanine and tryptophan) and leucine. Proline in the second position (…..-pro-leu**-**……) increases the rate of catalytic activity. Chymotrypsin does not hydrolyze bonds formed to the imino group of proline in proteins (Gráf *et al*., 2005)

Elastases (E.C. 3.4.21.36) are defined by their ability to release soluble peptides from insoluble elastin fibers by a proteolytic process. Elastase is a serine proteinase of the same family with chymotrypsin and trypsin whose catalytic site is composed of three hydrogen bonded amino acid residues, Asp102, His57, Ser195 which form the catalytic triad. They are also synthesized as inactive precursors called proelastases and activated

by trypsin. Primary specificity of elastase is towards nonbulky residues such as alanine, serine, glycine and valine in the first position (Bieth, 2005).

1.3.1.2 Cysteine Proteases

Peptidases in which the nucleophile that attacks the scissile peptide bond is the sulfhydryl group of a cysteine residue are known as cysteine-type peptidases. The catalytic mechanism is similar to that of the serine-type peptidases in that nucleophile and a proton donor (a general base) are required. The proton donor in all cysteine cysteine peptidases is a histidine residue, as in the majority of serine peptidases. In some families only the dyad of Cys and His seems to be essential for catalysis, whereas in other a third residue is required to orient the imidazolium ring of the His (a role analogous to that of the essential aspartate in some of the serine peptidases). (Rawlings and Barrett, 2005b).

The first clearly recognized cysteine peptidase was papain (E.C. 3.4.22.2) isolated from the latex of the tropical papaya fruit. Papain-like cysteine peptidases form the foundation of *clan CA* in which there is more than 20 families. The catalytic function is related to that of chymotrypsin (a serine peptidase) in that both enzymes have an acyl-enzyme intermediate on their reaction pathway. Papain is a single-chain non-glycosylated polypeptide containing three disulfide bonds. Papain is considered to possess a fairly broad specificity. The only selectivity is a bulky nonpolar side chain (*e.g*. phenylalanine) at P2 position (second amino acid at the N-terminal side of the site of cleavage) and valine is not accepted at P1 position. The enzyme is stable and active under a wide range of conditions from pH 4 to 10 and at temperatures up to 80°C. It retains its activity in 8 M urea (Ménard and Storer, 2005).

1.3.1.3 Aspartic Proteases

Aspartic and metallopeptidases differ importantly from serine and cysteine peptidases in that the nucleophile that attacks the scissile peptide bond is an activated water molecule rather than the nucleophilc side chain of an amino acid. Aspartic peptidases are so named because aspartate residues are ligands of the activated water molecule. In most of the aspartic peptidases a pair of aspartic residues act together to bind and activate the catalytic water molecule, but in some, residues of amino acids replace the second aspartate. All the aspartic peptidases described so far are endopeptidases and are characterized by their acidic pH optima. There are 5 clans of aspartic peptidases (Rawlings and Barrett, 2005c).

The most widely known aspartic peptidase is the pepsin. It is the principle protease of the stomach. Pepsin is an endoprotease with broad specificity. The pH range of peptide hydrolysis is from below 1 to about 6 (James, 2005).

1.3.1.4 Metalloproteases

Metalloproteases are peptidases in which nucleophilic attack on a peptide bond is mediated by a water molecule, as in the case of aspartic peptidases, but in the metallopeptidases a divalent metal cation, usually zinc but some times cobalt, manganese, nickel or copper, activates the water molecule. The metal ion is held in place by amino acid ligands, usually three in number. Metallopeptidases can be divided into two broad groups depending on the number of metal ions required for catalysis. In many metallopeptidases, only one zinc ion is required, but in some families there are two metal ions that act together or co-catalytically. All metallopeptidases in which cobalt or manganese is

essential require two metal ions, but there are also families of zinc-dependant metallopeptidases in which two zinc ions are co-catalytic. All the peptidases containing nickel require only one ion. In peptidases with co-catalytic metal ions, only five amino acid residues (rather than six) act as ligands, and one of them ligates both metal ions. Metallopeptidases with co-catalytic metal ions are exopeptidases, whereas metallopeptidases with one catalytic metal ion may be exopeptidase or endopeptidase. The known metal ligands in metallopeptidases are histidine, glutamate, aspartate or lysine. Besides the metal ligands, at least one other residue is required for catalysis. This is glutamate in many metallopeptidases, but lysine and arginine have been implicated in the activity of leucine aminopeptidase (Rawlings and Barrett, 2005d).

Leucine aminopeptidase, carboxypeptidase A and carboxypeptidase B are some members of metallopeptisases.

Leucine aminopeptidase (E.C. 3.4.11.1) is a zinc-dependent metallopeptidase that require two zinc ions acting co-catalytically. It prefers substrates with leucine residue at the N-terminal position, but may also hydrolyze peptides with different N-terminal L-amino acid residues. Peptides having proline after N-terminal residue are not cleaved. Leucine aminopeptidases were identified in animals, plants and bacteria (Sträter and Lipscomb, 2005; Walling, 2005).

Carboxypeptidase A (E.C. 3.4.17.1) prefers peptide and protein substrates with an aromatic or branched aliphatic (phenylalanine, tyrosine, tryptophan, leucine or isoleucine) C-terminall amino acid. It requires two zinc ions acting co-catalytically. Histidine or glutamate residues ligands to the zinc. It is secreted in an inactive form named procarboxypeptidase (Auld, 2005).

Carboxypeptidase B (E.C. 3.4.17.2) is highly specific for excising C-terminal lysine or arginine residues from peptides and proteins with a preference for arginine. It also acts, at a slower rate, on C-terminal valine, leucine, isoleucine, asparagine, glycine or glutamine. It is secreted as zymogen called procarboxypeptidase B which is activated upon trypsin digestion (Avilés and Vendrell, 2005).

1.3.2 Amylases

Amylases are enzymes that hydrolyze starch, the main carbohydrate supply of organisms. Starch may be found in the form of amylose (usually linear, α-1,4-glycosidic linkages of glucose residues) or amylopepctin (α-1,4 and α-1,6-linked glucose residues, highly branched, storage form). Amylases are classified according to which linkages the enzyme is capable of degrading, α-1,4 or α-1,6. Enzymes that cleave α-1,6-linked glucose units are called debranching enzymes, and have primary affinity for α-1,6 linkages present in amylopectin and related polymers. Enzymes that act on α-1,4 glycosidic linkages are α-amylases, β-amylases and glucoamylases.

1.3.2.1 α-Amylases

α-Amylase (α-1,4-glucan-4-glucanohydrolase, E.C. 3.2.1.1) catalyses the hydrolysis of α-(1,4)-glycosidic linkages of starch components, glycogen and various oligosaccharides. They are endo-acting enzymes and liberate glucose and malto-oligosaccharides in the α-anomeric form and rapidly reduce the molecular size of starch. α-Amylases are among the most widely distributed of the amylolytic enzymes. They are found in animals, plants and microorganisms.

The 3-D structures of α-amylases have revealed a monomeric, Ca-containing enzymes, with a single polypeptide chains folded into three

domains (A-C). The catalytic domain A of α-amylases fold into an $(\alpha/\beta)_8$-barrel protein. The active site is created by residues located at the C-terminus of the β strands and in the helix-connecting loops extending from these ends. Domain B, which varies most among species, protrudes from the middle of the barrel at the C-terminal side and forms a small separate, Ca-stabilized structural domain. The open active cleft is formed between domains A and B, so that residues from domain B also participate in substrate binding. The domain C, which is relatively conserved, folds into an antiparallel β-barrel. The orientation of domain C relative to domain A varies depending on the type and source of α-amylase (Brayer *et al.,* 1995). The function of this domain is not clearly known, however, it is reported that truncation of domain C more than 30 to 40 residues from C-terminus is deleterious to the proper folding of the α-amylase (Vihinen *et al.,* 1994, Franco *et al.*, 2002).

Different forms of α-amylases in insects has been observed (Franco *et al*., 2002). Their patterns vary depending on the different diets that insects feed on, and the presence of antimetabolic proteins like α-amylase inhibitors. Although the sequence of several insect α-amylases are known, the three-dimensional structure of only one α-amylase is resolved, which is the α-amylase from *T. molitor* (Buonocore *et al*., 1976). The structure of the enzyme is similar to other α-amylases. It is composed of a single polypeptide chain of 471 amino-acid residues, one calcium ion, one chloride ion and 261 water molecules. Similar to other α-amylases, *T. molitor* α-amylase is composed of 3 domains, domain A, B and C. Domain A contains the catalytic site and ligand binding and form a $(\beta/\alpha)_8$-barrel. Domain B is globular and inserted into domain A. It is formed by several extended segments and a short α-helix. This domains forms a cavity against the β-barrel of domain A in which the calcium ion is bound. Domain C is located opposite to domain B on the other side of domain A. The C domain comprises C terminal residues

380-471 and forms a separated folding unit, exclusively made of β-sheet.

1.3.2.2 β-Amylases

The naming of α-amylases and β-amylases is based on the configuration of product released. When the hydrolysis products formed are in α-D-configuration the enzymes are called α-amylases. Whereas β-amylases release products with β-D-configuration. α-Amylases are endoenzymes, whereas β-amylases are exo-enzymes acting from the non-reducing ends. Moreover, α-amylases can sometimes catalyse hydrolysis of α-1,6-glycosidic linkages in a low catalytic rate, while β-amylases cannot. β-Amylases cleave β-maltose from non-reducing ends of α-1,4-linked starch and malto-oligosaccharides in an exo-fashion. The enzyme is produced by higher plants and microorganisms.

β-Amylases (α-1,4-D-glucan maltohydrolase, E.C. 3.2.1.2) are usually tetrameric proteins composed of identical subunits. The monomeric enzymes are composed of a large core which folds into an $(\alpha/\beta)_8$-barrel, a smaller lobe formed of three long loops extending from the C-terminal end of the β-barrel, and a long C-terminal loop of 50 – 60 amino acids. The active site pocket on the protein surface involves residues from both lobe and core parts. One of the loops in the lobe region is flexible and moves during substrate binding (Mikami *et al.*, 1993, 1994).

1.4 Hydrolytic Enzyme Inhibitors

1.4.1 Protease Inhibitors

Protease inhibitors comprise a large and diverse group of plant proteins capable of forming reversible protein-protein complexes with enzymes resulting in their inactivation.

Protease inhibitors are classified according to the protease that they specifically inhibit. There are four protease inhibitor families: serine protease inhibitors, cysteine protease inhibitors, aspartic protease inhibitors and metalloprotease inhibitors. Serine protease inhibitors, the most diverse family of protease inhibitors, include soybean trypsin inhibitor family of Kunitz type, Bowman Birk Family, barley trypsin inhibitor family, potato inhibitor I family, potato inhibitor II family, squash inhibitor family, Ragi I-2/maize trypsin inhibitor family and serpin family (Koiwa *et al*., 1997). Cysteine protease inhibitors, also known as cystatins, are a superfamily of tight and reversibly binding inhibitors of papain-like cysteine proteases. The cystatin superfamily has been subdivided into three families; i) stefins, ii) cystatins and iii) kininogens, based on their sequence homology, the presence and position of intrachain disulfide bonds, and the molecular mass of the protein (Margins *et al*., 1998). Plant cystatins are known as phytocystatins, have been characterized from several plants; cowpea, potato, cabbage, ragweed, carrot, papya, apple, avocado, sunflower, rice, wheat, maize, soybean and sugarcane (Fan and Wu, 2005). Aspartic protease inhibitors are relatively rare in plants. There are two families of metalloprotease inhibitors, the metallo-carboxypeptidase inhibitor family in potato and tomato plants and cathepsin D inhibitor family in potatoes (Lawrence and Koundal, 2002).

All serine protease inhibitor families from plants are competitive inhibitors and all of them inhibit proteases with a similar standard mechanism (Laskowski and Kato, 1980). The inhibitor binds to the active site on the enzyme to form a complex with a very low dissociation constant (10^7 to 10^{14} at neutral pH values), thus effectively blocking the active site. A binding loop on the inhibitor, usually "locked" into conformation by a disulfide bond, projects from the surface of the molecule and contains a peptide bond (reactive site) cleavable by the

enzyme. This peptide bond may be cleaved in the enzyme inhibitor complex, but cleavage does not affect the interaction, so that a hydrolyzed inhibitor molecule is bound similar to an unhydrolyzed one. The inhibitor thus directly mimics a normal substrate for the enzyme, but does not allow the normal enzyme mechanism of peptide bond cleavage to proceed to completion *i.e*., dissociation of the product (Walker *et al*., 1998, Lawrence and Koundal, 2002).

Proteinase inhibitors are considered to be endogenous protease regulators, storage proteins, and plant defense agents blocking the insect, microbial and other consumer proteases (Mosolov *et al.,* 2001). Protease inhibitors regulate endogenous protease levels before and during seed germination for storage protein digestion and control of protein turnover. They also protect seed reserves from premature hydrolysis. The concentration of inhibitors is reduced during germination, facilitating the hydrolysis of protein for utilization in germination process (Ussuf *et al*., 2001). Protease inhibitor gene expression increases in response to wounding, suggesting their role in protecting plants from insect attack and microbial infection. During seed development, protease inhibitors accumulate relatively late, and rapidly increase in the desiccation phase, implying a role in protein stabilization. Dehydration-related stresses such as drought, salinity and abscisic acid induce the expression of trypsin inhibitors in developing seeds (reviewed in Ussuf *et al*., 2001). In some plants, an induction of a 22 kDa protein homologous to soybean trypsin inhibitor (SBTI) was observed under high salinity drought and conditions (Reviron *et al*., 1992). The mRNA levels of a Bowman-Birk inhibitor from pea roots was found to increase under drought conditions (Welham *et al.,* 1998). A new role for protease inhibitors in the modulation of apoptosis or programmed cell death has been identified in soybean (Kosslak *et al*., 1997). Cysteine protease plays an important role in the regulation of programmed cell death leading to hypersensitive reaction, following pathogen attack. It has

been shown that ectopic expression of cystatin (cysteine protease inhibitor) inhibits the induced cysteine protease activity, which in turn blocks programmed cell death. Enzymatic and signal transduction activities of protease inhibitors were also observed. As an example to enzymatic activity, SBTI in reduced (thiol) form was found to reduce dehydroascorbate, whereas, it acquires a trypsin inhibitor activity in the oxidized (disulfide) form (Trumper *et al*., 1994). A 20 kDa protein isolated from soybean was found to have both GTP-binding activity and trypsin inhibitory activity, suggesting a role of protease inhibitors in signal transduction (Hirata *et al.*, 1999).

Protease inhibitors having both protease and α-amylase inhibitory activity are known as bifunctional inhibitors. A bifunctional inhibitor was first isolated from ragi seeds (Indian finger millet, *Elausine coracana* Gaertn) in 1981 (Shivaraj and Pattabiraman, 1981). The protein forms tertiary complexes consisting of an inhibitor, trypsin and α-amylase. It has two independent binding sites for both enzymes at the opposite sites of the inhibitor globule. The primary structure of the protein was found homologous to that of the α-amylase inhibitor from wheat and trypsin inhibitor from barley and corn endosperm. All these proteins belong to the family of cereal α-amylase and trypsin inhibitors. Kernel proteins capable of suppressing both subtilisin and endogenous α-amylase comprise an important group of bifunctional inhibitors. Similar proteins were isolated from barley (Mundy *et al*., 1983), wheat (Mundy *et al*., 1984), rye (Mosolov and Shul'gin, 1986), and rice (Ohtsubo and Richardson, 1992). All are structurally related to the Kunitz soybean trypsin inhibitor (SBTI). The inhibitor was suggested to play a role in *in vivo* regulation of germination α-amylase (α-amylase II) (Weselake *et al*., 1983) and in protection of plant from phytopathogens by suppressing the microbial subtilisin-like serine proteases (Mosolov and Shul'gin, 1986). It has two independent binding sites for protease and α-amylase.

1.4.2 α-Amylase Inhibitors

Proteins that inhibit α-amylases have been isolated from plants and microorganisms. These inhibitors can have natural roles in the control of endogenous α-amylase activity or in defense against pathogens and pests. Plant seeds are rich sources of α-amylase inhibitors. The activity of these inhibitors against insect α-amylases brings about the aspects of transgenic protection of plants against insect predators, especially for those that feed on starchy seeds and depends on their α-amylase for survival.

Six types of proteinaceous α-amylase inhibitors are found in nature as classified by their tertiary structures. These are lectin-like, knottin-like, cereal type, Kunitz-like, *γ*-purothionin-like and thaumatin-like.

Lectin-like α-amylase inhibitors include αAI1 and αAI2 from common, white and black kidney beans. These two allelic forms have different inhibition specificities (Grossi de Sa *et al.*, 1997). Both inhibitors have a heterotetrameric structure ($\alpha_2\beta_2$), and are highly glycosilated at their mature form (Nakaguchi *et al.,* 1997). Formation of inhibitor-enzyme complex is dependent on pH, time and inhibitor concentration. Structural analysis for the elucidation of inhibitory mechanism of α-amylase inhibitors demonstrated that two hairpin loops of α-AI1 were inserted in to the active site of α-amylase blocking the substrate binding and establishing a hydrogen bond network with the residues of the active site (Bompard-Gilles *et al.*, 1996; Nahoum *et al*., 1998).

Knottin type α-amylase inhibitors isolated from *Amaranthus hypocondracus* seeds (AAI) are the smallest proteinaceous α-amylase inhibitors yet described, with 32 residues and three disulfide bonds (Chagolla-López *et al*., 1994). AAI specifically inhibits insect α-amylases

and is inactive against mammalian α-amylases (Pereira *et al*., 1999). Inhibition is through the blockage of active site, similar to the lectin-like inhibitors. The specific activity of AAI against insect α-amylases makes it an attractive candidate for the development of insect-resistant transgenic plants.

Cereal type α-amylase inhibitors are composed of 120-160 amino acid residues forming five disulfide bonds (Buonocore *et al.,*1977). These inhibitors are also known as sensitizing agents in humans upon repeated exposure, causing allergy, dermatitis and baker's asthma associated with cereal flour (Garcia-Casado *et al.,* 1996). Wheat α-amylase inhibitor coded 0.19 (named according to the electrophoretic mobility relative to bromophenol blue) and bifunctional inhibitor from Indian finger millet RBI are the most studied inhibitors of this type. Wheat α-amylase inhibitor 0.19 inhibits α-amylases from insects, birds, Bacilli and mammals. It is composed of 124 amino acids and acts as homodimers, while most of the other α-amylase inhibitors of this family act as monomers (Franco *et al*., 2000). The dimeric α-amylase inhibitor 0.19 contains five α-helices arranged in up and down manner with all 10 cysteine residues forming disulfide bonds (Oda *et al*., 1997). Bifunctional inhibitor from Indian finger millet RBI is another member of cereal inhibitor family. It is monomeric and composed of 122 amino acid residues with five disulfide bonds. Its three dimensional structure is very similar to 0.19 inhibitor, with a globular fold with four α-helices in a simple up-and-down topology and a small antiparallel β-sheet. It can competitively inhibit a variety of amylases. The inhibitor directly targets the enzyme active site of the enzyme, form hydrogen bonds with catalytic residues and prevent substrate binding (Strobl *et al.,* 1995; Oda *et al*., 1997).

Kunitz-like α-amylase inhibitors contain around 180 residues and four cysteines. They are present in cereals such as barley, wheat and rice

(Franco *et al*., 2002). The best-characterized α-amylase inhibitor from Kunitz class is the barley α-amylase/subtilisin inhibitor (BASI), a bifunctional double headed inhibitor with a fast tight inhibitory reaction with cereal α-amylase and serine proteinases of subtilisin family. The mechanism of inhibition is different from the cereal, knottin and lectin-like α-amylase inhibitors in that the inhibitor does not interact directly with any catalytic residues of the enzyme, as revealed by inhibition of barley α-amylase 2 (AMY2) by BASI (Vallé *et al*., 1998). Nevertheless, this inhibitor interacts strongly with both the A and B domains near the catalytic site, through the formation of 12 hydrogen bonds, two salt bridges and multiple van der Waal's contacts, and thereby prevents the substrate access. A cavity at the enzyme-inhibitor interface contains a trapped calcium ion whose presence is suggested to electrostatically enhance the network of water molecules at the complex interface and thereby raises the stability of the complex. BASI is involved in regulating the degradation of seed carbohydrate, preventing the endogenous α-amylase 2 from hydrolyzing starch during premature sprouting (Kadziola *et al.,* 1998). Additionally, it protects the seeds against exogenous proteinases and α-amylases produced by various pathogens and pests (Mundy *et al*., 1983). BASI inhibits AMY2 with a 1:1 stoichiometry, however, it cannot inhibit barley amylase 1 (AMY1), which bears 74% sequence identity to AMY2 (Garcia-Olmedo *et al*., 1992). Homologous proteins with 92% and 58% sequence identity are present in wheat (WASI) (Mundy *et al*., 1984) and rice (RASI) (Ohtsubo and Richardson, 1992), respectively.

Thaumatin-like α-amylase inhibitors are proteins with molecular masses around 22 kDa with significant sequence similarity to pathogenesis related group-5 (PR-5) proteins and to thaumatin, an intensively sweet protein from *Thaumatococcus danielli* fruit (Vigers *et al*., 1991). The best characterized inhibitor from this class is zeamatin, a bifunctional inhibitor from *Zea mays* that is homologous to sweet protein thaumatin.

Zeamatin has a total of 13 β strands, 11 of which form a β sandwich at the core of the protein. Several loops extend from this inhibitor core and secured by one or more of the eight disulfide bonds. The surface of the protein is heavily populated with Arg and Lys residues (Batalia *et al.,* 1996). Zeamatin is able to inhibit porcine pancreatic trypsin and digestive α-amylases of several insects (Blanco-Labra and Iturbe-Chinas, 1980; Schimoler-O'Rouke *et al.*, 2001). Other proteins from this class, such as thaumatin-like proteins R and S from barley seeds, did not show any inhibitory activity against trypsin or α-amylases despite their highly similar N-terminal sequences. Zeamatin is mainly known for its antifungal activity, but this is not related to inhibition of hydrolytic enzymes as this protein does not inhibit fungal α-amylases and fungi do not contain trypsin (Blanco-Labra and Iturbe-Chinas, 1980). Zeamatin binds to β-1,3-glucans and permeabilizes fungal cells leading to cell death (Abad *et al*., 1996).

γ-Purothionins-like α-amylase inhibitors contain 47 or 48 residues, are sulfur-rich and form part of the *γ*-thionin superfamily. Members of this superfamily are involved in plant defenses through a variety of mechanisms: modification of membrane permeability (Castro *et al*., 1996), inhibition of protein synthesis (Mendez *et al*., 1996) and proteinase inhibition (Wijaya *et al*., 2000). Inhibition of insect α-amylases has been observed by three isoforms from *Sorghum bicolor* called SIα-1, SIα-2 and SIα-3 (Bloch and Richardson, 1991). The structure of SIα-1 has been resolved by NMR revealing a α + β sandwich structure with nine residues helix packed tightly against the sheet. The helix is held in place by two disulfide bridges, which link sequential turns of the helix to residues in the middle of strand β3, so-called cysteine-stabilized helix motif (Bloch *et al*., 1998).

There are also nonproteinaceous α-amylase inhibitors which are diverse types of organic compounds like acarbose, isoacarbose, acarviosine-

glucose, hibiscus acid and cyclodextrins (Franco *et al.,* 2002). The inhibitory activity of these compounds against α-amylases is due in part to their cyclic structures, which resemble α-amylase substrates and therefore bind to α-amylase catalytic sites. The nonproteinaceous α-amylase inhibitors can be used in the field of medicine, both for treatment and in diagnostic procedures (O'Donnell *et al*., 1997). Nevertheless, the use of nonproteinaceous inhibitors for production of insect resistant transgenic plants is much more difficult, as several metabolic pathways are involved in their synthesis, and hence, the presence of multiple expressed transgenes would be required in order to confer protection.

1.5 Approaches for Developing Genetically Engineered Plants Resistant to Insects

Attack of variety of pests to crops costs losses about 100 billon dollars annually, all over the world. Methods available today for protecting plant crops against insect predation are heavily dependent on environmentally aggressive chemicals and were estimated to reduce losses in only about 7% (Oerke *et al*., 1994). Despite extensive plant breeding efforts, over $10 billion are spent worldwide each year on the management and chemical control of insect damage (Khush, 1999). This fact justifies the necessity for research and development of alternative approaches to this problem. Since farmlands are finite, the output of agriculture must raise concomitant to the increase of world population. Until recently, pest resistant varieties have been developed through the application of classical Mendelian genetics and conventional plant breeding methods. Recent advances in cellular and molecular biology have opened new avenues for production of genetically engineered (transgenic) plants with new genetic properties. The first report of this new agriculture was established about 20 years ago with the introduction into tobacco plant of the genetic material coding for an entomotoxic protein from

bacterium *Bacillus thruingiensis* (Andrews *et al*., 1987). Nowadays a number of *Bt*-containing products are in the market in United States (www.aphids.usda.gov) as well as in some other countries. However, there are some concerns regarding the biosafety of *Bt* proteins for mammals and ecological impact of this new class of bioinsecticides. An alternative strategy could be to take advantage of plant's own defense mechanisms, for example, by manipulating the expression of their endogenous defense proteins, or by introduction of an insect control gene derived from another plant.

1.5.1 *Bacillus thruingiensis* (*Bt*) Genes

Bacillus thruingiensis, commonly known as *Bt*, is a bacterium that occurs naturally in the soil. It has been used for more than 50 years as a biological insecticide. It produces a number of insect toxins, the most distinctive of which are protein crystals formed during sporulation. The crystal proteins are specifically toxic to Lepidopteran insects (Olsen and Daly, 2000) and act by disrupting the midgut cells of the insect. The crystal proteins are dissolved by alkaline gut juices in the midgut lumen, and are converted by gut proteases into toxic core fragments. Thereafter the midgut epithelia cells swell and eventually burst. This cytolysis leads to gut paralysis, cessation of feeding and finally (typically after 1-3 days) death from starvation and/or septicaemia.

140 genes coding for *Bt* toxins have been described and classified into 24 major groups, and 4 classes based on host range. These include *cry I* (active against Lepidoptera), *cry II* (Lepidoptera and Diptera), *cry III* (Coleptera), *cry IV* (Diptera). Transgenic plant carrying *Bt* genes have been produced in tobacco, potato, tomato, cotton, maize and rice with different crystal protein genes (Babu *et al.,* 2003). The critical factor following transformation is the desired expression of the insecticidal gene to provide adequate protection from the major pest species in the

field. Substantial increase in expression levels were required and these have been achieved through the use of strong promoters and enhancers and by engineering the codon usage to bring it more into line with plant-preferred codon usage, rather than the A+T rich *Bacillus* preferred usage, and to eliminate undesirable mRNA secondary structure and polyadenylation signals. *Bt* genes now in use for plant genetic engineering have been substantially modified with expression levels in transgenics much higher (100X) than was obtainable using *Bt* genes (Hilder and Boulter, 1999).

The high degree of specificity of Bts is often cited as one of the benefits of their use over synthetic pesticides. However most crops are not subjected to attack by a single pest species but rather by an entire complex of different pests, which are not always susceptible to Bts. For this reason there is a need to identify insect control genes for these unsusceptible pests (Hilder and Boulter, 1999). The specificity of Bt is such that it was expected to have no direct effects on predator populations, but some reports suggests that there may be a reduction in fitness of predatory chrysopid larvae preying on Bt-maize fed caterpillars. Any direct impact of transgenic Bt on predator population is likely to be much less marked than would be the case with sprayed insecticides; nevertheless such tritrophic interactions may be yet another area requiring careful management in the deployment of Bt transgenic crops (Hilder and Boulter, 1999).

Development of resistance to Bt is another problem. The widespread use of Bt transgenics is likely to exert so much selection pressure for resistant insects that this valuable control measure will be lost to them. Various resistance management strategies have been proposed for transgenics, including the use of more than a single resistance factor and provision of special and temporal refugia to ensure survival of susceptible genotypes, although there is still no consensus for the

optimal strategy and further field experience is required (Hilder and Boulter, 1999).

1.5.2 Plant Toxic Proteins with Insecticidal Properties

Plants have evolved sophisticated defense mechanisms most of which are concentrated in the seeds since these are the vehicles for propagation and survival of the species. The best known plant proteins supposed to involved in defense mechanisms are lectins, ribosome-inactivating proteins (RIPs), proteinase inhibitors, α-amylase inhibitors, arcelins, chitinases, canatoxin and modified forms of storage proteins. However the co-evolution of plants and their predators has lead to the adaptation of insects to inhibitors or other deterrent compounds present in the plants they feed on. The same inhibitors are on the other hand very efficient in blocking enzymes from other insects that do not feed on that plant (Chrispeels, 1996).

1.5.2.1 Lectins

Lectins are class of proteins of non-immune origin that possess at least one non-catalytic domain that specifically and reversibly binds to mono- or oligosaccharide. Seeds, particularly those of Leguminoseae, are rich sources of lectins. Various plant lectins have shown entomotoxic effects when fed to insects from Coleoptera, Homoptera and Lepidoptera orders. It has been demonstrated that some lectins bind to brush-border membrane of the insect's intestinal epithelial cells or, in the case of chitin-binding lectins, to the peritrophic membrane. Other possible toxic effect includes binding of lectins to glycosylated digestive enzymes. However, the precise mechanism of action of lectins in insects is still unknown. Genes coding for entomotoxic lectins have been introduced into different crops rendering the transgenic plants less susceptiple to the attack of insects. However, some lectins are highly toxic when

ingested by mammals, surviving gastrointestinal passage, and inducing a variety of systemic effects. Some lectins have also a potential negative tritrophic effects upon beneficial predatory insects feeding on the intoxicated pests. Thus the application of most lectins, known so far, to display entomotoxic activity for engineering insect resistant plants is largely restricted by their toxicity to mammals and/or non-target insects (Carlini and Grossi-de-Sá, 2002).

Lectins such as those purified from snowdrop or garlic are toxic to insects but not to mammals (Sharma *et.al.,* 2000). The most effective protein tested is lectin from snowdrop (*Galanthus nivalis agglutinin*; GNA), which gave approximately 80% mortality at a concentration of 1 g/l in the diet, when used in assays with first and third instar nymphs. GNA also had antimetabolic effect on brown plant hopper and green leafhopper pests of rice (Powell *et al.,* 1993). Rao *et al.* (1998) introduced snowdrop lectin gene (*gna*) to rice. GNA expressed transgenic plants showed decreased survival and fecundity of insects and retarded insect development. Effect of GNA on the sap-sucking insects of the order Hemiptera has also been investigated. Hilder *et al.* (1995) has introduced *gna* into tobacco and the transgenics expressing this lectin showed enhanced resistance to peach potato aphid, with degree of protection being shown to correlate with level of expression of the introduced lectin. However, transgenic potatoes expressing GNA resulted in reduced viability of beneficial, predatory ladybirds when fed on aphids from the engineered plants (Birch *et al.,*1999).

1.5.2.2 Ribosome Inactivating Proteins (RIPs)

Two ribosome inactivating proteins, ricin and abrin have been identified in plant seeds. They have been shown to inhibit protein synthesis in eukaryotic cells through inactivation of 60S ribosomal subunit. These toxic proteins exert their effects intracellularly and composed of two

domains with distinct biological properties. The haptomer or B- (binding) domain recognizes the target cell by interacting with a specific carbohydrate or glycoderivative present on the cell surface. The B-chain of plant toxic proteins known so far binds to D-galactose or *N*-acetyl-galactosamine residues. The effectomer or A activity subunit, is the toxic agent acting as an enzyme. The event that precede the toxic effects of these proteins are: (1) binding of the B-subunit to a receptor on the cell surface triggers receptor-mediated internalization of the holotoxin; (2) once inside the endocytic compartment, the protein may be processed by limited proteolysis and/or reduction of thiol groups and/or conformational changes due to acidification of the endocytic vesicles; (3) separation of the two domains of the toxin occurs, rendering the effectomer enzymatically active; (4) the effectomer domain then inserts itself into the vesicle membrane, reaching the cytoplasm; (5) the final toxic effect is dependent on the enzymatic action of the effectomer upon a cytoplasmic component (Carlini and Grossi-de-Sá, 2002). The ricin A-chain (30 kDa) and the effectomer chains of most plant toxic proteins specifically cleave *N*-glycosidic bond at adenosine 4324 of the eucaryotic 28S ribosomal RNA, thereby interrupting protein synthesis and leading to cell death. They also display polynucleotide: adenosine glycosidase activity upon DNA or RNA, which contribute to the toxic effect (Carlini and Grossi-de-Sá, 2002).

Ribosome inactivating proteins were isolated from various plant sources and vast majority of them are single chain proteins. According to the source, ribosomes show differential sensitivity to RIPs. The effect of RIPs in plant cells and isolated ribosomes has been poorly studied, while ribosomes of protozoans and fungi seem to be highly sensitive. Ricin was shown to be ineffective to a variety of insects of different orders, although it was able to inhibit protein synthesis by insect ribosomes in cell-free preparations. These results suggest that insects surviving a diet

containing ricin probably inactivate the protein in their digestive tract or do not bind the hemilectin (Carlini and Grossi-de-Sá, 2002).

Mainly due to their toxicity to mammals, these entomotoxic proteins are of little value for the development of insect resistant plants.

1.5.2.3 Proteinase Inhibitors (PIs)

Proteolytic enzymes catalyze the cleavage of peptide bonds in proteins. They are classified according to their mechanism of catalysis and the amino acid present in the active center: (1) Serine proteinases, with serine and histidine; (2) Cysteine proteinases, with cysteine; (3) Aspartic proteinases, with aspartate group and (4) metalloproteinases, with metallic ion (Zn^{2+}, Ca^{2+} or Mn^{2+}). Proteinase inhibitors have been described in animals, plants and microorganisms and have been extensively studied in order to elucidate their structural and functional properties.

In plants different roles for proteinase inhibitors have been suggested, including their action on storage proteins, as regulators of endogenous proteolytic activity; as participants in many developmental processes, including programmed cell death; and as components associated with resistance of plants against insects and pathogens. They may be synthesized constitutively during normal development or may be induced in response to insect and pathogen attacks (Koiwa *et al*., 1997; Carlini and Grossi-de-Sá, 2002).

Proteinaceous protease inhibitors (PIs) are generally categorized according to the class of protease that they inhibit. Plant serine protease inhibitors fall into a number of structurally distinct families based on their amino acid sequences. Bowman-Birk type inhibitors are small polypeptides (8 kDa), typically found in legume seeds. They are double

headed, binding simultaneous and independently to two separate proteinase molecules, such as trypsin and chymotrypsin (Bode and Huber, 1992). Kunitz-type inhibitors are 20 kDa proteins, usually made of one polypeptide chain or two disulphide-linked chains, with an arginine residue in their single reactive site. In general these inhibitory proteins behave as pseudo-substrates, with the amino acid at position P1 of the inhibitor determining the specificity for the enzyme, either trypsin or chymotrypsin (Bode and Huber, 1992). Some plant serine proteinase inhibitors are bifunctional molecules, being able to inhibit trypsin as well as α-amylase. Plant cysteine proteinase inhibitors are known as phytocystatins (5-87 kDa). They have been identified in a variety of monocot and dicot species, such as maize, rice, potato, soybean and apple. One group of phytocystatins contains a single domain and comprises the majority of phytocystatins (Pernas *et al.*, 1998), whereas a second group has multiple domains, such as the multicystatins found in potato tubers, tomato leaves and sunflower seeds. Some phytocystatins show homology with serine proteinase inhibitors that belong to Kunitz-type trypsin inhibitor family. The phytocystatins differ largely from animal cystatins by displaying high inhibitory activity towards insect gut proteinases, making them attractive as biological control agents of insect pests.

Disruption of pests' essential amino acid metabolism by inhibition of protein digestion has been a key target. Many insects, particularly members of the Lepidoptera, depend on serine proteinases (trypsin, chymotrypsin and elastase-like endoproteinases) as their primary protein digestive enzymes and the genes encoding various different serine proteinase inhibitors have been cloned and introduced into transgenic plants. Other pests rely on cysteine proteinases rather than serine proteinases as their primary digestive proteinase (Hilder and Boulter, 1999). DNA sequences encoding different proteinase inhibitors have been incorporated into the genome of different important plants

such as rice (Lee *et al*., 1999, Duan *et al.*, 1996), wheat (Altpeter *et al.*, 1998), tobacco (Masoud *et al.*, 1993) and potato (Kregar and Strukelj, 1999) and protective effects have been obtained in some cases, mainly against phytonematodes and Lepidopteran pests.

In spite of these effects, the use of protease inhibitors in plant protection has some limitations. These may include the degradation of the proteinaceous inhibitors by non-target proteinases and development of a complex proteolytic system through which they overcome the effects of inhibitors due to an upregulation of other proteinases that are not inhibited by them. Moreover, adaptation of insect pests to plants over-expressing proteinase inhibitors was demonstrated in many reports. Some species have ability to compensate for proteinase inhibition by switching to an alternative proteolytic activity (Jongsma *et al.*, 1995).

1.5.2.4 α-Amylase Inhibitors (αAIs)

α-Amylases (α-1,4-glucan-4-glucanohydrolases) are widespread hydrolytic enzymes found in microorganisms, animals and plants. They catalyze the initial hydrolysis of α-1.4-linked sugar polymers, such as starch and glycogen, in shorter oligosaccharides, an important step towards transforming sugar polymers into single units that can be assimilated by the organisms. These widely distributed molecules are the most important digestive enzymes of many insects that feed exclusively on seed products during larval and/or adult life. When the action of amylases is inhibited, nutrition of the organism is impaired causing shortness of energy.

In much the same way that pests' protein metabolism has been targeted with genes encoding proteinase inhibitors, so their carbohydrate metabolism has been targeted with α-amylase inhibitors. α-Amylase

inhibitors occur in many plants as part of natural defense mechanisms. They are particularly abundant in cereals and legumes. Particular attention has been focused on the lectin-like inhibitors present in common bean, *Phaseolus vulgaris,* seeds, which has been shown to have toxic effects to several insect pests (Ishimoto *et al.*, 1996, Fory *et al.*, 1996, Chrispeels, 1996). Complete resistance against bruchids, pea weevil and azuki bean weevil was found in transgenic pea and azuki bean seeds expressing the inhibitor, αAI-1, of the *P. vulgaris* (Ishimoto *et al.*, 1996, Morton *et al.*, 2000, Shade *et al.*, 1994).

In *Phaseolus* seeds, αAI is a member of protein family that includes two other defense proteins, the phytohemagglutinin (PHA) and arcelins (Arc). The members of these plant defense proteins are encoded by tightly linked genes in the *P. vulgaris* genome and their deduced amino acid sequence are highly homologous (45-85% identical) (Chrispeels and Raikhel, 1991). Therefore, it is likely that these genes arose by dublication of a single ancestral gene (Nodari *et al.*, 1993). These proteins display insecticidal activities and protect seeds against different predators through different mechanisms.

Phaseolus genus contains at least four phenotypes of αAIs; αAI-1, αAI-2, αAI-3 and the null type (Suzuki and Ishimoto, 1999). They show different specificities toward different α-amylases. αAI-1, found in most cultivated common bean varieties, inhibits mammalian α-amylases and insect larval α-amylases of *C. chinensis, C. maculatus* and *B. pisorum*, but it is not active against Mexican bean weevil (*Zabrotes subfsciatus*), which is an important storage pest of the common bean (Ishimoto and Chrispeels, 1996). The second variant αAI-2, which shares 78% amino acid homology with αAI-1, is found in wild accessions of common bean and specifically inhibits the *Z. subfasciatus* larval α-amylase (Ishimoto and Kitamura, 1993).

1.5.2.5 Arcelins

Arcelins are insecticidal proteins, found in some wild accessions of the common bean, which have shown to confer resistance against bruchid beetles. As mentioned earlier, amino acid sequence comparison shows that arcelins belongs to the bean lectin-like family which includes the two types of phytohemagglutinin subunits (PHA-L and PHA-E) and α-amylase inhibitors. Seven different allelic forms of arcelins have been defined with molecular weight in range of 27-42 kDa (Carlini and Grossi-de-Sá, 2002). Arc-1 and Arc-5 are the ones conferring the highest resistance levels to the bean bruchid pests (Cardona *et al.*, 1990). Despite the sequence homology with lectins, arcelins are themselves weak lectins. Their sugar binding specificity significantly differs from those of PHA-L and PHA-E. The mechanism of action of arcelins is not clear. The toxic properties of arcelins may be related to their interaction with glycoproteins or other constituents in the midgut. If arcelins behave as lectins, then two types of interactions could be possible: binding of arcelins to glycoconjugates exposed on the epithelial cells along the insect digestive tract, or to glycosylated digestive enzymes. In recent experiments it was observed the arcelin-1 disrupts the epithelial structure in some regions on the midgut of *Z. subfasciatus*, but not in the midgut of *A. obtectus* larvae (Paes *et al*., 2000).

1.5.2.6 Canatoxin-like Proteins and Ureases

Canatoxin was first identified in jackbeans as a potent neurotoxic protein (Carlini and Guimãraes, 1981). Canatoxin behaves as a non-covalently linked dimmer of a 95 kDa polypeptide chain, accounting for 0.5% of the seed dry weight. The toxin injected intraperitoneally induces convulsions and death in mice and rats; however it is inactive if given orally to the animals, perhaps due to its instability at low pH (Carlini and Guimãraes, 1991). Canatoxin interacts with complex glycoconjugates such as

gangliosides, in particular those that are polysialylated, resembling the bacterial neurotoxins, tetanus and botulinum.

Canatoxins have striking homology with urease present in the same seed. Canatoxin has about 30% of urease activity. Some biological properties of canatoxin are also displayed by urease, including lethality for insects.

The widespread presence of canatoxin-like proteins in other leguminous seeds, including edible ones, as well as accumulation pattern of the protein during seed maturation are suggestive of an important physiological role, may be related to plant defense. Canatoxin was found to be lethal to insects relying on cathepsins B and D as main digestive enzymes. Hemipteran pests, *Nezara viridula* and *Dysdercus perivianus* were also found to highly susceptible to entomotoxic effects of canatoxin. In contrast, insects with trypsin-based digestion were not affected by ingesting canatoxin (Carlini *et al.,* 1997). Ferreira-DaSilva *et al*. (2000) found that canatoxin is proteolytically activated by cathepsins A and D in the insect midgut to produce entomotoxic peptide(s). However there is no information yet as to the fate and mechanism of action of canatoxin's proteolytic fragments in the insects.

1.6 Scope of the Work

The aim of this study was to identify and characterize the proteolytic and amylolytic activities from the midguts of two important pests of Turkey, namely cotton bollworm (*Helicoverpa armigera*) and sunn pest (*Eurygaster integriceps*), for the improvement of transgenic plants using α-amylase and/or protease inhibitors active against the insect hydrolytic enzymes.

The studies conducted are outlined below:

- Identification of hydrolytic enzymes (proteases and α-amylases) from insect midgut
- Characterization of insect midgut hydrolytic enzymes in terms of their optimum pH, K_m and V_{max} values
- Inhibition studies using several synthetic and natural protease and α-amylase inhibitors to identify a potent inhibitor against insects

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical Materials

The chemicals used in this study were obtained from Sigma Chemical Company (N.Y USA) or Merck Chemical Company (Deisenhofen Deutschland). All solutions were prepared by distilled water.

2.1.2 Insect Materials

Sunn pests (*Eurygaster integriceps*) at fifth instar stage, were kindly supplied by Central Research Institutes of Crop Protection, Adana and Ankara, Turkey. They were collected from wheat fields around Southeast region of Turkey, during infestation period (summer) of the insect. Insects were dissected in the laboratory and midguts were isolated and stored in 0.15 M NaCl solution at -20 °C until use.

Cotton bollworm (*Helicoverpa armigera*) were kindly supplied by Dr. Sakine Uğurlu from Central Research Institute of Crop Protection, Ankara, Turkey. At mid-to-late instar larvae stage, the insects were

dissected and whole midgut contents were suspended in 0.15 M NaCl solution, and stored at -20 °C until use.

2.2 Methods

2.2.1 Insect Dissection and Midgut Isolation

Sunn pests were cut by a scissors from the ends of abdominal part and the midguts were taken out by a sterile forceps. Each 5 midgut sample were collected in an eppendorf tube containing 0.5 mL of 0.15 M NaCl solution.

Cotton bollworms were dissected by a blade from the abdomen and the whole midgut was taken out by a forceps. Each 5 midgut sample were collected in an eppendorf tube and suspended in 0.5 mL of 0.15 M NaCl solution. The samples were frozen by liquid N_2 and stored at -20 °C until use.

2.2.2 Homogenization of Midgut Samples

The midguts were homogenized by a glass-Teflon homogenizer in 0.15 M NaCl solution and the homogenates were centrifuged at 10.000 rpm for 5 min at 4°C using Thermo IEC minicentrifuge. The supernatants were used in the enzyme assays.

2.2.3 Protein Determination

Protein concentrations in the samples were determined by Bradford method (1976) using bovine serum albumin (BSA) (Sigma) as standard protein. Standard calibration curve was constructed by using 1 mg/mL stock solution of BSA. Dilutions were carried out in BSA solution to have 0.01 mg/mL to 0.16 mg/mL final concentrations with distilled water in a

final volume of 0.5 mL. Then, 5 mL of Bradford reagent (containing 0.01 % (w/v) Coomassie Brilliant Blue G-250, 4.7 (v/v) % ethanol, 8.5 % (w/v) phosphoric acid) was added and incubated at RT for 15 min for color formation and equilibration. The absorbance was read at 595 nm and calibration curve (OD_{595} versus mg/mL protein) was constructed. Protein concentrations in the samples were determined from the calibration curve after Bradford assay.

2.2.4 Spectrophotometric Enzyme Assays

2.2.4.1 α-Amylase Assay

Alpha-amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) activity was determined according to the method of Bernfeld (1955) using starch as a substrate. The midgut extract containing 50 µg protein was incubated with 0.25 mL of 1% (w/v) starch, prepared in 0.1 M sodium phosphate buffer pH 6.0 containing 20 mM NaCl and 0.1 mM $CaCl_2$, for 10 min at 30°C. The reaction was terminated by addition of 0.5 mL dinitrosalicilic acid reagent (1 g of 3-5 dinitrosalicilic acid dissolved in 100 mL solution containing 1.6 g of NaOH and 25 g of sodium-potassium tartarate.). The mixture was incubated in boiling water bath for 10 min for the reaction of dinitrosalicilic acid with free reducing sugars formed after α-amylase digestion. After incubation, 5 mL of distilled water was added into the reaction mixture and incubated at room temperature for 15 min. The absorbance was read at 546 nm, using Shimadzu UV-mini 1240 spectrophotometer, against the blank solution containing all the reagents except the midgut extract.

The α-amylase activity was calculated from a maltose calibration curve. Dinitrosalicilic acid reagent was incubated with 0.25 mL of maltose solution of different concentrations (0-3.5 mM) and the reaction was then carried out as described above. The graph of optical density at 546

nm versus maltose concentration (mmoles) was drawn and the slope was used in determination of α-amylase activity as mmoles maltose formed per minute per mg protein.

Alpha-amylase activity from *Helicoverpa armigera* midgut was determined similarly, except, midgut extract containing 100 µg protein was included in the assay medium and starch (1 %) was dissolved in 0.1 M Glycine-NaOH buffer containing 20 mM NaCl and 0.1 mM $CaCl_2$.

2.2.4.2 Trypsin Assay

Trypsin (EC 3.4.21.4) activity was measured spectrophotometrically according to the method of Ortego *et al.* (1996) using BApNa (Nα-benzoyl-DL-arginine p-nitroanilide) as substrate. Sunn pest midgut extract containing 200 µg protein was incubated with 0.1 M Tris-HCl pH 8.0 and 20 µl of 50 mM BApNa (dissolved in DMSO) in a final volume of 1 mL for 20 minutes at 37 °C. The reaction was terminated by addition of 0.2 mL of 30% acetic acid and centrifuged at 10,00 g for 5 min. The optical density of the supernatant was measured at 410 nm using Shimadzu UV-mini 1240 spectrophotometer. One enzyme unit was defined as the amount of protein that catalyzed cleavage of 1 nmole of substrate per minute. Extinction coefficient of BApNa is 8,800 M^{-1} cm^{-1}. The blank contained all the reagents except the enzyme extract.

H. armigera trypsin activity was measured as described above, except, 50 µg of midgut extract was incubated with 0.1 M Glycine-NaOH buffer, pH 10.5. The enzyme activity was defined as the amount of protein that catalyzed the cleavage of 1 µmole of substrate per minute.

2.2.4.3 Chymotrypsin Assay

Chymotrypsin (EC 3.4.21.1) activity was spectrophotometrically measured according to the method of Ortego *et al*. (1996) using SA_2PPpNa (N- succinyl – alanine – alanine – proline - phenylalanine p-nitroanilide) as substrate. Sunn pest midgut extract containing 200 µg protein was incubated with 0.1 M Tris-HCl pH 8.0 for 5 min for equilibration. Reaction was started by addition of SA_2PPpNa in a final concentration of 0.125 mM. Decrease in absorbance was monitored for 3 minutes at 410 nm, in a final reaction volume of 1.5 mL. One unit of chymotrypsin activity was defined as the amount of protein that catalyzed the cleavage of 1 µmole of substrate per minute. Extinction coefficient of SA_2PPpNa is 8,800 M^{-1} cm^{-1}. The blank contained all the reagents except the enzyme extract.

Chymotrypsin activity of *H. armigera* was measured similarly, except, midgut extract containing 50 µg of protein was included in the assay medium and assay was carried out in glycine-NaOH buffer, pH 10.5.

2.2.4.4 Elastase Assay

Elastase (EC 3.4.21.37) activity was measured spectrophotometrically according to the method of Ortego *et al*. (1996) using SA_3pNa (N-succinyl-alanine-alanine-alanine p-nitroanilide) as substrate. Sunn pest midgut extract containing 200 µg protein was incubated with 0.1 M Tris-HCl pH 8.0 buffer for 5 min for equilibration. The reaction was started by addition of SA_3pNa (dissolved in DMSO) in a final concentration of 0.25 mM in 1 mL final assay volume. The reaction was run at room 30 °C for 1 hour and terminated by addition of 500 µl of 30% acetic acid. The resulting mixture was centrifuged at 10,000 g for 5 min and the supernatant was read at 410 nm using Shimadzu UV-mini 1240 spectrophotometer. Elastase activity was calculated as amount of

protein that catalyzes the cleavage of 1 µmole of substrate per minute, under defined conditions. Extinction coefficient of SA_3pNa is 8,800 M^{-1} cm^{-1}. The blank contained all the reagents except the enzyme extract.

Helicoverpa armigera midgut elastase activity was measured similarly, except, midgut extract containing 50 µg of protein was used in the assay medium and assay was carried out using 0.1 M glycine-NaOH buffer, pH 10.5.

2.2.4.5 Leucine Aminopeptidase Assay

Leucine aminopeptidase (EC 3.4.11.1) activity was measured spectrophotometrically according to the method of Ortego *et al*. (1996) using LpNa (L-leucine p-nitroanilide) as substrate. Sunn pest midgut extract containing 100 µg protein was incubated with 0.1 M Tris-HCl pH 8.0 buffer for 5 min for equilibration. The reaction was started by addition of LpNa (dissolved in DMSO) in a final concentration of 2.5 mM in final reaction volume of 1.5 mL. The decrease in absorbance at 410 nm was monitored for 3 min at 30 °C. The enzyme activity was calculated from extinction coefficient of LpNa (8,800 $M^{-1}cm^{-1}$) and one unit of enzyme activity was defined as amount of protein that catalyze the cleavage of 1 µmole of substrate per minute under defined assay conditions. The blank contained all the reactants except the enzyme solution.

The leucine aminopeptidase activity from *Helicoverpa armigera* midgut was determined similarly, except, midgut extract containing 50 µg of protein was used in the assay medium.

2.2.4.6 Carboxypeptidase-A Assay

Carboxypeptidase-A (3.4.17.1) activity was measured spectrophotometrically according to the method of Folk and Schirmer (1963) using HPA (hippuryl-phenylalanine) as substrate. Sunn pest midgut extract containing 200 µg protein was reacted with HPA solution containing 1 mM HPA in 25 mM Tris-HCl, pH 7.5 with 0.5 M NaCl in a final volume of 1 mL. The rate of hydrolysis of HPA was determined by measuring the increase in absorbance at 254 nm for 3 minutes, at 30 °C. One unit of enzyme was defined as amount of protein that catalyzes the hydrolysis of 1 µmole of HPA per minute under defined assay conditions. Extinction coefficient of hippuric acid formed during the reaction is 0.36 mM^{-1} cm^{-1}. The blank contained all the reactants except the enzyme solution. Carboxypeptidase-A activity from *Helicoverpa armigera* midgut was determined similarly.

2.2.4.7 Carboxypeptidase-B Assay

Carboxypeptidase B (3.4.17.2) activity was measured spectrophotometrically according to the method of Folk *et al*. (1960) using HA (hippuryl-L-arginine) as substrate. Sunn pest midgut extract containing 200 µg protein was reacted with 1 mM HA in 25 mM Tris-HCl pH 7.5 containing 0.1 M NaCl in a final volume of 1 mL. The rate of hydrolysis of HA was determined by meausuring the increase in absorbance at 254 nm for 3 minutes, at 30 °C. One unit of enzyme activity was defined as amount of enzyme catalyzing the hydrolysis of 1 µmole of substrate per minute under specified assay conditions. Extinction coefficient of hippuric acid formed during the reaction is 0.36 mM^{-1} cm^{-1}. Blanks contained all the reactants except the enzyme solution. Carboxypeptidase-B activity from *Helicoverpa armigera* midgut was determined similarly.

2.2.4.8 Papain Assay

Papain activity was determined spectrophotometrically according to the method of Delledone *et al.* (2001) using azoalbumin as a substrate. Sunn pest midgut extract containing 200 µg protein was incubated with 200 µl of 10% azoalbumin solution (dissolved in 0.1 M Tris-HCl pH 8.0) for 30 min at 30 °C. The reaction was terminated by addition of 960 µl of 10 % (w/v) tri-chloro-acetic acid (TCA). The resulting mixture was kept in ice for 15 min and centrifuged at 8,000 g for 3 minutes, for the sedimentation of undigested substrate. The supernatant (1 mL) was mixed with 200 µl of 3.3 M NaOH solution to intensify the color of free azo dye. The absorbance of resulting solution was measured at 440 nm and the azoalbumin hydrolyzing activity was defined as amount of enzyme that cause increase in absorbance by 0.001 units per minute per mL assay medium. The blank contained all the reactants except the enzyme solution.

Helicoverpa armigera midgut papain activity was determined similarly, except, azoalbumin used in the assay medium was dissolved in 0.1 M glycine-NaOH buffer, pH 11.0)

2.2.4.9 Total Protease Assay

Total protease activity was determined spectrophotometrically according to the method of Wilhite *et al.* (2000) using azocasein as a substrate. The assay mixture contained 100 µg of sunn pest midgut extract, 10 µl of 2.4 % triton X-100, 10 µl of 6.8 mg/mL BSA (bovine serum albumin), 50 µl of 0.1 M Tris-HCl pH 8.0, and 160 µl of 2% azocasein (dissolved in 0.1 M Tris-HCl pH 8.0). The reaction mixture was incubated at 30 °C for 1 hour and the reaction was terminated by addition of 600 µl of 10 % tri-chloro-acetic acid (TCA) solution. The resulting mixture was

incubated on ice for 10 min and centrifuged at 16,000 g for 5 min at room temperature using Thermo IEC minicentrifuge. The resulting supernatant was read at 335 nm, and enzyme activity was calculated as amount of protein that cause increase in absorbance by 0.001 units per minute.

Total protease activity from *Helicoverpa armigera* was determined similarly, except, 20 µg of enzyme extract was included in the assay medium and assay was carried out using different buffer systems at pH 8.0 to pH 11.0 (0.1 M Tris-HCl buffer pH 8.0; 0.1 M Na-PO_4 buffer pH 9.0 and pH 10.0; 0.1 M glycine-NaOH buffer, pH 11.0), at 30 °C.

2.2.5 Inhibition Assays

Natural protease and α-amylase inhibitors and synthetic protease inhibitors used in the assays were tabulated in Table 2.1. All inhibition assays were carried out by incubating the midgut extracts with inhibitors (at concentrations indicated in Table 2.1) for 10 min at 30°C before measuring the enzyme activity. Enzyme activities were measured spectrophotometrically as mentioned at 2.2.4., except, enzymes were added into the assay media together with inhibitors.

Effect of inhibitors on enzyme activities were calculated in three different ways. For all inhibitors % Inhibition was calculated from the following formula:

% Inhibition = 100 – Inhibited Activity/Uninhibited Activity*100

Table 2.1 Protease and α-amylase inhibitors used in the assays

Inhibitor	Acts on	Solvent	Conc. / Assay Medium	Targeted Enzyme(s)	Insect
PMSF phenylmethylsulfonyl floride	Serine proteases, Irreversible	EtOH	0 – 2.5 mM	All proteases, Total protease	*H.armigera*, *E. integriceps*
TPCK 1-Chloro-3-tosylamido-4-phenyl-2-butanone	Papain, Chymo-trypsin Irreversible	DMSO EtOH	0 – 100 µM	Chymotrypsin, Trypsin, Elastase, Total protease	*H.armigera*, *E. integriceps*
Chymostatin	Chymo-trypsin, Reversible, Competitive	DMSO EtOH	0 – 30 nM	Chymotrypsin	*H.armigera*
E-64 L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane	Cysteine proteases, Irreversible	DMSO EtOH	0 – 40 µM	Papain, Total protease	*H.armigera*
EDTA Ethylenediamine tetraacetic acid	Metallo-proteases, Reversible	dH_2O	0 – 0.1 mM	Total protease	*H.armigera*
$CuCl_2$	Metallo-proteases, Irreversible	dH_2O Buffer	0 – 1.0 mM	LAP, Total protease	*H.armigera*, *E. integriceps*
$CdCl_2$	Metallo-proteases, Irreversible	dH_2O Buffer	0 – 60 µM	LAP, Total protease	*H.armigera*, *E. integriceps*
SBTI Soybean Kunitz trypsin inhibitor)	Serine proteases, Reversible, Competitive	dH_2O Buffer	0 – 2.25 µM	Trypsin, Elastase, Chymotrypsin Total protease	*H.armigera*, *E. integriceps*
Aprotinin Trypsin inhibitor from bovine lung	Serine proteases, Reversible, Competitive	dH_2O Buffer	0 – 10 µM	Trypsin, Elastase, Chymotrypsin Total protease	*H.armigera*, *E. integriceps*
MAAI Maize seed α-amylase inhibitor	α-Amylase	Aqueous Buffer	0 - 0.25 mg/mL	α-Amylase	*H.armigera*, *E. integriceps*
BAAI Kidney bean seed α-amylase inhibitor	α-Amylase	Aqueous Buffer	0 - 0.5 mg/mL	α-Amylase	*H.armigera*, *E. integriceps*
CpAAI Chickpea seed α-amylase inhibitor	α-Amylase	Aqueous Buffer	0 – 1 mg/mL	α-Amylase	*H.armigera*, *E. integriceps*
WAAI Wheat α-amylase inhibitor	α-Amylase	Aqueous Buffer	0 – 30 µM	α-Amylase	*H.armigera*, *E. integriceps*

In order to compare the effectiveness of inhibitors, IC_{50} (amount of inhibitor necessary to decrease the activity of the target enzyme by 50%) values were calculated by constructing V_o (as specific activity) versus [I] graphs from each inhibition data. The slope of the curve was used to calculate the IC_{50} values of inhibitors.

For the reversible inhibitors, K_i (inhibition constant) values were calculated from the following approximation:

Determination of K_i From Kinetic Data

The rate of substrate hydrolysis without inhibitor (V_o) is given by :

$$V_o = V_{max}.S / (K_m+S)$$

In a competitive system, the rate of substrate hydrolysis after addition of the inhibitor is :

$$V_i = V_{max}.S / (K_m(1+I/K_i)+ S)$$

The ratio V_o / V_i is:

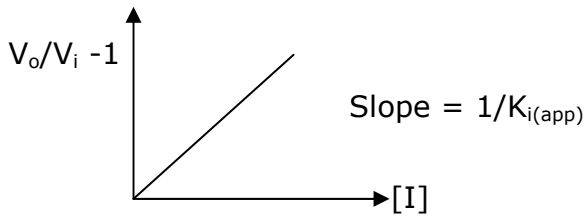
$V_o / Vi = (K_m(1+I/K_i) + S) / (K_m + S) = (K_m + (K_m.I)/K_i + S) / (K_m + S)$

$= (1 + I/K_i + S/K_m) / (1 + S/K_m) = (I/K_i + 1 + S/K_m) / (1 + S/K_m)$

$= I / (K_i(1+S/K_m) + 1 ===> V_o / V_i = 1 + I / K_{i(app)}$

$$K_{i(app)} = I / (V_o/V_i -1)$$

Drawing V_o/V_i -1 versus [I]



The true K_i value can be calculated from the following relationship :

$K_{i(app)} = K_i (1 + S/K_m)$ ⟶ $K_i = K_{i(app)} / (1 + S/K_m)$

2.2.6 Determination of K_m, V_{max} and Optimum pH

The presence of every enzymatic activity was determined by performing the spectrophotometric assays at different concentrations of midgut extracts. The results were used to construct V_o (units) versus [E] (mg) graphs, slope of which gave the amount of enzyme activity per mg of enzyme extract. Amount of enzyme extract necessary to measure the enzyme activity correctly was also evaluated from these results.

K_m and V_{max} values for each enzyme was determined though kinetic experiments and calculated through constructing Lineweaver-Burk plots (1/Vo versus 1/[S]). Substrate concentrations were varied at a constant enzyme concentration. The rates of each experiment were calculated and used to draw Lineweaver-Burk plots. Microsoft Excel was used in calculations.

Optimum pH values were calculated by performing each enzymatic assay in different buffer systems. The buffers used in the assays were tabulated in Table 2. The concentrations of substrates and enzymes were kept constant during the assays.

Table 2.2 Buffer solutions used in determination of optimum pH

pH	Buffer System
4.0 -5.0	0.1 M Ascorbic acid
6.0-7.0	0.1 M Potassium phosphate
8.0 – 9.0	0.1 M Tris- HCl
10.0-11.0	0.1 M Glycine-NaOH
12.0	0.1 M Sodium phosphate

2.2.7 Electrophoretic Methods

2.2.7.1 Casein Zymography

Protease activity of the midgut extracts were determined by non-reducing SDS-polyacrylamide gel electrophoresis (PAGE) using casein as substrate. Four percent stacking gel and 8% separating gel including 0.2 % casein were prepared according to a modified method of Oliver *et al.* (1999) using EC-120 mini-gel vertical electrophoresis chamber. Samples were prepared in sample dilution buffer (Appendix B) (1 unit buffer: 4 units sample) and incubated at room temperature for 10 minutes before loading. The gel was run on constant voltage (30 V for stacking gel, 100 V for separating gel) until blue tracking dye (bromophenol blue) reach to the bottom of the gel. The gel was removed and SDS was washed out by gently shaking the gels in wash solution containing 2.5 % Triton-X-100 for 1 hour, changing the wash solution at 30 min. After washing for two times with distilled water, the gel was incubated for 3 hours at 30°C for the proteolytic activity, using 0.1 M Tris-HCl buffer pH 8.0 for *H. armigera* midgut extracts. The gel was stained with Coomassie Blue R-250 (Appendix C), and proteolytic activities were detected as clear bands against a blue background. The gel was photographed using Vilber Gel Imaging System.

2.2.7.2 Starch-Polyacrylamide Gel Electrophoresis

Alpha amylase activity of the midgut extracts were determined in non-reducing polyacrylamide gel electrophoresis (PAGE) using starch as substrate. For *H. armigera* α-amylase activity, 4% stacking gel and 6% separating gel containing 0.5% soluble starch were prepared according to Titarenko and Chrispeels (2000) with minor modifications, using EC-120 mini-gel vertical electrophoresis chamber. Samples were prepared in 5X sample dilution buffer (Appendix B) and incubated at room

temperature for 10 minutes before loading. The gel was run on constant current (6 mA for stacking gel, 10 mA for separating gel) until blue tracking dye reach to the bottom of the gel. The gel was washed with 2.5% Triton-X-100 to remove the SDS and incubated in 0.1 M glycine-NaOH buffer, pH 10.0 containing 1% soluble starch for amylolytic activity, for 1 hour at 30°C. Undigested starch was stained with KI/I_2 solution (3% KI and 0.3% I_2) and excess dye was removed by washing with dH_2O for several times. Amylolytic activity was detected as clear bands against a black background. The gel was photographed using Vilber Gel Imaging System.

For *E. integriceps* midgut α-amylase activity, native polyacrylamide gel (without SDS) was prepared. The stacking gel and separating gel was prepared as before, excluding SDS from all solutions. Midgut extracts containing 0.5 µg protein was loaded to each well without any SDS or heat treatment. Gel was run at constant voltage (30 V for stacking gel, 100 V for separating gel) until blue tracking dye reach to the bottom of the gel. The gel was incubated in 0.1 M sodium phosphate buffer pH 6.0 for 1 hour at 30°C for amylolytic activity and stained with KI/I_2 solution (3% KI and 0.3% I_2) and excess dye was removed by washing with dH_2O for several times. Amylolytic activity was detected as clear bands against a black background. The gel was photographed using Vilber Gel Imaging System.

2.2.8 Partial Purification of Plant α-Amylase Inhibitors

2.2.8.1 Partial Purification of *Phaselous vulgaris* (Common Bean) and *Cicer arietinum* (chickpea) α-Amylase Inhibitors

Partial purification of chickpea and common bean α-amylase inhibitors were carried out by using the same method (Akyol, 2000). Two grams of dry seeds were ground in a cold mortar and pestle with liquid nitrogen until a fine powder was obtained. The powder was suspended in 40 mL of 0.02 M sodium phosphate buffer, pH 6.7, containing 20 mM NaCl and 0.1 mM $CaCl_2$. The suspension was stirred on a magnetic stirrer at 4°C for 1 hour and centrifuged at 11,900 g for 10 minutes using Sigma centrifuge. The supernatant was brought to 20% ammonium sulfate saturation and centrifuged at 15,500 g for 45 minutes at 4°C. After centrifugation the supernatant was brought to 60% ammonium sulfate saturation. The suspension was centrifuged at 15,500 g for 60 min at 4°C. The supernatant was discarded and the pellet was re-suspended in 20 mL of 0.02 M sodium phosphate buffer, pH 6.7. The suspension was dialyzed against 1.0 L of the same buffer overnight at 4°C. The extract was then collected in eppendorf tubes and stored at -20°C until use.

Dialysis bag treatment: Dialysis bag was boiled in a solution containing 0.1% sodium bicarbonate and 0.1% EDTA for 5 minutes and extensively washed with dH_2O.

2.2.8.2 Partial Purification of *Zea mays* (Maize) α-Amylase Inihibitor

Partial purification of maize seed α-amylase inhibitor was carried out according to slightly modified method of Figueira *et al.* (2003). Corn seeds were finely ground using mortar and pestle with liquid nitrogen, or using a mill (if available). The flour was defatted by shaking with acetone (1:1 v/v) for 15 minutes, 4-times and air-dried at room temperature. The corn flour (20 g) was mixed with 100 mL of 0.1 M acetate buffer, pH 6.0 and continuously stirred for 90 minutes at 4°C. The soluble protein was obtained by centrifuging at 30,000 x g for 20 minutes at 4°C. The supernatant was submitted to fractionation by ammonium sulfate precipitation (30% to 60% saturation). The precipitate was recovered by centrifuging at 30,00 x g for 20 minutes at 4°C and dissolving the pellet in 0.01 M Tris-HCl buffer, pH 8.0. The suspension was dialyzed against the same buffer overnight at 4°C and the partially purified maize seed extracts were stored at -20°C until use.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Sunn Pest (*Eurygaster integriceps*)

3.1.1 Hydrolytic Enzyme Profile of Sunn Pest Midgut

Hydrolytic enzymes α-amylase, trypsin, chymotrypsin, elastase, leucine aminopeptidase, carboxypeptidase-A, carboxypeptidase-B, papain and total protease activities from sunn pest midgut were identified by spectrophotometric methods using synthetic or natural substrates. The amount of enzyme activity was determined by enzyme titration assays by gradually increasing the protein concentration of the midgut extract in each assay medium. The resulting curves (Fig. 3.1) were used for calculating the amount of enzymes in terms of specific activities (Table 3.1).

Among the proteases tested, only trypsin, elastase and leucine aminopeptidase-like activity could be detected by spectrophotometric assays. The highest proteolytic activity was observed with a metalloprotease, leucine aminopeptidase, however, carboxypeptidase-like metalloprotease activities were not observed. Among the serine-like proteases, trypsin and elastase activities could be

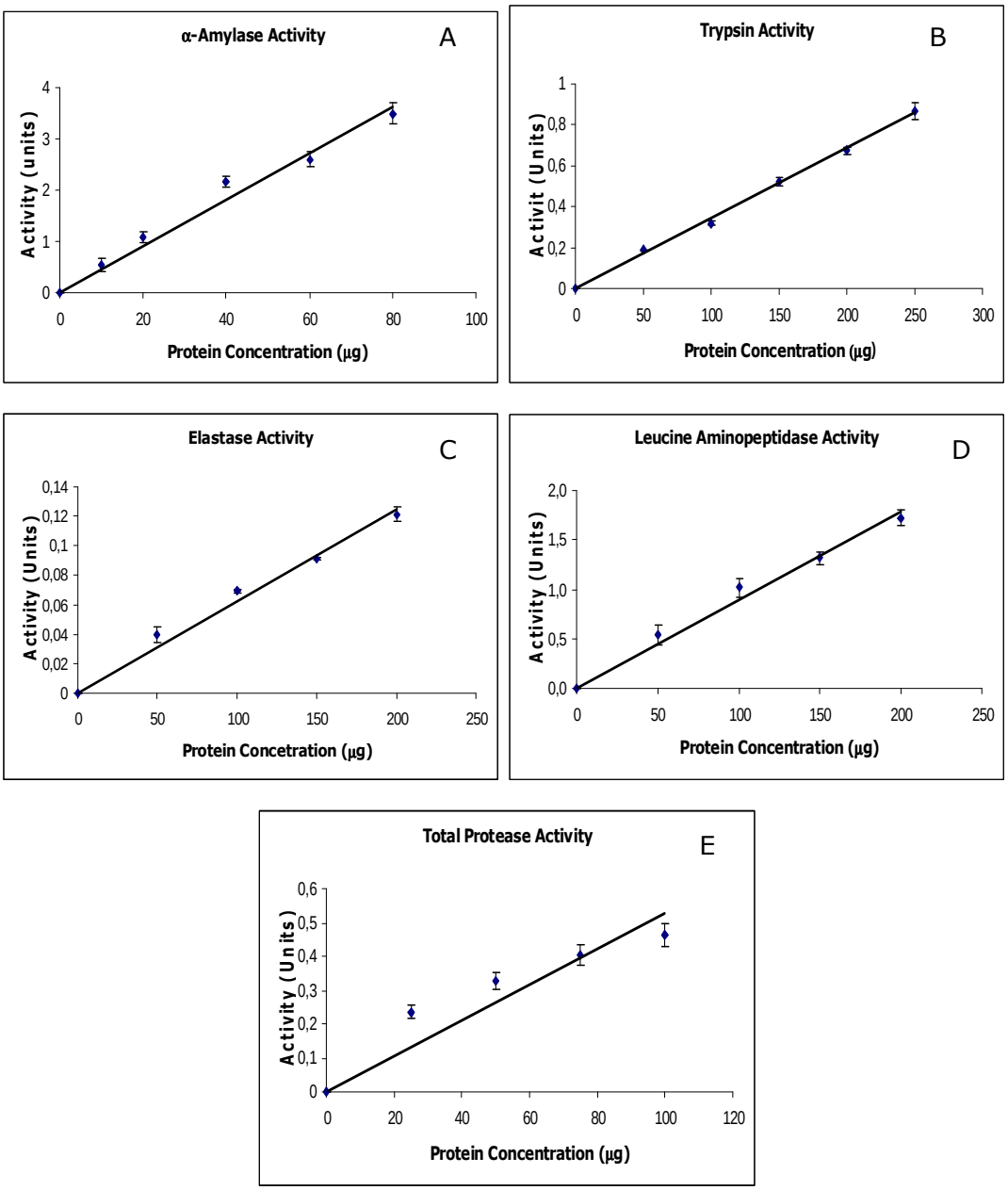


Figure 3.1 Hydrolytic enzymes from Sunn Pest midgut. A) α-Amylase activity (1 Unit activity = mmoles maltose produced/10 min); B) Trypsin activity; C) Elastase activity; D) Leucine aminopeptidase activity (1 Unit activity = µmoles *p*-nitroanilide produced /min); E) Total protease activity (1 Unit activity = 0.001 units change in OD_{335} /min).

Table 3.1 Hydrolytic enzymes of Sunn Pest midgut.

	Substrates	**Specific Activity**
α-Amylase	Starch	4.45 mmoles/min/mg
Trypsin	BApNa	3.0 ± 0.4 nmoles/min/mg
Elastase	SA_3pNa	0.66 ± 0.03 nmoles/min/mg
Leucine Aminopeptidase	LpNa	14.4 ± 0.8 nmoles/min/mg
Total Protease	Azocasein	7.9 ± 0.04 $m\Delta OD_{335}$ /min/mg

detected, the former being very low. Presence of serine type proteolytic enzymes in the Heteropteran insect midguts was previously reported by several researchers (Boyd *et al.,* 2002; Foissac *et al*., 2002; Zhu *et al*., 2003; Wright *et al.,* 2006). A low constitutive elastase activity was also reported by Zeng and Cohen (2001), who showed the up-regulation of elastase when the insect is fed by elastin. Boyd *et al.* (2002) also reported the presence of aminopeptidase-like activity in the midgut of an Heteropteran insect. The high leucine aminopeptidase (an exopeptidase) activity may be indicating the presence of relatively small peptides that can be easily digested by an exopeptidase activity. Presence of exopeptidase activity in the midgut of several Heteropteran insects was previously reported (Houseman *et al*., 1984; Houseman *et al*., 1985). However, carboxypeptidase-like exopeptidase activity could not be detected in the sunn pest midgut. There are contradicting reports on types of proteases present in Heteropteran insect midguts. Although no cysteine-like activity could be detected in the sunn pest midgut, Colebatch *et al.* (2001) reported the presence of serine and cysteine

type proteases in the *Creontiades dilutus*, an Heteropteran insect. Presence of aspartic-like proteases was also reported (Houseman and Downe, 1982). There are reports that in Heteropteran insects there may be considerable differences in the proteolytic enzyme profiles of insects belonging to the same family (Zeng *et al*., 2002, Wright *et al*., 2006). In fact, the Heteropteran insects are the most diverse group of the class Insecta and composed approximately of 35,000 species. There is a quite diversity in the feeding characteristics of these insects. There are facultative phytophagous (feeding only on plants), zoophytophagous (feeding on plants and animals) and facultative zoophagous (feeding exclusively on animals) insects in the Heretopteran order. Therefore, a significant diversity in the proteolytic enzymes, which adapt themselves according to the diet insect feeds, has been observed in this order. Diversity in the proteases in insects may have also been arisen from the presence of protease inhibitors in plant tissues. So, different proteolytic activity even in the same family of insects is an adaptation to the diet which may contain protease inhibitors that cause suppression of several types of proteases and induction of the others.

There was a low proteolytic activity in the sunn pest midgut which is in accordance with the previous findings on the midgut proteolytic activity of different Heteropteran insects (Colebatch *et al*., 2001; Boyd *et al.,* 2002; Boyd, 2003; Zhu *et al.,* 2003; Wright *et al.,* 2006;). Most reports indicate a higher proteolytic activity in the salivary glands compared to that of the midgut. Salivary gland and midgut proteolytic enzymes were shown to be significantly different in their types and characteristics and they may be complementary to each other (Wright *et al.,* 2006). It has been known that sunn pest carries out most of its protein digestion extra-orally by injecting its toxic saliva into the grain and degrading the gluten in the grain (Kretovich, 1944). The insect's saliva was shown to contain proteolytic enzymes by several reports (Every, 1990; Sivri *et al.,* 1999; Sivri *et al.,* 2004) which reveal the digestion of gluten proteins in

the wheat grain, however the complete characterization of the salivary proteases has not been carried out. A low proteolytic activity in sap sucking phytophagous insects of the Heteropteran order, like sunn pest, have also been attributed to the feeding characteristics of these insects. They are assumed not to carry out digestive proteolysis, but instead to rely on free amino acids which is found in high amounts in the phloem and xylem saps for their nutritional requirements (Foissac *et al.,* 2002). To better evaluate the protease activity profile of sunn pest, it is necessary to determine the salivary proteases. From present results it can be hypothesized that most of the proteolytic digestion of sunn pest completed outside the midgut, most probably by extra-oral digestion. When the oligopeptides reach the midgut, they are finally broken down by several serine proteases and exopeptidases (like leucine aminopeptidase).

When compared to proteolytic activities, the α-amylase activity was very high (10^6 order of magnitude higher). These results correlate well with the carbohydrate-rich diet of the sunn pest, and it is a characteristics for all Heteropteran insects having phytophagous feeding habits.

3.1.2 Characteristics of Hydrolytic Enzymes of Sunn Pest Midgut

Biochemical characterization of sunn pest midgut α-amylase and proteases in terms of optimum pH, K_m and V_{max} values were carried out. The enzyme activities were measured as previously defined, except, the pH of the buffers for determination of optimum pH, and concentration of substrates for the determination of K_m and V_{max} values were changed in each assay medium.

Optimum pH values for the enzymes were determined at pH range of 4.0 and 12.0, and shown in Figure 3.2. A broad pH optima was observed

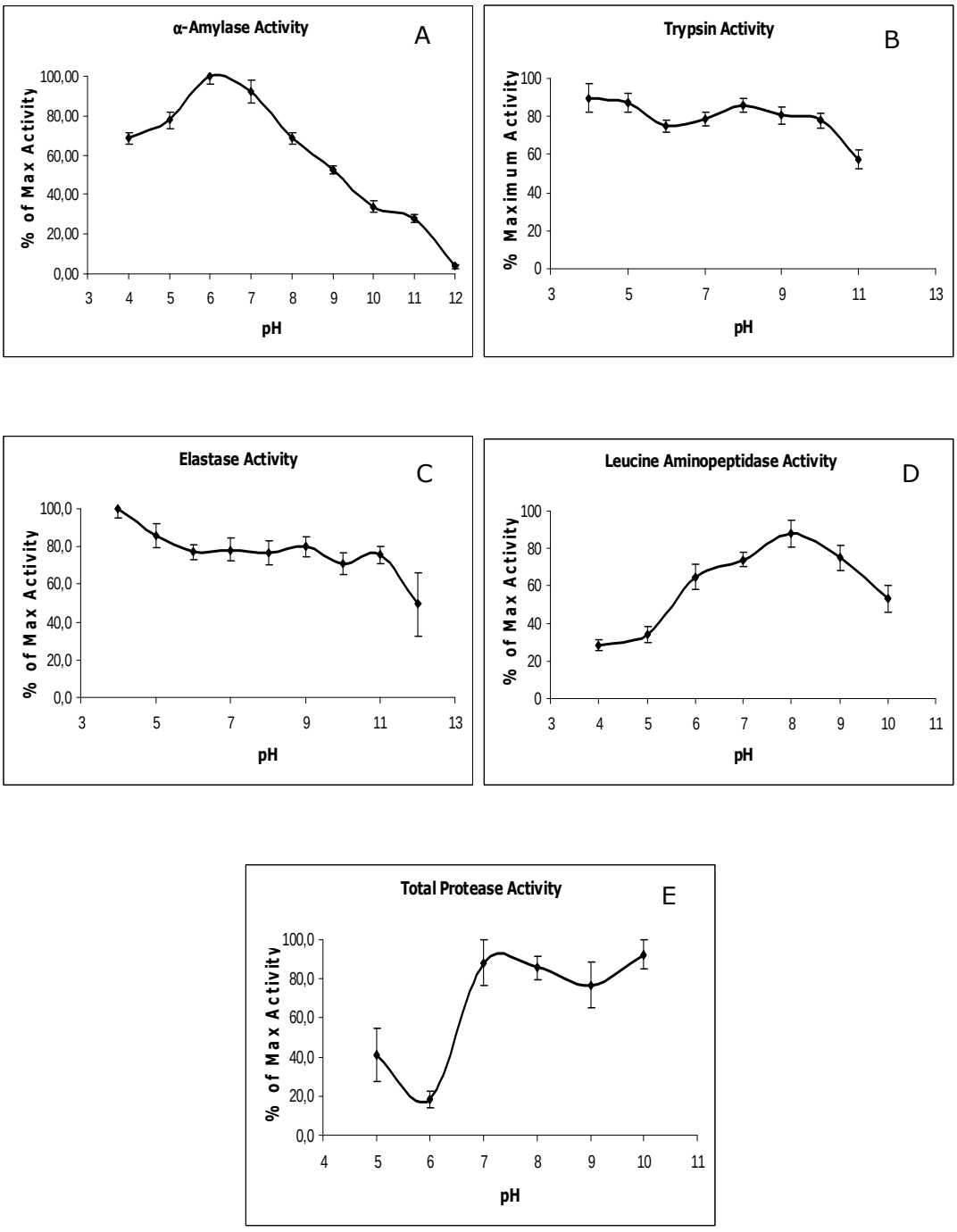
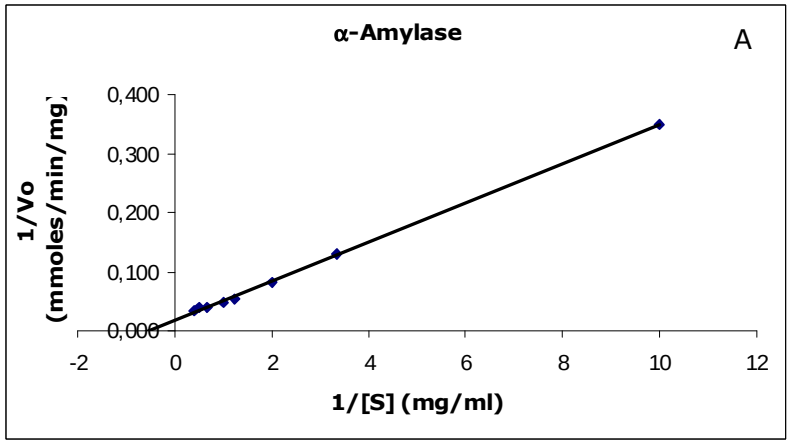
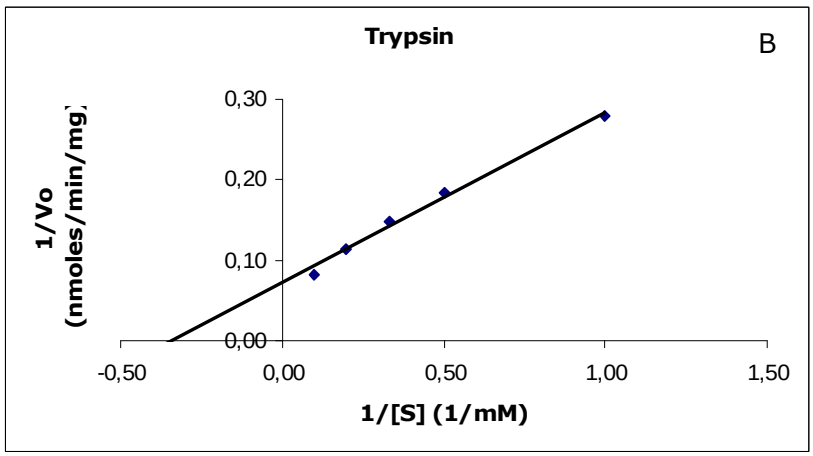


Figure 3.2 Effect of pH on hydrolytic enzymes of sunn pest. A) α-Amylase, B) Trypsin, C) Elastase, D) Leucine aminopeptidase and E) Total protease.

for the elastase-like activities between pH 4.0 and pH 11.0 and trypsin-like activities between pH 4.0 and pH 9.0. On the other hand, leucine aminopeptidase activity, which is the highest proteolytic activity detected in the sunn pest midgut, had a sharp pH optimum of 8.0. For total proteolytic activity, optimum pH was observed between pH 7.0 and pH 10.0. A wide pH optima for the proteolytic activity from midgut of heteropteran insects were previously reported (Colebatch *et al.,* 2001). The alkaline pH range for general protease activity is the indication of predominance of serine proteases. The reports concerning the proteolytic activities from midgut of Heteropteran insects indicate presence of both alkaline and acidic proteases. Boyd (2003) reported the predominance of alkaline proteases (serine type) from *Deraeocoris nigritulus* (Hemiptera: Miridae). Foissac *et al*. (2002) reported an alkaline pH and presence of trypsin and cathepsin-B-like proteases from the Heteropteran insect, *Nilaparvata lugens*. A number of other studies with the same family, however, indicated the presence of aspartic and cysteine proteases (Colebatch *et al.*, 2001; Terra and Ferreira, 1994) in the midgut juice. Zhu *et al.* (2003) reported an alkaline pH optima for BApNAase activity from midgut of *Lygus lineolaris*, but found a acidic pH optima against azocaseinolytic activity. In this study, both azocaseinolytic activity (total protease activity) and the activity against specific proteases showed a neutral-to-alkaline range of pH optimum, which reflects the predominance of serine type proteases in the sunn pest midgut.

K_m and V_{max} values for the enzymes were determined by Lineweaver-Burk plots (Figure 3.3) and the results were tabulated in Table 3.2. Among the proteases, the highest maximal rate was obtained with leucine aminopeptidase, which was the highest proteolytic activity in the sunn pest midgut. K_m value for the leucine aminopeptidase showed the highest affinity towards the *p*-nitroanilide substrates used in the measurement of specific proteolytic activities. For the determination of





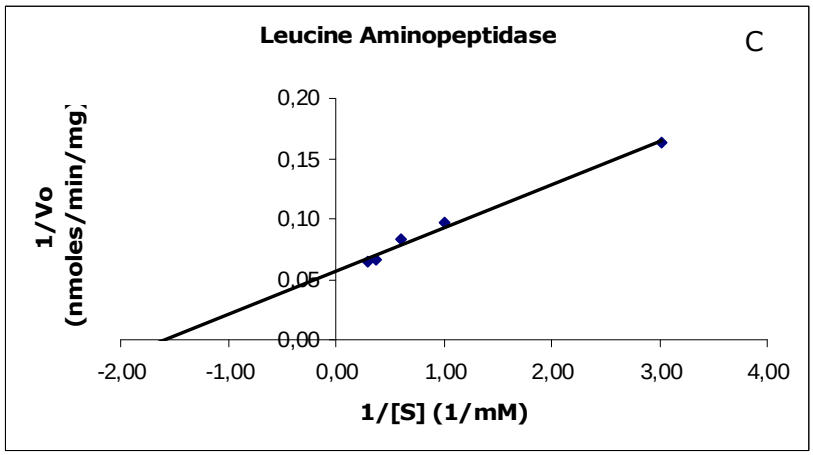


Figure 3.3 Lineweaver-Burk Plot for sunn pest midgut hydrolytic enzymes. A) α-amylase, B) trypsin and C) leucine aminopeptidase.

Table 3.2 K_m, V_{max} and optimum pH values of the hydrolytic enzymes from sunn pest midgut

	K_m	**V_{max}**	**Opt pH**
α-Amylase (starch)[1]	1.28 ± 0.57 mg/ml	53.2 ± 2.7 mmoles/min/mg	6.0
Trypsin (BApNa)[1]	2.97 ± 0.8 mM	14.0 ± 1.2 nmoles/min/mg (at pH 8.0)	4.0 – 9.0
Leucine aminopeptidase (LpNa)[1]	0.55 ± 0.08 mM	17.8 ± 0.3 nmoles/min/mg	8.0
Elastase (SA_3pNa)[1]	-	-	4.0 – 11.0
Total Protease (azocasein)[1]	-	-	7.0 – 10.0

(1) In the parenthesis are given the substrates used. All assays were carried out at 30 °C. Each data is the average of at least three independent experiments ± SEM

K_m and V_{max}, trypsin activity was measured at pH 8.0. Trypsin showed a lower affinity for its *p*-nitroanilide substrate compared to that of the leucine aminopeptidase. Its maximal rate is also lower than the rate of the leucine aminopeptidase. K_m and V_{max} values for the elastase-like activity could not be determined, due probably to its very low specific activity (Table 3.1).

The K_m and V_{max} of sunn pest midgut α-amylase activity was significantly different from that of the proteolytic activity (Table 3.2). As starch does not have a specific molecular weight, K_m was given as the mg/ml. V_{max} of α-amylase was found as 53.2 ± 2.7 mmoles maltose /min/mg, which

is 10^6 order of magnitude higher when compared to the proteolytic activities. As previously discussed, sunn pest is an phytophagous Heteropteran insect predominantly feeding on cereals seeds. Its diet depends largely on carbohydrates present in high amount in plant seeds. A high amylase activity reflects its adaptation to carbohydrate-rich diet. A high amylolytic activity compared to proteolytic activity was previously reported for other phytophagous insects.

3.1.3 Inhibition of Sunn Pest Midgut Hydrolytic Enzymes with Synthetic and Natural α-Amylase and Protease Inhibitors

3.1.3.1 Inhibition of Sunn Pest α-Amylase Activity

Sunn pest α-amylase activity was tested for inhibition with naturally occurring α-amylase inhibitors from bean (BAAI), chickpea (CpAAI), maize (MAAI) and wheat (WAAI). The experiments were carried out by incubating the sunn pest midgut extracts with different concentrations inhibitors prior to enzyme assays. The results were compared to the uninhibited reactions and shown as % inhibitions (Figure 3.4).

The α-amylase inhibitors from maize, bean and chickpea were partially purified extracts of plant seeds. The inhibitory activities in the extracts were tested against a commercial α-amylase (Sigma) and a considerable inhibition was observed for each (Table 3.3). Although it inhibited the commercial α-amylase activity by 100 %, BAAI caused no inhibition on sunn pest α-amylase activity. WAAI and MAAI caused only a partial inhibition on sunn pest midgut α-amylase activity. Inhibition by wheat α-amylase inhibitor was about 38 % at 30 µM inhibitor concentration and that of MAAI was about 40 % at when extract containing 50 µg of protein was included in the assay medium. CpAAI caused a 26 % inhibition when 100 µg of chickpea seed extract was included, but

inhibition decreased when concentration of inhibitor was increased. This may be due to the presence of a contaminating protein in the crude extract of chickpea seeds. To obtain more reliable results, pure inhibitors should be used in the assays.

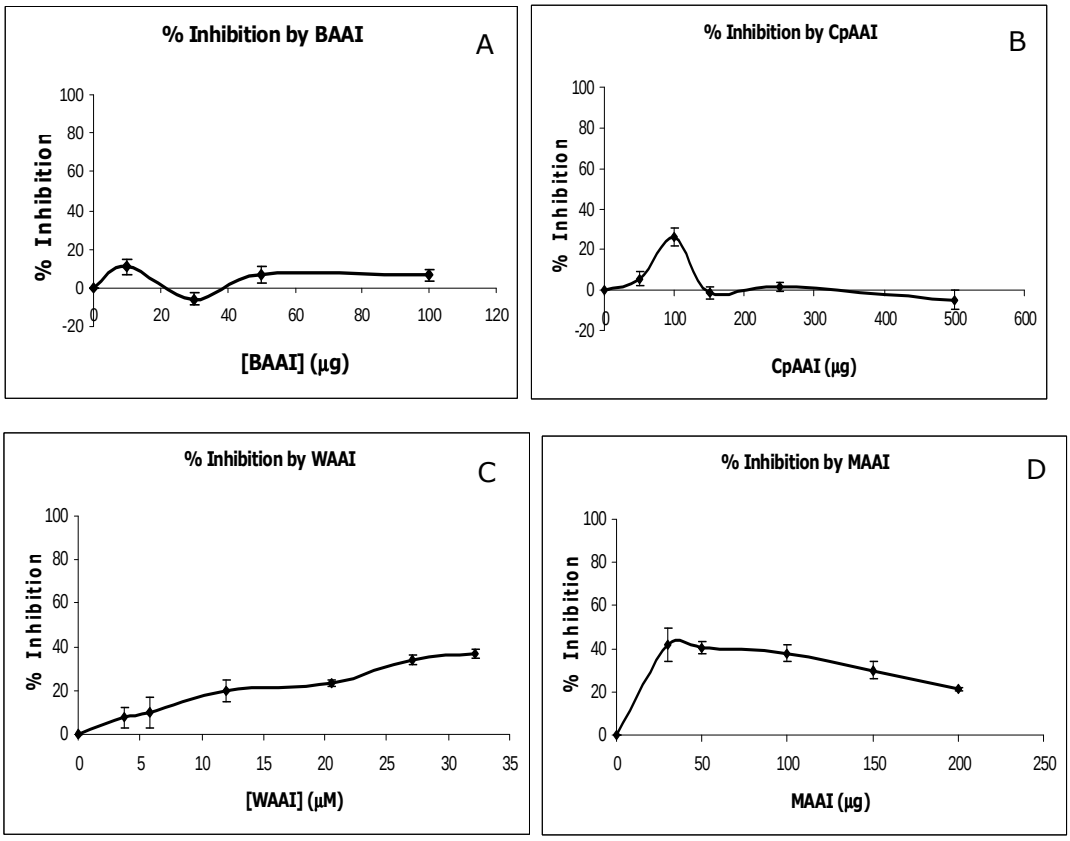


Figure 3.4 Percent inhibition of sunn pest midgut α-amylase activity by different concentrations of α-amylase inhibitors. A) bean α-amylase inhibitor (BAAI), B) chickpea α-amylase inhibitor (CpAAI), C) wheat α-amylase inhibitor (WAAI) and D) maize α-amylase inhibitor (MAAI). Values in x-axis indicates the amount of inhibitor included per assay

Table 3.3 Percent maximum inhibition of sunn pest α-amylase activity natural α-amylase inhibitors at given concentrations

	% Inhibition of sunn pest midgut α-amylase	% inhibition of commercial α-amylase
WAAI (30 µM)[1]	36.9 ± 1.8	100.0 ± 0.0
BAAI (100 µg)[1]	n.i.[2]	100.0 ± 0.0
CpAAI (100 µg)[1]	26.0 ± 4.5	86 ± 3.2
MAAI (50 µg)[1]	40.2 ± 2.8	80 ± 5.4

(1) Indicates the amount of inhibitor/assay medium at which maximal inhibition was obtained. (2) No inhibition.

It should be indicated that if the development of sunn pest resistant wheat species using α-amylase inhibitors was considered, a more potent inhibitor should be found. In this study, the highest inhibition was obtained with WAAI and MAAI, the former being effective at µM concentrations, and the latter at µg amounts (not pure). It is suggested that an effective inhibitor should inhibit the enzymatic activity at nM concentrations. Otherwise, a very high expression level is needed in the host plant which may have unwanted effects on normal development of the host plant and on the non-target organisms (Svensson *et al*., 2004).

3.1.3.2 Inhibition of Sunn Pest Trypsin Activity

Trypsin activity of sunn pest midgut was tested for inhibition by two natural serine protease inhibitors, soybean trypsin inhibitor (SBTI) and aprotinin (bovine lung serine protease inhibitor), and a synthetic protease inhibitor Phenylmethylsuphonyl fluoride (PMSF) (Figure 3.5).

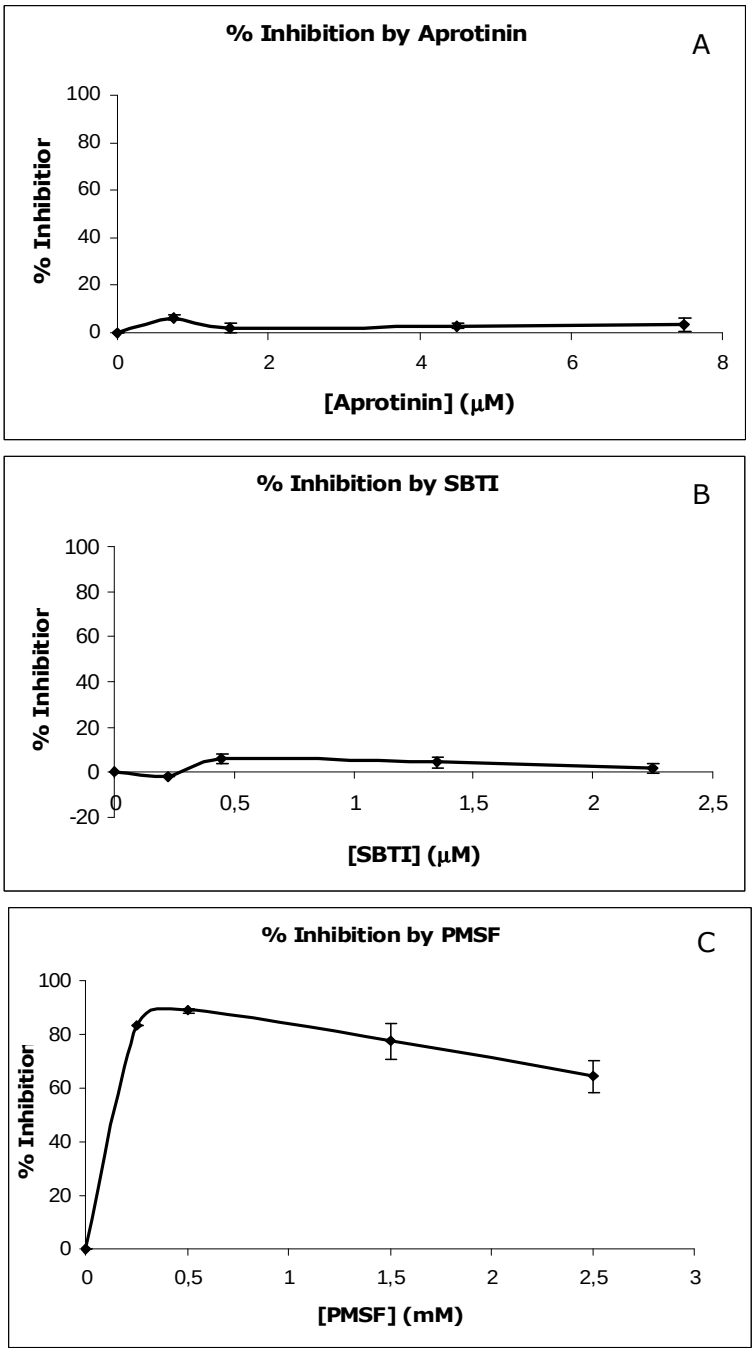


Figure 3.5 Inhibition of sunn pest midgut trypsin activity by different concentrations of protease inhibitors. A) soybean trypsin inhibitor (SBTI), B) aprotinin and C)PMSF.

The inhibitory activity was tested as previously described. No inhibition was obtained with natural protease inhibitors aprotinin and SBTI at µM concentrations (Figure 3.5 A-B). PMSF decreased the sunn pest trypsin activity by 90 % at 0.5 mM final assay concentration (Figure 3.5 C). However, increased PMSF concentration caused a slight decrease in inhibitory activity. This may be due to the interaction of other molecules in the crude midgut extracts with PMSF, or the ethanol, in which PMSF was dissolved, may have resulted in such an effect.

3.1.3.3 Inhibition of Sunn Pest Elastase Activity

Inhibition of sunn pest midgut elastase activity was tested with serine protease inhibitors SBTI, aprotinin, PMSF (Phenylmethylsuphonyl fluoride) and TPCK (Tosyl phenylalanyl chloromethyl ketone) at increasing concentrations (Figure 3.6). Sunn pest midgut elastase-like activity was found resistant to inhibition by natural protease inhibitors SBTI and aprotinin, but, it was significantly inhibited by PMSF and TPCK. Although TPCK was known as a chymotrypsin inhibitor, it caused a 50 % inhibition on elastase-like activity, too. PMSF decreased the elastase-like activity by 57 % at 3 mM concentration, but similar to trypsin inhibition, its inhibitory effect slightly diminished at higher concentrations.

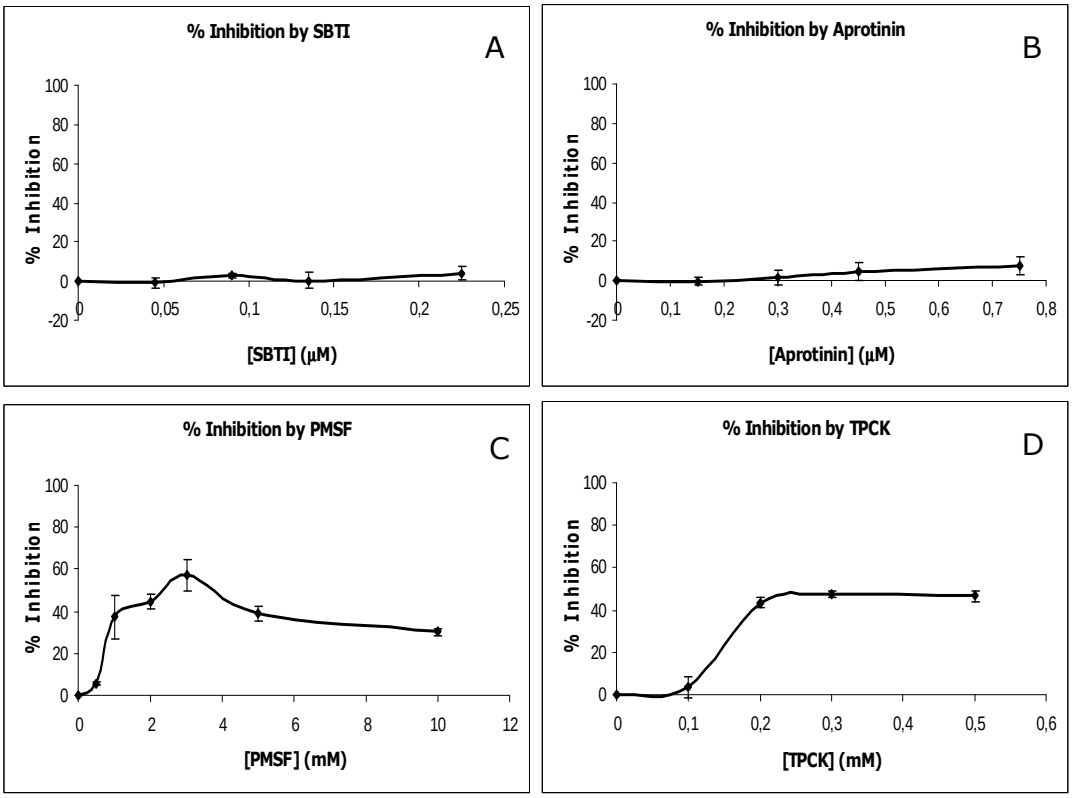


Figure 3.6 Inhibition of sunn pest midgut elastase activity by different concentrations of protease inhibitors. A) soybean trypsin inhibitor (SBTI), B) aprotinin, C)PMSF and D) TPCK.

3.1.3.4 Inhibition of Sunn Pest Leucine Aminopeptidase Activity

Leucine aminopeptidase-like activity from sunn pest midgut was significantly inhibited by PMSF, and two metalloprotease inhibitors $CdCl_2$ and $CuCl_2$ (Figure 3.7) Among the metalloprotease inhibitors, $CdCl_2$ was more effective, causing 100 % inhibition, while $CuCl_2$ inhibited the leucine aminopeptidase-like activity by about 75 %. PMSF caused 60 % inhibition at 150 µM concentration.

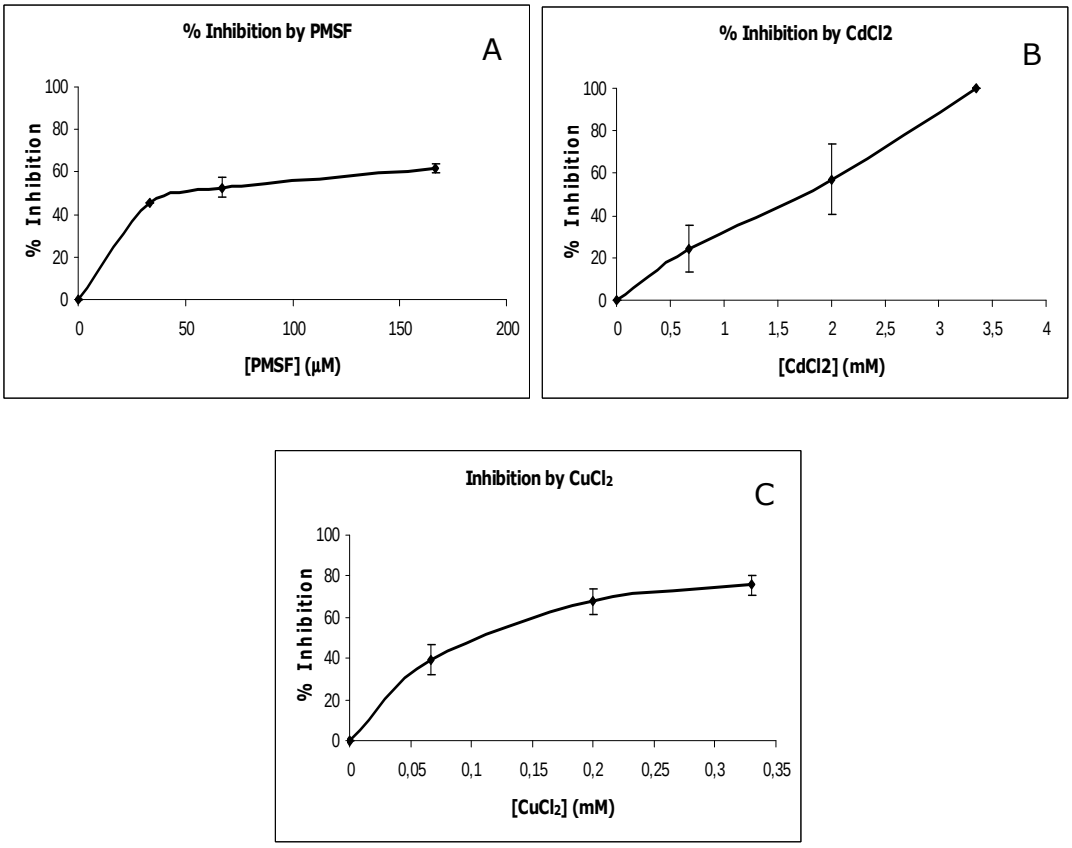


Figure 3.7 Inhibition of sunn pest midgut leucine aminopeptidase activity by different concentrations of protease inhibitors. A) PMSF, B) $CdCl_2$ and C)$CuCl_2$.

3.1.3.5 Inhibition of Sunn Pest Total Protease Activity

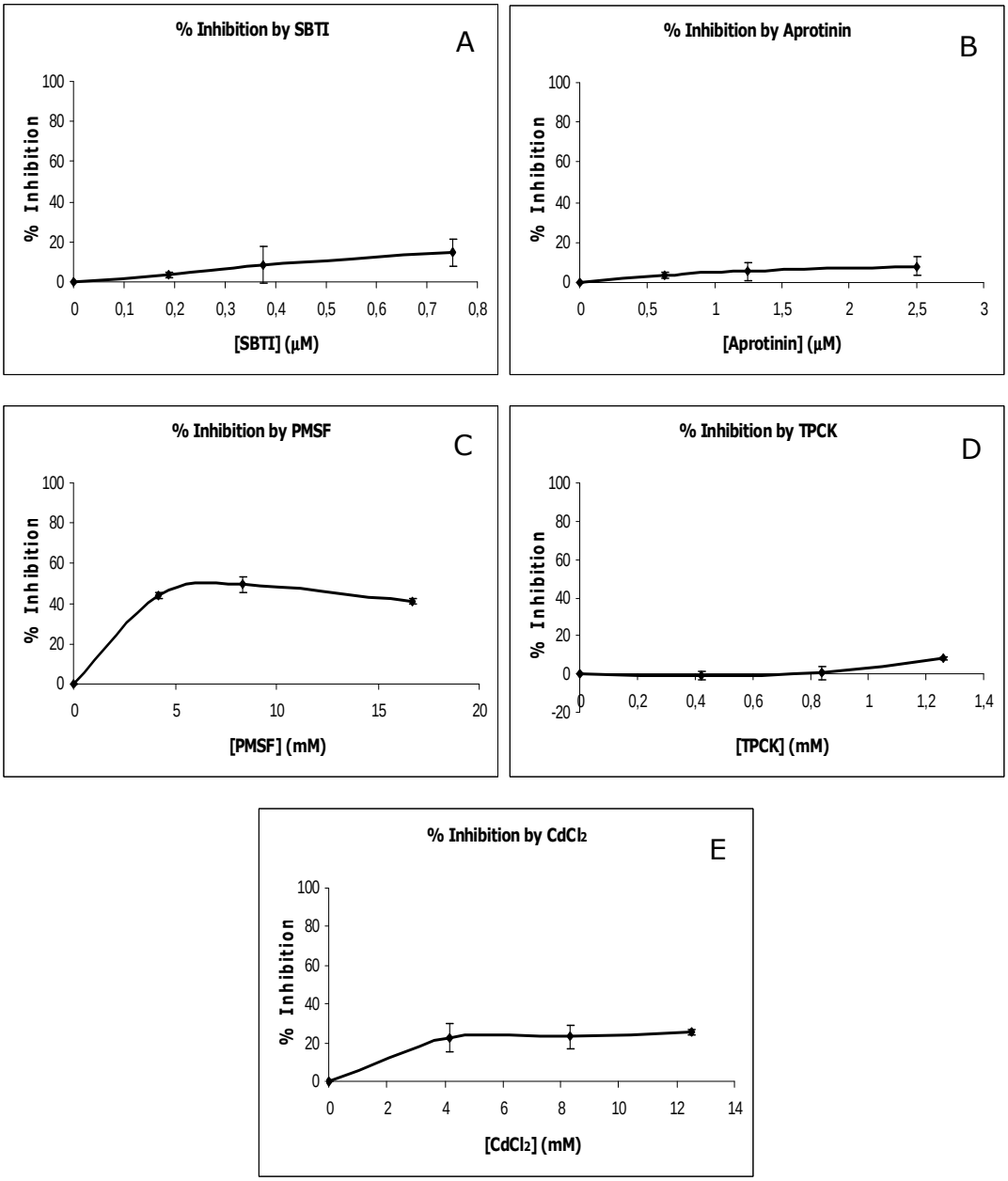


Figure 3.8 Inhibition of sunn pest midgut total protease (azoalbumin hydrolyzing) activity by different concentrations of protease inhibitors. A) soybean trypsin inhibitor (SBTI), B) aprotinin, C)PMSF D) TPCK and E) $CdCl_2$.

Inhibition of total proteolytic activity (or, azocaseinolytic activity) was tested with natural protease inhibitors SBTI and aprotinin, and synthetic inhibitiors PMSF, TPCK and $CdCl_2$ (Figure 3.8). The maximal percent inhibitions of sunn pest midgut proteases by given inhibitors were tabulated in Table 3.4.

Table 3.4 Percent maximum inhibition of sunn pest midgut proteases by natural and synthetic protease inhibitors.

	Trypsin	**Elastase**	**LAP**	**Total Protease**
SBTI	n.i.[1] (2.5 µM) [3]	n.i. (0.2 µM)	n.d.	n.i. (1 µM)
Aprotinin	n.i. (8 µM)	n.i (1 µM)	n.d.	n.i. (2.5 µM)
TPCK	n.d. [2]	46.7 ± 2.5 (0.5 mM)	n.d.	n.i. (1.2 mM)
PMSF	89.1 ± 0.8 (0.5 mM)	57.1 ± 7.1 (5mM)	61.7 ± 1.95 (0.15 mM)	41.1 ± 1.4 (15 mM)
$CuCl_2$	n.d.	n.d	75.6 ± 4.8 (0.3 mM)	n.d.
$CdCl_2$	n.d.	n.d.	100.0 ± 0.0 (3 mM)	25.3 ± 1.4 (10 mM)

Data were shown as % inhibition at specific inhibitor concentrations at which maximum inhibition is achieved. Values are the means of at least three independent experiments and indicate the % inhibition ± SEM. (1) n.i. No inhibition (Less then 10%). (2) n.d. Not determined. (3) Values in parenthesis are concentrations of respective inhibitors used in the assay medium.

In correlation with their effects on specific proteases, SBTI and aprotinin did not caused a significant inhibition on total proteolytic activity of sunn pest midgut. PMSF, a serine protease inhibitor, caused about 40 % inhibition, which may reflect the percentage of serine-type proteases in the sunn pest midgut. But we should consider that, PMSF did not caused a 100 % inhibition on specific proteolytic activities of serine-type proteases, trypsin and elastase. Therefore, the percentage of serine-type proteases may be higher than 40 %. TPCK caused no inhibition on total protease activity, although it inhibited the elastase-like activity by 46.7 %. This was not reflected on total protease activity due to a very low contribution of elastase-like activity on total proteolytic activity. This result also supports the absence of chymotrypsin-like activity in sunn pest midgut, as TPCK is a chymotrypsin inhibitor. $CdCl_2$ caused a 25 % inhibition on total protease activity. It was a very strong metalloprotease inhibitor, as observed by its effect on leucine aminopeptidase activity. So, total metalloprotease-like activity may contribute 25 % of the total proteolytic activity of sunn pest midgut.

3.2 Cotton Bollworm (*Helicoverpa armigera*)

3.2.1 Hydrolytic Enzyme Profile of Cotton Bollworm Midgut

Hydrolytic enzymes α-amylase, trypsin, chymotrypsin, elastase, leucine aminopeptidase, carboxypeptidase-A, carboxypeptidase-B, papain and total protease activities from cotton bollworm midgut were identified by spectrophotometric methods using synthetic or natural substrates. The amount of enzyme activity was determined by enzyme titration assays by gradually increasing the protein concentration of the midgut extract in each assay medium. The resulting curves (Fig. 3.9) were used to calculate the amount of enzymes in terms of specific activities (Table 3.5).

Among the proteases tested, trypsin, elastase, chymotrypsin, leucine aminopeptidase and papain activities were detected. The highest proteolytic activity was of serine type endoproteases trypsin, chymotrypsin and elastase. A high metalloprotease type exoprotease activity (leucine aminopeptidase) was also observed, but no carboxypeptidase-like exoprotease activity could be detected. There was also a cysteine-type protease as observed by papain-like activity. Presence of different types of proteases in the *Helicoverpa armigera* digestive system reflects its polyphagous nature. The insect infests many crops including cotton, tomato, sunflower, corn, pigeonpea and chickpea. Diversity of the proteolytic activity ensures the digestion of many different kinds of proteins present in the host plant. It was reported that the insect can regulate its proteolytic activity rapidly according to the diet it feeds on (Bown *et al*., 1997; Gatehouse *et al.,* 1997, Chougule *et al*., 2005; Srinivasan *et al.*, 2005). Although several reports indicate the presence of carboxypeptidase-like activity in the *H. armigera* midgut (Bown *et al*., 1998; Estebanez-Perpina *et al*., 2001), we could not define neither carboxypeptidase-A nor carboxypeptidase-B

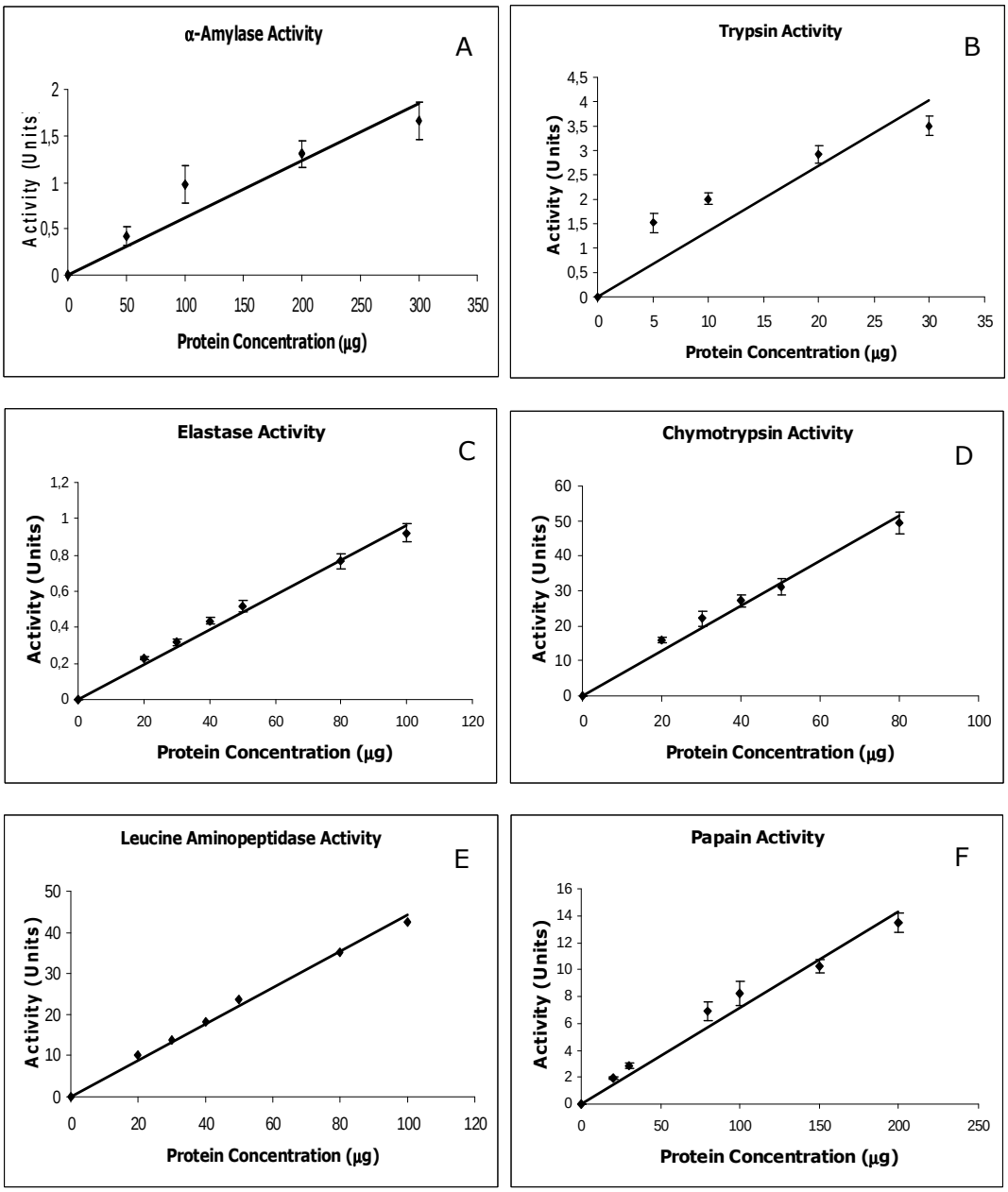


Figure 3.9 Hydrolytic enzymes from cotton bollworm midgut. A) α-Amylase activity (1 Unit activity = mmoles maltose produced/10 min); B) Trypsin activity; C) Elastase activity; D) Chymotrypsin Activity E) Leucine aminopeptidase activity (1 Unit activity = µmoles *p*-nitroanilide produced /min); F) Papain Activity (1 Unit activity = 0.001 units change in OD_{440} /min).

–like activities. As previously reported, changes in the diet may affect the expression patterns of proteolytic activities in the *H. armigera* midgut, which may result in the variation in types of proteases detected in different cultures of *H. armigera.*

Predominance of serine type proteases, especially the trypsin-like activity, is characteristics of Lepidopteran insects. Presence of trypsin, chymotrypsin and elastase-like activities in the *H. armigera* midgut was previously reported (Bown *et al.,* 1997;Patankar *et al*., 2001; Srinivasan *et al*., 2005; Chougule *et al*., 2005). Although most reports indicated the prevalence of trypsin-like activity, chymotrypsin-like activity was found to be much higher in this study. There are no reports available revealing

Table 3.5 Hydrolytic enzymes of *H. armigera* midgut.

	Substrates	**Specific Activity**
α-Amylase	Starch	620 ± 23 µmoles/min/mg
Trypsin	BApNa	221.5 ± 20.1 nmoles/min/mg
Chymotrypsin	SA_2PPpNa	643.7 ± 24.3 nmoles/min/mg
Elastase	SA_3pNa	9.7 ± 0.6 nmoles/min/mg
Leucine Aminopeptidase	LpNa	441 ± 27 nmoles/min/mg
Papain	Azoalbumin	71.3 ± 4.7 0.001 unit ΔOD/min/mg

the presence of cysteine type proteases in the *H. armigera* midgut, but our results indicated the presence of a papain-like activity. The accuracy of the result was re-evaluated by using a cysteine-protease specific inhibitor, E-64, which significantly decreased the papain-like proteolytic activity (discussed in part 3.2.3.6).

A very high α-amylase activity was detected in the *H. armigera* midgut. It was 10^3 orders of magnitude higher compared to the proteolytic activities. As it was discussed previously, phytophagous insects (insects that feed exclusively on plants) depend largely on carbohydrate-rich diet. Similar to sunn pest, *H. armigera* is a phytophagous insect, and a high amylolytic activity compared to proteolytic activity was observed in both insects. However, compared to sunn pest, the amount of amylolytic activity was lower in *H. armigera*. The reason is most probably the difference in the diets of the insects which feed on different parts of the plants and adapted their hydrolytic enzymes accordingly. Sunn pest predominantly feeds on seeds of cereals which have a very high carbohydrate content, while *H. armigera* feeds on almost all parts (stems, leaves, flowers, fruits) of plants.

3.2.2 Characteristics of Cotton Bollworm Hydrolytic Enzymes

Biochemical characterization of *H. armigera* midgut α-amylase and proteases in terms of optimum pH, K_m and V_{max} values were carried. The enzymes activities were measured as previously defined, except, the pH of the buffers for determination of optimum pH, and concentration of substrates for the determination of K_m and V_{max} values were changed in each assay medium.

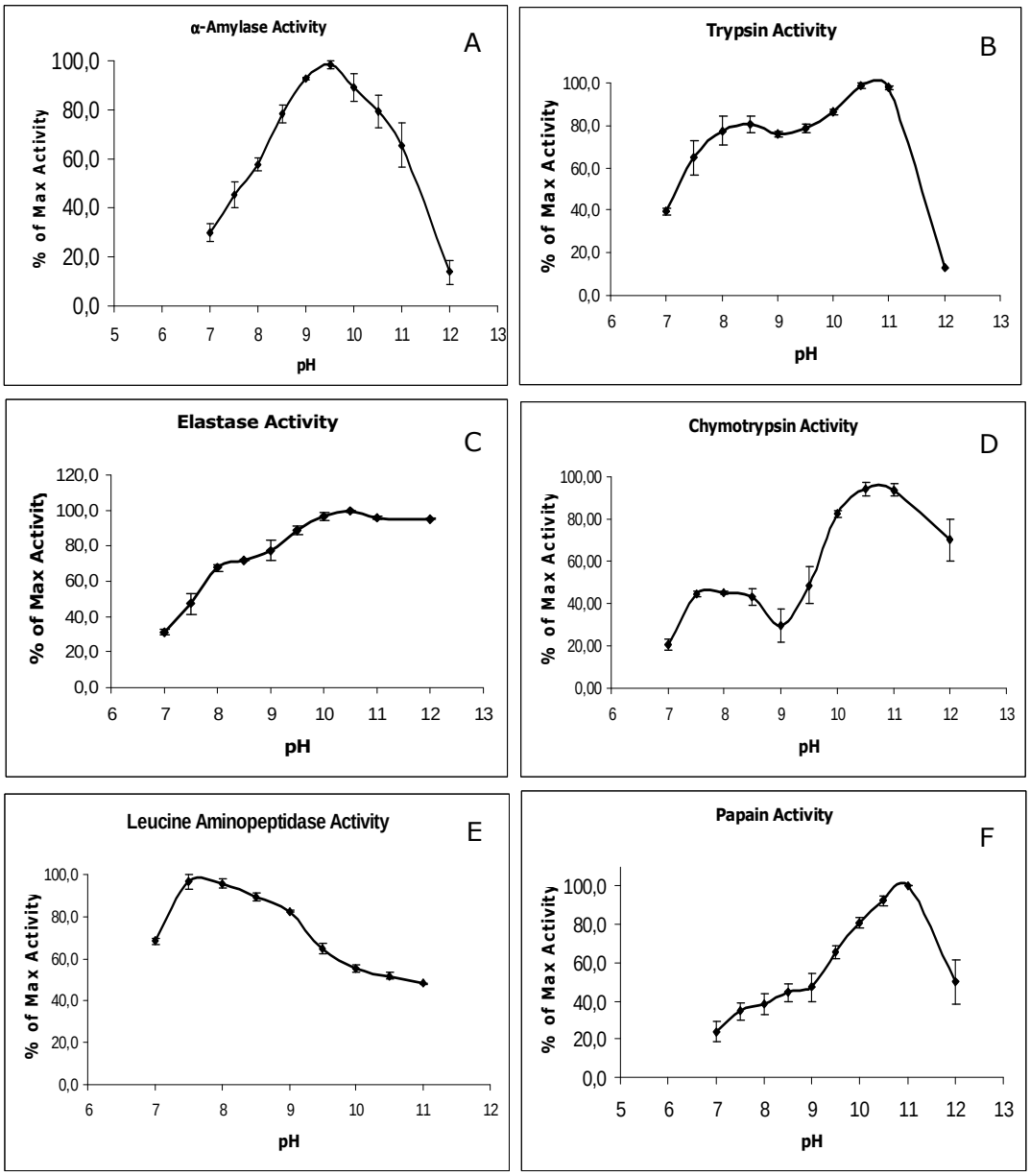


Figure 3.10 Effect of pH on *H. armigera* midgut hydrolytic enzymes. A) α-Amylase, B) Trypsin, C) Elastase, D) Chmotrypsin E) Leucine aminopeptidase and F) Papain.

The effect of pH on the hydrolytic enzymes of *H. armigera* was shown in Figure 3.10 and the optimum pH values were tabulated in Table 3.6. The optimum pH value for all hydrolytic enzymes were in the extreme alkaline range (pH 10.0 -11.0), except the leucine aminopeptidase which had an optimum pH of 8.0. Alkaline pH optimum is the characteristic of Lepidopteran insects, and this reflects predominance of the serine type proteolytic activity which is active at alkaline pH range (Johnston *et al*., 1991).

K_m and V_{max} values for the enzymes were determined by Lineweaver-Burk plots (Figure 3.11 and Figure 3.12) and the results were tabulated in Table 3.6. All enzymatic activities were measured at their optimum pH values. Among the proteases, the highest maximal rate (V_{max}) was obtained with chymotrypsin, leucine aminopeptidase, and trypsin which were the highest proteolytic activities in the *H. armigera* midgut. A low V_{max} for elastase-like activity was observed compared to that of the other proteolytic activities. Although V_{max} for papain-like activity seem to be high, we cannot compare it with the others due to the differences in the units of the enzymatic activity. K_m value for the chymotrypsin showed the highest affinity towards the *p*-nitroanilide substrates used in the measurement of specific proteolytic activities, except the papain-like activity in which azoalbumin was used as substrate. Trypsin showed a lower affinity for its *p*-nitroanilide substrate compared the other proteases. V_{max} for the α-amylase activity was very high compared to the proteases, which correlates with the high specific activity of the α-amylase in the *H. armigera* midgut.

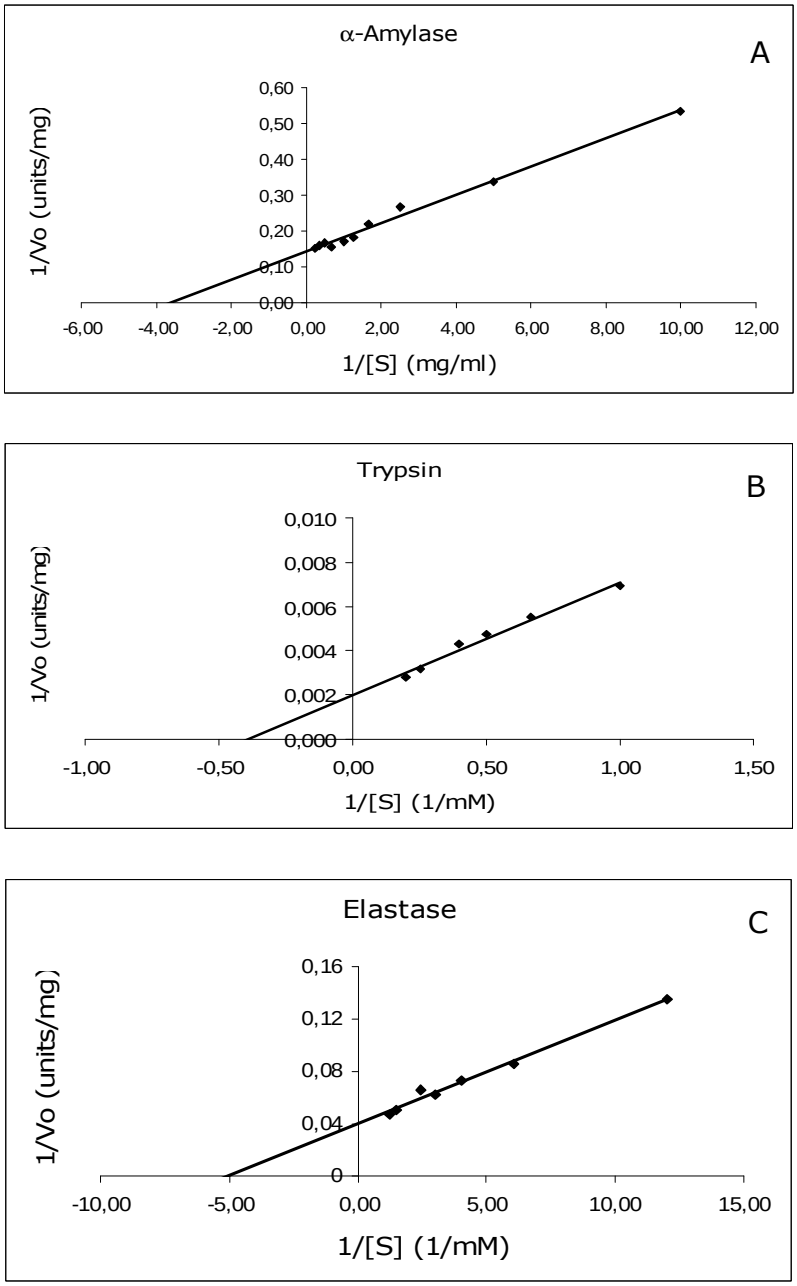


Figure 3.11 Lineweaver-Burk Plots for *Helicoverpa armigera* midgut hydrolytic enzymes. A) α-amylase, B) trypsin and C) elastase.

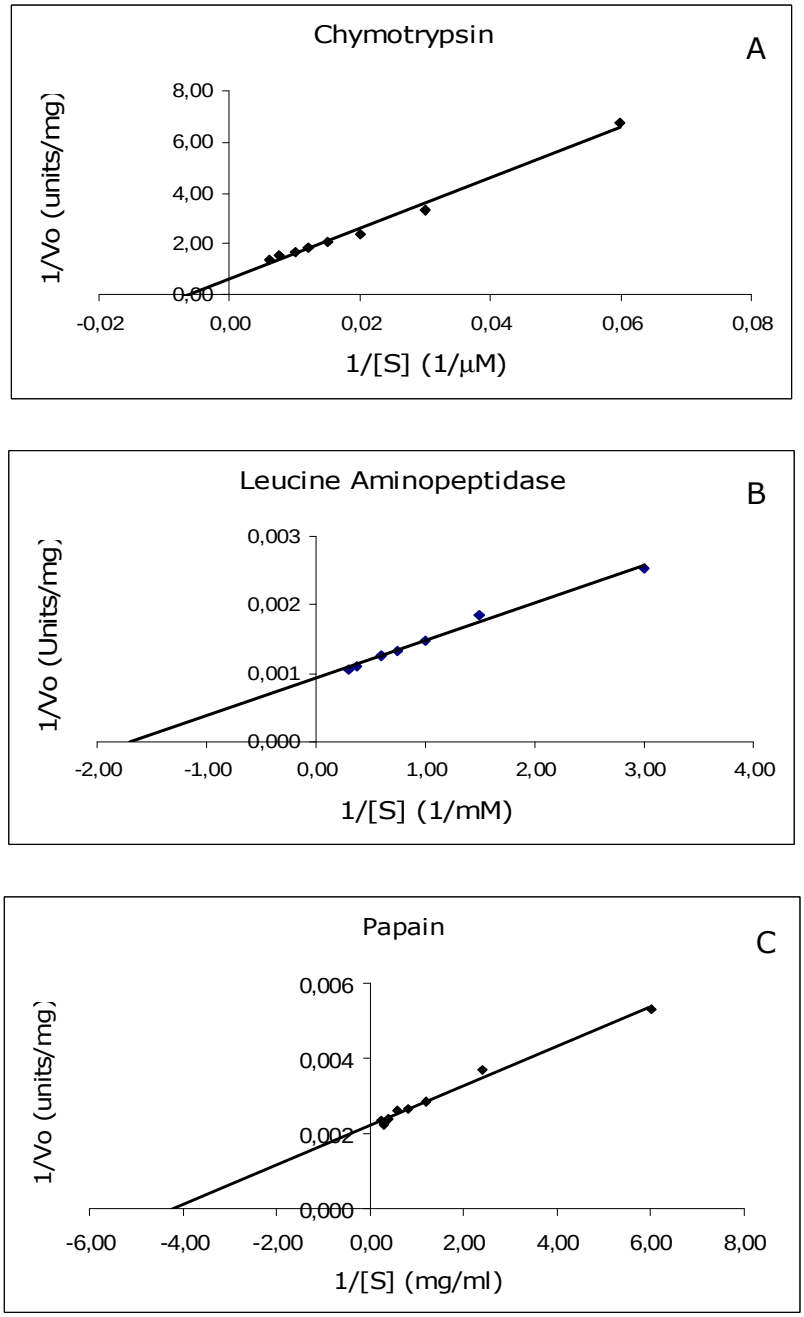


Figure 3.12 Lineweaver-Burk Plots for *Helicoverpa armigera* midgut hydrolytic enzymes. A) Chymotrypsin, B) leucine aminopeptidase and C) papain.

Table 3.6 K_m, V_{max} and optimum pH values of the hydrolytic enzymes from *H. armigera* midgut.

	K_m	**V_{max}**	**Opt pH**
α-Amylase (starch)[1]	0.3 ± 0.009 mg/ml	824.8 ± 80 µmoles/min/mg	9.5
Trypsin (BApNa)	2.27 ± 0.29 mM	428.21 ± 36.2 nmoles/min/mg	10.5
Chymotrypsin (SA_2PPpNa)	0.116 ± 0.020 mM	1332.5 ± 245 nmoles/min/mg	10.5
Elastase (SA_3pNa)	0.216 ± 0.047 mM	24.44 ± 2.32 nmoles/min/mg	11.0
Leucine Aminopeptidase (LpNa)	0.52 ± 0.04 mM	1216.9 ± 106 nmoles/min/mg	8.0
Papain (Azoalbumin)	0.25 ± 0.06 mg/ml	411.34 ± 80.7 0.001 unit ΔOD/min/mg	11.0
Carboxypeptidase A (HPA)	-	-	-
Carboxypeptidase B (HA)	-	-	-

(1) In parenthesis are the substrates used. Each data is the average of at least three independent experiments ± SEM

3.2.3 Inhibition of Cotton Bollworm Midgut Hydrolytic Enzymes with Synthetic and Natural α-Amylase and Protease Inhibitors

3.2.3.1 Inhibition of Cotton Bollworm α-Amylase Activity

α-Amylase activity of *H. armigera* midgut was tested for inhibition with several naturally occurring plant α-amylase inhibitors from seeds of bean, chickpea, maize and wheat. Equal amount of enzyme corresponding approximately to 1 unit of α-amylase activity was included in each assay medium. The inhibitors from the first three were crude seed extracts and the inhibitory activity in each extract was tested against the commercial α-amylase (Table 3.3). *H. armigera* α-amylase activity was found resistant to inhibition by bean, chickpea and maize α-amylase inhibitors, but it was significantly inhibited by wheat α-amylase inhibitor (Figure 3.13).

Starch-SDS-PAGE revealed three isoforms of α-amylases in the *H. armigera* midgut (Figure 3.14) which are arbitrarily named as Amy-1, Amy-2 and Amy-3 according to their mobilities in the PAGE. This is the first report revealing the isoforms of α-amylases in the *H. armigera* midgut.

The inhibitory effects of bean, chickpea, maize and wheat α-amylase inhibitors were also tested in the starch-SDS-PAGE which clearly showed the inhibition of Amy-1 and Amy-2 with wheat α-amylase inhibitor, while the other inhibitors had no effect on any of the isoforms (Figure 3.14).

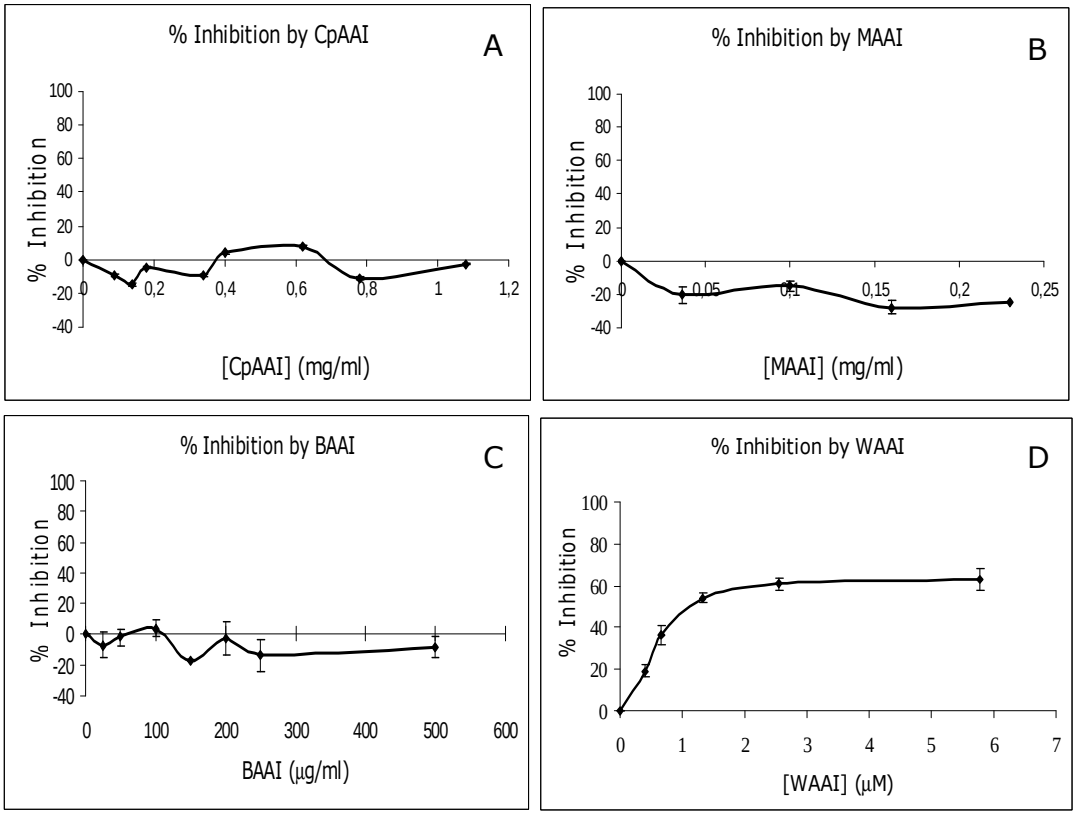


Figure 3.13 Percent inhibition of *H. armigera* midgut α-amylase activity by different concentrations of α-amylase inhibitors. A) chickpea α-amylase inhibitor (BAAI), B) maize α-amylase inhibitor (CpAAI), C) bean α-amylase inhibitor (WAAI) and D) wheat α-amylase inhibitor (MAAI). Values in x-axis indicates the amount of inhibitor included per assay medium.

Although the evaluation of the possible use of PIs for the development of *H. armigera* resistant host plants are widely studied, reports concerning the properties of *H. armigera* α-amylases and possible use of plant-derived α-amylase inhibitors for transgenic studies are not available. In this study it was found that legume seed α-amylase inhibitors (chickpea, maize and bean) were ineffective against the *H. armigera* midgut α-amylase activity, on the other hand, WAAI may be a good candidate for the transgenic studies as it inhibits α-amylase activity by 60 % at 2 µM concentration. However, to be used in the

transgenic studies, its effects on development of the insect should be revealed through the feeding tests. Moreover, other concerns on the application of transgenic plants producing inhibitors should be satisfied.

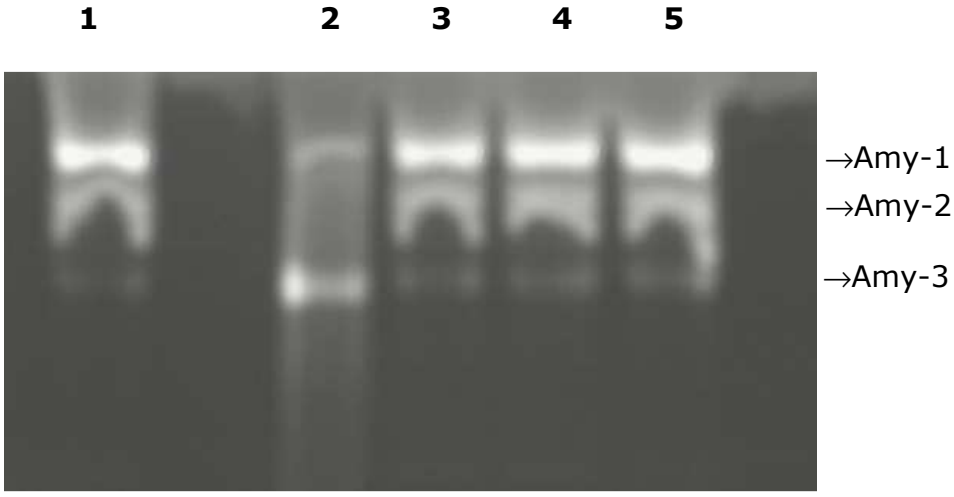


Figure 3.14 α-Amylase activity from midgut of *H. armigera* subjected to 6% SDS-PAGE containing 0.5% soluble starch. Samples were prepared by incubating in standard sample dilution buffer (without β-mercaptoethanol) for 10 min, at room temperature (no heat treatment). Midgut extracts containing 100 µg proteins (corresponding approximately to 1 unit of α-amylase activity) was loaded into each well. For inhibitions 100 µg of midgut extract was incubated with each inhibitor separately at room temperature and then treated with sample dilution buffer. Gels were run at 12 mA constant current and stained with KI/I_2 solution. Lane 1: 100 µg midgut extract without inhibitor, Lane 2: 100 µg extract + 5 µg WAAI; Lane 3: 100 µg midgut extract + maize extract containing 10 µg protein; Lane 4: 100 µg extract + Chickpea extract containing 40 µg protein; Lane 5: 100 µg midgut extract + Bean extract containing 40 µg protein.

3.2.3.2 Inhibition of Cotton Bollworm Trypsin Activity

Inhibition of cotton bollworm midgut trypsin-like activity was analyzed at increasing concentrations of two naturally occurring protease inhibitors SBTI and aprotinin and with a synthetic general serine protease inhibitor PMSF. Equal amount of protein corresponding approximately to 4 units of trypsin-like activity was included in each assay medium. Percent inhibitions were calculated from the maximal activity obtained without inhibitor, and the results were shown in Figure 3.25.

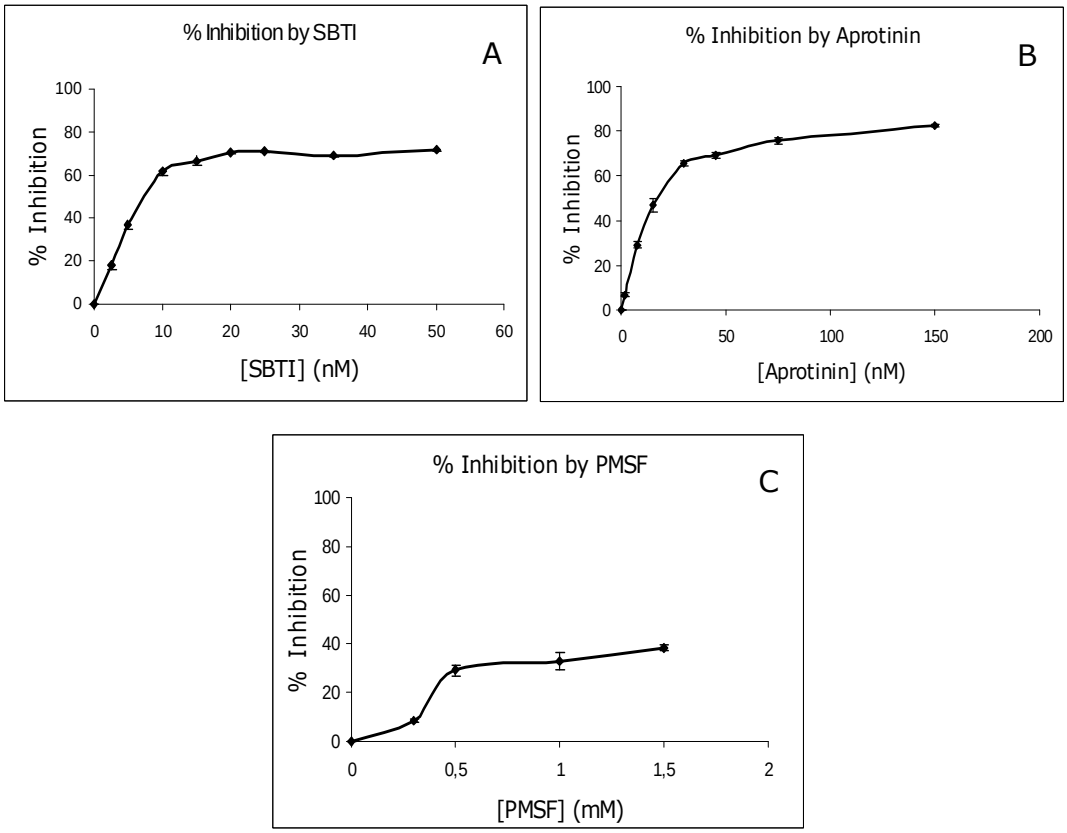


Figure 3.15 Inhibition of *H. armigera* midgut trypsin activity by different concentrations of protease inhibitors. A) soybean trypsin inhibitor (SBTI), B) aprotinin and C)PMSF.

Although SBTI and aprotinin had no effect on the sunn pest trypsin activity, they both significantly decreased the cotton bollworm trypsin activity (Figure 3.15 A and B). SBTI and aprotinin caused 70 % and 80 % inhibition of cotton bollworm trypsin activity, respectively, at nM concentrations. On the other hand, PMSF caused only 33 % inhibition of trypsin activity, at 1 mM final assay concentration.

A high level inhibition of cotton bollworm trypsin-like activity by SBTI was previously reported by Nandi *et al*. (1999). However, they indicated that SBTI has not been effective in reducing the growth and development of cotton bollworm when it is constitutively expressed in tobacco.

Although several non-host and host plant protease inhibitors were previously tested for inhibition of cotton bollworm trypsin-like activity, the effect of aprotinin (bovine lung serine protease inhibitor) was not reported before. Aprotinin caused a significant decrease in trypsin-like activity, *in vitro*. However, the effect of inhibitor should also be tested by feeding experiments.

The inhibition of cotton bollworm trypsin-like activity by several plant protease inhibitors was previously reported (Bown *et al*., 1997; Gatehouse *et al*., 1997; Patankar *et al.,* 2001; Chougule *et al*., 2005). It was indicated that some of the trypsin-like mRNAs were up-regulated while some were down regulated when cotton bollworm was fed with inhibitor proteins. Therefore the insect seems to be able to overcome the inhibitory effect of protease inhibitors by altering its midgut composition. Therefore, although our results indicated a strong inhibition of trypsin-like activity from cotton bollworm, feeding tests have to be carried out for more reliable results. To be used in development of cotton bollworm resistant transgenic plants, the inhibitor should posses

a certain degree of inhibition of proteolytic activity which should result in decrease in growth and development of the insect.

3.2.3.3 Inhibition of Cotton Bollworm Elastase Activity

Inhibition of cotton bollworm midgut elastase-like activity was analyzed at increasing concentrations of two naturally occurring protease inhibitors SBTI and aprotinin and with synthetic protease inhibitors PMSF and TPCK. Equal amount of protein corresponding approximately to 0.5 units of elastase-like activity was included in each assay medium. Percent inhibitions were calculated from the maximal activity obtained without inhibitor, and the results were shown in Figure 3.16.

No inhibitory activity was observed with natural protease inhibitors SBTI and aprotinin. On the other hand PMSF and TPCK significantly decreased the elastase-like activity by 73 % and 59 %, respectively. Although TPCK is a chymotrypsin-like serine protease inhibitor, it also inhibited the elastase-like activity.

Although natural protease inhibitors did not affect the elastase-like activity, it would have a little effect on inhibition of total proteolytic activity, because, as previously indicated, elastase activity was very low compared to the other proteolytic activities. However, presence of an insensitive protease in the insect midgut may have a negative effect on feeding tests. An insensitive protease may be up-regulated when the insect is fed by the inhibitor. However, previous studies with other plant protease inhibitors showed that, elastase-like activity, although it is insensitive to inhibition, was not up-regulated in Lepidopteran insects when they fed with protease inhibitors (Gatehouse *et al.,* 1997; Chougule *et al*., 2005).

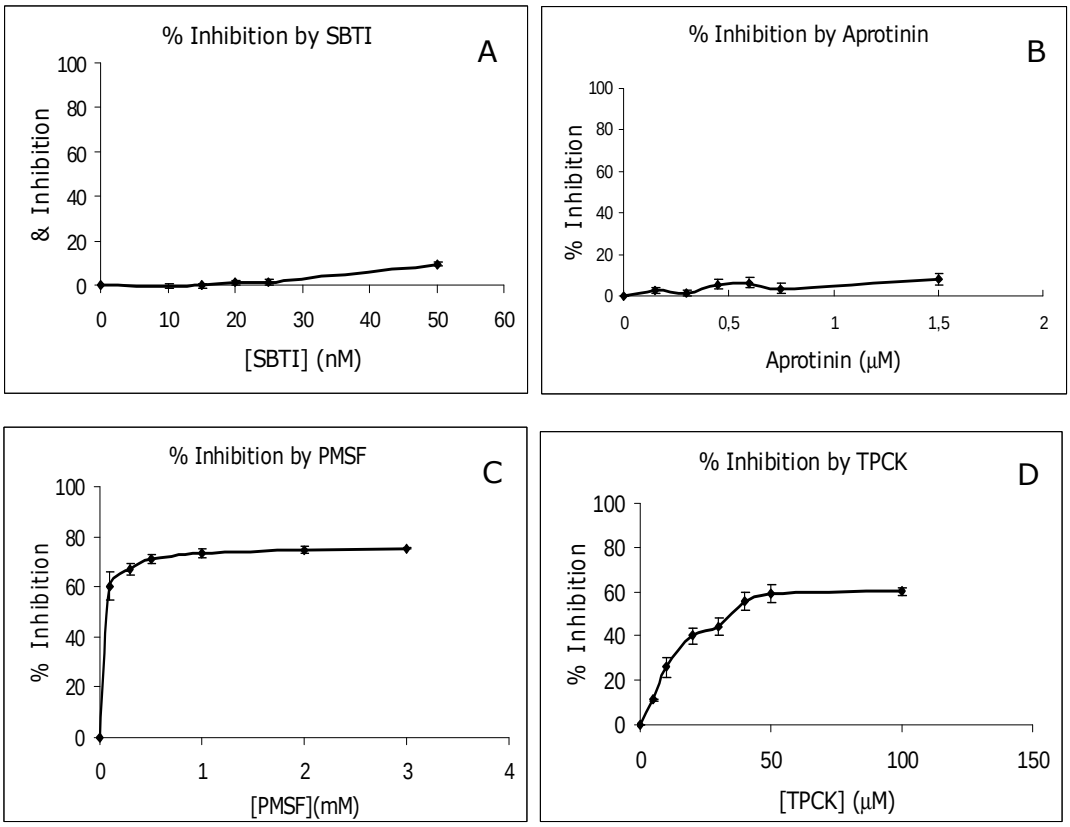


Figure 3.16 Inhibition of *H. armigera* midgut elastase activity by different concentrations of protease inhibitors. A) soybean trypsin inhibitor (SBTI), B) aprotinin C) PMSF and D) TPCK.

3.2.3.4 Inhibition of Cotton Bollworm Chymotrypsin Activity

Inhibition of cotton bollworm midgut chymotrypsin-like activity was analyzed at increasing concentrations of two naturally occurring protease inhibitors SBTI and aprotinin and with synthetic protease inhibitors chymostatin, PMSF and TPCK. Equal amount of protein corresponding approximately to 30 units of chymostatin-like activity was included in each assay medium. Percent inhibitions were calculated from the maximal activity obtained without inhibitor, and the results were shown in Figure 3.17.

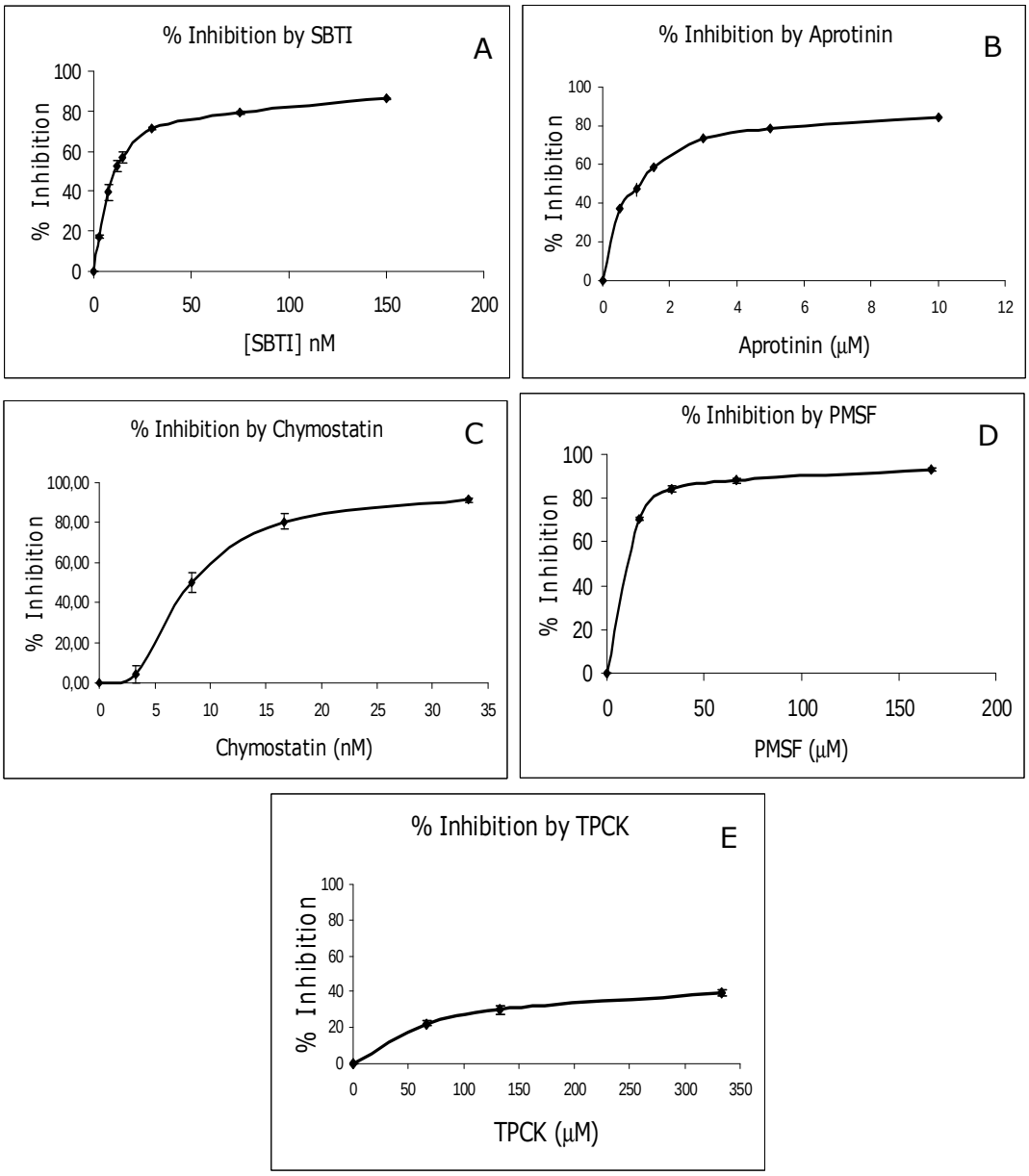


Figure 3.17 Inhibition of *H. armigera* midgut chymotrypsin activity by different concentrations of protease inhibitors. A) soybean trypsin inhibitor (SBTI), B) aprotinin C) PMSF and D) TPCK.

Chymotrypsin activity was significantly inhibited by both natural protease inhibitors, SBTI and aprotinin, by 85 % and 80 %, respectively. However, compared to aprotinin which was effective at µM concentrations, SBTI was much more potent as it showed its effect at nM concentration. PMSF and chymostatin caused around 90 % inhibition of chymotrypsin activity, while TPCK caused a smaller degree of inhibition. Being a specific inhibitor of chymotrypsin-like activity, chymostatin almost completely inhibited the cotton bollworm chymotrypsin-like activity at nM concentrations. The use of specific inhibitors against specific proteolytic activity proves that the measured activity belongs to that protease.

Chymotrypsin-like activity comprises a major proportion of proteases in the cotton bollworm midgut. Therefore the inhibition of the enzyme with natural protease inhibitors is important for the scope of this work. Both SBTI and aprotinin caused a considerable decrease in trypsin and chymotrypsin-like serine protease activities, *in vitro*. Thus, they can be used in further analysis for the development of cotton bollworm resistant transgenic plants.

3.2.3.5 Inhibition of Cotton Bollworm Leucine Aminopeptidase Activity

Inhibition of cotton bollworm midgut leucine aminopeptidase-like activity was analyzed at increasing concentrations of metalloprotease specific inhibitors $CuCl_2$ and $CdCl_2$, as well as, with a synthetic serine protease inhibitor PMSF. Equal amount of protein corresponding approximately to 20 units of leucine aminopeptidase-like activity was included in each assay medium. Percent inhibitions were calculated from the maximal activity obtained without inhibitor, and the results were shown in Figure 3.18.

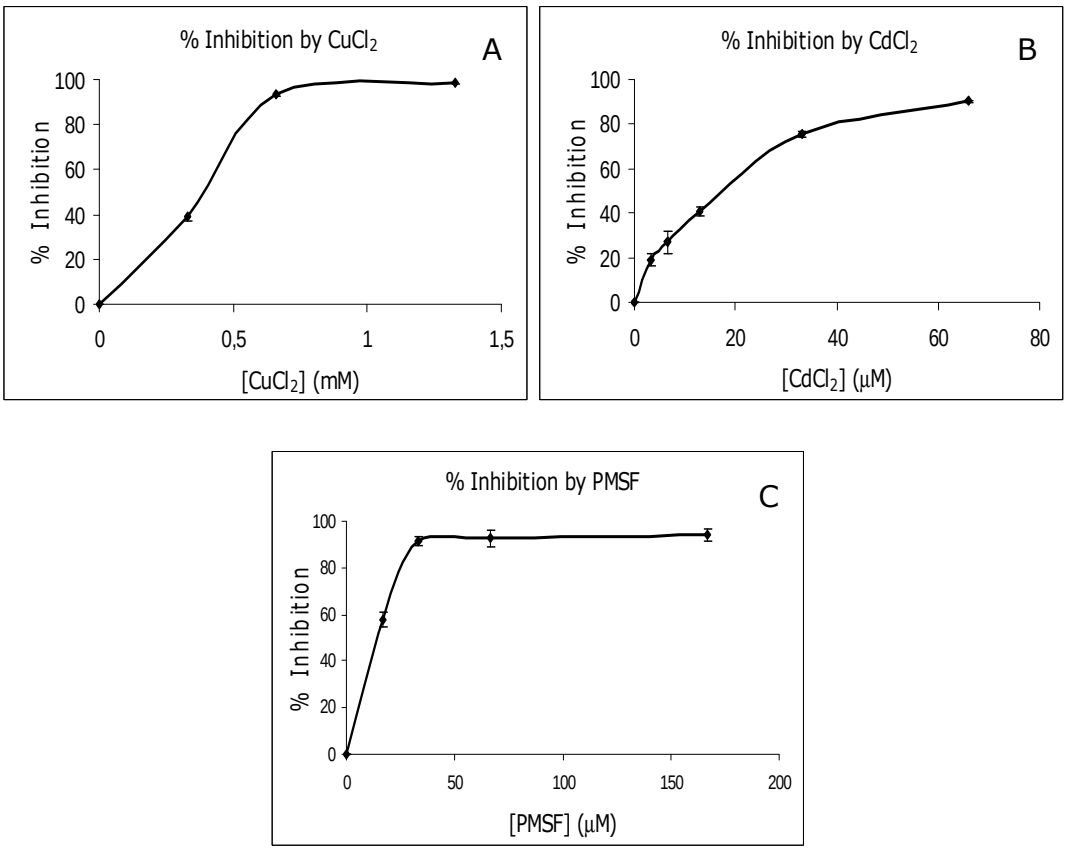


Figure 3.18 Inhibition of *H. armigera* midgut leucine aminopeptidase-like activity by different concentrations of protease inhibitors. A) $CuCl_2$ B) $CdCl_2$ and C) PMSF.

Leucine aminopeptidase, a metalloprotease type exopeptidase, cleaves N-terminal residues from the proteins. Metalloproteases are not affected by serine protease inhibitors *in vitro*, however, they were shown to display the highest response to the diet composition (Chougule *et al*., 2005). Lepidopteran insects fed on protease inhibitor rich diets showed the highest increase in expression of metalloproteases (Chougule *et al*., 2005; Bown *et al*., 1998). As we carried out the experiment *in vitro* only, we did not test the effect of natural serine protease inhibitors on leucine aminopeptidase-like activity.

Leucine aminopeptidase-like activity was almost completely inhibited by metalloprotease inhibitors $CuCl_2$ and $CdCl_2$, which confirms that the observed activity belongs to metalloprotease. CdCl2 was more potent than $CuCl_2$ as it exhibited its effect at µM concentrations. A general protease inhibitor PMSF also inhibited the leucine aminopeptidase-like activity by around 90 % at µM concentrations.

3.2.3.6 Inhibition of Cotton Bollworm Papain Activity

Inhibition of cotton bollworm midgut papain-like activity was analyzed at increasing concentrations of cysteine protease-specific inhibitor E-64 and with a general protease inhibitor PMSF. Equal amount of protein corresponding approximately to 3 units of papain-like activity was included in each assay medium. Percent inhibitions were calculated from the maximal activity obtained without inhibitor, and the results were shown in Figure 3.19.

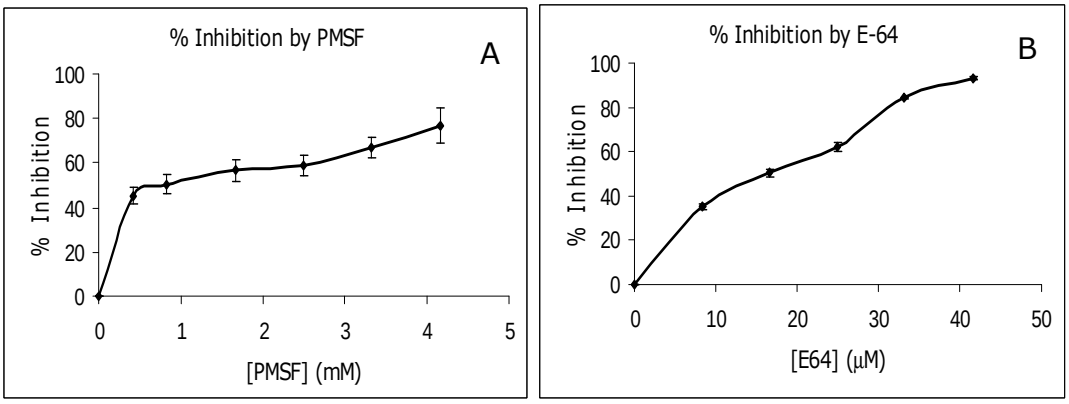


Figure 3.19 Inhibition of *H. armigera* midgut papain activity by different concentrations of protease inhibitors. A) PMSF and B) E-64.

Although cysteine protease type proteolytic activity was not reported in Lepidopteran insects, in *H. armigera* we have detected a cysteine protease, papain. The presence of cysteine type activity was confirmed by using a cysteine-protease specific inhibitor, E-64, which resulted in a 93 % inhibition at 40 µM final assay concentration. However, as will be discussed in part 3.2.3.7, the inhibitor caused no inhibition on total proteolytic activity, meaning that papain-like activity corresponds only a minor part of total proteolytic activity amount of total proteases in the insect midgut. Papain-like activity was also inhibited by a general protease inhibitor PMSF, significantly.

3.2.3.7 Inhibition of Cotton Bollworm Total Protease Activity

Total protease activity in the *H. armigera* midgut was measured using a general protease substrate, azocasein. The activity was measured at four different pH values (pH 8.0 to pH 11.0) and inhibition studies with natural and synthetic protease inhibitors were carried out separately at each pH value. The observed activities and the percent inhibitions were given in Table 3.7.

The total protease activity increased gradually as pH was increased, and the highest proteolytic activity was measured at pH 11.0. This result is in correspondence with the optimum pH values of the specific proteolytic activities most of which had an optimum pH value of 10.5 and 11.0. The results also supported the previous findings on Lepidopteran insects which indicated an alkaline pH optimum of midgut hydrolytic enzymes.

Both SBTI and aprotinin caused a significant decrease in total protease activity, especially at high pH values (Table 3.7), which correlates well with their effects on specific proteases which had high alkaline pH

Table 3.7 General protease activity of midgut extracts from *H. armigera* at four different pH values and the effects of protease inhibitors on the general protease activity.

	pH 8.0	**pH 9.0**	**pH 10.0**	**pH 11.0**
Control (units/mg)	298 ± 23[a]	320 ± 32[ab]	341 ± 22[ab]	400 ± 32[b]
	% Inhibition			
SBTI (5 nM)	23.0 ± 5.5	33.1 ± 6.1	34.4 ± 3.7	48.0± 6.02
Aprotinin (0.15 µM)	25.4 ± 9.5	39.8 ± 4.6	46.6± 10.2	55.0 ± 8.4
Chymostatin (1 µM)	47.8 ± 0.5	40.6 ± 0.2	38.4 ± 5.9	36.2 ± 6.6
TPCK (0.1 mM)	n.i	n.i	n.i	n.i
PMSF (1 mM)	61.6 ± 2.5	55.6 ± 1.2	54.5 ± 4.7	60.7 ± 5.7
EDTA (0.1 mM)	n.i.	n.i.	n.i.	n.i.
$CdCl_2$ (1 mM)	35.2 ± 0.6	20.1 ± 7.5	21.6 ± 4	13.4 ± 4.5
$CuCl_2$ (1 mM)	46.7 ± 4.2	27.5 ± 3.9	22.7 ± 5.9	15.4 ± 7.9
E-64 (0.1 mM)	n.i.	n.i.	n.i.	n.i.

Values are given as % inhibition $((1 - V_i/V_o)*100)$ ± SEM with respect to their corresponding controls without inhibitor, except the control values which are given as units/mg (1 unit azocaseinolytic activity was defined as 0.001units ΔOD_{335}/min/mg protein). Results marked with different letters are significantly different ($p<0.05$). Inhibitions lower than 10% was considered as no inhibition (n.i). Each data is the average of at least three independent experiments.

values. SBTI had affected two serine type proteases, trypsin and chymotrypsin by around 70 % and 80 %, respectively (Table 3.8). Therefore, its effect on total protease activity may be attributed to the decrease in trypsin and chymotrypsin-like activities. Thus, we can say that percentage of trypsin and chymotrypsin-like activities corresponds to more than half of total proteolytic activity in the *H. armigera*. Similar evidences were observed in the case of aprotinin inhibition, too. Moreover, a chymotrypsin-specific inhibitor, chymostatin, caused around 40 % inhibition in total protease activity, which indicates that at least 40 % of serine proteases is of chymotrypsin-type. This correlates well with the amount of chymotrypsin-like activity in the *H. armigera* midgut, which was the highest protease in the tissue. TPCK did not cause significant decrease in total protease activity, although it affected the chymotrypsin-like and elastase-like activities by around 40 % and 60 % respectively. The reason may be the very short half-life of the TPCK in alkaline pH values (reported by Sigma company), which causes decomposition of the inhibitor during long incubation periods for total protease assays. Moreover, due to low elastase activity and a low inhibitory effect of inhibitor on chymostatin activity, the inhibitory effect of TPCK might not be reflected in the total protease activity. A general serine protease inhibitor PMSF resulted in approximately 60 % inhibition at all pH values. This is consistent with our findings that serine proteases constitute more than 50 % of total proteolytic activity. However, PMSF also inhibited the metalloprotease and cysteine type proteases in the insect midgut. So, effect of PMSF on total protease activity may reflect the proportion of serine proteases in the insect midgut.

The metalloprotease inhibitors $CuCl_2$ and $CdCl_2$ caused 46.7 % and 35.2 % inhibition of total protease activity at pH 8.0, respectively. But their inhibitory effects decreased gradually as the pH was increased. This observation was in accordance with the optimum pH value of leucine

aminopeptidase (a metalloprotease) which was 8.0. Therefore, at pH 8.0, leucine aminopeptidase constitutes the major proteolytic activity in the insect midgut. When pH increases, its activity decreases. EDTA, another metalloprotease inhibitor, had no effect on total protease activity.

Although we have observed a papain-like cysteine protease activity in the insect midgut, use of a cysteine protease-specific inhibitor, E-64, during measurement of total protease activity, did not supported the previous results. We detected no inhibition in total protease activity when E-64 was included in the assay medium. This finding may be indicating the presence of very low papain-like activity, whose inhibition did not affect the total proteolytic activity significantly.

3.2.3.8 Summary of the Effect of Inhibitors on *H. armigera* Midgut Proteases

For the comparison, the inhibitory effects of all the inhibitors as percent inhibition on specific proteolytic activities were given in Table 3.8. When natural protease inhibitors SBTI and aprotinin are considered, SBTI seems to be more effective as it exhibited its inhibitory effect at nM concentrations. Although aprotinin decreased proteolytic activities at higher rate, it was effective at µM concentrations in inhibiting the chymotrypsin activity.

PMSF caused a significant inhibition on all proteolytic activities. But its effective concentration varied considerably for each protease. Other chemical protease inhibitors which were specific to specific types of proteases inhibited the corresponding protease significantly at varying concentrations. These results confirmed the type of protease present in the insect midgut.

Table 3.8 Summary of effect of inhibitors on *H. armigera* midgut protease activities.

Inhibitor	Protease type	Effective Concentration[a]	% Inhibition[b]
SBTI	Trypsin	20 nM	70.8 ± 0.8
	Chymotrypsin	75 nM	79.2 ± 0.6
	Elastase	-	n.i.
Aprotinin	Trypsin	150 nM	82.3 ± 0.7
	Chymotrypsin	10 µM	84.6 ± 1.25
	Elastase	-	n.i.
Chymostatin	Chymotrypsin	30 nM	91.2 ± 0.87
TPCK	Chymotrypsin	130 µM	29.8 ± 2.3
	Elastase	50 µM	59.1 ± 4.3
PMSF	Trypsin	1 mM	33.0 ± 3.9
	Chymotrypsin	50 µM	88.14 ± 1.24
	Elastase	1 mM	73.2 ± 1.7
	Leucine aminopeptidase	30 µM	91.6 ± 1.9
	Papain	4 mM	76.9 ± 7.8
$CuCl_2$	Leucine aminopeptidase	1.3 mM	98.4 ± 0.4
$CdCl_2$	Leucine aminopeptidase	66 µM	90.2 ± 0.4
E-64	Papain	40 µM	93.3 ± 0.8

[(a)] The values are the inhibitor concentrations that caused maximal inhibiton. [(b)] Values are given as maximum % inhibitions ((1 – V_i/V_o)*100). Each data is the average of at least three replicates ± SEM.

For a better evaluation of the potencies of each inhibitor, we have calculated the IC_{50} values (Concentration of inhibitor needed to decrease the enzymatic activity by 50%) (Table 3.9). The IC_{50} values also indicated that SBTI and aprotinin are very strong inhibitors of *H. armigera* midgut serine proteases, trypsin and chymotrypsin. They are both effective at nM concentrations. However, aprotinin is not as effective as SBTI in inhibiting the chymotrypsin-like activity. Elastase-like activity is insensitive to inhibition by both SBTI and aprotinin. IC_{50} values of PMSF varied significantly for each protease. Chymostatin is a very strong inhibitor of *H. armigera* midgut chymotrypsin-like activity as it had a very low IC_{50} value. Although both caused almost complete inhibition of the insect midgut leucine aminopeptidase-like activity, $CdCl_2$ is more potent than the $CuCl_2$ as it had a much lower IC_{50} value. E-64 had a IC_{50} value at μM concentrations.

The binding constants for the inhibitors (K_i) were also calculated for the competitive reversible inhibitors, SBTI, aprotinin and chymostatin, as well as for the wheat α-amylase inhibitor (WAAI) which was the only inhibitor in effecting the *H. armigera* midgut α-amylase activity (Table 3.10). Ki values indicate the concentration of inhibitors needed for the effective binding. In other words, it is the affinity of the enzyme for the inhibitor. For the protease inhibitors SBTI, aprotinin and chymostatin, the results correlated with the previous findings.

Table 3.9 Potencies of inhibitors against the hydrolytic enzymes of *H. armigera* midgut, given by IC_{50} values.

	Trypsin	Chymotrypsin	Elastase	LAP	Papain
			IC_{50}[a]		
SBTI (nM)	7.84 ± 0.54	12.5 ± 0.96	n.i.[b]	-[c]	-
Aprotinin (nM)	15.76 ± 1.08	1010 ± 140	n.i.	-	-
Chymostatin (nM)	-	9.96 ± 0.96	-	-	-
PMSF (µM)	1025.7 ± 79	17.93 ± 0.41	66.98 ± 4.73	17.93 ± 0.02	821.4 ± 114
$CdCl_2$ (µM)	-	-	-	21.6 ±0.37	-
$CuCl_2$ (µM)	-	-	-	359.6 ± 1.2	-
TPCK (µM)	-	166.9 ± 18.7	43.91 ± 7.16	-	-
E-64 (µM)	-	-	-	-	21.02 ± 0.36

[(a)] IC_{50} values indicate the amount of inhibitor causing 50% inhibition. [(b)] no inhibition. [(c)] not determined. Each value is the average of at least three independent experiments ± SEM.

Table 3.10 Binding constants (K_i) of SBTI, aprotinin and chymostatin against the midgut hydrolytic enzymes of *H. armigera*.

	Trypsin	**Chymotrypsin**	**Elastase**	**α-Amylase**
		K_i[a]		
SBTI (nM)	4.82 ± 0.36	6.84 ± 0.42	n.i.[b]	-[c]
Aprotinin (nM)	12.13 ± 0.4	610 ± 90	n.i.	-
Chymostatin (nM)	-	1.59 ± 0.27	-	-
WAAI (μM)	-	-	-	0.15 ± 0.02

([a]) K_i values were calculated for only reversible, competitive inhibitors, as described by Knight (1999). ([b]) n.i. no inhibition. ([c]) n.d. not determined. Each value is the average of at least three independent experiments ± SEM.

CHAPTER 4

CONCLUSION

The use of protease or α-amylase inhibitors in the development of insect resistant transgenic plant has been considered as good alternatives to pesticide use and *Bt*-transgenic plants, which exert a very high selection pressure on insect populations for resistant alleles. The protease and α-amylase inhibitors, on the other hand, are not toxic to insects but they exert a physiological stress by inhibiting the proteases or amylases of the insect midgut, resulting in inhibition of the growth. The retarded growth may reduce the use of toxic insecticides, as well as, makes the insects susceptible for predation by natural enemies, thus minimizes the crop damage (Lewis *et al*., 1997). As the strategy exploits plants' own defense response, it has little effect on the ecosystem.

For the development of insect resistant transgenic plants through the use of protease or α-amylase inhibitors, a detailed investigation and characterization of components of hydrolytic enzymes from the guts of the insect pests is a prerequisite, before selecting the appropriate inhibitor.

This study was conducted to identify and characterize the hydrolytic enzymes (proteases and α-amylase) of two important pests of Turkey

Eurygaster integriceps (sunn pest) and *Helicoverpa armigera* (cotton bollworm). The effect of several protease and α-amylase inhibitors on the enzyme activities were also tested for their potential use in the development of insect resistant transgenic plant.

In sunn pest midgut a very high α-amylase activity was detected, while, proteolytic activity was significantly low. This reflects the high carbohydrate diet of the sunn pest, which predominantly feeds on cereals seeds. α-Amylase activity was found resistant to bean α-amylase inhibitor, but it was affected by chickpea, maize and wheat α-amylase inhibitors, although the amount of inhibitions was not satisfactorily high. The natural protease inhibitors aprotinin and SBTI had no affect on the sunn pest midgut proteases. Due to very low proteolytic activity, and a high amylolytic activity, as a future work we propose the use of α-amylase inhibitors for the development of sunn pest resistant transgenic plants. However, a more potent inhibitor has to be found for use in transgenic studies.

Helicoverpa armigera had a high α-amylase and protease activities in the midgut. Compared to sunn pest, the proteolytic activity was much more diverse which reflects the wide range of host preference of the *H. armigera*, and adaptation of its proteolytic enzymes to different sources of proteins. The α-amylase activity was affected only by wheat α-amylase inhibitor, while the proteolytic activities were found significantly sensitive to both SBTI and aprotinin. Due to high protease and α-amylase activities, both strategies can be used for the development of *H. armigera* resistant transgenic plants. WAAI, SBTI and aprotinin seem to be good candidates for their use in transgenic studies. However, their *in vivo* effects on the insect growth and development should be considered before the transfer of the corresponding gene into the host plants. Because, insect pests may respond to changes in the diet by producing inhibitor-insensitive or inhibitor degrading proteases

(Jonsgma *et al*., 1995; Michaud, 1997). Therefore, the *in vivo* response of the insect proteases may be considerably different than the results obtained *in vitro*.

There are some points that should be analyzed carefully before the use of hydrolytic enzyme inhibitor genes in the insect management. The properties of a good inhibitor may be listed as follows: 1) it should have specific activity against insect hydrolytic enzymes, 2) it should remain stable to proteolysis during the food retention time in the insect midgut, 3) it should not have unwanted effects on non-target organisms such as human and animals, 4) it should be stable in the host plant environment and should not inhibit the germination of transgenic seeds by effecting the endogenous α-amylase or protease activities.

CHAPTER 5

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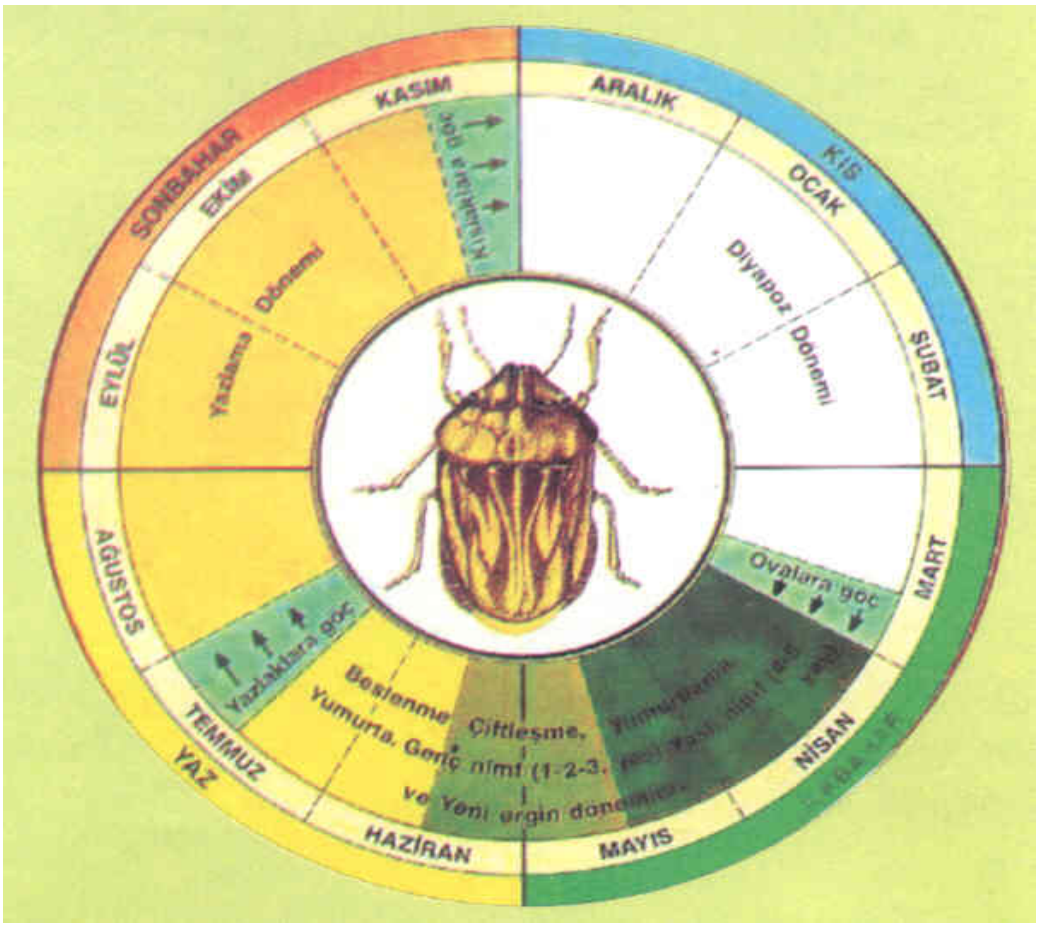
APPENDIX A

Life Cycle of Sunn Pest (*Eurygaster integriceps*)



APPENDIX B

Reagents for Non-Reducing Sodium Dedocyl Sulfate-Polyacrylamide Gel

1. **Separating Gel Buffer**

   1.5 M Tris-HCl pH 8.8

2. **Stacking Gel Buffer**

   0.5 M Tris HCl pH 6.8

3. **5X Sample Dilution Buffer**

   0.4 M Tris pH 6.8

   5% SDS

   20% Glycerol

   0.03% Bromophenol Blue

4. **10X SDS-Running Buffer**

   29 g Tris Ultrapure

   144 g Glycine

   10 g SDS (excluded in native PAGE)

   Dissolve in final volume of 1 L with dH_2O. Store at 4°C. Dilute 10X before use.

5. **10 % SDS**

   1 g SDS dissolved in 10 ml dH_2O

6. **10% APS**

   0.1 g ammonium persulfate dissolved in 1 ml of dH_2O

7. 30% Acrylamide/Bisacrylamide (200 ml)

58.4 g Acrylamide

1.6 g N'N'-bis-methylene-acrylamide

Dissolve in final volume of 200 ml. Store at dark. Max shelf life is 30 days.

8. 4% Stacking Gel Solution

30% Acrylamide-bisacrylamide	0.65 ml
dH_2O	3.05 ml
0.5 M Tris-HCl pH 6.8	1.25 ml
10% SDS	50 µl (excluded in native PAGE)
10% APS	25 µl
TEMED	5 µl

9. 8% Separating Gel Solution Containing 0.5% Casein

30% Acrylamide-bisacrylamide	2.66 ml
dH_2O	3.69 ml
0.5 M Tris-HCl pH 8.8	2.5 ml
5% Casein	1.0 ml
10% SDS	100 µl
10% APS	50 µl
TEMED	5 µl

10. 6% Separating Gel Solution Containing 0.5% Soluble Starch

30% Acrylamide-bisacrylamide	2.0 ml
dH_2O	4.3 ml
0.5 M Tris-HCl pH 8.8	2.5 ml
5% Starch	1.0 ml
10% APS	50 µl
TEMED	5 µl

APPENDIX C

Coomassie Blue R-250 Staining

Solutions

1. **Staining Solution**
   0.1 % Coomassie Blue R-250
   12% Glacial Acetic Acid
   50% Methanol

2. **Destaining Solution**
   30% Methanol
   7% Glacial Acetic Acid

Staining

The gel was incubated in the Coomassie Blue staining solution for 1 hour and destained with destaining solution several times until a clear bands appears.

APPENDIX D

Comparison of Hydrolytic Enzymes of Sunn Pest and Cotton Bollworm

	SUNN PEST		COTTON BOLLWORM	
HYDROLYTIC ENZYMES	**Specific Activity**	**Optimum pH**	**Specific Activity**	**Optimum pH**
α-Amylase	4.45 mmoles/min/mg	6.0	620 ± 23 µmoles/min/mg	9.5
Trypsin	3.0 ± 0.4 nmoles/min/mg	4.0 – 9. 0	221.5 ± 20.1 nmoles/min/mg	10.5
Chymotrypsin	Not Detected		643.7 ± 24.3 nmoles/min/mg	10.5
Elastase	0.66 ± 0.03 nmoles/min/mg	4.0 – 11.0	9.7 ± 0.6 nmoles/min/mg	11.0
Leucine Aminopeptidase	14.4 ± 0.8 nmoles/min/mg	8.0	441 ± 27 nmoles/min/mg	8.0
Papain	Not Detected		71.3 ± 4.7 mΔOD/min/mg	11.0
Carboxypeptidase A	Not Detected		Not Detected	
Carboxypeptidase B	Not Detected		Not Detected	

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1994-1995	School of Foreign Languages, M.E.T.U, Ankara, English Preparatory School
1988-1994	Bornova Mustafa Kemal Lisesi, Izmir, Turkey

Professional Experiences and Previous Employment

1999-2006	Teaching Assistantship, Department of Biology, M.E.T.U
1999-2001	M.Sc. Thesis on "Effect of NaCl Stress on Antioxidant Enzymes of Lentil"
2002-2006	Ph.D. Thesis on "Determination of Hydrolytic enzymes of Sunn Pest (*Eurygaster integriceps* Put.) and Cotton Boll Worm (*Helicoverpa armigera*)"

Courses Thought

- Bio310 Biochemistry Laboratory
- Bio101 General Biology Laboratory
- Bio442 Plant Biotechnology Laboratory

Languages

- English: fluent
  TOEFL: 260/300
  Foreign Language Proficiency Examination For State Employees: 88/100
- Turkish: native

Professional Skills

- Design and conduct experiments in biochemistry, molecular biology and biotechnology
- Good knowledge of basic techniques and equipments used in modern biological sciences Spectrophotometric techniques,Electrophoretic techniques (Agarose Gel Electrophoresis, 1-D and 2-D PAGE) Chromatographic techniques (Gel filtration, ion exchange, affinity, HPLC) Molecular Techniques (DNA isolation, PCR, Western / Northern /Southern Blots)

Professional Interests

- Plant Biochemistry
  Identification and characterization of enzymes involved in antioxidant response, Identification and characterization of protease inhibitors in plants
- Insect Biochemistry
  Identification and characterization of hydrolytic enzymes of insect pests

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Computer Skills

- Competent with all common Microsoft-Office based software and internet applications

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Technical Courses Attended

- II. Bioinformatics Workshop, İstanbul/Şile-Turkey, August, 2004.
- NANO-TRII Nanoscience and Nanotechnology, 3-5 May 2006, METU-Ankara Turkey

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Seminars Attended

- Speaker at 31st FEBS Congress: Molecules in Health and Disease. "Effect of Several Plant-Protease and α-Amylase Inhibitors on two important pests of Turkey; *Eurygaster integriceps* and *Helicoverpa armigera*" Istanbul/Turkey, June, 2006.
- Speaker at 14th National Biotechnology Congress "Determination and Characterization of hydrolytic enzymes of sunn pest (*Eurygaster integriceps* Put.) Eskişehir/Turkey, August, 2005.
- Speaker at 19th National Biochemistry Congress "Determination of Hydrolytic enzymes of *Helicoverpa armigera*) Antalya/Kemer, Turkey, April, 2005.
- Speaker at Seminar in Biochemistry "Effect of Salt Stress on Antioxidant System of Lentil Seedlings" METU, Department of Biology, May, 2002.
- Poster presentation in 12th Biotechnology Congress "Effect of Salt Stress on Antioxidant Enzymes of Lentil" Balıkesir/Ayvalık, Turkey, June, 2001.

Scientific Publications

- **Bandeoğlu, E.** (2001) "Effect of NaCl Stress on Antioxidant Enzymes of Lentil (*Lens culinaris* M." M.Sc. Thesis, METU, Ankara, Turkey.
- **Özgür, E.** (2006) "Identification and Characterization of Hydrolytic Enzymes of Cotton Bollworm (*Helicoverpa armigera*) and Sunn Pest (*Eurygaster integriceps*)" PhD. Thesis, METU, Ankara, Turkey.
- **Bandeoğlu E.**, Eyidoğan F.İ., Yücel M., Öktem H.A. 2004. "Antioxidant Response of shoots and roots of Lentil to NaCl Salinity Stress" *Plant Growth Regulation*. 42(1):69-77
- **Özgür, E.**, Yücel M., Öktem, H.A. 2006. "Effect of different plant protease and α-amylase inhibitors on two important pests of Turkey; *Eurygaster integriceps* and *Helicoverpa armigera*" The FEBS Journal. 273(1):76

Submitted/Manuscripts in preparation:

- **Özgür, E.** Yücel, M., Öktem, H.A. "Hydrolytic enzyme profiles from midgut of cotton bollworm, *Helicoverpa armigera* Hüber.(Lepidoptera:Noctuidae)" Submitted to Archives of Insect Biochemistry and Physiology by April, 2006.
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Awards

- 12th National Biotechnology Congress, 2nd Best Poster Award, 2002.
- The Scientific and Technological Research Council of Turkey, Scientific Publication Award, 2004.